Clinical Applications of Stem Cells in Ocular Surface Reconstruction

by

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"One way to open your eyes is to ask yourself, 'What if I had never seen this before? What if I knew I would never see it again?'"

- Rachel Carson (1907-1964)

"The most exciting phrase to hear in science, the one that heralds new discoveries is not 'Eureka!' (I found it!) but 'That's funny…'"

- Isaac Asimov (1920-1992)

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ABSTRACT

The central aim of this thesis is to improve current techniques for the *ex vivo* expansion of epithelial and endothelial cells on amniotic membrane for the treatment of ocular disorders. This thesis is primarily a microscope-based study and documents the findings of several investigations designed to refine tissue engineering of the cornea. Light, scanning and transmission electron microscopy were used to study cultured cell morphology. Observations were quantified and compared with data from control corneas, facilitating statistical analyses. Immunohistochemical techniques were also employed to determine the distribution of extracellular matrix proteins in processed amniotic membrane (AM) carriers.

Various components of the culture system (including carrier, media and cell type) were evaluated in a number of separate studies. The results of this thesis show that denuded amniotic membrane encourages better adhesion of *in vitro* cultured stem cells and as such is a more practical carrier than the cellular variant. A freeze-dried form of amniotic membrane (FD-AM) was produced and found to compare favourably with cryopreserved tissue. Immunohistochemical findings confirmed similar distributions of extracellular matrix proteins in both carriers and FD-AM was used as a culture substrate to create healthy-looking epithelial cell sheets. In the first study of its kind, amniotic membrane was successfully used as a supportive matrix for the culture of quiescent corneal endothelial cells. In addition, evidence from the preservation study suggests that polyphenol antioxidant (extracted from green tea) could be a useful addition to culture media for corneal organ storage as it was found to maintain endothelial morphology and prolong cellular adhesion. In an attempt to

remove the risk of allogeneic graft rejection, human oral mucous membranes were evaluated as potential sources of stem cells for autologous corneal grafts. Buccal and gingival epithelia cultured on AM formed well stratified and differentiated cell sheets which closely resembled *in vivo* corneal epithelium and were found to be useful in the treatment of bilateral limbal stem cell deficiencies. Co-culture with corneal stem cells was found to further induce differentiation of the oral mucosal cells into more corneal-like epithelium. To remove the risks of viral or prion transmission associated with human amniotic membrane, extracellular matrix protein coated gelatin hydrogels were examined as alternative carriers for the cultivation of *ex vivo* expanded corneal cells and produced some promising results. It was also found that human serum can be effectively used to replace foetal bovine serum in the culture medium, removing a potential risk of zoonose contamination. Morphological and quantitative analyses confirmed that the cells produced using human serum closely resembled those of control cornea.

Collectively, the findings described and discussed herein have fostered further understanding of the amniotic membrane carrier and contributed towards significant improvements in the suspension culture system for the *ex vivo* expansion of stem cells for ocular surface reconstruction. They have also shown that corneal endothelial cells can be cultured successfully on AM and have the potential to treat corneal dystrophies.

1 INTRODUCTION

1.1 "Ah, I see..."

This common statement, in which understanding is linked to sight, provides a prime example of how imperative vision is to man. Without doubt, vision is the most important of the five senses for most vertebrates; conveying information crucial to survival and providing a vital awareness of surroundings. Humans are arguably the most visually orientated and dependant of all the mammals. Although there are certainly situations in which other senses are important and there are examples of animals that have secondarily lost vision as a necessity, these are exceptions. Life that has evolved in and adapted to the spectrum of solar radiation can be expected to develop a means to use it for gathering information. It is not surprising then, that the human eye and those parts of the brain associated with the interpretation of visual information are well-developed and any loss of functionality is devastating.

Visual impairment, which may be defined as blindness (best vision of <20/400 in the better eye, according to the World Health Organization [WHO] definition) or low vision (<20/60), is one of the most common disabilities: an estimated 40 million people worldwide were blind nearly a decade ago (at the time of the last accurate assessment) and 110 million people had low vision (Thylefors *et al.*, 1995). Despite half a century of concerted effort, the global burden of blindness is mounting. Every five seconds a person goes blind; every minute a child goes blind. Projections suggest one hundred million people will lose their sight unnecessarily by 2020 if current trends continue (American Academy of Ophthalmology, September 2003).

The phenomenon of good vision is heavily dependent on the ocular surface, in particular the transparent cornea as the principal refractive component. Diseases affecting the cornea are a major cause of blindness worldwide, second only to cataract in overall importance. The epidemiology of corneal blindness is complicated and encompasses a multitude of infectious and inflammatory eye diseases that cause corneal scarring, ultimately leading to functional blindness (Whitcher *et al.*, 2001). While cataracts account for nearly half of global blindness, the next major cause is trachoma which blinds 4.9 million individuals annually, mainly as a result of corneal scarring and vascularization. Ocular trauma and corneal ulceration are significant causes of corneal blindness that may be responsible for 1.5 to 2 million new cases of monocular blindness every year. Hence, with over 6 million people blinded by diseases of the cornea, there is a tremendous requirement for effective treatments (Trinkaus-Randall, 2000).

The typical treatment for a damaged cornea is that of a transplant. According to National Health Service figures for the United Kingdom, around 2,000 people donate their corneas on death each year, yet demand still considerably exceeds supply. Given the dramatic shortfall of available donor corneas and the inherent problems of rejection, bioengineered replacements for the damaged tissue have long been sought. The ultimate aim is to produce an artificial cornea, designed to promote retention, minimize postoperative complications and restore vision to patients who cannot receive, or are unlikely to have a beneficial outcome from a human donor graft. This thesis seeks to address some of the issues involved in the creation of an artificial cornea, pivoting around the use of amniotic membrane as a carrier for suspension-cultured stem cells in ocular surface reconstruction.

1.2 The Eye

The human eye is a highly specialized organ of photoreception; a spheroid with a diameter of approximately one inch and a weight of approximately eight grams (Martini, 1998), containing some 70% of all the sensory receptors in the body. The eyeball has a complex anatomy (figure 1.1) and can be divided into two cavities by the ciliary body and the lens. There is a large posterior cavity containing the gelatinous vitreous body and a smaller anterior cavity filled with aqueous humour.

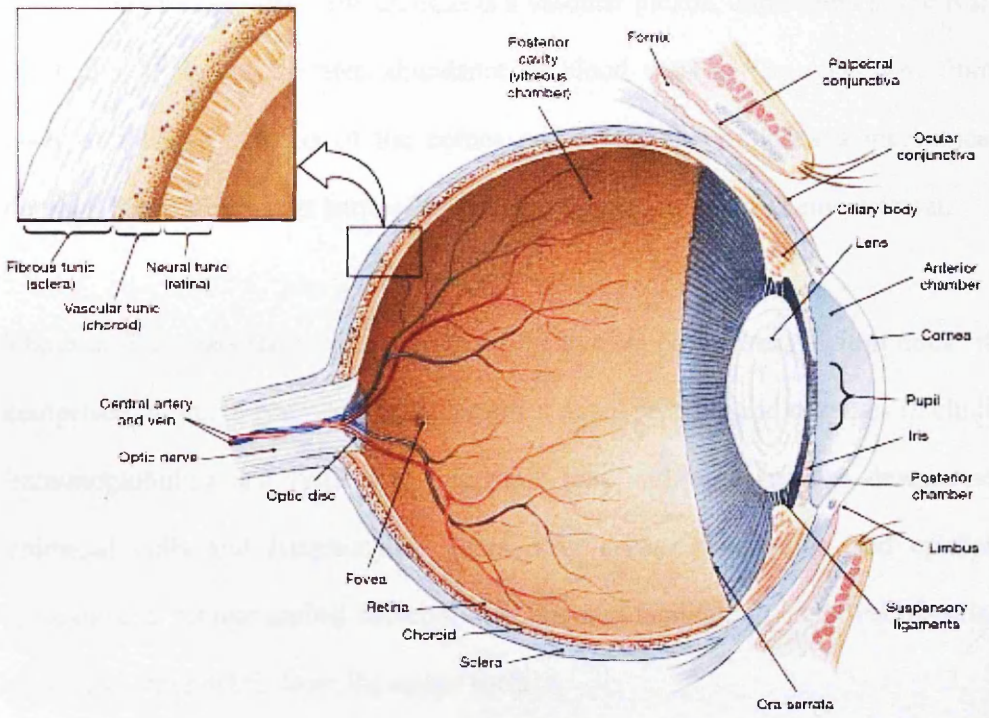


Figure 1.1: Sectional anatomy of the human eye. Inset (top left) illustrates the three tunics that constitute the wall of the eyeball. Taken from 'Anatomy and Physiology' (Martini, 1998).

The aqueous humour is produced by the ciliary body and provides a fluid cushion that helps retain the shape of the eye. Intraocular pressure is maintained by drainage of the excess through the trabecular meshwork into the canal of Schlemm (Hogan, 1971). The gelatinous vitreous humour which fills the larger posterior cavity gives additional support to the retina in stabilizing the shape of the eye. The wall of the eye consists of three layers or tunics (Martini, 1998): an outer fibrous layer, an intermediate vascular layer and an inner neural tunic or retina, containing the visual receptors and associated neurones. The vascular tunic, as well as enclosing blood vessels and lymphatics, holds the choroid and the intraocular muscles within the ciliary body and the iris. The choroid is a vascular plexus, unmatched in the rest of the body for its concentrated abundance of blood vessels. The outermost fibrous layer of the eye consists of the cornea and sclera and provides a mechanically durable, fairly impervious barrier between the eye and its external environment.

The tear film coats the visible surface of the eye. Approximately 7.5μm thick, it is comprised of mucin containing glucose, urea, many proteins and enzymes (including immunoglobulins and lysosyme), inorganic ions and cells such as desquamated epithelial cells and lymphocytes. Tears offer surface lubrication and epithelial nourishment, protect against bacterial infection and facilitate the removal of cellular waste and other debris from the ocular surface.

1.3 The Ocular Surface

The fibrous or corneoscleral tunic can be divided into two general regions, namely the cornea and the sclera. They merge at the periphery of the cornea, in the region of the limbus. The healthy ocular surface is composed of highly specialized conjunctival and corneal epithelia which are formed by two phenotypically distinct cell types (Wei *et al.*, 1996; Martini, 1998).

1.3.1 The Sclera

The sclera is the tough, opaque tissue, commonly known as 'the white of the eye', that serves as the eye's protective outer coat (Panjwani, 1997). It is composed mainly of collagen fibres, randomly orientated and interlaced while following the curve of the eyeball. The sclera extends from the corneal periphery to the very back of the eye, where it is meets the optic nerve. The human sclera is generally thickest at the posterior pole (1mm) and thinnest at the equator (0.4-0.5mm) (Maurice, 1962). Scleral functions include maintenance of the boundary of the eye, serving as a protective envelope for delicate internal structures and providing a site for attachment of the six extraocular muscles which connect via tendons to the orbit (the eye socket in the skull) and control movement of the eyeball (Stidham, 2000). The sclera is highly vascularized, containing blood vessels and lymphatics to drain any excess aqueous humour.

1.3.2 The Conjunctiva

The conjunctiva is the thin, transparent tissue forming the outer surface of the eye. It begins at the outer edge of the cornea, covering the visible part of the sclera and

lining the inside of the eyelids. The conjunctiva can be divided into three sections: *palpebral conjunctiva* covers the back of the eyelids, *bulbar conjunctiva* coats the anterior portion of the eyeball and the *fornix* forms the transition zone between the posterior eyelid and the eyeball. Like all mucous membranes, the conjunctiva has an epithelial layer and a submucosal lamina propria. As a layer of loose connective tissue, the lamina propria supports the delicate mucosal epithelium while allowing cells a degree of movement and providing immune defence. The conjunctival epithelium is vascularized and consists of loosely organized cell layers populated by mucus-secreting goblet cells (Pfister, 1975). Goblet cells are confined to the bulbar conjunctiva and are oval or round in shape, with a flattened nucleus near the base of the cell and a large intracellular collection of mucin. The apical surface of conjunctival epithelial cells is covered with microvilli which increase the surface area and hence promotes attachment of the tear film. Basal cells of the conjunctiva are attached to a typical basement membrane by hemidesmosomes. Two layers make up the conjunctival stroma; a superficial connective tissue matrix and a deeper fibrous layer through which run the conjunctival blood vessels and nerves. The conjunctiva possesses immunological defences to protect the surface of the eye.

1.3.3 The Cornea

The cornea is the transparent, normally avascular tissue which overlays the iris and the lens, forming the most anterior part of the eye (Panjwani, 1997). In conjunction with the sclera, it provides mechanical strength for the eye. As well as protecting the eye from ultraviolet radiation and providing a physical barrier to infection, the cornea is responsible for light transmittance and refraction. The curvature of the cornea (figure 1.2) is a major factor governing the extent to which it refracts light entering

the eye, providing most of the focus. Aberrations in the shape of the cornea can cause profound detriment to vision and surgical procedures to correct vision problems often involve adjusting corneal curvature.

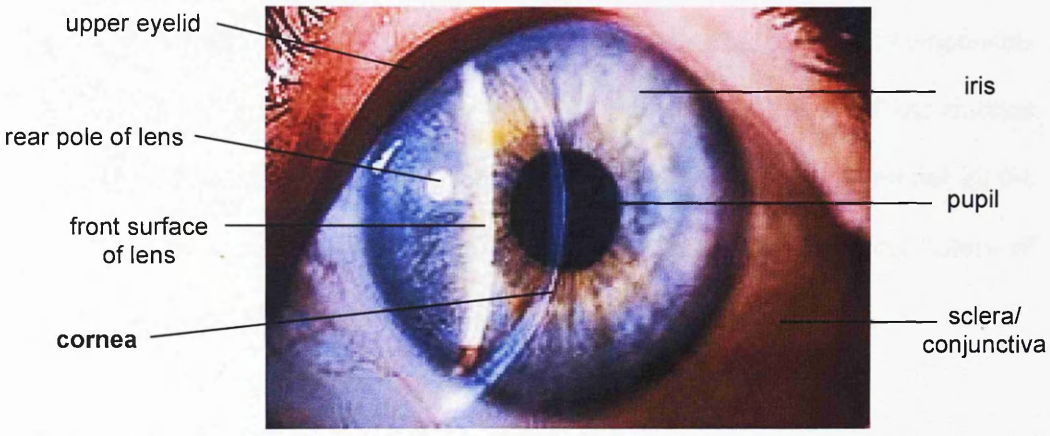


Figure 1.2: Slit lamp photograph showing a healthy eye with a clear cornea. The curvature of the cornea is indicated by the blue line [www].

Its physical isolation from the circulatory system (hence the immune system) has clinical implications. The cornea is effectively incapable of mounting a typical graft-rejection response so corneal transplants have a relatively high rate of success.

Structurally, the cornea is composed of five layers lying parallel to its surface (Maurice, 1962). There is a 5-6 cell layer thick, stratified, squamous, non-keratinized epithelium overlying an acellular condensation of stroma, so-called Bowman's layer. The vast majority of the cornea is the stroma, remarkably regular collagen lamellae continuous with the sclera. Descemet's membrane forms the basement membrane of the corneal endothelial monolayer. Each of the component layers is described in more detail in section 1.5, following an introduction to the extracellular matrix.

1.4 Extracellular Matrices

Extracellular matrix (ECM) molecules make up 90% of the cornea stroma, and the basal laminae of both the corneal epithelial and endothelial cells consist entirely of extracellular matrix molecules. In the following subsections, the major components and assembly of extracellular matrices are described along with a brief introduction to the basement membrane and associated terminology. Section 1.5 elaborates on the finer structure of the cornea and describes in more detail the specialized nature of extracellular matrices within this complex tissue.

1.4.1 An Introduction to the Extracellular Matrix

The development and normal functioning of all cell types in an organism depends upon molecules in their environments. The term 'extracellular matrix' essentially includes all secreted macromolecules that are immobilized outside cells. Major components of extracellular matrices include collagens, adhesive glycoproteins and proteoglycans; the insoluble collagen fibres being embedded in a hydrated gel-like ground substance. The ECM provides order in this extracellular space and has functions associated with the adhesion, differentiation, proliferation and organization of epithelial cells. The basement membrane is a specialized form of extracellular matrix and is very important in the cornea (Yurchenco and Schittny, 1990).

1.4.2 The Basement Membrane Zone

The basement membrane zone is a thin, continuous, sheet-like extracellular matrix structure composed of hemidesmosomes, basement membrane and anchoring fibrils. Basement membranes are specialized structures located at the boundary between

epithelial cells and underlying connective tissue (Madri *et al.*, 1984). A proteoglycan and glycoprotein sheet secreted by cells to form the extracellular matrix, the basement membrane comprises two distinct layers: the *basal lamina* and the *reticular lamina*. Figure 1.3 depicts the basement membrane zone, as seen by transmission electron microscopy.

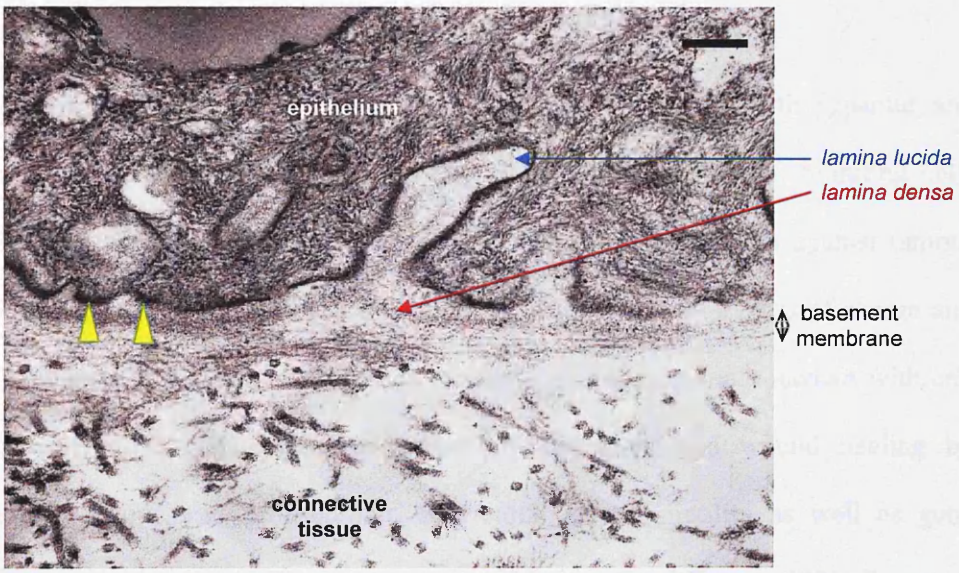


Figure 1.3: Transmission electron micrograph illustrating the basement membrane zone of human corneal epithelial cells grown on denuded amniotic membrane. The basement membrane effectively separates the underlying collagenous and proteoglycan-rich stroma from the epithelial cells, providing a surface for anchorage. Yellow arrows indicate hemidesmosomes. The basal lamina (uppermost delicate layer of the basement membrane) is visible as two layers (laminae lucida and densa) [scale bar = 200nm].

Immediately subjacent to the epithelial cells, the basal lamina is a product of the epithelial cells themselves. Approximately 80-100nm thick, it contains mainly collagen type IV and laminin networks, connected with proteoglycans. The reticular lamina contains fibrillar collagen and is produced by fibroblasts of the underlying connective tissue. As defined by electron microscopy, the basal lamina also comprises two layers: a *lamina lucida* and *lamina densa*. The lamina lucida is the

electron-lucent layer located closest to the cell membrane while the lamina densa is discernible as an electron-dense layer next to the connective tissue. It is possible the lamina lucida does not exist *in vivo* and could be an artefact resulting from dehydration during tissue processing (Chan *et al.*, 1993). Mechanically the basal lamina anchors epithelial and connective tissue and as such helps stabilize and orientate the tissues.

Basement membranes have many important functions. They both separate and connect two distinct tissue compartments, allowing the passage of migrating cells under certain physiological conditions while also acting as barriers against tumour cell invasion. They regulate the movement of molecules on the basis of charge and molecular weight. Many components of the basement membrane interact with cell surface receptors, influencing epithelial cell behaviour and wound healing by regulating cell shape, proliferation, differentiation and motility as well as gene expression and apoptosis (Boudreau *et al.*, 1996; Timpl and Brown, 1996; Burgeson and Christiano, 1997). The more important of these components are described below.

1.4.3 Collagens

The collagens constitute a superfamily of ECM proteins with a structural role as their primary function (Lodish *et al.*, 1995). Based on the exon structures of their genes and the configuration of the protein, they can be divided into several groups (Olsen and Ninomiya, 1998). All collagenous proteins have domains with a triple helical conformation, in which three α chain subunits form a right-handed super helix (figure 1.4). The presence of such domains provides collagens with regions of rigidity. Collagen fibrils contain only one such domain, accounting for almost the

entire length of the molecule, whereas others such as FACIT collagens (fibril-associated collagens with interrupted triple helices), basement membrane collagens and those with transmembrane domains have several triple helical regions separated by non-helical sequences. In order to accommodate for this helical conformation, every third residue in each α chain along the helical axis is a glycine.

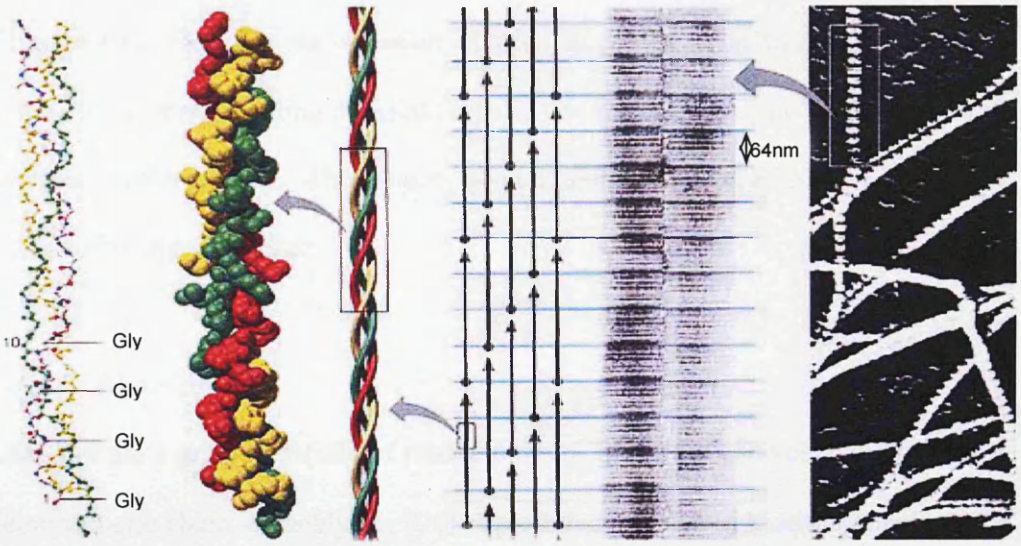


Figure 1.4: Basic structural elements and higher organization of collagen. The primary structure of collagen consists of a left handed α-helix with glycine (Gly) as every third residue. Three α chains are then joined by hydrogen bonds to form the right handed, triple helical quaternary structure. Collagen fibrils have a characteristic 64nm banded appearance under the electron microscope (Mathews *et al.*, 2000).

Collagenous proteins usually form aggregates, either alone or with other ECM components (Aumailley and Gayraud, 1998). Their major roles are in providing structural integrity and helping cells anchor to the matrix. In the corneal ECM, the most important collagens are types I, III and V (fibrillar, banded stromal collagens, accounting for 75% of the corneal total), type IV (monomeric collagen, major component of the basement membrane) and type VII (anchoring fibrils which secure epithelial cells to the matrix) (Marshall *et al.*, 1991a; Marshall *et al.*, 1991b).

1.4.4 Adhesive Glycoproteins

Glycoproteins are widely distributed in all forms of life. They occur in cells, both in soluble and membrane-bound forms, as well as in the extracellular matrix and fluids. As the name suggests, a *glycoprotein* is a compound containing carbohydrate (or glycan) covalently linked to protein. The carbohydrate may be in the form of a monosaccharide, disaccharide(s), polysaccharide(s) or any of their derivatives. Glycoproteins facilitate the adhesion of cells to the ECM in that they possess a number of different binding domains; usually one for collagen, one for integrins and one for proteoglycans. The major glycoproteins of the ECM are laminins, fibronectins and vitronectin.

1.4.4.1 Laminins

Laminins are a growing family of related proteins (400-1000kDa) characterized by a heterotrimeric chain assembly (α, β, γ), a preferred location in basement membranes and a multitude of biological activities. To date, eleven different assembly forms have been identified (Sasaki and Timpl, 1998). Electron microscopic visualization of laminins usually shows a cross-shaped structure consisting of a long arm (80nm, comprising C-terminal portions) and two or three short arms (25-40nm, N-terminal chain ends). Laminins boast a number of high-affinity binding sites for other components of the basement membrane and for cell-adhesion molecules on the cell surface (Beck *et al.*, 1990; Filenius *et al.*, 2001). The centre of the laminin molecule is thought to be located near the junction of the lamina densa and lamina lucida while its long arm favours three major orientations; one close to the cell surface, suggesting cell receptor binding; the other two, towards internal matrix structures (Schittny *et al.*, 1988). The first laminin to be isolated, laminin-1 (Timpl *et al.*, 1979), is present

along the entire length of the epithelial basement membrane in human cornea where it is thought to be involved in wound healing (Suzuki *et al.*, 2000) and corneal epithelial cell adhesion (Kurpakus *et al.*, 1999).

1.4.4.2 Fibronectins

Fibronectins are another important class of soluble multi-adhesive matrix proteins whose principal role is attaching cells to collagen matrices. This family of high molecular weight glycoproteins are produced from alternative splicing of a single gene (Ingham, 2003). Some forms remain soluble and are found in blood plasma (where they have a role in blood clotting), others associate into insoluble disulphide-bonded fibrils in the extracellular matrix (Hynes, 1998). Like laminins, these peptide dimers contain binding sites for a variety of other molecules such as heparan sulphate proteoglycan, integrins and collagens. By virtue of their attachments, fibronectins help regulate the shape of cells and the organization of the cytoskeleton and are essential for cell migration and differentiation. In the corneal epithelium, fibronectin is secreted following injury and is present in the basement membrane under migrating corneal epithelium during re-epithelialization (Fujikawa *et al.*, 1984).

1.4.4.3 Vitronectin

Vitronectin is predominantly a blood plasma glycoprotein, but is also found in ECMs where it binds collagens and heparin-like glycosaminoglycans and promotes cell adhesion and spreading. Vitronectin adsorbs strongly to tissue culture glass/plastic and mediates cell attachment (via integrin receptors) to these substrates (Steele *et al.*, 1997; Hall, 1998). Vitronectin is a secreted protein and exists in either a single chain form or a cleaved, two-chain form held together by a disulphide bond.

1.4.5 Proteoglycans

Proteoglycans (PGs) are ubiquitous glycoproteins whose carbohydrate moieties consist of long, unbranched chains of alternating residues of hexosamine and uronic acid or galactose, often sulphated. They are found predominantly in the extracellular matrix, within intracellular vesicles or associated with the cell surface of most eukaryotic cells where they have a variety of functions (Lander, 1998). They can be bound by glycoproteins in the ECM or by receptors on cells, promoting cell adhesion (Wight *et al.*, 1992). Unlike most proteins which are grouped on the basis of amino acid similarities, proteoglycans are defined by glycosaminoglycan (GAG), a common post-translational modification. Glycosaminoglycans are polysaccharides which strongly influence the structure and molecular interactions of the proteins to which they are attached, thus making the proteoglycans extremely diverse macromolecules. The basic structure of a proteoglycan is illustrated in figure 1.5.

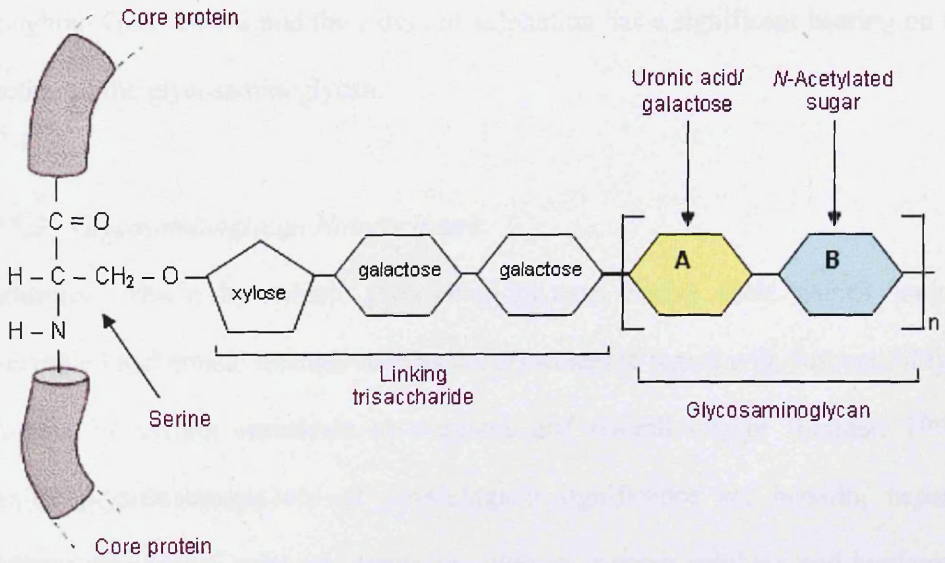


Figure 1.5: Schematic diagram of the basic *O*-linked proteoglycan structure. Proteoglycans consist of one or more glycosaminoglycan chains covalently linked via a chain of sugars and an *O*-glycosidic bond, to the serine [or threonine] residues of a protein core.

The *in vivo* roles of proteoglycans in the cornea are not well understood, however functions have been ascribed to some (Iozzo, 1998). Stromal oedema has been associated with a reduction in proteoglycans, particularly those containing keratan sulphate (Kangas *et al.*, 1990; Quantock *et al.*, 1991). Reference to specific proteoglycans found in the cornea can be found in section 1.5.3, which provides a more in depth description of corneal ultrastructure and composition.

1.4.5.1 Glycosaminoglycan Structure

Glycosaminoglycans are linear polymers with no branches. Except for the short linkage region by which they attach to serine residues in protein cores, each GAG is synthesized from just two strictly alternating monosaccharides. There are only three such disaccharide repeat units that can be polymerized onto proteins in this fashion; it is the subsequent enzymatic modification of these chains that generates complexity. The addition of negatively charged sulphate groups occurs sporadically throughout GAG chains and the extent of sulphation has a significant bearing on the function of the glycosaminoglycan.

1.4.5.2 Glycosaminoglycan Nomenclature

Traditional criteria by which glycosaminoglycans derive their names include observable biochemical features such as the disaccharide repeat unit, susceptibility to digestion by certain chemicals or enzymes and overall charge (Lander, 1998). Specific glycosaminoglycans of physiological significance are heparin, heparan sulphate, chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronic acid:

➢ Heparin and heparan sulphate [HS] both derive from the repeating disaccharides: glucuronic acid and glucosamine (Murdoch *et al.*, 1992). Heparin is less heavily sulphated than heparan sulphate.

➢ Chondroitin sulphate [CS] and dermatan sulphate [DS] also share a common polymer derivative: glucuronic acid and galactosamine. The distinction between CS and DS involves replacement of glucuronic acid with iduronic acid; if this modification occurs at high frequency, the chain is labelled dermatan rather than chondroitin sulphate.

➢ Keratan sulphate [KS] has alternating units of glucosamine and galactose and is unique amongst GAGs as it can be synthesized not only as an *O*-linked sugar attached to serine, but also as an *N*-linked sugar. Keratan sulphate I in the cornea is attached to the protein core via a *N*-acetylglucosamine-asparaginyl bond, while KS II in cartilage is *O*-linked (Funderburgh, 2000).

➢ Hyaluronic acid is unique among the GAGs in that it does not contain any sulphate and is not found covalently attached to proteins as a proteoglycan. It does however form complexes with proteoglycans in the ECM. Hyaluronic acid polymers have high molecular weights and can displace large volumes of water, making them excellent lubricators and shock absorbers.

1.5 Corneal Ultrastructure

As indicated previously and depicted in figure 1.6 below, the cornea has a complex ultrastructure with five layers comprising [anterior to posterior]: a differentiated epithelium, Bowman's layer, stroma, Descemet's membrane and an endothelial monolayer.

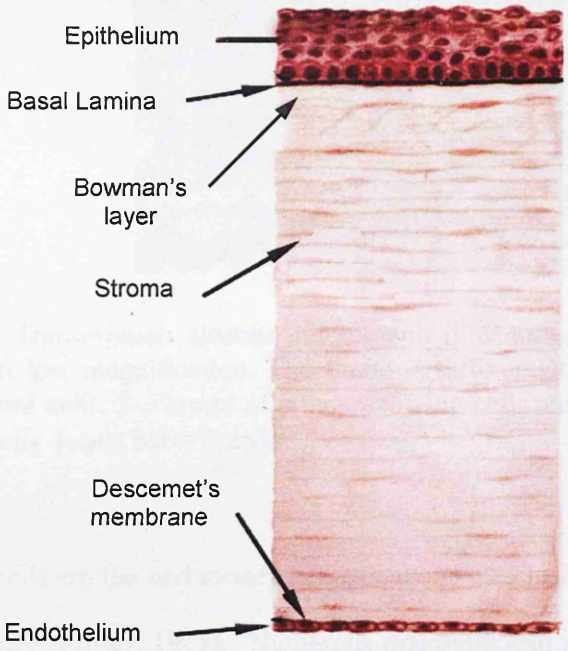


Figure 1.6: Diagrammatic representation of a cross section through the human cornea, showing the five component layers [www].

1.5.1 Corneal Epithelium

Corneal epithelium is a highly differentiated, stratified squamous epithelium which is devoid of goblet cells and therefore non-secretory. The epithelium, at 50-60μm thick, constitutes 10% of the total corneal thickness and is composed of 5-7 layers which include three groups of cells; flattened squamous superficial cells, wing or polygonal cells and a single row of basal columnar cells (figure 1.7 overleaf).

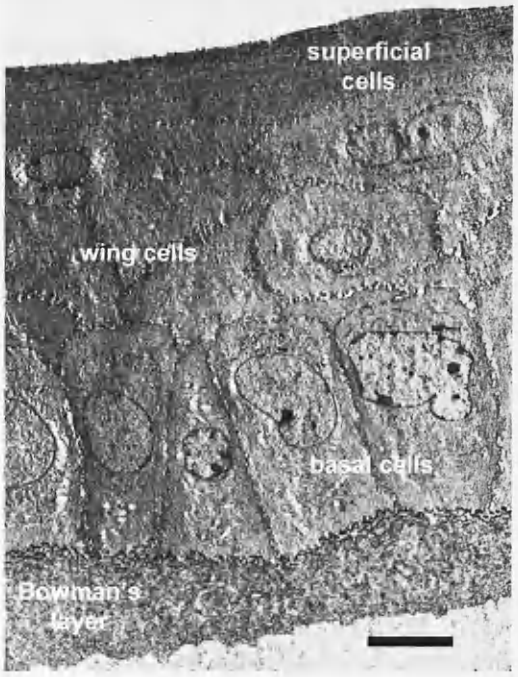


Figure 1.7: Transmission electron micrograph illustrating cadaver human corneal epithelium at low magnification. The tissue is differentiated into a single layer of columnar basal cells, 2-3 layers of polygonal wing cells and 2-3 layers of squamous superficial cells [scale bar = 5μm].

Superficial cells are flat and mostly hexagonal and attached to each other by straight cell boundaries (Pfister, 1973). Numerous microvilli and microplicae are found on their apical surfaces to enhance adherence of the tear film to the glycocalyx. Tight junctions are present around the entire lateral borders of each cell to function as an anatomic barrier to the passage of substances into the intercellular space (Huang *et al.*, 1989). The wing cell region is composed of approximately three layers of polygonal cells with dense cytoplasm and numerous desmosomal contacts. The deeply situated basal cells make up a single layer of columnar cells that rest on the basement membrane. These cells are rounded at their anterior surface with oval nuclei. They are mitotically active, with daughter cells migrating towards the surface to become wing cells (Thoft and Friend, 1983). Basal cells interdigitate laterally

with neighbouring cells and are joined by desmosomes. Hemidesmosomal junctions attach basal cells to the basement membrane where they secrete the basal lamina (Garrod, 1993; Ohji *et al.*, 1994).

1.5.1.1 Junctions of the Corneal Epithelium

The epithelium, as the outermost layer of the cornea, is subjected to the adverse conditions of the external environment. In order to prevent invasion by micro-organisms and to maintain barrier function, it must be able uphold its integrity and withstand the shearing forces of blinking. To this end, a number of junctional complexes are found in the corneal epithelium. Most important for mechanical strength are desmosomes, adherens junctions and hemidesmosomes (Garrod, 1993; Green and Jones, 1996). While desmosomes and adherens junctions mediate strong adhesion between cells, hemidesmosomes form the anchor between basal cells and the underlying basal lamina. The transmembrane domains of the desmosome and adherens junction belong to the cadherin family of calcium-dependent adhesion molecules (Braga, 2002; Garrod *et al.*, 2002), whereas those in the hemidesmosome include the integrin class of cell matrix receptors (Quaranta and Jones, 1991).

Desmosomes are configured in such a way that any stress on the cell is conveyed to its neighbours via intermediate keratin filaments, thus affording strength and rigidity to the entire epithelial sheet (figure 1.8 overleaf). Thickened cytoplasmic plaques in adjacent cell membranes are bound by interlocking transmembrane linker proteins desmoglein and desmocollin. Keratin intermediate filaments bind these plaques and form part of the internal structural framework of the cell, bestowing shape and rigidity.

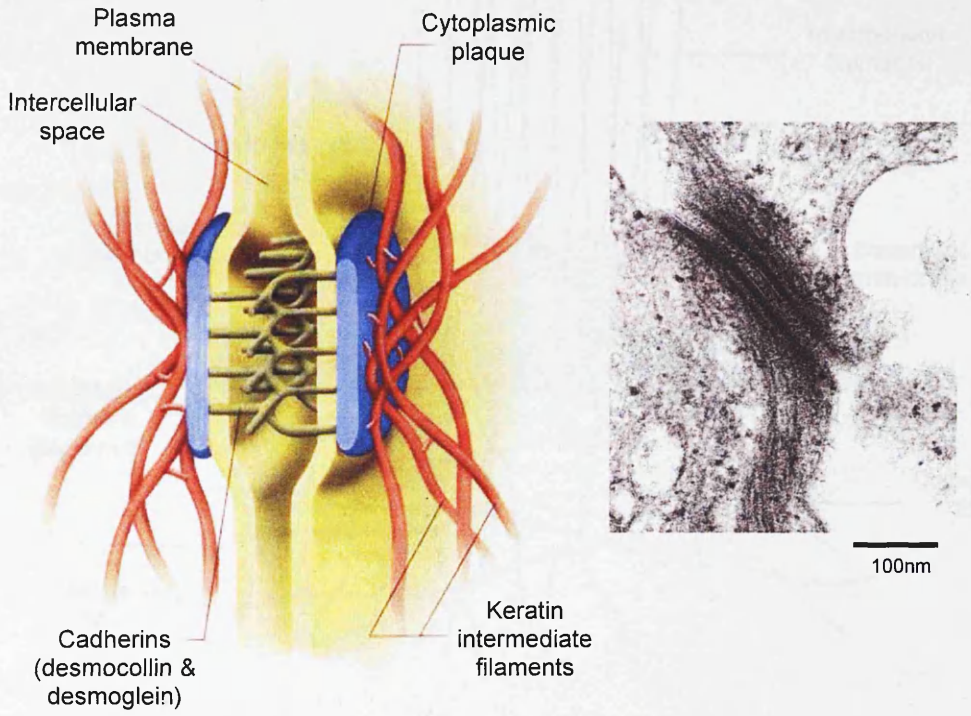


Figure 1.8: [Left] Schematic model showing components of a desmosomal junction (Gumbiner, 1993). Transmembrane linker proteins desmoglein and desmocollin (both cadherins) attach via cytoplasmic plaques to keratin intermediate filaments which traverse the cell interior. [Right] Transmission electron micrograph depicting a desmosome joining adjacent corneal epithelial cells at high magnification.

The schematic in figure 1.9 overleaf illustrates a hemidesmosomal junction (or half desmosome). Hemidesmosomes firmly secure basal epithelial cells to the extracellular matrix by way of interconnecting cytoskeletal keratin filaments with collagen VII fibres in the basal lamina (Gipson, 1992). Intermediate filaments are attached to proteinaceous plaques of the plasma membrane. Integrin $\alpha_6\beta_4$ (Garrod, 1993) binds the plaque to the basal lamina protein laminin. Since cells are anchored to the cell-cell and cell-substrate contacts through cytoplasmic intermediate filaments, these junctions are clearly identifiable by electron microscopy, which makes them ideal markers for visually gauging the mechanical strength of an epithelial sheet.

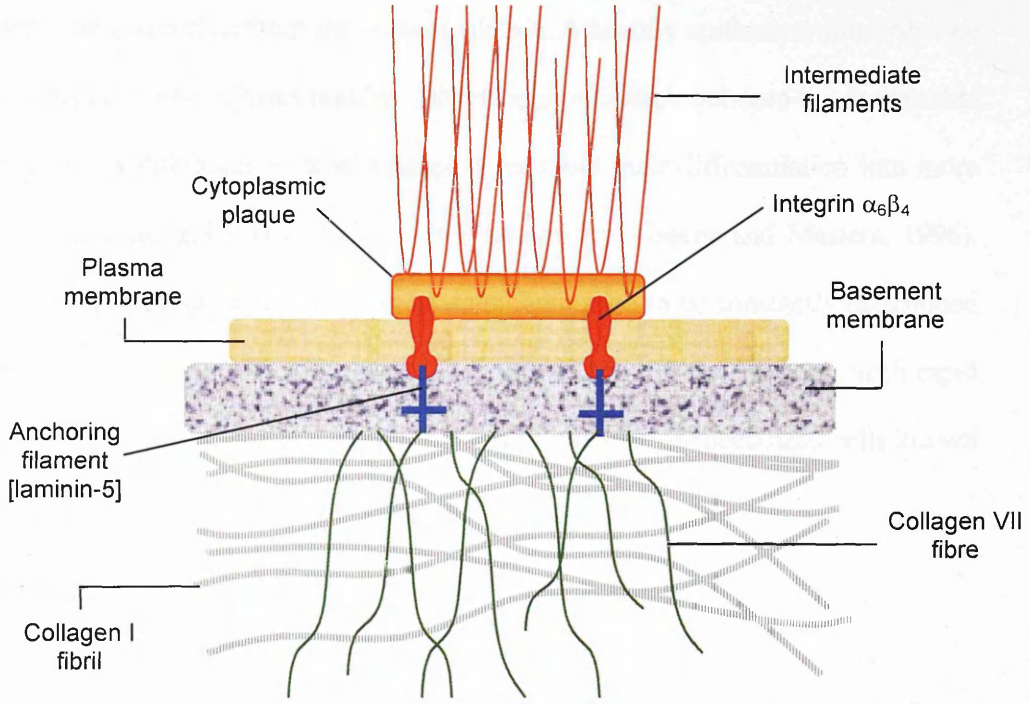


Figure 1.9: Schematic illustration of a hemidesmosome attaching an epithelial cell to the basement membrane. Integrins bind proteins in cellular cytoplasmic plaques as well as laminin-5 anchoring filaments in the basal lamina (adapted from Quaranta and Jones, 1991).

1.5.1.2 Role of Corneal Epithelium

The role of the corneal epithelium is basically fourfold: [1] provision of a functional barrier between the tear film and the intraocular environment by way of superficial tight junctions, [2] creation and maintenance of a smooth, transparent optical surface by adsorption of the tear film, [3] mediation of the diffusion of water, solutes and drugs at the anterior surface (Trinkaus-Randall, 2000) and [4] transmission and refraction of light.

1.5.1.3 Epithelial Turnover

Precise functioning of the cornea is essential for good vision, and depends on the continual production of new epithelial cells, their appropriate differentiation and

eventual desquamation from the corneal surface. A healthy epithelium must also be able to repair ocular injuries rapidly. Effective coordination between the factors that control the proliferation of basal epithelial cells and their differentiation into more superficial wing and surface cells is therefore critical (Beebe and Masters, 1996). The functional properties of the corneal epithelium need to be constantly maintained through differentiation as cells continuously stream towards the surface. Such rapid self-renewal of this tissue is achieved by a small number of specialized cells known as limbal stem cells (described in detail in section 1.7.1).

1.5.2 Bowman's Layer

Lying directly beneath the basement membrane of the epithelium is an acellular, transparent sheet of tissue known as Bowman's layer. It derives its name from the ophthalmologist, anatomist and physiologist Sir William Bowman (1816-1892). Bowman's layer is a basically a modified condensation of the anterior stroma, 8-12μm thick, terminating abruptly at the limbus (Hogan, 1971). It consists mostly of densely packed, randomly arranged collagen fibrils (20-30nm diameter, types I, III, V and VI) (Marshall *et al.*, 1991a). On histological sections, it appears amorphous and separate from the underlying collagen lamellae and keratocytes. Bowman's layer provides an anchor for the corneal epithelial cells. While the posterior border merges with the stroma, the anterior surface is well delineated and is separated from the epithelium by a thin, yet prominent basal lamina on which the basal cells rest directly. The basal lamina consists mainly of adhesive glycoproteins; laminin and fibronectin, and heparan sulphate proteoglycans such as perlecan (Fujikawa *et al.*, 1984; Schittny *et al.*, 1988; Murdoch *et al.*, 1992; Ljubimov *et al.*, 1995; Fukuda *et al.*, 1999). Corneal epithelial adhesion to Bowman's layer is achieved by way of a

basement membrane complex which anchors the epithelium via a complex mesh of collagen type VII anchoring fibrils (Gipson *et al.*, 1987) and plaques of type VI collagen, interacting with the lamina densa and the collagen fibrils of Bowman's layer (figure 1.10). Bowman's layer is incapable of regeneration and once injured, can form a scar as it heals. If large enough and centrally located, these scars can lead to some vision loss.

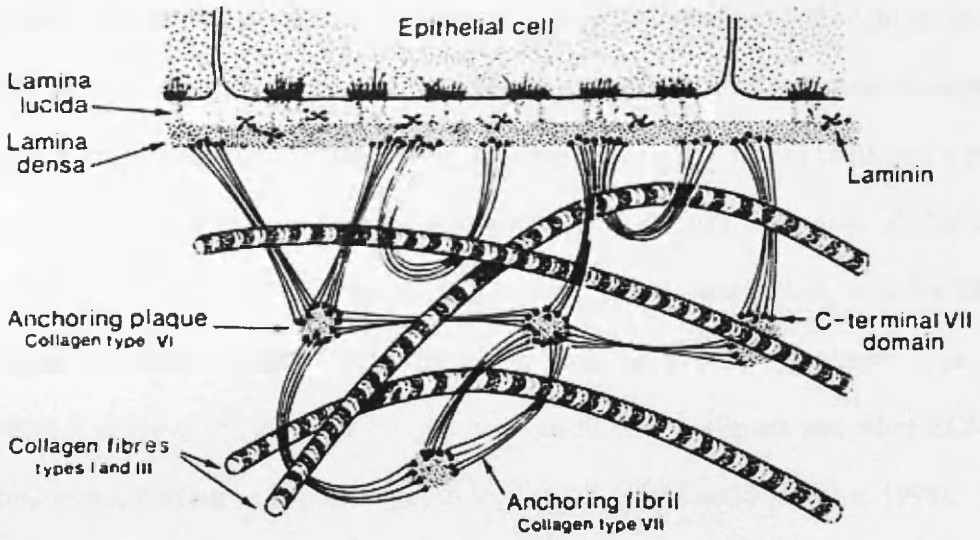


Figure 1.10: Schematic illustration of Bowman's layer, showing interactions between the various collagens and glycoproteins of this subepithelial membrane-like zone [www].

1.5.3 The Corneal Stroma

In humans, the corneal stroma is approximately 500μm thick and represents almost 90% of corneal thickness. It primarily consists of collagen embedded in a hydrated matrix rich in proteoglycans, interspersed with specialized fibroblasts called keratocytes. The organisation of collagen fibrils as well as the level of hydration of the stroma is important in ensuring corneal transparency.

1.5.3.1 Stromal Collagens

Stromal collagen has a hierarchical organization (Komai and Ushiki, 1991; Freund *et al.*, 1995; Fukuda *et al.*, 1999; Bron, 2001; Meek and Boote, 2004). Triple helical α-collagen molecules approximately 300nm long and 1.5nm in diameter are held together by interpeptide hydrogen bonds to form fibres. Several thousand of these fibres align parallel to each other thus forming a collagen fibril, 25-31nm in diameter. In humans, stromal fibrils are hybrids of collagen type I, III and V, each differing slightly in amino acid sequence. Inclusion of type V within a fibril is thought to limit fibril diameter due to steric hindrance (Birk *et al.*, 1990). It is the structural collagen type I which constitutes the majority of the corneal stroma however collagens type VI and XII have also been detected (Marshall *et al.*, 1991b; Marchant *et al.*, 2002; Young *et al.*, 2002). These latter collagens are largely non-helical, non-fibrillar telopeptides termed FACIT collagens which bind the termini of collagen type I helices and serve as molecular bridges between fibrillar collagens and other ECM components, playing an important physiological role (Olsen and Ninomiya, 1998).

The majority of stromal collagen is nevertheless fibrillar and assembled into broad flat sheets called lamellae, approximately 200-250 of which comprise the human corneal stroma (Maurice, 1957). The anterior and posterior stromas differ in a number of ways. Generally the posterior stroma is more ordered (Freund *et al.*, 1995), more hydrated, more easily swollen (Muller *et al.*, 2001) and has a lower refractive index (Meek *et al.*, 2003a) than the anterior third of the stroma. The posterior lamellae are also wider and thicker (100-200μm wide and 1.0-2.5μm thick) than the anterior (0.5-30μm wide and 0.2-1.2μm thick) (Komai and Ushiki, 1991). In the anterior stroma, collagen lamellae run in random directions often oblique to the

corneal surface and occasionally split, branch and interweave in an irregular fashion while those in the posterior stroma tend to run parallel to the surface. Each collagen lamella is composed of thin, parallel collagen fibrils organized in a pseudo-hexagonal oriented array (figure 1.11). X-ray diffraction studies have calculated the average centre-centre collagen fibril spacing in the hydrated human cornea to be approximately 62nm (Leonard and Meek, 1997; Quantock, 2000). Collagen fibrils within a single lamella lie parallel to each other in the plane of the cornea, but are rotated with respect to fibrils in adjacent lamellae.

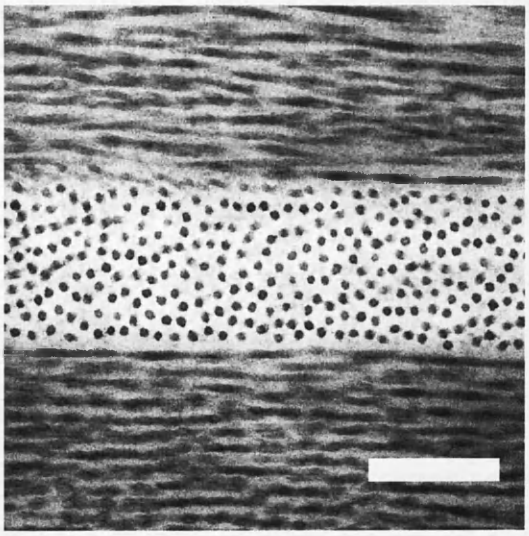


Figure 1.11: Transmission electron micrograph showing the arrangement of collagen in the corneal stroma. Central grey circles (approximately 25nm in diameter) indicate fibrils of one lamella in cross section, sandwiched between fibrils of adjacent lamellae, sectioned along their length [scale bar = 200nm] (reproduced with permission from Fullwood, 2004).

1.5.3.2 Stromal Proteoglycans

Collagen in the corneal stroma is embedded within a matrix rich in proteoglycans consisting of long glycosaminoglycan chains. Glycosaminoglycans have vital roles in the hydration of the cornea and therefore affect the spacing of the collagen fibrils. It has been observed that the corneal clouding associated with Scheie's syndrome

(skeletal dysplasia) is linked to an accumulation of GAGs and the corresponding swelling of fibrils and disruption to the collagen stroma (Quantock *et al.*, 1993). The human corneal stroma ideally consists of 78% water, while collagen and proteoglycans account for the remaining 12-15% and 1-3% of the net tissue weight respectively. Approximately 60% of all proteoglycans in the stroma comprise KS (i.e. lumican, keratocan and mimecan) and the remainder, DS (i.e. decorin). Studies on keratocan-deficient mice have shown a correlation between the observed increase in collagen fibril diameter and decrease in KS, suggesting a role for KS proteoglycans in the regulation of collagen fibril diameter (Meek *et al.*, 2003b). Lumican is the most abundant KSPG in the cornea and has an important role in corneal transparency. Murine models carrying a knockout for the lumican gene manifest highly disorganised stromas and corneal opacity (Chakravarti *et al.*, 1998; Chakravarti *et al.*, 2000; Quantock *et al.*, 2001). In addition, macular corneal dystrophy is characterized by a loss of transparency, thought to be related to the abnormal sulphation of KS (Hassell *et al.*, 1980; Meek *et al.*, 1989). Decorin is thought to be involved in tissue assembly in the cornea and to participate in the control of interfibrillar spacing and the lamellar adhesion properties of stromal collagens (Hahn and Birk, 1992; Danielson *et al.*, 1997).

1.5.3.3 Corneal Transparency

A number of features help to ensure the cornea's light-conducting transparency including avascularity, regularity of the epithelium and crucially the degree of spatial order of collagen fibrils within the stromal matrix, as regulated by proteoglycans. The healthy corneal stroma contains collagen fibrils of uniform diameter with consistent interfibrillar spacing. Maurice laid the foundations for many of the current

x-ray diffraction studies on cornea. He ventured that the regular spacing of collagen fibrils prevented light scattering from the primary visual axis and that light dispersal at all other angles is cancelled out by scattering in the opposite direction, thus enabling transmission of light through the cornea (Maurice, 1957). More recently, advances in x-ray diffraction have furthered understanding of fibril organization and its implications with regard to corneal transparency (Meek *et al.*, 2003b) and it is widely acknowledged that significant disruption to collagen fibril arrangement renders the corneal stroma opaque. In addition to the regulatory role of PGs, it has been proposed that stroma keratocytes may preferentially express water-soluble proteins, often enzymes, for controlling their optical properties (Jester *et al.*, 1999). Corneal endothelial cells (Fayet *et al.*, 2001) contain active transport pumps which maintain the 78% state of stromal hydration optimal for vision. The role of the endothelium in maintaining transparency is detailed in section 1.5.5.

1.5.4 Descemet's Membrane

Descemet's membrane (so-called after the French physician Jean Descemet, 1732-1810) bounds the inner surface of the stroma, from which it is easily separated. It is secreted by the corneal endothelial cells as a resilient barrier for protection against infection and trauma. Approximately 5-10μm thick in adult humans, this specialized basement membrane appears structureless under the light microscope however examination under the electron microscope reveals the extent of its organization. Three processes are required for the formation of adult Descemet's membrane (Murphy *et al.*, 1984). The initial prenatal step is the synthesis of a very thin basement membrane which is thickened by the deposition of a series of collagen units, superimposed to form a lamellar structure (at least 30 layers by the end of

gestation). Differentiation of the membrane also occurs in the prenatal period and leads to the formation of a striated structure through the addition of cross-linking bridges perpendicular to the lamellae. The final thickening process occurs after birth when non-striated, non-lamellar, homogenous material is deposited posterior to the striated prenatal layer. Descemet's membrane is rich in glycoproteins (fibronectin and laminin), entactin/nidogen, proteoglycans (Katz *et al.*, 1994) and type IV collagen (Marshall *et al.*, 1991a; Leung *et al.*, 2000).

1.5.5 The Corneal Endothelium

The endothelium is the single layer of hexagonal cells covering the posterior surface of the cornea and facing the anterior chamber. Endothelial cell nuclei are characteristically large and oval. Cell boundaries appear to be principally hexagonal in outline, forming a mosaic as depicted in figure 1.12 below.

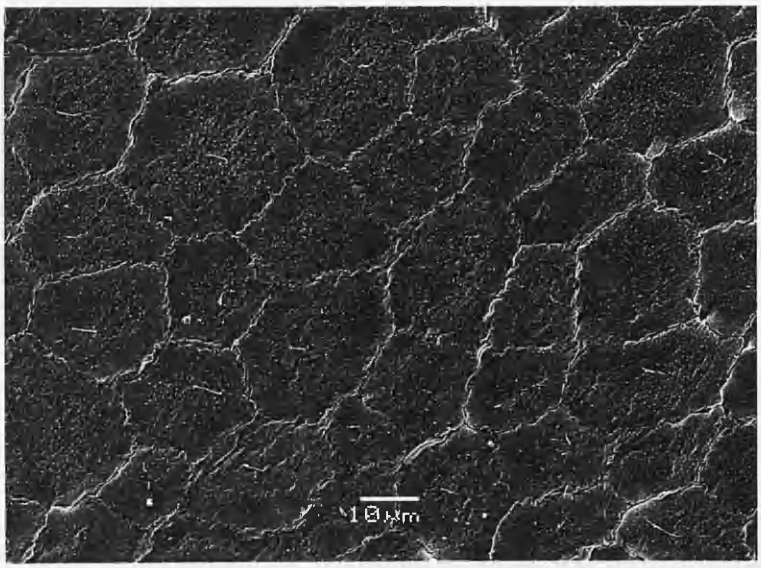


Figure 1.12: Scanning electron micrograph of cadaver human corneal endothelium, routinely fixed and processed, illustrating the mosaic pattern of the hexagonal cells with interdigitated membranes [scale bar = 10μm].

The basal surface of the endothelial cell is flat against Descemet's membrane yet no special adhesion junctions operate in this region. There is however extensive interdigitation of the walls of adjacent cells which exhibit desmosomes and occasional tight junctions. Corneal endothelial cells have large numbers of mitochondria, indicative of their crucial role in active fluid transport (Waring *et al.*, 1982). The intact endothelium acts as a semi-permeable barrier between the stroma and the aqueous humour, and this integrity is a prerequisite for corneal transparency (Joyce, 2003). In order to regulate corneal hydration and prevent swelling, the corneal endothelial cells are equipped with sodium/potassium-ATPase and bicarbonate-dependent magnesium-ATPase ionic pumps (Maurice, 1957; Hodson and Miller, 1976). Intrinsically leaky, the endothelium is also the principle source of glucose and other nutrients for the cornea. In allowing paracellular percolation of aqueous humour into the cornea while preventing bulk flow and actively pumping out any excess liquid, the endothelium is essential for maintaining corneal transparency.

Crucially, endothelial cells show very little mitotic activity after birth and the 400,000 or so cells present at birth literally have to last a lifetime (Joyce *et al.*, 1996). Endothelial cells are inevitably lost with age (Yee *et al.*, 1985; Bourne *et al.*, 1997), so in order to prevent stromal oedema, adjacent cells spread to cover the denuded areas. Consequently, the thickness of the endothelium and its corresponding cell density decrease dramatically with age; from around 4,000 cells/mm^2 at birth, to approximately 2,500 cells/mm^2 in the adult (Svedbergh and Bill, 1972). Despite the endothelial layer's capacity for vast enlargement, corneal oedema and loss of vision occur if cell densities drop below the region of 300-600/mm^2 (Green, 1991). For this

reason, processes of cell adhesion and migration are vital in maintaining a healthy endothelium and hence, also a healthy cornea.

1.5.5.1 Corneal Endothelial Disorders

Approximately half of all corneal transplantations carried out are due to damaged endothelium. As well as damage caused by age and trauma, endothelial disease is a major causative factor. The most common corneal endothelial diseases are Fuchs' dystrophy (Adamis *et al.*, 1993) and Iridocorneal Endothelial [ICE] Syndrome (Morris and Dunbar, 2004) though the former is better understood. Late hereditary Fuchs' dystrophy is usually seen in the fifth or sixth decade of life, and affects more women than men. Typically of dominant inheritance and bilateral, the dystrophy stems from primary malfunction of the corneal endothelium and ultimately causes disruption of the corneal dehydration system and optically compromised tissue. Fuchs' dystrophy occurs as endothelial cells gradually deteriorate; first transforming into fibroblast-type cells and finally apoptosing (Borderie *et al.*, 2000), thereby no longer able to regulate stromal hydration (Mandell *et al.*, 1989). Eventually the epithelium also swells leading to bullous keratopathy in which fluid-filled blebs (or bullae) form on the corneal surface, hampering vision. Ruptures cause a foreign-body sensation and allow bacterial invasion causing corneal ulcers, resulting in pain and severe visual impairment. As a chronic condition, most treatment is purely palliative and involves simple measures to remove moisture from the eye. Corneal grafts for Fuchs' dystrophy account for approximately 10 percent of all corneal grafts performed and generally, if surgical intervention occurs prior to involvement of the peripheral cornea, there is a high likelihood that the graft will remain clear for two years.

1.6 Stem Cells

"When Prometheus transgressed the law of the ancient Gods and stole fire for humankind to teach them civilisation and the arts, his punishment was typically brutal. Jupiter had the great Titan chained to the side of Mount Caucasus, where a vulture preyed daily on his liver, which was renewed as quickly as it was devoured"

(Rosenthal, 2003)

This legend captures well the body's remarkable capacity for repairing wounds; restoring tissues to their original form and function by recruiting proliferating cells with a retained collective memory of the complex developmental processes by which the tissue was initially compiled. Many tissues undergo rapid and continuous cell turnover and thankfully for Prometheus, the liver is one of the most highly regenerative organs. Cells ultimately responsible for repopulation are termed *stem cells* and can be defined as any cell with a high capacity for self-renewal extending throughout adult life.

1.6.1 Stem Cell Types and Characteristics

There are distinctions to be made when describing stem cells. *Totipotent* cells contain all the genetic information required to create all body cells plus the placenta. They only have this capacity during the first 3-4 divisions of a fertilized egg, after which time the cells become increasingly specialized. The next stage of division results in *pluripotent* cells, which are highly versatile and can give rise to any cell type except those of the placenta or supporting tissues for foetal development. Embryonic stem (Mejia *et al.*, 2000) cells fall into this category, originating from undetermined early

embryos (specifically the inner cell mass of the blastocyst) with no history of differentiation. Adult stem cells are less well understood but are believed to represent a population of reserve stem cells typically less versatile and hence more restricted in their developmental potential, conceivably set aside during gestation for coercion into regenerative services in later life (Verfaillie, 2002). They can be either *multipotent* as in the case of haematopoetic stem cells which produce several blood cell types or *unipotent* as in the cornea. Stem cells can theoretically divide without limit, so long as the host is still alive. These extraordinary cells are both clonogenic (capable of unlimited self renewal) and able to undergo asymmetric cell division, a process that allows one daughter cell to retain stem cell characteristics, while the other daughter cell becomes slightly more differentiated (Zieske, 1994). The latter cells have been termed transient amplifying cells, have a limited proliferative potential and are considered the initial step of a pathway resulting in terminal differentiation (Schermer *et al.*, 1986). While stem cells are by definition unspecialized, under certain physiologic or experimental conditions, they can be induced to perform specialized functions. It is these unique characteristics by which stem cells can be distinguished from other body cells and which makes them the focus of intense scientific study.

1.6.2 Therapeutic Potential

Despite the difficulties of location, isolation and propagation, through the replacement of damaged cells, stem cells have tremendous therapeutic potential. While studies on embryonic stem cells have caused great optimism in the scientific community, ethical concerns, uncertainties about immunological compatibility and the propensity to form teratomas following transplantation are significant problems

to be overcome before this approach can be used clinically. With fewer risks involved and cause for ethical concern, the search for stem cells in the adult has recently been intensified (Orkin and Morrison, 2002). Adult stem cells, such as those of the cornea, which have undergone some degree of differentiation are perhaps a more feasible source for tissue engineering (Heath, 2000) yet with no predictable location for stem cells in most adult tissues and limited reliable methods of identification, they are notoriously difficult to isolate.

The next sections describe the limbus (thought to be the most likely location for corneal stem cells) and limbal stem cells specifically. There follows a description of some of the more common limbal stem cell deficiencies and an account of the progress to date in treating these disorders, the continuation of which this thesis is primarily concerned.

1.7 The Limbus

The transitional zone of the cornea, bordering the sclera/conjunctiva shows differences in structure microscopically from the rest of the tissue (Maurice, 1962). The limbus has an increased number of cell layers and most notably in humans, Bowman's layer terminates abruptly (figure 1.13). The basement membrane zone of the limbal epithelium differs from that of the cornea, being rich in collagen IV (Kolega *et al.*, 1989) with a rough, undulating surface consisting of stromal pegs and anchoring fibrils to promote the adhesion of limbal basal cells (Gipson, 1989). Descemet's membrane splits and forms part of the trabecular meshwork, together with the endothelium. Collagen fibrils are continuous between the corneal stroma and the sclera but thicken at the limbus. In addition, a rich vascular plexus surrounds the cornea and marginal capillary loops enter a short way into the stroma, allowing for increased levels of epithelial nutrition and cytokine interactions.

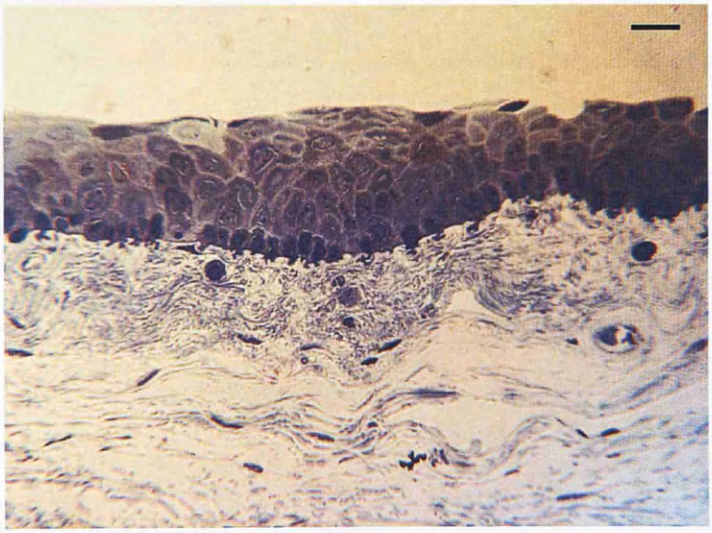


Figure 1.13: Light micrograph of the apical region of the limbal zone (human cadaver corneal tissue; routinely fixed, processed and stained); note the thickened epithelium, the absence of Bowman's layer, and the stromal blood cells. Limbal basal cells (possibly stem cells) are more densely stained and attached firmly to the basement membrane by stromal pegs [scale bar = 10μm].

1.7.1 A Stem Cell Niche

The limbus is thought to serve as a stem cell niche, an optimal microenvironment able to support the undifferentiated stem cell state (Boulton and Albon, 2004). The stem cells which perpetuate the cornea are believed to reside in the basal cell layer of the limbal epithelium (figure 1.16). In particular, the presence of stromal pegs along with the interaction of anchoring fibrils, in promoting adhesion of basal cells, protects them from injury and limits movement away from the microenvironment, thereby protecting the stem cells from entering the pathway of terminal differentiation (Zieske, 1994).

1.7.2 Limbal Stem Cells

Visual acuity is dependent on the corneal epithelium, the integrity of which is maintained by the centripetal migration of stem-cell-derived transient amplifying cells (Cotsarelis *et al.*, 1989; Wolosin *et al.*, 2000) (figure 1.14). Davanger and Evensen (1971) first hypothesized that the epithelial cells of the limbus may be involved in the renewal of the cornea, having observed migration of pigmented cells from the limbus towards the central cornea.

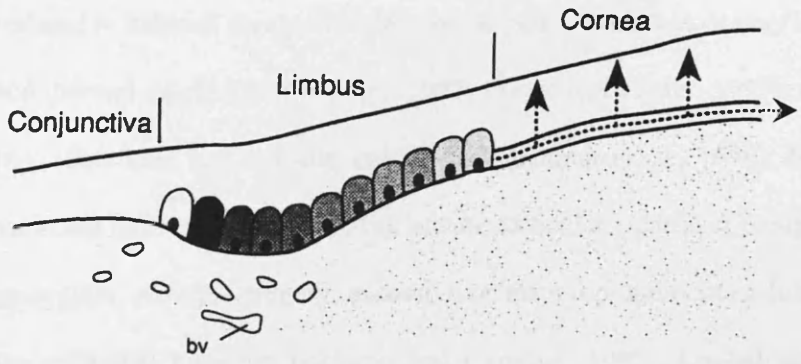


Figure 1.14: Diagrammatic representation of the limbal zone. Blood vessels [bv] are located subjacent to the limbal basal cells. Stem cells are shown in black, with shading marking a corresponding increase in cell differentiation and decrease in stem cell-like characteristics. Transiently amplifying cells move centripetally and apically to populate the central cornea (adapted from Zieske, 1994)

Schermer *et al* presented further support for the limbal location of corneal stem cells in 1986 with their keratin expression data (Schermer *et al.*, 1986). Keratin 3 (K3) is expressed in the basal cells of the corneal epithelium yet is lacking in those of the limbus, suggesting a lesser degree of differentiation. Other evidence in support for the localization of stem cells in the limbal basal layer includes: [1] retention of tritiated thymidine labels for long periods indicating a long cell cycle (Cotsarelis *et al.*, 1989); [2] limbal basal cells have a higher proliferative potential in culture than cells of the corneal epithelium (Ebato *et al.*, 1987; Lindberg *et al.*, 1993); [3] surgical removal of the limbus results in invasion of cells from the conjunctiva (Chen and Tseng, 1991); [4] grafting of cells from the limbus regenerates corneal-like epithelium (Tsai and Tseng, 1994; Dua and Azuara-Blanco, 1999a; Henderson *et al.*, 2001); and [5] limbal basal cells divide in response to wounds of the central cornea, as would be expected of stem cells (Cotsarelis *et al.*, 1989).

1.7.3 Limbal Stem Cell Deficiencies

The limbal stem cell theory formed the basis for identifying and reclassifying a multitude of corneal blinding diseases. Limbal stem cell deficiency (LSCD) is most frequently related to external factors that destroy the cells. Such factors may include: chemical and thermal injury (Bourne *et al.*, 1997; Pfister and Pfister, 1997b; Sridhar *et al.*, 2000), ultraviolet and ionising radiation (Fujishima *et al.*, 1996), Stevens-Johnson syndrome (Koizumi *et al.*, 2001a), advanced ocular cicatricial pemphigoid, recurrent pterygium, multiple surgery, excessive or inappropriate contact lens wear, or extensive microbial infection (Achong and Caroline, 1999). Limbal stem cell deficiencies are characterized by conjunctival epithelial invasion, neovascularization, chronic inflammation and stromal inflammation (keratitis and scarring) (figure 1.15).

Consequently, patients experience severe irritation, photophobia, and decreased vision, making them poor candidates for conventional corneal transplantation (Lavker *et al.*, 2004).

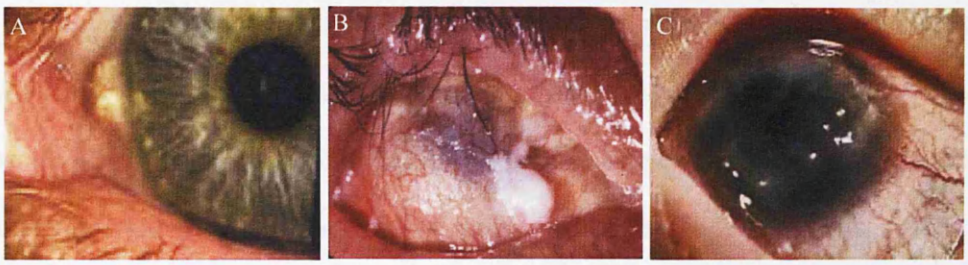


Figure 1.15: Common disorders affecting the ocular surface and leading to limbal stem cell deficiency: (A) pterygium, (B) advanced cicatricial pemphigoid and (C) Stevens-Johnson syndrome [www].

1.7.3.1 Pterygium

A pterygium is a pink, triangular-shaped conjunctival growth on the cornea (figure 1.15A). Some expand slowly throughout life, while others peak after a certain point, rarely reaching a size so large that the pupil is covered. It is thought that UV light from the sun may be a risk factor since pterygia are more common in sunny climates (Roh and Weiter, 1994). Pterygia are surgically removed when vision is affected, but rarely for cosmetic reasons since once excised they may grow back, particularly if the patient is less than 40 years of age. Lubricants are often prescribed to reduce redness and provide relief from the chronic irritation.

1.7.3.2 Ocular Cicatricial Pemphigoid (OCP)

Ocular cicatricial pemphigoid is a rare systemic autoimmune inflammatory disease which primarily involves the oral and ocular mucous membranes (Foster and Rashid, 2003). Characterized by the development of blisters, the initial presentation is often

that of reddened, painful, tearing and light-sensitive eyes in a patient 60 to 70 years of age. These inflammatory lesions of the ocular surfaces can result in scarring, loss of tear film, adhesions of the lids to the eye, corneal ulceration, perforation (figure 1.15B) and in the most relentlessly progressive or untreated cases, loss of the eye. The mouth and skin often suffer similar blistering lesions. General treatments involve corticosteroids and immunosuppressive agents. The ocular surface requires additional lubrication, antibiotics and sometimes surgical procedures, in attempt to maintain vision. Nevertheless, many patients will experience severe visual loss due to ocular surface scarring, despite the most aggressive management.

1.7.3.3 Stevens-Johnson Syndrome (SJS)

Stevens-Johnson syndrome (SJS) is also known as erythaema multiforme major and is characterized by painful, blistery lesions on the skin and the mucous membranes of the mouth, throat, genital region and eyelids. Stevens-Johnson syndrome can cause serious eye problems; the ocular complications often being disabling and leading to severe vision loss. This sight-threatening disease can occur at any age and is considered very difficult to treat, especially in children (Tsubota and Shimazaki, 1999). The aetiology is unknown, but an abnormal immunologic reaction is thought to be the cause. Often precipitated by drugs or viral infection, any mucous membrane may be affected by the disease. When the acute phase is controlled, patients usually recover good function of most organs, but nevertheless permanent bilateral blindness is common due to severe keratoconjunctivitis and a lack of corneal stem cells. With corneal stem cells limited to the limbus and representing only a small portion of the mucosal epithelium, severe immunologic reactions can deplete the supply and leave the cornea covered with conjunctival cells or even eyelid skin (as in figure 1.15C).

1.7.4 Treatment of Limbal Stem Cell Deficiencies

Persistent epithelial defects can be caused by various ocular disorders and trauma, as outlined previously. Conventional corneal transplantation cannot restore vision in the case of LSCDs, as the requirement is for limbal stem cells to restore the corneal epithelium. Traditional therapies involve correcting the underlying cause, suppressing inflammation and augmenting the tissue-repair processes (He *et al.*, 1999) but despite appropriate use of these measures, established epithelial defects may persist (Sridhar *et al.*, 2000). Improvements in surgical procedures over recent years have enabled failed ocular surfaces to be replaced with exogenous healthy epithelium. According to the type and source of tissue removed to transplant the stem cell-containing limbal epithelium, several surgical procedures have been devised (Lavker *et al.*, 2004). Prior to the realization that conjunctival transdifferentiation did not occur (Dua, 1998), conjunctival transplantation was performed to treat limbal stem cell deficiency related ocular surface disease (Thoft, 1977). Thoft later performed keratoepithelioplasty, whereby lenticles of peripheral cornea were placed at the corneoscleral limbus and successfully recovered the cornea (Thoft, 1984). Kenyon and Tseng (1989) reported the first conjunctival-limbal autograft. With the advent of the limbal stem cell concept, Kinoshita modified this technique to transplant limbal stem cells (Kinoshita *et al.*, 1991). In unilateral disorders, the stem cell source is the healthy contra-lateral eye (Dua and Azuara-Blanco, 2000a) while in bilateral disorders, stem cell allografts are obtained from either living tissue-matched donors or non-matched cadaver eyes (Tsai and Tseng, 1994). As allogeneic transplants, it is mandatory to administer systemic immunosuppression in these instances and long term success rates vary tremendously. Tsubota *et al.* (1999) and Solomon *et al.* (2002) reported a 40-50%

success rate based on a 3-5 year follow up, while Ilari and Daya (2002) document that only 21.2% of eyes remained clear and stable after 5 years. This limited success has been attributed to aqueous tear-deficient dry eye (Shimazaki *et al.*, 2000), lid abnormalities (Solomon *et al.*, 2002) and chronic, persistent inflammation. Signs of graft rejection include corneal vascularization, epithelial rejection lines, invasion of conjunctiva and inflammation. The practice of limbal allograft transplantation is not only limited by these risks of complications (Thoft and Sugar, 1993) but also by donor tissue availability. Given the grave shortage of cadaver corneas available for transplant, alternatives are being sought. The use of biomaterials and tissue engineering in the treatment of limbal stem cell deficiencies is a rapidly expanding area of research which is reviewed in the following section.

1.8 Biomaterials and Tissue Engineering

A biomaterial is by definition 'any material that is designed to contact living tissue for a therapeutic or medical purpose' (Piskin, 1992). In general terms, the field of biomaterials covers both biological tissue transplantation and the implantation of non-biological, artificial devices. Similarly, with regards the eye, ocular biomaterials can be biological, such as amniotic membrane or cultured cells, or artificial. Artificial ocular biomaterials include such diverse devices as contact lenses, punctal plugs and intraocular lenses. With such an array of eye diseases and the shortage of readily available tissue for transplantation, the need for biomaterials is obvious. The field of tissue engineering emerged in response to the growing need for tissues and organs for transplantation. Success hinges on regeneration-competent cells, a supportive carrier and an environment conducive to cell growth, differentiation and eventual integration. In many respects, amniotic membrane is an ideal carrier.

1.8.1 Amniotic Membrane

Mammalian embryos are contained in a fluid-filled sac of foetal membranes, arising from extra-embryonic tissues (figure 1.16). The outer layer (or chorion) contacts maternal cells while the inner layer (or amnion/amniotic membrane) is bathed by amniotic fluid. This innermost layer of the placenta is a thin, semitransparent tissue comprising a single layer of ectodermally derived columnar cells, a thick and continuous basement membrane and a subjacent avascular stromal matrix containing large amounts of collagen (van Herendael *et al.*, 1978; Fukuda *et al.*, 1999). The amniotic epithelium serves to protect the foetus from traumas during development as well as having a secretory role (van Herendael *et al.*, 1978).

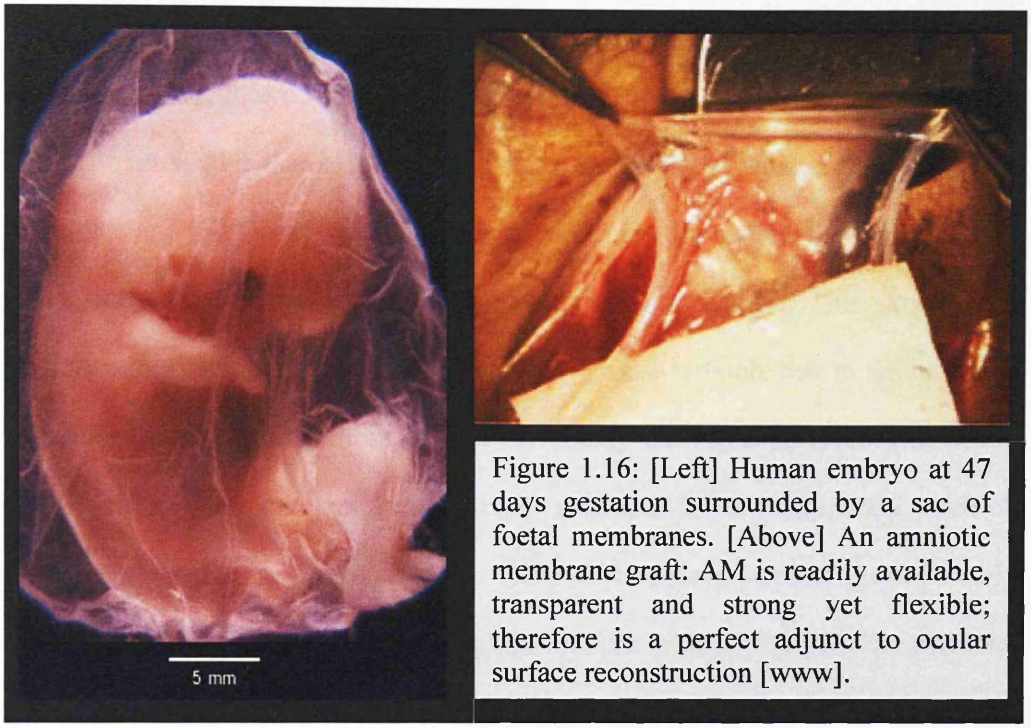


Figure 1.16: [Left] Human embryo at 47 days gestation surrounded by a sac of foetal membranes. [Above] An amniotic membrane graft: AM is readily available, transparent and strong yet flexible; therefore is a perfect adjunct to ocular surface reconstruction [www].

1.8.2 Amniotic Membrane: A History of Use

The first documented use of amniotic membrane in medical science was in 1910 when Davis used foetal membranes as surgical material to treat burned and ulcerated skin surfaces (Davis, 1910). Amniotic membrane has long been used in skin transplantation, as a biological dressing for burns (Stern, 1913; Bose, 1979), skin wounds and leg ulcers (Troensagaard-Hansen, 1950; Trelford and Trelford-Sauder, 1979; Ward *et al.*, 1989). The ensuing decades have seen great scientific interest in amniotic membrane and it has been used in a wide variety of procedures, including as an adjunctive tissue for artificial vagina reconstruction (Dhall, 1984). In addition, AM has also proved useful in preventing tissue adhesion in surgery of the abdomen, pelvis, head, vagina and otolarynx (Trelford-Sauder *et al.*, 1978). However, it is the use of AM in ocular surface reconstruction which is possibly the most active area of research in this field and with which this thesis is primarily concerned.

1.8.3 Amniotic Membrane in Ocular Surface Reconstruction

Foetal membranes were first used on the eye in 1940 by de Rotth as a conjunctival graft to treat symblepharon (de Rotth, 1940). De Rotth recognized the qualities of this tissue and felt that it would be ideal for replacing the conjunctiva in that it was thin, strong, flexible, transparent, sterile and readily available. This surgery had limited success (only one out of the six cases), almost certainly due to the inclusion of live cells and the chorion. In 1946, Sorsby and Symons used chemically processed 'dry' amniotic membrane termed 'amnioplastin' as a temporary patch on burnt eyes (Sorsby and Symons, 1946). Despite remarkable success given an early intervention, amniotic membrane for ophthalmic use disappeared from the literature until 1995 when it was reintroduced by Kim and Tseng (Kim and Tseng, 1995b). They utilized a preserved form of AM to reconstruct damaged ocular surfaces in a rabbit model and noted positive outcomes in 40% of cases. These encouraging results, coupled with improved methods of AM processing, have prompted a recent surge of interest in this surgical application.

Amniotic membrane has proved useful for surgical reconstruction of the ocular surface and has been successfully used in the treatment of ocular surface disorders including LSCDs such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, thermal and chemical burns, and severe pterygium (Shimazaki *et al.*, 1997; Tseng *et al.*, 1998; Tsubota and Shimazaki, 1999). This non-antigenic basement membrane facilitates migration of epithelial cells, reinforces adhesion of basal cells, and promotes epithelial differentiation. Collectively, these are the thought to be the characteristic actions by which amniotic membrane permits rapid epithelialization (Lee and Tseng, 1997).

1.8.3.1 Action Mechanisms of Amniotic Membrane

When appropriately processed and preserved, AM can be used as either a graft to replace the damaged stromal matrix, as a patch to prevent inflammatory insults from further damaging the ocular surface, or a combination of both. Human amniotic membrane transplantation is used for a widening spectrum of ophthalmic indications and has been found to confer many beneficial effects; these and potential action mechanisms include those summarized in table 1.1 below.

Action Mechanisms
Prolonged life span and maintenance of epithelial progenitor cell clonogenicity
Promotion of non-goblet cell epithelial differentiation
Exclusion of inflammatory cells with anti-protease activities
Suppression of the transforming growth factor β signalling system and myofibroblast differentiation of normal fibroblasts
Observed Clinical Effects
Facilitation of epithelialization
Maintenance of normal epithelial phenotype
Reduced inflammation
Reduced vascularization
Reduced scarring

Table 1.1: Potential action mechanisms and reported clinical effects of amniotic membrane transplantation (adapted from Tseng, 2001)

The basement membrane component of the amniotic membrane resembles that of the conjunctiva, expressing collagen IV, V and VII, laminin and fibronectin (Fukuda *et al.*, 1999) and is therefore an ideal substrate for supporting the growth of epithelial progenitor cells (limbal stem cells and TACs), specifically in prolonging life span and maintaining their clonogenicity (Tseng *et al.*, 1998). Amniotic membrane has been found to promote epithelial differentiation, to reinforce cell adhesion and to prevent apoptosis (Boudreau *et al.*, 1996; Wang *et al.*, 2001). Moreover, amniotic

membrane has anti-angiogenic (Kim and Tseng, 1995a; Ma *et al.*, 2004) and anti-inflammatory properties (Chen *et al.*, 2000; Zhou *et al.*, 2003) and expresses numerous growth factors (Koizumi *et al.*, 2000c). Amniotic membrane has also been found to inhibit fibrosis (Shimazaki *et al.*, 1998) and display anti-bacterial properties (Talmi *et al.*, 1991). In rabbits, amniotic membrane transplantation has elicited a reduction in corneal haze and scarring (Wang *et al.*, 2001). Collectively these properties make AM an ideal carrier for the *ex vivo* expansion of limbal stem cells and their subsequent transplantation onto the ocular surface.

1.8.4 *Ex Vivo* Expansion of Limbal Stem Cells on AM

Tissue engineering is a multi-disciplinary field that combines the principles of engineering and biological sciences in the development of bioartificial tissues and organs (Heath, 2000). The concepts of tissue engineering and cell culture have developed rapidly over the past three decades, so that it is now possible to manipulate cells both *in vivo* and *in vitro* by controlling their proliferation and differentiation through the addition of exogenous growth factors and hormones (Vacanti and Vacanti, 2001). Pellegrini and co-workers (1997) were the first to successfully generate and engraft multi-layered cultured corneal epithelium to resurface damaged eyes. As described in the previous section, amniotic membrane appears to serve as an ideal substrate for restoring the limbal stem cell niche for *ex vivo* expansion (Grueterich *et al.*, 2003a) and has subsequently been used by many researchers, with or without 3T3 fibroblast feeder layers for autologous (Schwab *et al.*, 2000; Tsai *et al.*, 2000; Grueterich *et al.*, 2002b) or allogeneic (Koizumi *et al.*, 2001a; Koizumi *et al.*, 2001b) limbal stem cell transplantation for treating LSCD. This latter technique has proved very effective in reinstating a corneal epithelial

phenotype, with the further advantage that only a small limbal biopsy is required, thus minimizing the risk of depleting the donor eye. In addition, *ex vivo* expanded cells are considered preferential to limbal allografts since antigen-presenting Langerhans cells are eliminated from the transplant, reducing the risk of allograft rejection (Sano *et al.*, 2000).

Various methods are currently employed in generating new tissue for transplantation, however it is the 'cell-seeded matrix' or 'cell suspension' approach which, though the most complex, seems to be the most promising route to tissue regeneration and it is this method which is used in this thesis (schematic shown below in figure 1.17).

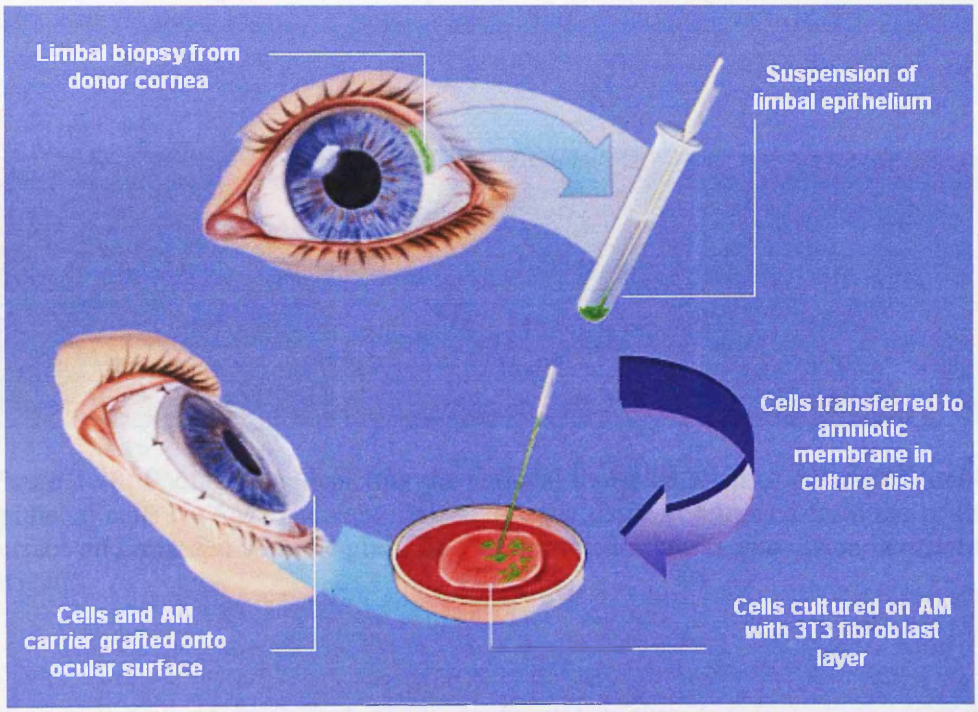


Figure 1.17: Schematic diagram of the suspension culture system. Epithelial cells are isolated from the limbus and then seeded onto denuded amniotic membrane and co-cultured with inactivated 3T3 fibroblasts (based on Koizumi *et al.*, 2002)

Colleagues in Shigeru Kinoshita's research team at the Kyoto Prefectural University of Medicine, Japan have pioneered a highly successful technique for the repair of damaged corneal epithelium based on the work of Tsai and co-workers (Tsai *et al.*, 2000) in which cells from a biopsy of the limbal region are disaggregated and seeded onto a matrix derived from human amniotic membrane (Koizumi *et al.*, 2002; Ban *et al.*, 2003). During a highly controlled culture regime involving lowering the growth medium to expose the uppermost cell layers to air, the limbal epithelial cells reorganize themselves into stratified sheets which closely resemble normal corneal epithelium. Within three to four weeks, this sheet (along with the amniotic membrane carrier) can then be sutured onto the damaged ocular surface where it should hopefully restore vision. The graft is covered with a therapeutic contact lens for protection (figure 1.18).

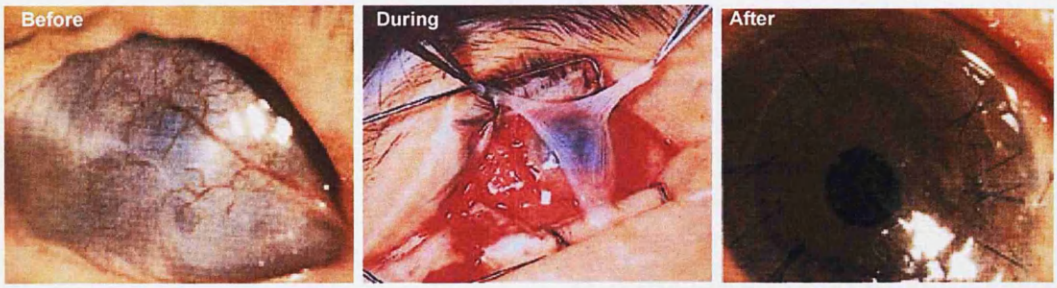


Figure 1.18: Photographs depicting the surgical transplantation of cultivated limbal epithelial cells on AM. The vascularized, opaque tissue is removed from the ocular surface and replaced with the graft. The cornea regains clarity and vision is restored [www].

Transplantation of biological tissues onto and within the eye is not always straightforward. Preferably, tissue damage ought to be surgically reconstructed using autologous tissue grafts from the host. In many cases however, such as bilateral limbal stem cell disorders, autografting is not an option and an alternative source of

tissue has to be established. This generally takes the form of a same species donor (usually of cadaver origin, but increasingly from living human donors) and is termed an allograft. As well as the severe shortage of tissue available, the major disadvantage to transplanting allogeneic tissue is the need for life-long immunosuppressive drug treatments to prevent graft rejection, postoperative inflammation and infection.

Many researchers have documented the clinical success of such surgery and in some cases as many as 80% of grafted eyes (Garrod *et al.*, 2002) were restored to good vision (Koizumi *et al.*, 2001a). As with any technique, this is far from perfect and there remains considerable scope for improvement. This thesis builds on the progress made to date and uses microscopic techniques to evaluate potential enhancements, in particular optimizing the carrier and culture technique, and investigating alternative sources of autologous stem cells for culture which would potentially remove the need for prolonged immunosuppression.

1.9 Central Aims

The *ex vivo* expansion of limbal stem cells on amniotic membrane for ocular surface reconstruction, while largely successful, is not infallible. Limitations with this technique include a shortage of donor materials, risks of allograft rejection and the need for prolonged immune suppression (which has its own complications). The central aim of this thesis is to use microscopic and immunohistochemical techniques to refine tissue engineering of the cornea and as such, covers several potential areas of improvement to include:

- An investigation into the comparative merits of cellular and denuded amniotic membrane as a carrier for cultivated human limbal stem cells.
- An evaluation of amniotic membrane as a supportive matrix for the culture of corneal endothelial cells for grafting into dystrophic eyes. Similarly, an investigation into the use of polyphenol antioxidant for preserving corneal endothelial cells, pre-transplant.
- The ultrastructural and immunohistochemical characterization of freeze-dried amniotic membrane and evaluation of its potential as an alternative substrate to conventionally cryopreserved tissue.
- Analysis of the human oral mucosa as a potential source of stem cells for autologous corneal grafts.
- An examination of extracellular matrix protein coated-gelatin hydrogels as alternative carriers for limbal stem cell cultures.
- An investigation into the feasibility of replacing foetal bovine serum in the culture medium with that of human origin.

2 MATERIALS AND METHODS

This chapter is divided into sections that detail the materials used and methods employed in fulfilling the main objectives of this thesis.

2.1 Comparison of Cellular and Denuded Amniotic Membrane as Carriers for Human Limbal Stem Cell Cultivation

This section describes the materials and methods utilized in the comparison of cellular and denuded human amniotic membranes as culture substrates for limbal stem cells for use in ocular surface reconstruction. The control in this investigation (and all subsequent human cell studies) was donor human corneal tissue obtained from the Northwest Lions Eye Bank and preserved on ice prior to fixation in 4% glutaraldehyde and routine processing as outlined in sections 2.1.5 and 2.1.6.

2.1.1 Preparation of the Cell Suspension Culture System

All cell cultures were carried out by project collaborators at the Kyoto Prefectural University of Medicine in Japan and transported to Lancaster University for analysis. Figure 2.1 overleaf shows a schematic of the stem cell suspension culture system used in each of the investigations in this thesis. Briefly, limbal stem cells were co-cultured with mitomycin [MM]C-treated 3T3 fibroblasts, using a modified keratinocyte culture system (Rheinwald and Green, 1975). Confluent 3T3 fibroblasts were incubated with 4mg/ml of MMC for two hours at 37°C under 5% CO_2. These were then trypsinized and plated onto plastic dishes with a density of 2×10^4 cells/cm^2. Cellular and denuded membranes (prepared as outlined in section 2.1.2)

were spread, amniotic epithelial/basement membrane side-up, on culture plate inserts (Corning, NY, USA) in dishes containing the treated 3T3 fibroblasts.

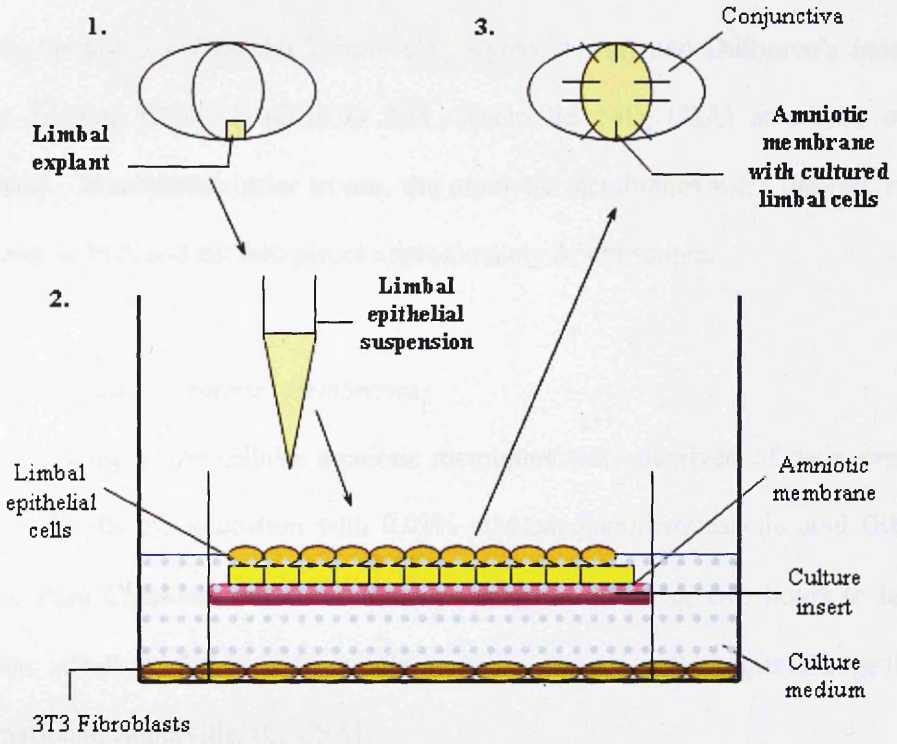


Figure 2.1: Schematic diagram of the suspension culture system. Epithelial cells are isolated from the limbus and then seeded onto denuded amniotic membrane and co-cultured with inactivated 3T3 fibroblasts (adapted from Koizumi *et al.*, 2002).

2.1.2 Preparation of Amniotic Membrane

All amniotic membrane samples were initially prepared at the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Japan). In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human amniotic membranes were obtained at the time of elective caesarean section in volunteers seronegative for human immunodeficiency virus, hepatitis B and C and syphilis.

2.1.2.1 Cellular Amniotic Membranes

Membranes were washed under sterile conditions with phosphate buffered saline (PBS) containing antibiotics (0.3% ofloxacin, 5ml) and stored at -80°C for twelve months in glycerol (Nacalai Tesque Co., Kyoto, Japan) and Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL, Rockville, MD, USA) at a ratio of 1:1 (vol:vol). Immediately prior to use, the amniotic membranes were thawed, rinsed with sterile PBS and cut into pieces approximately 2.5cm square.

2.1.2.2 Denuded Amniotic Membranes

Several pieces of the cellular amniotic membrane were deprived of their amniotic epithelial cells by incubation with 0.02% ethylenediaminetetraacetic acid (EDTA) (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for two hours to loosen cellular adhesion, followed by gentle scraping using a cell scraper (Nalge Nunc International, Naperville, IL, USA).

2.1.3 Origin of Limbal Cells for Culture

Human corneal tissue supplied by Cologne University Tissue Bank (Cologne, Germany) was used for epithelial cell culture in this instance.

2.1.4 Cell Suspension Culture of Human Limbal Epithelial Cells

Suspension culture was carried out by project collaborators from the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Japan), following approval from the Institutional Review Board, with cultures transported to Lancaster University for subsequent evaluation. Immediately after the central corneal button had been used for corneal transplantation, the limbal ring was washed with sterile

PBS. Following removal of up to two thirds of the scleral and corneal stroma, the limbal ring was cut into 2-3 pieces and incubated at 37°C for 1 hour with 1.2IU dispase (Roche Molecular Biochemicals, Manheim, Germany) as described by Gipson and Grill (Gipson and Grill, 1982). The reported method was modified slightly with regard to the duration of the incubation. Limbal epithelial cells (5-10×10^4 cells) were scraped with fine forceps and suspended in 3ml of culture medium and seeded onto pieces of cellular and denuded amniotic membrane spread on culture inserts and co-cultured with MMC-inactivated 3T3 fibroblasts. The culture medium used was DMEM and Ham's F12 media (1:1 mixture) and included foetal bovine serum (or FBS), insulin (5µg/ml), cholera toxin (0.1nmol/l), epidermal growth factor (10ng/ml) and penicillin-streptomycin (50IU/ml). Cultures were incubated at 37°C in a 5% CO_2 enriched incubator, submerged in medium for two weeks and then exposed to air by lowering the medium level (airlifting) for a further two weeks. The culture medium was changed every two days.

2.1.5 Sample Processing for Scanning Electron Microscopy [SEM]

Limbal cultures on cellular and denuded amniotic membrane were initially fixed in 4% glutaraldehyde in PBS buffer for a minimum of two hours. Specimens were then washed in PBS for 3 × 15 minutes and post-fixed in 2% aqueous osmium tetroxide for two hours. They were washed again in PBS before being dehydrated through a graded alcohol series, in which samples were placed in ethanol solutions at 50%, 70%, 80%, 90%, 95% and 100% concentrations for 20 minutes each. Samples were then transferred to hexamethyldisilazane (HMDS) (TAAB Laboratories, UK) for 20 minutes and left overnight to air-dry. The evaporation of HMDS is an alternative method to critical-point drying of samples for scanning electron microscopy (Nation,

1983) and is documented to provide results of the same standard (Braet *et al.*, 1997). Cultures were then mounted on aluminium specimen stubs and sputter-coated with gold using an Edwards S150A sputter-coater prior to examination on a JEOL JSM 5600 scanning electron microscope (Japanese Electron Optical Limited, Tokyo, Japan).

2.1.6 Sample Processing for Transmission Electron Microscopy [TEM]

Cultures were fixed in 4% glutaraldehyde in PBS buffer for a period of at least 2 hours prior to processing for transmission electron microscopy. They were then washed in PBS, post-fixed in 2% aqueous osmium tetroxide for 2 hours, washed again in PBS before being passed through a graded alcohol series (50%, 70%, 80%, 90%, 95% and 100%). Samples were infiltrated with propylene oxide twice for 20 minutes, then left overnight in a 1:1 mixture of propylene oxide and araldite resin (Agar Scientific, UK). The following day, samples were transferred to pure araldite resin and left to infiltrate overnight, under agitation. Finally, samples were embedded in moulds containing fresh resin and polymerized at 60°C for 24-36 hours. Ultrathin sections (50-70nm thick) were cut on a Reichert Ultracut E microtome, collected on naked copper grids and counterstained for 1 hour each with 1% aqueous uranyl acetate and phosphotungstic acid, then for 20 minutes with Reynolds' lead citrate (Reynolds, 1963) prior to examination on a JEOL JEM 1010 transmission electron microscope (Japanese Electron Optical Limited, Tokyo, Japan).

2.1.7 Collection of Quantitative Data for Cellular and Denuded Cultures

To quantify and statistically analyse the microscopic observations, the following protocols were applied (adapted from Koizumi *et al.*, 2002; Ban *et al.*, 2003):

2.1.7.1 Thickness of Cell Layers

Transmission electron micrographs of the cultured epithelial cells on cellular and denuded AM were digitized using an Epson Perfection 1240U scanner and the thickness of cell layers was measured at various randomly selected points (n=14) using AnalySIS® (Soft Imaging System GmbH) software. The scale bar on each of the micrographs was used for calibration and so the results obtained were in micrometers and required no calculation (as in figure 2.2).

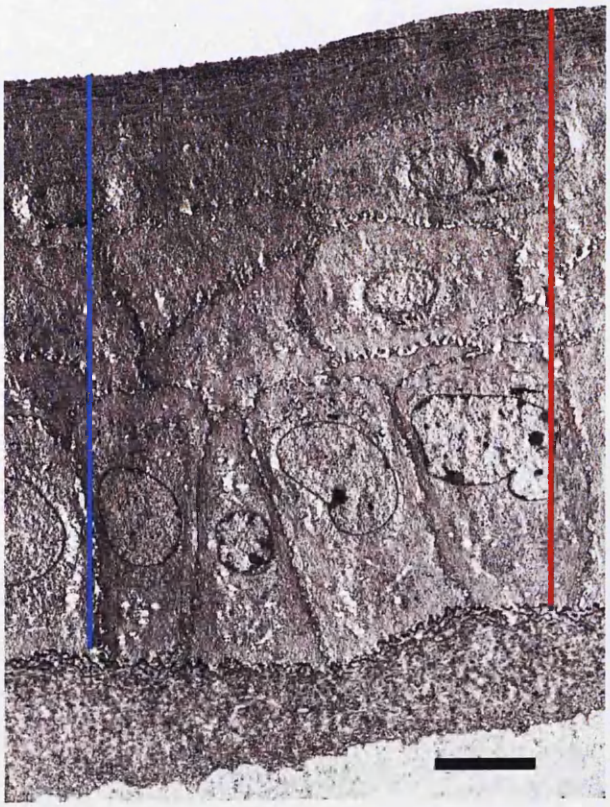


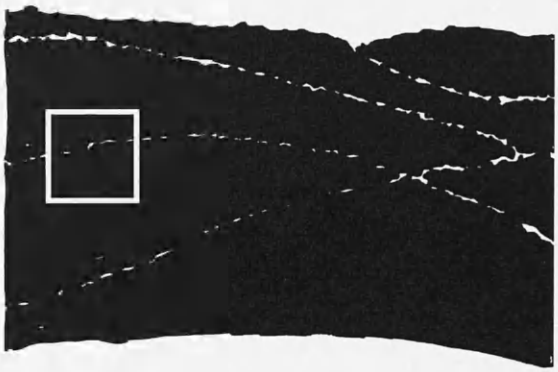
Figure 2.2: Average thickness was calculated using AnalySIS® software and transmission electron micrographs calibrated using the scale bars. Regions were selected at random (see coloured lines) for measurement. [Micrograph illustrates control human corneal epithelium, routinely processed and stained for TEM].

2.1.7.2 Number of Cell Layers

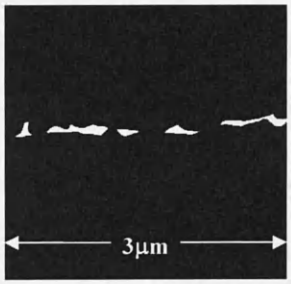
To determine the mean number of cell layers in each of the cultures, 14 areas were selected at random and the number of cells (basal, wing and superficial) was counted manually under the transmission electron microscope.

2.1.7.3 Area of Intercellular Space

[1] An outline of the cell morphology was made from the electron micrograph



[2] Inter and intracellular areas were digitised and a 3μm lateral distance was selected at random



[3] The area of intercellular space over this 3μm length was calculated using AnalySIS®

[4] The number of desmosomes over the same 3μm length was manually counted

Figure 2.3: Diagrammatic representation of the protocol for determination of intercellular space areas to statistically compare cells cultured on cellular and denuded amniotic membranes. [Micrograph illustrates control human corneal epithelium, routinely processed and stained for TEM].

To calculate the areas of space between adjacent cells, outlines of cell morphology were made on acetates from TEM micrographs. Images were again digitized using an Epson Perfection 1240U scanner. Regions of the interface of adjacent cells (corresponding to a 3μm distance) cultivated on both cellular (n=14) and denuded (n=14) amniotic membranes were selected at random by a person independent of this project. AnalySIS® software was used to calculate the intercellular area, while the number of desmosomes in the same randomly selected area was manually counted (refer to figure 2.3).

2.1.7.4 Number of Desmosomes

As described above, mean numbers of desmosomes in each of the cell cultures were calculated from fourteen regions at random, across 3μm lateral distances at cell-cell interfaces (figure 2.3).

2.1.7.5 Number of Basal Junctions/Hemidesmosomes

The number of hemidesmosomal junctions at the basement membrane in the denuded culture (n=14) and that of desmosomal junctions attaching cultivated cells to AM epithelial cells on the cellular substrate (n=14) were similarly quantified over randomly selected 3μm lateral distances using TEM micrographs.

2.1.7.6 Superficial Cell Surface Area

Average cell surface areas on denuded (n=50) and cellular (n=50) membranes were calculated using SEM micrographs taken at low magnification. Cell peripheries were manually outlined and once calibrated, the AnalySIS® software calculated superficial cell surface areas (figure 2.4 overleaf).

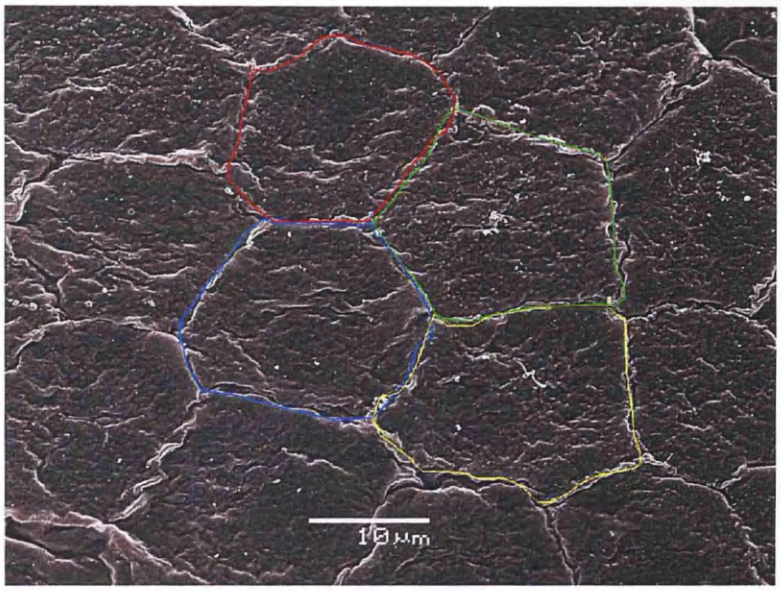


Figure 2.4: Mean cell surface areas were calculated using AnalySIS® software and scanning electron micrographs calibrated using the scale bars. Regions were selected at random (see coloured lines) for measurement. [Micrograph illustrates control human corneal endothelium, routinely processed for SEM].

2.1.8 Presentation of Quantitative Data

Mean values (± standard deviation, S.D.) were determined for each of the parameters and for ease of interpretation these were plotted in a chart in the form of percentage difference from control, which in this case was normal human cornea. The scale of the vertical axis was kept constant for every study in this thesis.

2.1.9 Statistical Data Analysis

Statistical analysis was carried out on the raw data obtained for each substrate using SigmaStat® v2.03 (SPSS Inc., UK). Where the data passed normality and equal variance tests, t-tests were performed and where the data failed one or both of these, a Mann-Whitney Rank Sum test was used. The level of significance was taken to be 0.050 in both instances. Statistical comparisons were drawn between the cultivated stem cells and the control human corneal epithelium as well as between the cellular and denuded culture systems themselves.

2.2 Cultivation of HCEC on Amniotic Membrane

This section describes the materials and methods used to assess the usefulness of denuded amniotic membrane as a carrier for the cultivation of human corneal endothelial cells.

2.2.1 Origin of Endothelial Cells for Culture

A human donor cornea (47 years old, male, endothelial cell density: 3065cells/mm^2) obtained from Northwest Lions Eye Bank, USA, was preserved in Optisol GS (Chiron Vision, Irvine, CA, USA) and transported on ice to the Kyoto Prefectural University of Medicine. After using its centre for human corneal transplantation, the residual limbal tissue was used, at day 7 after death, in this study.

2.2.2 Cell Suspension Culture of Human Corneal Endothelial Cells

Suspension culture was carried out at the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Japan), following approval from the Institutional Review Board, with cultures transported to Lancaster University for subsequent evaluation. Limbal tissue was placed in a petri dish containing DMEM (Invitrogen Corp., Carlsbad, CA, USA), 50U/ml penicillin and 50μg/ml streptomycin. Under a dissecting microscope, Descemet's membrane with its attached corneal endothelium was stripped from the stroma and placed in a 35mm dish containing 1.2IU/ml dispase in PBS. The tissue was incubated for 1 hour at 37°C and the cells were rinsed gently with a sterile pipette. The dispase was then inactivated by suspending the cells in a medium containing DMEM, penicillin (50IU/ml) and streptomycin (50μg/ml). After gentle centrifugation (3 minutes at 180g), the cells were re-suspended in culture

medium containing DMEM, penicillin (50IU/ml), streptomycin (50μg/ml), 10% foetal bovine serum (ICN Biomedicals, Inc. Aurora, Ohio, USA) and 2ng/ml basic fibroblast growth factor (Invitrogen Corp., Carlsbad, CA, USA).

The endothelial cells were then incubated in wells of a collagen IV-coated 24-well plate at 37°C in a 5% carbon dioxide humidified atmosphere. The medium was changed every other day. Cells reached confluence in 10-20 days and were then sub-cultured by treatment with trypsin and EDTA (Invitrogen Corp., Carlsbad, CA, USA) and seeded at a ratio split of 1:2 to 1:8.

2.2.3 Preparation of Amniotic Membrane

Human AM obtained at the time of caesarean section was stored at -80°C in DMEM and glycerol (Nacalai Tesque, Kyoto, Japan) after washing with PBS containing antibiotics (5ml 0.3% ofloxacin). Immediately before use, the thawed AM was deprived of amniotic epithelial cells by incubation with 0.02% EDTA (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 2 hours, followed by gentle cell scraping with a cell scraper (Nalge Nunc International, Naperville, IL, USA). The tissues were then washed twice with sterile PBS.

2.2.4 Seeding Corneal Endothelial Cells on Denuded Amniotic Membrane

Confluent monolayers of human corneal endothelial cells from passage five were trypsinized, centrifuged and re-suspended at a final cell seeding concentration of 6.0×10^3 cells/mm^2. Re-suspended cells were gently seeded on denuded AM spread, basement membrane side up, on polyester culture inserts (Corning, NY, USA) in wells of a 12-well plate and incubated at 37°C in a 5% carbon dioxide humidified

atmosphere. Three days later, the culture medium was changed and then changed every other day for two weeks.

2.2.5 Sample Processing for Scanning Electron Microscopy

Briefly, day 14 cultures on amniotic membrane were fixed in 2.5% glutaraldehyde in 0.1M PBS, washed three times for 15 minutes in PBS, post-fixed for 2 hours in 2% aqueous osmium tetroxide and washed three more times in PBS. Following dehydration through a graded ethanol series (50, 70, 80, 90, 95 and 100%) specimens were transferred to HMDS (Agar Scientific, London, UK) twice for 10 minutes and allowed to air-dry. When dry, specimens were mounted on aluminium stubs and sputter-coated with gold before examination on a JEOL JSM 5600 scanning electron microscope.

2.2.6 Sample Processing for Transmission Electron Microscopy

Briefly, day 14 cultures on AM were fixed in 2.5% glutaraldehyde in 0.1M PBS, post-fixed in 2% aqueous osmium tetroxide, dehydrated through a graded ethanol series, and embedded in araldite resin (Agar Scientific, UK). Ultrathin (70nm) sections were collected on copper grids and stained for 1 hour each with uranyl acetate and 1% phosphotungstic acid and for 20 minutes with Reynold's lead citrate prior to examination on a JEOL JEM 1010 transmission electron microscope.

2.3 Morphological Analysis of Polyphenol-Treated Rat Corneal Endothelium in Long-Term Storage

This section describes the methodology used to examine the effects of polyphenol treatment on rat corneal endothelial cells over a 4 week time course, with the aim of improving cell preservation in long-term storage.

2.3.1 Corneal Tissue Preparation

Six week old female Wistar rats were purchased from Shimazu Laboratory Supplies (Kyoto, Japan) and sacrificed by injecting an overdose of sodium pentobarbital. Eyes were enucleated and corneal tissues excised at the limbus. Tissues were washed twice in Optisol-GS before storage. All animals were treated in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research

2.3.2 Polyphenol and Optisol-GS

Green tea extract polyphenol, containing (-)- epigallo-catechin-3-O-gallate, was purchased from PFI Inc., (Kyoto, Japan) with a purity in excess of 90%. The polyphenol was dissolved in PBS at a concentration of 2mg/ml and stored at -80°C until use. Optisol-GS (containing 2.5% chondroitin sulphate, 1% dextran, 100μg/ml gentamicin and 200μg/ml streptomycin sulphate) was purchased from Bausch & Lomb Surgical Inc., Irvine, CA.

2.3.3 Tissue Storage

Corneal tissues were stored in Optisol-GS containing 500μg/ml polyphenol at 4°C for 24 hours. After washing twice in pure Optisol-GS, the tissues were stored in polyphenol-free Optisol-GS for up to 28 days at 4°C. The medium was changed every seven days. The control samples were treated with Optisol-GS solution only. At days 7, 14 and 28 corneal samples were fixed with 4% glutaraldehyde solution in phosphate buffer. All of the above was carried out by project collaborators at the Kyoto Prefectural University of Medicine, Kyoto, Japan and the samples were shipped to Lancaster University for preparation and examination by SEM.

2.3.4 Scanning Electron Microscopy

Corneal samples were prepared for analysis by SEM exactly as outlined in section 2.1.5. The corneal tissues were mounted endothelial side-up on aluminium specimen stubs and sputter coated with gold prior to examination.

2.4 Evaluation of Sterilized, Freeze-Dried Amniotic Membrane

This section describes the materials and methods used in the ultrastructural and immunohistochemical evaluation of freeze-dried amniotic membrane (FD-AM) as an alternative carrier for corneal cells in ocular surface reconstruction.

2.4.1 Amniotic Membrane Samples

Samples of human amniotic membrane were all obtained from the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Japan). Freeze-dried amniotic membrane was evaluated alongside frozen, routinely preserved amniotic membrane for comparison purposes. For ultrastructural examination, amniotic membranes were conventionally processed for scanning and transmission electron microscopy. For immunohistochemical characterization, cellular and denuded, frozen and freeze-dried membranes were labelled with antibodies directed to extracellular matrix proteins and glycosaminoglycans. In a final study, denuded freeze-dried amniotic was used as a culture substrate for rabbit corneal epithelial cells.

2.4.2 Freeze-Drying of Amniotic Membrane

All amniotic membrane samples were prepared at the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Japan) and shipped to Lancaster University for subsequent analysis. On approval by the Institutional Review Board of Kyoto Prefectural University of Medicine and in accordance with the tenets of the Declaration of Helsinki for research involving human subjects, amniotic membrane was obtained at the time of elective caesarean section. Under sterile conditions the

membrane was washed with sterile PBS containing antibiotic-antimycotic liquid and cut into small pieces. Several pieces of AM were then deprived of amniotic epithelial cells as previously described (section 2.1.2.2). Amniotic membranes were freeze-dried under vacuum conditions and vacuum-packed at room temperature as soon as possible. Finally, γ-irradiation (25kGy) was used to sterilize the resultant freeze-dried amniotic membrane and bacteriological tests were carried out to confirm sterilization.

2.4.3 Preparation of Frozen Amniotic Membrane

As described previously (section 2.1.2), human amniotic membranes were obtained at the time of caesarean section, washed under sterile conditions with PBS containing antibiotics and stored at -80°C for five months in glycerol and DMEM at a ratio of 1:1 (vol:vol). Immediately prior to use, the amniotic membranes were thawed, rinsed with sterile PBS and cut into small pieces. Several pieces of amniotic membrane were then deprived of their amniotic epithelial cells as detailed earlier (section 2.1.2.2).

2.4.4 Immunohistochemical Characterization of Amniotic Membrane

Antibodies were directed against several extracellular matrix molecules in freeze-dried amniotic membrane. Frozen amniotic membrane was also examined for comparison.

2.4.5 Histochemicals

2.4.5.1 Primary

For the primary labelling of fibronectin, a monoclonal mouse antibody (IST-4) immunospecific for the human epitope was obtained from Sigma Chemical Company (Poole, UK). For labelling of laminin, a monoclonal rabbit affinity-isolated antibody, again specific for the human epitope, was obtained from Sigma Chemical Company (Poole, UK). Vitronectin was labelled with a monoclonal mouse-derived antibody (VIT-2) obtained from Sigma Chemical Company (Poole, UK). For collagen type IV labelling, a mouse monoclonal antibody (NLI/53) was purchased from Biogenesis (Poole, UK). This antibody is reactive to native and denatured forms of type IV collagen and shows no cross-reactivity with collagen types I, II, III, or V. Collagen type I was labelled with a mouse-derived antibody (COL-1) obtained from Sigma Chemical Company (Poole, UK) which recognizes the native helical form of type I collagen. Cross-reactivity has not been found with collagen types II, III, IV, V, VI, VII, IX, X and XI. For heparan sulphate [HS] labelling, the monoclonal mouse antibody F58-10E4 was obtained from the Seikagaku Corporation (Japan). The epitope for this particular antibody is known to contain one or more N-sulphated glucosamine residue(s). For labelling of chondroitin sulphate [CS], the monoclonal mouse IgM CS-56 was obtained from Sigma Chemical Company (Poole, UK). CS-56 recognizes an epitope present in the interior region of native chondroitin 4- and 6-sulphate chains (Avnur and Geiger, 1984; Sorrell *et al.*, 1993). For keratan sulphate [KS], the antibody 5-D-4 was obtained from the Seikagaku Corporation (Japan). 5-D-4 binds to large oligosaccharides of sulphated polyN-acetyllactosamine (Mehmet *et al.*, 1986).

2.4.5.2 Secondary

For primary antibodies to be visualized by transmission electron microscopy, goat anti-mouse IgG, goat anti-mouse IgM and goat anti-rabbit IgM secondary antibodies conjugated to 5nm colloidal gold were used. All secondary antibodies were obtained from British Biocell International.

Antigen	Category	Clone	Supplier	2° Antibody
Fibronectin	Mouse (ascites) IgG	IST-4	Sigma, UK	Goat anti-mouse IgG
Laminin	Rabbit IgG	-	Sigma, UK	Goat anti-rabbit IgG
Vitronectin	Mouse (ascites) IgM	VIT-2	Sigma, UK	Goat anti-mouse IgM
Collagen type IV	Mouse IgM	NLI/53	Biogenesis, UK	Goat anti-mouse IgM
Collagen type I	Mouse (ascites) IgG	COL-1	Sigma, UK	Goat anti-mouse IgG
Heparan sulphate	Mouse IgM	F58-10E4	Seikagaku, Japan	Goat anti-mouse IgM
Chondroitin sulphate	Mouse (ascites) IgM	CS-56	Sigma, UK	Goat anti-mouse IgM
Keratan sulphate	Mouse IgG	5-D-4	Seikagaku, Japan	Goat anti-mouse IgG

Table 2.1: Summary of immunohistochemicals used in the characterization of the freeze-dried and frozen amniotic membranes.

2.4.5.3 Controls

As a negative control, primary antibodies were replaced with non-specific primary antibodies (Serotec, Oxford, UK) at equivalent dilutions. This determined the level of non-specific background labelling on a sample.

2.4.6 Tissue Processing for TEM (Bairaktaris *et al.*, 1998)

Extracellular matrix proteins in freeze-dried and frozen, cellular and denuded amniotic membranes were studied via immunoelectron microscopy. Frozen membrane samples were thawed and freeze-dried samples were rehydrated in PBS buffer prior to fixation in 4% paraformaldehyde in phosphate buffer at pH 7.2 for a period of at least two hours. The samples were washed three times in phosphate

buffer containing 0.1M glycine before being dehydrated through a graded ethanol series (20 minutes in each of 50%, 70%, 80% and 90% ethanol solutions). Specimens were then agitated for 1 hour in London Resin (LR) White resin (TAAB Laboratories). Subsequent infiltrations in fresh resin were for one hour and then overnight. Amniotic membranes were then embedded in moulds with fresh resin and polymerized at 50°C for 24 hours. Ultrathin sections were cut on a Reichert Ultracut E microtome and collected on G400 gilded copper grids (Agar Scientific, UK).

2.4.7 Immunogold Labelling of Basement Membrane Components

Sections to be labelled for examination under the transmission electron microscope underwent the following protocol with all steps carried out at room temperature unless otherwise stated. Labelling for the primary antibodies was carried out by first placing grids in droplets of 0.1M glycine in PBS for two 10 minute periods, then in droplets of goat serum for 20 minutes to prevent non-specific labelling. Excess goat serum was removed before grids were transferred to a grid box loaded with 15µl of primary antibody diluted 1:50 in PBS buffer at pH 7.4 containing 1% bovine serum albumin (BSA) and 1% Tween® 20 (buffer 1). To prevent evaporation of the primary antibody, grids were incubated overnight at 4°C in a moist chamber. The primary antibody was replaced by a non-specific antibody for negative controls.

The next day, grids were washed for 5 × 8 minutes in droplets of buffer 1 solution. This was followed by five 8 minute washes in distilled water. Grids were transferred to a grid box loaded with 15µl of the appropriate 5nm gold-conjugated secondary antibody (British Biocell International, Cardiff, UK), diluted 1:50 in PBS at pH 8.2 containing 1% BSA, 1% Tween® 20, 1% normal goat serum, 1% fish gelatin and 2%

sodium chloride (buffer 2). Sections were incubated for 3 hours at room temperature after which the grids were washed for 8 minutes each time in 5 droplets of buffer 2 solution followed by five washes in distilled water. Sections were counterstained in aqueous uranyl acetate for one hour before being examined on a JEOL JEM 1010 transmission electron microscope.

2.4.8 Culture of Rabbit Corneal Epithelial Cells on Freeze-Dried AM

Corneal epithelial cells were cultured at the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Japan) and transported to Lancaster University for subsequent examination.

2.4.8.1 Extraction of Rabbit Limbal Biopsies

Limbal biopsies (each 4mm^2 in size) were taken from 8 adult albino rabbits (2-2.5kg), anaesthetized by intramuscular injection of xylazine hydrochloride (5mg/ml) and ketamine hydrochloride (50mg/ml). Animals were treated in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and with the experimental procedure approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine. Corneal endothelium and half the corneal stroma were removed with scissors to the extent possible; the cells in the resulting sample were then disaggregated using dispase for use in suspension culture.

2.4.8.2 Epithelial Suspension Culture on FD-AM

Cells were co-cultured with MMC-inactivated 3T3 fibroblasts as previously described (section 2.1.1). Denuded freeze-dried AM were spread; epithelial basement membrane side up, on culture inserts (Corning Inc., NY, USA) in dishes containing

treated 3T3 fibroblasts. The disaggregated rabbit limbal cells were seeded onto these membranes and cultured using the method detailed in section 2.1.4. The culture was submerged in medium for two weeks and then exposed to air by lowering the medium level for one week; the medium was changed every day.

2.4.9 Sample Processing for Scanning Electron Microscopy

Freeze-dried amniotic membranes (both cellular and denuded) as well as rabbit corneal epithelial cell cultures on freeze-dried AM were processed for examination by SEM. All samples were fixed in 4% glutaraldehyde in PBS buffer for a period of at least 2 hours prior to processing. Samples followed the exact protocol outlined in section 2.1.5.

2.4.10 Sample Processing for Transmission Electron Microscopy

Similarly, freeze-dried amniotic membranes (both cellular and denuded) as well as rabbit corneal epithelial cell cultures on freeze-dried AM were processed for examination by TEM. All samples were fixed in 4% glutaraldehyde in PBS buffer for a period of at least 2 hours prior to processing, as detailed in section 2.1.6.

2.4.11 Quantitative Analysis of Corneal Cells Cultivated on Freeze-dried AM

Rabbit corneal cultures on AM were quantitatively analysed using the same parameters as outlined in section 2.1.7. Data were collated and mean values calculated. Average values for thickness/numbers of cell layers, numbers of desmosomes/hemidesmosomes and cell surface area/intercellular space areas were plotted as percentage differences from those of control rabbit cornea. Statistical analysis on the raw data was carried out as detailed in section 2.1.9.

2.5 Cultivation of Human Oral Mucosal Epithelial Cells on Denuded Amniotic Membrane

This section details the materials and methods used to evaluate the autologous transplantation of human oral mucosal epithelial cells for ocular surface reconstruction.

2.5.1 Samples for Ultrastructural Examination

All samples for this study were derived from the Department of Ophthalmology at the Kyoto Prefectural University of Medicine (Japan) and sent to Lancaster University for processing and analysis. Human mucosal epithelial cell biopsies from the buccal (oral cavity-lining) and gingival (masticatory) regions of the mouth were retained along with a corneal biopsy for comparison. In addition, buccal and gingival mucosal cells were seeded onto denuded amniotic membrane and cultured using a similar protocol to that designed for the corneal epithelium. For ultrastructural examination, biopsies and resulting cultures were conventionally processed for scanning and transmission electron microscopy.

2.5.2 Mucosal Cell Biopsies

2.5.2.1 Preoperative Oral Management

Given the potential for contamination of the cell samples from indigenous oral bacteria, prior to surgery all patients were given comprehensive oral hygiene instructions and teeth were scaled for the purpose of good plaque control. X-rays were taken and oral examinations carried out. Under medical supervision, patients

were encouraged to brush teeth and use a povidone-iodine mouthwash solution (Isodine Gargle, Meiji Seika Kaisha, Ltd., Tokyo) after every meal. All dental caries were treated, removable dentures cleaned and smokers were urged to abstain. Patients were advised by a dental surgeon about the possibility of complications from the extraction, including swelling, pain and oral dysfunction. Antibiotics were administered to the patient preoperatively.

2.5.2.2 Preparation for Surgery

To minimize bacterial contamination, a number of disinfection procedures were carried out in preparation for surgery. The extraoral region was twice cleansed with chlorhexidine gluconate (Stericlon W solution, Ken-Ei Pharmaceutical Co., Ltd., Osaka) twice. Scaling was performed and teeth and gingival were brushed with chlorhexidine hydrochloride (Gelcoat F, Sumoka Sima Co, Ltd., Osaka). Finally the mouth was rinsed with chlorhexidine gluconate (Gargule Sunstar 100G, Sunstar, Inc., Osaka).

2.5.2.3 Oral Stem Cell Sampling

Following careful examination, a sampling site was located (either the mucogingival junction of the lower molar area or buccal mucosa). Infiltration anaesthesia comprising 2% lidocaine hydrochloride containing epinephrine (Fujisawa Pharmaceutical Co. Ltd., Osaka) was injected around the sampling site and 5mm^2 incisions were made in the locality (surgical blade no.11; Feather Safety Razor Co. Ltd., Osaka) enabling removal of a cell sample along with submucosal connective tissue. The wound was closed with 3-0 surgical silk sutures (Nescosuture, Azwell Inc., Osaka).

2.5.2.4 Postoperative Oral Management

Antibiotics were administered to prevent postoperative infection and analgesics were prescribed if needed. Surgical site was regularly evaluated for signs of infection and irrigated with povidone-iodine daily. All sutures were removed after one week.

2.5.3 Sample Preparation for Light Microscopy

Human gingival, buccal and corneal biopsies were routinely prepared for examination by light microscopy. All samples were fixed in 4% glutaraldehyde in PBS buffer for a minimum of two hours prior to processing. Tissues were processed as for TEM and embedded in araldite resin (as in section 2.1.6). Semi thin sections (1-1.5μm thick) were cut on a Reichert Ultracut E microtome, collected on glass slides and conventionally stained using toluidene blue, prior to coverslipping and examination on a Reichart light microscope. Images were captured digitally.

2.5.4 Preparation of Amniotic Membrane

For oral epithelial cell cultures, amniotic membrane was prepared and denuded as detailed in section 2.1.2. Briefly, human amniotic membranes were obtained at the time of elective Caesarean section. Under sterile conditions, the membranes were cryopreserved at -80°C. Membranes were deprived of their amniotic epithelial cells by incubation with EDTA at 37°C for 2 hours to loosen cell adhesion, followed by gentle scraping with a cell scraper.

2.5.5 Primary Cultures of Human Oral Epithelial Cells on AM

Human oral epithelial cultures were produced in the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Kyoto, Japan) as previously reported for

rabbit oral mucosal stem cells (Nakamura *et al.*, 2003a) using a similar yet slightly modified method to that for limbal stem cells (section 2.1.4). Oral mucosal biopsy specimens (from section 2.5.2) each measuring approximately 2-3mm^2, were taken from each patient under local anaesthesia 2-3 weeks prior to transplantation. Submucosal connective tissues were removed with scissors to the extent possible; the resulting samples were cut into small explants that were immersed three times for 10 minutes in PBS solution containing antibiotics (50 IU/ml penicillin-streptomycin and 5μg/ml amphotericin B). The explants were then incubated at 37°C for 1 hour with 1.2 IU dispase then treated with 0.25% Trypsin-EDTA solution for 10 minutes at room temperature to separate the cells. Enzyme activity was stopped by washing with DMEM and Ham's F12 medium (1:1) containing 10% foetal bovine serum, insulin (5μg/ml), cholera toxin (0.1nmol/l), human recombinant epidermal growth factor (10ng/ml), and penicillin-streptomycin (50 IU/ml). The oral epithelial cells (1×10^5 cells/ml) were then seeded onto denuded AM spread on the bottom of culture inserts and co-cultured with MMC-inactivated 3T3 fibroblasts. The culture was submersed in medium for 2 weeks and then exposed to air by lowering the level of the medium over the course of one week. Cultures were incubated at 37°C in a 5%CO_2-enriched incubator and the medium changed every day.

2.5.6 Sample Preparation for Scanning Electron Microscopy

Mucosal cell biopsies and cultures on amniotic membrane were routinely prepared for examination by SEM. All samples were fixed in 4% glutaraldehyde in PBS buffer for a period of at least 2 hours prior to processing. Samples underwent the protocol outlined in section 2.1.5.

2.5.7 Sample Processing for Transmission Electron Microscopy

Similarly, mucosal epithelial biopsies and cultures were processed for examination by TEM. All samples were fixed in 4% glutaraldehyde in PBS buffer for a minimum of two hours prior to routine processing, as detailed in section 2.1.6.

2.5.8 Quantitative Analysis of Mucosal Cell Cultures

Oral mucosal cultures on AM were analysed quantitatively. Data were collated as outlined in section 2.1.7 and mean values calculated and plotted as percentage differences from normal human cornea control. Statistical analysis of the raw data was also carried out exactly as outlined previously in section 2.1.9.

2.6 Clinical Outcomes of Amniotic Membrane/Oral Mucosal Stem Cell Transplants

This section details the materials and methods used to evaluate two human oral mucosal epithelial grafts used in ocular surface reconstruction, both of which were removed after 5-6 months to allow for conventional penetrating keratoplasty with donor corneal tissue.

2.6.1 Samples for Ultrastructural Examination

Oral mucosal epithelial cells were cultivated on denuded amniotic membrane exactly as outlined in section 2.5.5. The resultant culture sheets were used to reconstruct fifteen eyes of twelve patients with acute and chronic phase ocular surface disorders, all procedures being carried out by project collaborators at the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Nakamura *et al.*, 2004a). In all eyes, the ocular surface was covered with cultivated oral epithelium 48 hours after transplantation, remained free from epithelial defects and visual acuity was reportedly improved. During the long-term observation period however two eyes became opaque and displayed some of the symptoms of graft rejection. These grafts were removed and replaced with donor corneal tissue in conventional PKP then shipped to Lancaster University for further analysis, with full consent. The primary diagnoses of the two case studies were acute phase chemical injury *[case one]* and acute phase Stevens-Johnson Syndrome, SJS *[case two]*. In the first case the graft was removed 5 months after placement. The second graft (SJS patient) was removed at 6 months.

2.6.2 Sample Preparation for Scanning Electron Microscopy

Tissues were routinely prepared for examination by SEM. Both samples were fixed in 4% glutaraldehyde in PBS buffer for a period of at least 2 hours prior to processing. Samples then underwent the protocol outlined in section 2.1.5.

2.6.3 Sample Processing for Transmission Electron Microscopy

Similarly, both tissues were processed for examination by TEM. Samples were fixed in 4% glutaraldehyde in PBS buffer for a minimum of two hours prior to routine processing, as detailed in section 2.1.6.

2.7 Rabbit Oral Mucosal/Corneal Epithelial Hybrid Cultures on Amniotic Membrane

This section details the materials and methods used to create and analyse hybrid cultures of rabbit oral mucosal stem cells and corneal epithelial cells on amniotic membrane.

2.7.1 Origin of Cells for Culture

All samples for this study were derived from the Department of Ophthalmology at the Kyoto Prefectural University of Medicine (Japan) and sent to Lancaster University for processing and analysis. Corneal and oral mucosal biopsies for hybrid culture were taken from the same donor albino rabbit. Biopsies were collected under local anaesthesia using a similar method to that outlined in section 2.5.2.

2.7.2 Preparation of Amniotic Membrane

For hybrid cell cultures, amniotic membrane was prepared and denuded as detailed in section 2.1.2.

2.7.3 Co-Cultures of Rabbit Oral/Corneal Epithelia on Amniotic Membrane

Hybrid oral and corneal epithelial cell cultures on amniotic membrane were created at the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Kyoto, Japan) using a combination of methods previously reported (Koizumi *et al.*, 2002; Nakamura *et al.*, 2003a) and described in sections 2.1.4 and 2.5.5. Briefly, corneal and oral biopsies were collected from an albino rabbit under local anaesthesia. Resulting samples were treated with dispase and trypsin/EDTA to

dissociate the cells. The resulting cell suspensions were mixed together in equal volumes (1:1 ratio, corneal:oral) and then seeded onto denuded amniotic membrane spread on the bottom of culture inserts and co-cultured with MMC-inactivated 3T3 fibroblasts. The culture was submersed in culture medium for 2 weeks and then exposed to air for one week. Cultures were incubated at 37°C in a 5%CO_2-enriched incubator and the medium changed every day.

2.7.4 Sample Preparation for Scanning Electron Microscopy

Hybrid rabbit cell cultures on amniotic membrane were routinely prepared for examination by SEM. Samples were fixed in 4% glutaraldehyde in PBS buffer for a period of at least 2 hours prior to processing and then underwent the protocol outlined in section 2.1.5.

2.7.5 Sample Processing for Transmission Electron Microscopy

Similarly, hybrid cultures were processed for examination by TEM. All samples were fixed in 4% glutaraldehyde in PBS buffer for a minimum of 2 hours prior to routine processing (section 2.1.6).

2.7.6 Quantitative Analysis of Hybrid Cell Cultures

Rabbit hybrid oral mucosal/corneal cell cultures on AM were analysed quantitatively. Data were collated as outlined in section 2.1.7 and mean values calculated and plotted in terms of percentage difference from normal rabbit cornea control. Statistical analysis of the raw data was also carried out exactly as outlined previously in section 2.1.9.

2.8 Extracellular Matrix Protein-Coated Gelatins as Carriers for Human and Rabbit Limbal Stem Cell Cultivation

The following section describes the materials and methods used in the ultrastructural characterization of extracellular matrix protein coated gelatin as a potential alternative carrier to amniotic membrane in ocular surface reconstruction. All samples for this study were derived from the Department of Ophthalmology at the Kyoto Prefectural University of Medicine (Japan) and sent to Lancaster University for processing and analysis.

2.8.1 Preparation and Coating of Gelatins

A 10% solution of gelatin was prepared with distilled water, incubated at 37°C for 30 minutes and then left to air dry overnight at room temperature, cast in the moulds of a six-well dish. Cross-linking of the gelatin sheets was achieved by 72 hours of exposure to 160°C heat under vacuum conditions. Ten percent solutions of extracellular matrix proteins (collagen type IV, fibronectin and a mixture of both) were spread on top of the gelatin sheets (5μg/cm^2) and left to infiltrate for two hours at room temperature. Any excess was removed by washing with PBS solution. Samples of the coated gels were then sent to Lancaster University for SEM and TEM examination, while the remainder were used as corneal epithelial cell culture substrates.

2.8.2 Origin of Corneal Epithelial Cells for Culture

Human corneal tissue supplied by the US Eye Bank was used for epithelial cell culture in this instance. Immediately after the central corneal button had been used

for corneal transplantation, the limbal ring was washed with sterile PBS. Following removal of up to two thirds of the scleral and corneal stroma, the limbal ring was cut into 4 pieces and incubated at 37°C for 1 hour with 1.2IU dispase. For the rabbit corneal epithelial cell culture, limbal biopsies were taken from adult albino rabbits and prepared exactly as described in section 2.4.8.1.

2.8.3 Culture of Corneal Epithelial Cells on Coated Gelatins

Suspension culture was carried out by project collaborators from the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Japan), following approval from the Institutional Review Board, with cultures transported to Lancaster University for subsequent evaluation. Rabbit and human corneal epithelial cells were cultured on coated gelatin sheets using exactly the same methods. The suspension culture technique, previously documented (Koizumi *et al.*, 2002; Nakamura *et al.*, 2004b) and outlined in section 2.1.4, was used with minor modifications. Epithelial cells were seeded onto the collagen type IV and fibronectin coated gelatin hydrogels and co-cultured with MMC-inactivated 3T3 fibroblasts, submersed in medium for 2 weeks then air-lifted for 2-3 days.

2.8.4 Sample Preparation for Scanning Electron Microscopy

Extracellular matrix protein coated gelatin samples and corneal epithelial cultures on coated gelatin were routinely prepared for examination by scanning electron microscopy. Samples were fixed in 4% glutaraldehyde in PBS buffer for at least two hours prior to processing as outlined in section 2.1.5.

2.8.5 Sample Processing for Transmission Electron Microscopy

Similarly, coated gelatins and corneal epithelial cultures on gelatin were processed for examination by transmission electron microscopy. All samples were fixed in 4% glutaraldehyde in PBS buffer for a minimum of two hours prior to routine processing, as detailed in section 2.1.6.

2.8.6 Quantitative Analysis of Corneal Cells Cultivated on Gelatin

Data was collected from both human and rabbit corneal cultures on gelatin exactly as outlined in section 2.1.7. Mean values were calculated and plotted as percentage differences from control human/rabbit cornea respectively. In addition, statistical analysis was carried out on the raw data, as outlined in section 2.1.9.

2.9 Human Serum in Corneal Epithelial Cell Culture

The following section describes the materials and methods used in the evaluation of a culture system using human serum as an alternative to foetal bovine serum for the culture of human corneal epithelial cells on denuded amniotic membrane. All samples for this study were derived from the Department of Ophthalmology at the Kyoto Prefectural University of Medicine (Japan) and sent to Lancaster University for processing and analysis.

2.9.1 Origin of Corneal Epithelial Cells for Culture

Donor human corneal tissue was supplied by the US Eye Bank. Immediately after the central corneal button had been used for corneal transplantation, the limbal ring was washed with sterile PBS. Following removal of excess scleral and corneal stroma, the limbal ring was cut into pieces and incubated at 37°C for 1 hour with 1.2IU dispase to disaggregate the cells prior to suspension culture.

2.9.2 Preparation of Human Serum for Culture System

Human serum was extracted from the blood of the patients scheduled to undergo cultivated corneal epithelial cell grafts. Thirty millilitre blood samples were taken and centrifuged at 3000 r.p.m. for 10 minutes. The supernatant was centrifuged for a second time to isolate the serum (second supernatant fraction). The serum was incubated at 56°C for 30 minutes to inactivate the complement and then decontamination was performed by passage through a Millipore filter of a 0.22μm pore size. After testing for bacterial contamination, aliquots were cryopreserved at -30°C until needed.

2.9.3 Culture of Corneal Epithelial Cells on Denuded AM

Corneal epithelial cells were cultured at the Department of Ophthalmology, Kyoto Prefectural University of Medicine (Kyoto, Japan) using the suspension method as previously reported (Koizumi *et al.*, 2002) and described in section 2.4.8, the only modification being the replacement of foetal bovine serum with human serum in the culture medium of all but the control system.

2.9.4 Sample Preparation for Scanning Electron Microscopy

Corneal epithelial cultures on denuded AM were routinely prepared for examination by scanning electron microscopy. Samples were fixed in 4% glutaraldehyde in PBS buffer for at least two hours prior to processing as outlined in section 2.1.5.

2.9.5 Sample Processing for Transmission Electron Microscopy

Similarly, corneal epithelial cultures on AM were processed for examination by transmission electron microscopy. All samples were fixed in 4% glutaraldehyde in PBS buffer for a minimum of two hours prior to routine processing, as detailed in section 2.1.6.

2.9.6 Quantitative Analysis of Corneal Cells Cultivated with Human Serum

Data pertaining to human corneal epithelial cell cultures on AM were collected exactly as outlined in section 2.1.7. Mean values were calculated and plotted in terms of percentage difference from control human cornea. The statistical significance of these observed differences was again determined using the raw data, as described in section 2.1.9.

3 RESULTS

3.1 Comparison of Cellular and Denuded Amniotic Membrane as Carriers for Human Limbal Stem Cell Cultivation

Human limbal cells were cultivated on both cellular and denuded amniotic membranes. Their relative usefulness as a carrier was morphologically analysed using scanning and transmission electron microscopy. A quantitative study was undertaken to compare various parameters including areas of intercellular space, the number of desmosomal junctions between cells and the number of basal attachments to the substrate.

3.1.1 Scanning Electron Microscopy

3.1.1.1 Limbal Cells on Denuded Amniotic Membrane

Examination of the apical surface of the limbal cells cultivated on denuded AM showed a continuous layer of flat polygonal epithelial cells (plate 3.1). The cells averaged 50-60 microns in diameter and were similar in appearance to normal human corneal epithelial cells. In some areas, epithelial cells appeared to be undergoing the process of desquamation, as would be expected in a healthy epithelial sheet. Cultivated cells were closely attached to each other with tightly opposed cell junctions and distinct cell boundaries (plate 3.5) and the apical surface of the cells were covered in short microvilli (plate 3.7).

3.1.1.2 Limbal Cells on Cellular Amniotic Membrane

With regard to cells cultured on cellular amniotic membrane, not all of the AM was covered with limbal epithelial cells. The AM epithelial cells appeared disrupted (plate 3.2) and had large intercellular spaces and long, distended microvilli (plate 3.8). Where limbal cells were present on top of the AM epithelial cells, they did not appear to be well attached to the cells beneath them (plate 3.3). In places the limbal cells appeared to be burrowing beneath the AM epithelial cells and making direct contact with the basement membrane (plate 3.4). Adjacent limbal cells were not tightly attached to each other, and there were prominent intercellular spaces (plate 3.6).

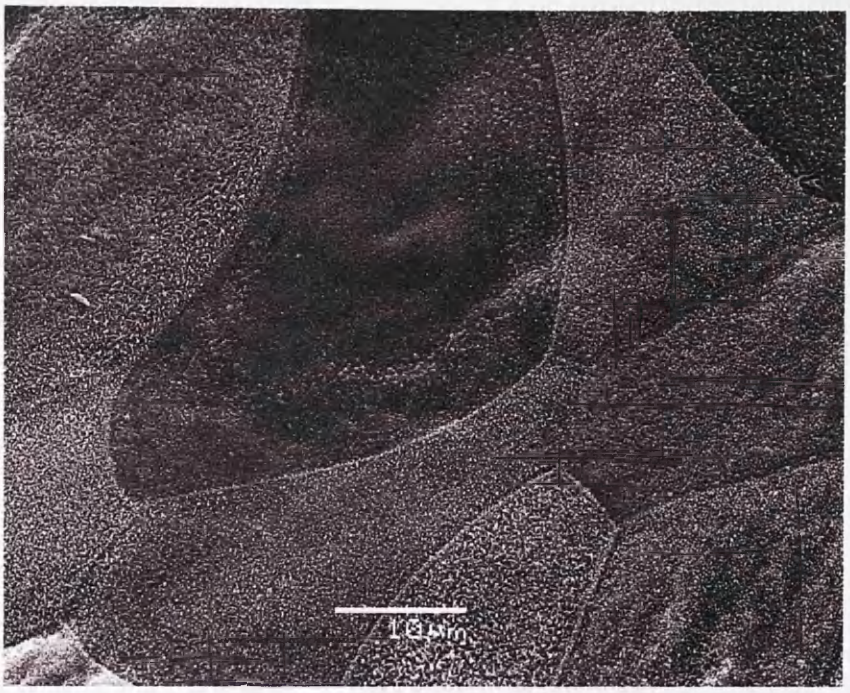


Plate 3.1: Scanning electron micrograph of human limbal epithelial cells on denuded amniotic membrane. Limbal epithelial cells formed a confluent layer, with prominent cell borders. Cells appeared healthy and well-developed, similar to *in vivo* corneal epithelium. [Scale bar = 10µm]

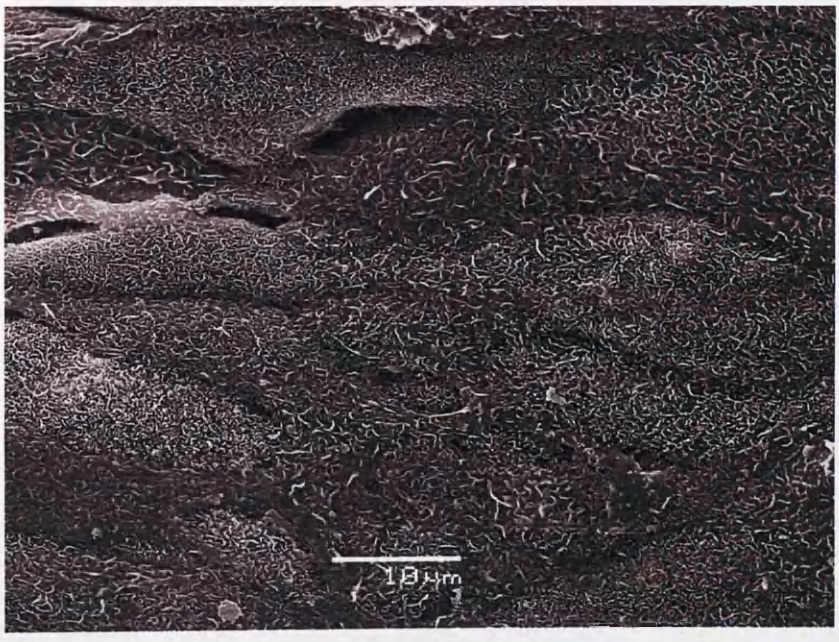


Plate 3.2: Scanning electron micrograph of human limbal epithelial cells on cellular amniotic membrane. In contrast to those grown on denuded AM, limbal epithelial cells did not appear to be in very good condition and had irregular cell borders. [Scale bar = 10µm]

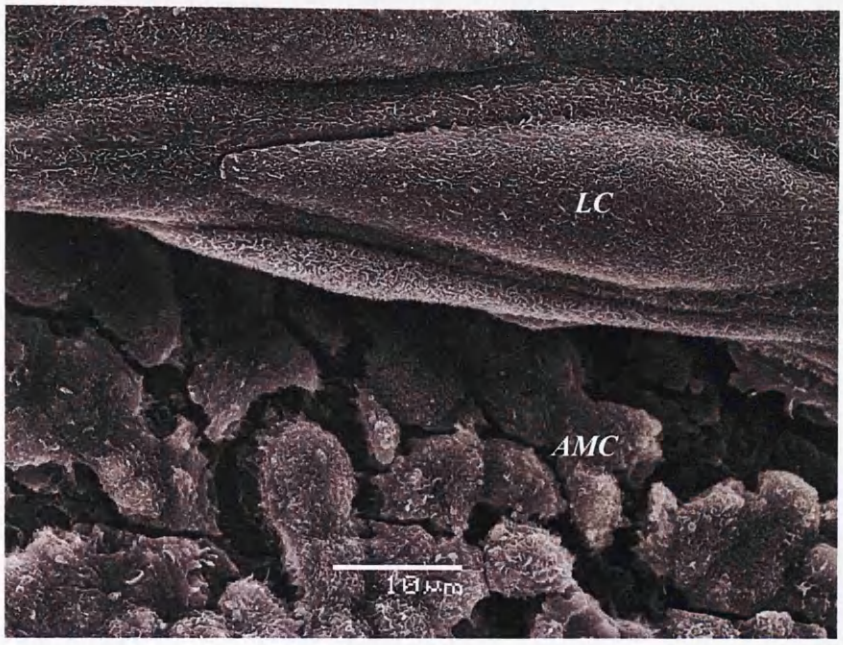


Plate 3.3: Scanning electron micrograph of human limbal epithelial cells cultivated on cellular amniotic membrane. In some areas, the limbal cells *[LC]* appeared to grow over the top of the amniotic epithelial cell debris *[AMC]* with poor basal attachment. [Scale bar = 10μm]

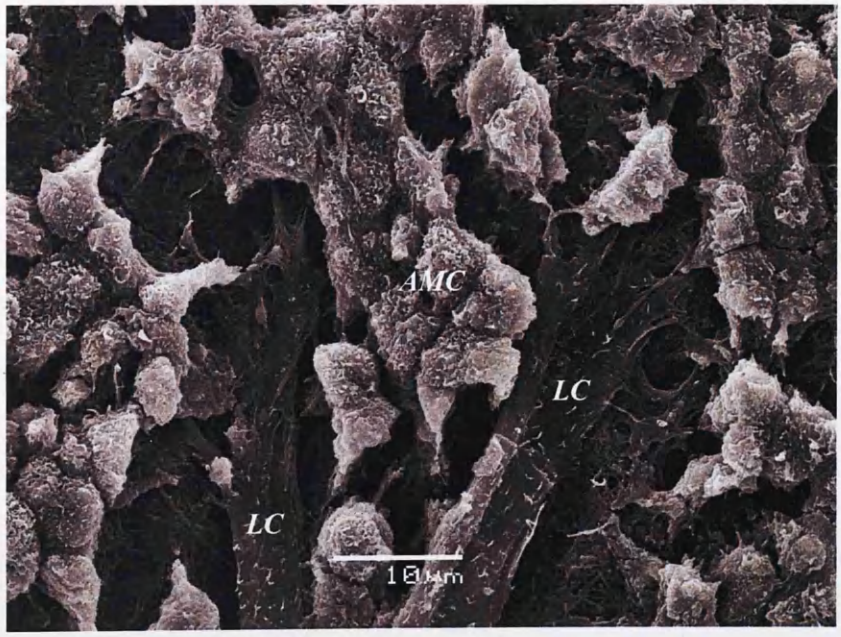


Plate 3.4: Scanning electron micrograph of cultivated human limbal epithelial cells on cellular amniotic membrane. In other places, cultivated limbal cells *[LC]* spread beneath the degraded AM epithelial cells *[AMC]* apparently making contact with the basement membrane itself. [Scale bar = 10μm]

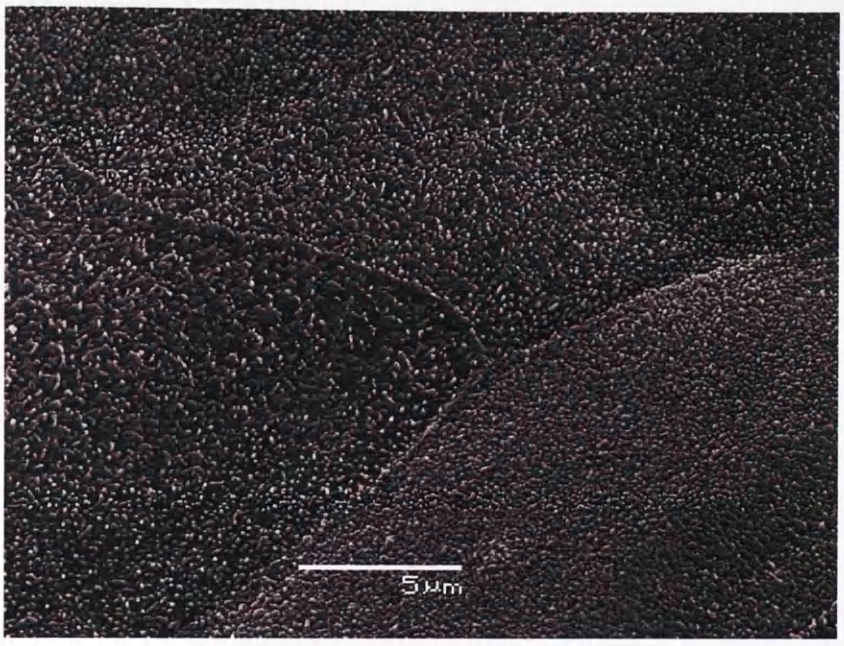


Plate 3.5: Scanning electron micrograph of human limbal epithelial cells on denuded amniotic membrane. Cultivated epithelial cells were tightly opposed with distinct ridges at cell boundaries. [Scale bar = 5µm]

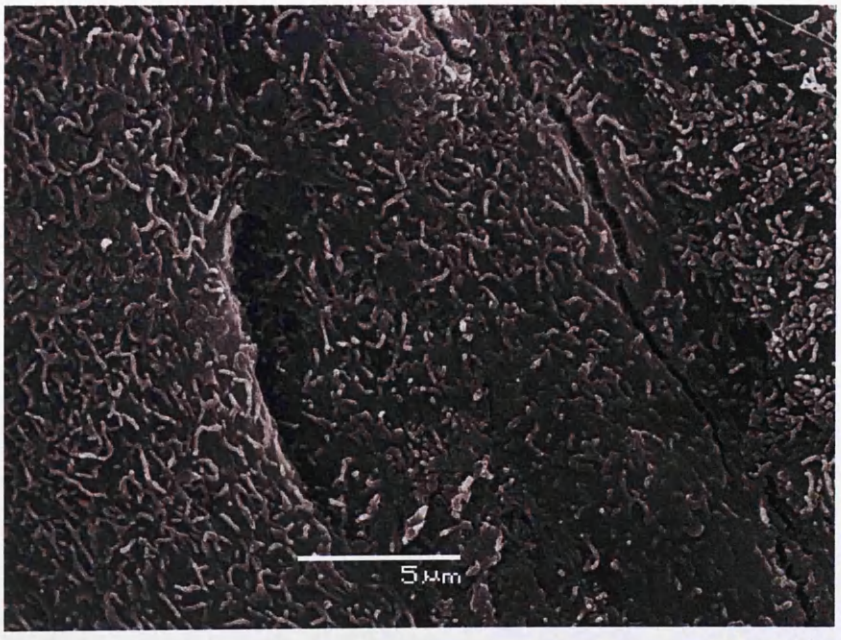


Plate 3.6: Scanning electron micrograph of cultivated human limbal epithelial cells on cellular amniotic membrane. Cell borders were less pronounced and not as tightly opposed as those in the denuded AM culture. [Scale bar = 5µm]

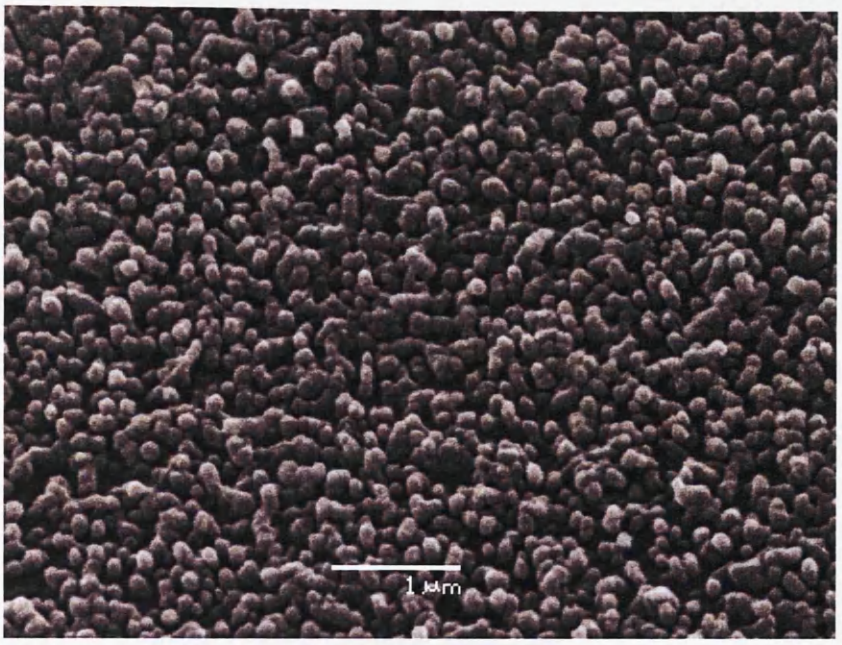


Plate 3.7: Scanning electron micrograph of human limbal epithelial cells on denuded amniotic membrane. The apical surface of the cells was covered in short, regular microvilli. [Scale bar = 1µm]

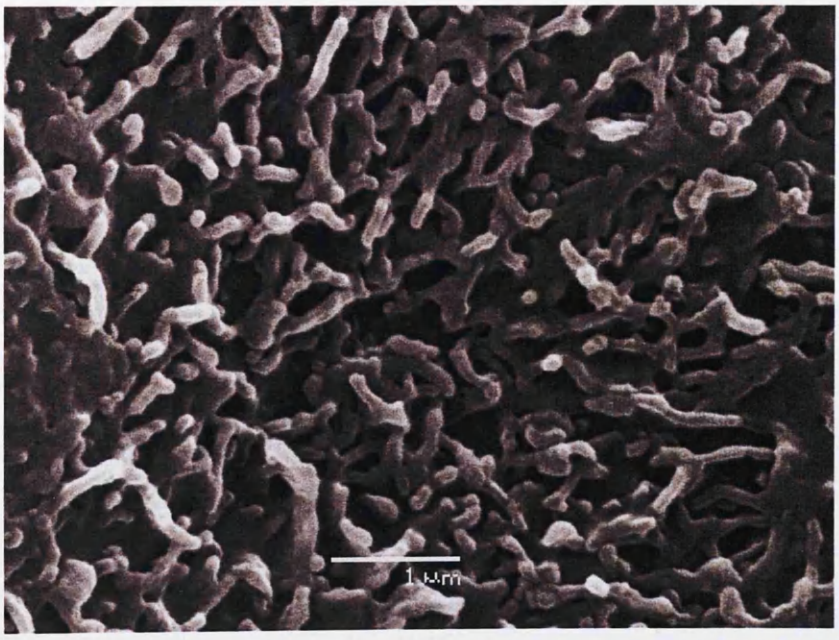


Plate 3.8: Scanning electron micrograph of human limbal epithelial cells on cellular amniotic membrane. Superficial cells were covered in long, distended microvilli. [Scale bar = 1µm]

3.1.2 Transmission Electron Microscopy

3.1.2.1 Limbal Cells on Denuded Amniotic Membrane

Limbal epithelial cells cultivated on denuded amniotic membrane were well-stratified and differentiated into 4-5 distinct cell layers (plate 3.9). Adjacent cells were joined by numerous desmosomes and intercellular spaces were minimal (plate 3.11). Basal epithelial cells adhered well to the AM substrate with hemidesmosomal attachments and produced basement membrane material (plate 3.13).

3.1.2.2 Limbal Cells on Cellular Amniotic Membrane

In contrast, limbal epithelial cells cultivated on the cellular/intact amniotic membrane did not appear to be in good condition. The amniotic epithelial cells were necrotic, with degraded external membranes and large intercellular spaces between adjacent cells of the monolayer (plate 3.10). These cells were not well adhered to the AM matrix and there was little evidence of any basement membrane material (plate 3.15). The limbal epithelial cells were at most three layers thick and in some areas formed a monolayer; as such they were neither well-stratified nor differentiated. Spaces between the cells were large and there were very few desmosomal attachments (plate 3.12). Attachment of the basal limbal cells to the AM epithelial cells was via desmosomal junctions, though these were infrequent (plate 3.14). Not all of the AM was covered with limbal cells, and in places there were no cells at all (plate 3.16).

Plate 3.9: Transmission electron micrograph of human limbal epithelium *[LC]* cultivated on denuded amniotic membrane *[AM]* taken at low magnification. Cells were well-stratified and differentiated and formed 4-5 layers. [Scale bar = 2μm]

Plate 3.10: Transmission electron micrograph of human limbal epithelium *[LC]* cultivated on cellular amniotic membrane *[AM]* taken at low magnification. Amniotic epithelial cells *[AMC]* appeared to be necrotic and were not well adhered to the AM. Corneal cells formed a monolayer in places and were neither well-stratified nor differentiated. [Scale bar = 2μm]

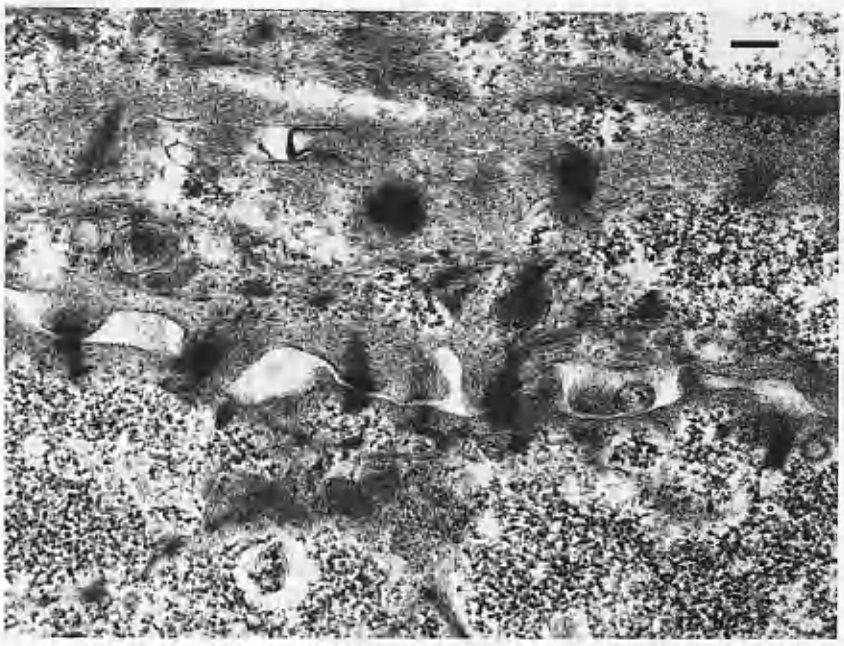


Plate 3.11: Transmission electron micrograph depicting cultivated human limbal epithelium on denuded AM at high magnification. Numerous desmosomes joined adjacent cells in all cell layers. [Scale bar = 200nm]

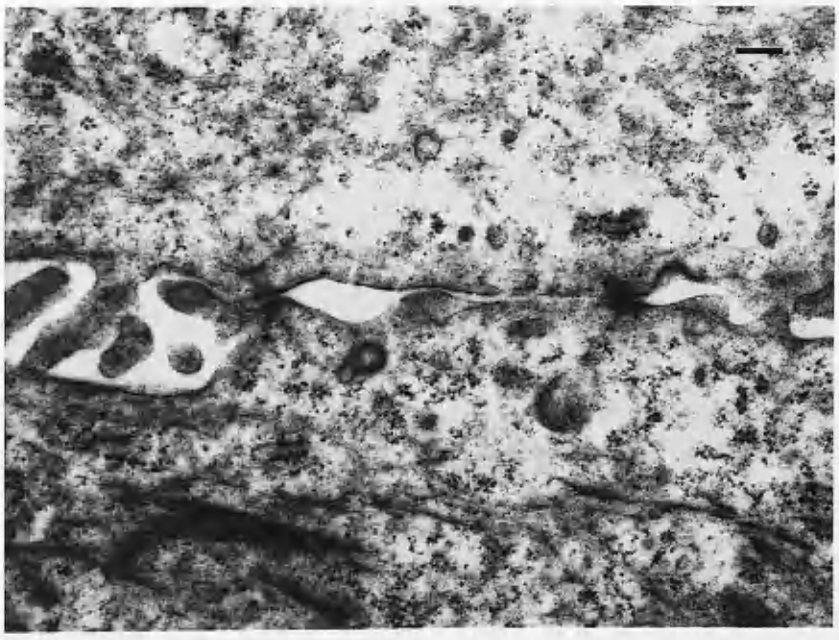


Plate 3.12: Transmission electron micrograph of human corneal epithelium on cellular AM at high magnification, showing infrequent desmosomal junctions that joined adjacent cells. [Scale bar = 200nm]

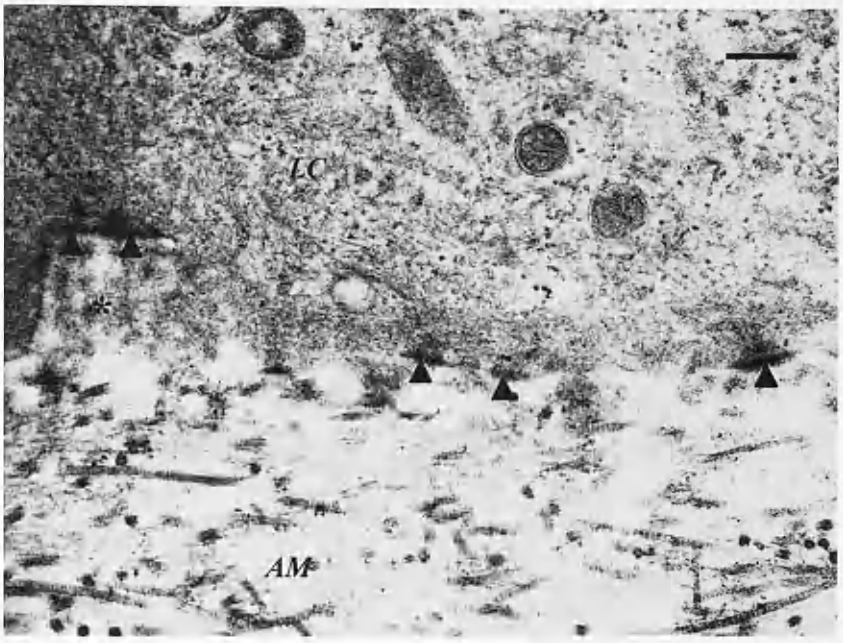


Plate 3.13: Transmission electron micrograph of the human limbal epithelium *[LC]* cultivated on denuded amniotic membrane *[AM]*. Basal corneal cells were very well adhered to the substrate via numerous hemidesmosomal junctions (arrowheads) and appeared to be secreting basement membrane material (*). [Scale bar = 500nm]

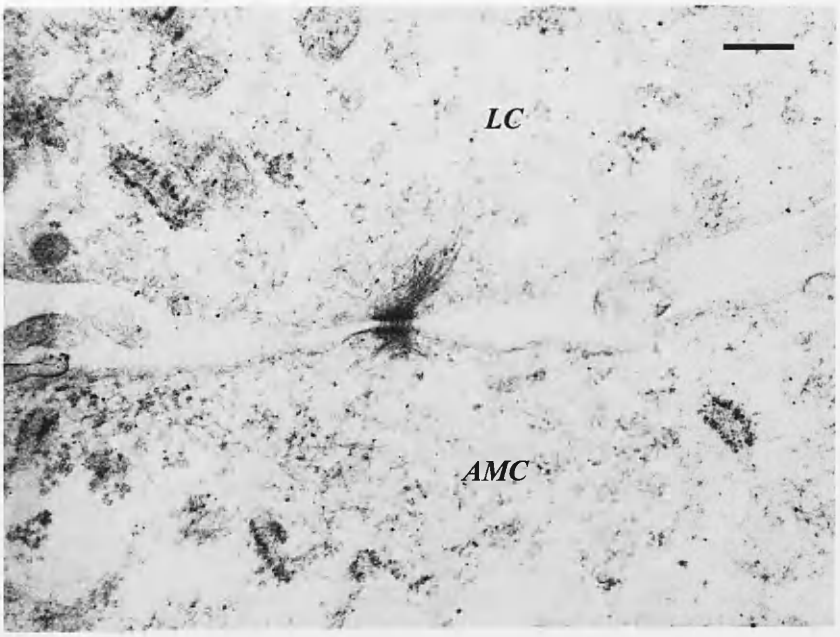


Plate 3.14: Transmission electron micrograph showing human limbal epithelium on cellular AM at high magnification. Basal limbal cells *[LC]* were attached the underlying degraded amniotic epithelial cell layer *[AMC]* by infrequent desmosomal junctions. [Scale bar = 500nm]

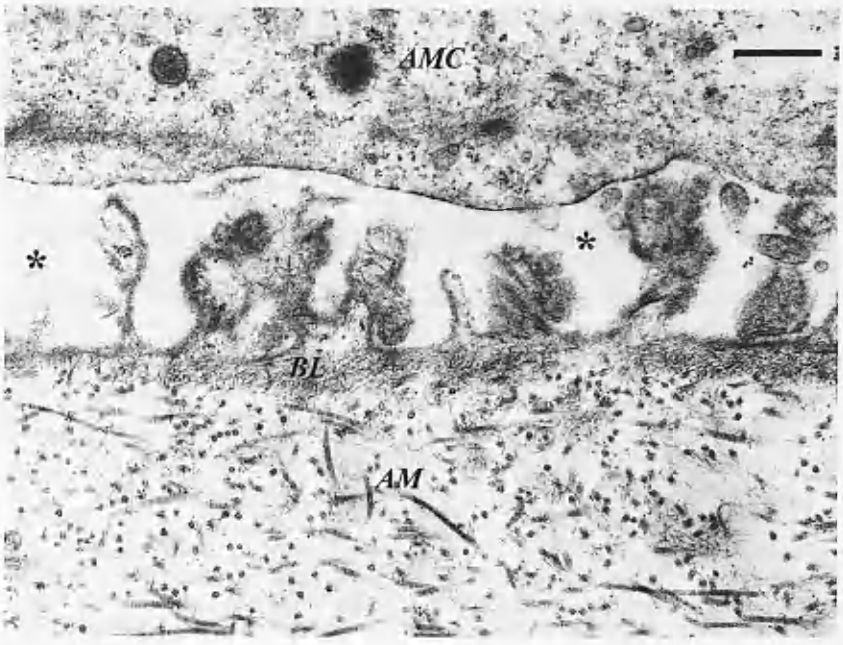


Plate 3.15: Transmission electron micrograph of the basement membrane region of the cellular AM. Necrotic amniotic epithelial cells *[AMC]* were not well attached to the basal lamina *[BL]*. There were large spaces, as indicated by the asterisks and there was little evidence of hemidesmosomal junctions. [Scale bar = 1μm]

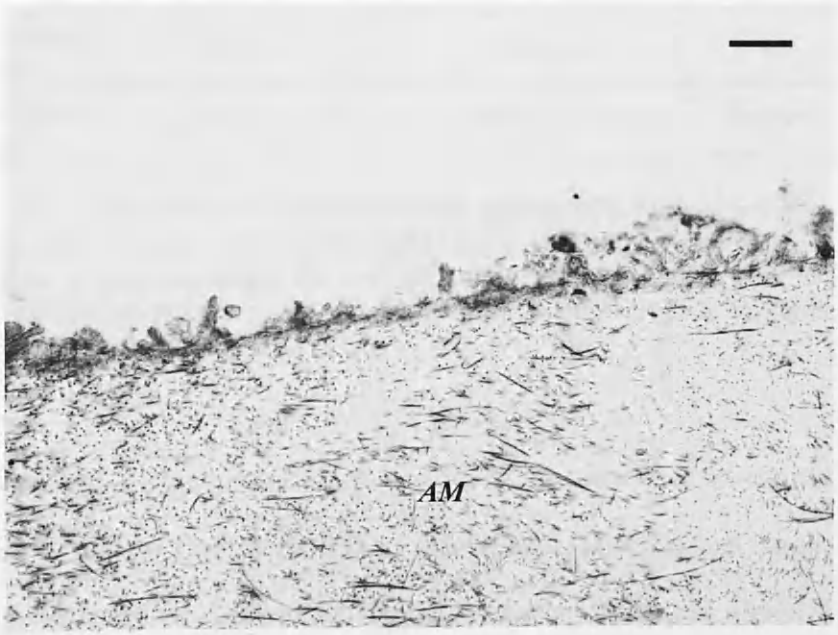


Plate 3.16: Transmission electron micrograph showing cellular amniotic membrane *[AM]* taken at high magnification. There were some areas of membrane completely devoid of a cell layer. [Scale bar = 1μm]

3.1.3 Quantitative Study

A number of parameters were quantitatively compared between the limbal cells on denuded AM and those on intact AM, the mean data (±S.D.) are shown in table 3.1 below. Fourteen data points were collected for each parameter, except for the cell surface area which was calculated using scanning electron microscopy and therefore facilitated more measurements (n=50). Normal human donor corneal epithelium from Northwest Lions Eye Bank was included in this study as a control.

	Limbal cells cultivated on denuded AM	**Limbal cells cultivated on cellular AM**	**Human corneal epithelium**
Thickness of cell layers (μm)	18.65 ± 2.33	11.10 ± 2.53	29.02 ± 2.29
Number of cell layers	4.21 ± 0.89	1.79 ± 0.80	6.64 ± 0.74
Intercellular space area (μm^2)	0.18 ± 0.11	1.15 ± 1.11	0.11 ± 0.08
Number of desmosomes	2.29 ± 1.33	0.43 ± 0.65	3.21 ± 1.37
Number of basal junctions	2.00 ± 1.11	0.43 ± 0.51	4.71 ± 1.44
Cell surface area (μm^2)	525.99 ± 264.43	488.05 ± 317.79	1093.89 ± 335.43

Table 3.1: Comparison of cultivated human limbal cells on denuded and cellular AM with normal human cornea. Mean data (± S.D.) are shown. Fourteen data points were taken at random, except for the cell surface areas in which case n=50. The averages for intercellular area and number of desmosomes are given for random 3μm long interfaces between adjacent cells.

Cultivated limbal cells on denuded AM were fairly comparable to the corneal control epithelium. Though there were fewer desmosomal junctions and larger spaces between cultivated cells, these differences were not statistically significant. The epithelium cultivated on denuded AM had statistically fewer cell layers (hence a thinner epithelium, $P<0.001$), smaller cells ($P<0.001$) and fewer basal junctions

(P<0.001) than the control cornea. It is important to note that for the cellular AM cultures; only the limbal cells were counted. By contrast, highly significant differences (P<0.001) were observed for every parameter when comparing cultivated limbal cells on cellular amniotic membrane with the control. When compared to normal cornea, there was an almost 10 fold increase (935%) in intercellular spaces in the cellular AM culture system, as well as fewer mechanical attachments both between cells (87% reduction) and with the underlying amniotic epithelium (91% fewer junctions than in the control epithelium). The limbal cells on cellular AM also had significantly fewer cell layers (73%) than control corneal epithelium. As such, cells cultivated on denuded AM were generally found to more closely resemble the normal human cornea, with significantly smaller intercellular spaces and more numerous mechanical attachments to each other and to the underlying basement membrane.

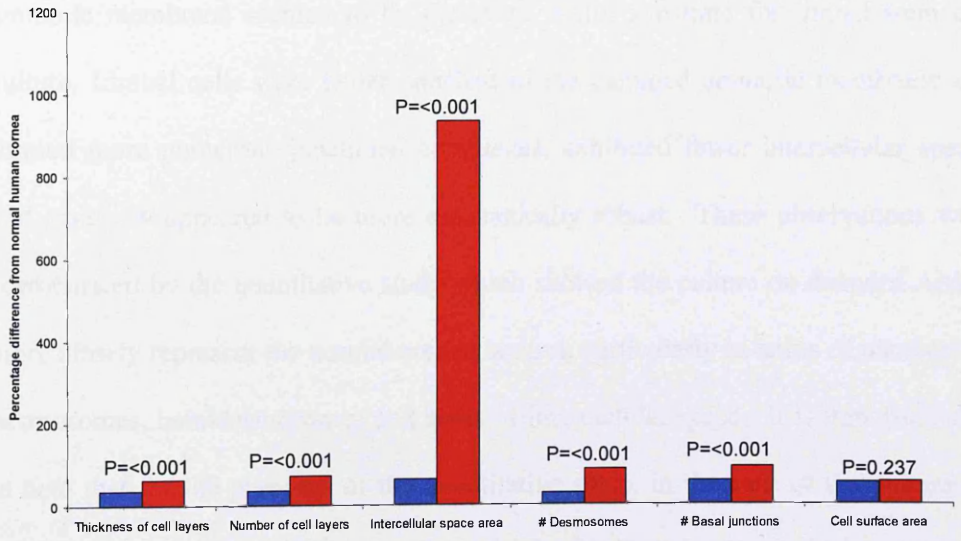


Chart 3.1: Comparison of cultivated human limbal epithelial cells on denuded (blue) and cellular (red) amniotic membrane, expressed as percentage difference from normal human cornea control. Raw data were used to calculate statistical significance (P values) of observed differences between denuded and cellular culture systems and these are stated above the bars.

For ease of interpretation, mean data were plotted as percentage differences from the control (normal human cornea) and the two culture systems compared with each other (chart 3.1). There were highly significant differences ($P<0.001$) for all parameters except cell surface area. The most obvious difference was in terms of intercellular space areas which were vastly increased by the cellular AM culture system. There was also a highly significant difference ($P<0.001$) in the number of desmosomes between neighbouring epithelial cells between cultures. In terms of basal attachments of the cultivated epithelial cells to the substrates, there was again a highly significant ($P<0.001$) difference between the number of hemidesmosomes at the basement membrane in the denuded culture and that of desmosomal junctions at the basal limbal cell-AM epithelial cell interface in the culture on intact AM.

3.1.4 Summary Interpretations

Examination of the cultured epithelial cells by SEM and TEM revealed that denuded amniotic membrane seemed to be the more useful substrate for limbal stem cell culture. Limbal cells were better attached to the denuded amniotic membrane and formed more numerous junctional complexes, exhibited fewer intercellular spaces and generally appeared to be more mechanically robust. These observations were corroborated by the quantitative study which showed the culture on denuded AM to more closely represent the normal cornea control, particularly in terms of numbers of desmosomes, hemidesmosomes and areas of intercellular space. It is important also to note that for the purposes of the quantitative study, in the case of the culture on cellular AM, only the small area of the sheet with limbal cells could be analysed. Scanning electron microscopy revealed vast areas of the sheet to be devoid of limbal cells, probably due to their poor attachment to the necrotic amniotic epithelium.

4 RESULTS

4.1 Cultivation of Human Corneal Endothelial Cells on Denuded Amniotic Membrane

The transplantation of cultivated human corneal endothelial cells (HCEC) could potentially be very useful for the treatment of diseases caused by corneal endothelial disorders. In this investigation, the feasibility of using denuded AM as a carrier for this application was examined using scanning and transmission electron microscopy. Cultivated HCEC were compared to *in vivo* human corneal endothelium.

4.1.1 Scanning Electron Microscopy

4.1.1.1 Control Human Corneal Endothelial Cells

Scanning electron microscopy of control HCEC revealed a continuous layer of flat squamous polygonal cells, fairly uniform in size with an average cell surface area of 345.2μm^2 (S.D.±100.l) (plate 4.1). Adjacent cells were tightly opposed with pronounced ridges at cell boarders (plate 4.3). Corneal endothelial cells had flattened surfaces (plate 4.5), lacking the microvilli seen in corneal epithelial cells.

4.1.1.2 Human Corneal Endothelial Cells Cultivated on Denuded AM

Human corneal endothelial cells cultured on denuded AM had an average cell surface area of 309.9μm^2 (S.D.±84.7) (plate 4.2). The interdigitations at the cell boundaries were less pronounced than those of *in vivo* corneal endothelium however the cells appeared to be healthy-looking, well-developed and were tightly opposed (plate 4.4). Cultivated cells had flattened apical surfaces (plate 4.6) as did the control cells.

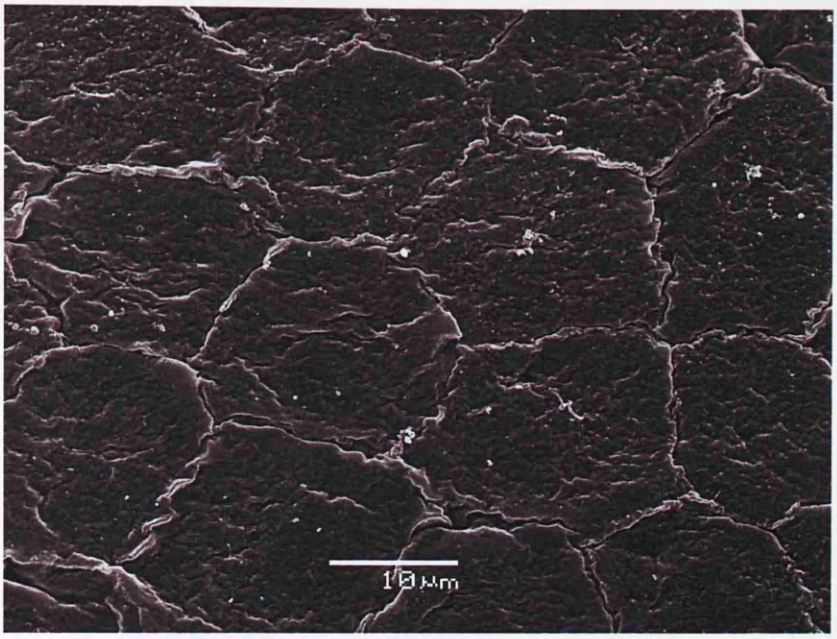


Plate 4.1: Scanning electron micrograph of control human corneal endothelial cells taken at low magnification. Cells were polygonal, fairly uniform in size and had distinct ridges at cell borders. [Scale bar = 10µm]

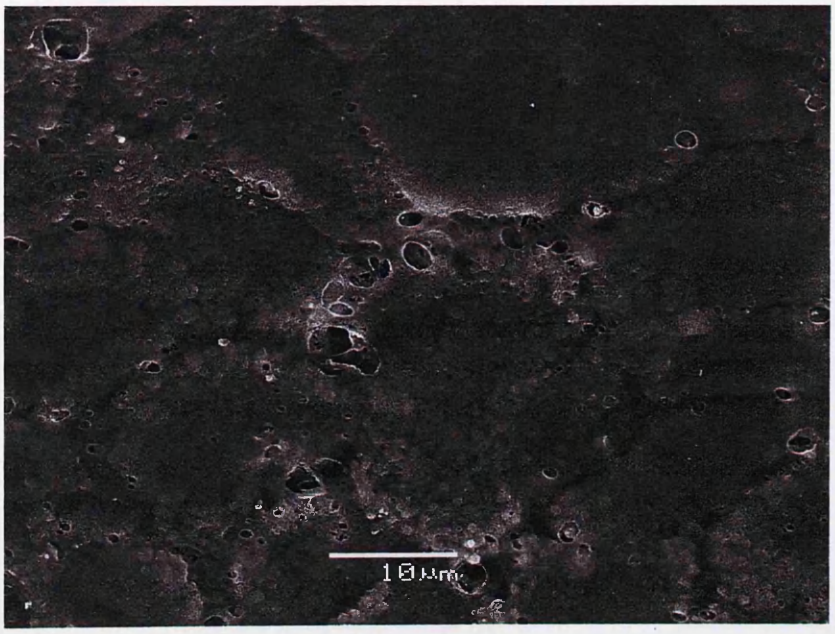


Plate 4.2: Scanning electron micrograph of cultivated human corneal endothelial cells on denuded amniotic membrane. Cells formed a confluent cell layer and appeared healthy and well-formed, despite a lack of distinct cell borders. [Scale bar = 10µm]

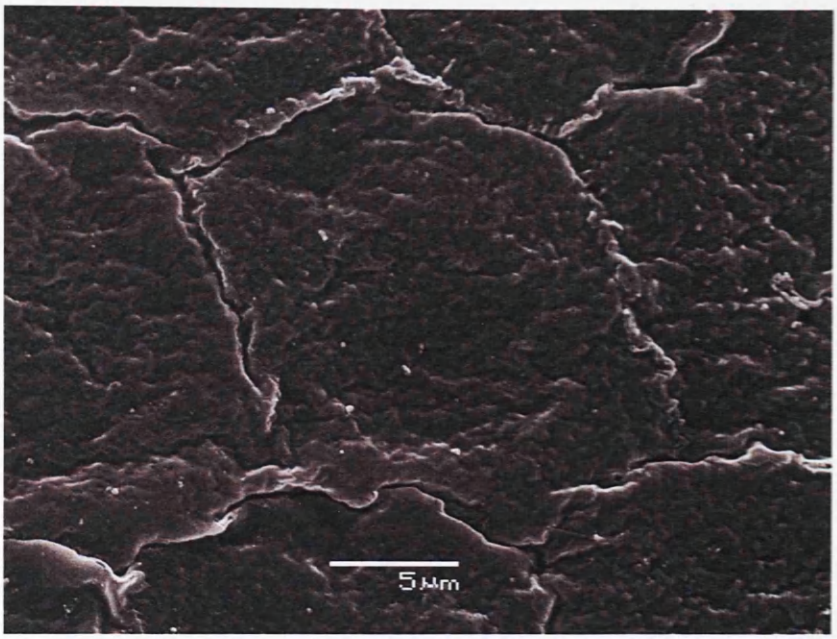


Plate 4.3: Scanning electron micrograph of control human corneal endothelial cells taken at high magnification. There were very few intercellular spaces between neighbouring cells and cell borders were well-pronounced. [Scale bar = 5µm]

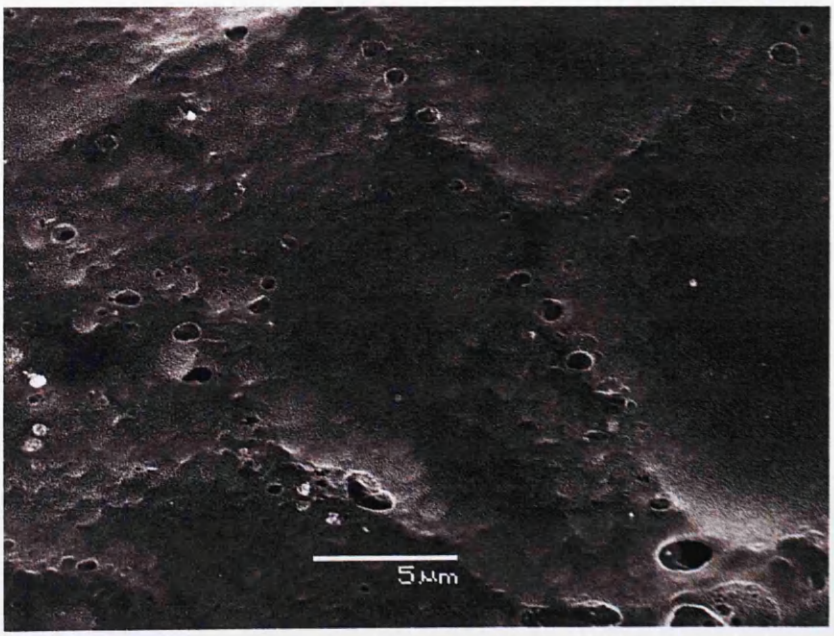


Plate 4.4: Scanning electron micrograph of cultivated human corneal endothelial cells on denuded amniotic membrane. Cultivated cells formed a confluent cell layer, with few intercellular spaces, yet lacked the distinct cell borders seen in the control. [Scale bar = 5µm]

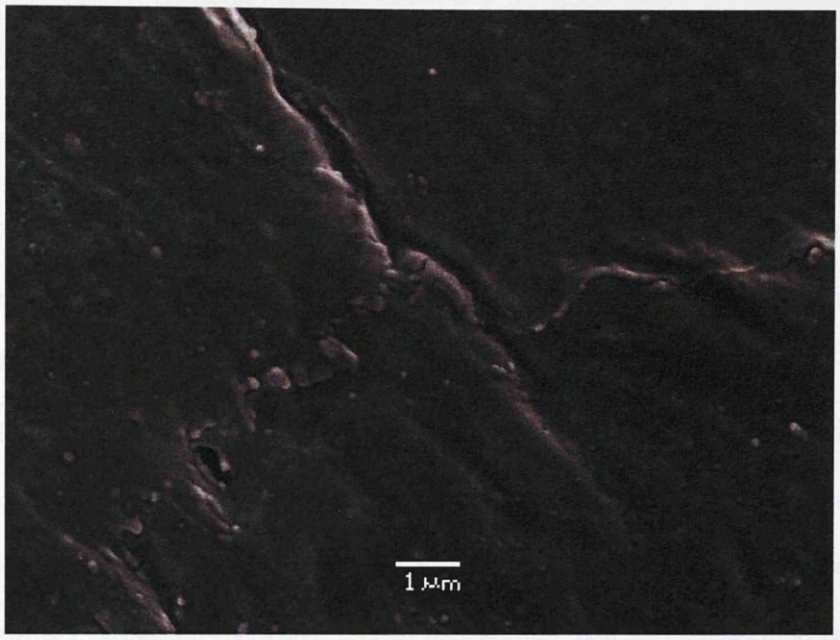


Plate 4.5: Scanning electron micrograph of control human corneal endothelial cells, taken at very high magnification. The endothelial cells had a very flat surface, lacking microvilli seen on corneal epithelial cells. [Scale bar = 1µm]

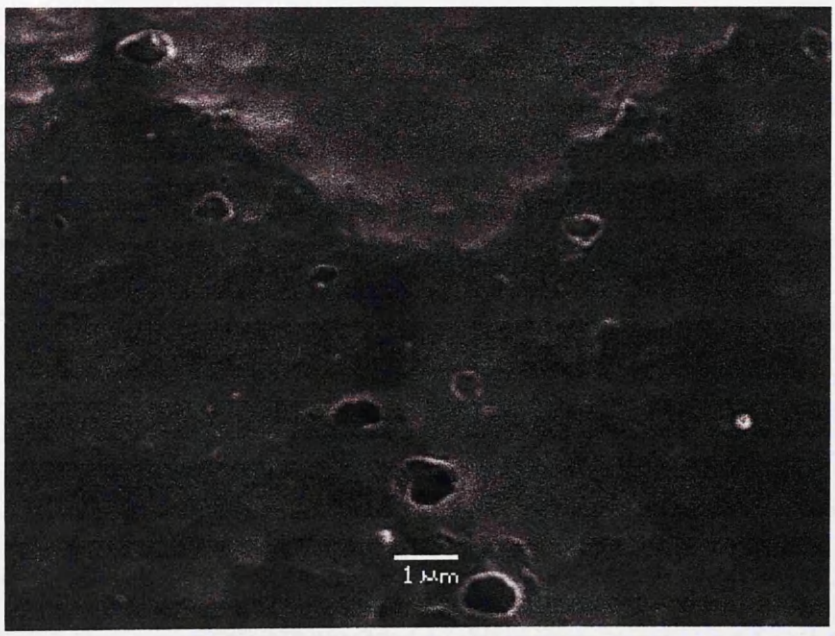


Plate 4.6: Scanning electron micrograph of cultivated human corneal endothelial cells on denuded amniotic membrane. High magnification confirmed that the cultivated cells had smooth upper surfaces, with little or no microvilli. [Scale bar = 1µm]

4.1.2 Transmission Electron Microscopy

4.1.2.1 Control Human Corneal Endothelial Cells

Transmission electron microscopy of control cells showed a monolayer of densely stained human corneal endothelium on a thick Descemet's membrane (plate 4.7). The cells appeared to be in good condition and were well attached both to each other (plate 4.9) and to the underlying extracellular matrix (plate 4.11). Any intercellular spaces between cells were minimal and there appeared to be interdigitations and folds in the cell membranes at the cell borders. Cells had prominent nuclei. The apical surface of the cells appeared largely smooth and there did not seem to be many microvilli-like protrusions (plate 4.9).

4.1.2.2 Human Corneal Endothelial Cells Cultivated on Denuded AM

Examination of the cultivated endothelial cells on AM by transmission electron microscopy revealed a fairly continuous monolayer of flat squamous polygonal endothelial cells (plate 4.8). Cells appeared fairly uniform in size. The condition of the culture sheets varied. In places, the cultured cells looked healthy, well-developed, and were closely attached to each other with tightly opposed cell junctions (plate 4.10). Adjoining cells were found to overlap each other slightly to maintain maximal contact, as would be expected and is seen in the control endothelium (plate 4.10). Other areas of the culture sheet appeared less healthy, with some prominent intercellular spaces. The endothelial cells adhered well to the denuded amniotic membrane substrate and also produced basement membrane material (plate 4.12).

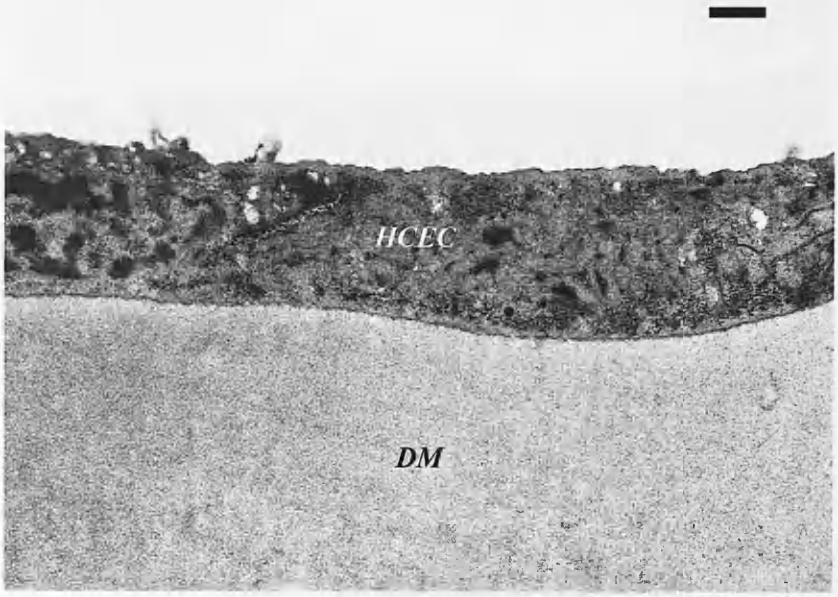


Plate 4.7: Transmission electron micrograph of control human corneal endothelium. A monolayer of endothelial cells *[HCEC]* rested on a thick Descemet's membrane *[DM]*. Adjacent cells were tightly opposed and overlapped slightly. [Scale bar = 2μm]

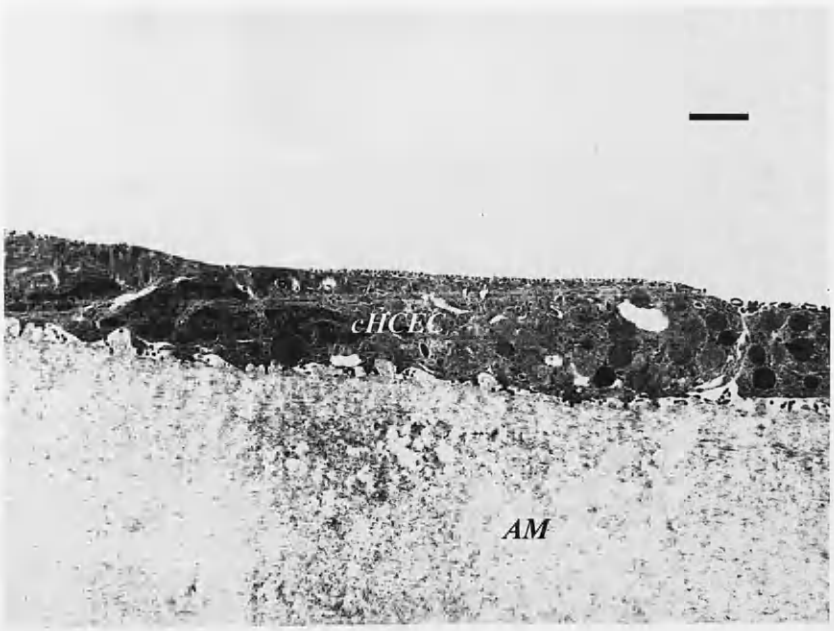


Plate 4.8: Transmission electron micrograph of cultivated human corneal endothelial cells *[cHCEC]* on denuded amniotic membrane *[AM]*. Cells appeared to be in good condition and formed a confluent monolayer. [Scale bar = 2μm]

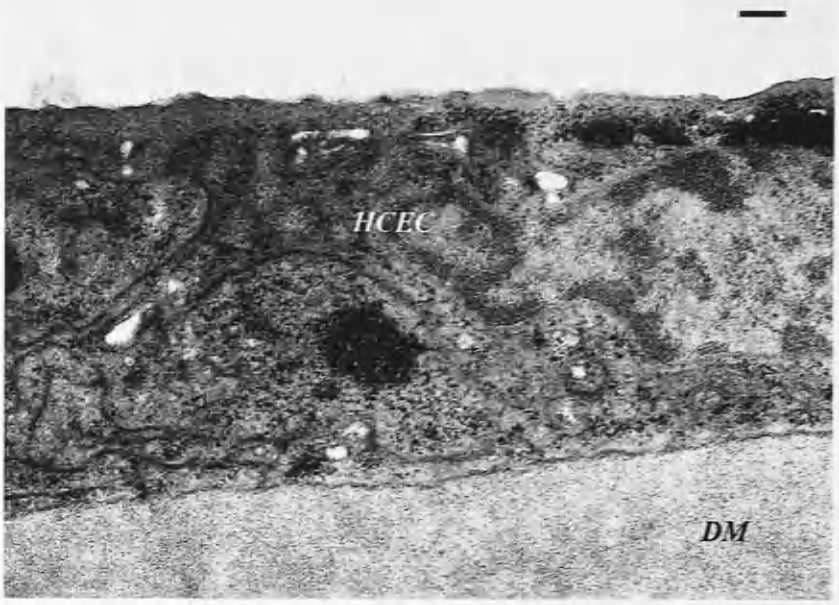


Plate 4.9: Transmission electron micrograph of control human corneal endothelium *[HCEC]* on Descemet's membrane *[DM]*. Adjoining cells overlapped each other slightly to maintain good contact and cell membranes were interdigitated at the cell borders. [Scale bar = 500nm]

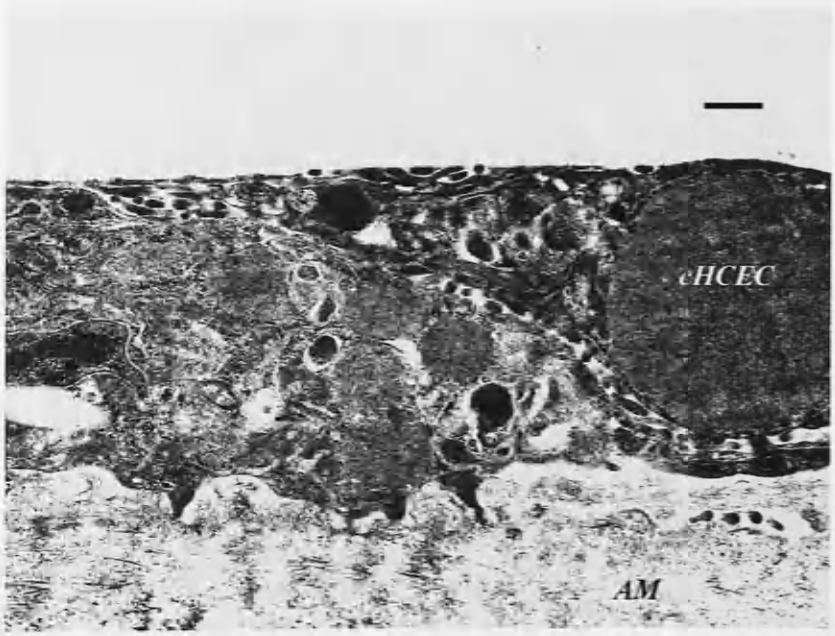


Plate 4.10: Transmission electron micrograph of cultivated human corneal endothelial cells *[cHCEC]* on denuded amniotic membrane *[AM]*. As seen with the control cells, there was considerable overlapping of adjacent cells and little in the way of intercellular spacing. [Scale bar = 500nm]

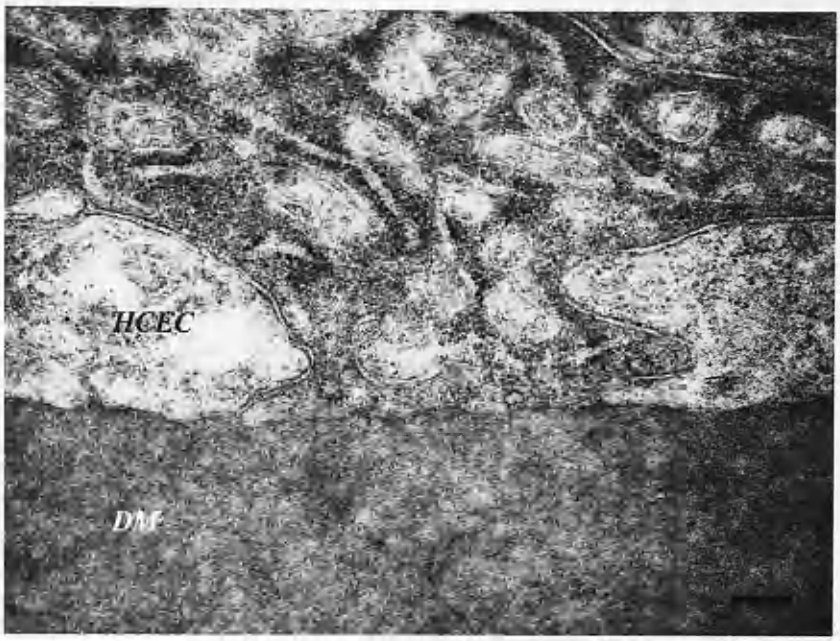


Plate 4.11: Transmission electron micrograph showing the basal region of the control human corneal endothelium *[HCEC]*. The cells were well attached to the underlying Descemet's membrane *[DM]*. [Scale bar = 200nm]

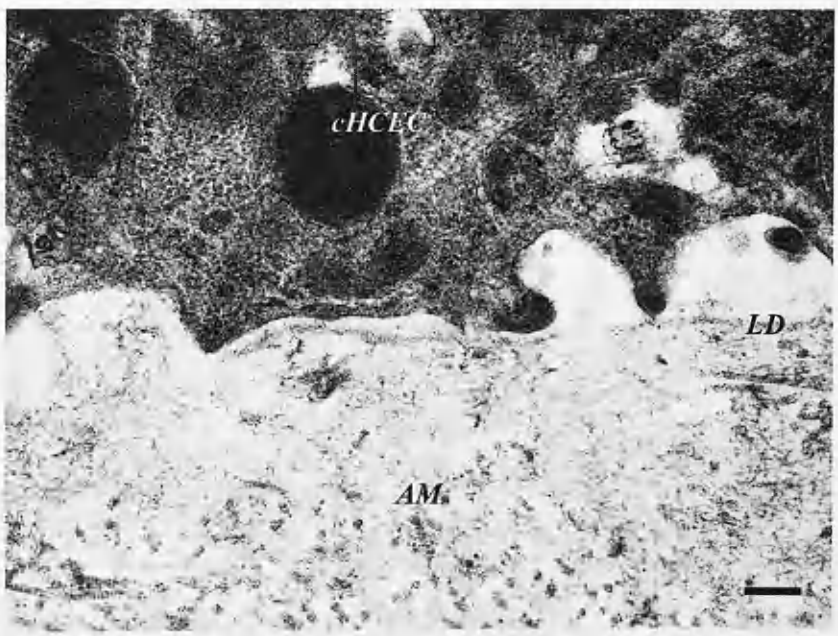


Plate 4.12: Transmission electron micrograph of the basal region of the cultivated human corneal endothelial cells *[cHCEC]* on denuded amniotic membrane *[AM]*. The AM appeared to be in good condition, with an intact lamina densa *[LD]*. The endothelial cells were well attached to the substrate and appeared to produce basement membrane material. [Scale bar = 200nm]

4.1.3 Summary of Observations

Scanning electron microscopy of HCEC cultivated on amniotic membrane revealed a continuous layer of flat squamous polygonal endothelial cells, uniform in size yet lacking the interdigitations at cell boundaries present in normal endothelium. Transmission electron microscopic images showed a monolayer of flat endothelial cells which appeared healthy and well-formed with tightly opposed cell junctions.

Although epithelial cells are frequently cultivated on amniotic membrane, this is a novel substrate for the culture of corneal endothelial cells. This study produced some promising results and demonstrates that tightly opposed human corneal endothelial cells can be cultivated on denuded AM with some success *in vitro*.

4.2 Morphological Analysis of Polyphenol-Treated Rat Corneal Endothelium in Long Term Storage

The storage of corneal tissue in the period following removal and prior to transplantation is one of the most important factors in determining a successful outcome to corneal transplantation. In this study, polyphenol was used with Optisol-GS to try to improve corneal endothelial cell viability and increase storage times. Polyphenol-treated rat corneal endothelial cells were analysed by SEM after 1, 2 and 4 weeks in storage and compared to those stored in Optisol-GS only (control).

4.2.1 Scanning Electron Microscopy

4.2.1.1 Untreated Rat Corneal Endothelial Cells

After one week in storage, the control (untreated) corneal endothelial cells were in fairly good condition, despite some expulsion of cell nuclei onto the surface of the cells (plate 4.13). By two weeks, the external cell membranes were degraded and the cell nuclei had become exposed (plate 4.15). At four weeks, the control endothelial cells had detached completely from Descemet's membrane in places (plate 4.17).

4.2.1.2 Polyphenol-Treated Rat Corneal Endothelial Cells

Compared with control cells, the polyphenol-treated endothelial cells appeared to be generally better preserved. There was little difference in appearance between cells at 1, 2 and 4 weeks in storage (plates 4.14, 4.16 and 4.18 respectively). While some shrinkage had occurred, cell membranes remained largely intact and adjacent cells were closely attached and had prominent cell borders. There was some surface debris on the treated cells which was almost certainly of polyphenol origin and not cellular.

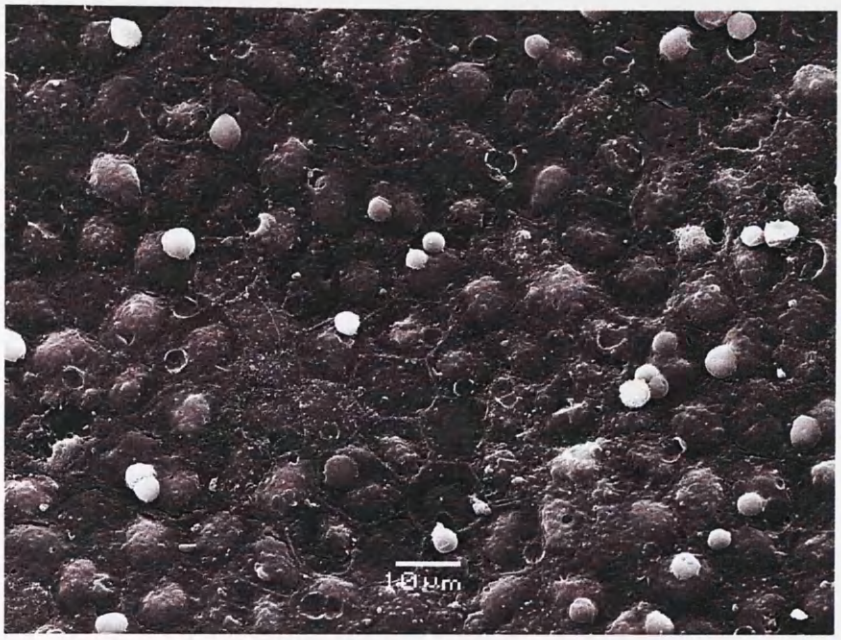


Plate 4.13: Scanning electron micrograph depicting control (untreated) rat corneal endothelium after 1 week in storage. Cells were generally in good condition, except for some regions where membranes were disrupted and there were exposed nuclei on the cell surface. [Scale bar = 10μm]

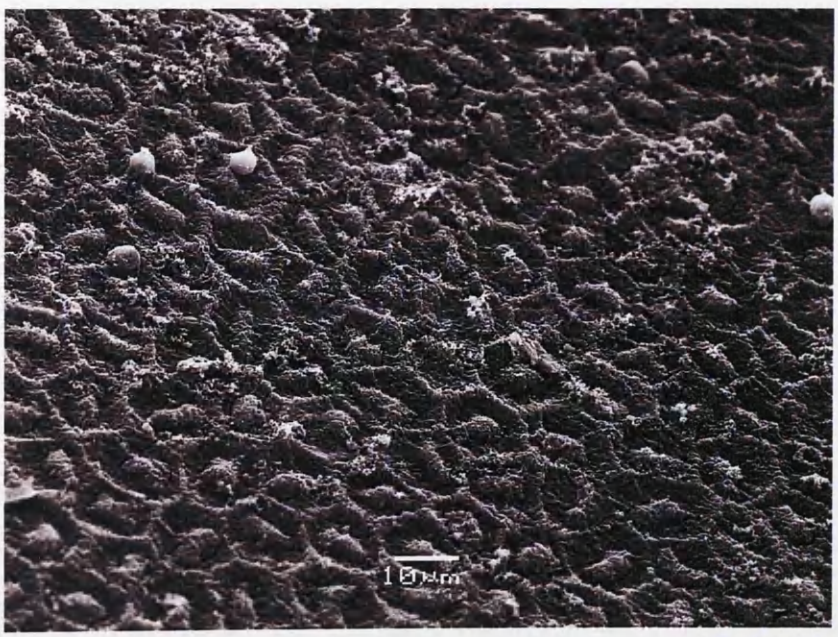


Plate 4.14: Scanning electron micrograph showing polyphenol-treated rat corneal endothelium after 1 week in storage. The endothelial membranes were still intact and whilst some shrinkage had occurred, there were fewer ruptured cells than in the control sample. The debris on the surface appeared to be of polyphenol origin. [Scale bar = 10μm]

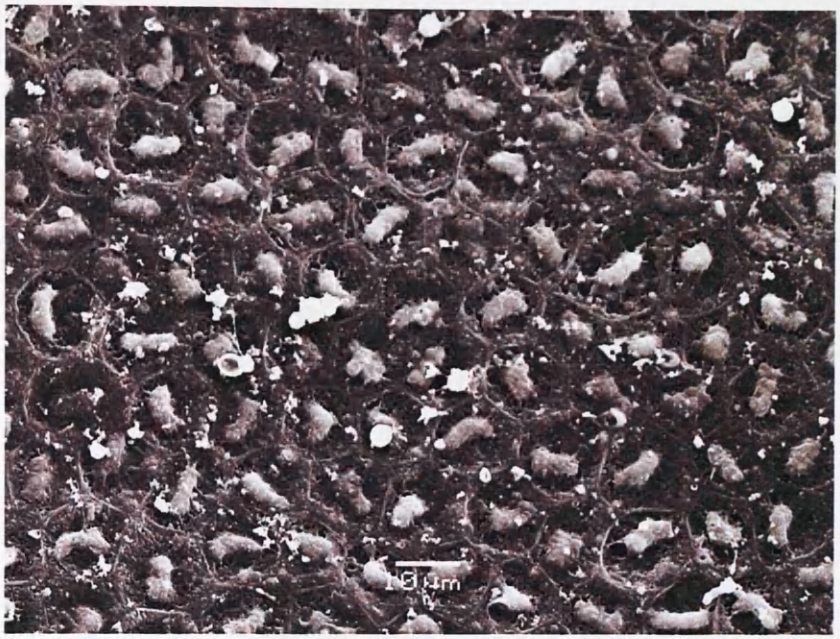


Plate 4.15: Scanning electron micrograph depicting control (untreated) rat corneal endothelium after 2 weeks in storage. Endothelial cell membranes were markedly disrupted and the nuclei were prominently exposed. [Scale bar = 10μm]

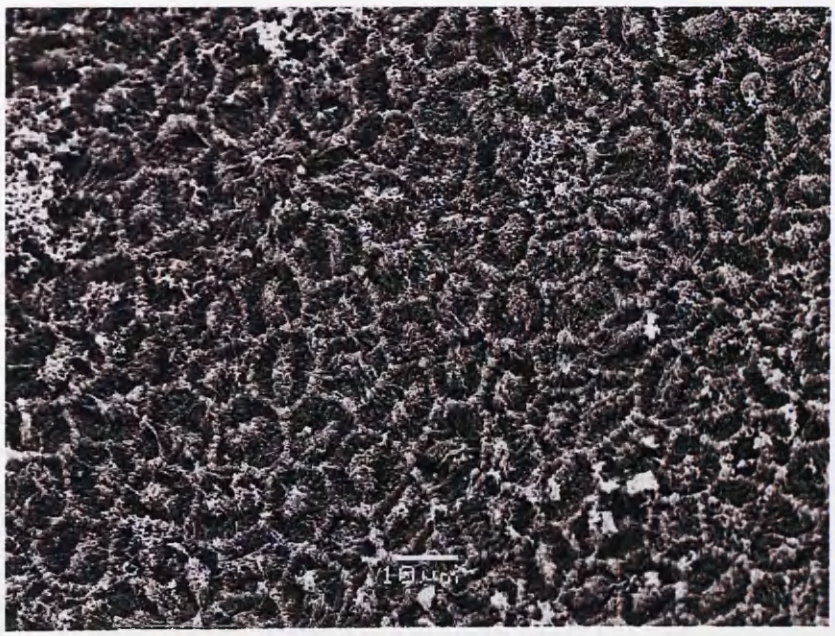


Plate 4.16: Scanning electron micrograph illustrating polyphenol-treated rat corneal endothelium after 2 weeks in storage. Most of the endothelial cell membranes were still intact although some shrinkage was evident. Cell nuclei and borders were quite pronounced. [Scale bar = 10μm]

Plate 4.17: Scanning electron micrograph showing control (untreated) rat corneal endothelium after 4 weeks in storage. There were some large areas (asterisk) where the endothelial cells had detached from Descemet's membrane, as depicted above. [Scale bar = 10µm]

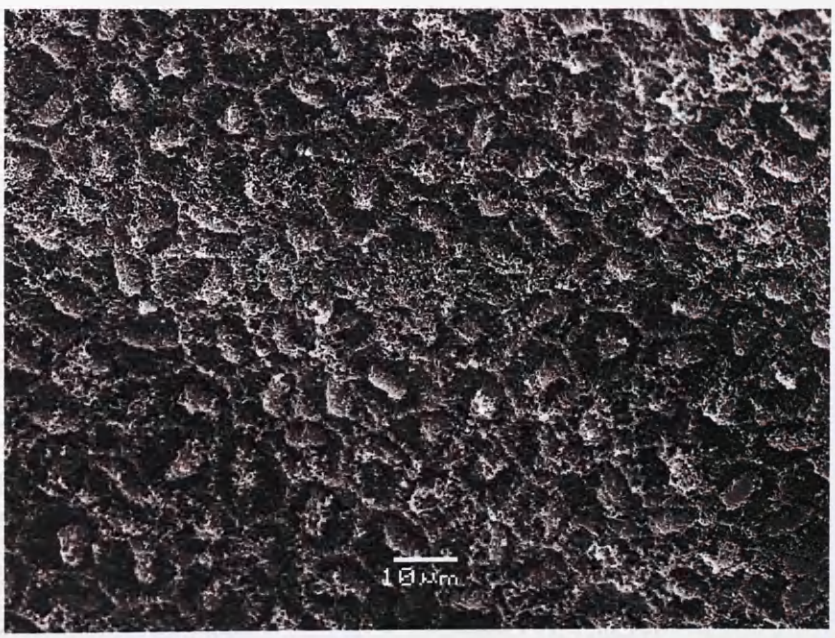


Plate 4.18: Scanning electron micrograph depicting polyphenol-treated rat corneal endothelium after 4 weeks in storage. These treated cells were in better condition than the control cells and even at 4 weeks, cell membranes remained intact. [Scale bar = 10µm]

4.2.2 Summary of Observations

Treatment of rat corneal endothelial cells with polyphenol for 24 hours prior to storage in Optisol-GS appears to help with preservation. Polyphenol treated cells had more intact membranes and more defined cell borders than the control cells. They also appeared to be better attached to Descemet's membrane, since it was only in the control samples that cells detached from the membrane.

The collapse of the cell membranes in the polyphenol-treated cells may be an artefact of the SEM preparation. However, even if it is genuine, it is clear that the condition of the cell membranes is much better in the polyphenol-treated cells than the controls.

5 RESULTS

5.1 Evaluation of Sterilized, Freeze-Dried Amniotic Membrane

A variety of unique characteristics make amniotic membrane a useful tool in the *ex vivo* expansion of limbal stem cells and in ocular surface reconstruction. Currently, either fresh or preferably frozen/cryopreserved AM is used for such applications (Kruse *et al.*, 2000). In view of the attention focused on various pathogenic organisms in recent years proper sterilization of the AM prior to use is vital. In this chapter, the feasibility of using sterilized, freeze-dried amniotic membrane (FD-AM) as an alternative substrate for corneal epithelial stem cell cultivation is examined.

5.1.1 Scanning Electron Microscopy

Freeze-dried amniotic membranes, both cellular and denuded, were examined by scanning electron microscopy alongside their frozen counterparts for comparison.

5.1.1.1 Cellular Amniotic Membranes

Examination of freeze-dried cellular amniotic membranes by scanning electron microscopy revealed a continuous layer of flat squamous polygonal epithelial cells, morphologically resembling corneal endothelial cells (plate 5.1). The cells appeared to be intact and fairly well-preserved. Adjacent cells were well attached to each other with tightly opposed cell boundaries and distinct ridges at cell borders. Amniotic epithelial were very smooth in appearance and had no obvious microvilli on their apical surface.

Examination of the surface of frozen cellular membranes revealed similar findings. The membrane was completely covered with a continuous layer of polygonal epithelial cells (plate 5.2). Amniotic epithelial cells appeared to be fairly intact with prominent cell boundaries and an apical covering of short microvilli.

5.1.1.2 Denuded Amniotic Membranes

Examination of freeze-dried denuded amniotic membranes by scanning electron microscopy revealed a fairly flat and smooth layer of extracellular matrix (plate 5.3). There were no intact cells remaining on the membrane, however there were areas with epithelial cell debris, appearing as rough projections on the surface. At high magnification, it was clear that the basal lamina and in particular the delicate lamina densa had remained intact.

The denuded frozen samples were similar in appearance (plate 5.4). Examination of the apical surface of these membranes confirmed the complete removal of the amniotic epithelial cell layer. There was an exposed smooth and featureless extracellular matrix and again, an intact basal lamina.

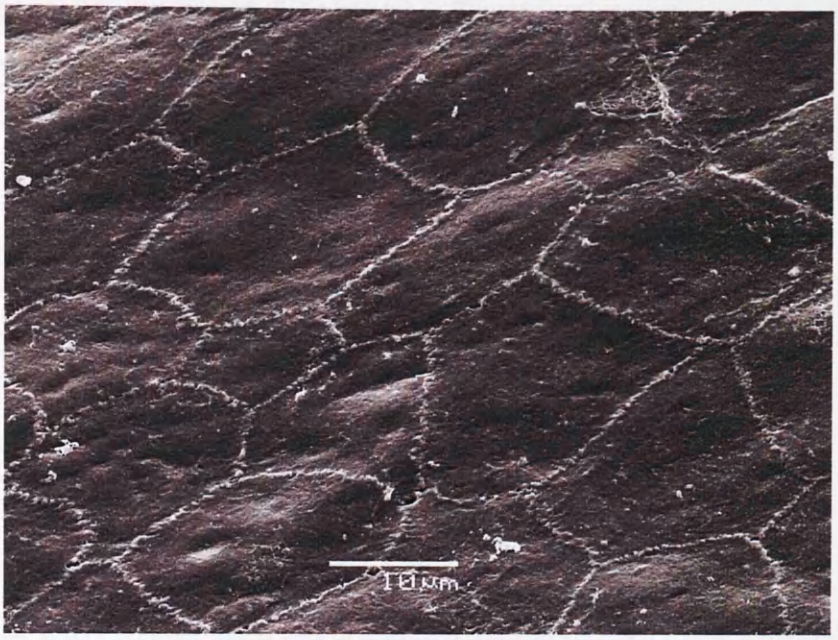


Plate 5.1: Scanning electron micrograph depicting freeze-dried cellular amniotic membrane. Polygonal amniotic epithelial cells formed a confluent layer, with prominent cell borders. [Scale bar = 10μm]

Plate 5.2: Scanning electron micrograph of frozen cellular amniotic membrane. Amniotic epithelial cells formed a confluent layer, with prominent cell borders and numerous microvilli on their apical surface. [Scale bar = 10μm]

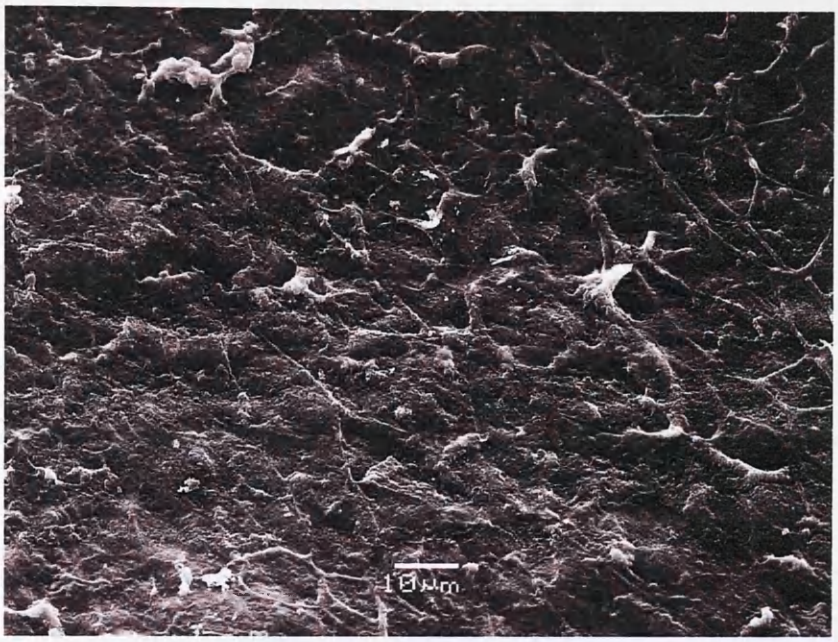


Plate 5.3: Scanning electron micrograph of freeze-dried denuded amniotic membrane. All intact epithelial cells had been removed to expose the extracellular matrix basal lamina. There was some cell debris on the surface. [Scale bar = 10µm]

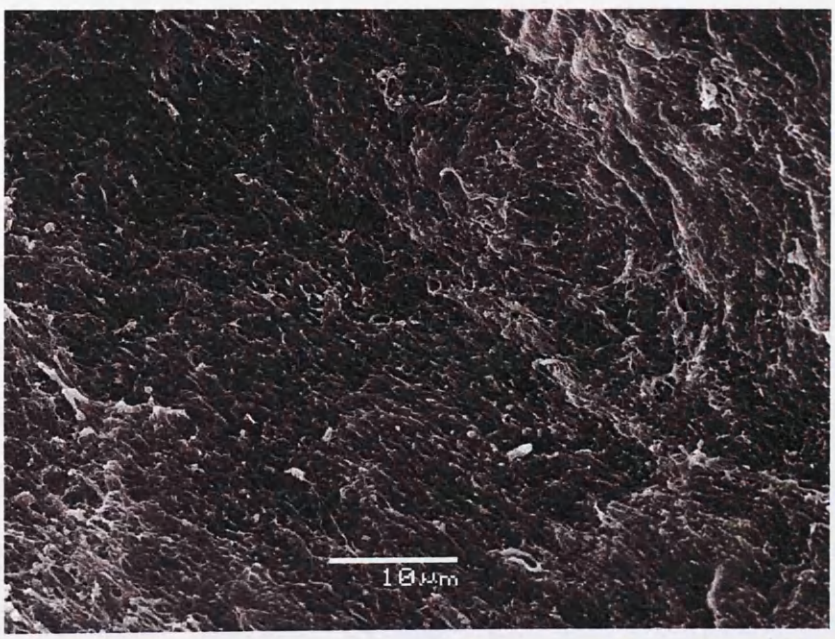


Plate 5.4: Scanning electron micrograph of frozen denuded amniotic membrane. The amniotic epithelial cell layer was successfully removed revealing a smooth intact basement membrane. [Scale bar = 10µm]

5.1.2 Transmission Electron Microscopy

Freeze-dried amniotic membranes, both cellular and denuded, were examined by transmission electron microscopy along with frozen membranes for comparison.

5.1.2.1 Cellular Amniotic Membranes

Examination of freeze-dried cellular amniotic membranes by transmission electron microscopy revealed a continuous monolayer of cuboidal epithelial cells attached to a stromal matrix via a basement membrane (plate 5.5). The cells themselves were compact and very densely stained. Cell membranes seemed to be intact however the apical cell surface was flattened. This is likely to be due to the vacuum packing of the membranes. Amniotic epithelial cells appeared to be well attached to the membrane (plate 5.6). Due to the compaction of the AM, it was difficult to see any detail in the stroma and basement membrane area.

Examination of the frozen cellular membrane showed a single layer of amniotic epithelial cells with centrally located nuclei and prominent surface microvilli (plate 5.7). The cells appeared to be in a necrotic state with multiple cytoplasmic vacuoles. The low temperature conditions in which the tissue is kept prior to processing are likely to result in ice crystal damage that would explain the poor state of these cells. Amniotic epithelial cells were adhered to a basal lamina in which collagen type IV fibrils formed a dense meshwork (plate 5.8). The epithelial cells were anchored to the lamina densa region of the basal lamina by way of hemidesmosomal junctions (plate 5.8). A matrix of loosely arranged type I collagen fibrils made up the stroma of the amniotic membrane.

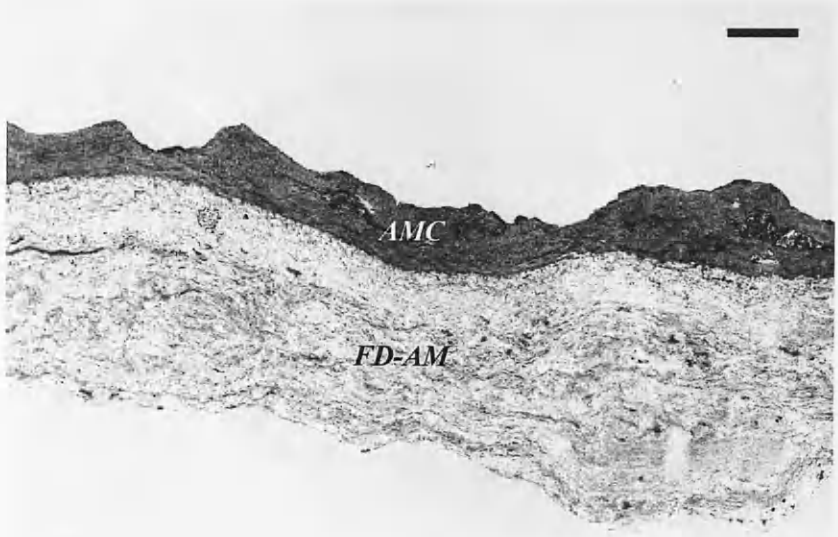


Plate 5.5: Transmission electron micrograph of cellular freeze-dried amniotic membrane *[FD-AM]* taken at low magnification. A compacted, densely-stained monolayer of amniotic epithelial cells *[AMC]* was supported by a stromal matrix of fibrillar collagen. [Scale bar = 2μm]

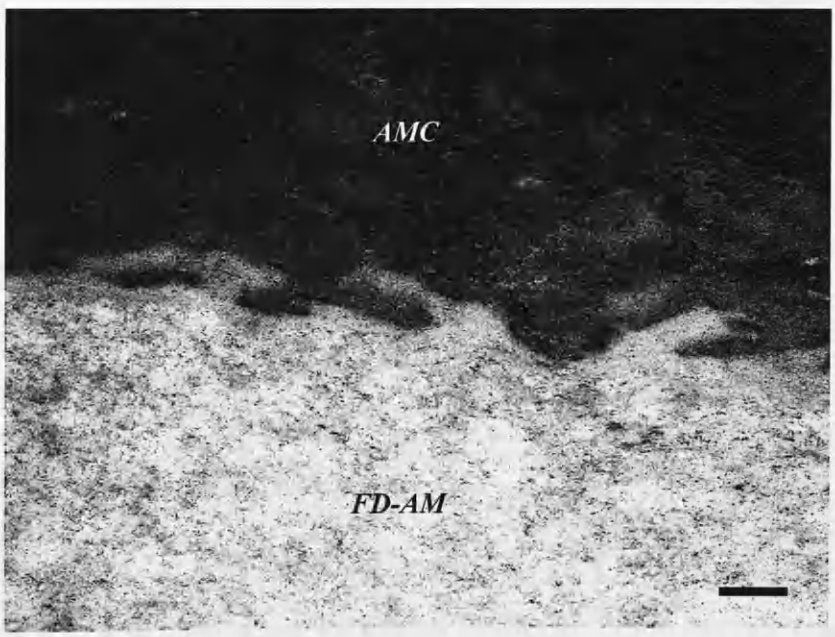


Plate 5.6: Transmission electron micrograph of cellular freeze-dried amniotic membrane *[FD-AM]* taken at high magnification. Epithelial cells *[AMC]* were well attached to the underlying basal lamina. [Scale bar = 200nm]

Plate 5.7: Transmission electron micrograph of cellular frozen amniotic membrane *[AM]* at low magnification. A monolayer of cuboidal epithelial cells *[AMC]* rested on a stromal matrix of fibrillar collagen. [Scale bar = 2μm]

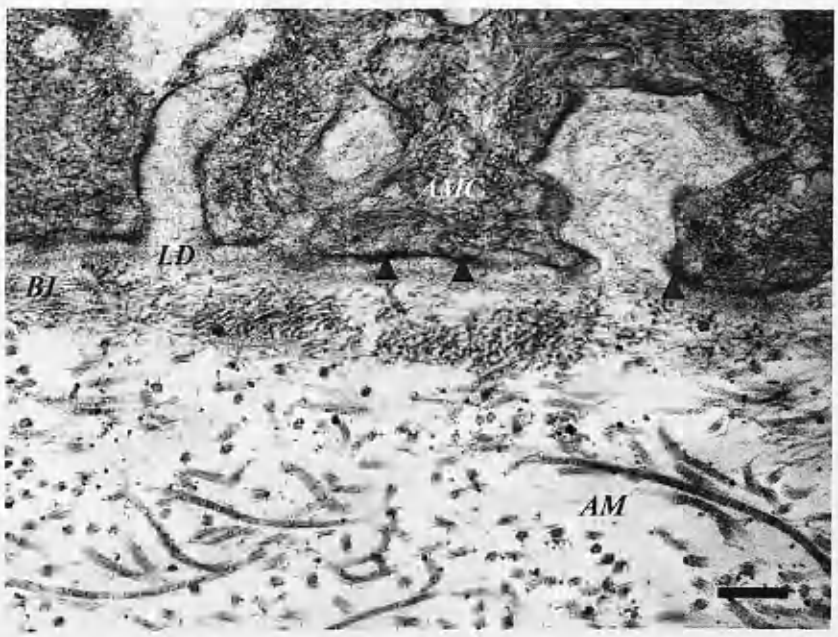


Plate 5.8: Transmission electron micrograph of cellular frozen amniotic membrane *[AM]* at high magnification. Interdigitations of the amniotic epithelial cells *[AMC]* protruded into the basal lamina *[BL]* where they attached to the membrane via hemidesmosomes (arrowheads). The lamina densa *[LD]* was visible as an electron dense layer overlying the basal lamina. Type I collagen fibrils made up the stroma. [Scale bar = 200nm]

5.1.2.2 Denuded Amniotic Membranes

In contrast to cellular amniotic membrane, examination of the freeze-dried denuded samples revealed only small remnants of cell debris on the basal lamina with no evidence of any intact residual epithelial cells (plate 5.9). As with the cellular tissue, these freeze-dried amniotic membranes appeared to have been compacted, possibly by the vacuum packing. It was clear however that the basal lamina had not been damaged by the denuding process (plate 5.10). The collagen stroma also appeared to have been well-preserved and had collagen type I fibres in a typically random arrangement.

The denuded frozen samples were comparable with the freeze-dried tissue. The frozen membranes were far less compact than the freeze-dried samples and as such it was easier to resolve the finer detail (plate 5.11). There was little or no structural damage to the fine meshwork of delicate type IV collagen fibrils that made up the basal lamina. The collagen stroma was characterized by a loosely arranged matrix of fibres as in the cellular membranes. Removal of the epithelial cells appeared to have had no detrimental affect on the amniotic stroma, the basal lamina, or the electron dense lamina densa (plate 5.12).

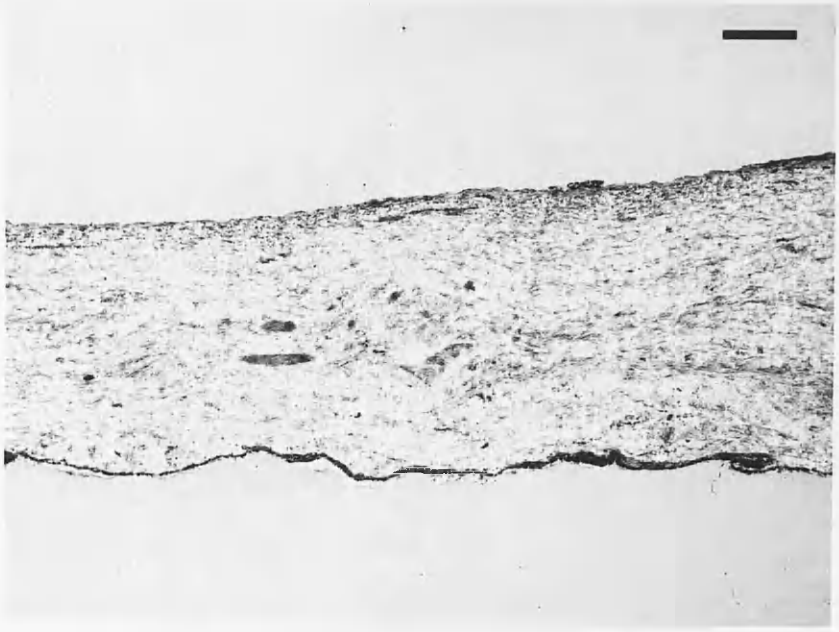


Plate 5.9: Transmission electron micrograph of denuded freeze-dried amniotic membrane. Residual amniotic epithelial cells were successfully removed, leaving only a little cell debris. The amniotic stroma was composed of loosely arranged collagen fibrils. [Scale bar = 2μm]

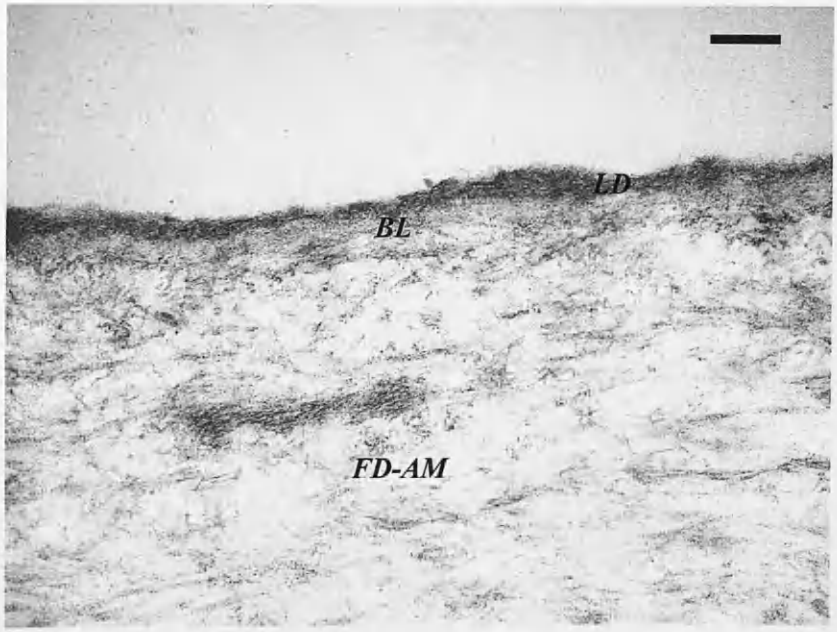


Plate 5.10: Transmission electron micrograph of denuded freeze-dried amniotic membrane *[FD-AM]* at high magnification. There was a small amount of cell debris remaining after the denuding process. The thin basal lamina *[BL]* and lamina densa *[LD]* appeared to be undamaged. [Scale bar = 200nm]

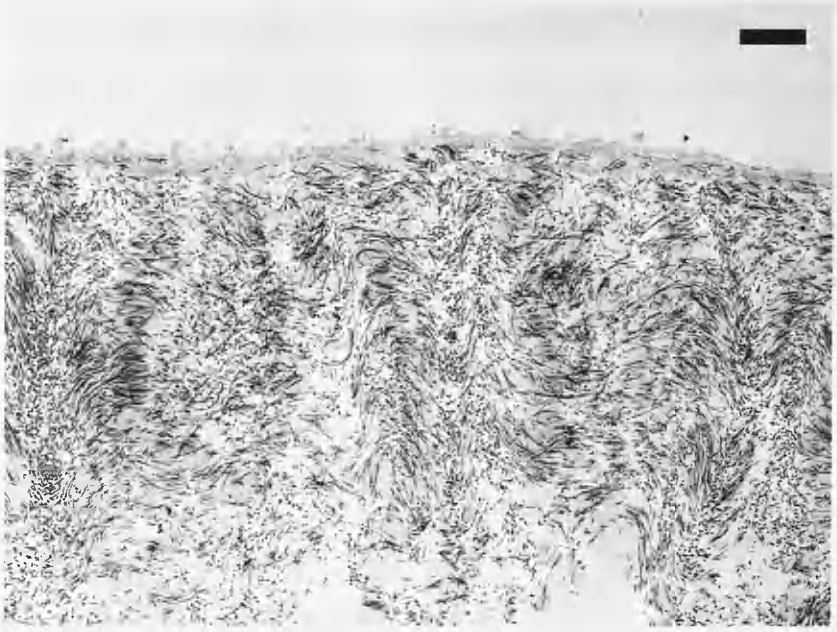


Plate 5.11: Transmission electron micrograph of denuded frozen amniotic membrane taken at low magnification. There were no residual amniotic epithelial cells following the denuding process and the basal lamina remained intact. [Scale bar = 2μm]

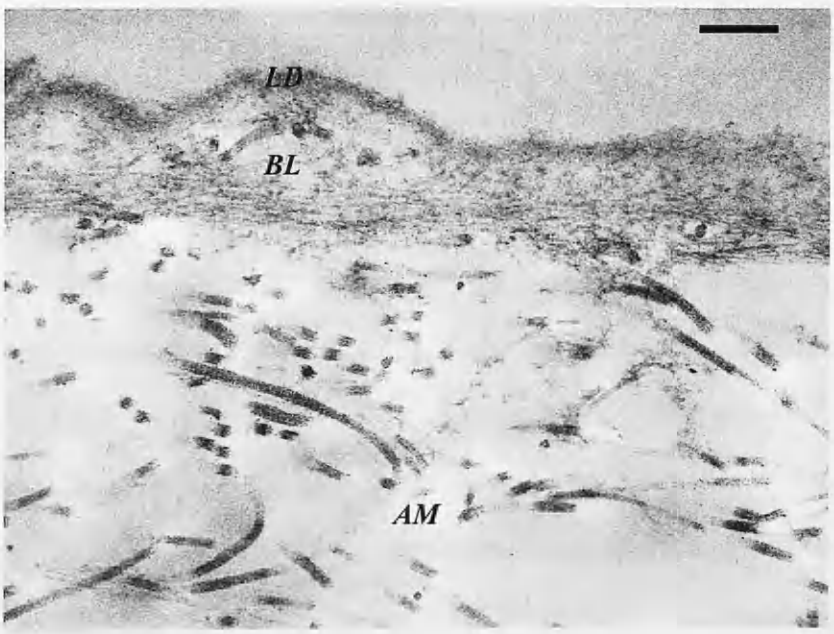


Plate 5.12: Transmission electron micrograph of denuded frozen amniotic membrane *[AM]* taken at high magnification. The electron dense lamina densa *[LD]* remained intact at the apex of the basal lamina *[BL]*. Below this is the amniotic stromal matrix of collagen fibrils. [Scale bar = 200nm]

5.1.3 Transmission Electron Microscopy: Immunogold Labelling

Both cellular and denuded freeze-dried amniotic membranes were labelled for several extracellular matrix molecules. Frozen membranes were also examined for comparison. The distributions of matrix molecules are described in detail in the sections below and summarized in table 5.1. It is important to note that in the processing of these samples, ultrastructural preservation was compromised in order to maintain antigenicity.

5.1.3.1 Fibronectin (IST-4)

In cellular membranes, there were moderate levels of labelling with anti-fibronectin (plates 5.13A-D). This was localized in the basal lamina region just below amniotic epithelial cells and concentrated in the lamina densa. Equivalent levels of labelling were observed in the lamina densa of denuded samples. There was a little labelling in the amniotic membrane stroma. Levels of labelling in freeze-dried membranes were comparable to those seen in frozen membranes.

5.1.3.2 Laminin

The antibody to laminin reacted strongly with the basal lamina area when compared to the control antibody. Laminin appeared to be concentrated in the lamina densa region, with high levels being observed directly beneath amniotic epithelial cells in the cellular samples (plates 5.14A and B). Denuding appeared to have little or no effect on the presence of laminin in the lamina densa, as staining was found in equally high levels in denuded samples (plates 5.14C and D). There was no labelling in the stroma of any of the samples. Again, the levels and locations of laminin labels were comparable in the freeze-dried and frozen membranes.

5.1.3.3 Vitronectin (VIT-2)

Labelling patterns for vitronectin were very similar to those seen for fibronectin. Vitronectin was concentrated in the lamina densa, with low levels of staining in the basal lamina and stroma (plates 5.15A-D). There were no discernable differences in the levels of labelling between freeze-dried and frozen membranes, nor cellular and denuded tissues.

5.1.3.4 Collagen type IV (NLI/53)

Type IV collagen was observed in high levels continuously along the basal lamina region, slightly more concentrated in the lamina densa (plates 5.16A-D). Small amounts of staining were found in the stroma of all samples studied. It appeared that neither the freeze-drying nor denuding process had any detrimental effect on collagen IV distribution.

5.1.3.5 Collagen type I (COL-1)

Collagen type I was seen in moderate levels in the stroma of the amniotic membrane with little or no labelling in the basal lamina area. Labelling seemed to be associated with the collagen fibrils of the stroma, as would be expected. Distributions were very similar in freeze-dried and frozen, cellular and denuded membranes (plates 5.17A-D).

5.1.3.6 Heparan Sulphate (F58-10E4)

In all amniotic membranes studied, the monoclonal antibody for heparan sulphate reacted strongly with the lamina densa and with the subjacent basal lamina to a lesser extent (plates 5.18A-D). There was little or no labelling in the stroma. Neither the

freeze-drying nor the denuding process appeared to have had any affect on the distribution of HS.

5.1.3.7 Chondroitin Sulphate (CS-56)

Low levels of chondroitin sulphate labelling were found in the stroma of cellular and denuded amniotic membranes (plates 5.19A-D). Labels were associated with collagen fibrils in the stroma with negligible levels in the basal lamina region. Again, the levels of staining seen in the freeze-dried membranes were comparable to those in the frozen tissues.

5.1.3.8 Keratan Sulphate (5-D-4)

Keratan sulphate labelling was observed in low-moderate levels in the stroma of all amniotic membrane samples (plates 5.20A-D). Labelling was distributed in a similar manner to that of collagen type I and chondroitin sulphate, in close proximity to the stromal collagen fibrils with little or no labelling in the basal lamina.

5.1.3.9 Control (non-specific IgG)

The control non-specific antibody showed negligible levels of reactivity in all amniotic membrane samples under study (plates 5.21A-D).

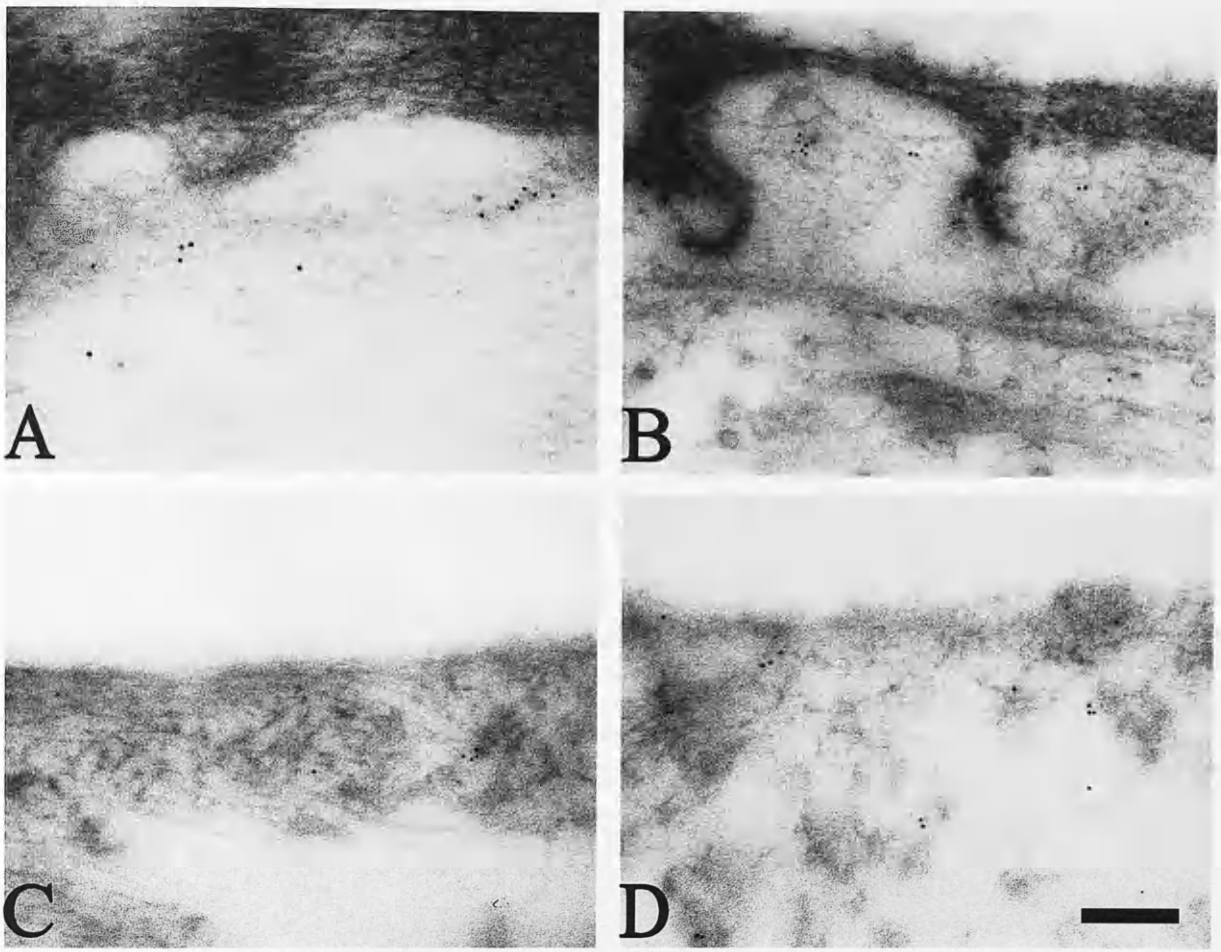


Plate 5.13: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-fibronectin**. Figures A and B represent the interface between amniotic epithelial cells and the basal lamina of cellular amniotic membranes, while C and D show the basal lamina region of denuded membranes. The 5nm gold particles appear as black dots. In all samples there were moderate levels of labelling in the lamina densa and low levels in the underlying basal lamina and stroma. [Scale bar = 100nm]

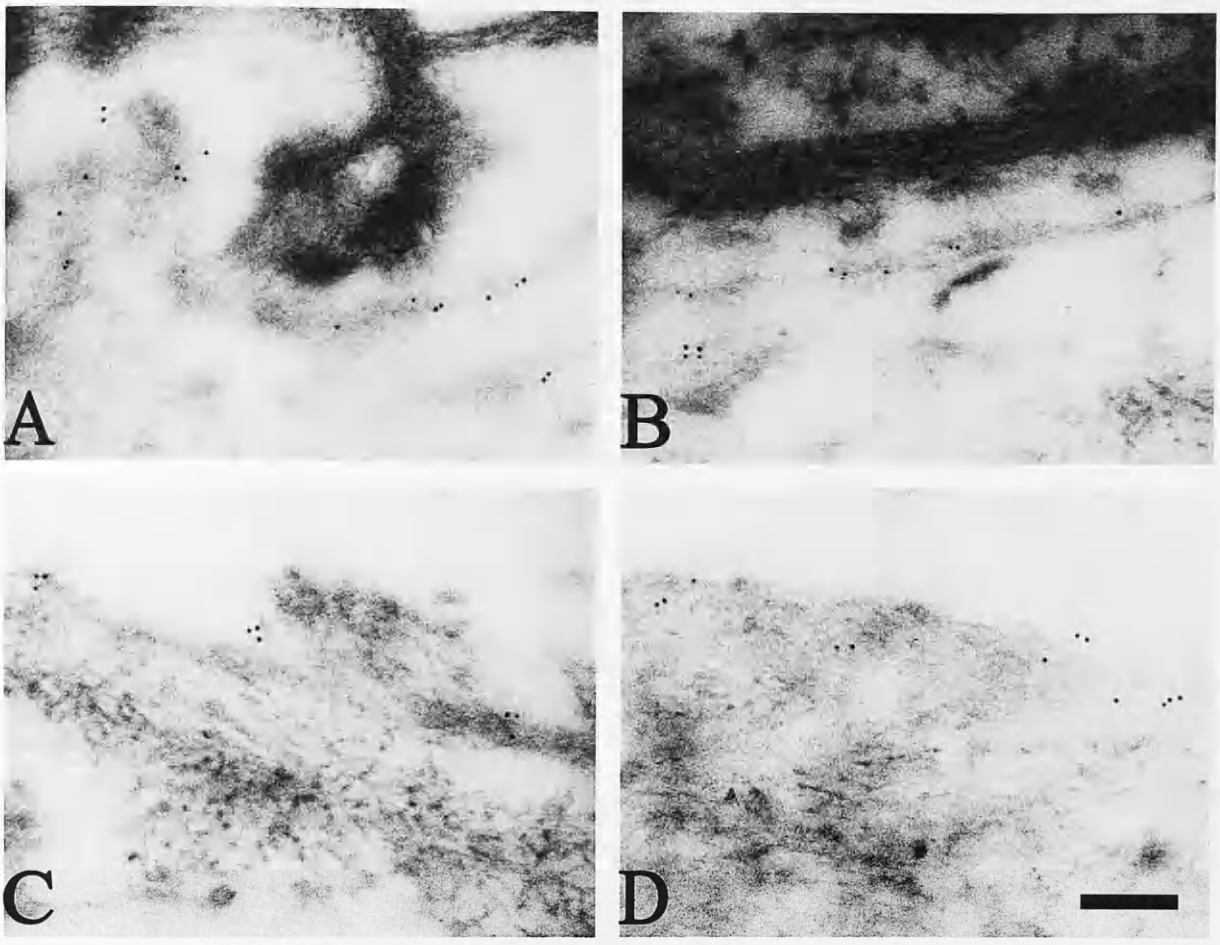


Plate 5.14: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-laminin**. Figures A and B represent the interface between amniotic epithelial cells and the basal lamina of cellular amniotic membranes, while C and D show the basal lamina region of denuded membranes. The 5nm gold particles appear as black dots. In all samples there were high levels of labelling in the lamina densa and low levels in the underlying basal lamina. [Scale bar = 100nm]

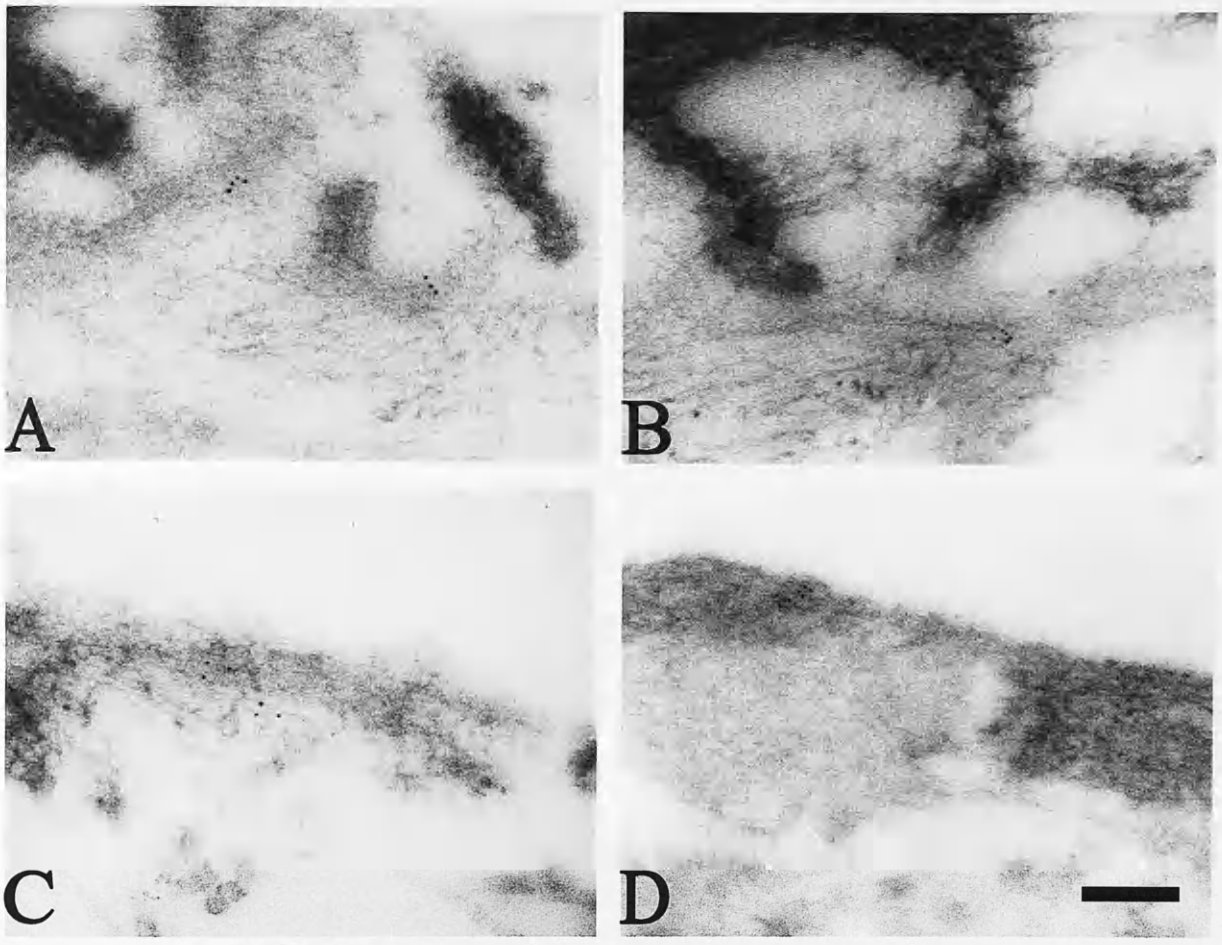


Plate 5.15: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-vitronectin**. Figures A and B represent the interface between amniotic epithelial cells and the basal lamina of cellular amniotic membranes, while C and D show the basal lamina region of denuded membranes. The 5nm gold particles appear as black dots. In all samples there were moderate levels of labelling in the lamina densa and low levels in the underlying basal lamina and stroma. [Scale bar = 100nm]

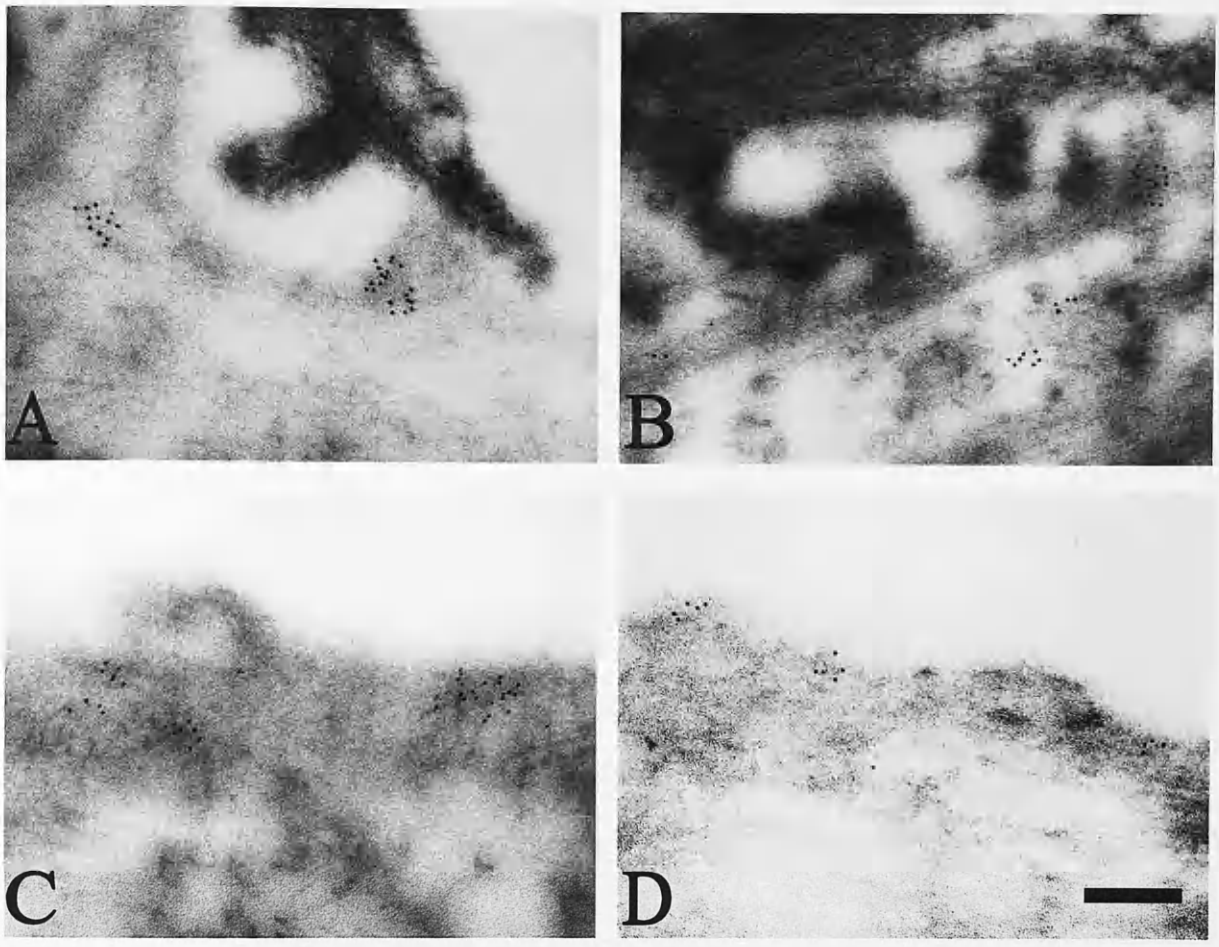


Plate 5.16: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-collagen type IV**. Figures A and B represent the interface between amniotic epithelial cells and the basal lamina of cellular amniotic membranes, while C and D show the basal lamina region of denuded membranes. The 5nm gold particles appear as black dots. In all samples there were high levels of labelling in the lamina densa, moderate levels in the underlying basal lamina and low levels in the stroma. [Scale bar = 100nm]

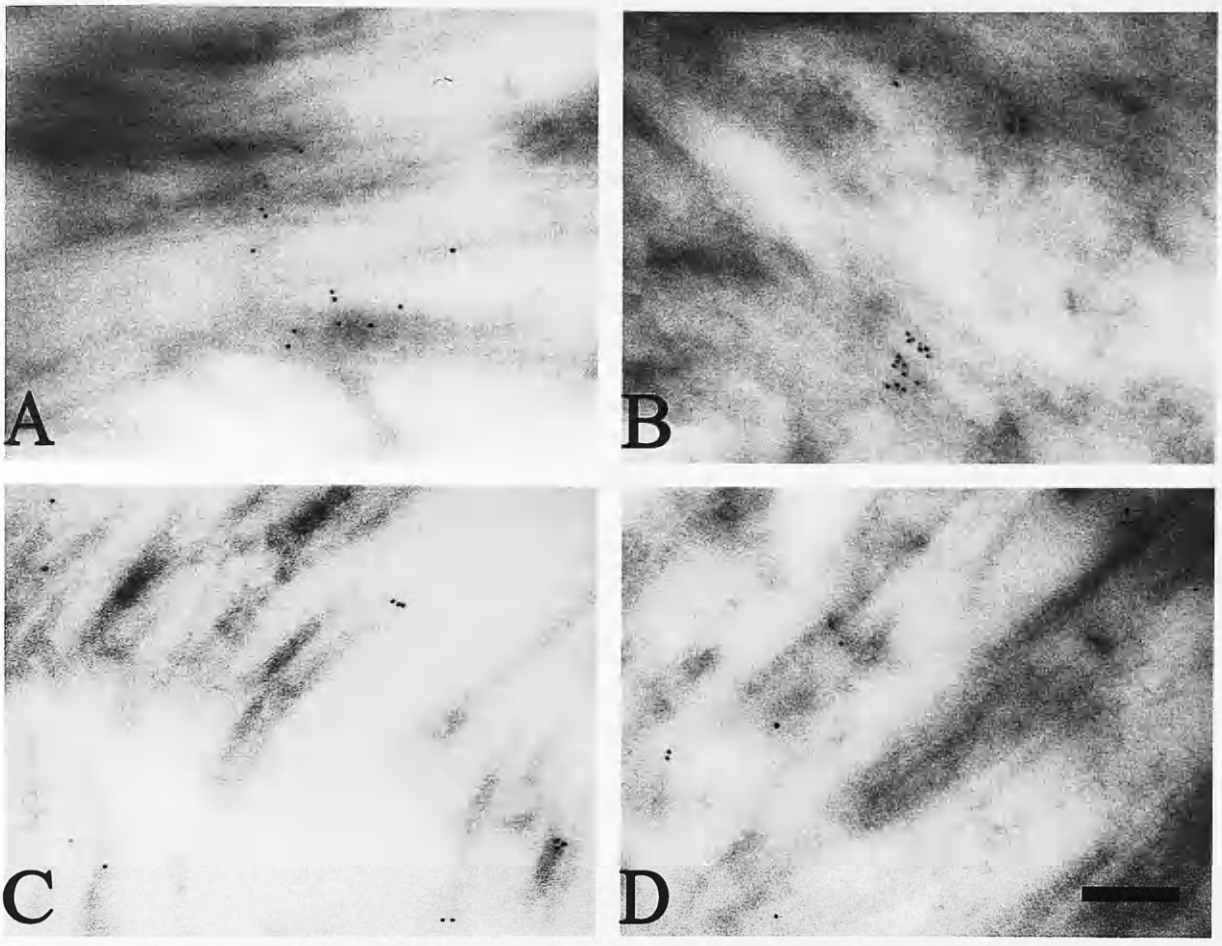


Plate 5.17: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-collagen type I**. The 5nm gold particles appear as black dots. Moderate levels of labelling were seen in the stroma of all samples, in close proximity to the collagen fibrils. There was little or no labelling in the basal lamina region. [Scale bar = 100nm]

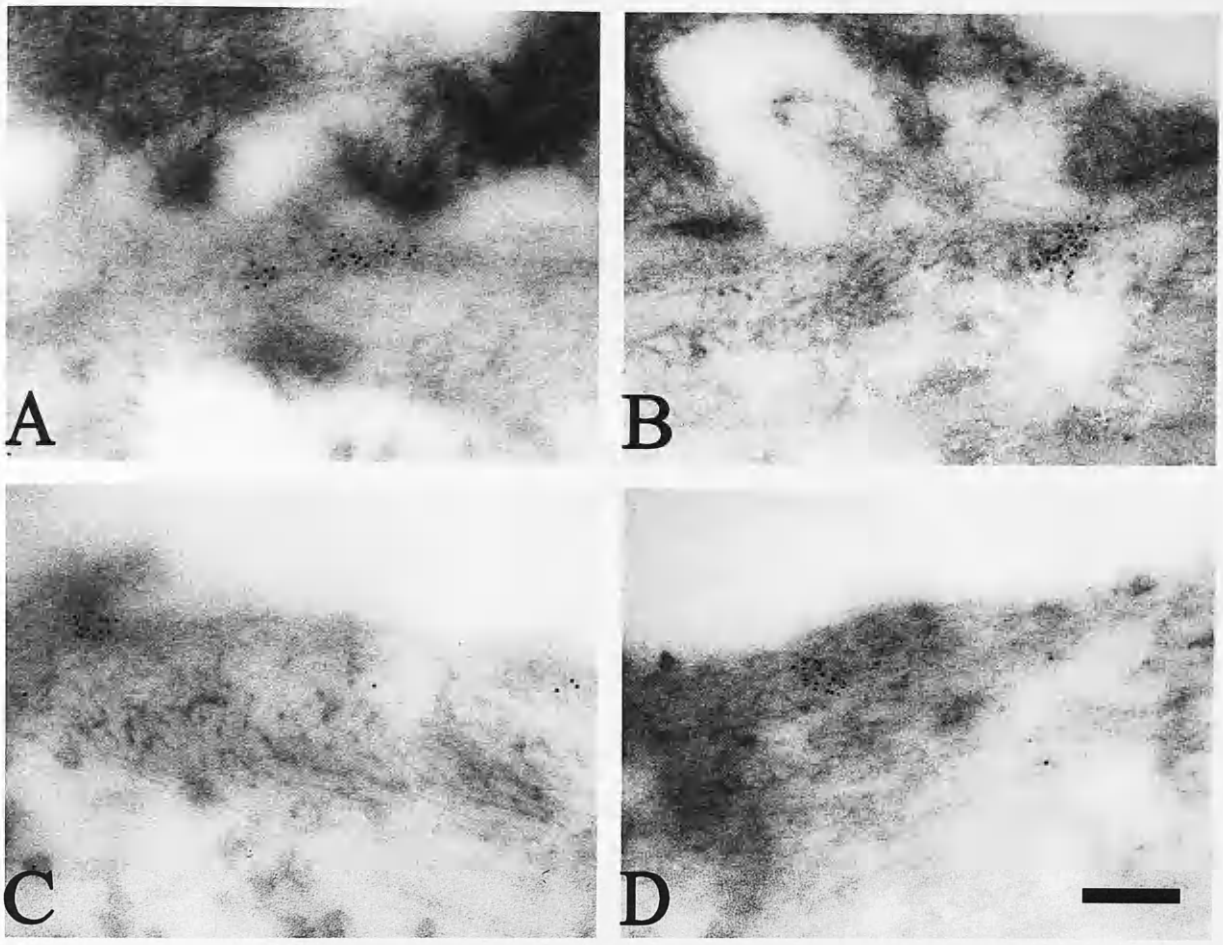


Plate 5.18: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-heparan sulphate**. Figures A and B represent the interface between amniotic epithelial cells and the basal lamina of cellular amniotic membranes, while C and D show the basal lamina region of denuded membranes. The 5nm gold particles appear as black dots. In all samples there were high levels of labelling in the lamina densa and low levels in the underlying basal lamina. [Scale bar = 100nm]

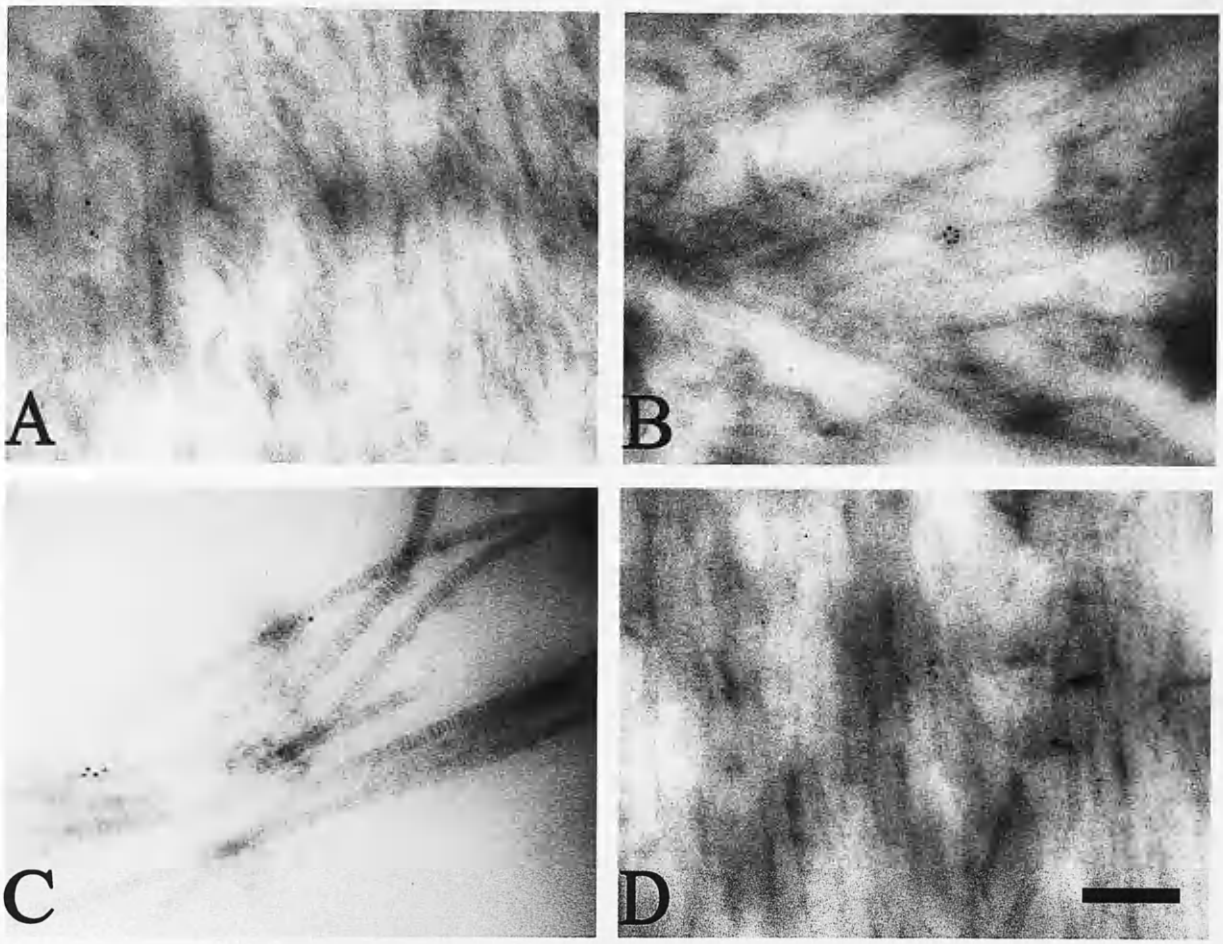


Plate 5.19: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-chondroitin sulphate**. The 5nm gold particles appear as black dots. In all samples, there were only low levels of labelling in the stroma, in close proximity to the collagen fibrils. There was little or no labelling in the basal lamina region. [Scale bar = 100nm]

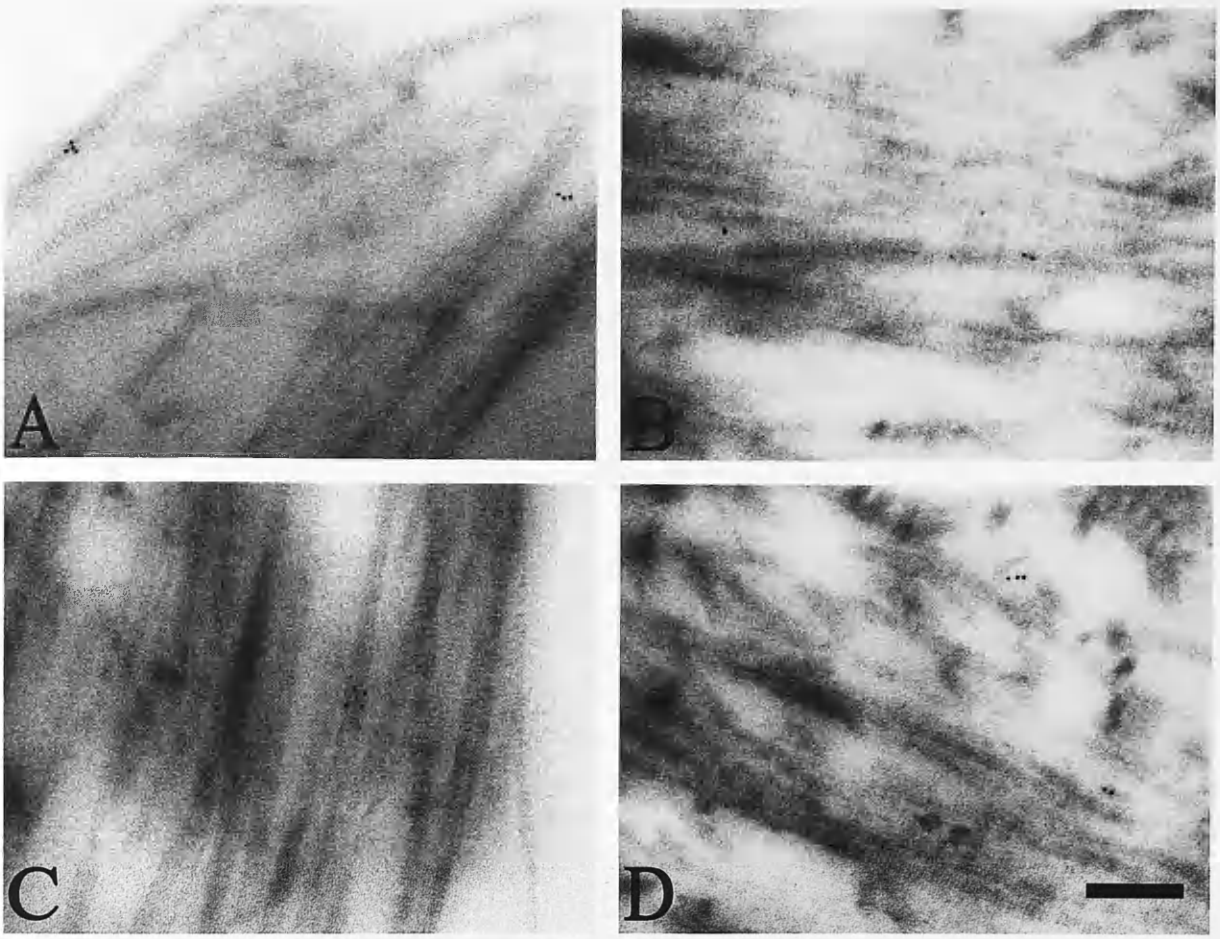


Plate 5.20: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with **anti-keratan sulphate**. The 5nm gold particles appear as black dots. In all samples, there were low-moderate levels of labelling in the stroma, in close proximity to the collagen fibrils. There was little or no labelling in the basal lamina region. [Scale bar = 100nm]

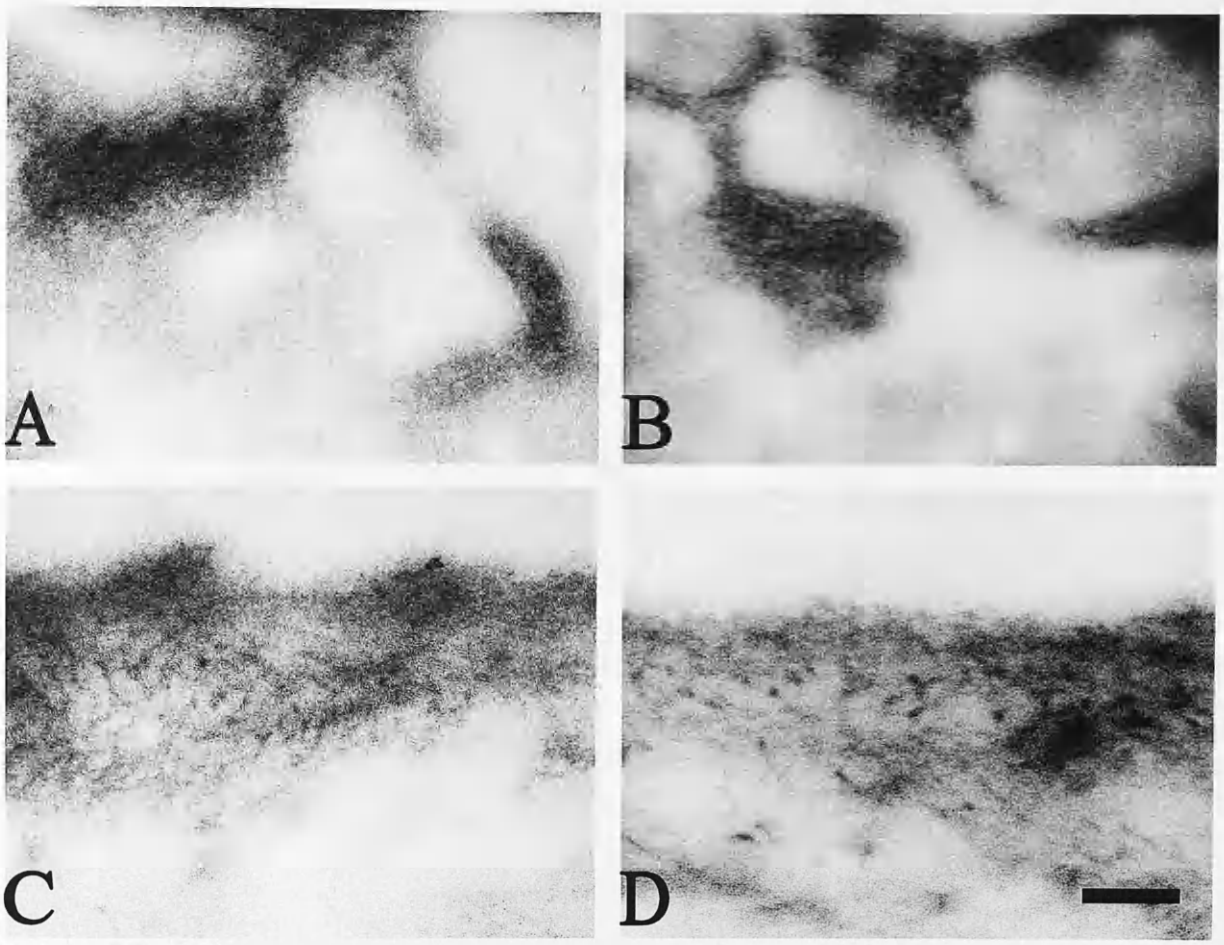


Plate 5.21: Transmission electron micrographs illustrate cellular frozen (A) and freeze-dried (B) and denuded frozen (C) and freeze-dried (D) amniotic membranes, immunolabelled with a control **non-specific antibody**. Figures A and B represent the interface between amniotic epithelial cells and the basal lamina of cellular amniotic membranes, while C and D show the basal lamina region of denuded membranes. Negligible levels of labelling were seen in each sample. [Scale bar = 100nm]

	Frozen cellular AM			Freeze-dried cellular AM		
	Lamina densa	Basal lamina	Stroma	Lamina densa	Basal lamina	Stroma
Fibronectin	++	+	+	++	+	+
Laminin	+++	+	-	+++	+	-
Vitronectin	++	+	+	++	+	+
Collagen IV	+++	++	+	+++	++	+
Collagen I	-	-	++	-	-	++
HS	+++	+	-	+++	+	-
CS	-	-	+	-	-	+
KS	-	-	+	-	-	+
	Frozen denuded AM			**Freeze-dried denuded AM**		
	Lamina densa	Basal lamina	Stroma	Lamina densa	Basal lamina	Stroma
Fibronectin	++	+	+	++	+	+
Laminin	+++	+	-	+++	+	-
Vitronectin	++	+	+	++	+	+
Collagen IV	+++	++	+	+++	++	+
Collagen I	-	-	++	-	-	++
HS	+++	+	-	+++	+	-
CS	-	-	+	-	-	+
KS	-	-	+	-	-	+

Table 5.1: Relative distribution of extracellular matrix molecules in freeze-dried and frozen amniotic membranes, both cellular and denuded. Level of labelling: +++ high, ++ moderate, + low, - none.

5.2 Cultivation of Rabbit Corneal Epithelial Cells on Denuded Freeze-Dried Amniotic Membrane

In an attempt to assess the usefulness of freeze-dried AM as a culture substrate, it was used to grow rabbit corneal epithelium. Project collaborators at the Kyoto Prefectural University of Medicine who carried out the culture experiments observed that cells began to form colonies on the FD-AM within 3 days. After 7 days a confluent primary culture of corneal epithelial cells had been established that covered the entire membrane. At 3 weeks, the cultivated corneal epithelial cells were fixed and couriered to Lancaster University for detailed analysis.

5.2.1 Scanning Electron Microscopy

Scanning electron microscopic examination of the cultivated rabbit corneal epithelial cells on freeze-dried amniotic membrane revealed a fairly continuous layer of flat squamous polygonal epithelial cells with a mean cell surface area of 402.2μm^2 (S.D.$\pm$184.9μm^2) (plate 5.22). In some areas the cells appeared healthy and well-developed and were closely attached to each other with tightly opposed cell junctions though cell borders were not very defined (plate 5.24). In places, epithelial cells appeared to be undergoing the process of desquamation. There were some less healthy-looking areas of the culture where cells appeared to be rounded and abnormal (plate 5.23). The apical surface of the cells was covered in short, regular microvilli (plate 5.25).

Plate 5.22: Scanning electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane, taken at low magnification. Cells formed a confluent layer but had less prominent cell borders than *in vivo* corneal epithelium. [Scale bar = 10μm]

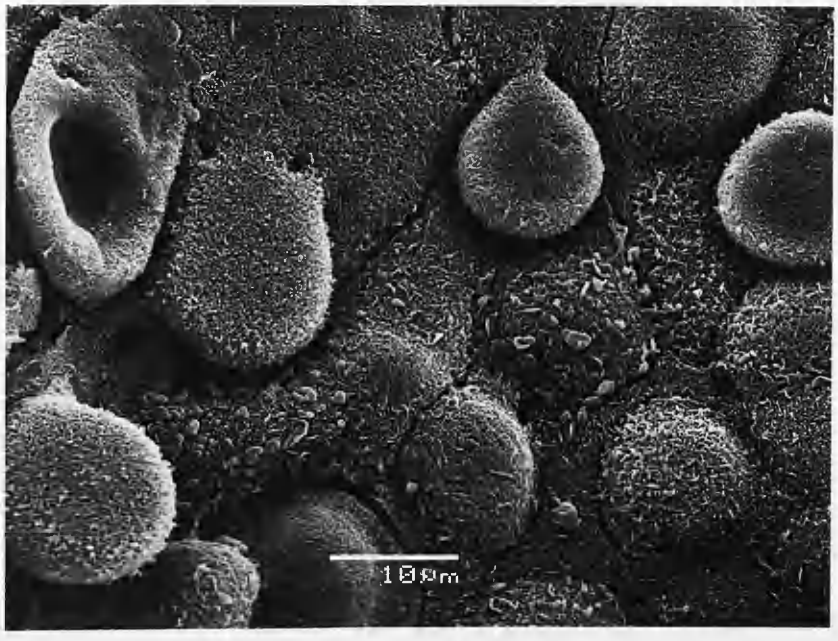


Plate 5.23: Scanning electron micrograph of rabbit corneal epithelial cells grown on freeze-dried denuded amniotic membrane. Some areas of the culture sheet exhibited rounded, abnormal cells as in the image above. [Scale bar = 10μm]

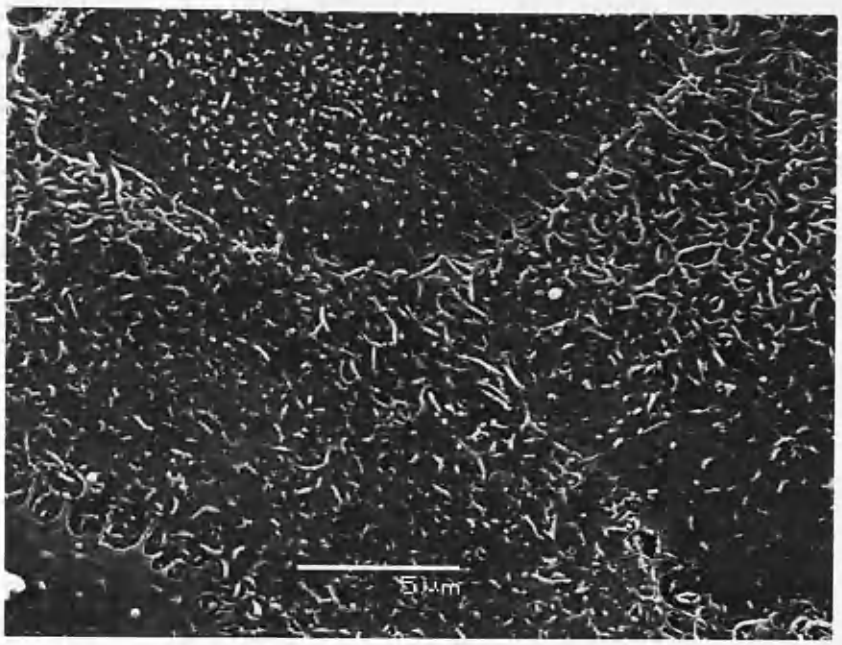


Plate 5.24: Scanning electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane, taken at high magnification. Adjacent cells were well attached to each other. [Scale bar = 5μm]

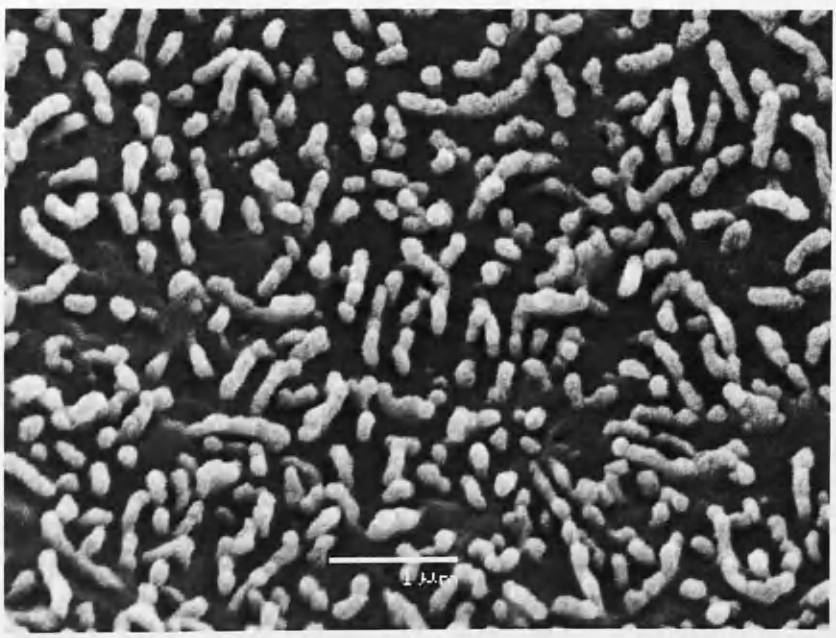


Plate 5.25: Scanning electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane taken at very high magnification. The apical surface of the superficial cells was covered in short regular microvilli. [Scale bar = 1μm]

5.2.2 Transmission Electron Microscopy

TEM examination of the corneal epithelial culture sheet showed that the cells produced 5-6 layers of well-stratified epithelium (plate 5.26), appeared healthy and were differentiated into basal columnar cells, suprabasal wing cells and flat squamous superficial cells (plate 5.27). In all cell layers the epithelial cells were closely attached to neighbouring cells by numerous desmosomal junctions (plate 5.28). The epithelial cells in the basal cell layers (plate 5.29) were well attached to the FD-AM substrate with hemidesmosomal attachments and produced basement membrane material (plate 5.30).

Plate 5.26: Transmission electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane *[FD-AM]*. The culture formed 5-6 well-stratified and differentiated cell layers. [Scale bar = 2μm]

Plate 5.27: Transmission electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane. Superficial cells were approximately 2-3 layers thick and squamous in appearance. There was evidence of cell desquamation (*). [Scale bar = 1μm]

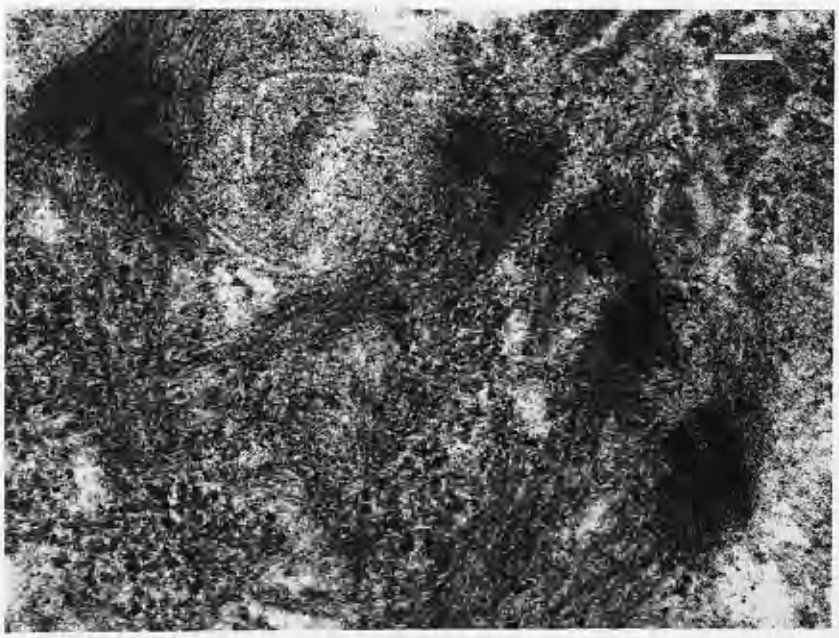


Plate 5.28: Transmission electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane taken at very high magnification. Adjacent cells in all cell layers were joined by numerous desmosomes. [Scale bar = 100nm]

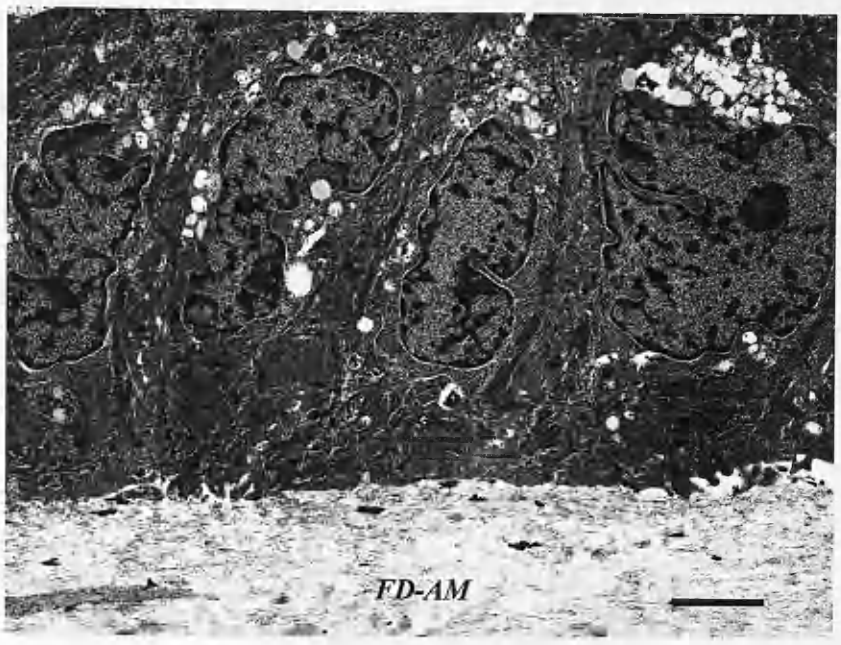


Plate 5.29: Transmission electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane *[FD-AM]*. The basal cells were columnar in appearance and were tightly opposed. [Scale bar = 1μm]

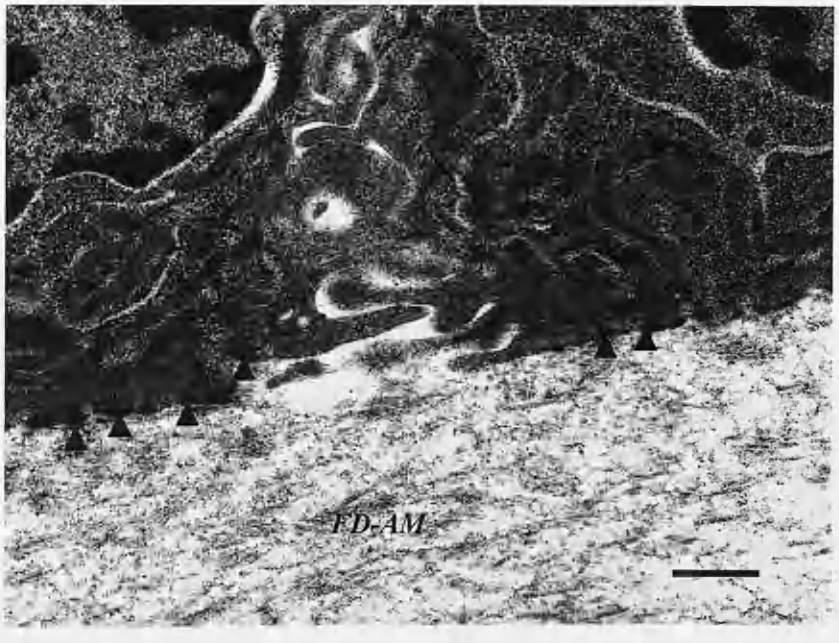


Plate 5.30: Transmission electron micrograph of rabbit corneal epithelial cells on freeze-dried denuded amniotic membrane *[FD-AM]*, taken at high magnification. Basal cells were generally well attached to the amniotic membrane by way of hemidesmosomal junctions (arrowheads). [Scale bar = 500nm]

5.2.3 Quantitative Study

Further to the microscopic examination of the cultivated corneal epithelial cells on FD-AM, a quantitative study was undertaken to compare these cells with those of the normal rabbit cornea. Mean data (±S.D.) are given in table 5.2 below.

	Corneal cells cultivated on freeze-dried denuded AM	**Control rabbit corneal epithelium**
Thickness of cell layers (μm)	22.29 ± 8.77	20.55 ± 2.99
Number of cell layers	6.57 ± 1.34	7.79 ± 1.31
Intercellular space area (μm^2)	0.18 ± 0.24	0.09 ± 0.07
Number of desmosomes	1.57 ± 1.02	2.57 ± 1.60
Number of basal junctions	2.64 ± 1.74	8.29 ± 1.68
Cell surface area (μm^2)	402.21 ± 184.87	788.19 ± 200.53

Table 5.2: Comparison of cultivated rabbit corneal epithelial cells on freeze-dried AM with normal rabbit cornea. Mean data (± S.D.) are shown. Fourteen data points were taken at random for each of the parameters, except for the cell surface areas in which case n=50. The averages for intercellular area and number of desmosomes are given for random 3μm long interfaces between adjacent cells.

Raw data were compared with that of the control and a percentage difference value calculated for each of the parameters, thus facilitating interpretation of the overall effect of the culture technique on the epithelial cells. The raw data were again used to calculate the statistical significance of these results. Depending on the normality and variance of the data, either a t-test or a Mann-Whitney Rank Sum test was used. P values indicate the level of significance. The cultivated epithelial cells closely resembled the control cornea. In terms of percentage differences, the most apparent was in terms of 92% larger intercellular spaces in the cultivated cells however this

difference was found to be statistically not significant, undoubtably as result of the high standard deviation. Statistically, there were smaller but more significant differences between the number of cell layers ($P=0.023$), the number of hemidesmosomal junctions ($P<0.001$) and the cell surface area ($P<0.001$) of the cultivated cells compared with the rabbit corneal epithelium control. Differences in thickness of cell layers, area of intercellular space and numbers of desmosomes were found to be not statistically significant.

5.2.4 Summary Interpretations

Examination of the freeze-dried amniotic membrane by SEM and TEM showed that it was similar to conventionally frozen amniotic membrane. Immunohistochemistry also revealed no remarkable differences in the location or abundance of extracellular matrix proteins. The freeze-dried AM appeared to be a useful alternative culture substrate for rabbit corneal epithelial cells. When compared to the control (normal rabbit corneal epithelium), cultivated cells were significantly smaller (in terms of superficial cell surface area), had fewer hemidesmosomal junctions (for basal attachment) and fewer cell layers. However, in spite of these differences, overall impressions of the cultured cells were very good.

6 RESULTS

6.1 Cultivation of Human Oral Mucosal Epithelial Cells on Denuded Amniotic Membrane

This chapter describes an investigation into the culture of human oral mucosal epithelial cells on denuded amniotic membrane to serve as potential new sources of autologous epithelium for ocular surface reconstruction. Both gingival (gum) and buccal (cheek) cell cultures were analysed morphologically using scanning and transmission electron microscopy. A quantitative study compared the two cell types with each other and with the same control human cornea as used in previous investigations (donor tissue from Northwest Lions Eye Bank).

6.1.1 Light Microscopy

6.1.1.1 Gingival Mucosal Biopsies

Examination of the small gingival biopsy sample revealed well in excess of 40 layers of well-stratified epithelium, differentiated into columnar basal, suprabasal wing and squamous superficial epithelial cells (plate 6.1). Adjacent cells seemed to be closely attached, with only some small spaces in the more basal regions. Subepithelially, there was a mass of what appeared to be vascularized connective tissue

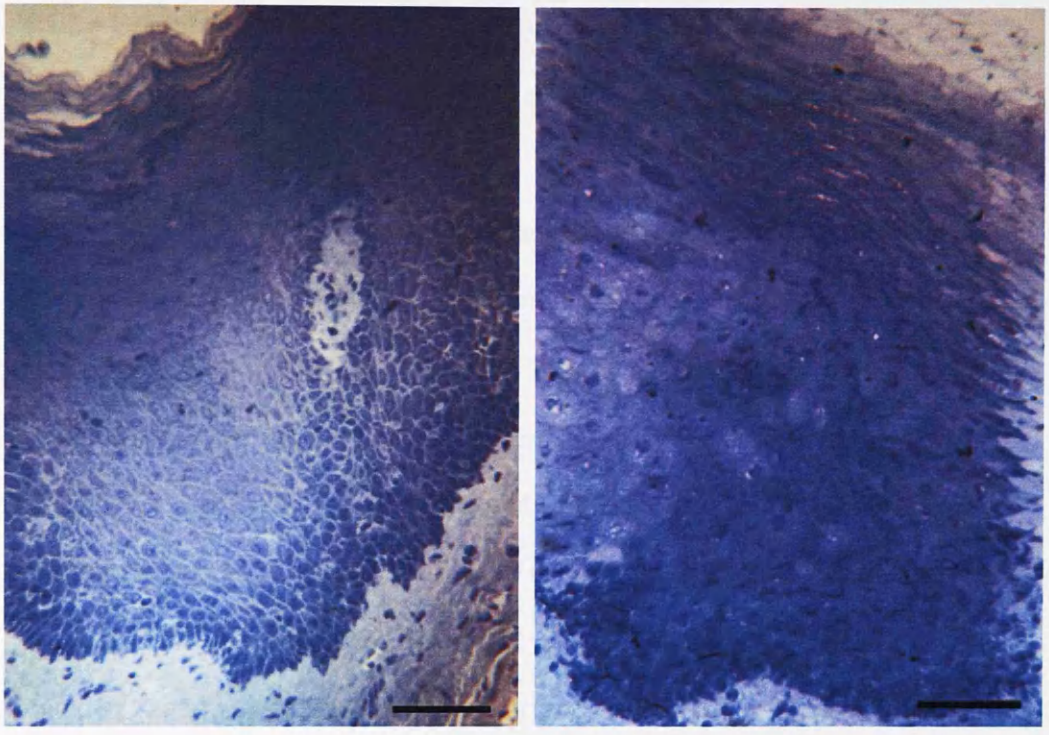
6.1.1.2 Buccal Mucosal Biopsies

The buccal mucosal biopsies were similar in appearance to the gingival ones. There appeared to be more than 40 layers of stratified and differentiated epithelial cells

resting on a mass of connective tissue (plate 6.2). Superficial cells formed many layers and were tightly opposed. Again, there were some small intercellular spaces between cells of the basal layers. Basal cells were well attached to the underlying tissue.

6.1.1.3 Control Human Corneal Biopsy

In contrast to the oral mucosal biopsies, light microscopy of the corneal biopsy showed a thick stroma with cells at the anterior and posterior on their respective basement membranes. There was only a thin layer of differentiated epithelial cells, forming approximately 4-5 layers (plate 6.3). The cells of the epithelium were tightly opposed and attached to the underlying Bowman's layer. The corneal stroma was thick and strewn with keratocytes. In the region of the posterior cornea, there was a Descemet's membrane and some residual flattened corneal endothelial cells in a monolayer.



Plates 6.1 and 6.2: Light micrographs of human oral mucosal biopsies stained with toluidene blue. Gingival [left] and buccal [right] epithelial cells formed in excess of 40 layers. Superficial cells were squamous and appeared to be desquamating in places. Basal cells were well attached to the underlying vascularized connective tissue. [Scale bars = 50μm].

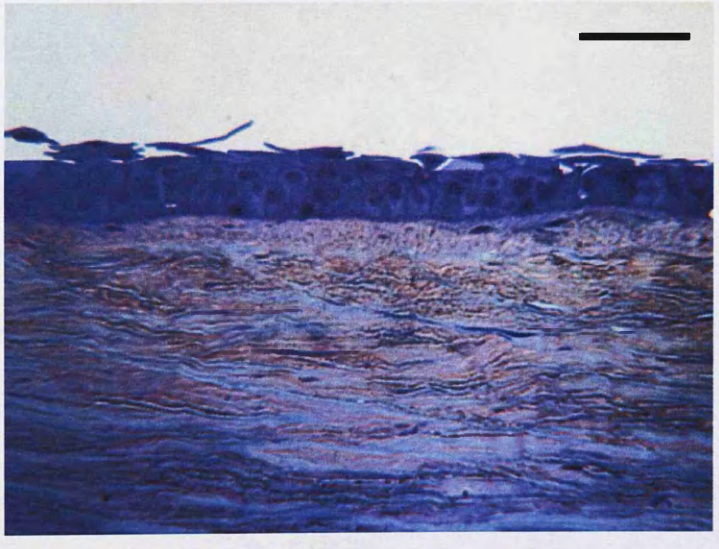


Plate 6.3: Light micrograph showing human control corneal epithelium, stained with toluidene blue. The epithelium had 4-5 differentiated cell layers and there was evidence of superficial cell desquamation from the surface. The corneal stroma was thick, only a fraction is visible in this micrograph. [Scale bar = 50μm]

6.1.2 Scanning Electron Microscopy

6.1.2.1 Gingival Mucosal Biopsies

Examination of the gingival mucosal epithelium by SEM revealed an undulated, yet continuous layer of squamous, polygonal epithelial cells with an average cell surface area of 519.6μm^2 (S.D.±168.3) (plate 6.4). As would be expected in a healthy epithelial sheet, some cells appeared to be undergoing the process of desquamation. Neighbouring cells were tightly opposed and had distinct and well-defined cell borders (plate 6.5). The apical surface of the mucosal epithelial cells was generally covered with long parallel ridge-like folds, quite different from the surface microvilli found on superficial corneal epithelial cells (plate 6.6). In other areas the microplicae appeared less regular and had a more random arrangement. The surface of other cells was pitted and resembled a series of holes (plate 6.7).

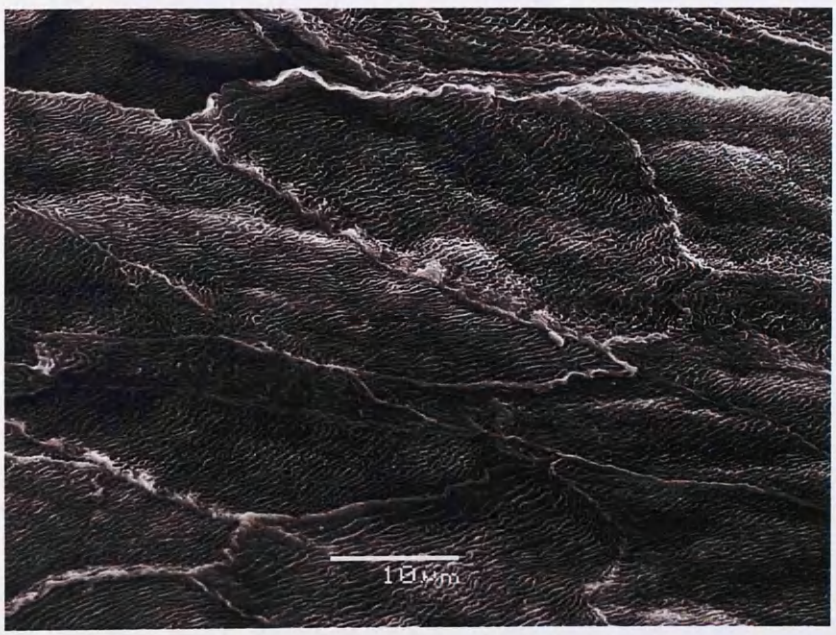


Plate 6.4: Scanning electron micrograph of biopsy-derived human gingival mucosal cells, taken at low magnification. Cells were polygonal and squamous in appearance and formed a continuous layer. [Scale bar = 10μm]

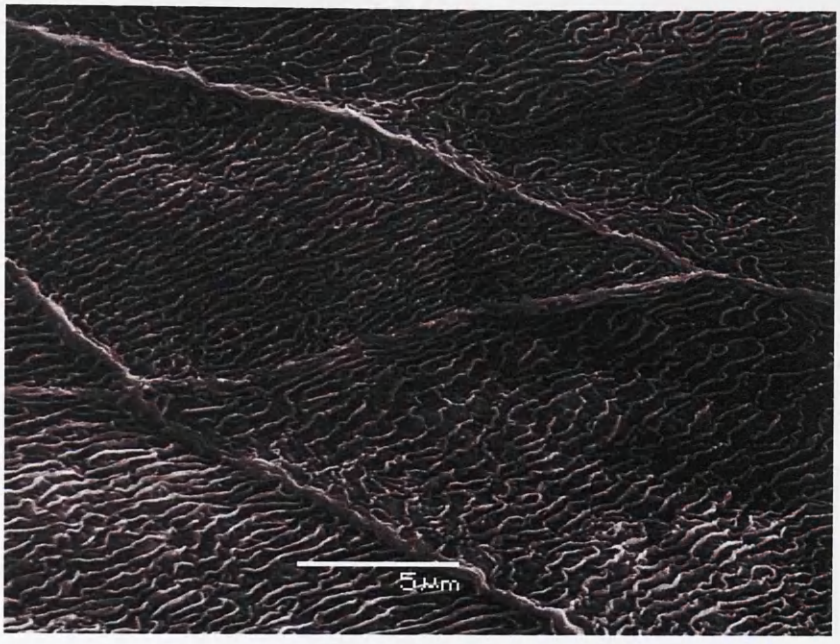


Plate 6.5: Scanning electron micrograph of biopsy-derived human gingival mucosal cells, taken at high magnification. Adjacent cells were closely attached to each other with tightly fitting and well-formed cell junctions. [Scale bar = 5µm]

Plate 6.6: Scanning electron micrograph of the apical surface of gingival mucosal cells at very high magnification. The surface of the superficial cells often revealed parallel ridge-like surface folds, quite unlike the microvilli found on corneal epithelial cells. [Scale bar = 1µm]

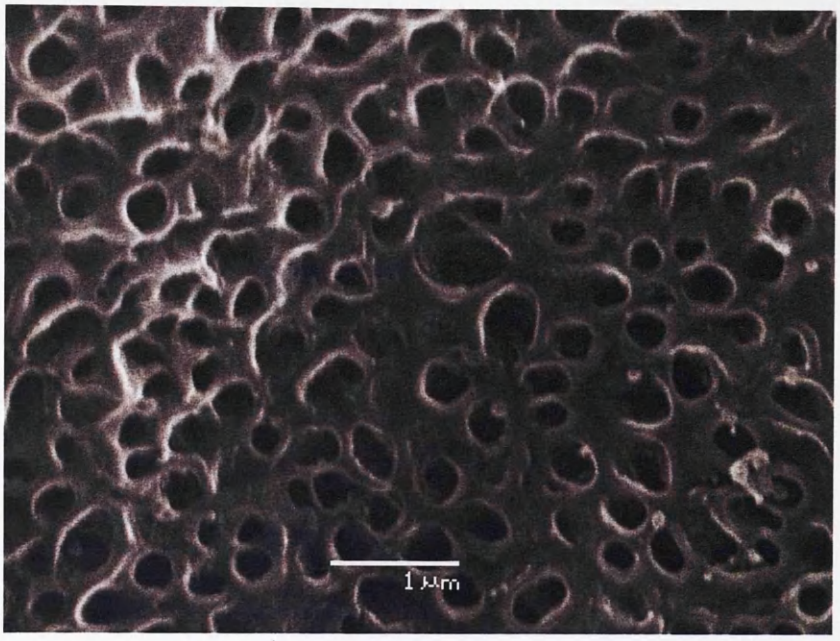


Plate 6.7: Scanning electron micrograph of the apical surface of gingival mucosal cells at very high magnification. While most of the cells were covered in ridge-like folds, some demonstrated a pitted appearance, as illustrated above. [Scale bar = 1µm]

6.1.2.2 Buccal Mucosal Biopsies

Examination of the buccal mucosal epithelium by SEM revealed an undulated, yet continuous layer of squamous, polygonal epithelial cells with an average cell surface area of 556.1µm^2 (S.D.±288.5) (plate 6.8). Some cells appeared to have very prominent nuclei. In places, cells appeared to be undergoing the process of desquamation. Neighbouring cells were tightly opposed and had distinct cell borders (plate 6.9). The apical surface of the mucosal epithelial cells was covered with long parallel ridge-like microvilli (plate 6.10) similar to those seen on the gingival mucosal cells but quite different from the surface microvilli found on superficial corneal epithelial cells. In some areas the microvilli had a slightly different morphology and were shorter and less ridge-like (plate 6.11).

Plate 6.8: Scanning electron micrograph of biopsy-derived human buccal mucosal cells, taken at low magnification. Cells were polygonal and squamous in appearance and formed a continuous layer. The cell surface was undulated, unlike that of corneal epithelial cells which is flat by nature. [Scale bar = 10µm]

Plate 6.9: Scanning electron micrograph of junctions between biopsy-derived human buccal mucosal cells, taken at high magnification. Cells were polygonal and squamous in appearance and formed a continuous layer. [Scale bar = 5µm]

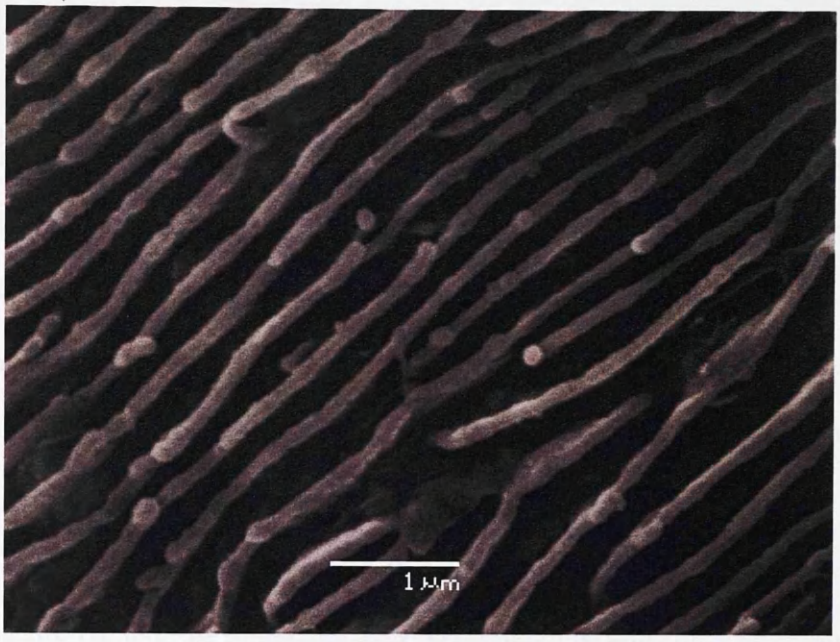


Plate 6.10: Scanning electron micrograph of the apical surface of buccal mucosal cells at very high magnification. The surface of the superficial cells often revealed parallel ridge-like surface folds, similar to those on the gingival mucosa but quite unlike the microvilli found on corneal epithelial cells. [Scale bar = 1µm]

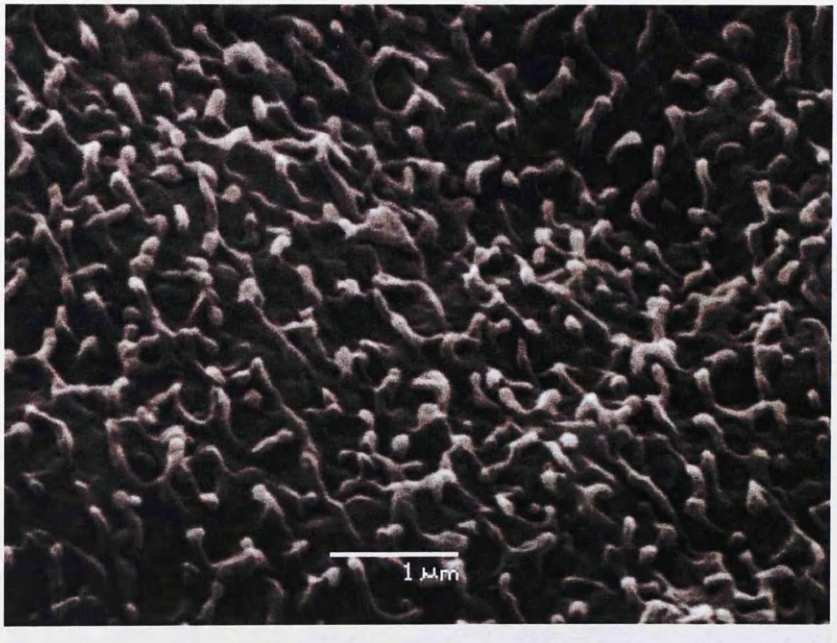


Plate 6.11: Scanning electron micrograph of the apical surface of buccal mucosal cells at very high magnification. Some of the superficial cells had short microvilli-like projections on their surface. [Scale bar = 1µm]

6.1.2.3 Control Corneal Biopsy

Human corneal epithelium was used in this study as a control against which to compare the oral (gingival and buccal) mucosal cells. Human tissue was obtained from Northwest Lions Eye Bank, preserved and processed as the mucosal cells. Examination of corneal epithelium by scanning electron microscopy revealed a continuous layer of squamous, polygonal epithelial cells with an average cell surface area of 1093.9μm^2 (S.D.±335.4) (plate 6.12). There were a number of cells undergoing the process of desquamation, as would be expected in a healthy epithelial sheet. Adjoining cells were tightly opposed and had distinct cell borders (plate 6.13). The apical surface of the superficial cells was covered in short finger-like microvilli (plate 6.14).

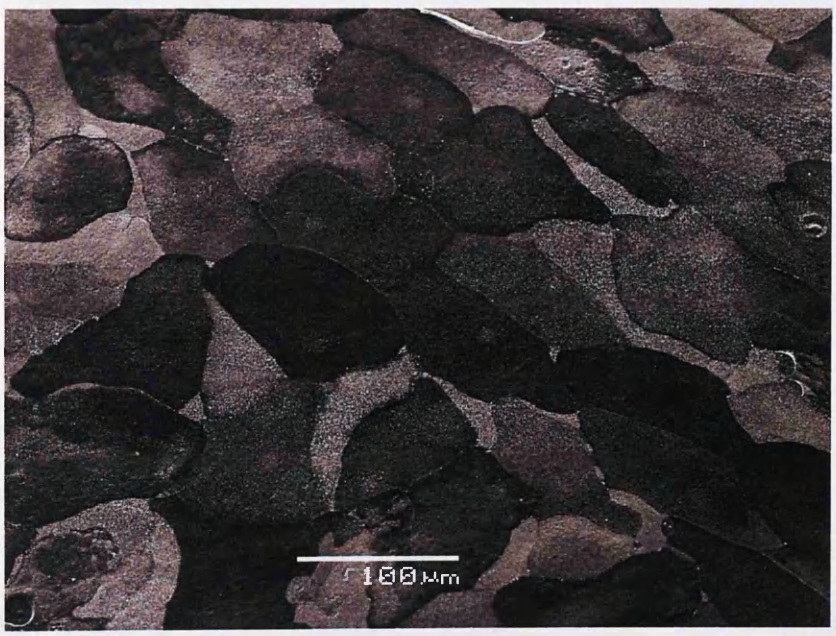


Plate 6.12: Scanning electron micrograph of human control corneal epithelial cells, taken at very low magnification. Cells were polygonal and squamous in appearance and formed a continuous layer. [Scale bar = 100µm]

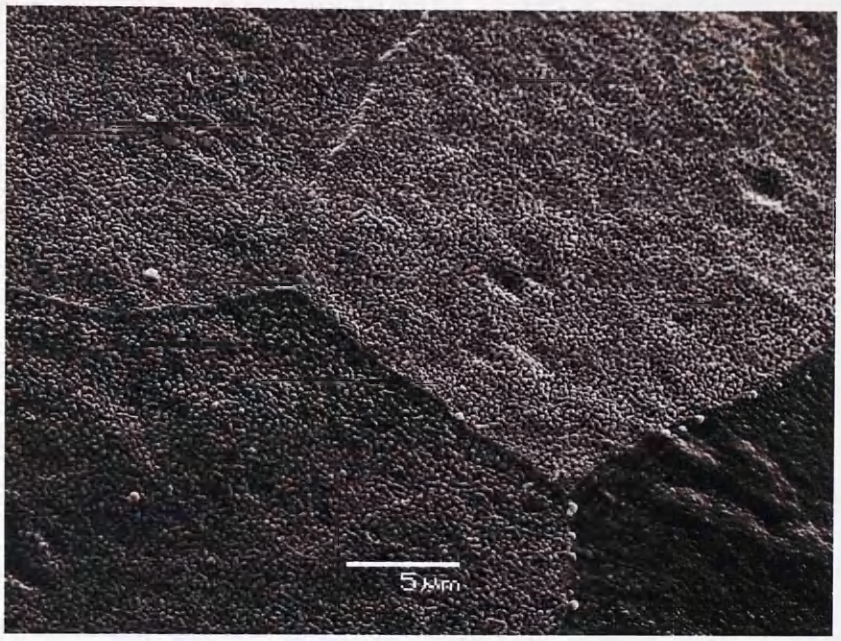


Plate 6.13: Scanning electron micrograph of junctions between human control corneal epithelial cells, taken at high magnification. Adjacent cells were tightly opposed and had distinct and well-defined cell borders. [Scale bar = 5µm]

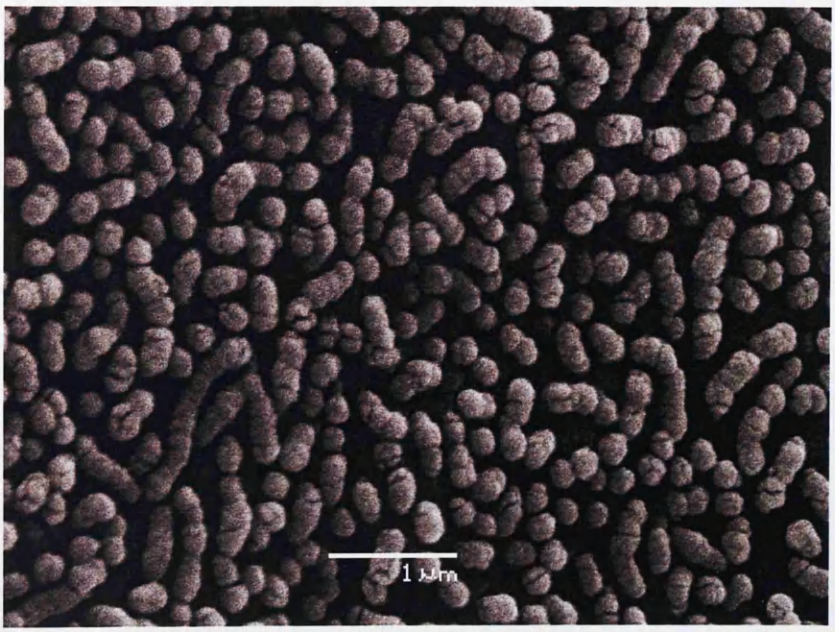


Plate 6.14: Scanning electron micrograph of the apical surface of human corneal epithelial cells at very high magnification. Superficial cells had short microvilli-like projections on their surface. [Scale bar = 1µm]

6.1.3 Transmission Electron Microscopy

6.1.3.1 Gingival Mucosal Biopsies

Examination of the biopsy-derived gingival epithelial cells by TEM revealed many layers (40+) of epithelial cells. These appeared to be well-stratified and well-differentiated. There were many layers of superficial squamous epithelial cells with very few intercellular spaces (plate 6.15). The apical surface of the most superficial cells was covered with short, regular microvilli and in places a glycocalyx-like substance (plate 6.16). There were several layers of cuboidal wing cells (plate 6.17). Adjacent cells were attached by way of desmosomal junctions and cell membranes were highly interdigitated (plate 6.18). Basal cells were columnar in appearance (plate 6.19) and were attached to the basement membrane by hemidesmosomal junctions (plate 6.20). There were fairly large intercellular spaces between cells in the basal region. Beneath the epithelial cell layer there was also a mass of connective tissue. This was vascularized and contained an abundance of fibrillar collagen, as well as fibroblast cells.

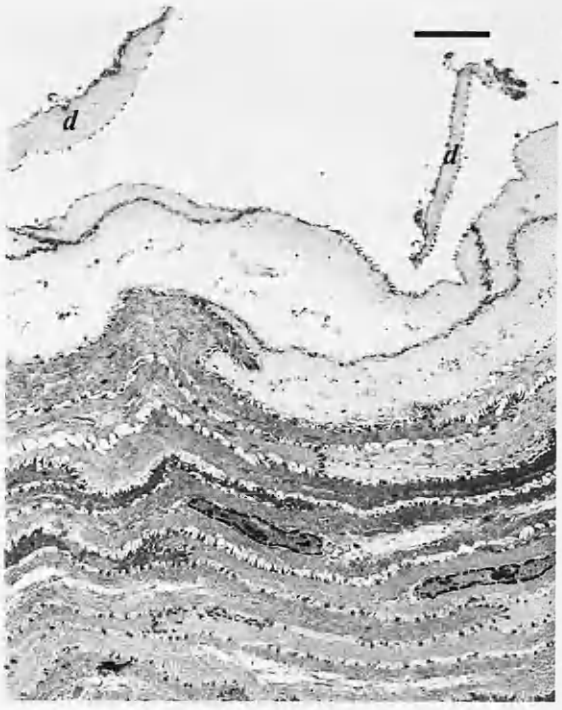


Plate 6.15: Transmission electron micrograph of the gingival mucosal cells at low magnification. Squamous superficial cells formed many layers, and there was evidence of cell desquamation *[d]* from the surface. [Scale bar = 5μm]

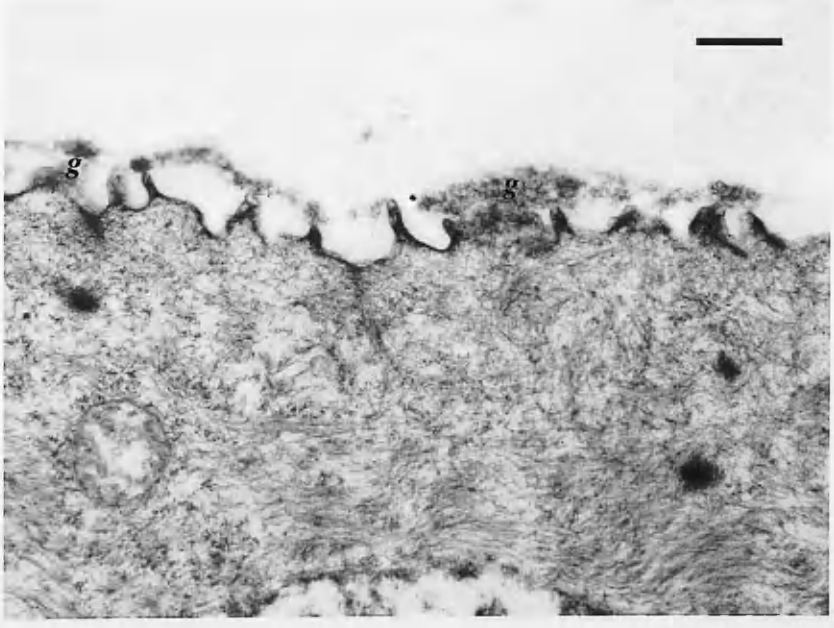


Plate 6.16: Transmission electron micrograph of the superficial gingival mucosal cells at high magnification. There were small projections on the apical surface and in places, a thin covering of glycocalyx-like substance *[g]*. [Scale bar = 500nm]

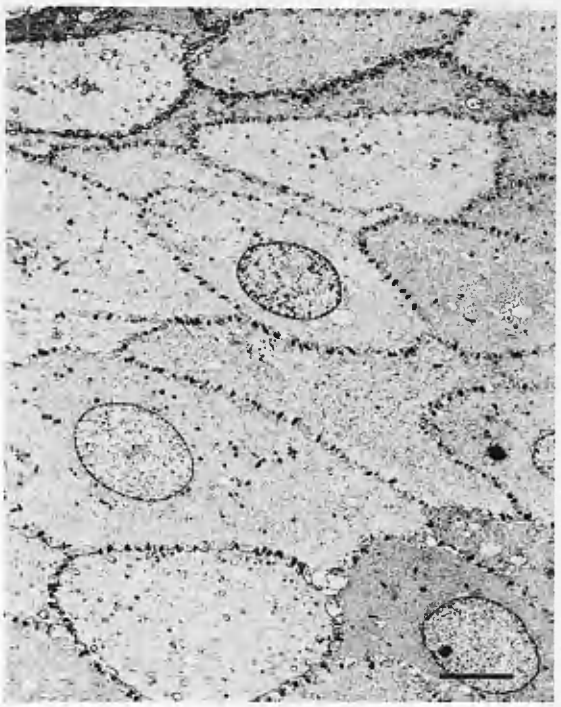


Plate 6.17: Transmission electron micrograph of the gingival mucosal cells at low magnification. There were several layers of wing cells as depicted above. These cells were tightly packed and joined by numerous desmosomal junctions. [Scale bar = 5μm]

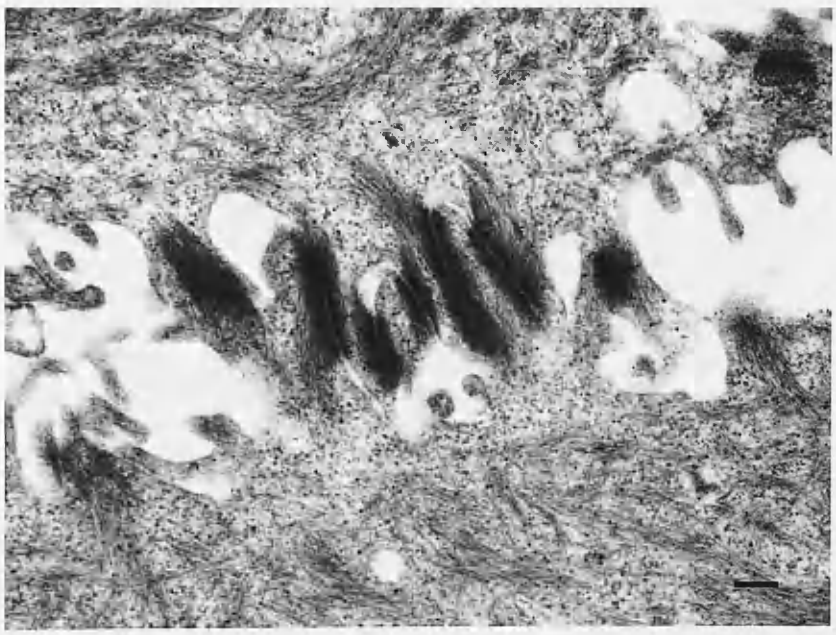


Plate 6.18: Transmission electron micrograph of junctions between the gingival mucosal cells at high magnification. There were numerous desmosomes joining cells at all cell layers. [Scale bar = 200nm]

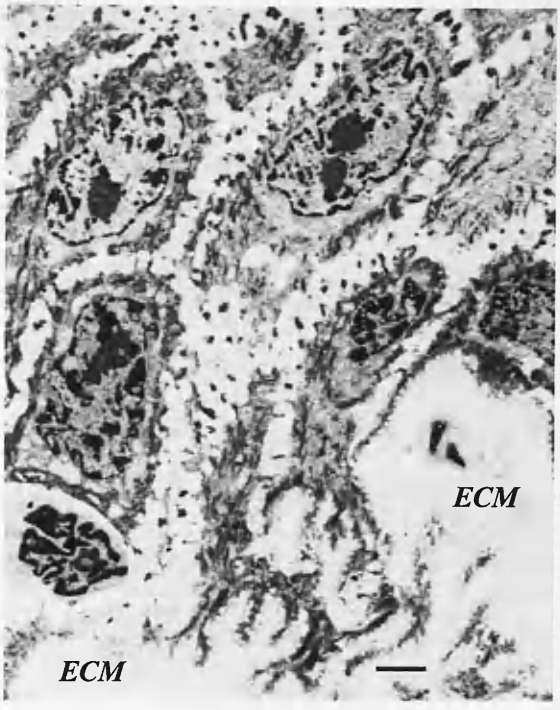


Plate 6.19: Transmission electron micrograph of the gingival mucosal cells at low magnification. Basal cells were columnar in shape, though not tightly opposed. There were some large spaces between adjacent cells however basal cells were well attached to the underlying extracellular matrix *[ECM]*. [Scale bar = 2μm]

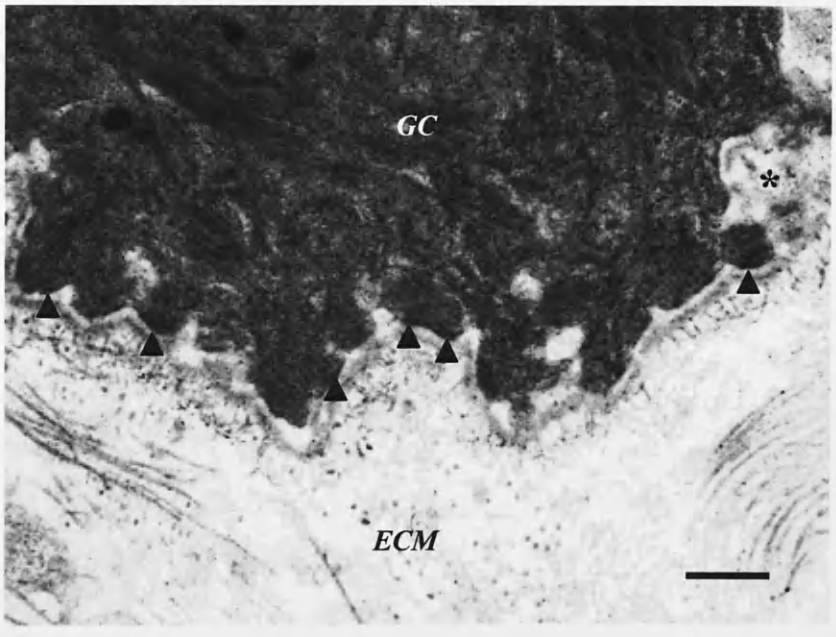


Plate 6.20: Transmission electron micrograph of the gingival mucosal cells *[GC]* at high magnification. Basal cells were well attached to the basement membrane via frequent hemidesmosomes (arrowheads) and were secreting basement membrane material (*). [Scale bar = 500nm]

6.1.3.2 Buccal Mucosal Biopsies

Examination of the buccal epithelial cells by TEM revealed in excess of 40 layers of epithelial cells. The buccal epithelium was similar in appearance to that of the gingival. Cells were well-stratified and differentiated. Superficial epithelial cells had highly interdigitated cell membranes however were not very regular in shape and were not as squamous as those found in the corneal epithelium (plate 6.21). Some cells appeared to be undergoing the process of desquamation. The apical surface of the most superficial cells was covered with short, regular microvilli (plate 6.22). There were several layers of cuboidal wing cells (plate 6.23). Adjacent cells in all layers were attached by way of numerous desmosomal junctions (plate 6.24). Intercellular spaces were more prominent in the more basal regions of the epithelium. Basal cells were columnar (plate 6.25) and attached to each other by way of desmosomal junctions. In some areas, there were large intercellular spaces between adjacent basal cells. The basal cells were attached to the basement membrane by hemidesmosomal junctions (plate 6.26). There was a submucosal mass of extracellular matrix and connective tissue. This was quite heavily vascularized and both red and white blood cells were observed within capillaries (plate 6.27).

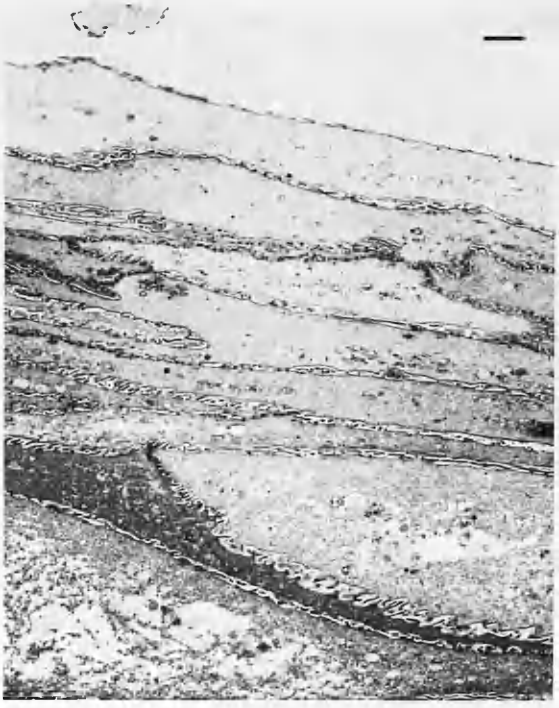


Plate 6.21: Transmission electron micrograph of the buccal mucosal cells at low magnification. Squamous superficial cells formed many layers and there was evidence of cell desquamation from the surface. [Scale bar = 2μm]

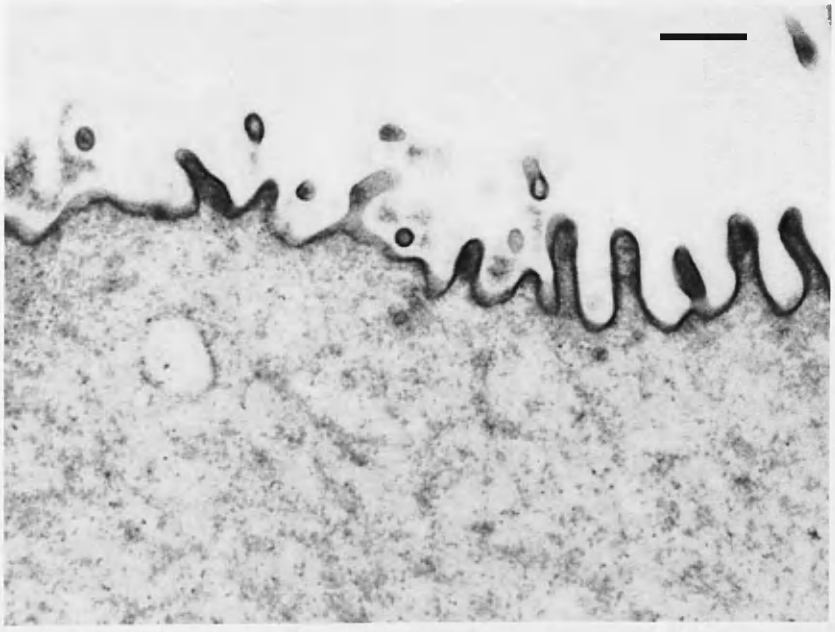


Plate 6.22: Transmission electron micrograph of the buccal mucosal cells at high magnification. Superficial cells were covered in microvilli-like projections. [Scale bar = 500nm]

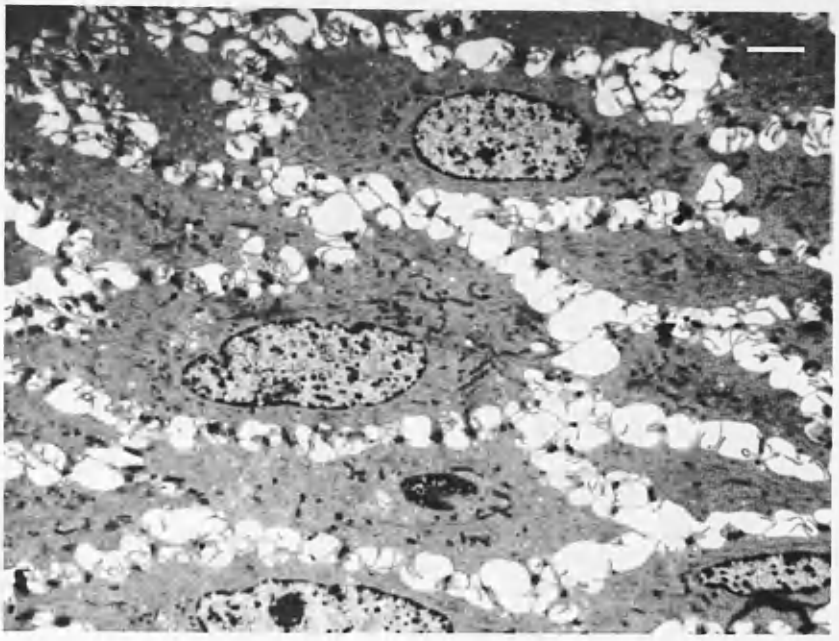


Plate 6.23: Transmission electron micrograph of the buccal mucosal cells at low magnification. There were several layers of wing cells with prominent nuclei. The wing cell layer had fairly large spaces between cells. [Scale bar = 2μm]

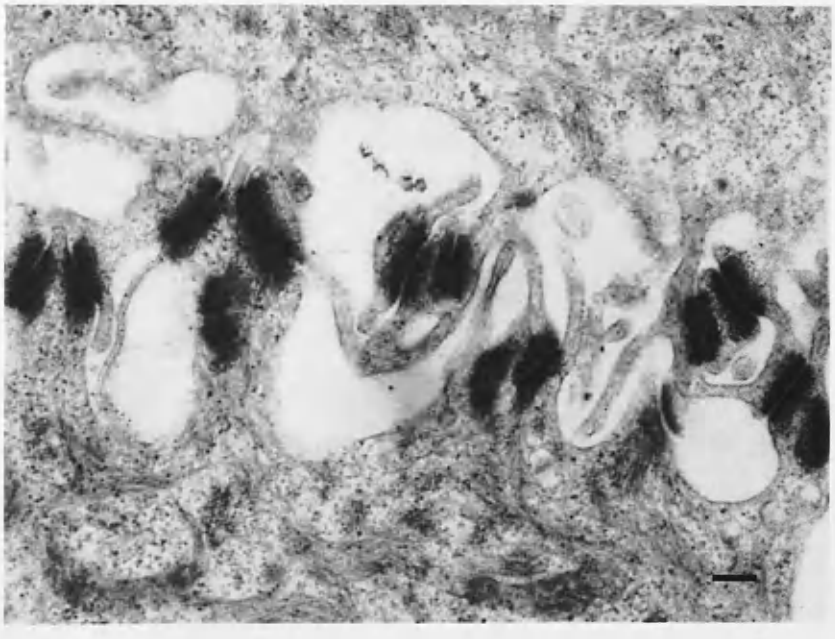


Plate 6.24: Transmission electron micrograph of junctions between the buccal mucosal cells at high magnification. There were numerous desmosomes joining cells at all cell layers. [Scale bar = 200nm]

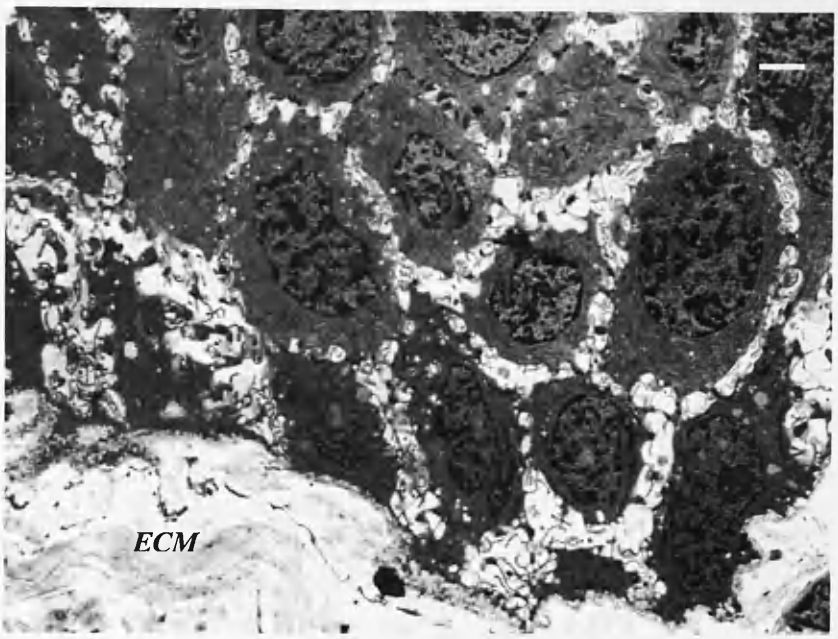


Plate 6.25: Transmission electron micrograph of the buccal mucosal cells at low magnification. Basal cells were slightly more columnar in shape than cells in other areas, though not they were not very tightly opposed. [Scale bar = 2μm]

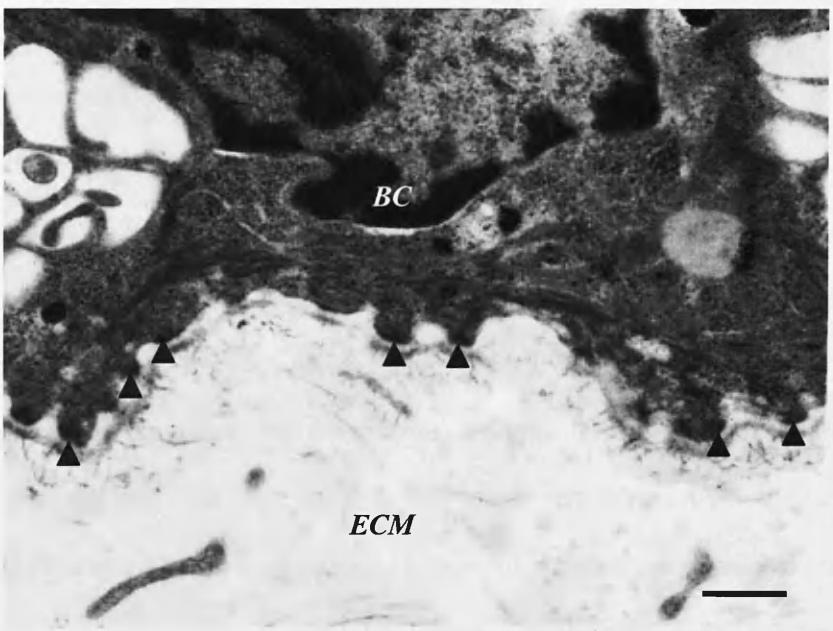


Plate 6.26: Transmission electron micrograph of the buccal mucosal cells *[BC]* at high magnification. Basal cells were well attached to the basement membrane via frequent hemidesmosomes (arrowheads). [Scale bar = 500nm]

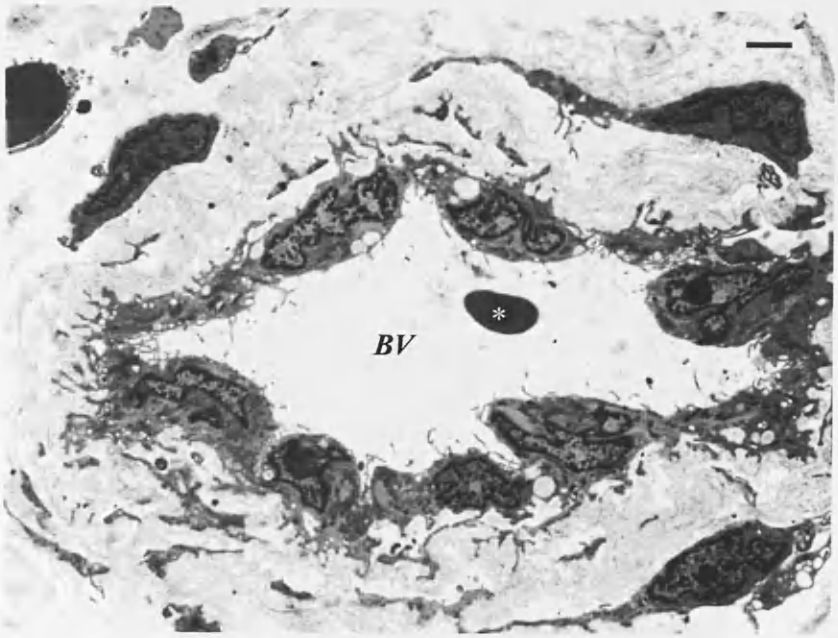


Plate 6.27: Transmission electron micrograph of the submucosal connective tissue. Beneath the basal cell layers the connective tissue was heavily vascularized. This micrograph illustrates a red blood cell (*)-containing capillary *[BV]*. There were also many white blood cells which did not seem to be contained within vessels. [Scale bar = 2μm]

6.1.3.3 Control Corneal Biopsy

Examination of the control corneal epithelial cells (human donor tissue from Northwest Lions Eye Bank) by TEM revealed 6-8 layers of well-stratified epithelial cells (plate 6.28). The epithelium was differentiated into columnar basal cells, cuboidal suprabasal wing cells and 3-4 layers of squamous superficial cells. Some cells appeared to be undergoing the process of desquamation. The apical surface of the most superficial cells was covered with short, regular microvilli (plate 6.29). Adjacent cells in all layers were attached by way of numerous desmosomal junctions and there were negligible intercellular spaces. Basal cells were columnar and attached to the basement membrane by numerous hemidesmosomal junctions (plate 6.30).

Plate 6.28: Transmission electron micrograph of human corneal epithelial cells at low magnification. Cells were well-stratified and differentiated and formed 6-8 layers. The epithelium was adhered to Bowman's layer *[BL]* via a thin basement membrane. [Scale bar = 5μm]

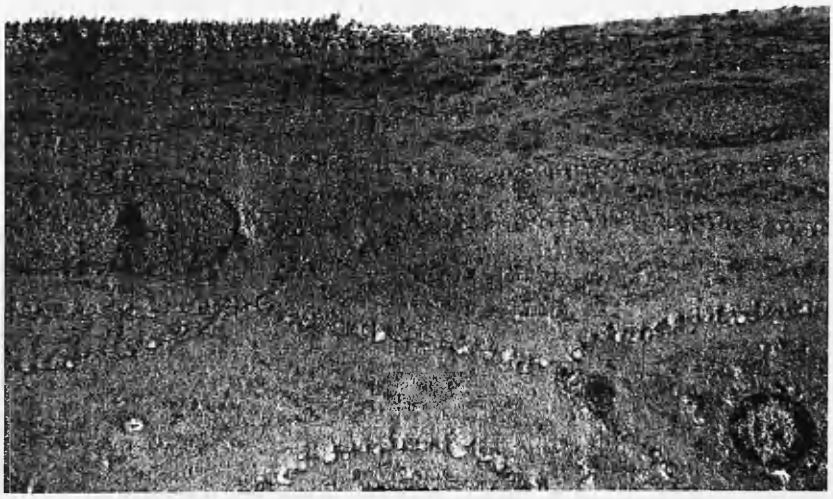


Plate 6.29: Transmission electron micrograph of human corneal epithelium. Superficial cells were squamous and covered with short microvilli. [Scale bar = 2μm]

Plate 6.30: Transmission electron micrograph of human corneal basal epithelial cells. Basal cells were columnar and were well attached to the underlying Bowman's layer *[BL]* with numerous hemidesmosomes. [Scale bar = 2μm]

6.1.4 Scanning Electron Microscopy

6.1.4.1 Cultivated Gingival Mucosal Epithelial Cells on AM

Examination of the gingival epithelial culture by SEM revealed a continuous layer of flat, squamous polygonal epithelial cells with an average cell surface area of 613.1μm^2 (S.D.±294.8) (plate 6.31). The cells seemed to be in good condition and were similar in appearance to *in vivo* human corneal epithelium. In places, cells appeared to be undergoing the process of desquamation (plate 6.31). Adjacent cells were closely attached with tightly opposed cell junctions and fairly distinct cell boundaries (plate 6.32). The apical surfaces were covered in short microvilli resembling those seen on the surface of normal corneal epithelial cells (plate 6.33).

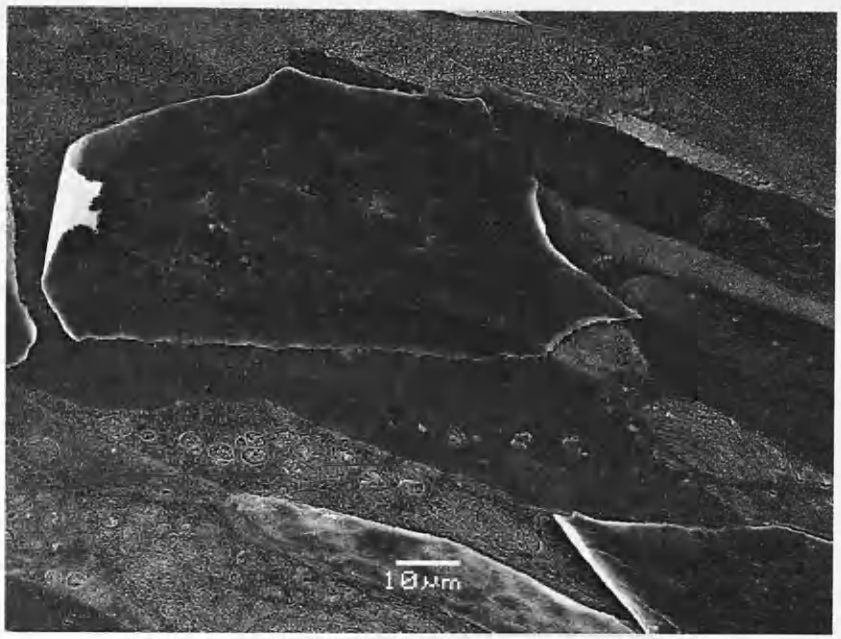


Plate 6.31: Scanning electron micrograph of cultivated human gingival epithelial cells on denuded amniotic membrane. The cells appeared healthy and well-formed with distinct cell boundaries. There were many cells desquamating from the surface. [Scale bar = 10µm]

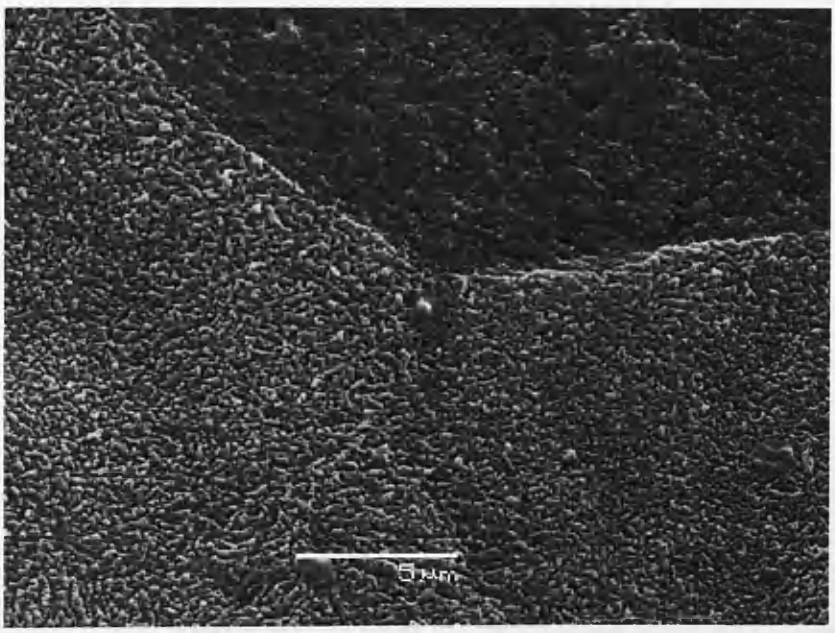


Plate 6.32: Scanning electron micrograph of cultivated human gingival epithelial cells on denuded amniotic membrane, taken at high magnification. Adjacent cells were tightly opposed with distinct cell borders. [Scale bar = 5µm]

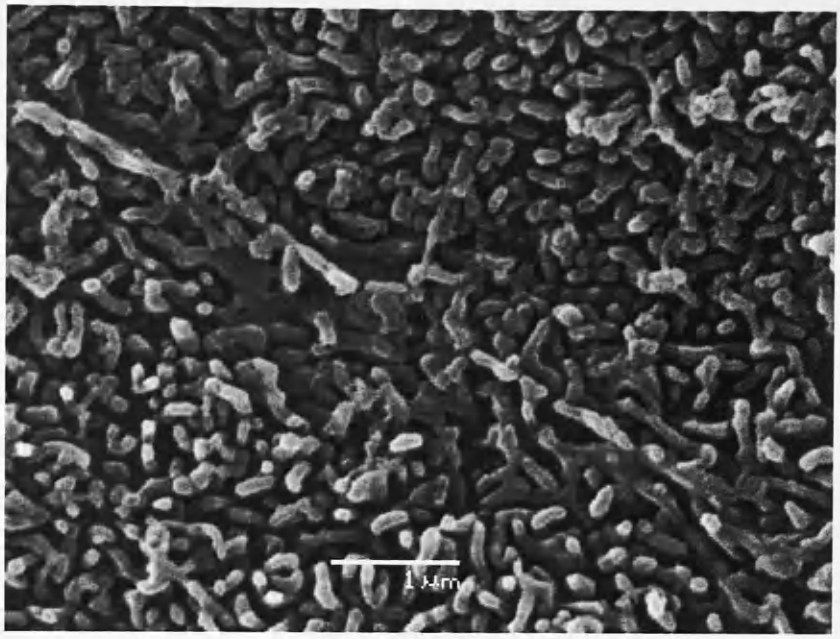


Plate 6.33: Scanning electron micrograph of cultivated gingival mucosal cells at very high magnification. The apical surface of the cells was covered with short microvilli. This micrograph shows microvilli at the border of two adjacent cells. [Scale bar = 1µm]

6.1.4.2 Cultivated Buccal Mucosal Epithelial Cells on AM

Scanning electron microscopy of the buccal epithelial culture on amniotic membrane revealed a continuous layer of flat, squamous polygonal epithelial cells (plate 3.34). The cultivated cells had an average cell surface area of 491.4µm^2 (S.D.±173.7) and were healthy-looking and well-developed. The cultivated mucosal cells were very similar in appearance to corneal epithelium. In places, cells appeared to be undergoing the process of desquamation. Adjacent cells were closely attached with tightly opposed cell junctions and distinct cell boundaries (plate 3.35). The apical surface of the cells was covered in short microvilli (plate 3.36) which were very different in appearance to the surface folds seen on the mucosal cells.

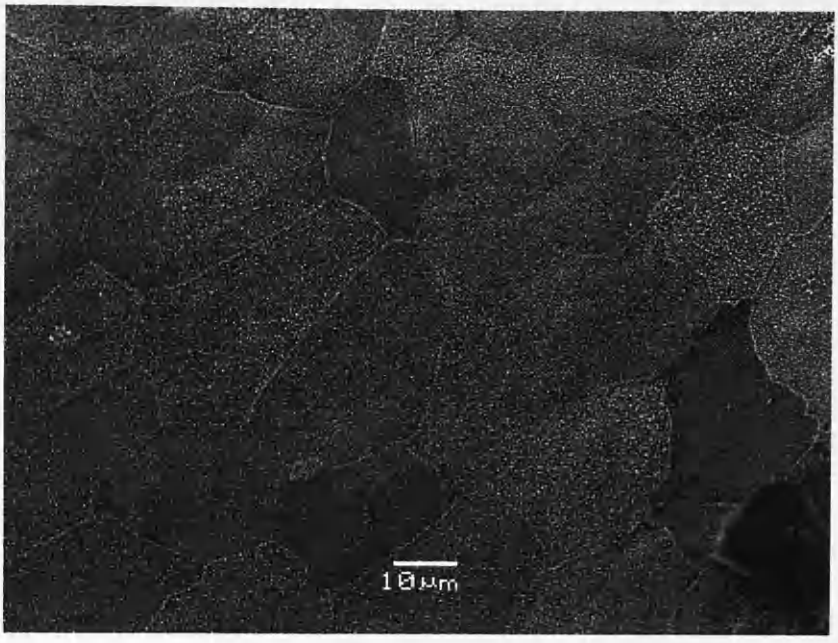


Plate 6.34: Scanning electron micrograph of cultivated human buccal epithelial cells on denuded amniotic membrane. The cells appeared healthy and well-formed with distinct cell boundaries. [Scale bar = 10µm]

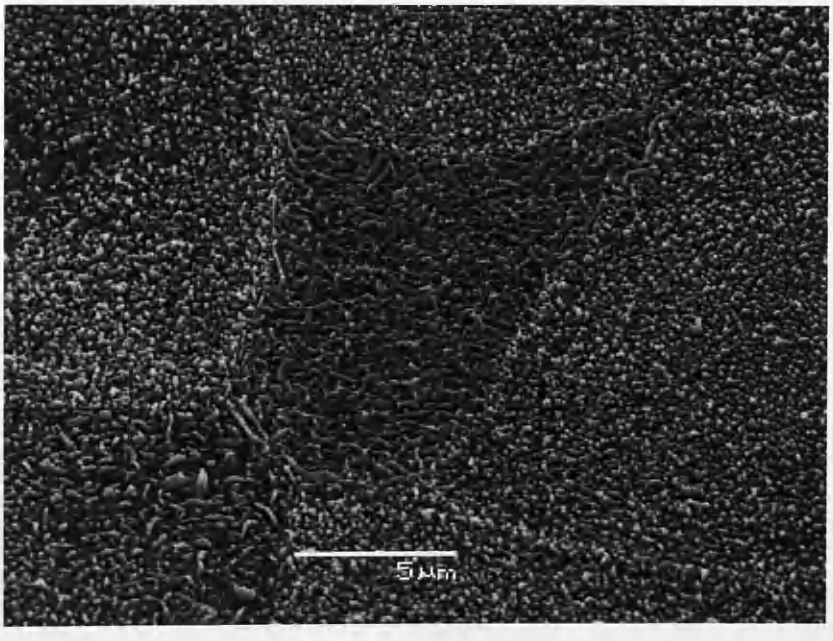


Plate 6.35: Scanning electron micrograph of cultivated human buccal epithelial cells on denuded amniotic membrane. The cells appeared to be in good condition and were closely attached to each other with tightly fitting junctions. [Scale bar = 5µm]

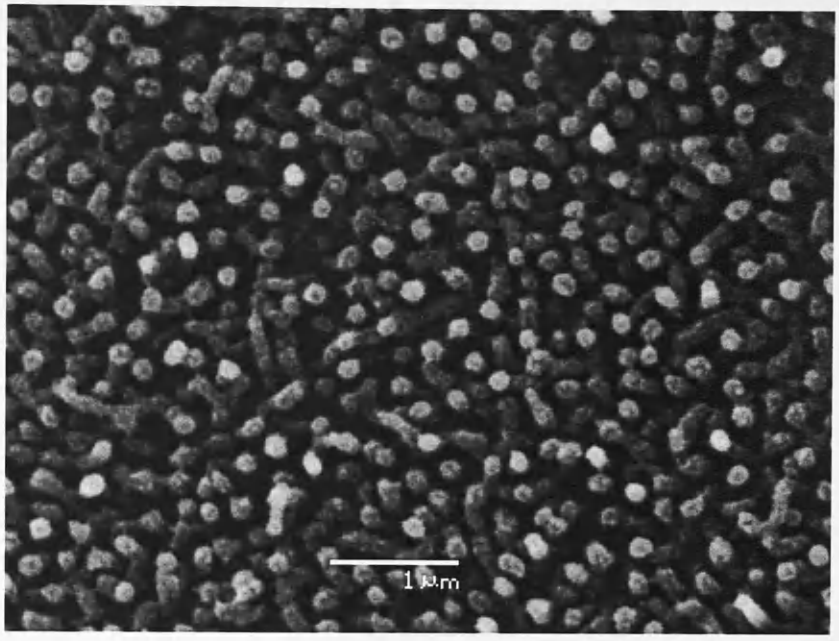


Plate 6.36: Scanning electron micrograph of cultivated human buccal epithelial cells on denuded amniotic membrane. The apical surfaces of the cells were covered with numerous microvilli like projections. [Scale bar = 1 µm]

6.1.5 Transmission Electron Microscopy

6.1.5.1 Cultivated Gingival Mucosal Epithelial Cells on AM

Examination of the cultivated human gingival epithelial cells by TEM revealed approximately 10-14 layers of fairly well-stratified epithelium (plate 6.37). The cells appeared quite healthy and were differentiated into basal columnar cells, suprabasal wing cells and flat squamous superficial cells. There was evidence of cell desquamation at the surface, as would be expected in a healthy epithelial sheet. Superficial cells were often found to contain intracellular vacuoles containing a granular-looking substance, possibly glycogen (plate 6.38). There was an apical covering of microvilli–like projections (plate 6.39). Basal cells (plate 6.40) adhered well to the amniotic membrane substrate with hemidesmosomal junctions and

appeared to produce basement membrane material (plate 6.41). Interdigitations into the AM were quite pronounced in some regions. There were however large intercellular spaces between cells in all cell layers and these were most prominent in the basal region. Epithelial cells were attached to neighbouring cells by infrequent desmosomal junctions.

Plate 6.37: Transmission electron micrograph of cultivated human gingival epithelial cells on amniotic membrane. Cells formed 10-14 layers of fairly well-stratified and differentiated epithelium. There were substantial spaces between adjacent cells at all layers (*). [Scale bar = 10μm]

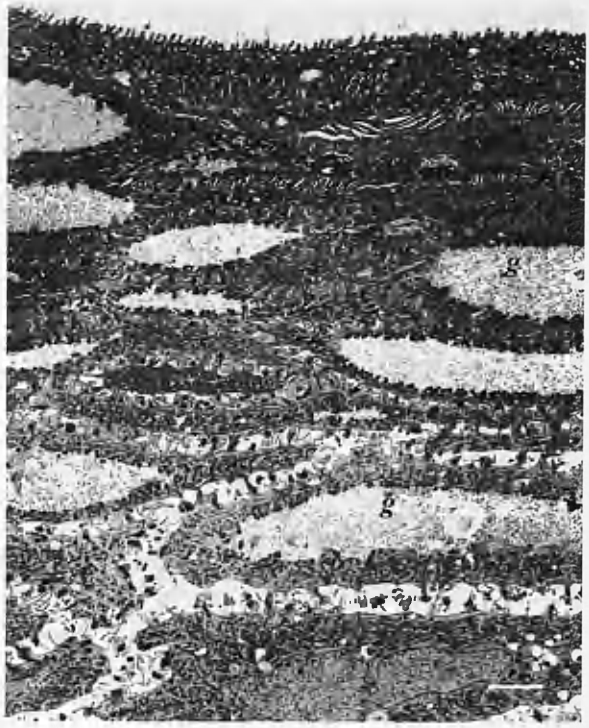


Plate 6.38: TEM micrograph of cultivated superficial gingival cells. Many cells contained inclusions of granular substance, possibly glycogen *[g]*. [Scale bar = 2μm]

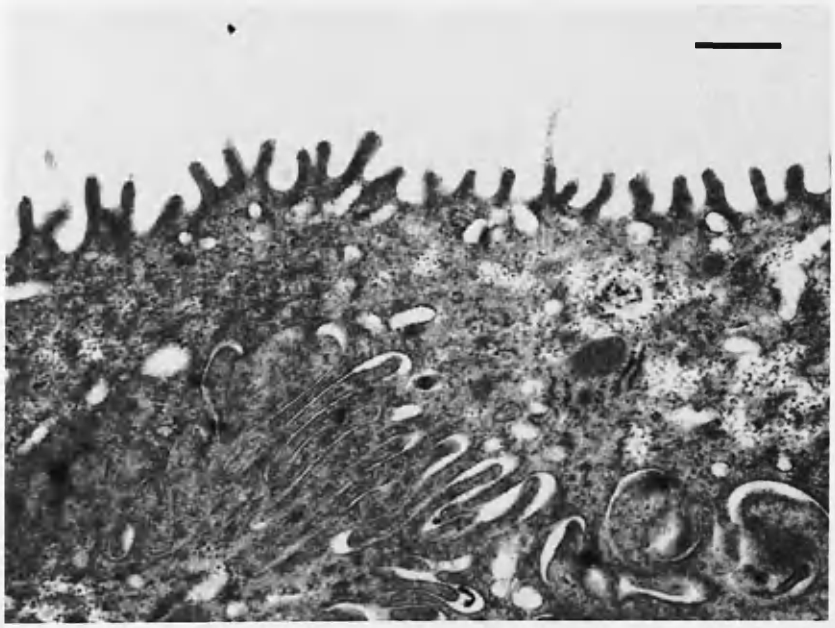


Plate 6.39: Transmission electron micrograph of cultivated human gingival epithelial cells on amniotic membrane. Superficial cells had an apical covering of short microvilli-like projections. [Scale bar = 500nm]

Plate 6.40: Transmission electron micrograph of cultivated human gingival epithelial cells on amniotic membrane *[AM]*. Basal cells were slightly columnar in shape though not as differentiated as normal corneal basal epithelial cells. There were large spaces between adjacent cells. [Scale bar = 2μm]

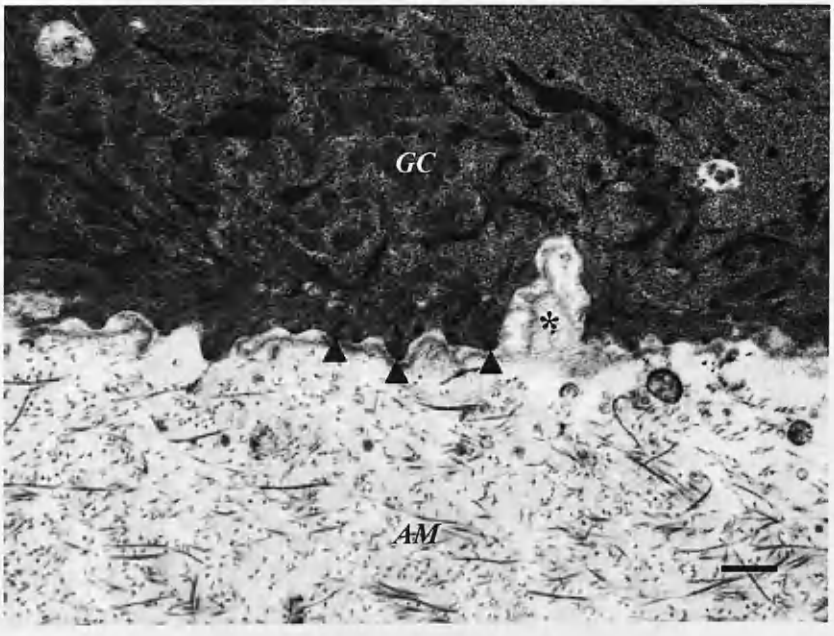


Plate 6.41: TEM micrograph of cultivated human gingival epithelial cells *[GC]* on amniotic membrane *[AM]*. Basal cells were attached well to the amniotic membrane by hemidesmosomes (arrowheads) and produced basement membrane material (*). [Scale bar = 500nm]

6.1.5.2 Cultivated Buccal Mucosal Epithelial Cells on AM

Transmission electron microscopy of the cultivated human buccal epithelial cells revealed approximately 6-10 layers of fairly well-stratified epithelium (plate 6.42). The cells appeared quite healthy and were differentiated into basal columnar cells, suprabasal wing cuboid cells and flat squamous superficial cells (plate 6.43). In places the basal cells adhered well to the amniotic membrane substrate (plate 6.44) and appeared to produce basement membrane material (plate 6.46). In other areas the cells had completely come away from the amniotic membrane (plate 6.45). There were large intercellular spaces between cells in all cell layers however epithelial cells were attached to neighbouring cells by desmosomal junctions (plate 6.47). The surface of the cells was covered with short microvilli, though in places these were longer than those normally present on corneal epithelial cells.

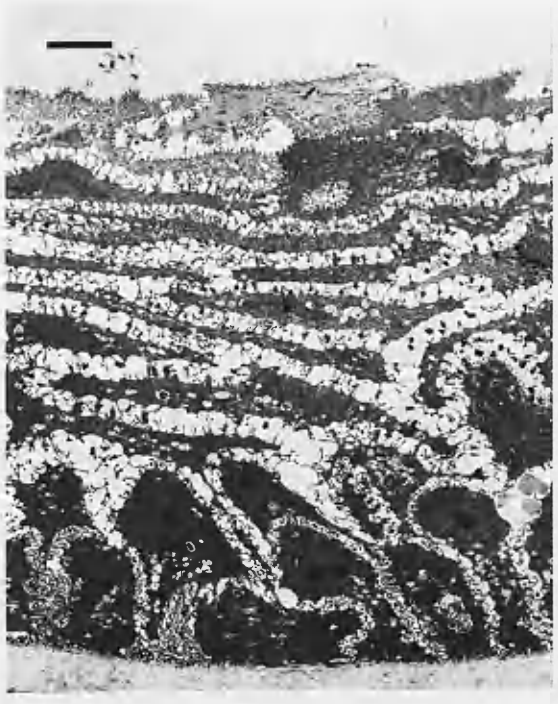


Plate 6.42: TEM micrograph of cultivated human buccal epithelial cells on amniotic membrane. Cells formed 6-10 layers of well-stratified and differentiated epithelium. There were fairly large spaces between adjacent cells at all layers. [Scale bar = 7μm]

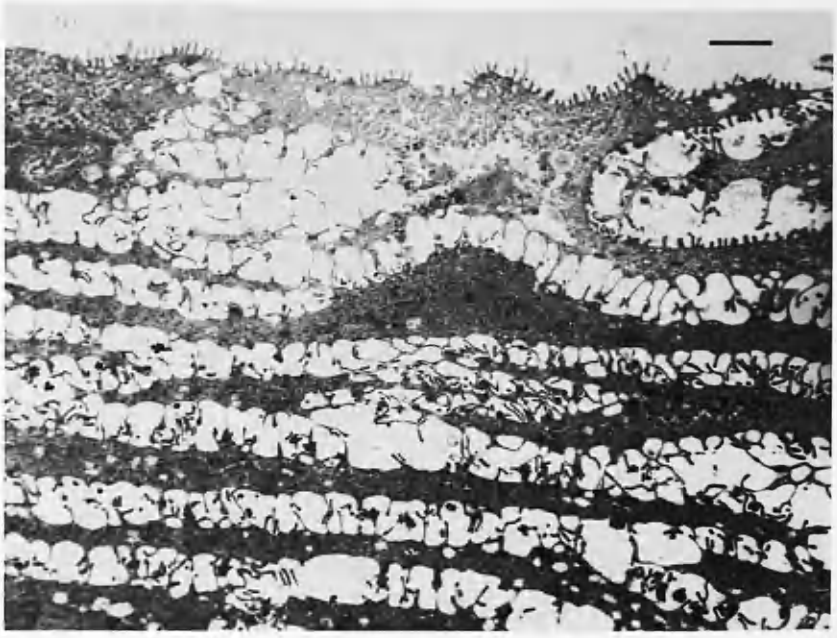


Plate 6.43: TEM micrograph of cultivated superficial buccal cells. Superficial cells formed several layers, and were generally squamous in appearance. There was an apical covering of microvilli-like projections. Intercellular spaces were prominent in the superficial regions. [Scale bar = 2μm]

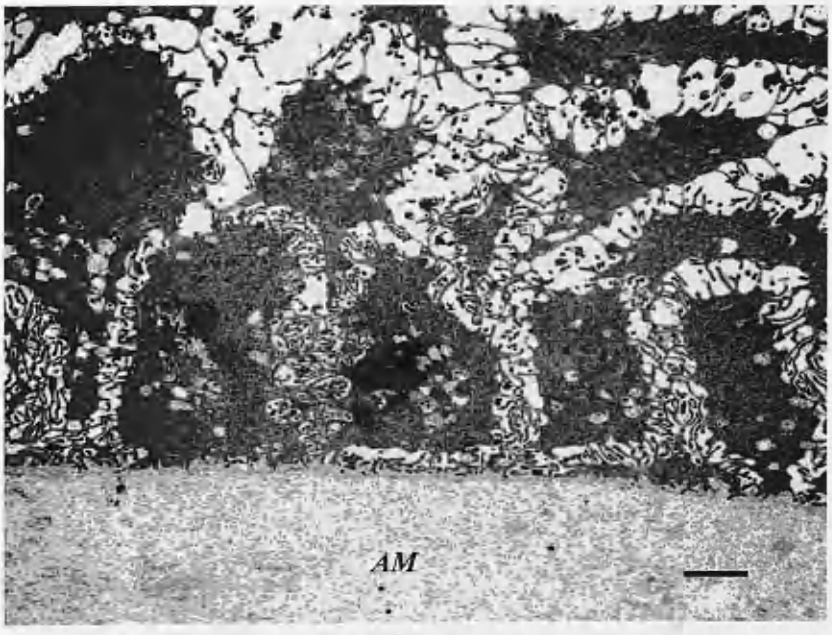


Plate 6.44: Transmission electron micrograph of cultivated human buccal cells on amniotic membrane *[AM]*. Basal cells were columnar in shape and were, for the most part, well attached to the substrate. [Scale bar = 2μm]

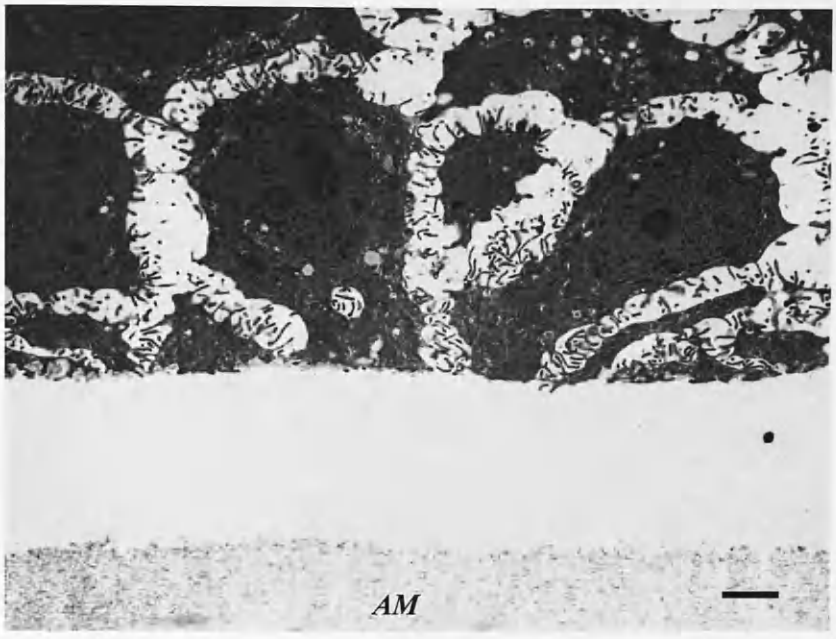


Plate 6.45: Transmission electron micrograph of cultivated human buccal cells on amniotic membrane *[AM]*. In some areas of the culture sheet (as pictured above), the epithelial layer had detached from the amniotic membrane. [Scale bar = 2μm]

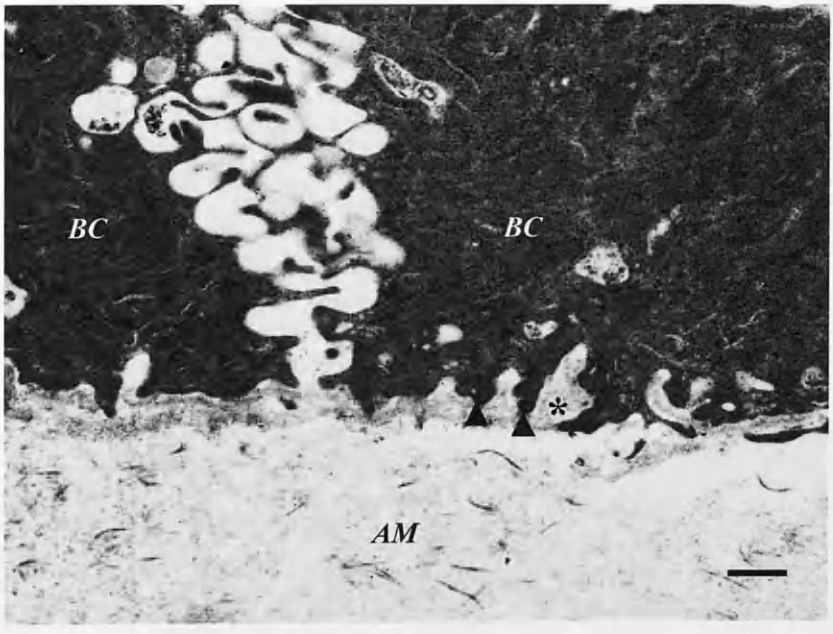


Plate 6.46: TEM micrograph of cultivated human buccal epithelial cells *[BC]* on amniotic membrane *[AM]*. This figure represents good attachment of the basal cells to the amniotic membrane substrate by hemidesmosomes (arrowheads). These cells appeared to be producing basement membrane material (*). [Scale bar = 500nm]

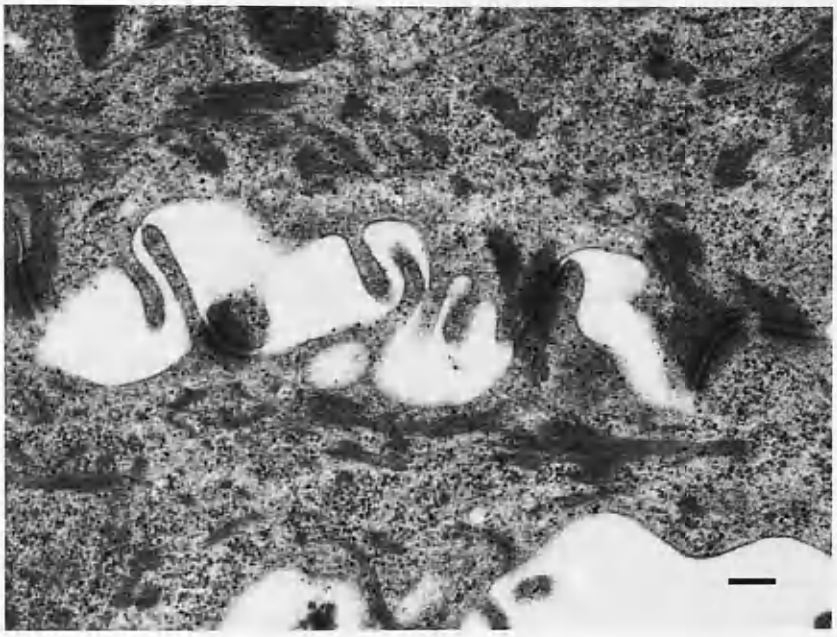


Plate 6.47: TEM micrograph of the junctions between adjacent cultivated human buccal epithelial cells. Though there were a number of large intercellular spaces between cells, mechanical strength was maintained by way of many desmosomal junctions. [Scale bar = 200nm]

6.1.6 Quantitative Study

A number of parameters were quantitatively compared between the two mucosal cell types and the control corneal epithelium. Mean data (±S.D.) are shown in table 6.1 below. Fourteen data points were collected for each parameter, except for the cell surface area which was calculated using scanning electron microscopy and therefore facilitated more measurements (n=50).

	Gingival mucosal cells cultivated on AM	**Buccal mucosal cells cultivated on AM**	**Human corneal epithelium**
Thickness of cell layers (μm)	73.42 ± 2.70	46.98 ± 12.57	29.02 ± 2.29
Number of cell layers	10.29 ± 1.33	8.57 ± 1.70	6.64 ± 0.74
Intercellular space area (μm^2)	1.08 ± 1.16	1.40 ± 0.89	0.11 ± 0.08
Number of desmosomes	2.07 ± 1.64	1.29 ± 0.91	3.21 ± 1.37
Number of hemidesmosomes	3.43 ± 1.45	2.21 ± 1.53	4.71 ± 1.44
Cell surface area (μm^2)	613.09 ± 294.75	491.44 ± 173.67	1093.89 ± 335.43

Table 6.1: Comparison of cultivated human buccal and gingival mucosal epithelial cells on denuded AM with normal human cornea. Mean data (± S.D.) are shown. Fourteen data points were taken at random, except for the cell surface areas in which case n=50. The averages for intercellular area and number of desmosomes are given for random 3μm long interfaces between adjacent cells.

For ease of interpretation, mean data for the gingival and buccal epithelia were plotted as percentage differences from the control (normal human corneal epithelium). Neither of the cultivated cell types accurately resembles corneal epithelium. Cultivated human gingival epithelial cells had a significantly thicker cell layer (153% increase in cell thickness compared with control, $P<0.001$) and significantly smaller surface areas ($P<0.001$). There was also a large increase (873%) in the area of intercellular space in the cultivated mucosal cells, though the large

standard deviation rendered this finding not statistically significant. The observed discrepancy in numbers of desmosomes was found to be not statistically significant (P=0.056). There were 27% fewer hemidesmosomal junctions in the cultivated gingival cells than in the control cornea, and this difference was found to be significant (P=0.026). Cultivated buccal cells differed more drastically from the control corneal epithelium. The observed differences were all statistically highly significant (P<0.001). Most notable was the 1159% increase in intercellular spacing in the cultivated buccal cells. Chart 6.1 compares the two cultivated cell types with each other, using control cornea as the benchmark. It appears that the cultivated gingival cells more closely resemble the corneal epithelium than the cultivated buccal cells on AM, having a slightly thicker cell layer, smaller intercellular spaces, more desmosomes for mechanical strength and more hemidesmosomes for basal attachment. Only the differences in thickness of cell layers (P<0.001) and number of hemidesmosomes (P=0.036) were deemed statistically significant.

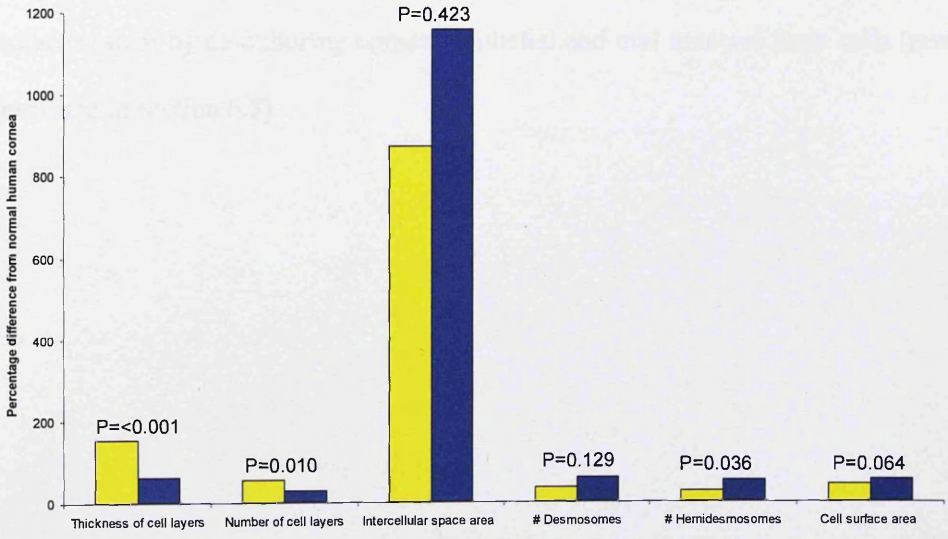


Chart 6.1: Comparison of cultivated human gingival (yellow) and buccal (blue) stem cells on amniotic membrane, expressed as percentage difference from normal human cornea control. Raw data were used to calculate statistical significance (P values) of observed differences between mucosal cell types and these are stated above the bars.

6.1.7 Summary Interpretations

Examination of the cultivated epithelial cells by SEM and TEM revealed that both gingival and buccal cells seemed to have become more corneal-like under culture conditions, yet still differed significantly from control *in vivo* human corneal epithelium in a number of parameters. Scanning electron microscopy of the mucosal biopsies showed the distinctive ridges that characterize mucosal epithelium. These were absent in the cultivated cells. Transmission electron microscopy showed very thick cell layers in the mucosal epithelial biopsies. The thickness of the cell layers was dramatically reduced in culture, both of buccal and gingival cells. From the results of the quantitative study, gingival cells seemed to be the better choice of the two and more closely resembled control corneal epithelium. They had smaller intercellular spaces and formed more junctions, both basal and mechanical, than the buccal cells in culture. While encouraging, there remain some discrepancies between the oral cultured cells and control corneal epithelium. Following a number of clinical trials using cultured mucosal tissue, an attempt was made to further improve this culture system by co-culturing corneal epithelial and oral mucosal stem cells (results described in section 6.3).

6.2 Clinical Outcomes of Amniotic Membrane/Oral Mucosal Stem Cell Transplants

Given the encouraging results of the cell culture experiments of the previous section, mucosal sheets were used in fifteen ocular surface reconstruction procedures by colleagues in Japan, with varying degrees of success (Nakamura *et al.*, 2004a). One of the benefits of this technique is that the stem cell graft and underlying amniotic membrane can be removed if necessary. This section describes the morphology of two grafts, removed after 5-6 months on the ocular surface, which were replaced by donor corneal tissue in conventional PKP surgery.

6.2.1 Clinical Observations

Clinical examination by project collaborators at the Kyoto Prefectural University of Medicine indicated that despite initial signs of success (acceptance of the oral graft with no epithelial defects) and a reported improvement in visual acuity, two of the grafted eyes began to display symptoms of allograft rejection, characterized by vascularization and conjunctival invasion, 2 months after surgery. In both cases there was considerable loss of epithelial transparency and the grafts were replaced with donor tissue. The removed grafts were prepared for examination by scanning and transmission electron microscopy.

6.2.2 Scanning Electron Microscopy

6.2.2.1 Case 1: Chemical Injury

Examination of the rejected oral mucosal graft by SEM revealed a central area of corneal-like epithelial cells (plate 6.48). These cells appeared to be in good condition

with an average surface area of 218.8μm^2 (S.D.±74.7). Adjacent cells were tightly opposed, with distinct cell borders (plate 6.49). Surface microvilli were short and corneal-like in appearance. Around this central zone of corneal-like cells, there appeared to be a transition towards a more conjunctival-like phenotype (plate 6.50). Cells in this region were intermediate in size, cell boundaries were quite defined and surface microvilli were more clumped together. Most of the amniotic membrane however, was covered with conjunctival epithelial cells (plate 6.51). These cells were much smaller than the corneal-like cells, with an average surface area of 38.4μm^2 (S.D.±14.0). In addition their cell borders were more prominent and the apical microvilli showed gross clumping (plate 6.51). Numerous inflammatory cells and red blood cells were found on the surface of the epithelial cells (plate 6.52).

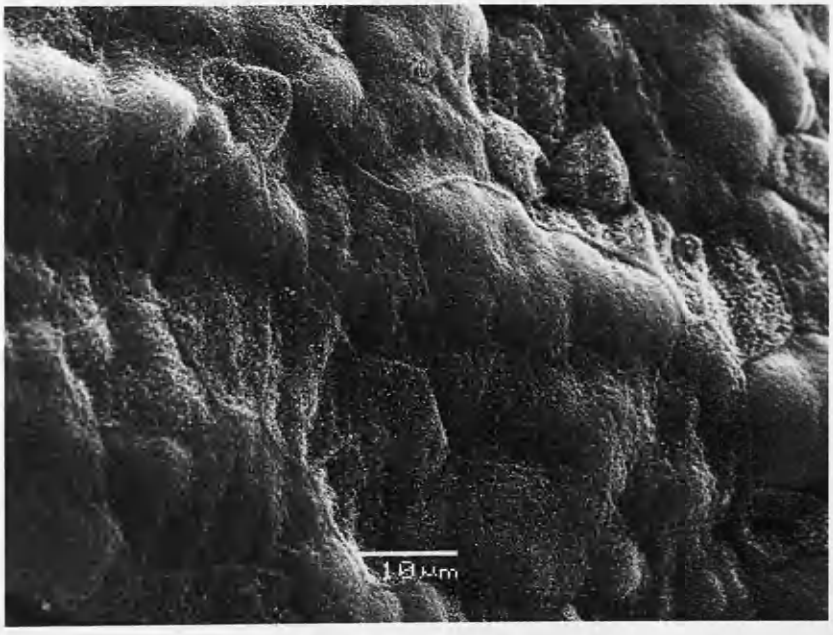


Plate 6.48: Scanning electron micrograph of the corneal epithelial-like cells on the rejected mucosal graft. Cells appeared to be in good condition. Adjacent cells had tightly opposing cell junctions and apical microvilli were short and corneal-like in appearance. [Scale bar = 10μm]

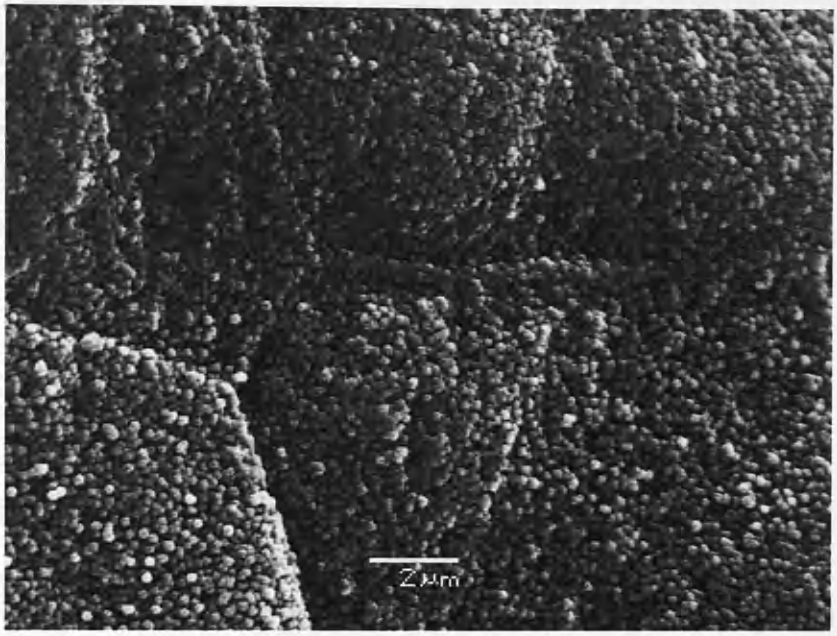


Plate 6.49: Scanning electron micrograph of the corneal epithelial-like cells on the rejected mucosal graft at high magnification. Adjacent cells had tightly opposing cell junctions with distinct cell boundaries. Apical microvilli were corneal-like in appearance. [Scale bar = 2µm]

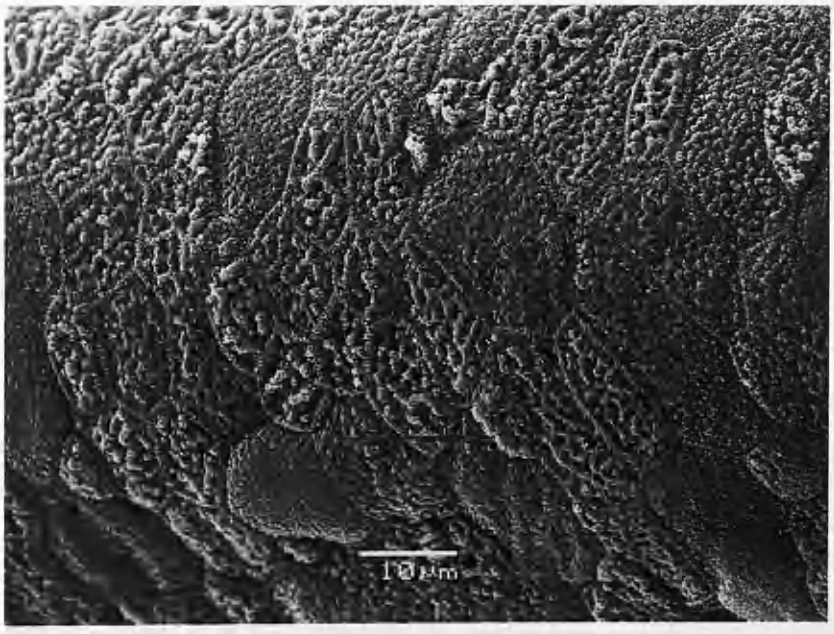


Plate 6.50: Scanning electron micrograph of the transition zone of the rejected mucosal graft where epithelial cells become more conjunctival in appearance. In this region, surrounding the central corneal-like epithelium, cells were intermediate in size, borders more prominent and surface microvilli more clumped together. [Scale bar = 10µm]

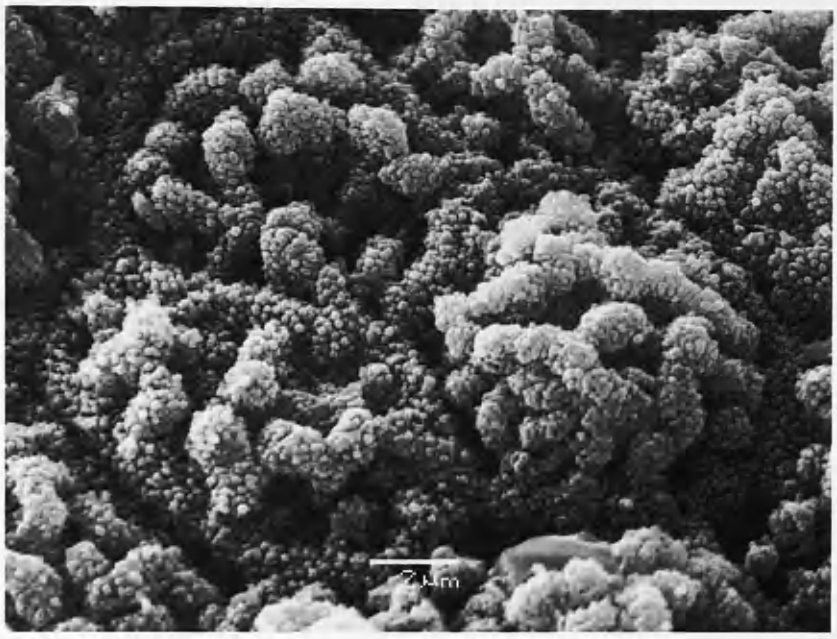


Plate 6.51: SEM micrograph of the conjunctival cells that invaded the mucosal stem cell graft. The conjunctival cells were smaller than the corneal-like cells, with more pronounced cell borders and grossly-clumped microvilli. [Scale bar = 2μm]

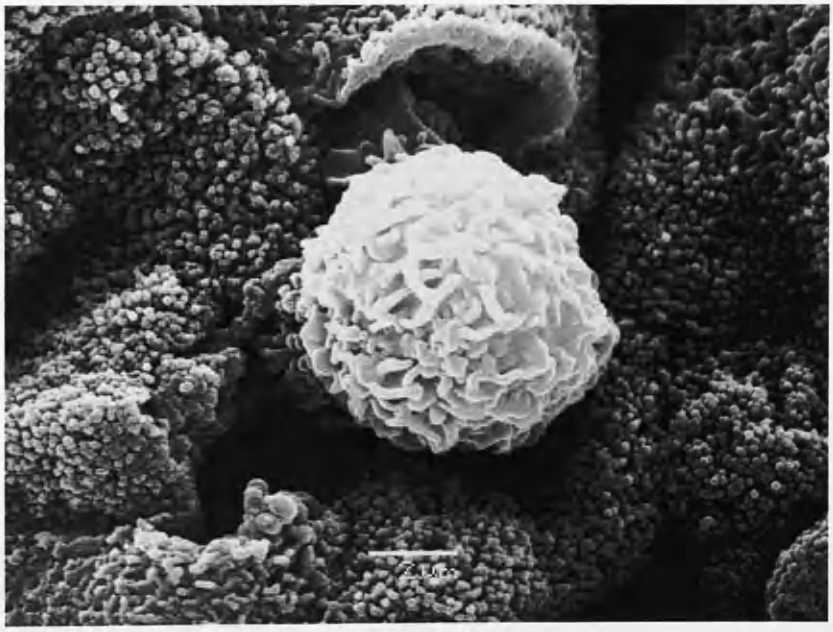


Plate 6.52: Scanning electron micrograph of the inflammatory cells found on the rejected graft, taken at very high magnification. Inflammatory cells appeared to be destroying the grafted epithelium. [Scale bar = 2μm]

6.2.3 Transmission Electron Microscopy

6.2.3.1 Case 1: Chemical Injury

Two samples of a rejected oral mucosal stem cell graft were isolated and shipped to Lancaster University for analysis; these represented the lower nasal area, and the upper temporal/nasal region. Both were similar in appearance and so are described together. Only small areas of corneal-like (oral mucosal) epithelial cells remained and these had become necrotic (plate 6.53). There was an obvious invasion of conjunctival epithelium (plate 6.54), characterized by their dense cytoplasm and clumped microvilli (plate 6.56). Most of the epithelium on the removed graft appeared to be of conjunctival origin (plate 6.55). The amniotic membrane seemed to have been integrated into the corneal stroma as there was no distinct boundary between the two. The amniotic membrane/stroma had become partially vascularized and there were a number of blood vessels in evidence (plate 6.57). In addition to red blood cells there were numerous inflammatory cells both within the amniotic membrane/stroma and amongst the epithelial cells (plate 6.58).

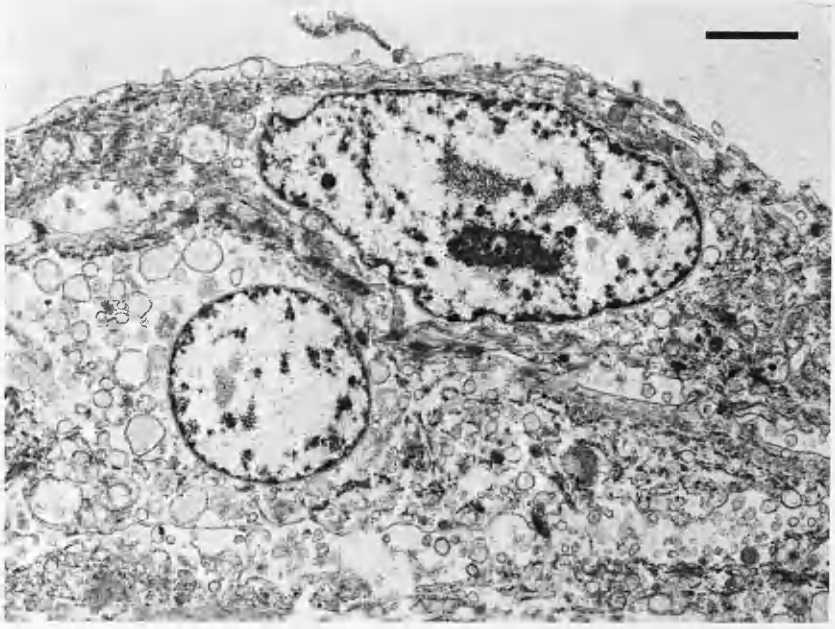


Plate 6.53: Transmission electron micrograph of necrotic corneal-like cells found on the rejected graft, taken at high magnification. There was only a small area of corneal-like epithelia remaining on the removed graft. [Scale bar = 2μm]

Plate 6.54: Transmission electron micrograph depicting an invasion of conjunctival epithelial cells. Conjunctival epithelial cells *[C]* appeared to be replacing residual necrotic grafted cells *[N]*, growing over them [Scale bar = 2μm]

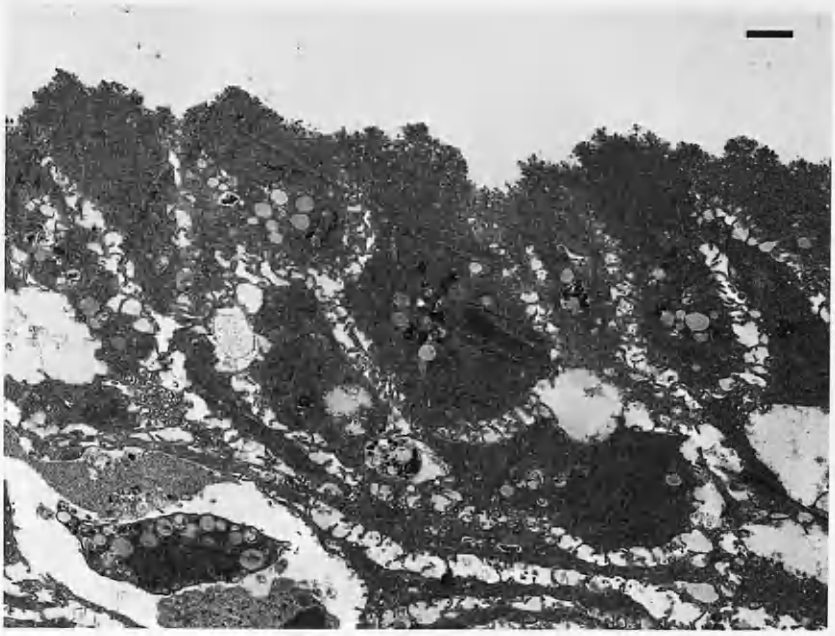


Plate 6.55: Transmission electron micrograph of a rejected oral mucosal epithelial graft. Conjunctival epithelial cells covered most of the amniotic membrane and appeared to have largely replaced the original grafted cells. [Scale bar = 2μm]

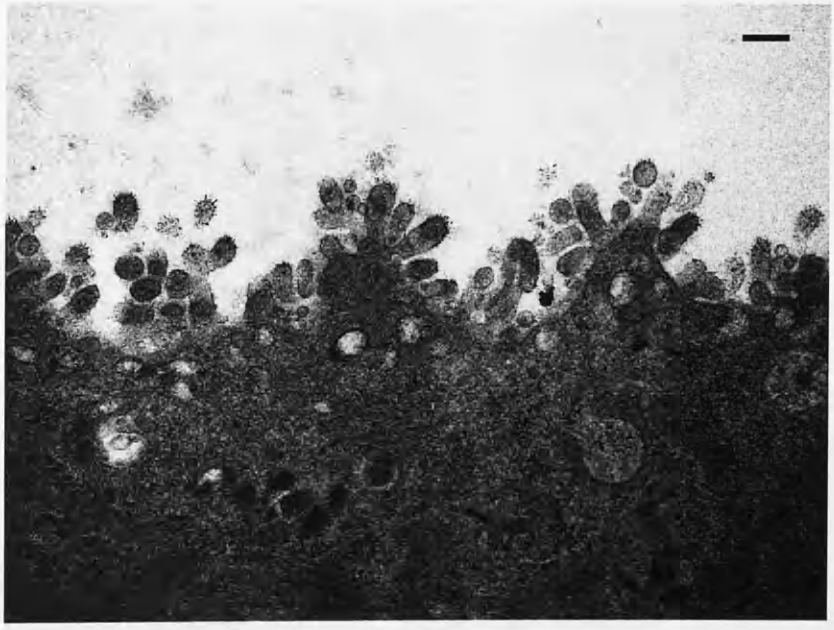


Plate 6.56: Transmission electron micrograph, taken at high magnification, depicting the grossly clumped microvilli characteristic of conjunctival epithelial cells which covered most of the amniotic membrane in the rejected mucosal epithelial graft. [Scale bar = 200nm]

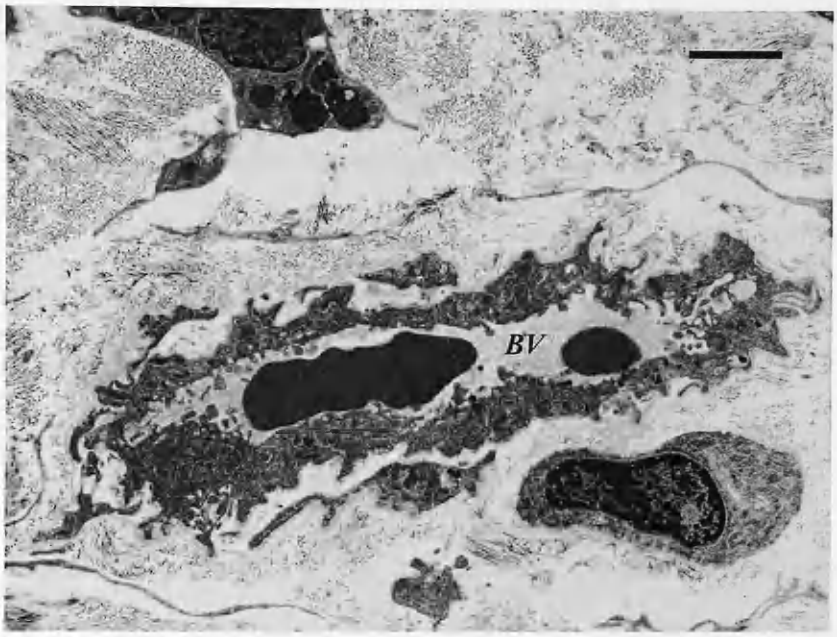


Plate 6.57: Transmission electron micrograph of a rejected oral mucosal epithelial graft. The amniotic membrane/underlying stroma had become vascularized with many blood vessels *[BV]* located directly beneath the invading conjunctival epithelial cell layer. [Scale bar = 2μm]

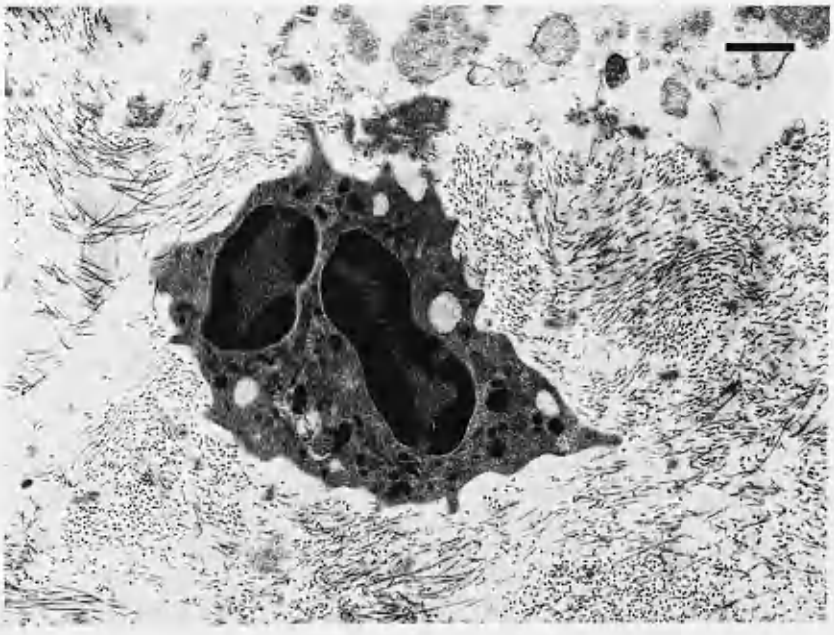


Plate 6.58: Transmission electron micrograph of a rejected oral mucosal epithelial graft. Inflammatory white blood cells, such as the granulocyte depicted above, were found throughout the necrotic grafted epithelium and within the underlying AM/stroma. [Scale bar = 1μm]

6.2.4 Scanning Electron Microscopy

In a second case study, an autologous cultivated oral mucosal graft removed from the ocular surface of an SJS patient 6 months after transplant surgery was analysed. The patient underwent conventional penetrating keratoplasty using donor cornea at the time of graft removal. Due to the very small size of the sample received from Japan, only scanning electron microscopy was used to examine the tissue.

6.2.4.1 Case 2: Stevens-Johnson Syndrome

Six months after grafting onto the human ocular surface, scanning electron microscopy of the removed corneal button revealed fairly healthy looking epithelium not unlike that of the normal cornea. The central area of epithelium appeared to be corneal-like (plate 6.59) and there was evidence of cell desquamation from the surface, indicating healthy epithelial turnover. In the more peripheral regions; epithelial cells were more conjunctival in appearance (plate 6.60). The conjunctival epithelial cells were smaller than the corneal-like cells, had more prominent cell borders, clumped microvilli, and were interspersed with mucin-secreting goblet cells (plate 6.60). Adjacent cells, both of corneal and conjunctival appearance were well attached to each other, with distinct ridges at cell borders and very little intercellular spacing (plate 6.61).

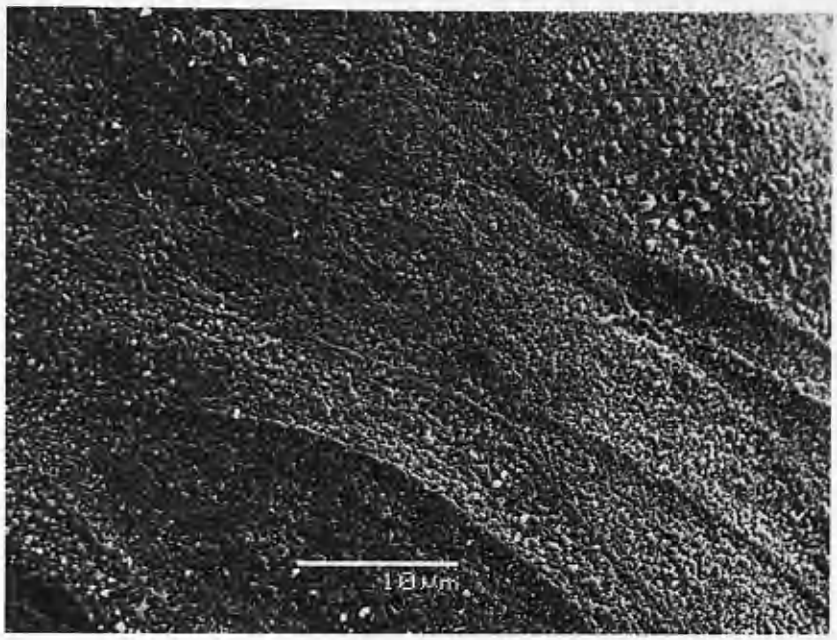


Plate 6.59: Scanning electron micrograph showing the epithelium of a human corneal button removed six months after an oral mucosal epithelial cell transplant. The cells in the central region of the corneal button were corneal-like in appearance. [Scale bar = 10μm]

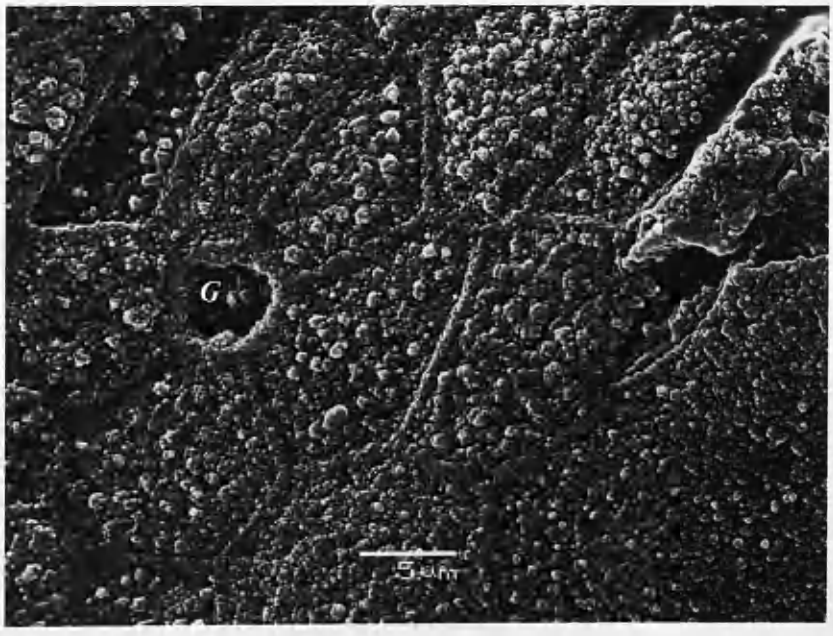


Plate 6.60: Scanning electron micrograph showing the epithelium of a human corneal button removed six months after an oral mucosal epithelial cell transplant. Towards the periphery, a conjunctival phenotype was evident. Conjunctival epithelial cells were interspersed with mucin-secreting goblet cells *[G]*. [Scale bar = 5μm]

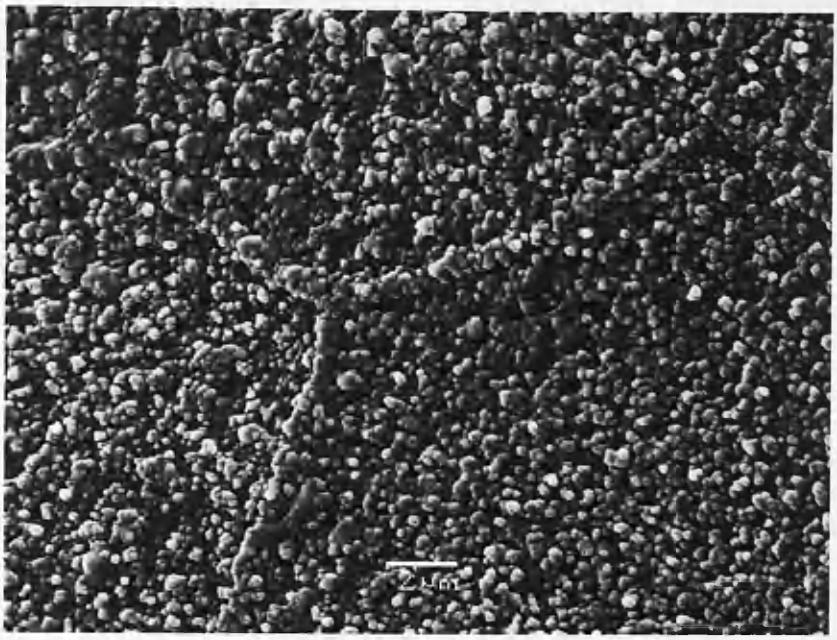


Plate 6.61: Scanning electron micrograph showing the epithelium of a human corneal button removed following oral mucosal epithelial cell transplant, at high magnification. Adjacent cells were well attached to each other, with tightly opposed cell boundaries and distinct ridges at cell-cell borders. [Scale bar = 2μm]

6.2.5 Summary Interpretations

In both removed grafts, examination by SEM revealed a small central region of corneal-like epithelium surrounded by a large area of conjunctival cells, much smaller in size and with more prominent cell borders. In the first case study, there were numerous inflammatory and red blood cells associated with the rejected graft. Transmission electron microscopy revealed that the AM appeared to have become integrated into the stroma and that the remaining few corneal-like cells appeared necrotic. Most of the epithelium was of conjunctival origin. In places the conjunctiva were growing over the corneal-like cells and in other areas they were displacing them from underneath. There were numerous inflammatory cells within both the stroma and the epithelial cell layers, as well as many blood vessels within the stroma.

6.3 Examination of Hybrid Cultures of Rabbit Corneal and Oral Mucosal Epithelial Cells on Denuded Amniotic Membrane

Previous investigations (described in chapter 6.1) showed that cultured oral epithelial cells still retain some mucosal characteristics. In an attempt to induce differentiation into a more corneal-like phenotype, this project incorporated corneal stem cells in the culture. Hybrid mixtures of rabbit corneal and oral mucosal epithelial cells (1:1 ratio) were seeded onto AM and cultured for 21 days by project collaborators at the Kyoto Prefectural University of Medicine then fixed and sent to Lancaster University for analysis. Samples from both before and after transplantation onto the rabbit ocular surface were examined in this investigation, to determine whether any changes occurred in the cell morphology following a 3 week period on the eye.

6.3.1 Scanning Electron Microscopy

6.3.1.1 Corneal & Oral Mucosal Epithelial Hybrid Cultures on AM

Though it was not possible to distinguish between the two cell types by SEM, the cultured epithelial cells appeared to be in fairly good condition, formed a confluent layer and were, in places, undergoing the process of desquamation (plate 6.62). Some of the epithelial cells appeared to be flattened and had distended microvilli at the cell boundaries (plate 6.63). Adjacent cells were well attached to each other with tightly opposed cell borders (plate 6.64). Oral mucosal cells appeared to have differentiated into corneal-like cells in culture, as there was no evidence of the parallel ridges or folds on the surface of the superficial cells which characterize oral mucosal epithelia. Superficial cells had an apical covering of short, regular microvilli (plate 6.65).

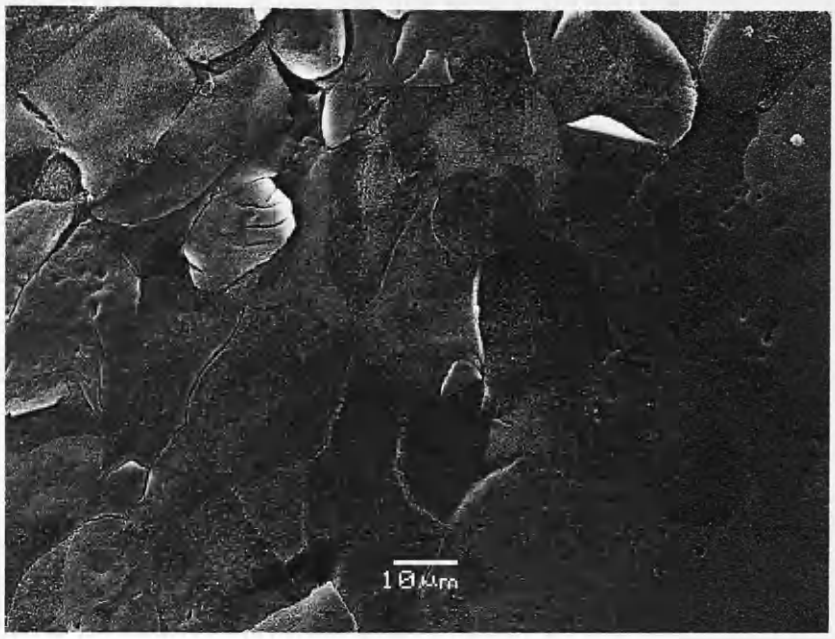


Plate 6.62: Scanning electron micrograph showing a rabbit oral mucosal/corneal epithelial cell culture on AM at low magnification. It was difficult to distinguish between the two epithelial cell types and the cultured epithelium generally resembled the morphology of normal cornea. There was evidence of cell desquamation. [Scale bar = 10µm].

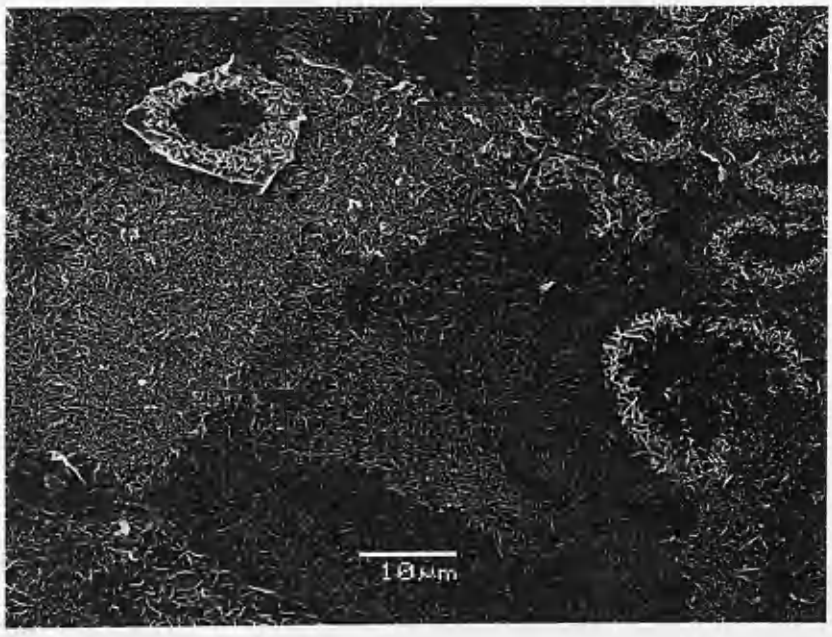


Plate 6.63: SEM micrograph showing rabbit oral mucosal/corneal epithelial cells cultured on AM. The condition of the cells was inconsistent in that some appeared to have a very flat surface with long microvilli at the cell borders. [Scale bar = 10µm]

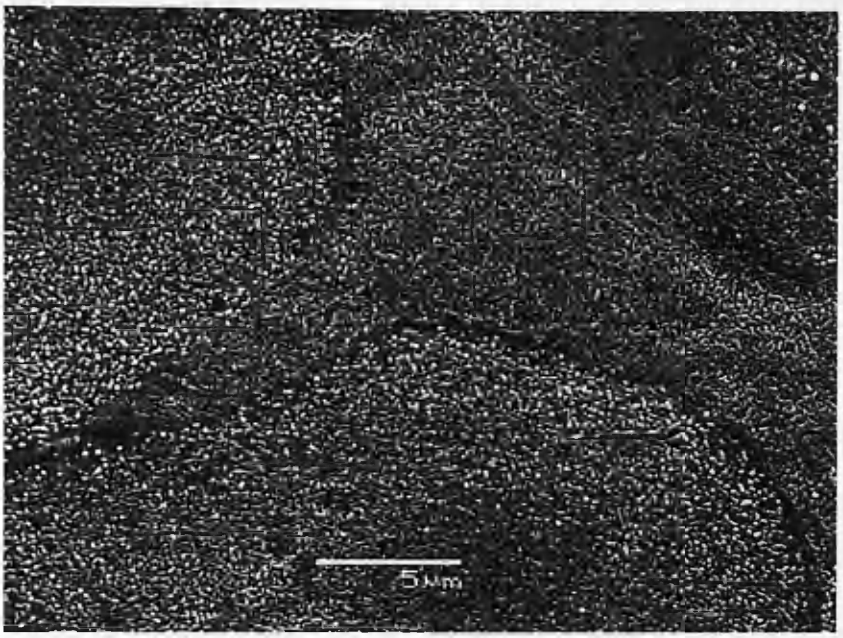


Plate 6.64: Scanning electron micrograph showing a rabbit oral mucosal/corneal epithelial hybrid culture on AM. Adjacent cells were well attached to each other, with minimal intercellular spacing. In places, there were distinct ridges at cell-cell borders. [Scale bar = 5µm]

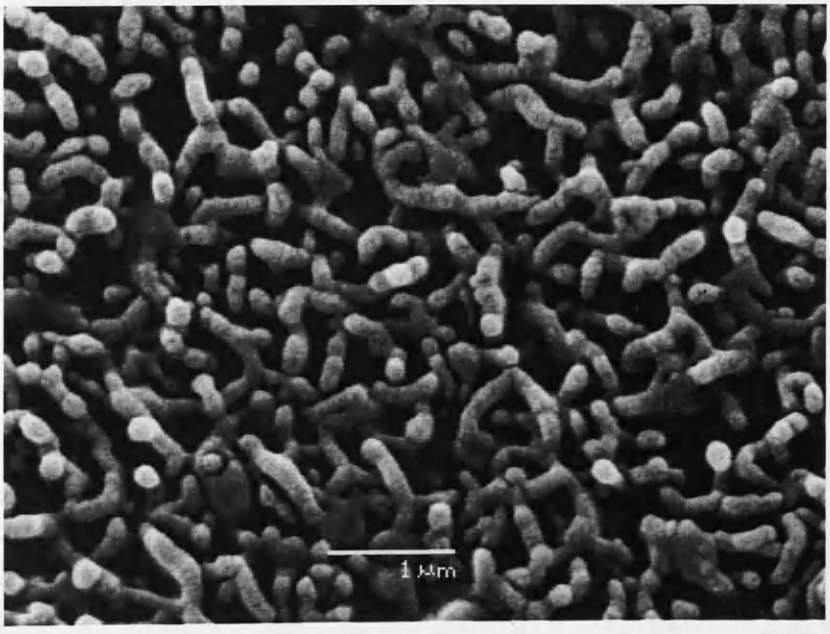


Plate 6.65: Scanning electron micrograph showing short, regular microvilli present on the surface of most of the cultivated hybrid epithelial cells, taken at high magnification. [Scale bars = 1µm].

6.3.1.2 Corneal & Oral Mucosal Epithelial Hybrid Cultures Post-Transplant

Examination of rabbit corneal buttons removed following transplantation of hybrid oral mucosal and corneal epithelial culture sheets by SEM revealed confluent layers of epithelium not unlike that of the normal cornea (plate 6.66). Epithelial cells appeared to be in very good condition and there was evidence of cell desquamation from the surface, as would be expected in a healthy epithelial sheet (plate 6.66). It seemed that the oral mucosal cells had differentiated into corneal-like cells in culture as there was no sign of the parallel ridges and folds on the surface of the superficial cells which characterize oral mucosal epithelia. Adjacent cells were well attached to each other, with tightly opposed junctions and distinct ridges at cell borders (plate 6.67). Superficial cells were covered in short, regular microvilli (plate 6.68).

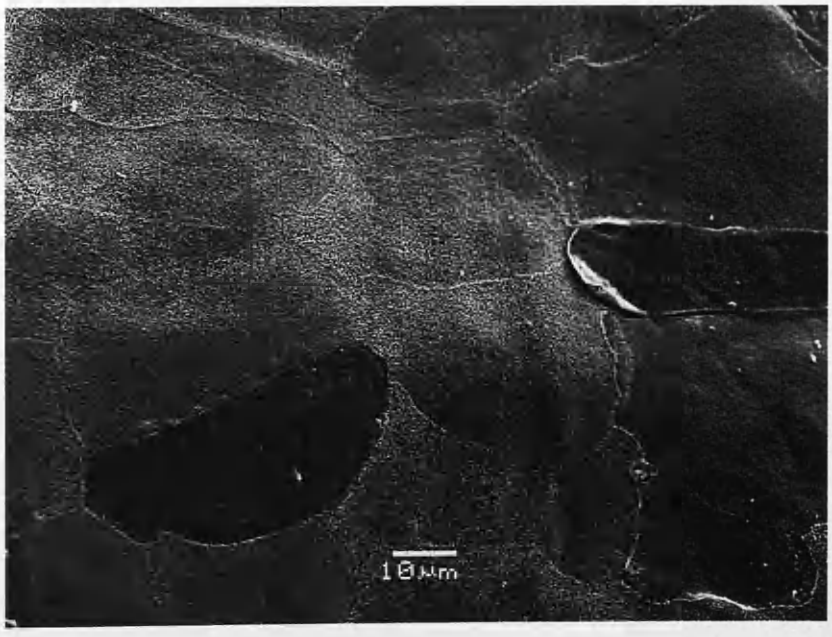


Plate 6.66: SEM micrograph showing the corneal epithelium of a rabbit hybrid cell culture sheet, removed after three weeks on the cornea. The epithelium closely resembled that of the normal cornea. There was evidence of cell desquamation. [Scale bar =10µm]

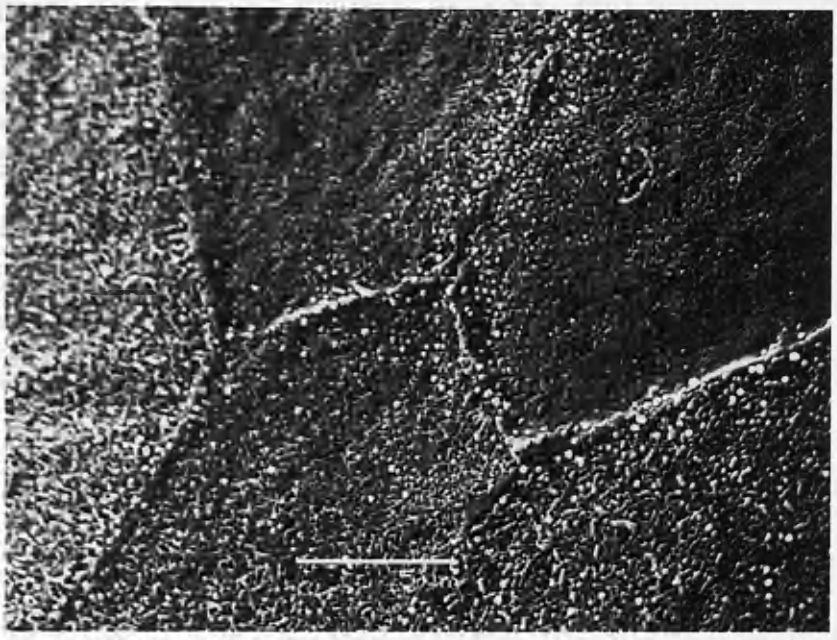


Plate 6.67: SEM micrograph showing a rabbit oral mucosal/corneal epithelial hybrid culture on AM, removed following transplantation onto the ocular surface. Adjacent cells were well attached to each other, with little or no intercellular spacing and there were distinct ridges at cell-cell borders, as in the normal corneal epithelium. [Scale bar = 5μm]

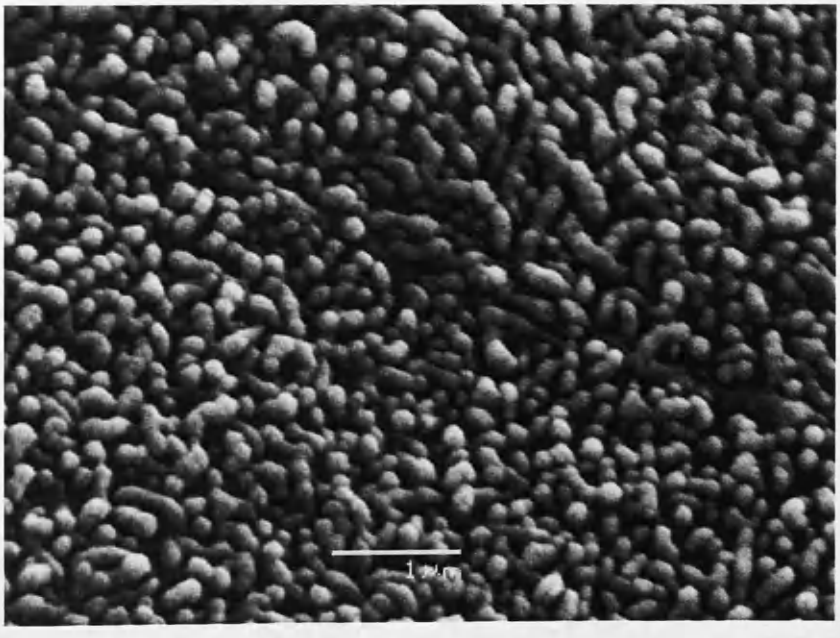


Plate 6.68: Scanning electron micrograph showing a post-transplant hybrid oral mucosal/corneal culture sheet. There were short, regular microvilli present on the surface of the epithelial cells. [Scale bar = 1μm]

6.3.2 Transmission Electron Microscopy

6.3.2.1 Corneal & Oral Mucosal Epithelial Hybrid Cultures on AM

Microscopic examination of the rabbit hybrid epithelial cell culture on denuded AM revealed 4-8 layers of stratified epithelium (plate 6.69). Cells appeared to be in good condition and were differentiated into basal columnar shaped cells, suprabasal wing cells and flat squamous superficial cells (plate 6.70). It was not possible to tell the two epithelial cell types apart. Basal epithelial cells (plate 6.71) adhered well to the amniotic membrane substrate and produced basement membrane material (plate 6.72). Superficial cells were squamous in appearance and had a covering of short microvilli. Cells in all layers were attached to each other by numerous desmosomal junctions (plate 6.73).

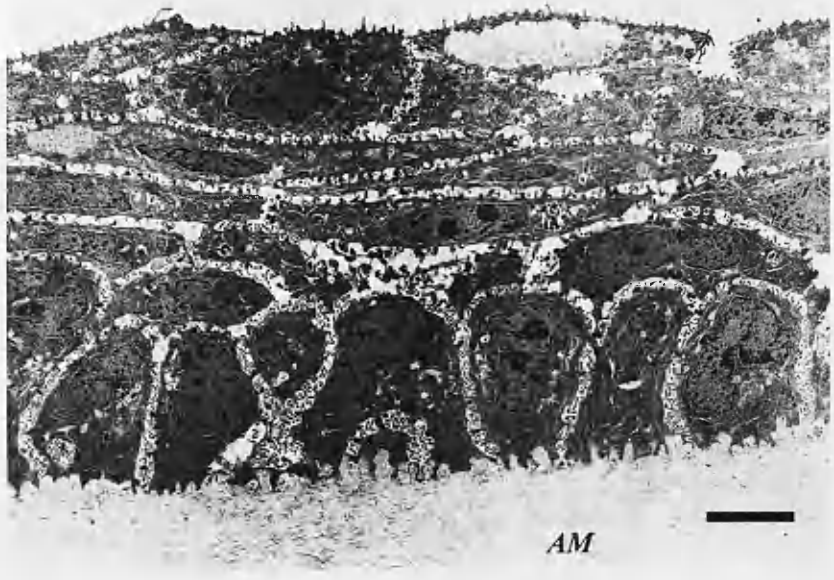


Plate 6.69: TEM micrograph of cultivated hybrid corneal and oral mucosal epithelial cells on denuded amniotic membrane *[AM]*. The epithelial cells formed 4-8 well-differentiated cell layers and the two cell types were indistinguishable. [Scale bar = 5μm]

Plate 6.70: TEM micrograph of cultivated rabbit corneal and oral mucosal hybrid epithelial cells. Superficial cells were squamous in appearance and had short regular microvilli on their apical surface. [Scale bar = 2μm]

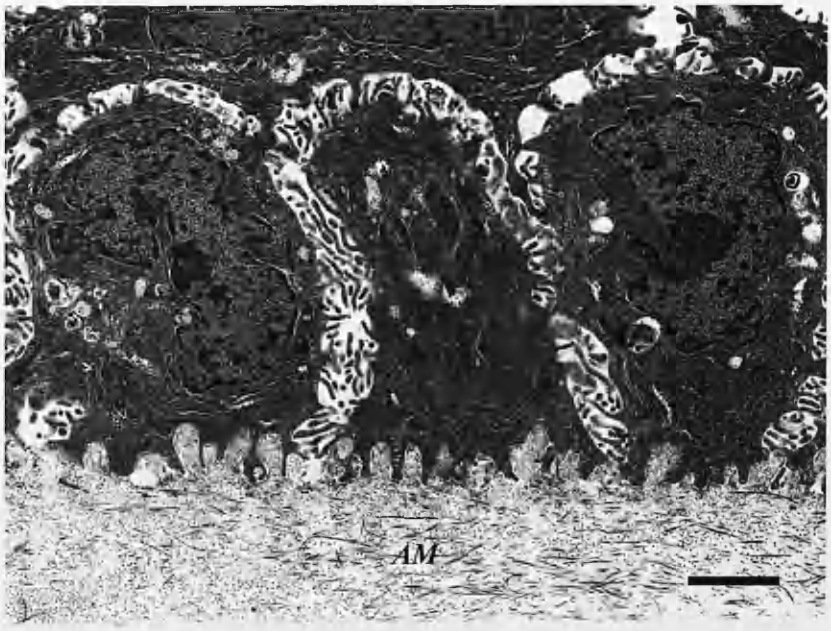


Plate 6.71: TEM micrograph showing the basal region of a rabbit oral mucosal/corneal epithelial hybrid culture on amniotic membrane *[AM]* at high magnification. Basal cells were columnar and well attached to the substrate. [Scale bar = 2μm]

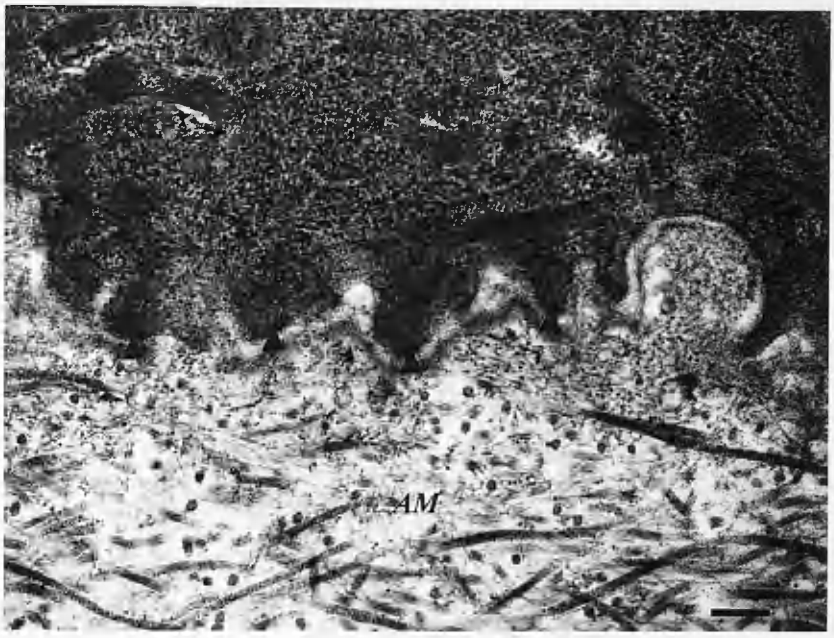


Plate 6.72: TEM micrograph showing the basal region of a rabbit oral mucosal/corneal epithelial hybrid culture on amniotic membrane at high magnification. Basal cells were attached to the substrate by hemidesmosomes (arrowheads). [Scale bar = 500nm]

Plate 6.73: TEM micrograph illustrating the numerous desmosomal junctions joining adjacent cells in all cell layers of the rabbit hybrid cultures. [Scale bar = 200nm].

6.3.2.2 Corneal & Oral Mucosal Epithelial Hybrid Cultures Post-Transplant

After 3 weeks on the ocular surface, the hybrid transplanted epithelium appeared to be in very good condition and closely resembled *in vivo* corneal epithelium. The epithelium formed 6-8 well-differentiated and stratified cell layers (plate 6.74). There were 3-4 layers of superficial cells, covered in short microvilli (plate 6.75). Basal cells were highly differentiated (plate 6.76) and were well attached to the amniotic membrane (plate 6.77). Adjacent cells in all cell layers were closely attached to each other by way of numerous desmosomal junctions (plate 6.78). There was little or no intercellular spacing. As with the original cultures, it was not possible to distinguish between the oral mucosal and corneal epithelial cell types on the basis of morphology. The resulting cell layer closely resembled that of normal corneal epithelium.

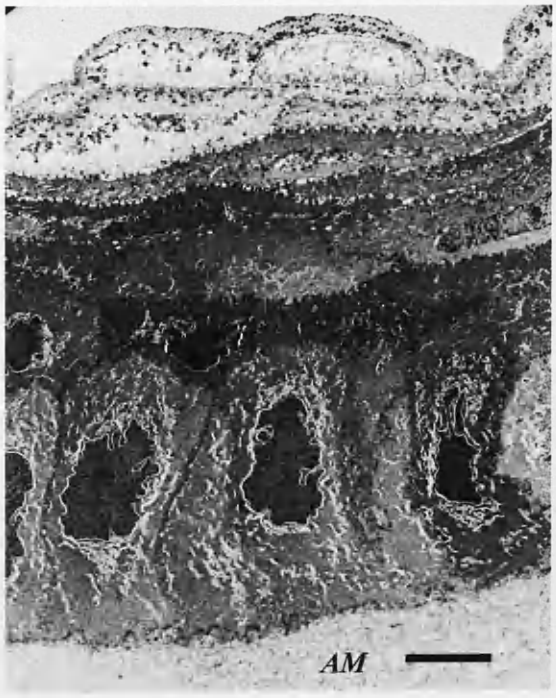


Plate 6.74: TEM micrograph showing the corneal epithelium of a rabbit hybrid cell culture sheet, removed after 3 weeks on the cornea. The epithelium appeared to be in very good condition and closely resembled that of normal cornea, having formed 6-8 well-differentiated and stratified cell layers. [Scale bar = 7μm]

Plate 6.75: TEM micrograph showing the corneal epithelium of a rabbit hybrid cell culture sheet, removed after 3 weeks on the cornea. The superficial cells were squamous and had short microvilli on their surface. [Scale bar = 1μm]

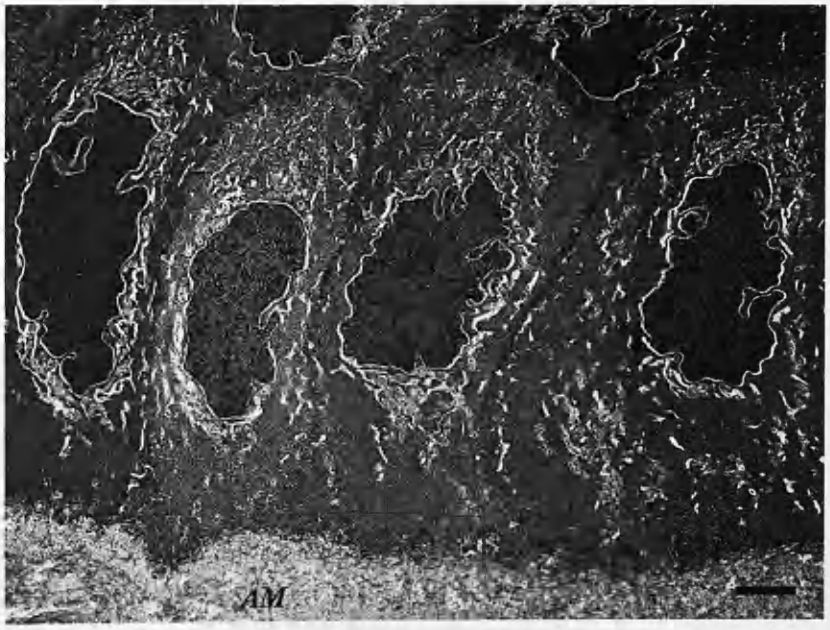


Plate 6.76: TEM micrograph showing the corneal epithelium of a rabbit hybrid cell culture sheet, removed after 3 weeks on the cornea. Basal cells were columnar and tightly opposed to each other. [Scale bar = 2μm]

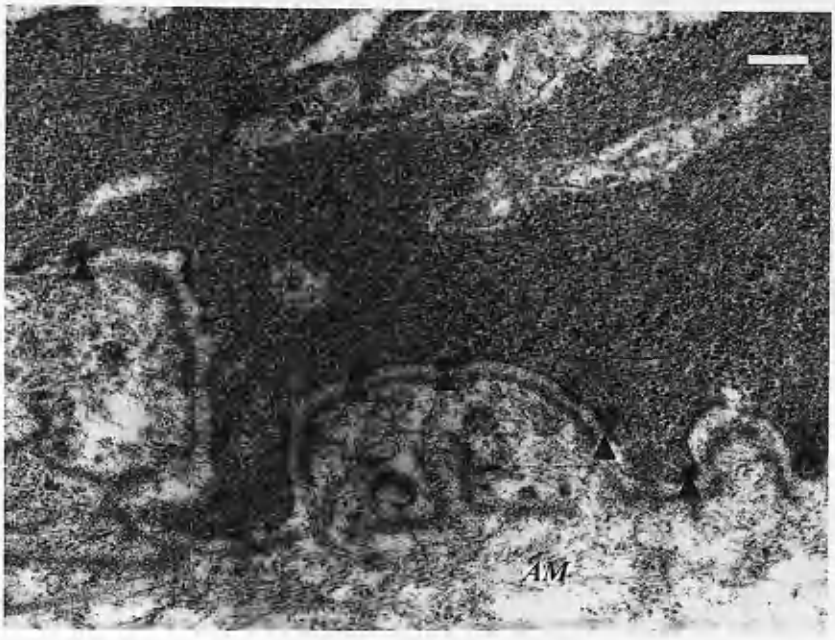


Plate 6.77: TEM micrograph showing the corneal epithelium of a rabbit hybrid cell culture sheet, removed after 3 weeks on the cornea. Basal cells were well attached to the underlying amniotic membrane *[AM]*, via hemidesmosomes (arrowheads). [Scale bar = 200nm]

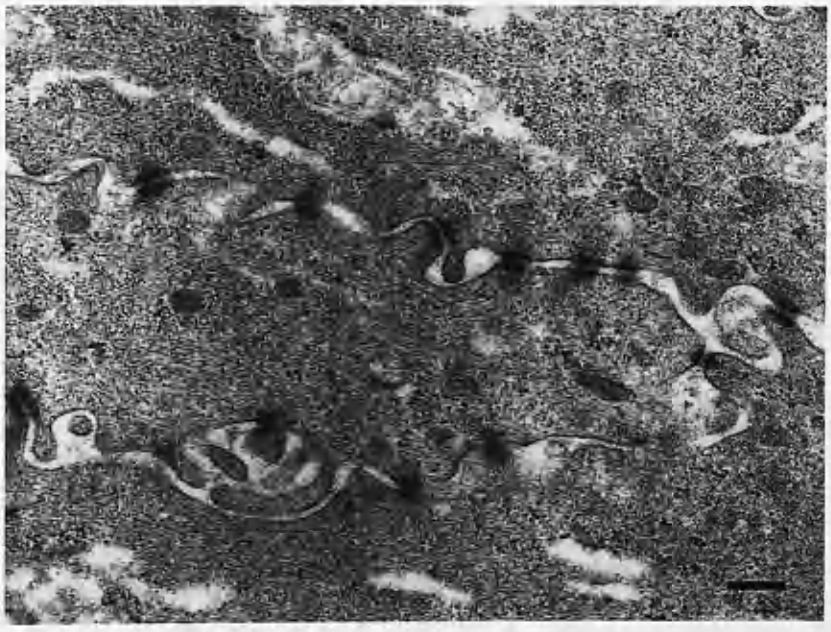


Plate 6.78: TEM micrograph showing the corneal epithelium of a rabbit hybrid cell culture sheet, removed after 3 weeks on the cornea. Cells in all cell layers were tightly opposed and intercellular spacing was negligible. Mechanical strength was maintained by desmosomal junctions, as pictured above. [Scale bar = 200nm]

6.3.3 Quantitative Study

A number of parameters were quantitatively compared between the hybrid cell culture pre-transplant and after 3 weeks on the rabbit ocular surface. Mean data (±S.D.) are shown in table 6.2 below. Fourteen data points were collected for each parameter except for the cell surface area which was calculated using scanning electron microscopy and therefore more measurements were permitted (n=50).

	Hybrid oral/corneal cells on AM (pre-transplant)	**Hybrid oral/corneal cells on AM (post-transplant)**	**Rabbit corneal epithelium**
Thickness of cell layers (μm)	21.36 ± 8.31	33.58 ± 13.75	20.55 ± 2.99
Number of cell layers	5.14 ± 1.03	7.21 ± 1.81	7.79 ± 1.31
Intercellular space area (μm^2)	0.50 ± 0.24	0.08 ± 0.07	0.09 ± 0.07
Number of desmosomes	1.64 ± 1.01	2.79 ± 1.42	2.57 ± 1.60
Number of hemidesmosomes	2.71 ± 1.59	4.21 ± 1.53	8.29 ± 1.68
Cell surface area (μm^2)	189.03 ± 132.56	794.11 ± 446.84	788.19 ± 200.53

Table 6.2: Comparison of cultivated hybrid oral mucosal/corneal epithelial cells on AM before and 3 weeks after transplant onto the ocular surface. Both were compared with normal rabbit cornea and mean data (± S.D.) are shown. Fourteen random data points were taken, except for the cell surface areas in which case n=50. The averages for intercellular area and number of desmosomes are given for random 3μm interfaces between adjacent cells.

For ease of interpretation, mean data for the 'before' and 'after' transplant hybrid sheets were plotted as percentage differences from the control (normal rabbit corneal epithelium) and statistically compared. The hybrid cell cultures more closely resemble cornea than the oral mucosal cell cultures of the previous study. Prior to placement on the cornea, the rabbit corneal/oral mucosal hybrid culture was in fairly good condition. In terms of thickness of cell layers, the hybrid culture very similar to

that of normal cornea (P=0.982). There was however a significant (P<0.001) 33% decrease in the number of cells making up the epithelial layer. There was also a substantial increase (442%) in intercellular spacing, which would explain the above observation. The difference in number of desmosomes was not significant but there were significantly fewer hemidesmosomes attaching the basal cells to the AM than were found in the control cornea (P<0.001). Following a 3 week period on the cornea, the removed culture sheet even more closely resembled *in vivo* rabbit corneal epithelium. The only parameter in which there was a significant difference when compared with control epithelium was that of numbers of hemidesmosomes (P<0.001). Chart 6.2 below compares the hybrid culture sheets before and after the transplant period.

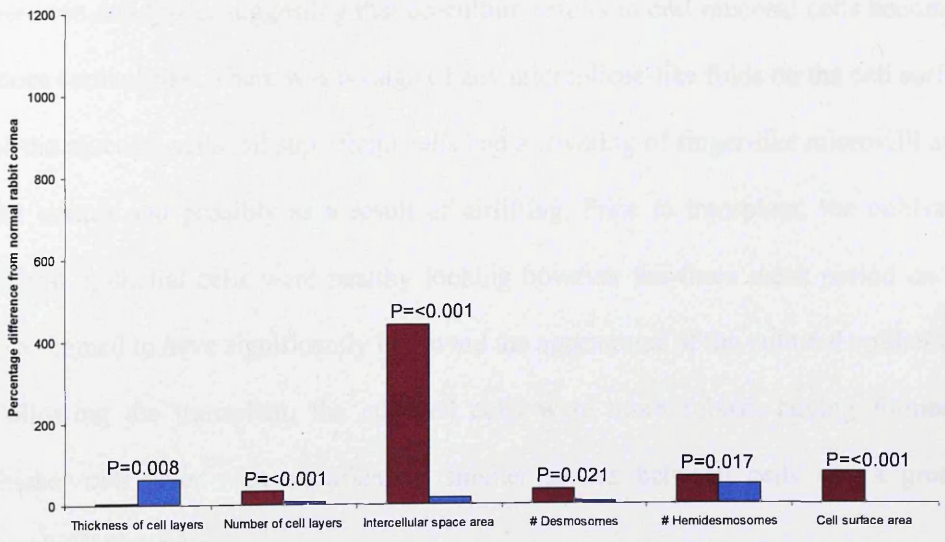


Chart 6.2: Comparison of cultivated rabbit hybrid cultures before transplant (purple) and after 3 weeks on the eye (blue), expressed as percentage difference from normal rabbit cornea control. Raw data were used to calculate statistical significance (P values) of observed differences between mucosal cell types and these are stated above the bars.

The 3 week placement on the rabbit cornea appeared to have improved the cells in most of the parameters under study. The cell layer was 60% thicker following transplant ($P=0.008$) and the number of cell layers was also significantly increased, bringing it more in accordance with that of rabbit cornea (mean of 7.21 compared with 7.79 for control). Post-transplant, cells had formed a more compact layer, as indicated by the highly significant ($P<0.001$) reduction in intercellular space areas. Numbers of desmosomes were increased, as were numbers of hemi desmosomes, though neither were statistically significant improvements. Superficial hybrid cells post-transplant had almost identical cell surface areas to those of the control cornea.

6.3.4 Summary Interpretations

Examination of the cultivated hybrid oral mucosal and corneal epithelial cells by scanning and transmission electron microscopy revealed no noticeable difference between cell types, suggesting that co-culture results in oral mucosal cells becoming more corneal-like. There was no sign of any microplicae-like folds on the cell surface of the mucosal cells; all superficial cells had a covering of finger-like microvilli as in the cornea and possibly as a result of airlifting. Prior to transplant, the cultivated hybrid epithelial cells were healthy looking however the three week period on the eye seemed to have significantly improved the appearance of the cultured epithelium. Following the transplant, the cultured cells were more robust, having formed a thicker cell layer with significantly smaller spaces between cells and a greater number of junctions.

7 RESULTS

7.1 Use of Extracellular Matrix Protein-Coated Gelatins as Carriers for Human and Rabbit Limbal Stem Cell Cultivation

Gelatin hydrogel sheets were coated with extracellular matrix (ECM) proteins (fibronectin, collagen IV and a combination of both) and their usefulness as a potential new substrate for stem cell culture was analysed morphologically using scanning and transmission electron microscopy. A quantitative study was also carried out to compare the cultivated limbal cells on gelatin with normal human/rabbit corneal epithelia.

7.1.1 Scanning Electron Microscopy

7.1.1.1 Coated Gelatin Sheets

Eight gelatin hydrogel sheets were analysed using scanning electron microscopy; two each of uncoated gelatin, gelatin coated with fibronectin, gelatin with collagen type IV, and gelatin with both coatings together. At low magnifications it was difficult to see any apparent differences between the samples. Examination at high magnifications however revealed that the uncoated gelatin appeared porous and had distinct pits on its surface (plate 7.1). The ECM-coated samples appeared generally smoother and had less well-defined pores (plates 7.2-7.4). There were no evident differences in the morphology of the coatings.

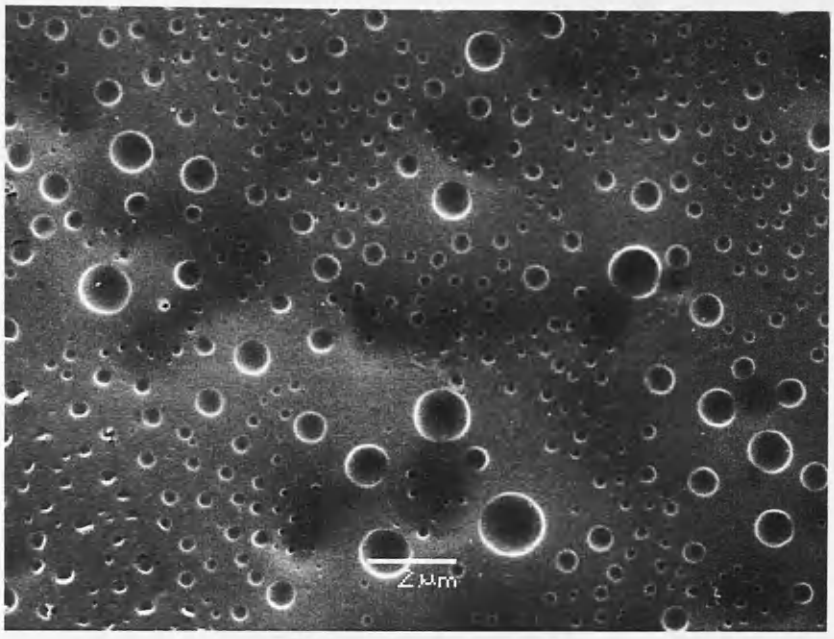


Plate 7.1: Scanning electron micrograph of an uncoated gelatin hydrogel sheet, taken at very high magnification. Surface pores are quite distinct. [Scale bar = 2μm]

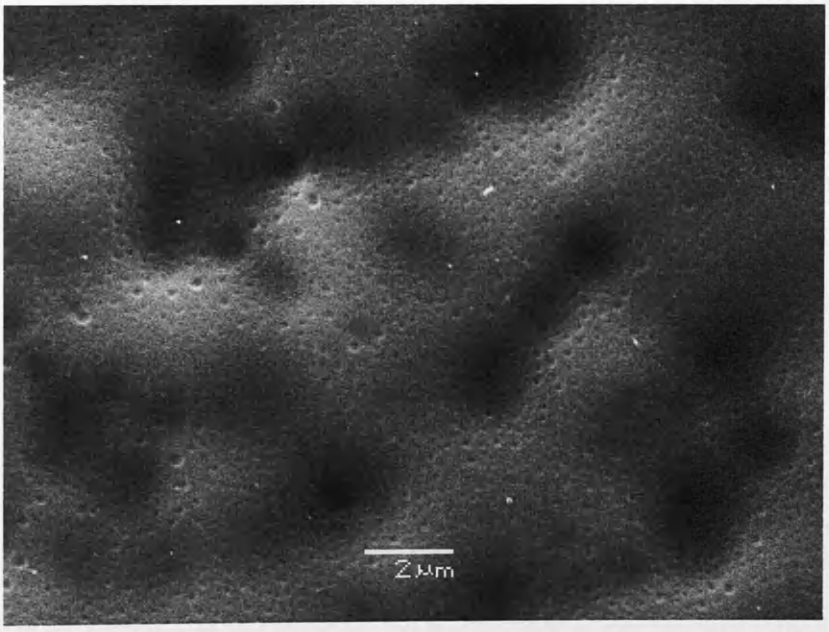


Plate 7.2: Scanning electron micrograph of gelatin hydrogel coated with type IV collagen, taken at very high magnification. Surface pores are less defined than in the uncoated sheet. [Scale bar = 2μm]

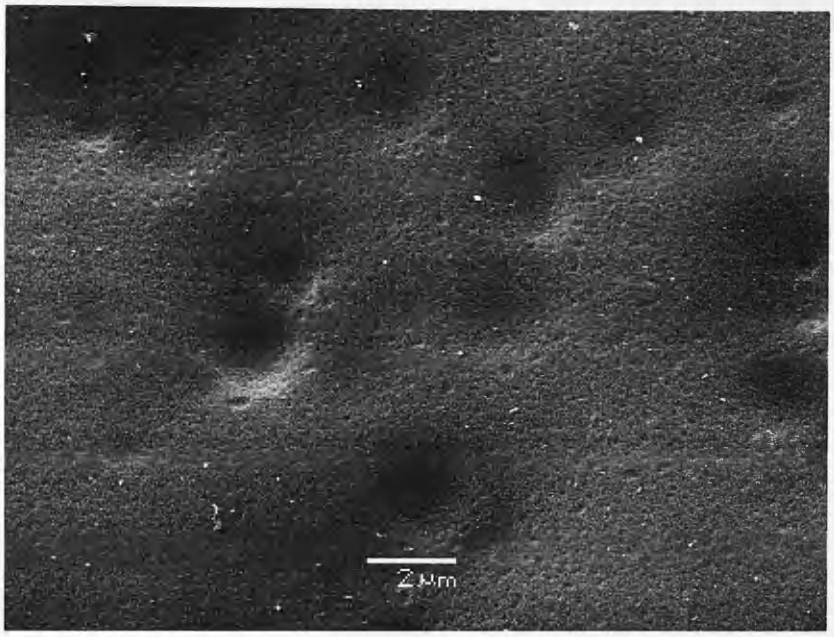


Plate 7.3: Scanning electron micrograph of gelatin hydrogel coated with fibronectin, taken at very high magnification. Surface pores are less defined than in the uncoated sheet and the coating seemed to form a smooth layer. [Scale bar = 2μm]

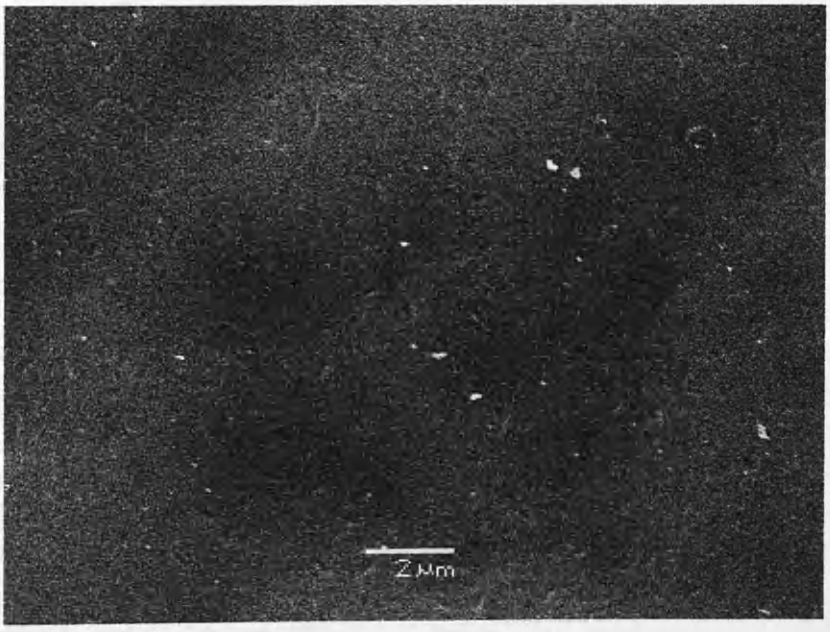


Plate 7.4: Scanning electron micrograph of gelatin hydrogel coated with both type IV collagen and fibronectin, taken at very high magnification. Surface pores were hardly visible on this sample. [Scale bar = 2μm]

7.1.2 Transmission Electron Microscopy

7.1.2.1 Coated Gelatin Sheets

It was evident from examination by transmission electron microscopy that there was a difference between the control (uncoated) gelatin and those with an ECM-coating. There were distinct pores/indentations on one surface of the control (plate 7.5). These pores were not particularly obvious when the gelatin was coated, however there were variations in thickness of coating along the length of the section. There seemed to be only a very thin layer of collagen IV (plate 7.6). The fibronectin layer was fairly uneven and the coating appeared patchy, varying from 50-200nm in thickness (plate 7.7). When both proteins were used to coat the surface a thick and even electron dense layer resulted which was up to 500nm thick (plate 7.8).

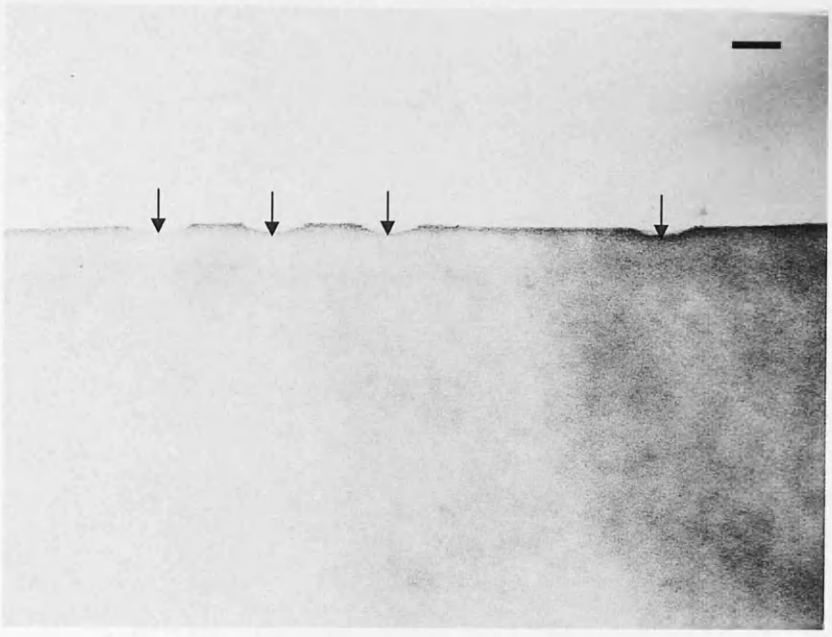


Plate 7.5: Transmission electron micrograph of uncoated gelatin hydrogel, taken at high magnification. With no coating, the pits (indicated by arrows) on the surface appeared well-defined. [Scale bar = 200nm]

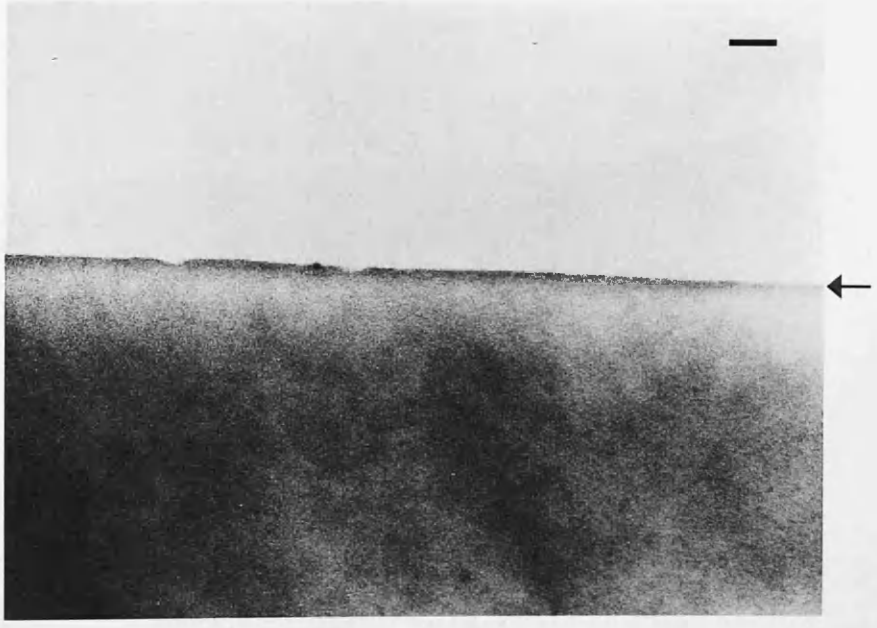


Plate 7.6: Transmission electron micrograph of collagen type IV coated gelatin hydrogel, taken at high magnification. There seemed to be a thin layer of collagen type IV (arrow) which effectively reduced the pore size of the gelatin. [Scale bar = 200nm]

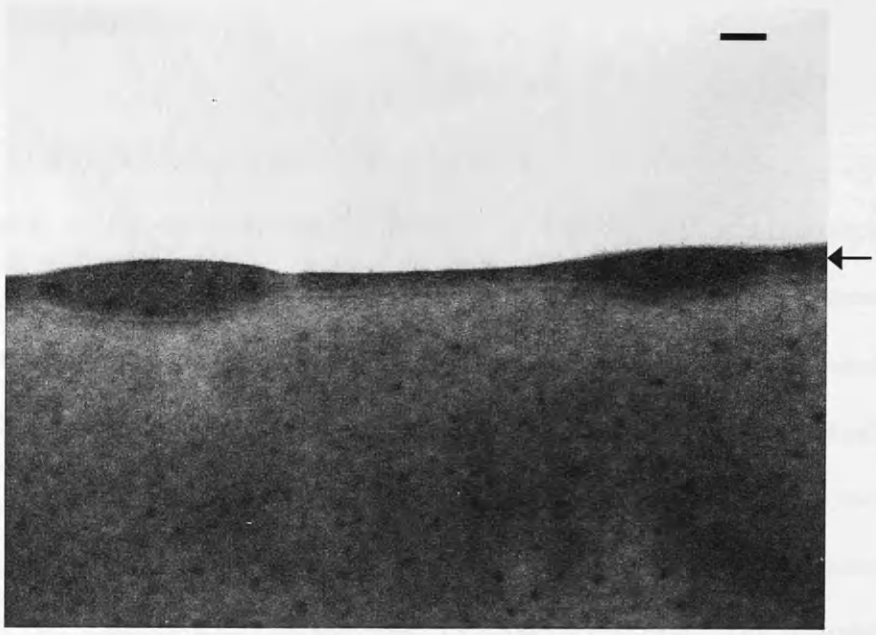


Plate 7.7: Transmission electron micrograph of fibronectin coated gelatin hydrogel, taken at high magnification. The fibronectin layer (arrow) was fairly uneven and the coating appeared patchy, varying from 50-200nm in thickness. [Scale bar = 200nm]

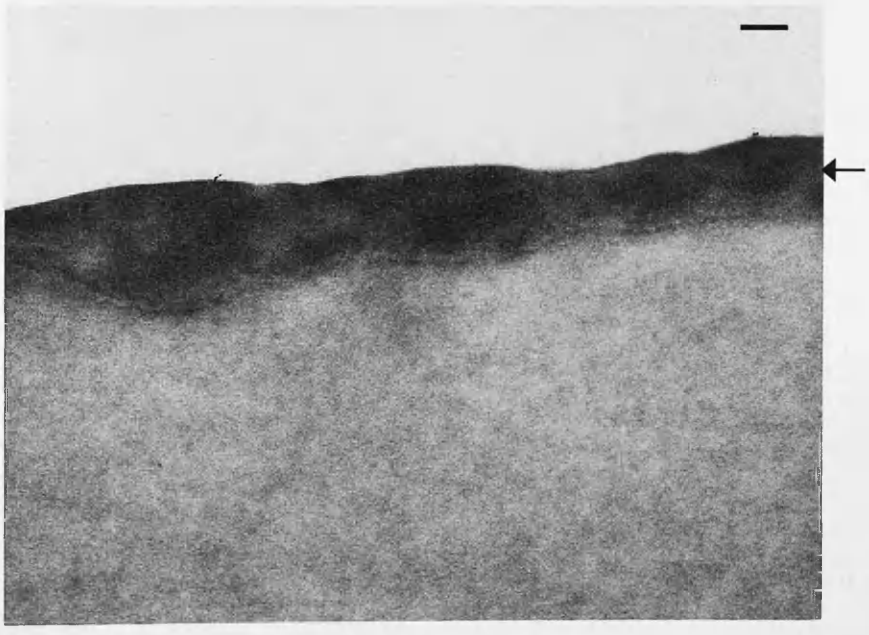


Plate 7.8: Transmission electron micrograph of collagen IV and fibronectin coated gelatin hydrogel, taken at high magnification. The ECM layer (arrow) was fairly evenly distributed was up to 500nm thick in places. [Scale bar = 200nm]

7.1.3 Scanning Electron Microscopy

7.1.3.1 Cultivated Human Corneal Epithelial Cells

The human corneal epithelial cells cultivated on ECM (collagen IV and fibronectin)-coated gelatins were generally in good condition (plate 7.9). There were however some areas devoid of cells, where the gelatin was exposed. Many cells appeared to be undergoing the process of desquamation, as would be expected in a healthy epithelial sheet (plate 7.9). Adjacent epithelial cells seemed to be well attached to each other, with little intercellular spacing and some distinct ridges at cell boundaries (plate 7.10). Apical microvilli were short and finger-like (plate 7.11), resembling those of *in vivo* corneal epithelium.

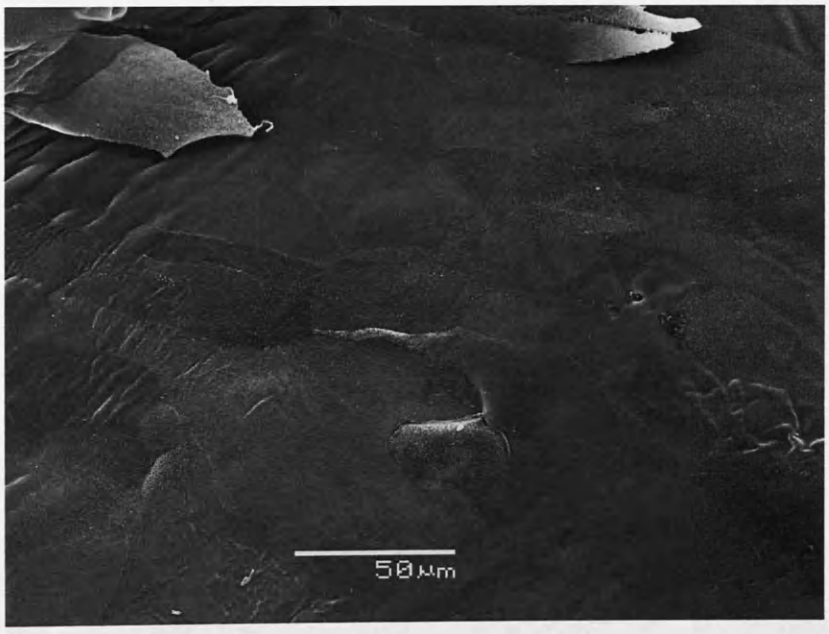


Plate 7.9: Scanning electron micrograph of human corneal epithelial stem cells on ECM-coated gelatin hydrogel, taken at very low magnification. Cultured cells were in good condition. [Scale bar = 50µm]

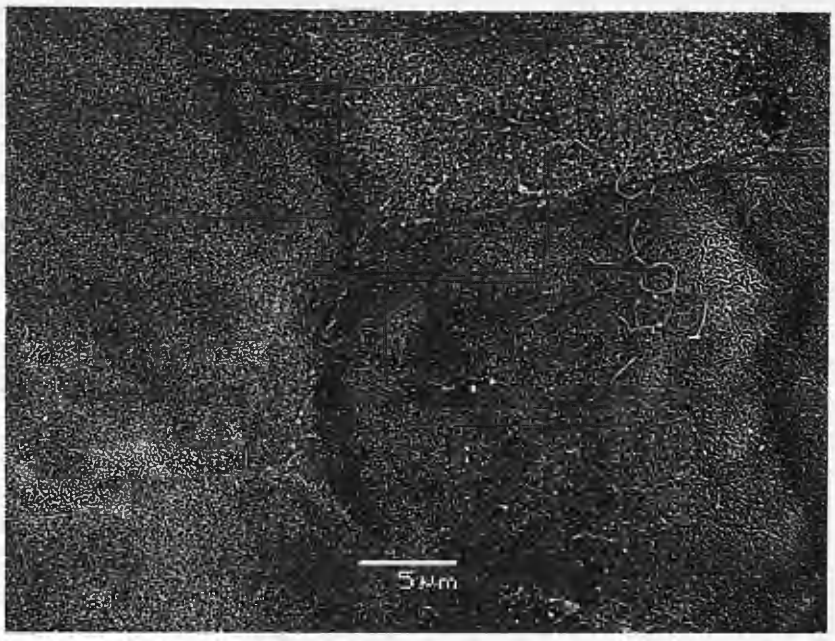


Plate 7.10: Scanning electron micrograph of human corneal epithelial stem cells on ECM protein coated gelatin hydrogel, taken at high magnification. Adjacent cells were well attached, with tightly opposed cell boundaries and distinct cell borders. [Scale bar = 5μm]

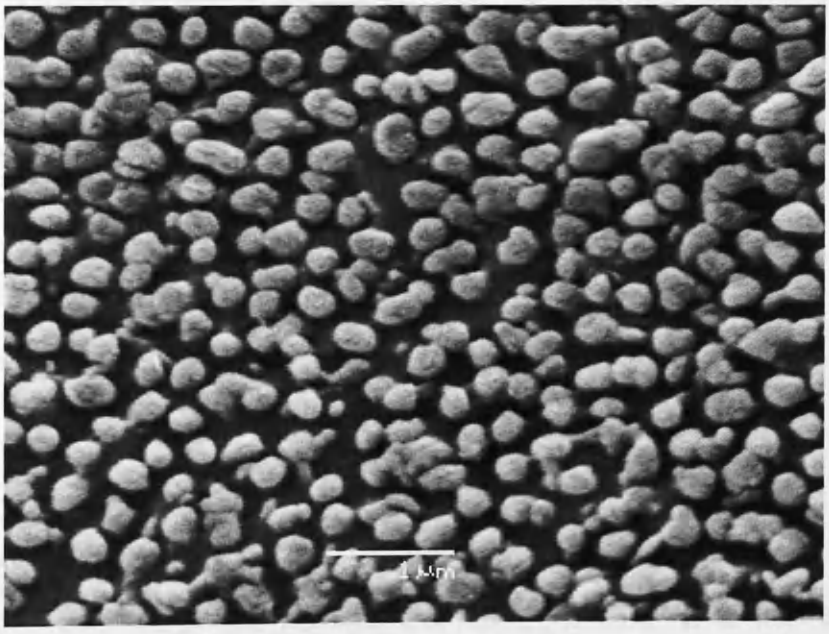


Plate 7.11: Scanning electron micrograph of human corneal epithelial stem cells on ECM protein coated gelatin hydrogel, taken at very high magnification. Superficial cells had an apical covering of short microvilli. [Scale bar = 1μm]

7.1.3.2 Cultivated Rabbit Corneal Epithelial Cells

Examination by scanning electron microscopy revealed the rabbit corneal epithelial cells cultivated on extracellular matrix protein coated gelatin to be generally in reasonable condition (plate 7.12). There was however a large number of rounded (perhaps apoptotic) cells on the surface of the cell sheet (plate 7.12). Adjacent epithelial cells again seemed to be well attached to each other (plate 7.13). In places there were distinct ridges at cell boundaries. Superficial epithelial cells were covered in short and regular microvilli (plate 7.14)

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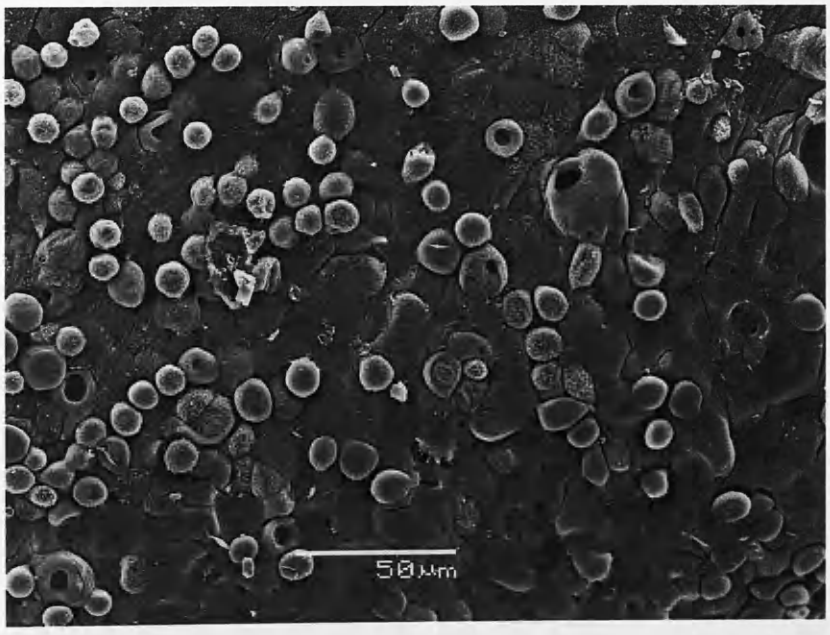

Plate 7.12: Scanning electron micrograph of rabbit corneal epithelial stem cells on ECM protein coated gelatin hydrogel, taken at very low magnification. While cells formed a confluent layer, there were some rounded cells on the surface. [Scale bar = 50µm]

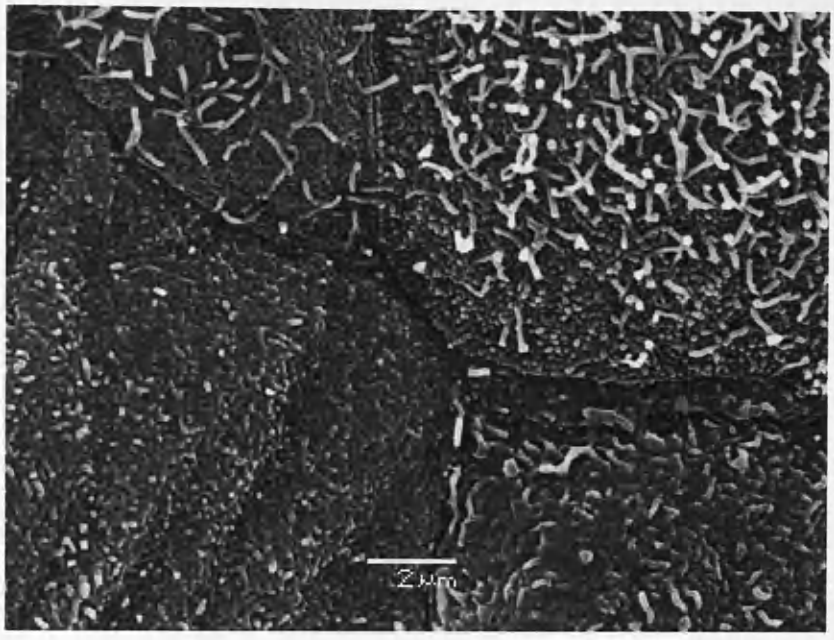

Plate 7.13: Scanning electron micrograph of rabbit corneal epithelial stem cells on ECM protein coated gelatin hydrogel, taken at high magnification. Adjacent cells were well attached to each other, with distinct ridges at cell-cell borders. [Scale bar = 2µm]

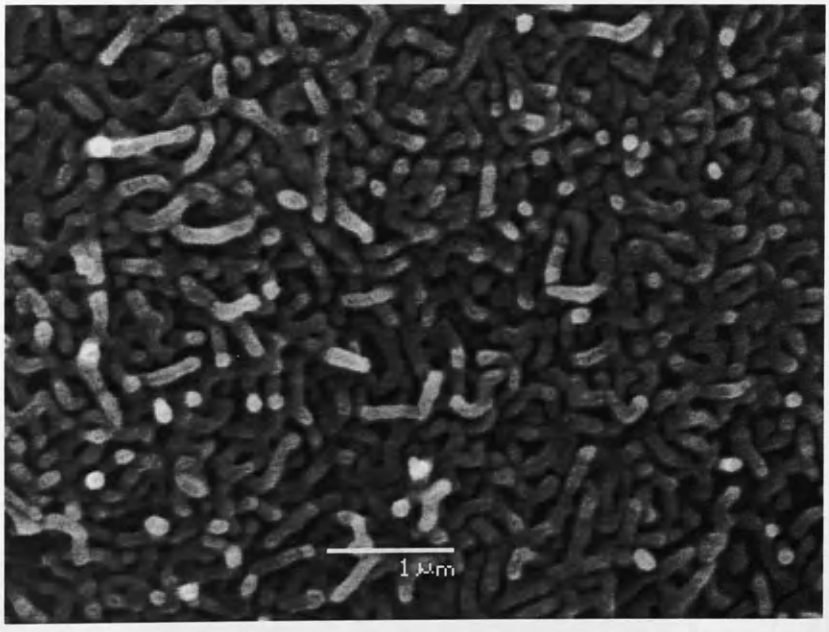

Plate 7.14: Scanning electron micrograph of rabbit corneal epithelial stem cells on ECM protein coated gelatin hydrogel, taken at high magnification. The apical surface of the superficial cells was covered in microvilli. [Scale bar = 1µm]

7.1.4 Transmission Electron Microscopy

7.1.4.1 Cultivated Human Corneal Epithelial Cells

Human corneal epithelial cells cultivated on ECM protein coated gelatin were in fairly good condition. There were 4-6 layers of well-stratified and differentiated epithelial cells (plate 7.15). Columnar basal cells appeared to be fairly well attached to adjacent cells, despite some rather large intercellular spaces. Basal cells seemed to be firmly attached to the gelatin though it was difficult to find any hemidesmosomal junctions and there did not appear to be a layer of secreted basement membrane material (plate 7.16). Superficial cells formed approximately 2-3 cell layers, again with some large spaces between cells (plate 7.17).

Plate 7.15: Transmission electron micrograph of human corneal cells cultivated on gelatin hydrogel, taken at low magnification. The cells appeared to be in fairly good condition and formed approximately 6 well-stratified and differentiated cell layers. There were some large intercellular spaces. [Scale bar = 2μm]

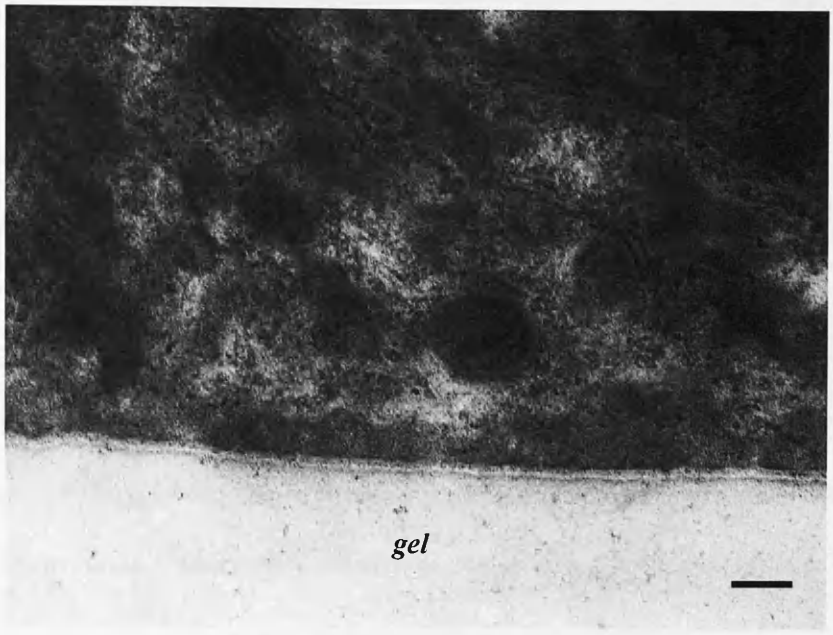

Plate 7.16: Transmission electron micrograph showing the basal region of a human corneal epithelial cell culture on gelatin hydrogel. Basal cells appeared to be firmly adhered to the substrate though it was difficult to find any evidence of hemidesmosomes or basement membrane material secretion. [Scale bar = 200nm].

Plate 7.17: Transmission electron micrograph of human corneal cells cultivated on gelatin hydrogel, taken at low magnification. Superficial cells were squamous and formed 2-3 layers with some large spaces between cells. Apically, there was a covering of short microvilli. [Scale bar = 1μm]

7.1.4.2 Cultivated Rabbit Corneal Epithelial Cells

The rabbit corneal epithelial cells cultivated on ECM protein coated gelatin were in good condition and formed 8-10 layers of well-stratified and differentiated epithelium (plate 7.18). There were some fairly large spaces between adjacent cells in all cell layers. Basal cells were columnar and well attached to adjacent cells. Attachment to the gelatin was by way of numerous hemidesmosomal junctions and there appeared to be secretion of basement membrane material (plate 7.19). Superficial cells were squamous in appearance and formed approximately 3-4 cell layers (plate 7.20). Desmosomes joined adjacent cells in all cell layers (plate 7.21).

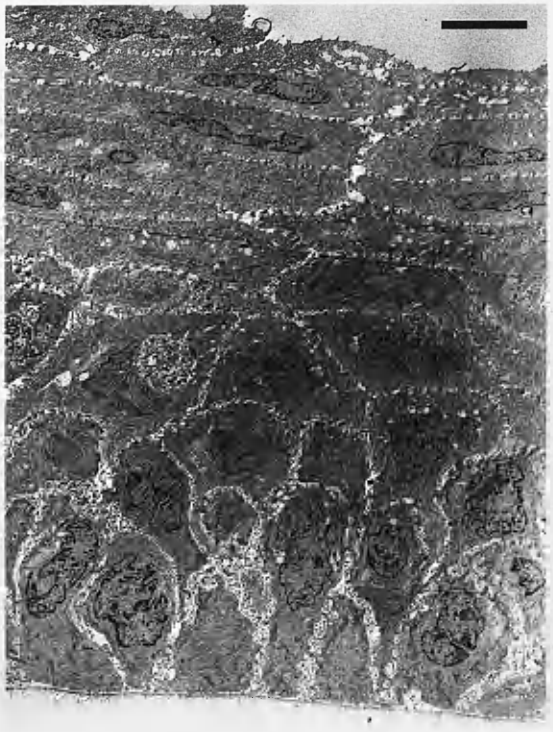

Plate 7.18: Transmission electron micrograph of rabbit corneal cells cultivated on gelatin hydrogel, taken at low magnification. The cells formed approximately 8-10 well-stratified and differentiated cell layers. [Scale bar = 5μm]

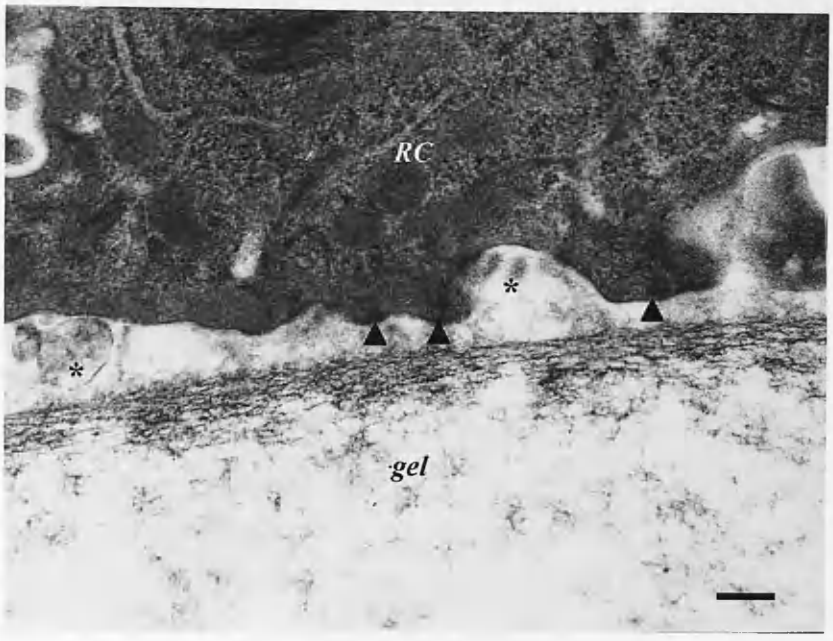

Plate 7.19: Transmission electron micrograph showing the basal region of cultivated rabbit corneal epithelial cells *[RC]* on gelatin. Basal cells were well attached to the substrate with numerous hemidesmosomes (arrowheads). They appeared to be secreting basement membrane material (*). [Scale bar = 200nm].

Plate 7.20: Transmission electron micrograph of rabbit corneal cells cultivated on gelatin hydrogel, taken at high magnification. Superficial cells formed 3-4 layers and had microvilli on their apical surface. [Scale bar = 2μm]

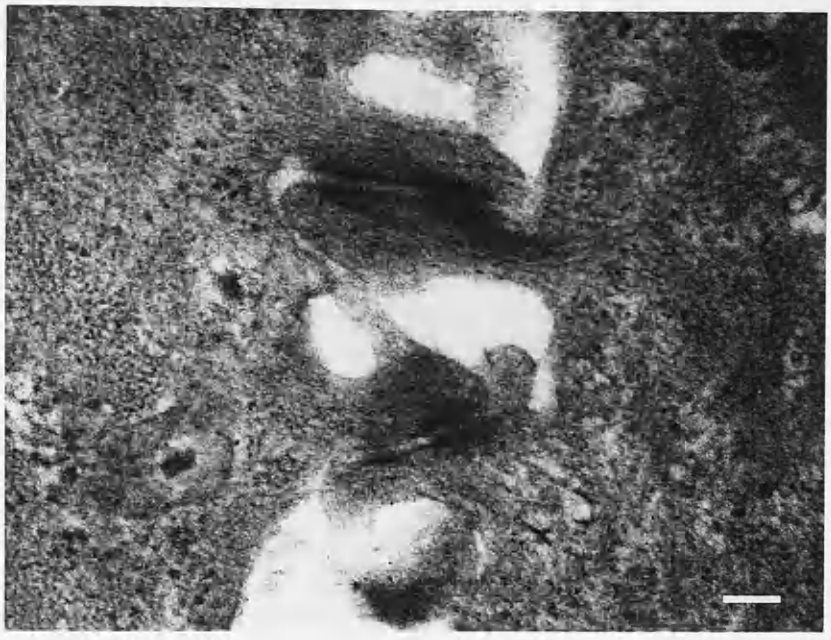

Plate 7.21: Transmission electron micrograph of rabbit corneal cells cultivated on gelatin hydrogel, taken at high magnification. Adjacent cells in all cell layers were joined by numerous desmosomal junctions, as depicted above. [Scale bar = 100nm]

7.1.5 Quantitative Study

Corneal cells cultivated on collagen IV and fibronectin-coated gelatins were compared to control corneal epithelium quantitatively across a number of parameters and the mean data (±S.D.) are shown in table 7.1 overleaf. Fourteen data points were collected for each parameter except for the cell surface area which was calculated using scanning electron microscopy and therefore facilitated more measurements (n=50). Percentage differences from control cornea and statistical significances of the observed differences were calculated.

	Cultivated human corneal cells on gelatin	Human corneal epithelium	Cultivated rabbit corneal cells on gelatin	Rabbit corneal epithelium
Thickness of cell layers (μm)	21.96 ± 2.45	29.02 ± 2.29	41.00 ± 9.09	20.55 ± 2.99
Number of cell layers	5.71 ± 1.27	6.64 ± 0.74	9.14 ± 1.23	7.79 ± 1.31
Intercellular space area (μm^2)	1.06 ± 0.94	0.11 ± 0.08	0.60 ± 0.36	0.09 ± 0.07
Number of desmosomes	1.93 ± 1.64	3.21 ± 1.37	1.86 ± 1.17	2.57 ± 1.60
Number of hemidesmosomes	0.36 ± 0.84	4.71 ± 1.44	4.57 ± 1.95	8.29 ± 1.68
Cell surface area (μm^2)	956.29 ± 474.51	1093.89 ± 335.43	241.00 ± 170.19	788.19 ± 200.53

Table 7.1: Comparison of cultivated human and rabbit corneal cells on ECM-coated gelatin with *in vivo* controls. Mean data (± S.D.) are shown. Fourteen data points were taken at random, except for the cell surface areas in which case n=50. The averages for intercellular area and number of desmosomes/hemidesmosomes are given for random 3μm long interfaces between adjacent cells.

Human corneal epithelial cells on a gelatin carrier were fairly comparable to the corneal control epithelium. The most apparent differences were an 852% increase in intercellular spaces and a 92% reduction in numbers of basal junctions in the cultured cells as compared with control. Whilst there were slight observed reductions in cell surface area and numbers of cell layers, these differences were not statistically significant. Differences that were deemed highly significant included a reduction in the thickness of the cell layers ($P<0.001$), an increase in intercellular space areas ($P<0.001$) between cells as well as fewer desmosomes ($P=0.033$) and hemidesmosomes ($P<0.001$). Since human cells available for culture are not always in good condition, rabbit cells were cultivated on the gelatin hydrogel to act as a comparison and to help more accurately gauge the usefulness of gelatin as a culture substrate.

With the exception of numbers of desmosomes, the rabbit cell culture on gelatin hydrogel had highly significant differences for each parameter when compared to the rabbit cornea control. The rabbit cell culture was approximately twice the thickness of the control corneal epithelium (99% thicker, $P<0.001$), and there was a corresponding 550% increase in intercellular spaces ($P<0.001$) between cultured cells, when compared with the control. In addition, the cultured cells had significantly fewer hemidesmosomes ($P<0.001$) attaching the basal cells to the gelatin than were present in the corneal control.

7.1.6 Summary Interpretations

Gelatin samples appeared to be in good condition and were successfully coated with extracellular matrix proteins. Examination by SEM showed that there were very obvious pits in the surface of the uncoated gelatins which became less apparent when ECM coatings had been applied. Transmission electron microscopy revealed that the coatings varied in thickness along the length of the sections, possibly as a result of the pores on the surface. It seemed that the collagen type IV coating was the thinnest while the thickest ECM layer resulted when both proteins were applied together. Human and rabbit corneal epithelial cells cultivated on coated gelatins appeared to be in good condition. The human cells formed 4-6 layers while the rabbit culture formed 8-10 layers. Both cell types were well attached to the gelatin substrate though rabbit cells appeared to be in better condition than the human cells with smaller intercellular spaces, more desmosomes and more hemidesmosomal junctions.

8 RESULTS

8.1 Human Serum in Corneal Epithelial Cell Culture

Foetal bovine serum is conventionally used in the culture of corneal epithelial cells for transplant. Given the concerns over the use of animal sera in human cell cultures, an investigation was launched into the feasibility of replacing the bovine serum with that of human origin. To this end, human corneal epithelial cells cultivated with human serum were examined by SEM and TEM and quantitatively compared to those cultured using bovine serum as well as to control *in vivo* human cornea.

8.1.1 Human Corneal Cell Cultures on AM: Experimental Samples

Human corneal epithelial cells were cultivated on amniotic membrane by project collaborators at Kyoto Prefectural University of Medicine (Kyoto, Japan) and transported to Lancaster University for analysis. Cells from four different donors were used in this investigation (eye bank tissue); three were cultured using human serum in the culture medium (termed 'serum 1-3' respectively) and one using foetal bovine serum (FBS). In addition to the foetal bovine serum control culture (current standard), normal human corneal epithelium was used as the target against which each of the cultured epithelia could be quantitatively compared.

8.1.2 Scanning Electron Microscopy

8.1.2.1 Human Serum-Cultivated Corneal Cells on AM

All three cultures of human corneal epithelial cells created using a human serum culture system appeared to be in very good condition (plates 8.1A-C). Superficial cell surface areas were similar in all cases; mean values (±S.D.) were 502.8μm^2 (±238.4; serum 1), 536.6μm^2 (±205.7; serum 2) and 647.1μm^2 (±324.7; serum 3). Adjacent cells were closely attached to each other with tightly opposed borders and distinct ridges at cell boundaries (plates 8.2A-C). At high magnification and on all samples, there was an apical covering of short, regular microvilli (plates 8.3A-C). Scanning electron microscopy revealed no discernible differences between the cultured tissues from different donors.

8.1.2.2 FBS-Cultivated Human Corneal Cells on AM

Since foetal bovine serum is frequently used in cell culture experiments, it was used in this study as a benchmark for the current technique. Examination of FBS-cultivated human corneal cells on amniotic membrane revealed a continuous layer of polygonal epithelial cells with a mean surface area of 630.3μm^2 (S.D.±319.6) (plate 8.1D). The appearance of these cells was not dissimilar to those cultivated with human serum. Adjacent cells were tightly opposed with distinct cell borders (plate 8.2D) and all cells had an apical covering of short, regular microvilli (plate 8.3D).

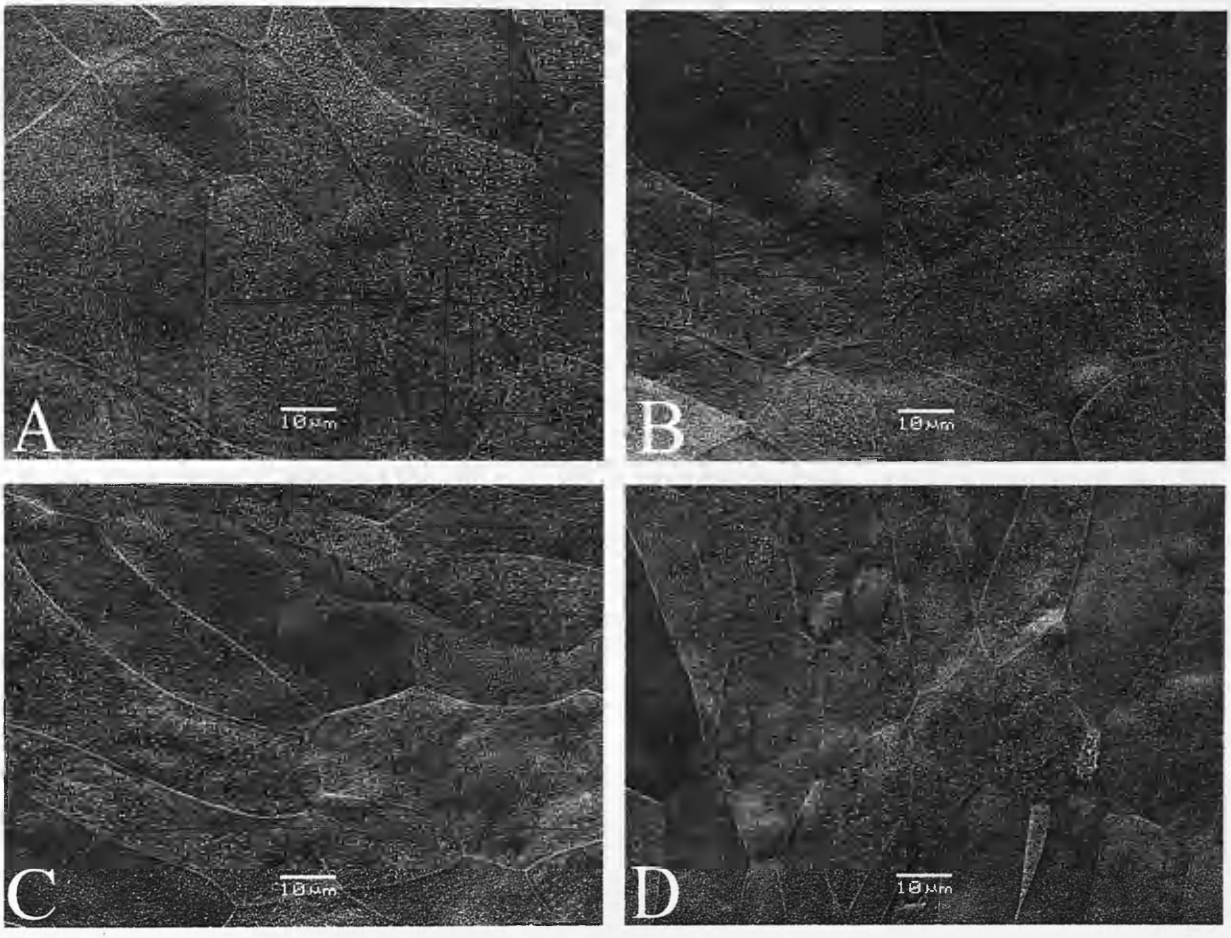

Plate 8.1: Scanning electron micrographs of human corneal cells cultivated using human serum, taken at low magnification. Figures represent cultures as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). All cultures appeared very to be in very good condition, with tightly opposed adjacent cells. There were some cells undergoing the process of desquamation in each of the samples. [Scale bars = 10µm]

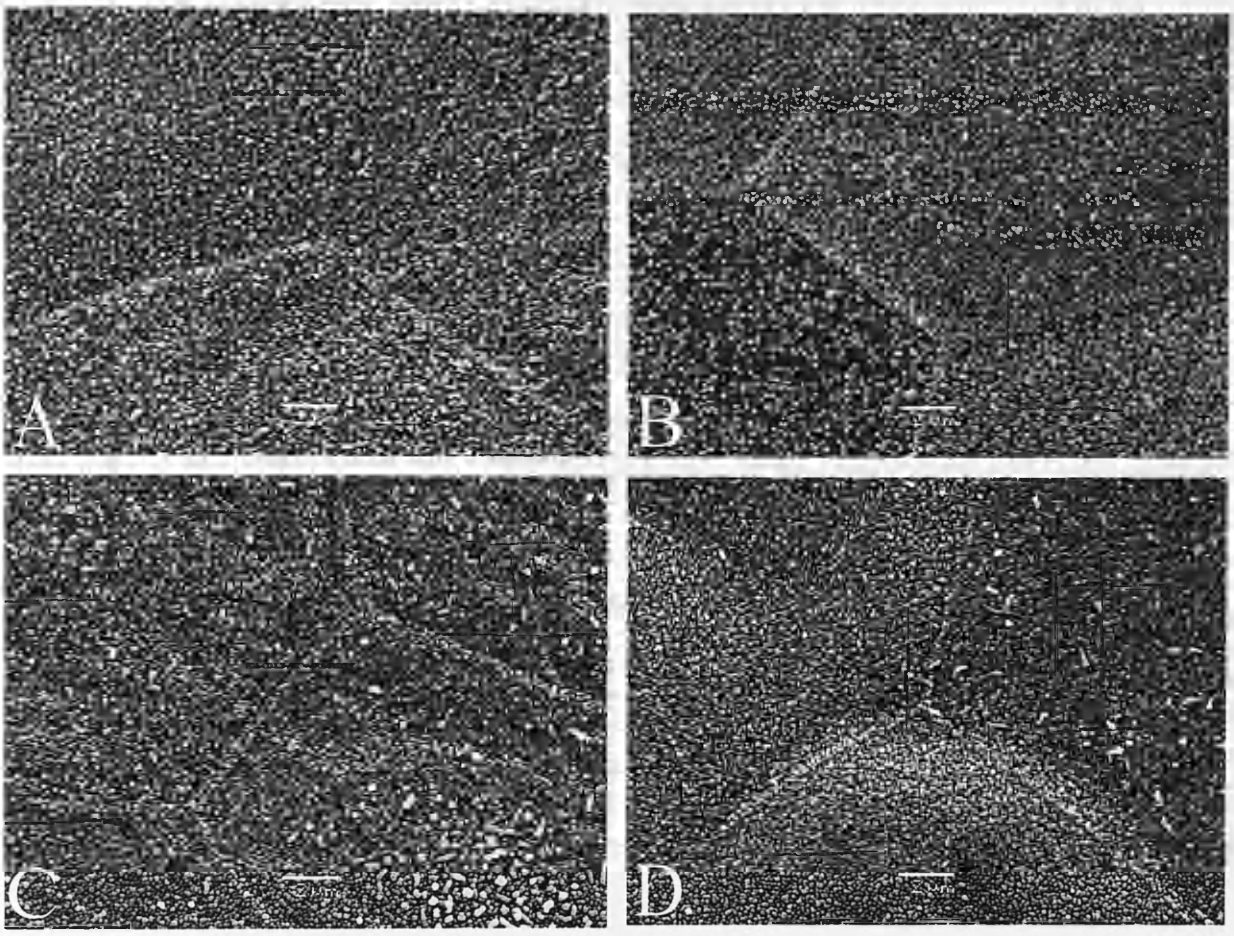

Plate 8.2: Scanning electron micrographs of human corneal cells cultivated using human serum, taken at high magnification. Figures represent cultures as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). In all cultures, adjacent superficial cells were closely attached to each other and exhibited distinct cell boundaries with very little intercellular spacing. [Scale bars = 2μm]

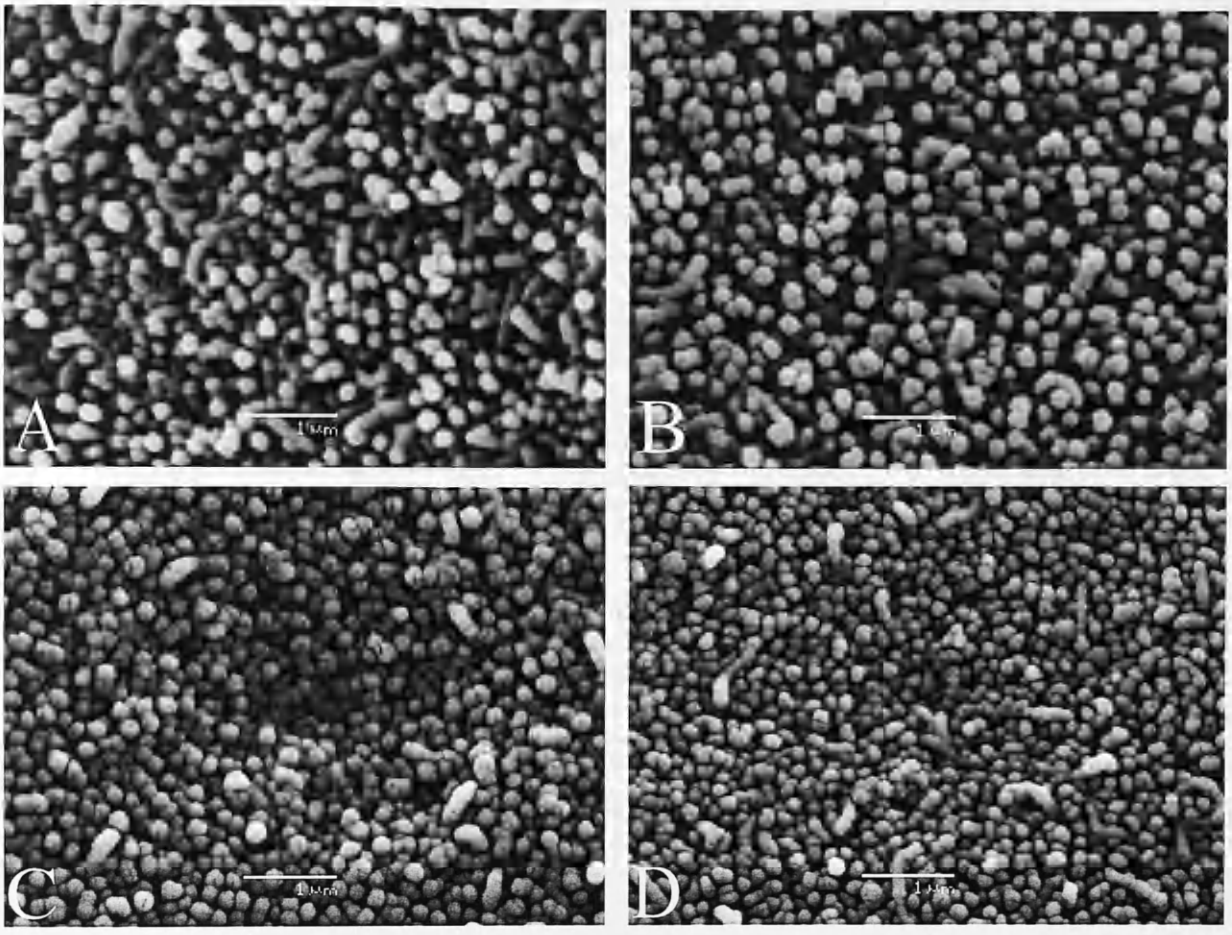

Plate 8.3: Scanning electron micrographs of human corneal cells cultivated using human serum, taken at very high magnification. Figures represent cultures as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). In all samples, the epithelium had an apical covering of short, regular microvilli. [Scale bars = 1μm]

8.1.3 Transmission Electron Microscopy

8.1.3.1 Human Serum-Cultivated Corneal Cells on AM

The human limbal cells cultured on denuded AM using human serum were generally in good condition and appeared to be in a better state than the culture using foetal bovine serum (described in section 8.1.3.2). Cells formed 4-6 well-stratified and differentiated cell layers, varying in thickness between cultures (plates 8.4A-C). Basal cells (plates 8.5A-C) were well attached to the underlying AM with numerous hemidesmosomal junctions and appeared to be secreting basement membrane material (plates 8.6A-C). There were some fairly large intercellular spaces between cells in the 'serum 2' culture, especially in the basal regions (plate 8.5B). Cell cultures using sera 1 and 3 were more compact. Superficial cells were squamous in appearance and had short regular microvilli on their apical surface (plates 8.7A-C). Cells in all layers were joined with frequent desmosomal junctions (plates 8.8A-C).

8.1.3.2 FBS-Cultivated Human Corneal Cells on AM

Human limbal cells were cultured on denuded AM using foetal bovine serum (FBS) for comparison. Cells formed 4-6 layers, though they were less well differentiated than would be expected (plate 8.4D). There were large intercellular spaces between adjacent cells at all cell layers, though they were particularly prominent in the basal regions (plate 8.5D). Basal cells were well attached to the underlying AM and appeared to be secreting basement membrane material (plate 8.6D). Hemidesmosomal junctions were less frequent than in the human serum cell cultures. Squamous superficial cells had short regular microvilli on their apical surface (plate 8.7D). Cells in all layers were joined with desmosomal junctions (plate 8.8D) though these were less abundant than in the human serum cultures.

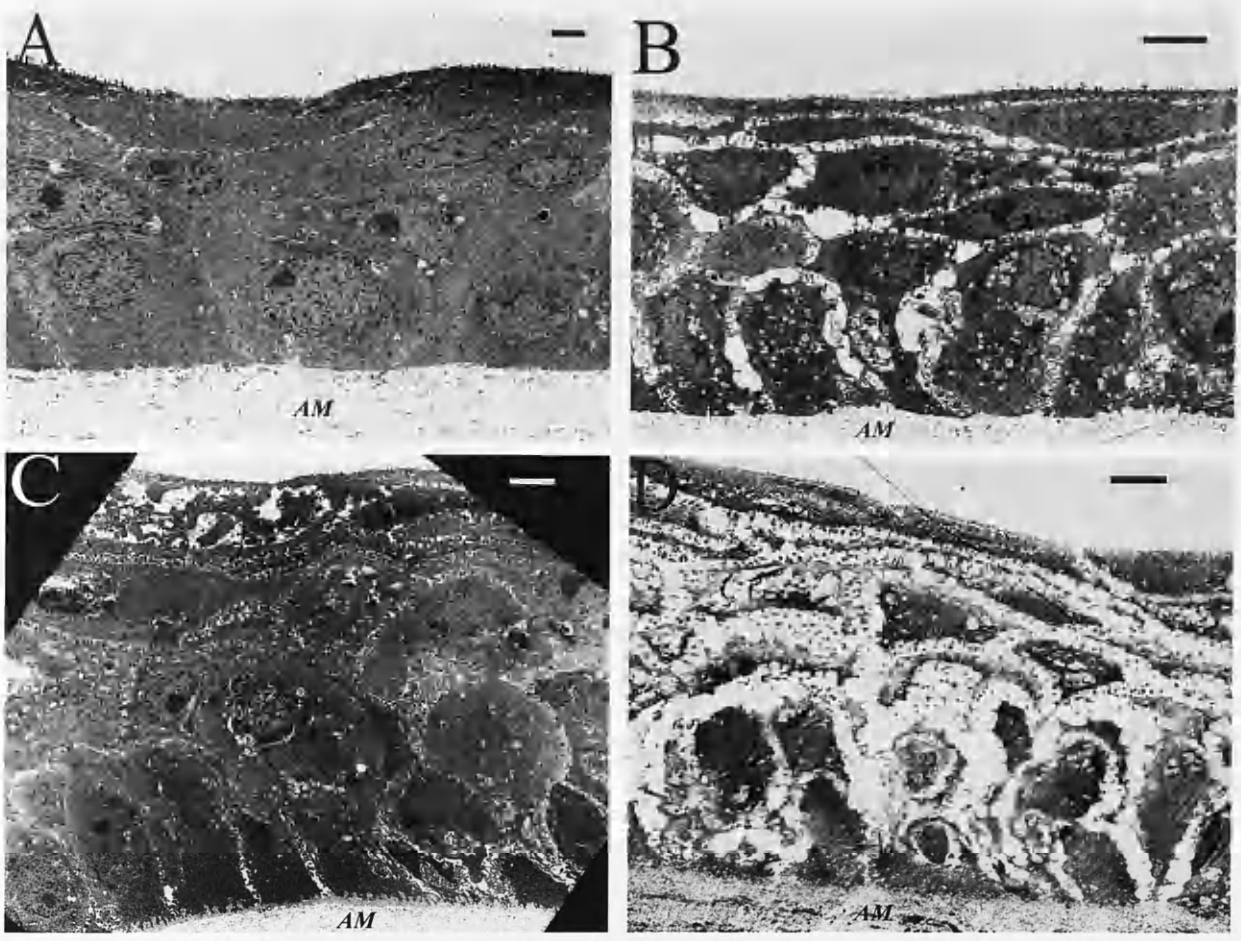

Plate 8.4: Transmission electron micrographs of human corneal cells cultivated on amniotic membrane *[AM]* using human serum, taken at low magnification. Figures represent cultures as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). Each culture produced 4-6 layers of well-stratified and differentiated cells. The culture using foetal bovine serum (D) was less healthy-looking than the human serum cultures and had large intercellular spaces between cells in all cell layers. [Scale bars: A, B and D = 2μm, C = 5μm]

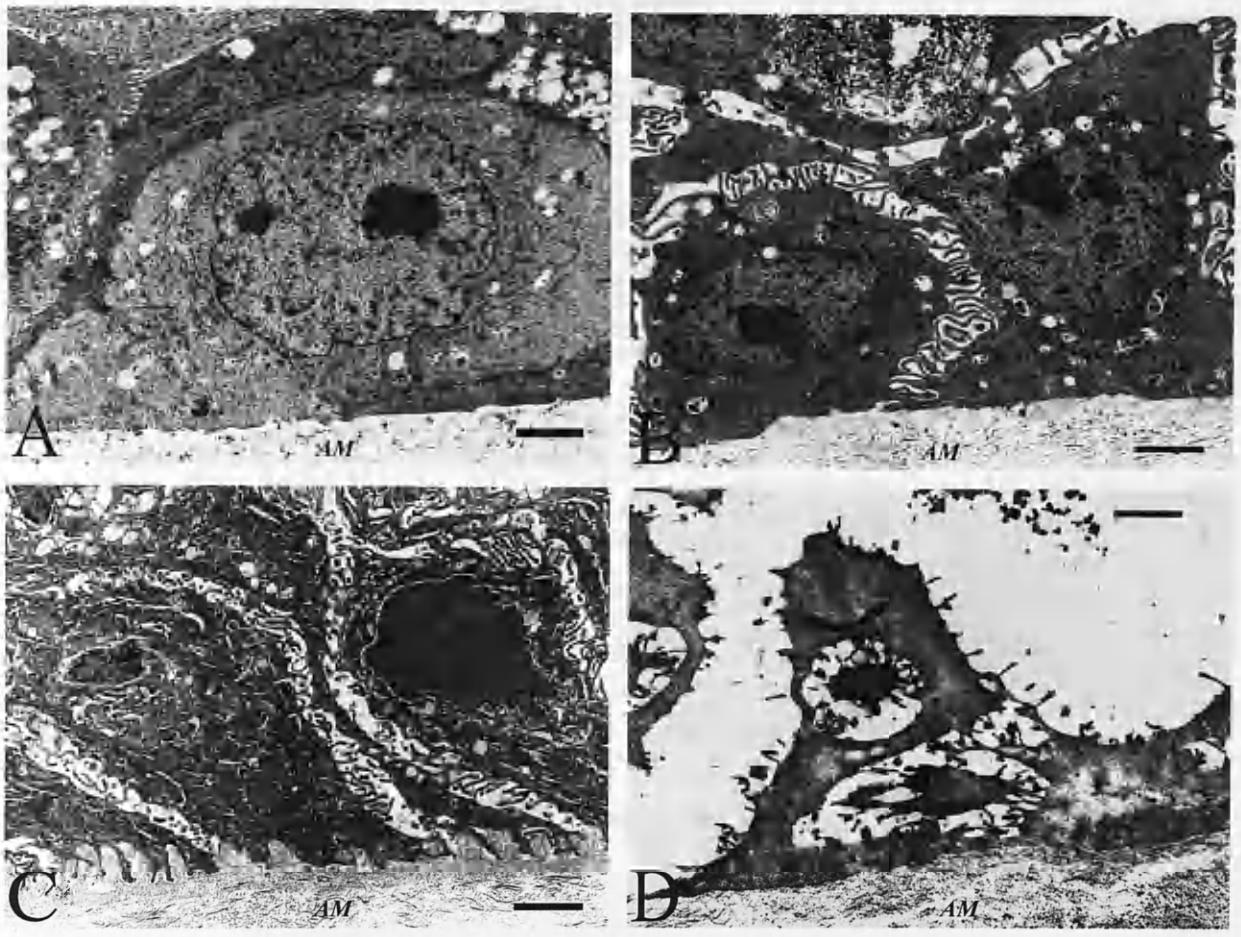

Plate 8.5: Transmission electron micrographs of human corneal cells cultivated on amniotic membrane *[AM]* using human serum, taken at high magnification. Figures represent cultures as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). Most of the basal cells were columnar in shape, though some areas of the cultures were better differentiated than others. There were large intercellular spaces between basal cells of the FBS culture (D). [Scale bars = 2μm]

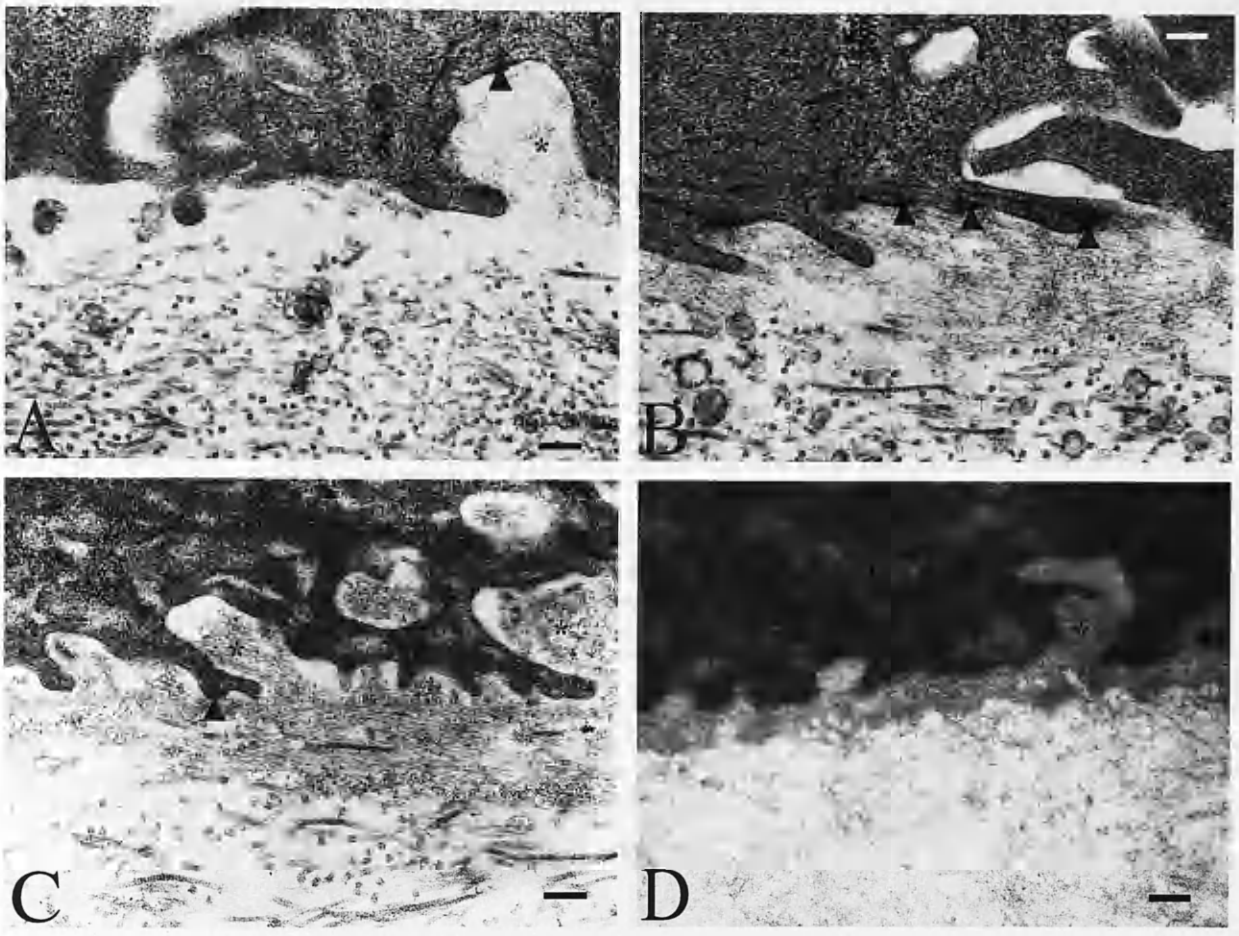

Plate 8.6: Transmission electron micrographs at very high magnification to illustrate the basement membrane regions of human corneal cells cultivated using human serum. Figures represent samples as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). Limbal cells were well adhered to the denuded amniotic membrane, and extended interdigitations into the substrate. There also appeared to be hemidesmosomal junctions (arrowheads) and secretion of basement membrane material (asterisk) in each of the cultures. [Scale bars = 500nm]

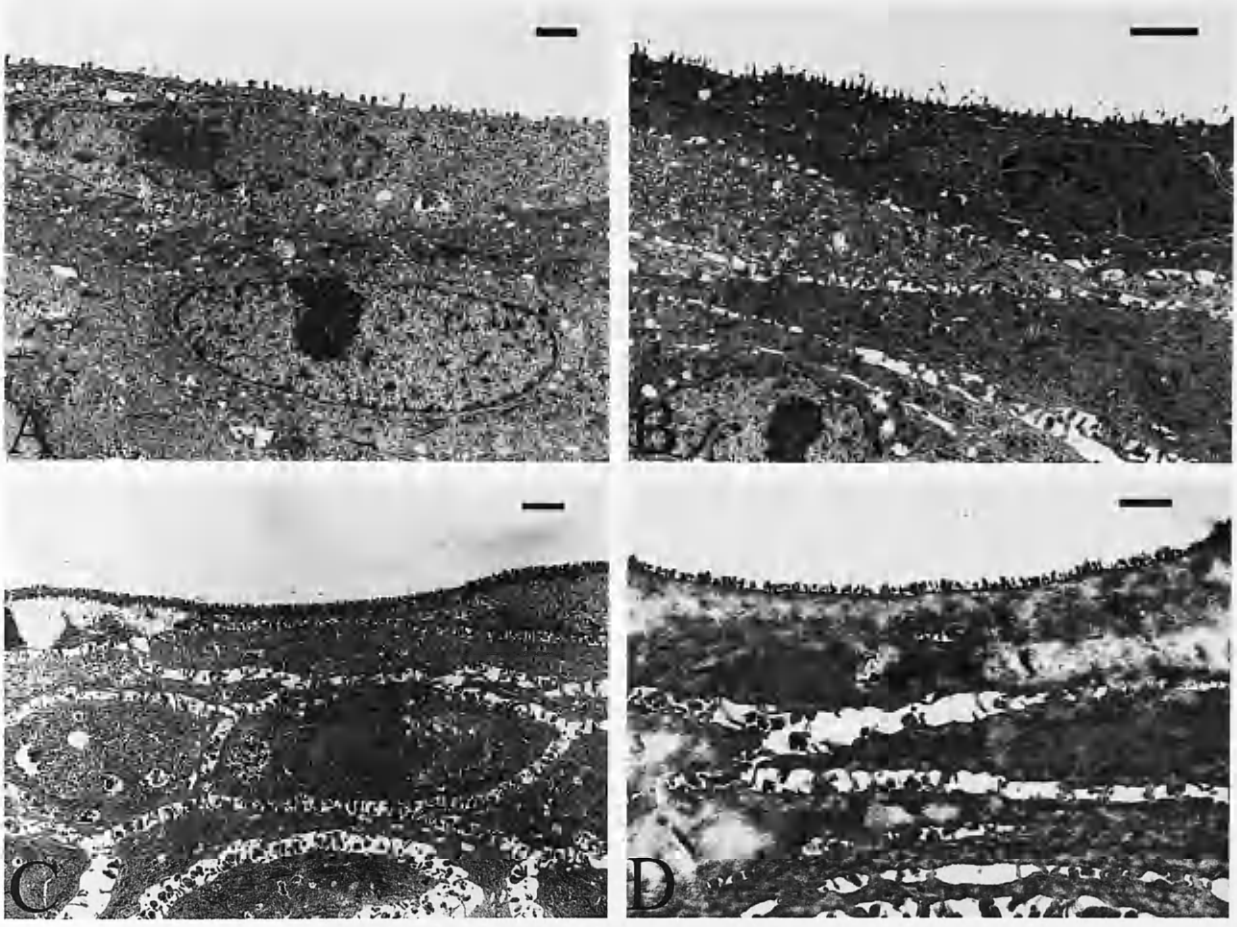

Plate 8.7: Transmission electron micrographs taken at high magnification to show the superficial cell layers of human serum cultivated human limbal epithelial cells. Figures represent samples as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). Superficial cells were squamous in appearance and there were generally smaller intercellular spaces in this region than basally. [Scale bars: A =1μm, B-D = 2μm]

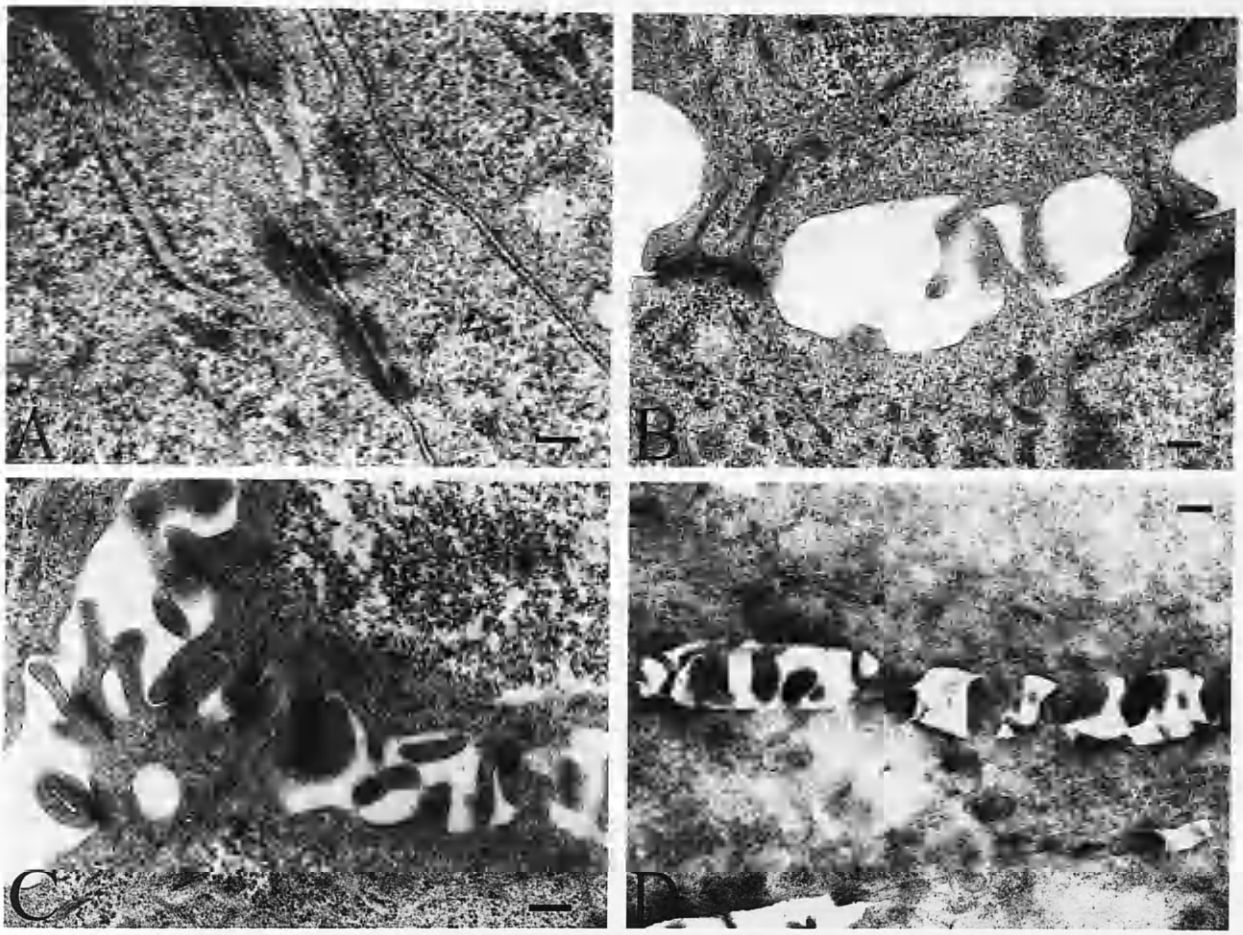

Plate 8.8: Transmission electron micrographs at high magnification show cultivated human limbal epithelial cells. Figures represent samples as follows; *serum 1* (A), *serum 2* (B), *serum 3* (C) and *FBS* (D). Cells in all layers were joined by numerous desmosomal junctions, though there were bigger intercellular spaces and fewer junctions in the culture using bovine serum than those using human sera. [Scale bars = 200nm]

8.1.4 Quantitative Study

Both human serum and FBS-cultivated corneal cells on amniotic membrane were compared to normal human corneal epithelium (post-mortem tissue, identical to previous studies) quantitatively across a number of parameters. The mean data (±S.D.) are shown in table 8.1 below. Fourteen data points were collected for each parameter, except for the cell surface area which was determined using scanning electron microscopy and as such more measurements were facilitated (n=50).

	Human serum culture 1	**Human serum culture 2**	**Human serum culture 3**	**FBS culture**	**Human corneal epi**
Thickness of cell layers (μm)	17.73 ± 1.35	25.21 ± 2.38	47.40 ± 3.06	33.20 ± 10.52	29.02 ± 2.29
Number of cell layers	5.21 ± 0.89	5.71 ± 0.91	7.21 ± 1.42	6.21 ± 1.12	6.64 ± 0.74
Intercellular space area (μm^2)	0.15 ± 0.18	0.79 ± 0.52	0.94 ± 0.67	1.12 ± 0.76	0.11 ± 0.08
Number of desmosomes	1.64 ± 1.01	1.57 ± 0.94	1.93 ± 1.54	1.14 ± 0.95	3.21 ± 1.37
Number of hemidesmosomes	3.07 ± 1.69	2.00 ± 1.57	3.50 ± 1.40	1.79 ± 1.42	4.71 ± 1.44
Cell surface area (μm^2)	502.80 ± 240.77	536.59 ± 207.75	647.07 ± 327.96	630.25 ± 322.88	1093.89 ± 335.43

Table 8.1: Comparison of human and foetal bovine serum-cultivated corneal cells on AM with normal human cornea. Mean data (± S.D.) are shown. Fourteen data points were taken at random, except for the cell surface areas in which case n=50. The averages for intercellular area and number of desmosomes/hemidesmosomes are given for random 3μm long interfaces between adjacent cells.

For ease of graphical illustration, mean data were plotted as percentage differences from the control (human cornea). Charts were plotted for each of the human serum cultures (1-3) and for the foetal bovine serum cultivated cells, to enable comparison with the target; control cornea.

8.1.4.1 Human Serum (1) Cultivated Corneal Epithelial Cells

From the morphological analyses, it was apparent that of all the human serum cultures, 'serum 1' cells most closely resembled control cornea; in terms of stratification, differentiation and intercellular spacing. The quantitative study added further credence to these observations. Intercellular spacing was increased by 38.5% in the cultured cells (P=0.414). In all other parameters, observed differences were found to be statistically significant. The cell layer was 38.9% thinner (P<0.001) with a 21.5% reduction in cell layers (P<0.001), when compared to control cornea. In addition there were significantly fewer desmosomes (P=0.002) and hemidesmosomes (P=0.010) in the cultivated cells than in the control human corneal epithelium.

8.1.4.2 Human Serum (2) Cultivated Corneal Epithelial Cells

Human 'serum 2' cultivated cells differed more significantly from the control than the 'serum 1' cells (section 8.1.4.1). Most significant was the 608.3% increase in intercellular spacing in this culture (P<0.001), when compared to the control corneal epithelium. In fact, the human 'serum 2' cultivated cells differed significantly from the control in each parameter under study. Even the relatively small reductions in the thickness (13.1%, P<0.001) and the number of cell layers (14.0%, P=0.018) were statistically significant. When compared to normal human cornea, there were 51.1% fewer desmosomes (P=0.001) joining adjacent cells and 57.6% fewer hemidesmosomes (P<0.001) attaching the basal cells to the underlying matrix. In addition, the superficial epithelial cells in this culture had a 51% smaller surface area than the control.

8.1.4.3 Human Serum (3) Cultivated Corneal Epithelial Cells

The most notable statistically significant difference in the 'serum 3' cultivated cells when compared to control cornea, is that of a 746.8% increase in intercellular spaces ($P<0.001$). The 8.6% increase in number of cell layers was not a statistically significant difference ($P=0.195$) however all other parameters were. In this instance, the cell layer was significantly thicker than the control (by 63.3%, $P<0.001$). Previously described cultures using human serum (sections 8.1.4.1 and 8.1.4.2) had thinner epithelial cell layers than the control. As well as having more cell layers, this increase in thickness is probably attributable to the comparatively large areas of intercellular space. There were significantly fewer desmosomes ($P=0.028$) and hemidesmosomes ($P=0.032$) in the cultured epithelium than in the control, however percentage wise, these differences were smaller than in each of the other cultures.

8.1.4.4 FBS Cultivated Corneal Epithelial Cells

In terms of thickness of cell layers, the FBS-cultivated corneal cells were marginally (14.4%) thicker than those in the control cornea, despite having slightly (6.5%) fewer cell layers. Neither of these differences was statistically significant. In all other parameters studied, the observed differences were statistically highly significant. The FBS-cultivated cells had the biggest intercellular spaces of all the cultured cells, with a 9-fold increase compared with the control corneal epithelium ($P<0.001$). With regard to numbers of desmosomes and hemidesmosomes, again, differences with the control were more marked in this culture than for the human serum cultures. There was a significant 64.4% reduction in numbers of desmosomes ($P<0.001$) and 62.1% fewer hemidesmosomes ($P<0.001$) in the FBS-cultured cells. Superficial cells were 42.4% smaller than in the control corneal epithelium.

8.1.4.5 Comparisons of Human Serum and FBS Cultivated Cells

Since foetal bovine serum is routinely used in cell culture media and represents the most readily available option to date, each of the human serum cultures were quantitatively compared to the FBS-cultured corneal epithelial cells to gauge its relative usefulness in culture. Separate charts were plotted to compare each of the human serum-cultured corneal epithelial sheets with that using FBS.

Chart 8.1 shows the comparison between human 'serum 1' cultured cells and those using FBS. Observed differences were all statistically significant, with the exception of desmosome counts (P=0.188). The 'serum 1' culture had significantly fewer cell layers (P=0.024) and therefore a thinner epithelium (P<0.001) than the FBS culture. Superficial cell surface areas were also slightly smaller in the human serum cultivated cells (P=0.023). Most obvious was the highly significant reduction in intercellular spaces in the human serum-cultivated cells, when compared to those cultured with bovine serum. There were almost twice the number of hemidesmosomes in the human serum cultivated cells than in the FBS culture (P=0.038).

In chart 8.2, comparing human 'serum 2' with FBS-cultivated corneal epithelial cells, it is evident that the cultures were more comparable. Only that the human serum cultured epithelium was thinner was a significant difference (P=0.037). All other quantified differences were not significant however the human serum cultivated cells had fewer cell layers (P=0.207) and smaller cells (P=0.088), fewer junctions (desmosomes, P=0.240; hemidesmosomes, P =0.708) and smaller intercellular spaces (P=0.197) than the FBS-cultivated epithelium.

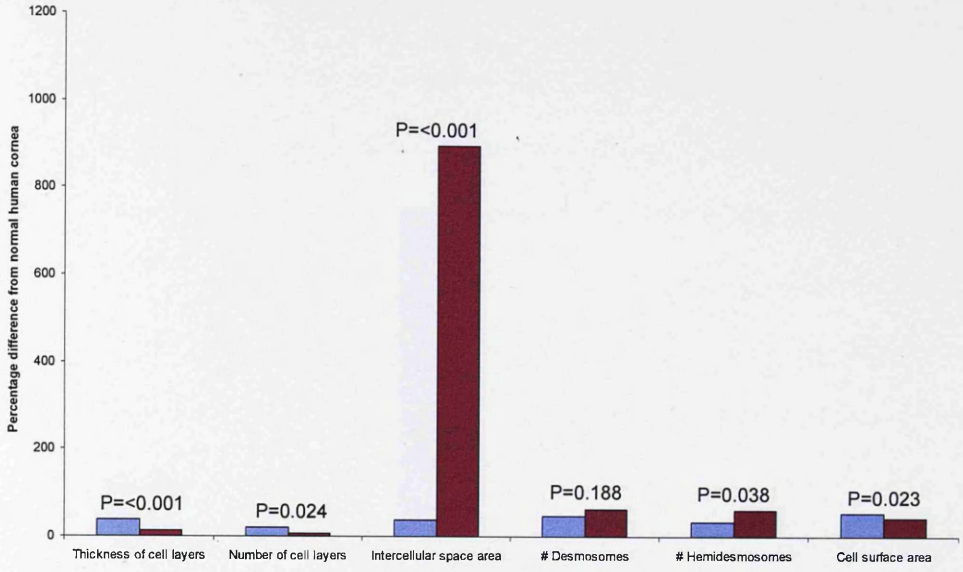

Chart 8.1: Comparison of human serum (1, blue) and FBS (purple) cultivated human corneal epithelial cells on AM, expressed as percentage difference from normal human cornea control. Raw data were used to calculate statistical significance (P values) of observed differences between the culture systems and these are stated above the bars.

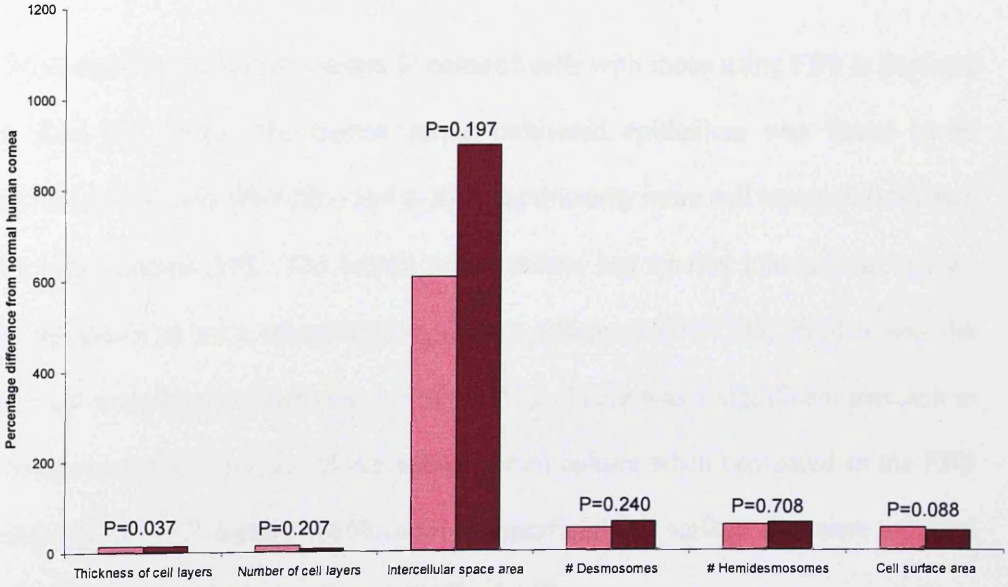

Chart 8.2: Comparison of human serum (2, pink) and FBS (purple) cultivated human corneal epithelial cells on AM, expressed as percentage difference from normal human cornea control. Raw data were used to calculate statistical significance (P values) of observed differences between the culture systems and these are stated above the bars.

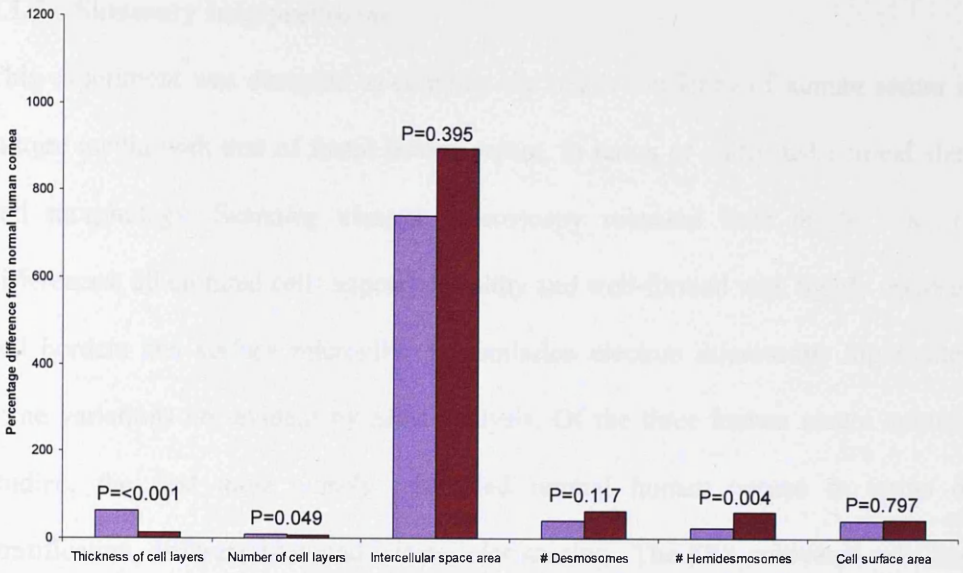

Chart 8.3: Comparison of human serum (3, lilac) and FBS (purple) cultivated human corneal epithelial cells on AM, expressed as percentage difference from normal human cornea control. Raw data were used to calculate statistical significance (P values) of observed differences between the culture systems and these are stated above the bars.

The comparison of human 'serum 3' cultured cells with those using FBS is depicted in chart 8.3 above. The human serum cultivated epithelium was found to be significantly thicker ($P<0.001$) and to have significantly more cell layers (0.049) than the FBS cultured cells. The human serum culture had smaller intercellular spaces, though this was not a statistically significant difference ($P=0.395$), neither was the increase in numbers of desmosomes ($P=0.117$). There was a significant increase in hemidesmosomal junctions in the human serum culture when compared to the FBS cultivated cells ($P=0.004$). Differences in superficial cell surface area were minimal between cultures and were not statistically significant.

8.1.5 Summary Interpretations

This experiment was designed to compare the relative efficacy of human serum in culture media with that of foetal bovine serum, in terms of cultivated corneal stem cell morphology. Scanning electron microscopy revealed little in the way of differences; all cultured cells appeared healthy and well-formed with tightly opposed cell borders and surface microvilli. Transmission electron microscopy highlighted some variations not evident by SEM analysis. Of the three human serum cultures studied, the first most closely resembled normal human cornea in terms of stratification, differentiation and intercellular spacing. The FBS-cultivated cells had the biggest intercellular spaces of all the cultured cells, as well as the smallest numbers of desmosomes and hemidesmosomes. To summarize, the cells cultured using bovine serum were least like the control human cornea.

9 DISCUSSION

Overview of the Aims of this Thesis

While *ex vivo* expansion of limbal stem cells on amniotic membrane for ocular surface reconstruction has proven largely successful, it is not an infallible technique. Limitations include shortage of donor materials, risk of allograft rejection and the need for prolonged intensive immune suppression. The central aim of this thesis was to use microscopic and immunohistochemical techniques to refine tissue engineering of the cornea in the pursuit of more superior cell sheets for transplantation onto the ocular surface. Each of the results chapters in this thesis has described a potential area of improvement, specifically:

- An investigation into the comparative merits of cellular and denuded amniotic membrane as a carrier for cultivated human limbal stem cells (chapter 3).
- An evaluation of amniotic membrane as a supportive matrix for the culture of corneal endothelial cells for grafting into dystrophic eyes. In addition, an investigation into the use of polyphenol antioxidant for preserving corneal endothelial cells, pre-transplant (chapter 4).
- The ultrastructural and immunohistochemical characterization of freeze-dried amniotic membrane and evaluation of its suitability as an alternative substrate to conventionally cryopreserved tissue (chapter 5).
- Analysis of the human oral mucosa as a potential source of stem cells for autologous corneal grafts (chapter 6).

- An examination of extracellular matrix protein coated gelatin hydrogels as alternative carriers for limbal stem cell cultures (chapter 7).

- An investigation into the feasibility of replacing foetal bovine serum in the culture medium with that of human origin (chapter 8).

A number of techniques were employed to evaluate the success or otherwise of these adaptations to the culture technique. Light, scanning electron and transmission electron microscopy were used to study cell morphology and observed differences were quantified to the best possible extent. The parameters chosen for quantification were considered important for the following reasons. The thickness of epithelial cell layers determines the extent of protection from bacteria and other contaminants of the ocular surface, however too thick a cell layer restricts oxygen and tear film permeability as well as acting as a physical hindrance. Likewise the number of cell layers offers some indication of the extent of stratification and differentiation which is more easily quantifiable (5-6 cell layers is considered optimal for the corneal epithelium). Calculation of areas of intercellular space and corresponding numbers of desmosomes provides information about the mechanical strength of the epithelial sheet; the more densely packed the cells in the tissue and the more intercellular junctional complexes, the better equipped it is to withstand the rigours of transplantation. Similarly basal junctions were quantified since good attachment to the carrier is vital for ensuring the cells do not simply extricate when the graft recipient blinks. Scanning electron microscopy enabled the accurate quantification of cell surface areas and thus provides information about the rate of cell proliferation and differentiation; small cells indicative of a lack of differentiation or perhaps too rapid a rate of division. Morphological and quantitative results from each of the studies described will be discussed in turn in the following sections.

9.1 Comparison of Cellular and Denuded Amniotic Membrane as Carriers for Human Limbal Stem Cell Cultivation

9.1.1 Background

The corneal epithelium is a self-renewing tissue maintained by the proliferation of stem cells located at the limbus (Schermer *et al.*, 1986; Cotsarelis *et al.*, 1989). Severe ocular surface disorders can deplete the limbal stem cell supply, resulting in invasion of the neighbouring opaque conjunctival epithelium and a loss of visual acuity (Shapiro *et al.*, 1981; Tseng, 1996). In order to reconstruct such damaged ocular surfaces a number of surgical techniques have been developed in the past 20 years including conjunctival transplantation (Thoft, 1977), keratoepithelioplasty (Thoft, 1984) and the grafting of donor (Tsai and Tseng, 1994) or autologous (Dua and Azuara-Blanco, 2000a) limbal epithelium. Since the pioneering work of Pellegrini *et al.* (1997) in which *ex vivo* expanded biopsy-derived limbal epithelial cells on a petrolatum gauze carrier were transplanted in patients with severe unilateral disorders, attention has been focused on finding appropriate substrates on which to culture limbal epithelial cells and on refining existing procedures for ocular surface reconstruction.

A suitable substrate/carrier ought to be biocompatible, flexible, mechanically strong, permeable for nutrient transport and able to support surface epithelial cell growth. Mechanical or tensile strength is essential for any graft to survive the rigors of transplantation, not least onto the ocular surface. Following a transplant operation, the cultured epithelium must be able to withstand the physical forces of blinking and eyeball movement, as well as potential blows and straining effects. Equally important

in a corneal graft is the provision of a source of stem cells for adequate self-renewal and long-term graft survival, especially in patients presenting total limbal stem cell deficiencies. Precise functioning of the cornea is essential for good vision and relies upon the continual production of new epithelial cells, their appropriate differentiation and eventual desquamation from the ocular surface. Functional properties need to be maintained through differentiation as cells constantly stream towards the surface, hence the need for a supply of specialized stem cells (Kruse, 1994; Dua and Azuara-Blanco, 2000b). A delicate balance is required between supplying sufficient stem cells for renewal and providing enough terminally differentiated corneal-like cells to maintain essential corneal functions.

The human amniotic membrane (AM) has been used by many investigators, both with and without limbal epithelial transplantation to reconstruct severely damaged ocular surfaces (Shimazaki *et al.*, 1997; Tseng *et al.*, 1998; Azuara-Blanco *et al.*, 1999; Gabric *et al.*, 1999; Chen *et al.*, 2000; Hanada *et al.*, 2001). It consists of a collagenous stroma and thick basement membrane of predominantly type IV collagen and laminin which closely resembles that of the conjunctival and corneal epithelium (Fukuda *et al.*, 1999). In addition, AM has been reported to promote epithelialization after transplantation via intrinsic growth factors (EGF, HGF and KGF) (Koizumi *et al.*, 2000c) and to exhibit anti-inflammatory effects by way of its suppression of epithelial interleukins (Solomon *et al.*, 2001), prevention of polymorphonuclear cell infiltration into the corneal stroma (Park and Tseng, 2000) and inhibition of protease activity (Kim *et al.*, 2000). It is widely accepted as a useful adjunct for ophthalmic surgery, though the optimal conditions for its use have yet to be established. In the pursuit of an effective method for reconstructing the damaged ocular surface, several

research groups have developed culture systems which use human AM as a carrier. Project collaborators at the Kyoto Prefectural University of Medicine developed a limbal epithelial culture system based on that established by Rheinwald and Green (1975) using denuded (acellular) AM as a carrier, supported by a 3T3 fibroblast feeder layer and airlifting techniques. They have produced well-stratified and morphologically differentiated cellular multilayers which closely resemble *in vivo* corneal epithelium and have been successfully used surgically to cover the severely damaged ocular surfaces of patients with SJS, OCP and chemical injury (Koizumi *et al.*, 2001a; Koizumi *et al.*, 2001b). Another established culture system is that using limbal explants on cellular AM, performed without the 3T3 feeder layer and airlifting. Recently, Grueterich and co-workers have shown that this system preserves stem cells or limbal epithelial progenitor cells and have produced cultured cell sheets which exhibit slow cell cycling, label-retaining characteristics and do not express keratins 3 and 12 or connexin 43 (Meller *et al.*, 2002; Grueterich *et al.*, 2002a); features which are found in the stem cell-containing limbal basal epithelium.

Given these developments, this investigation aimed to compare human limbal epithelial cells cultured on denuded AM (in which the basement membrane is exposed by the enzymatic removal of host epithelial cells) with those on cellular AM (where amniotic epithelial cells are retained) using a cell-suspension culture method and to ultimately determine the more suitable carrier for use in transplantation, in terms of morphological appearance and overall mechanical strength. Earlier investigations to compare substrates in this manner have only involved the use of animal tissue (Koizumi *et al.*, 2000b; Koizumi *et al.*, 2001a).

9.1.2 General Discussion of Results

This study was designed to compare the morphology of human limbal cells cultured on denuded and cellular membranes, using microscopic techniques. While AM has been successfully used in many surgical applications over the years, there is still some debate as to optimal conditions for use. The results of SEM and TEM examinations, presented in chapter 3, were quite conclusive. Limbal cells cultivated on denuded AM were well stratified and differentiated (plate 3.9), very similar in appearance to in vivo corneal epithelium. Additionally, basal limbal cells were attached directly to the basement membrane with numerous hemi-desmosomal junctions and appeared to be secreting basement membrane material. By contrast, limbal cells grown on cellular AM typically formed a monolayer (plate 3.10) and attachment to the underlying amniotic epithelial cells was via infrequent desmosomal junctions (plate 3.14); an observation which has also been made in rabbit cultures (Koizumi *et al.*, 2000a). The need for a mechanically robust cell sheet for clinical application in ocular surface reconstruction cannot be understated and over the years a number of different culture systems have been employed to achieve this end. The cell-suspension system used in this project was developed in 2002 (Koizumi *et al.*, 2002) and typically uses denuded AM as the substrate of choice. TEM examination of the current samples confirmed the supposition that exposure of the basement membrane by denuded AM promotes epithelial growth and encourages better cellular adhesion and stratification, probably as a result of the direct contact between the growth factors/ECM adhesion proteins in the basal lamina and the cultivated cells.

In terms of the cellular substrate, amniotic epithelium appeared to have been severely disrupted by the process of cryopreservation and the cells were clearly no longer

viable (plate 3.10). This process, the most commonly used method of preserving AM for use in ocular surface reconstruction, involves placing tissue on nitrocellulose paper in a part glycerol solution and subsequent storage in liquid nitrogen fumes at -80°C until surgery. Ice crystals form, causing irreversible damage to cellular organelles and as such, amniotic epithelial cells are not viable after preservation. Kruse and co-workers, in a study on the effects of cryopreservation on membranes, found that cells removed from preserved AM were not capable of proliferation in culture (Kruse *et al.*, 2000) and conclude that amniotic membrane grafts seem to function primarily as matrix and not by virtue of transplanted functional cells. The results of this thesis corroborate the findings of Kruse. It is likely that the poor structural condition of the necrotic amniotic epithelial cells would account for their weak attachment both to the underlying membrane (plates 3.15-16) and to the superior limbal epithelial cells (plate 3.3). Though junctional complexes were very few in number in the limbal culture on cellular AM (plate 3.14), it is surprising that they were able to form at all and the exact mechanism through which desmosomes are able to form between viable and non-viable cells remains a mystery.

Previous studies by project collaborators in the Kyoto group using rabbits (Koizumi *et al.*, 2000a) alongside clinical studies of cultivated limbal epithelial transplantation (Koizumi *et al.*, 2000b; Koizumi *et al.*, 2001a) have illustrated the need for good cellular attachment to the AM extracellular matrix. Without this, early survival of the transplanted graft cannot be expected. It has been reported that the presence of devitalized cryopreserved amniotic epithelial cells compromises the growth and attachment of limbal epithelial cells and in turn, detriments the integrity of the transplanted epithelium (Koizumi *et al.*, 2000a), an observation further supported by

the results of this thesis (plate 3.3). Animal experiments by Ti *et al.* (2002) describe long-term observations of limbal epithelial cells cultivated on cellular AM in rabbits and also corroborate the results of this thesis, suggesting that insufficient adhesion complexes are formed on the cellular substrate which result in susceptibility to such trauma as exposure, blinking and explant removal at the time of transplantation. Strong attachments both between adjacent epithelial cells and between basal cells and the AM matrix are crucial for the graft to survive the mechanical stresses inherent in transplantation.

It is generally accepted that outgrowth is slower on intact AM than on epithelially denuded AM (Koizumi *et al.*, 2000a; Grueterich and Tseng, 2002) therefore it has been speculated that amniotic epithelial cells directly hinder growth and impair attachment by forming a barrier between the membrane and the limbal cells. Cellular AM used on its own has been shown to be beneficial when spread on bare sclera in inhibiting conjunctival overgrowth (Koizumi *et al.*, 2001a), which would imply that cellular AM does not encourage cell growth. In the investigations of this thesis, TEM coupled with quantitative data analysis showed that denuded AM produced significantly smaller intercellular spacing and an increase in desmosomal junctions between adjacent limbal cells (chart 3.1). Both factors help to maintain the integrity of the epithelial layer and are important for visual acuity. Basal attachments were also significantly better in the denuded culture with more hemidesmosomal junctions at the basal cell-basement membrane interface than desmosomes joining the limbal cells to the AM epithelium in the intact culture (chart 3.1), suggesting that limbal cells on denuded AM would be better equipped to survive transplantation in the long-term.

Having established superior mechanical strength in the denuded culture, the issue of differentiation ought to be addressed. As alluded to in the background to this study, there are concerns that stripping the membrane of its epithelial cells may cause limbal stem cells to terminally differentiate in culture, hence reducing the life span of the cell sheet once transplanted onto a stem-cell deficient eye (Grueterich and Tseng, 2002; Grueterich *et al.*, 2002a). Though the results in this thesis have not shown the existence of stem cells or progenitor cells in the culture sheets, long-term clinical studies by the Kyoto group have shown that the recovered ocular surfaces of patients originally diagnosed with total limbal stem cell loss remain clear more than 5 years after allo-cultivated limbal epithelial transplantation (*unpublished clinical data*). In addition, amniotic epithelial cells appear to be in such poor condition that any abilities they may have once had in terms of influencing stemness will inevitably be short-lived; a theory endorsed in a recent paper comparing culture systems, in which just 7 days after transplantation, the dead amniotic epithelial cells of the cryopreserved cellular AM upon which limbal epithelium had been expanded were no longer discernible (Grueterich *et al.*, 2003b).

9.1.3 Conclusions

In conclusion, with morphology more closely resembling control cornea, smaller intercellular spaces and superior mechanical strength, denuded AM appears to be the more practical of the two substrates for human limbal epithelial cell culture, not only by the explant culture system but also in cell-suspension.

9.2 Cultivation of Human Corneal Endothelial Cells on Amniotic Membrane

9.2.1 Background

An intact corneal endothelium is essential for the maintenance of normal corneal hydration, thickness and transparency. Corneal transparency in particular is critically dependent on corneal endothelial cells, sufficient enough in number to actively pump fluid from the stroma and maintain a homeostatic environment. Endothelial cells lack regenerative capabilities, posing significant clinical problems since many corneal diseases, as well as ageing and trauma, are accompanied by substantial endothelial cell loss (Joyce, 2003). Typically, *in vivo* cell loss is compensated by cell enlargement and migration (Waring *et al.*, 1982; Schultz *et al.*, 1992) however in the disrupted cornea, irreversible corneal oedema results. Currently a corneal transplant (penetrating keratoplasty or PKP) is the only available mechanism for the replacement of the damaged or diseased endothelium.

In the western world, most keratoplasties are performed using organ cultured donor corneas, stored in culture medium for the mid to long-term. The European Cornea Banks prepare and culture an average of 25,000 corneas per year, of which only approximately 60% meet the quality criteria for keratoplasty (EEBA, 2003), the remainder being discarded due to their low endothelial cell density (Aboalchamat *et al.*, 1999). This represents a huge problem since there is already a major shortage in donor cornea supply. Several factors may give rise to low endothelial cell counts in donor organs prior to transplant including old age of the donor, high post mortem time, or mechanical damage during preparation for storage or surgical handling. Additionally, chronic endothelial cell loss has been observed post-keratoplasty,

presumably due to a low grade allograft rejection process (Waring *et al.*, 1982; Boisjoly *et al.*, 1993; Harper *et al.*, 1998). In an attempt to reduce the wastage of donor tissue and to improve the visual outcome for patients with corneal dystrophies (who account for approximately half of all recipients), a number of scientific strategies have been investigated. These include the reconstruction of poor quality donor organs by replacing endogenous cells with cultivated endothelium (Jumblatt *et al.*, 1978; Engelmann *et al.*, 1999), the development of new surgical techniques to allow grafting of just the endothelium rather than full-thickness cornea (which would facilitate the use of autologous cultivated tissue) (Melles *et al.*, 1998) and the examination of more readily- available alternative carriers for such an eventuality (McCulley *et al.*, 1980; Engelmann and Friedl, 1989; Mohay *et al.*, 1994).

An inevitable prerequisite for corneal endothelial cell transplantation is to supply enough vital, differentiated, functional cells. Difficulties in the isolation and cultivation of human corneal endothelial cells (HCEC) became obvious in the initial *in vitro* research over forty years ago (Stocker *et al.*, 1958). Initial efforts to establish cultures of HCEC reinforced the clinical observation that the cells did not mitose readily. Only cultures obtained from embryonic or young donors were sustainable in the long-term (Baum *et al.*, 1997; Pistov *et al.*, 1998) and those established from the corneas of older donors showed no growth at all or early signs of senescence (Nayak and Binder, 1984). Endothelial cells could however be incited to undergo mitosis *in vitro* in response to appropriate chemically-defined stimuli (Schultz *et al.*, 1992). Another problem was encountered in isolating the cells from Descemet's membrane, their natural substratum, to which they exhibit very strong adhesion (Engelmann *et al.*, 2004). The provision of suitable extracellular matrix components is a critical

factor in ensuring successful endothelial cell culture. Engelmann and Friedl (1989) determined suitable substrates were bovine Descemet's membrane, collagen type IV, laminin, fibronectin or chondroitin sulphate matrices. Though amniotic membrane fulfils the criterion of such a substrate, it has not previously been employed as a carrier for endothelial cells. For this reason, an investigation was launched to determine whether human AM, widely used as a surgical adjunct and as a carrier for corneal epithelial cells (Trelford and Trelford-Sauder, 1979; Koizumi *et al.*, 2001a; Koizumi *et al.*, 2001b; Solomon *et al.*, 2002), could also serve as a carrier for cultivated human corneal endothelial cells and whether the established and successful suspension culture technique could be used to grow these quiescent cells.

9.2.2 General Discussion of Results

9.2.2.1 Ultrastructural Examination

Given this suspension culture technique represents a novel approach for stimulating the growth of these quiescent cells, the SEM and TEM observations of chapter 4 were quite encouraging. Scanning electron microscopy revealed that the cultured cells formed a continuous layer of polygonal endothelial cells (plate 4.2) which were uniform in size and fairly closely resembled control endothelium (plate 4.1). Transmission electron microscopy confirmed good contact between adjacent cultivated cells and the characteristic overlap also seen in the *in vivo* endothelial monolayer (plates 4.9-10). While TEM observations indicated that attachment to the AM substrate was not quite as good as in the control (plate 4.11), the cultured cells exhibited satisfactory adhesion and appeared to produce basement membrane material (plate 4.12), indicating that amniotic membrane does indeed provide a

compatible basement membrane for endothelial cell cultivation. In view of the availability and well-established effectiveness of AM in various clinical and ophthalmic applications, these culture sheets could have great potential in restoring the vision of dystrophic eyes while also reducing the strain on donor cornea supply when used to culture cells from living donors.

9.2.2.2 Amniotic Membrane as a Carrier

For practical corneal endothelial cell transplantation *in vivo*, a carrier of some description is obviously necessary. Previous studies have used transparent gelatin membranes (McCulley *et al.*, 1980; Jumblatt *et al.*, 1980; Schwartz and McCulley, 1981) and coated hydrogel lenses (Mohay *et al.*, 1994) as synthetic carriers for these cells. Descemet's membrane (often of bovine origin) is the most frequently used non-synthetic carrier. Studies reporting the transplantation of normal (Lange *et al.*, 1993; Bohnke *et al.*, 1999; Engelmann *et al.*, 1999; Chen *et al.*, 2001) and immortalized SV40-transformed (Feldman *et al.*, 1993; Aboalchamat *et al.*, 1999) cultured HCEC on Descemet's membrane have described an ultrastructure closely resembling that of corneal endothelial cells *in vivo* and confirmed the presence of functional Na^+/K^+-ATPase-dependent pumps (Aboalchamat *et al.*, 1999). The study described in this thesis is the first to use human amniotic membrane as a culture substrate for HCEC. With the theoretical risk of transmission of zoonose infection associated with the use of animal tissue in a graft, human AM represents a much safer alternative. The TEM micrographs of chapter 4 showed that cultivated endothelial cells produced basement membrane material and were well adhered to the denuded AM (plate 4.12). Since human AM is more widely available than Descemet's membrane yet appears to be equally supportive of endothelial cell growth and adhesion under culture conditions,

the use of denuded AM in endothelial cell cultures could revolutionize treatment of corneal endothelial dystrophies.

9.2.2.3 Clinical Potential for Cultured Endothelial Cell Sheets

As alluded to in the background to this study, there is as yet no specific medical treatment for endothelial dysfunction and loss of normal vision due to low endothelial cell density can only be restored by PKP. Though successful to a degree, there remains considerable risk of allograft rejection and subsequent graft failure. Crucially, patients with corneal oedema secondary to endothelial dysfunction, diagnosed prior to the development of scarring or vascularization, need only have the corneal endothelium replaced to restore visual clarity. Were it possible, the replacement of just the endothelium (on a suitable carrier) would be less invasive and consequently cause fewer adverse outcomes. In 1998, Melles and co-workers described such a surgical technique (termed posterior lamellar keratoplasty) for the treatment of bullous keratopathy (Melles *et al.*, 1998); a method which, they claim, has several advantages over conventional PKP for treating corneal endothelial dystrophies. It requires a shorter operation time, causes fewer traumas and solves the problems associated with corneal surface circular incision and suturing (astigmatism, vascularization and wound rupture). With less risk of complications, fewer post-operative follow-ups are necessary, making better use of doctors' time while also enabling the more efficient use of donor tissue. There are potentially immunological advantages to transplanting just the endothelium since highly antigenic corneal epithelial cells and keratocytes are not involved. Endothelial cells are thought to have low antigenicity (Hori *et al.*, 2000) as does denuded AM (Ueta *et al.*, 2002). Additionally, the anterior chamber of the eye is an immune-privileged site,

permitting the long-term acceptance of histoincompatible tissue grafts that would be rejected at various other anatomic sites (Streilein and Niederkorn, 1981; Streilein *et al.*, 1996).

Posterior lamellar keratoplasty could quite easily be adapted for the cultivated HCEC sheets described in this thesis and would have the added benefit that surgery could be scheduled (the availability of fresh eye bank eyes no longer being a deciding factor). Unlike native corneal endothelium which must be used within days of harvesting, cultured corneal endothelium can be frozen and thawed for later use. Potentially, cultured cells from a single donor could provide enough cells for hundreds of recipients and there also is the possibility that cells from a small biopsy of a patient's healthy corneal tissue could be cloned and later transplanted back without risk of rejection. The use of autologous cultured tissue in such practice would overcome problems of donor shortfalls, reduce the risk of allograft rejection and combined with AM transplantation, could provide a practical alternative to PKP (Amano, 2002; Ishino *et al.*, 2004).

9.2.2.4 Transplantation in Animal Models

To overcome the shortage of human donor tissue, several studies have involved the use of cells of animal origin (Jumblatt *et al.*, 1978; Gospodarowicz *et al.*, 1979; McCulley *et al.*, 1980; Lange *et al.*, 1993; Koizumi *et al.*, 2005). The feasibility of using cultured cells as transplants has also been reported in *in vivo* animal models (Insler and Lopez, 1991; Mohay *et al.*, 1994; Mimura *et al.*, 2004) but this technique has yet to be applied clinically in humans. Insler and Lopez (1991) conducted experiments whereby cultured HCEC were seeded onto Descemet's membrane and

transplanted into African green monkeys. Their cells retained viability, morphologic characteristics and pumping function and 75% of the transplanted corneas were reportedly clear after 12 months. In a more short-term study, project collaborators at the Kyoto Prefectural University of Medicine used the cultured endothelial cell sheets examined in this to thesis to graft rabbit corneas stripped of native endothelium and compared the outcomes with those grafted with denuded AM and Descemet's membrane (Ishino *et al.*, 2004). As illustrated by the slit lamp images of figure 9.1 below (kindly provided by Dr. Ishino), corneas transplanted with cultivated HCEC remained transparent and maintained corneal thickness much better than the controls and the cells remained functional for at least one week. The recent observations of Mimura *et al.* (2004) in a very similar study using commercially-available collagen carriers, substantiated these findings. Their grafted rabbit eyes remained free from oedema in the 28 day observation period and showed only mild stromal opacity.

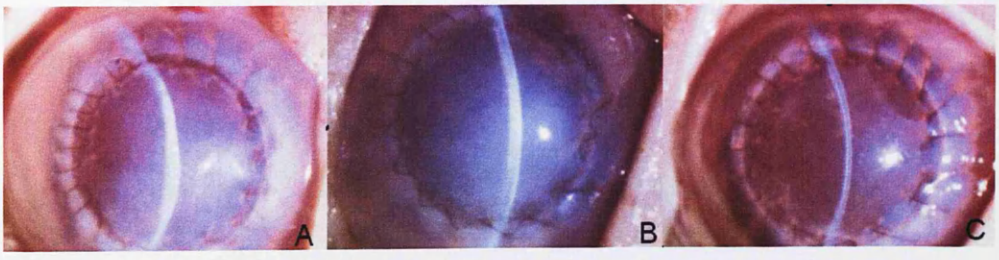

Figure 9.1: At day seven after transplant, the control rabbit eyes consisting of stripped Descemet's membrane (A) and denuded AM (B) were highly oedematous. The grafts of cultured HCEC on denuded AM (C) had little oedema and demonstrated excellent transparency (adapted from Ishino *et al.*, 2004).

9.2.3 Conclusions and Future Directions

This project introduced the concept of cultured HCEC sheets and demonstrated the feasibility of using denuded AM as a carrier. Results were very encouraging yet as a novel technique, it is likely that the system could benefit from some refinements. Future plans are to optimize plating density and culture conditions to ensure maximal endothelial coverage on the graft. In addition, the potential of SV40-transfected cell lines ought to be examined (Wilson *et al.*, 1993; Feldman *et al.*, 1993). Initial reports have shown that these immortalized cells give the best results in transplantation procedures yet there are concerns over controlling proliferation once optimal density has been reached, as well as the potential for tumour development and anxiety over the introduction of genetically-manipulated tissues into the human body. Additional project plans include replacing mature HCEC with pluripotent stem cells and manipulating the culture system to induce differentiation, with the ultimate aim of transplanting the resulting cells along with the denuded AM carrier for the treatment of corneal endothelial dystrophies.

9.3 Morphological Analysis of Polyphenol-Treated Rat Corneal Endothelium in Long-Term Storage

9.3.1 Background

The storage of corneas prior to transplantation is one of the single most important determining factors for success. The primary objective of corneal storage is the maintenance of endothelial viability from the time of corneal harvest to transplant, while a secondary objective is to ensure the efficient use of donor tissue, facilitated by optimizing the period of storage without loss of endothelial integrity. Over the years, various storage conditions have been utilized to achieve these goals (Lindstrom *et al.*, 1992). In 1974, McCarey and Kaufmann modified a basic culture medium (TC-199) with the addition of the osmotic agent, dextran to produce 'M-K medium' which permitted 4-7 days of corneal storage without significant deterioration (McCarey and Kaufman, 1974). The addition of chondroitin sulphate was subsequently found to further prolong the storage period, and lead to the development of assorted commercially-available media such as Corneal Storage Media (Lindstrom, 1984), K-Sol (Kaufman *et al.*, 1985), Dexsol (Lindstrom, 1990). At present, the most widely used storage medium is Optisol-GS (containing 2.5% chondroitin sulphate, 1% dextran, 100μg/ml gentamicin and 200μg/ml streptomycin sulphate) which allows the cold storage of corneas for up to two weeks (Lindstrom *et al.*, 1992; Smith *et al.*, 1995).

In an attempt to prolong the period of corneal storage and to maintain endothelial viability, new additions to the culture media have long been sought. Green tea has long been recognized for its health-promoting properties and as the freshest and least

processed form of tea, it is rich in polyphenol antioxidants, a broad term covering all manner of flavonoids and catechins (Lin *et al.*, 1996). Antioxidants scavenge and bind harmful oxygen-containing molecules; free radicals and peroxides, thereby preventing lipid peroxidation and damage to DNA and other cell components. Flavonoids are nutrient antioxidants, of which there are 12 types, found in most plants and common in the human diet. Catechins are flavonoid phytochemical compounds that appear predominantly in green tea leaves. Major variants in green tea include gallocatechin [GC], epigallocatchin [EPG], epicatechin [EC] and epigallocatechin gallate [EPGC]. As very potent antioxidants, up to 100 times more powerful than vitamins A, C and E at combating free radicals, these polyphenol catechins are being investigated for their chemoprotective effects. Studies have shown polyphenols to display anti-carcinogenic activity (Yang, 1997), anti-angiogenic (Cao and Cao, 1999), antimicrobial and antiviral properties (Nakayama *et al.*, 1993). More recently, Hyon and Kim (2001b) reported the polyphenol-assisted maintenance of pancreatic islet morphology in long term preservation, prompting this investigation into the effectiveness of EGCG (the most potent of the antioxidants in green tea) for long-term corneal storage.

9.3.2 General Discussion of Results

Several studies have shown that endothelial cell loss after keratoplasty is related to the length of corneal storage prior to surgery in that the longer the period, the greater the detriment to function (Geeraets *et al.*, 1977; Bourne, 1986; Kim *et al.*, 1994; Means *et al.*, 1995). One of the most critical functions of a corneal storage medium is to preserve the viability and function of the endothelium. Optisol-GS is currently the best available and most widely-used storage medium. It has been shown to preserve

endothelial barrier function for 14 days (Kim *et al.*, 1994) and to maintain viability in more than 80% of cells for up to 21 days, after which time the extent of deterioration increases significantly (Means *et al.*, 1995). The SEM results of this thesis (chapter 4.2) confirmed the observations of other investigators, demonstrating severe degradation of the endothelial cell membranes and exposure of nuclei at just 14 days storage in Optisol-GS (plate 4.15). At 28 days storage in Optisol alone, control corneal endothelium was found to have completely detached from Descemet's membrane in some areas (plate 4.17). Polyphenol-treated endothelial cells, by contrast, exhibited a superior morphology throughout the 28 day study and were found to more closely resemble that of fresh corneal tissue. While some shrinkage had occurred, cell membranes remained largely intact and adjacent cells were closely attached and had prominent cell borders (plate 4.18).

The mechanisms by which exposure to EGCG seems to preserve endothelial cells are not fully understood. As alluded to in the background to this study, polyphenols comprise one of the largest and most ubiquitous groups of plant metabolites, formed to protect against photosynthetic stress and reactive oxygen species (Yang *et al.*, 2001). They have been documented to protect against cancers (Yang *et al.*, 1998; Fujiki *et al.*, 1998; Suganuma *et al.*, 1999; Benelli *et al.*, 2002) and their antioxidant properties are well-recognized (Ho *et al.*, 1992; Lin *et al.*, 1996; Park *et al.*, 2003; Sang *et al.*, 2003). Reddy *et al.* (1989) used SEM to study the preservative effects of ascorbic acid, reduced glutathione, retinol acetate and α-tocopherol in storage media and concluded that these antioxidants assume an important role in the preservation of endothelial cell morphology. In a similar way, EGCG may implement its preservative effects by way of antioxidative activities. The successful long-term preservation of

pancreatic islet cells (Hyon and Kim, 2001b) and peripheral nerve cells (Ikeguchi *et al.*, 2003) by polyphenol solution were reported recently. Each of the studies reports a time lag between the removal of polyphenol solution and the resumption of cell function. In their article, Hyon and Kim (2001b) found preservation periods in excess of two months were possible with polyphenol treatment, whereas untreated cells deteriorated within two weeks. They noted an early decline in the insulin secretory capacity of treated islets though an enhancement was seen at day 40, which could suggest a possible polyphenol-induced period of physiological hibernation. A similar mechanism of reversibly blocking cellular enzyme activity could explain the improved morphology of the polyphenol-treated endothelial cells examined in this thesis. In additional, polyphenol has amphipathic properties and as such is able to easily pass through the ECM and cell membrane. While readily absorbed into proteins, the desorption process is very slow (Hyon and Kim, 2001a), possibly explaining why the initial 24 hour treatment has an effect which lasts for 28 days.

9.3.3 Conclusions and Future Directions

The results shown in chapter 4.2 demonstrated that exposure to EGCG has a beneficial effect on the maintenance of rat corneal endothelial morphology for up to 28 days storage in Optisol-GS. While encouraging, it represents only the initial phase of ongoing research and offers no real insight into whether treated cells also retain physiological functions. Polyphenol has proved useful in the long-term storage of rabbit corneal epithelial cells, not only in terms of preserving morphology, but also tight junctions and cell migration activity (Tsuzuki *et al.*, 2002). Future studies aim to include human corneal cells and to further characterize the effects of this treatment in long-term storage.

9.4 Evaluation of Sterilized, Freeze-Dried Amniotic Membrane

9.4.1 Background

Since 1910 when Davis applied foetal membranes to dress burned skin (Davis, 1910), amniotic membrane has been used as surgical material in a multitude of applications (Stern, 1913; Troensagaard-Hansen, 1950; Trelford-Sauder *et al.*, 1978; Trelford and Trelford-Sauder, 1979; Dhall, 1984) including reconstruction of the ocular surface (Tsubota *et al.*, 1996; Shimazaki *et al.*, 1997; Tseng *et al.*, 1998). A number of extensively documented characteristics make AM ideally suited for this purpose (Tseng, 2001). Easily obtainable and in abundant supply, AM has anti-microbial, anti-fibroblastic, anti-inflammatory, anti-angiogenic properties and also very limited immunogenicity. Ten years of thorough research has lead to the general acceptance of AM in ophthalmic surgery, however there remain some concerns.

Fresh AM, though its usefulness in ocular surface reconstruction has been documented (Mejia *et al.*, 2000; Adds *et al.*, 2001; Ucakhan *et al.*, 2002), is not always available on demand and its use is not generally advised due to concern over pathogenic organisms and the potential for disease transmission (Khokhar *et al.*, 2001). Human AM is obtained at the time of elective caesarean section and generally cryopreserved under sterile conditions at -80°C for a period of at least six months prior to use. Despite the best aseptic technique; even this procedure cannot guarantee complete sterility due to the biological origins of the tissue. Various methods of preservation have been tried over the years including cryopreservation in liquid nitrogen and preservation in silver nitrate, antibiotic solutions and glycerol (Maral *et al.*, 1999; Kruse *et al.*, 2000; Xu *et al.*, 2001; Ravishanker *et al.*, 2003) however,

each has its drawbacks, either due to expense, deterioration over the long term, or problems of sterility (Marangon *et al.*, 2004). Ideally, for clinical use, AM should be sterile, easy to obtain, transport and store for long periods without deterioration. The aim of this project therefore was to analyse and compare 'freeze-dried' amniotic membrane (FD-AM) preserved in the dry state under vacuum and sterilized using γ-irradiation at room temperature with the cryopreserved variant.

9.4.2 General Discussion of Results

9.4.2.1 Membrane Preparation Methods

Human AM can be procured from commercial sources and is usually cryopreserved (John, 2003). As with any biomaterial, the complete sterility of amniotic membrane is vital however, the expense incurred in preservation tends to restrict accessibility to more developed countries. Typically, donor placenta is obtained after informed consent during an elective caesarean section. Serological assays, performed on the donor at the time of procurement and again 6 months later, include HIV, hepatitis B, C and syphilis. The amnion is separated from the chorion, irrigated and then flattened onto nitrocellulose paper, epithelial side up prior to storage at -80°C in medium containing cryoprotectants and antibiotics. This preservation and later thawing devitalizes the amniotic epithelial cells (Kruse *et al.*, 2000; Zhong *et al.*, 2001), making the membrane non-viable yet still biologically active, an important consideration given that viable amniotic epithelial cells have been associated with low-grade inflammatory responses (Akle *et al.*, 1981). The idea of drying amniotic membrane is not a new one (Rao and Chandrasekharam, 1981; Waikakul *et al.*, 1990; Onerci, 1991; John and John, 2002a; John and John, 2002b; Rodriguez-Ares *et al.*,

2004). In 1981, Rao and Chandrasekharam produced an air-dried form of bovine AM for dressing burned skin. Their simple method of preparation involved laying the cleaned membranes on a plastic sheet and allowing them to air-dry before sealing in polythene and sterilizing by ultraviolet or gamma rays (Rao and Chandrasekharam, 1981). While they report some success in healing skin burns using these membranes, they also encountered severe infection and were replacing the grafts every few days, finding UV radiation to be an inadequate method of sterilization in that it failed to eradicate aerobic spore-bearing organisms in 70% of cases. In a similar study, Waikakul *et al.* (1990) applied a form of freeze-dried AM to flesh wounds of 65 patients, most of whom reported a reduction in pain. The FD-AM used in this thesis (chapter 5.1) is dried at room temperature under vacuum, irradiated and vacuum-packed for storage, requiring only rehydration prior to use in the operating room. Membranes were found to retain flexibility, strength and smoothness better when dried under vacuum than in ambient conditions (Nakamura *et al.*, 2004b). The physical properties of the FD-AM used in this thesis are illustrated in figure 9.1.

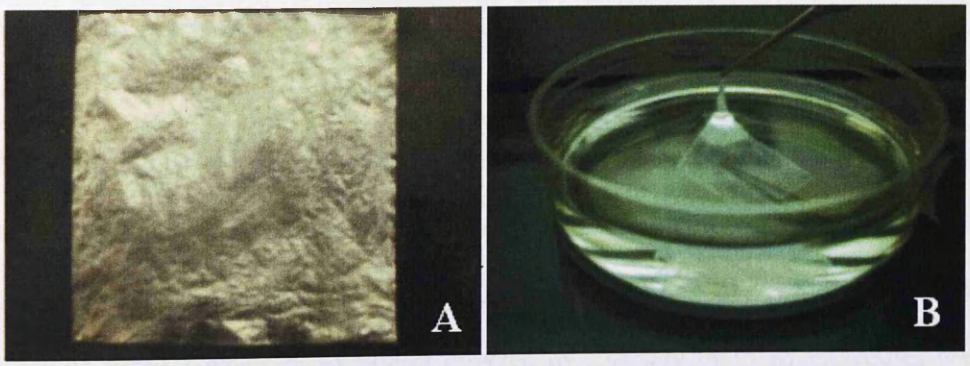

Figure 9.2: The sterilized, freeze-dried amniotic membrane was wafer-like, very light and thin (A). It became smooth and flexible on hydration, similar to cryopreserved AM (B) (reproduced from Nakamura *et al.*, 2004b).

9.4.2.2 Ultrastructural Examination and Immunohistochemistry

Scanning and transmission electron microscopy were used to compare the morphology of freeze-dried and frozen membranes. Examination of cellular membranes by SEM revealed that the amniotic epithelia appeared to have been fairly well-preserved, though the vacuum packing of the freeze-dried substrate seemed to have caused some flattening of the cells (plates 5.1-2). The denuded substrates were practically indistinguishable by SEM, with both retaining intact basal laminae (plates 5.3-4). Examination by TEM clearly showed that the FD-AMs (both cellular and denuded) were more compact than their frozen counterparts, possibly a product of the vacuum packing. As a result, it was difficult to discern any internal features in the freeze-dried amniotic epithelial cells (plate 5.5). Though not evident by SEM, frozen amniotic epithelial cells clearly appeared necrotic (plate 5.7), having multiple cytoplasmic vacuoles. This is likely to be a result of ice crystal damage, caused by the cryopreservation procedure and is corroborated by the findings of Zhong *et al.* (2001) who observed that after 90 days of frozen storage, AM epithelial chromatin dissolved, organelles deteriorated and mitochondria became vacuolated. The collagen fibres of the stroma appeared to have been undisrupted by the freeze-drying process (plate 5.10) and there were clearly visible and intact basal laminae in all membranes.

The extracellular matrix, including the basement membrane, forms the architectural supportive framework of tissues. Since the organization of ECM macromolecules is so instrumental to the physical and biological properties of AM, immunogold-labelling using a panel of antibodies directed to a variety of ECM molecules was used to verify that the freeze-drying process causes no major alterations to the normal state. Indeed, the composition of the extracellular matrix in FD-AM was

found to be practically identical to that of conventional frozen AM (as summarized in table 5.1). Labelling for both fibronectin and vitronectin was concentrated in moderately high levels in the lamina densa (plates 5.13 and 5.15), with low levels being observed in the basal lamina and stroma. This is corroborated by the findings of Fukuda *et al.* (1999) who found that the basal lamina and stromal regions of AM reacted strongly to antibodies raised against fibronectin. Fibronectin in the AM is likely to have originated from the overlying amniotic epithelium as previous studies have shown that in culture on plastic, these cells deposit ECM components including fibronectin (Aplin *et al.*, 1985). The basal laminae of cellular membranes were also found to contain large amounts of laminin; in particular, the lamina densa region directly beneath amniotic cells (plate 5.14). Fibronectin, laminin and vitronectin glycoproteins are involved in cell adhesion (Steele *et al.*, 1997), migration during wound healing (Fujikawa *et al.*, 1984) and epithelial growth and differentiation (Yamada and Kleinman, 1992) and are most likely to be involved in limbal epithelial attachment to AM in cell culture.

Collagen type IV was found to be located continuously along the basal lamina in high levels (plate 5.16). The majority of the basal lamina is comprised of type IV collagen, a scaffolding protein that has been visualised in the AM by previous investigators (Yurchenco and Schittny, 1990; Fukuda *et al.*, 1999). There is however, thought to be a subtle difference between the ECM composition of corneal and AM basal laminae, as indicated by the presence of a subchain of type IV collagen which is only present in that of the cornea (Fukuda *et al.*, 1999). The AM stroma labelled moderately for type I collagen (plate 5.17), known to form the bulk of the tissue (Fukuda *et al.*, 1999) and deposited by amniotic epithelial cells in culture (Aplin *et*

al., 1985). Stromal regions were also found to contain KS and CS, both of which were mainly associated with collagen type I fibrils and detected in low to moderate quantities (plates 5.19-20). Immunolabelling for HS revealed it to be concentrated in high levels in the lamina densa (plate 5.18); an observation confirmed by previous studies on AM and the cornea (King, 1985; Bairaktaris *et al.*, 1998).

While it has been suggested that denuding AM may damage the basement membrane and cause a reduction in ECM proteins, the results of this thesis confirmed that the removal of amniotic epithelial cells caused no detriment to the basal lamina and ECM distribution. This is also supported by the work of Cooper *et al.* on frozen AM (Cooper *et al.*, 2005). The results described herein strongly indicate that the processes of drying, irradiation and denuding do not impair the physical or biological properties of AM any more than the currently favoured cryopreservation process.

9.4.2.3 FD-AM as a Culture Substrate

Cryopreserved AM has been widely used as a substrate for cultivating corneal and conjunctival epithelium (Schwab *et al.*, 2000; Tsai *et al.*, 2000; Koizumi *et al.*, 2000b). Having successfully characterized freeze-dried AM and found it to physically and biologically resemble its frozen counterpart, the effectiveness of FD-AM as a substrate for the cultivation of rabbit corneal epithelial cells was examined. Drawing on their experiences of cell culture on cryopreserved AM, project collaborators at the Kyoto Prefectural University of Medicine were able to adapt the suspension culture system for this novel substrate, to great effect. After 3 weeks, the cultivated corneal epithelial cells showed 5-6 layers of stratification and were well-differentiated (plate 5.26), closely resembling corneal epithelium *in vivo* with results

comparable to those seen using cryopreserved substrates (Koizumi *et al.*, 2000b; Koizumi *et al.*, 2001a; Nakamura *et al.*, 2003a). Strong basal cell attachment to the underlying membrane is of fundamental importance in ensuring a successful outcome to transplantation so it was reassuring that the cultivated cells were firmly attached to the FD-AM and evidently producing basement membrane material (plate 5.30). When compared to *in vivo* rabbit corneal epithelium, the only significant differences were smaller superficial cell surface areas, fewer cell layers and fewer hemidesmosomes in cultured cells. Together, the encouraging results of the quantitative and morphological analyses prompted colleagues to transplant these cultured cell sheets, along with the novel carrier onto damaged rabbit corneal surfaces. Two days later, the grafted corneal surfaces were in good condition while surrounded by conjunctival epithelial defects (Nakamura *et al.*, 2004b), indicating survival of the transplanted cells on FD-AM and no contamination by host conjunctival epithelium. Conjunctival inflammation rapidly subsided and just 10 days after transplantation, the area covered by the cultivated corneal cells had expanded and began to make contact with the healing conjunctival epithelium.

9.4.3 Conclusions

Sterilized, FD-AM seems to retain all the characteristics of cryopreserved AM in terms of physical properties, levels and distributions of extracellular matrix proteins, biocompatibility and an ability to act as a substrate for the culture of corneal epithelial cells. As such FD-AM could successfully rival cryopreserved AM as the clinical material of choice and has the potential to extend the availability of this valuable biomaterial into developing countries where it could be easily stored and would represent a safer alternative to fresh membranes (Khokhar *et al.*, 2001).

9.5 Cultivation of Human Oral Mucosal Epithelial Cells on Denuded Amniotic Membrane

9.5.1 Background

Treatment of severe, bilateral ocular surface disease is one of the most challenging problems faced by ophthalmologists. The consequences of these conditions are devastating and conventional management is usually only palliative in nature. Surgical reconstruction of the ocular surface has been greatly advanced by the introduction of amniotic membrane and limbal stem cell transplantation (Tseng *et al.*, 1998). While generally successful, typical surgery involves the grafting of allogeneic cells from donors. Even with the necessary prolonged immunosuppressive therapy to combat risk of rejection, with severely inflamed eyes and in the most acute cases, the outcome is often poor. Transplantation of autologous limbal cells is a preferable option, yet is only feasible in situations where only one eye is affected and a limbal biopsy can be taken from the contralateral eye (Pellegrini *et al.*, 1997; Dua and Azuara-Blanco, 2000a). Diseases such as SJS and OCP are characterized by the complete and bilateral loss of limbal stem cells, making autografting impossible (Tsubota and Shimazaki, 1999) so with these in mind, an alternative source of autologous tissue was sought.

Epithelial cells of the oral mucosa were considered potential substitutes since they are generally considered less differentiated than skin keratocytes, have a short cell turnover time and are able to be maintained long-term under culture without keratinization (Hata *et al.*, 1995; Ueda *et al.*, 1995; Thompson *et al.*, 2001; Nishida *et al.*, 2004b). Mucosal grafts have been long used in ophthalmic applications in the

reconstruction of the ocular surface, eyelids and fornices (Denig, 1912; Ballen, 1963; Gipson *et al.*, 1986; Shore *et al.*, 1992; Kuckelkorn *et al.*, 1996). In 1912, Denig suggested the use of buccal mucosal grafts as a substitute for excised tissue when treating alkali burns of the eye (Denig, 1912). Ballen later described a study designed to determine whether non-keratinized, stratified squamous epithelium of non-ocular surface origin (termed 'mucous membrane') could be used as an autograft in the reconstruction of ocular surface defects caused by chemical injuries (Ballen, 1963). The lip was used as a tissue source and both epithelium and the underlying lamina propria were transplanted onto damaged rabbit and human eyes. Grafts remained adherent so long as necrotic tissue was completely removed and one edge remained in contact with a blood supply. While they were reported to be heavily vascularized, four of the six human eyes treated retained their grafts (Ballen, 1963). Though these grafts were useful in treating corneal ulcers, perforations and lid abnormalities, they were not suitable for improving vision since they retained opaque subepithelial fibrous tissue. Gipson and co-workers incorporated dispase II into the methodology, releasing the epithelial sheet from the underlying connective tissue and reported the successful treatment of peripheral wounds *in vivo* and failure when grafted into central, non-vascularized areas (Gipson *et al.*, 1986). On the basis of these investigations (and given that the oral mucosa is both accessible and ideally situated for biopsy in that the resulting scar is inconspicuous) project collaborators in Japan adapted their cell-suspension culture technique for rabbit oral mucosal cells and successfully managed to recreate corneal morphology in 2003 (Nakamura *et al.*, 2003a). This study of the human oral mucosa, the results of which are described in chapter 6, aimed to build on these initial results and to determine whether gingival and/or buccal cells cultivated on AM could be suitable autograft tissue for transplant.

9.5.2 General Discussion of Results

9.5.2.1 Ultrastructural Examination of Mucosal Biopsies

In this study, gingival and buccal stem cells were examined by light, scanning and electron microscopy, both as biopsies and post-culture on AM. The mucosal biopsies were found to have in excess of 40 layers of well stratified and differentiated epithelial cells (plates 6.1-2). By contrast, the corneal epithelium had only 4-5 cell layers (plate 5.3). Examination by SEM revealed that the superficial mucosal cells (both gingival and buccal) were characterised by apical undulating surfaces, covered in long parallel ridge-like folds (plates 6.6 and 6.10) quite different from the finger-like microvilli projections that cover corneal epithelium (plate 6.14); an observation which has been confirmed by other researchers (Chomette *et al.*, 1986; Kullaa-Mikkonen, 1987; Moreu *et al.*, 1993). TEM analysis resolved the finer morphological details. As in the corneal epithelium, oral mucosal cells were joined by numerous desmosomal junctions (plates 6.18 and 24) and were well attached to the underlying connective tissue (plates 6.20 and 26), both factors indicative of mechanical strength and useful in a potential ocular surface graft. There was little to differentiate between the buccal and gingival epithelial cells.

9.5.2.2 Ultrastructural Examination of Mucosal Epithelium Cultivated on AM

The culture conditions brought about some significant changes to cell morphology, resulting in an epithelial sheet which more closely resembled that of the healthy cornea than the oral mucosa. This has since been corroborated by the findings of Nishida and co-workers who used a temperature-responsive culture surface to create carrier-free confluent sheets of human (Nishida *et al.*, 2004b) and rabbit oral mucosal

epithelium (Hayashida *et al.*, 2005). The number of epithelial cell layers, once cultured, was reduced to 6-10 [buccal] and 10-14 [gingival] from an original 40; more in accordance with *in vivo* corneal epithelium, which typically has 6-8 cell layers. Since the mucosal cells were disaggregated enzymatically for use in suspension culture, this reduction in number of cell layers (and hence sheet thickness) is likely to be related directly to the quantities of cells seeded. In terms of transplant potential of the cultivated sheet, the most important considerations are good attachment of the basal cells to the amniotic membrane carrier and a functional tear-ocular surface interface. Corneal epithelial cells form desmosomes to ensure firm adhesion to neighbouring cells and hemidesmosomes for bonding to the ECM (Green and Jones, 1996). Both these types of distinctive cell junction were evident in the cultivated oral mucosa (plate 6.47), indicating a similar specialization to *in vivo* corneal cells. The anterior surface of superficial corneal epithelial cells is covered in finger-like projections known as microvilli or microplicae (Pfister, 1973), whose role is to stabilize the tear film and increase cell surface area thereby aiding absorption of nutrients and removal of cellular waste (Collin and Collin, 2000). Examination by SEM showed the superficial cells of the cultivated oral epithelium to have corneal-like microvilli (plates 6.33.and 36), rather than the parallel ridge-like folds observed on the surface of the source biopsies, adding further credence to the supposition that oral epithelial cells on AM have the ability to differentiate into corneal-like epithelia under culture conditions. The results of the quantitative comparison (chart 6.1) indicated that gingival cells seemed to be the better choice of mucosa since they more closely resembled *in vivo* corneal epithelium. Gingival cells (plate 6.37) had smaller intercellular spaces and formed more junctions, both basal and mechanical, than the buccal cells (plate 6.42) in culture.

9.5.2.3 Subsequent Clinical Applications

In 2003, Nakamura and co-workers successfully achieved ocular surface reconstruction using cultivated oral mucosal cells in a rabbit model (Nakamura *et al.*, 2003a). It was noted that 10 days after grafting, most of the keratectomized corneal surfaces were free from epithelial defects and conjunctival invasion, indicating early survival of the transplanted oral epithelium. Based on their animal studies and the morphological results described in this thesis, project collaborators applied this same method to 15 eyes of 12 human patients with severe ocular surface disorders. Preoperative diagnoses included chemical injury (4 patients), thermal injury (1 patient), SJS (5 patients), pseudo-OCP (1 patient) and an unknown idiopathic ocular surface disorder (1 patient). Despite two cases where signs of rejection prompted graft replacement by donor corneal tissue (discussed in the following section), there has been some astounding success in the long-term and the clinical success rate is currently 87% for follow-up periods ranging from 3 to 34 months (Nakamura, *unpublished data*). The following clinical photographs (figures 9.3 and 9.4) supplied for inclusion in this thesis by Dr. Nakamura of the Kyoto Prefectural University of Medicine, represent successful cases two years after ocular surface reconstruction using cultivated autologous oral mucosal epithelium.

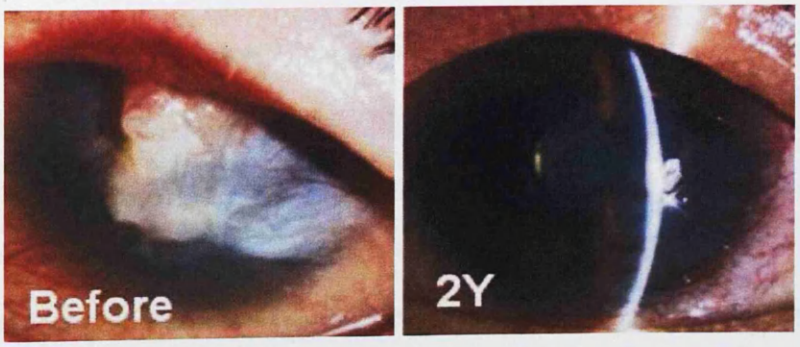

Figure 9.3: The ocular surface of a 14-year-old SJS patient before and after a cultivated autologous oral epithelial transplant. Prior to surgery, vision was restricted to hand motions. Two years later (2Y) her visual acuity had improved to 20/200.

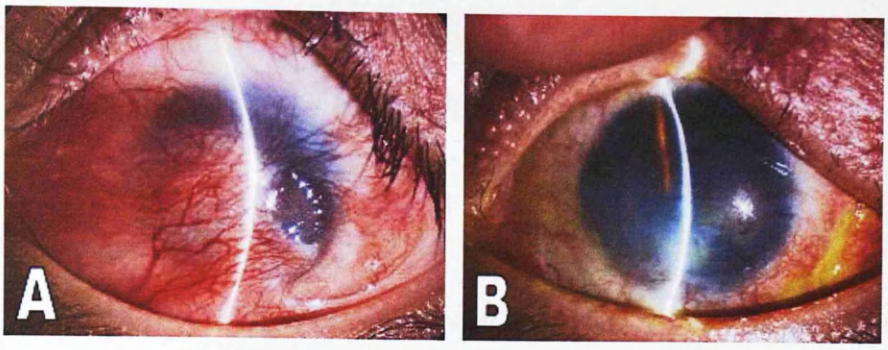

Figure 9.4: Ocular surface reconstruction of a 27-year-old chemical burn patient using cultivated oral mucosal epithelium. Prior to surgery the corneal surface was vascularised, heavily scarred and invaded by conjunctiva [A]. The patient was only able to detect hand movements. Two years after reconstructive surgery using autologous oral epithelium, the cornea was clearer and the patient reported 2/200 vision [B].

9.5.3 Conclusions and Future Directions

The use of tissue engineered corneal epithelial replacements holds great promise for the surgical reconstruction of stem cell-deficient eyes (Kinoshita *et al.*, 2004; Kinoshita and Nakamura, 2004). While cultivated autologous corneal epithelium is the safest and most reliable method, this is not an option for the treatment of bilateral ocular surface disorders. In these instances, a choice must be made between allogeneic corneal epithelium with which there is significant risk of rejection (Ilari and Daya, 2002) and autologous cultivated oral mucosal epithelium which is of different origin and may have as yet undetermined consequences in the long-term. Section 9.7 discusses the results of experiments in which oral and corneal stem cells were co-cultured, the aim being to maintain a population of corneal epithelium and encourage further differentiation of the oral mucosa. As a relatively new technique, there are still questions to be addressed about the longevity and mobility of the oral epithelial cells on the host eye and whether the grafts contain enough stem cells. It is also very important to determine whether the human oral mucosal cell type has the appropriate characteristics to act as a substitute for human corneal epithelium.

9.6 Clinical Outcomes of Amniotic Membrane/Oral Mucosal Stem Cell Transplants

9.6.1 Background

The transplantation of cultivated oral mucosal epithelial cells on AM onto severely damaged ocular surfaces is a novel technique and therefore not fully refined. As indicated in the previous section, the long-term clinical outcomes of this procedure were generally very good (13 of 15 grafted eyes remaining clear up to 34 months later) yet, possibly due to the existing severity of the inflammation, were not without complications. This section describes two cases where the grafted sheet was removed after several months having stabilized the cornea and replaced with donor corneal tissue using conventional penetrating keratoplasty. These tissues were examined using electron microscopy to determine the exact fate of the cells once grafted and to provide some insight into the mechanism of rejection.

9.6.2 General Discussion of Observations

Frequently with bilateral ocular surface disorders, the patient presents with chronically inflamed, vascularized, conjunctivalized and scarred corneas. The prognosis of corneal transplantation in the acute phase is poor due to the difficulties in overcoming the severe inflammation and allograft rejection (Koizumi *et al.*, 2001b; Shimmura, 2004). The application of autologous tissue to the ocular surface removes any risks of rejection, yet it is still a challenge to control the persistent inflammation, dry eye and trichiasis which induce epithelial defects. It is not surprising therefore, that in some cases the initial graft is insufficient and has to be replaced at a later date.

9.6.2.1 Case 1: Chemical Injury

Chemical and thermal burns ultimately lead to corneal scarring, opacification and vision loss (Dua *et al.*, 2000). Acids react with the superficial tissues to form proteinates which limit penetration into deeper corneal tissues (Pfister and Pfister, 1997a). Alkali burns, as in this case, are generally more debilitating and can cause recurrent epithelial breakdown, stromal cell death, inflammatory cell infiltration and endothelial dysfunction through saponification of lipoprotein cell membranes (Pfister and Pfister, 1997b). Alkali burns remain difficult to treat and frequently lead to sight-threatening complications (Nishida, 1997).

Examination of the removed mucosal graft using electron microscopy confirmed a typical rejection reaction; previously well-characterised by Cooper *et al.* (2004). Only a small central area of corneal-like epithelium (plate 6.48) remained and TEM showed that these cells had become necrotic (plate 6.53). The majority of the ocular surface was covered in conjunctival cells (plate 6.51); smaller in size and characterized by more prominent cell borders and the presence of mucin-secreting goblet cells (Pfister, 1975; Galbavy and Foster, 1985). Graft rejection appears to have resulted in the loss or destruction of almost all the original cultivated mucosal cells, allowing conjunctival invasion over the exposed AM initiating vascularization in the stroma (plate 6.57). Additionally, it is possible that the graft may not have contained adequate numbers of stem cells at the outset, resulting in colonization of conjunctiva once the stem cells depleted. Numerous inflammatory cells including lymphocytes, plasma cells, macrophages and granulocytes were associated with the epithelium and amniotic membrane (plate 6.58) and numerous red blood cells provided evidence of vascularization. It is impossible to determine whether the vascularization seen here

arose from the already existing host blood vessels or whether they appeared as a result of angiogenic factors secreted from the cultured epithelial cells (given the mucosal substantia propria is rich in vessels). Anti-angiogenic factors such as thrombospondin produced by stromal keratocytes, would no doubt have been acting to limit the extent of neovascularization in any case (Hiscott *et al.*, 1996).

The clinical photographs below (figure 9.5) were taken by project collaborators in Japan and kindly donated by Dr. Takahiro Nakamura. They authenticate the findings of the ultrastructural evaluation in this thesis, illustrating that the corneal surface displayed signs of a typical rejection with severe vascularization and conjunctival invasion. While the outcome was not ideal, in that the graft was eventually replaced with tissue of allogeneic origin, it is apparent from these images that the mucosal graft had a stabilizing effect on the cornea and prepared the ocular surface for the subsequent graft in suppressing inflammation and reducing vascularization to a certain extent. This was possibly aided by the fact that there was no underlying immunologically-mediated disease in this case.

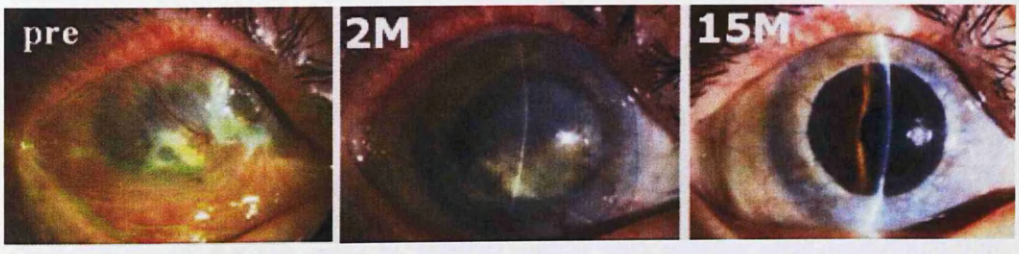

Figure 9.5: Clinical course photographs of *case 1*: a chemically burned eye before and after transplant surgery. The patient presented with a chronically inflamed, vascularized eye (pre). Cultivated autologous oral mucosal cells were grafted to stabilize the ocular surface (photo taken 2 months, 2M, after surgery). At 5 months, the oral mucosal graft was removed and replaced with full thickness cornea from a donor. Fifteen months (15M) after initial surgery the ocular surface was much clearer and free from epithelial defects. (Photographs courtesy of Dr. Nakamura)

9.6.2.2 Case 2: Stevens-Johnson Syndrome

Stevens-Johnson syndrome (SJS) is characterized by painful, blistery lesions on the mucous membranes and as such, the ocular complications are often disabling and lead to severe vision loss. This sight-threatening disease can occur at any age and is considered very difficult to treat (Tsubota and Shimazaki, 1999) and permanent bilateral blindness is common due to severe keratoconjunctivitis and a lack of corneal stem cells. With corneal stem cells limited to the relatively small area of the limbus, severe immunologic reactions can deplete the supply and leave the cornea covered with conjunctival cells or eyelid skin – as in the instance of this case study.

Examination of the cultivated cells 6 months after grafting onto the ocular surface by SEM revealed a small central region of fairly healthy looking epithelium not unlike that of the normal cornea (plate 6.59), surrounded by conjunctival cells (plate 6.60). The sample received for analysis was unfortunately too small to permit further analysis by transmission electron microscopy, so only surface features were noted. While the tissue appeared to be quite heavily conjunctivalized, there were neither inflammatory cells nor evidence of vascularization in the regions examined. Since conjunctival tissue is vascular in nature, this is not to say blood vessels were not present, just that they were not visible by SEM. Again, clinical course photographs supplied by Dr Takahiro Nakamura validate these observations (figure 9.6 overleaf). Comparison of the eye prior to surgery and at two months after transplantation indicates a certain degree of stabilization of the ocular surface by the cultivated cells. Following the second graft (conventional PKP) there was further significant improvement and the patient reports a drastically enhanced quality of life.

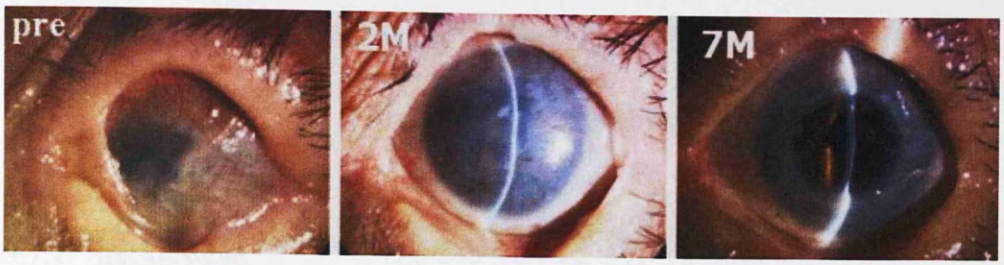

Figure 9.6: Clinical course photographs of *case 2*: the eye of an SJS patient, before and after surgery. The patient presented with a chronically inflamed, conjunctivalized eye (pre). Cultivated autologous oral mucosal cells were grafted to stabilize the ocular surface (photo taken 2 months, 2M, post-surgery). At 6 months, the oral mucosal graft was removed and replaced with full thickness cornea from a donor. Just a month after PKP the ocular surface was remarkably improved (7M). (Photographs reproduced with the kind permission of Dr. Nakamura).

It is well documented that the success rate of stem cell transplantation in patients with immunologically-mediated diseases (such as SJS and OCP) is much lower than in those with non-inflammatory ocular surface diseases (Samson *et al.*, 2002; Shimmura, 2004). Ultimately, the likelihood of rejection is largely dictated by host factors; most especially underlying inflammation and vascularization. One major benefit of cultivated epithelial transplantation on AM is that the graft can be easily removed and replaced if necessary (Nakamura *et al.*, 2003b).

9.6.3 Conclusions

The most plausible explanation for the long-term failure of these autologous cultivated oral mucosal grafts is the presence of acute inflammation at the outset. It appears that the more severe the existing condition, the greater the chance a graft will fail and require repeat surgery. Despite the poor prognosis, the cultivated cells appear to have stabilized the ocular surface sufficiently for the second grafts to have succeeded. All patients who underwent autologous cultivated oral mucosal stem cell transplants continue to be carefully monitored by colleagues in Japan and their current prognoses are excellent.

9.7 Rabbit Oral Mucosal/Corneal Hybrid Stem Cell Cultures on Amniotic Membrane

9.7.1 Background

The previous sections have detailed the cultivation and transplantation of autologous cultivated oral mucosal epithelial cells on amniotic membrane and described a range of clinical outcomes. Reconstruction of the ocular surface with autologous oral cells offers substantial advantages over allogeneic transplantation for the treatment of disease in which there is an underlying immunological cause, most significantly in eliminating the need for immunosuppression (Kinoshita *et al.*, 2004). As yet there are no determining stem cell markers for the oral epithelium and so it is difficult to confirm the presence of these cells in grafted sheets (Dua *et al.*, 2003). It is however, quite revealing that the grafts of cultivated oral epithelial cells are capable of remaining transparent for more than two years following transplantation (Nakamura, *unpublished clinical results* depicted in figures 9.1 and 9.2). The life span of transient amplifying cells which are committed to epithelial differentiation is thought to be one year (Kinoshita *et al.*, 1981). This would suggest that there are in fact progenitor cells present in the mucosal autograft which have the potential to differentiate into the corneal epithelial phenotype.

Graft failures (such as those documented in the previous section) are characterized by conjunctival invasion of the cornea. Whether this occurs due to a graft rejection reaction and the assault of inflammatory cells or as a result of stem cell depletion is unknown. In this investigation, rabbit stem cells from two different sources (oral mucosa and limbus) were co-cultured to create a hybrid epithelial sheet for grafting

onto the damaged rabbit ocular surface. The aims of this experiment were two-fold; [1] to increase the quantity of stem cells present on the graft, drawing on the well-documented success of *ex vivo* expansion of autologous limbal cells on AM (Tseng *et al.*, 1998; Dua and Azuara-Blanco, 2000a; Koizumi *et al.*, 2001a; Du *et al.*, 2003; Grueterich *et al.*, 2003a; Nakamura *et al.*, 2003b), while also retaining a quantity of oral mucosal epithelial stem cells and [2] to improve the morphology of the cultivated oral mucosal epithelial cells and induce differentiation into more corneal-like epithelium. This study attempted to establish whether the cells were capable of being cultured simultaneously and to determine whether they were morphologically distinguishable. The hybrid epithelial sheets were analysed by SEM and TEM both prior to transplant and after 3 weeks placement on the damaged rabbit ocular surface.

9.7.2 General Discussion of Results

9.7.2.1 Evaluation of Hybrid Culture Sheets Pre-Transplant

Microscopic examination revealed that the oral and corneal stem cells formed a confluent epithelial sheet and that the cells were indistinguishable from each other after three weeks under cell-suspension culture conditions. Oral mucosal cells appeared to have differentiated into corneal-like cells, much more so than when cultured without corneal stem cells. Examination by SEM showed that the co-cultured epithelial cells appeared to be in fairly good condition and formed a confluent layer (plate 6.62). Some of the epithelial cells were flattened centrally and had distended microvilli at the cell boundaries (plate 6.63). It is possible that these cells represent a stage of differentiation, intermediate between that of oral mucosal and corneal morphology. Superficial cells had apical coverings of short, regular

microvilli (plate 6.65) and there was no evidence of the parallel ridges or folds on the surface of the superficial cells which characterize oral mucosal epithelia.

TEM examination of the rabbit hybrid epithelial cell culture on denuded AM revealed 4-8 layers of stratified epithelium (plate 6.69). Cells appeared to be in good condition and were differentiated into basal columnar shaped cells, suprabasal wing cells and flat squamous superficial cells, as in *in vivo* rabbit cornea. Corneal cell junctions, crucial for epithelial integrity and very important in a potential graft, were revealed by TEM, joining adjacent cells to each other (plate 6.73) and to the underlying amniotic membrane (plate 6.72). The results of the quantitative study were equally encouraging; the hybrid cell cultures were found to more closely resemble *in vivo* rabbit cornea than the previously described mucosal cell-only cultures, in almost all parameters. This would suggest that the incorporation of corneal stem cells in the cell-suspension mix more accurately recreates the limbal niche in culture and induces differentiation of the oral mucosal stem cells, making them more corneal-like.

9.7.2.2 Evaluation of Hybrid Culture Sheets Post-Transplant

Following a three week period on the cornea, the culture sheet was further improved; exemplifying morphology very similar to that of normal rabbit cornea (plate 6.66). Again, there was no sign of the parallel ridges and folds on the surface of the superficial cells which characterize oral mucosal epithelia, superficial cells were all covered in short corneal-like microvilli (plate 6.68). There were also no flattened cells with peripheral microvilli, as were seen in the original hybrid culture. Perhaps this would suggest that placement on the cornea provides all the stimuli required for

corneal differentiation. TEM confirmed the hybrid transplanted epithelium to be in very good condition and quite indistinguishable from *in vivo* corneal epithelium. The epithelium formed 6-8 well-differentiated and stratified cell layers and there was little or no intercellular spacing (plate 6.74). As with the original cultures, it was not possible to distinguish between the oral mucosal and corneal epithelial cell types on the basis of morphology. When compared to the original graft, the post-transplant epithelial sheet was found to be thicker, with an increase in the number of cell layers and a significant reduction in intercellular spaces (chart 6.2). It also demonstrated an increase in the numbers of cell junctions.

Together, these results are very encouraging though they only represent the initial stage of these investigations. Further studies would be required in order to fully comprehend the nature of the interactions between oral and corneal stem cells and the mechanisms by which mucosal stem cells from the mouth are able to differentiate into corneal-like epithelia under culture conditions. The results described in this chapter provide evidence that mucosal stem cells, once considered to be unipotent, may have the potential to be at least partially transformed into a new cell type, given the right conditions. Obviously three weeks represents only a short period of clinical study. More data would be required before drawing any conclusions as to whether there is in fact any increase in the proportion of stem cells grafted and the affects on long-term viability of these hybrid grafts. A very recent study by Daya *et al.* (2005) used DNA fingerprinting techniques to analyse the outcomes of *ex vivo* expanded stem cell allografts in 10 patients. Over a mean follow-up period of 28 months in which seven were considered successful, DNA fingerprinting revealed no evidence of donor stem cell DNA beyond 9 months. Though this brings into question the

origin of the host corneal epithelium, when viewed in conjunction with the findings described in chapter 6.3, would suggest great potential for co-cultured epithelial grafts in ocular surface reconstruction. The inclusion of allogeneic corneal tissue in culture may induce the differentiation of autologous oral mucosal epithelial into a corneal-like phenotype and with a possible expected life-span of less than a year for the donor cells; there may be no need for prolonged immunosuppression.

9.7.3 Conclusions and Future Directions

This study represents the first of its kind in creating hybrid epithelial sheets using stem cells from different sources. Though involving a more complicated procedure, hybrid cell has the potential to combine the benefits of amniotic membrane transplantation with the *ex vivo* expansion of limbal epithelial cells, and also to incorporate an alternative source of stem cells, should there be insufficient limbal tissue available as in the case of severe bilaterally-affected ocular surface disorders. The incorporation of an alternative autologous stem cell source also reduces the need for allogeneic tissue and would possibly limit the risk of graft rejection responses. Future plans are to use human cells in similar hybrid constructs.

9.8 Extracellular Matrix Protein-Coated Gelatins as Carriers for Human and Rabbit Limbal Stem Cell Cultivation

9.8.1 Background

Cellular supports, scaffolds or carriers that provide an environment conducive for cell migration, growth and differentiation are important components of tissue-engineered grafts since rapid integration with the host is essential for long term graft survival (Piskin, 1992; Hodde, 2002). Amniotic membrane has been well characterized as a naturally occurring scaffold and is widely accepted as a useful support for epithelial cell transplant surgery. It not only accurately mimics the native corneal epithelial basement membrane but has a number of therapeutic characteristics and can be processed in such as way as to retain these qualities (Dua and Azuara-Blanco, 1999b; Tseng, 2001; Nakamura *et al.*, 2004b). However, despite comprehensive donor screening there remains the theoretical risk of transmission of infection by some unknown viral or prion agent. Also, the equipment required for cryopreservation is both bulky and expensive, restricting availability of the tissue. Alternatives have thus been sought which carry less risk, offer greater ease of preparation and are universally available on demand.

Gelatin hydrogels are simple, versatile, biocompatible and biodegradable matrices of denatured cross-linked collagen. The hydrophilic nature of these gels makes them fine candidates for biomedical applications and the excellent transparency, light transmission properties and permeability to small molecular weight nutrients makes them particularly useful for ophthalmic use (Jumblatt *et al.*, 1980; Zeltinger *et al.*, 2001; Griffith *et al.*, 2002). Gelatins have assorted uses in the surgical domain and

have been incorporated into a variety of ophthalmic procedures over the years. Viscous gelatin solutions have been applied in to the fluid-filled chambers of the eye in vitrectomy procedures where, along with sodium chloride solution, they maintain intraocular pressure (Takki-Luukainen and Tuovinene, 1961; Krassimir and Stefan, 1996). In dressing form, cross-linked gelatin sponges have been applied to full thickness skin wounds in the rat where they demonstrated good healing properties (Choi *et al.*, 2001). Additionally, when strategically placed so as to separate raw conjunctival surfaces, gelatin sponges have proven to be simple and effective tools in preventing symblepharon formation after alkali burns of the eye (Yamada *et al.*, 1997). A recent study by Hori *et al.* reports the preparation of biodegradable gelatin hydrogels for use on the ocular surface which have the ability to physically and chemically interact with epidermal growth factor (EGF), enabling controlled release upon degradation and thus facilitating wound healing (Hori *et al.*, 2005). There have been several reports of the use of gelatin hydrogels as cell culture substrates (Rosan *et al.*, 1965; Jumblatt *et al.*, 1980; Zhang and Zhao, 1991; Altankov *et al.*, 1991; Tao *et al.*, 2003; Amano *et al.*, 2005). Altankov *et al.* (1991) used collagen type I coated gelatin microspheres to cultivate Vero (African green monkey kidney) cells. In comparison with pure gelatin beads, they found cells attached more rapidly and grew faster on collagen coated beads, approximately 90% of cells having adhered after just one hour. In a more recent study, gelatin was used as a culture substrate for multipotent precursor cells from human corneal stroma and subsequently, to reconstruct the rabbit corneal matrix (Amano *et al.*, 2005). To date, there have been no reports of the use of gelatin as a culture substrate for limbal stem cells so for the purposes of this thesis, a simple gelatin matrix was prepared as a substrate for cell-suspension limbal epithelial culture and evaluated by electron microscopy.

9.8.2 General Discussion of Results

9.8.2.1 Ultrastructural Examination of Gelatin Hydrogels

Native gelatin hydrogels were found to be very porous in nature and despite the rather rigorous processing for electron microscopic examination; they retained visible indentations at their surface (plate 7.1). Other research groups have illustrated the importance of surface pores in a tissue scaffold as they determine total surface area and the distribution of ligands presented to cells for adhesion (Zeltinger *et al.*, 2001). ECM protein coatings appeared to have been accurately applied and well adsorbed, appearing slightly uneven in distribution due to the natural variation in pore size of the gel. The thickness of the individual protein coatings (~50-200nm) was approximately equivalent to that of the corneal epithelial basal lamina (~80-100nm). Applied together, fibronectin and collagen IV formed an electron dense layer reaching peaks of 500nm in thickness (plate 7.8). The healthy corneal epithelial basement membrane is composed primarily of type IV collagen and laminin (Fukuda *et al.*, 1999), while fibronectin dominates during the wound-healing process (Fujikawa *et al.*, 1984). It has been shown that surface integrins of migrating epithelial cells bind to all three therefore surface modifications with these proteins or their derivatives have been widely used in an effort to promote epithelial adhesion to synthetic polymer surfaces. Hydrogels coated with collagens type I and IV have shown a more rapid rate of *in vitro* cell growth when compared with those surfaces coated with either fibronectin or laminin (Trinkaus-Randall *et al.*, 1988; Kobayashi and Ikada, 1991a; Kobayashi and Ikada, 1991b) while adsorption of fibronectin to a collagen-modified surface has been found to accelerate corneal epithelial attachment (Xie *et al.*, 1997). Encouraged by these reports and the findings of the ultrastructural

examination of chapter 7, project collaborators used gelatin hydrogel carriers coated with collagen type IV and fibronectin in the *ex vivo* expansion of rabbit and human corneal epithelial cells, the results of which are discussed in the following section.

9.8.2.2 Ex Vivo Expansion of Limbal Cells on Gelatin Hydrogels

A major problem with carrying out studies using human tissue is the shortage of available material. Frequently the corneal cells available for culture are in poor condition and so in this study, rabbit cells were also cultured to help more accurately gauge the usefulness of gelatin as a culture substrate. Examination by SEM highlighted a number of rounded cells on the surface of the rabbit culture (plate 7.12) whereas TEM inspection showed the cells to be in good condition with intact membranes and cellular organelles (plate 7.18). This would suggest the anomaly could perhaps be an artefact of SEM processing. Both human and rabbit cells appeared to be firmly attached to the gelatin substrate however hemidesmosomes were much more apparent in the rabbit epithelial culture (plate 7.19). Cultivated human and rabbit cells were compared to control corneal epithelium from the relevant species. Human limbal cells cultured on gelatin were found to have significantly larger intercellular spaces and fewer cell junctions (both desmosomal and basal) than control epithelium while cultured rabbit cells differed significantly from their control in every parameter. That said, in terms of morphology and actual values, the rabbit cells appeared to be better suited to the culture conditions and the gelatin substrate than the human corneal cells. It is likely that the human limbal cells were in poor condition prior to culture so before any firm conclusions can be drawn as to the usefulness of coated gelatin as a scaffold for human cell culture, these conditions ought to be recreated with more human tissue.

9.8.3 Conclusions and Future Directions

These experiments are still in the preliminary stages and further laboratory investigations are required to fully refine the culture conditions; not least of all to obtain a hydrogel of optimal consistency and the best possible ECM-coating levels. Clinical trials involving the transplantation of confluent corneal epithelial sheets onto the ocular surface would then enable more accurate assessment of its potential as a replacement carrier for AM. Gelatin hydrogels have the advantage over AM in that they are universally available, quick and cheap to prepare. While studies into such alternatives are crucial, amniotic membrane remains a valuable adjunct in ocular surface reconstruction and practical carrier for cultivated cells. The beneficial qualities it displays in the suppression of infection, inflammation and scarring, the promotion of epithelial cell migration, differentiation and adhesion, and the inhibition of apoptosis, fibrosis and angiogenesis are not to be overlooked (Dua and Azuara-Blanco, 1999b; Tseng, 2001). While there is a theoretical danger of prion or viral transmission, it is necessary to weigh this minimal yet potentially serious risk against these extraordinary benefits when considering a choice of carrier.

Nishida and co-workers have since investigated the possibility of completely excluding any carrier from the epithelial graft (Nishida *et al.*, 2004a; Nishida *et al.*, 2004b). To this end, they have developed a novel temperature-responsive harvesting technique which, following a 30 minute drop in temperature from 37 to 20°C, releases an intact transplantable epithelial sheet, capable of being directly applied to the ocular surface without the need for a scaffold or sutures (Yamato and Okano, 2004). Since postoperative complications arise from infection due to more invasive procedures, this has tremendous potential for revolutionizing ocular surgery.

9.9 Human Serum in Corneal Epithelial Cell Culture

9.9.1 Background

As in the previous chapter which was concerned with the replacement of biological tissue of non-host origin in the tissue engineering procedure, so this investigation looks at the development of a culture technique for human corneal epithelial cells in which foetal bovine/calf serum (FBS) in the media is exchanged for that of human origin, preferably the graft recipient his/herself. Bovine serum is a complex mixture of components (proteins, lipids, hormones, growth factors and inorganic trace elements) required by cells for growth and maintenance, some of which are as yet undetermined. Such is its complexity that it is difficult to isolate the effect of any specific cytokine. Despite its well-documented usefulness, there are some notable concerns - namely cost, variability in the composition of the serum between batches and concerns over the potential for disease transmission (Ayoubi *et al.*, 1996; Bednarz *et al.*, 2001; Rieck *et al.*, 2003).

To study proliferation and differentiation of progenitor epithelial cells *in vitro*, two basic culture systems have been established. In the first system, reported by Rheinwald and Green (1975) and abbreviated herein as the 3T3 system, cells are cultured on feeder layers of 3T3 mouse embryonic fibroblasts in a DMEM medium containing FBS. This system is utilized by project collaborators in Japan and has successfully grown many types of epithelial cells including rabbit (Koizumi *et al.*, 2000b) and human limbal (Koizumi *et al.*, 2002) and oral mucosal epithelium (Nakamura *et al.*, 2004a). The 3T3 system is unique in permitting serial propagations and promoting the survival of various types of cultivated epithelial cell by

maintaining a stem cell population. In the second system, reported by Boyce and Ham (1983), epithelial cells are cultured in a serum-free medium supplemented with growth-promoting agents. This system has also supported the growth of rabbit and porcine corneal epithelial cells (Hackworth *et al.*, 1990; Kruse and Tseng, 1991), but seems to primarily promote the clonal growth of transient amplifying cells (Kruse and Tseng, 1992). The addition of FBS was subsequently found to stimulate proliferation of limbal stem cells and suppress that of corneal TACs (Kruse and Tseng, 1993). When comparing the two systems, Tseng *et al.* (1996) reached a number of conclusions, as listed: 1) the 3T3 system preferentially promoted stem cell growth, 2) 3T3 fibroblasts stimulated colony size only in the presence of FBS, 3) growth-promoting activity was present in serum-containing fibroblast-conditioned media but not at the cell surface or in the extracellular matrix of the 3T3 fibroblasts, 4) 3T3 fibroblast-derived anti-apoptotic activity helped maintain the undifferentiated state of limbal colonies. Thus it appears that the inclusion of serum and fibroblast-derivatives in culture media are important as they provide a mandatory source of mitogens.

In addition to its inclusion in cell culture media, almost two thirds of all human corneas processed in Europe for corneal grafting purposes are stored for up to five weeks in organ culture media containing FBS, in concentrations ranging from 2-10% (EEBA, 2000). The endothelial cell density is the main criterion determining quality of a donor cornea and is known to decrease by approximately 10-20% during storage (Pels and Schuchard, 1983; Bourne, 1986). Efforts to reduce endothelial cell loss have been directed towards media supplementation with mitogenic agents such as FBS, that primarily protect the cells from endotoxin-mediated damage during the

preservation period (Schultz *et al.*, 1992; Ayoubi *et al.*, 1996; Sobottka Ventura *et al.*, 1999) however unpredictable variations in the quality and composition of the different commercially available sera may severely compromise endothelial vitality and hence graft performance (Engelmann and Winter, 1993). In addition, FBS bears at least a theoretical risk as a potential infectious source (Molander *et al.*, 1971; Kniazeff *et al.*, 1975; Schuurman *et al.*, 1991; Erickson *et al.*, 1991; Yanagi *et al.*, 1996; Vilcek, 2001). Kniazeff *et al.* employed a viral screening method to detect endogenous bovine virus contaminants in commercially supplied foetal bovine serum. Of 51 batches from 14 suppliers, over 30% were found to contain bovine viruses (such as bovine viral diarrhoea virus, parainfluenza type3-like virus, bovine herpesvirus-1, bovine enterovirus type 4 and an unidentified cytopathogenic agent) including 25% which had been pre-tested by the supplier and designated free from known contaminants (Kniazeff *et al.*, 1975). As yet there is no concrete evidence that these are infectious to humans, yet strains have been isolated in human cell lines which bear a remarkable resemblance (Giangaspero *et al.*, 1997). Further concerns were highlighted by the recent bovine spongiform encephalitis (BSE) epidemic which mainly affected European cattle. There have been over 70 reports of iatrogenic transmission of Creutzfeldt-Jakob disease (CJD) through dura mater grafts in the literature (Lang *et al.*, 1998) and over 120 reports through human growth hormone preparations from human pituitary tissue (Markus *et al.*, 1992; de Billette *et al.*, 1996). Furthermore, there have been three reported incidences of CJD transmission by corneal transplantation, one of which has been proven unequivocally (Duffy *et al.*, 1974; Uchiyama *et al.*, 1994; Hogan and Cavanagh, 1995; Heckmann *et al.*, 1997; Hogan *et al.*, 1999). In 1996, a new variant of CJD was discovered (termed nvCJD) and molecular strain typing studies confirmed direct links with BSE in that the same

prion strain was responsible for the disease in both humans and cattle. With no reliable serological method of screening for BSE or CJD (Hogan *et al.*, 1999) and while FBS remains a standard component of corneal storage/culture media, the possibility of transmitting prion disease by transplanting corneal tissue cannot be negated (Moffatt, 2001).

All of the above reasons emphasize the need to find alternate sources of mitogenic agents. Different approaches have been taken to eliminate the need for a serum supplement. Some investigators have attempted to adapt cells to media without serum or to purify the factors responsible for cell growth and differentiation. Others have investigated nutrient combinations or experimented with hormone supplements. As a result, a selection of serum-free and reduced culture/storage media such as Eurosol, Endothelial-SFM, Minimal Essential Medium, Medium F99 and M199 have been developed and analysed for their effectiveness in preventing endothelial cell loss in storage, which were varied (Engelmann and Friedl, 1995; Ayoubi *et al.*, 1996; Bednarz *et al.*, 2001; Moller-Pedersen *et al.*, 2001; Hempel *et al.*, 2001; Stoiber *et al.*, 2001; Rieck *et al.*, 2003). Most researchers concluded that serum-free organ cultures were possible but that endothelial cell deterioration was rapid after the first week in storage and that there was a clear advantage gained from the presence of serum, even at low concentrations (Engelmann and Friedl, 1995; Ayoubi *et al.*, 1996). During organ culture, as in tissue culture systems, the composition of the medium changes over time as a result of cellular metabolic activity. In tissue culture, a stable environment is maintained for the cells by routinely changing the medium every couple of days. Eye banks do not typically follow this practice since the rate of substrate utilization and accumulation of waste is likely to be lower in organ

culture where the purpose is one of cell maintenance rather than proliferation. As such, organ culture conditions are not directly comparable to those of tissue culture systems, where serum seems to play a more major role prior to cells reaching confluence (Ayoubi *et al.*, 1996; Tezel and Del Priore, 1998). In terms of serum-free epithelial tissue culture, Kruse and Tseng (1991) as detailed above, seem to have produced the better results, demonstrating clonal growth of corneal epithelial stem cells however their success was limited in that epithelia did not stratify and even the basal cells stained positive for terminal differentiation-linked keratin 3.

Given the clear benefits offered by serum in corneal storage and culture, this study was designed to assess the practicality and effectiveness of autologous human serum as a replacement for the bovine variant in a limbal epithelial cell culture system.

9.9.2 General Discussion of Results

A medium that does not require components of bovine origin would reduce the risk of transmission of infectious diseases such as bovine spongiform encephalitis (BSE) and would add an additional safety feature to organ and tissue culture. This experiment was designed to compare the relative efficacy of human serum in culture media with that of FBS, in terms of cultivated corneal stem cell morphology. Examination by SEM revealed little in the way of differences between the cultivated cells (plate 8.1), while TEM highlighted variations not apparent by surface analysis (plate 8.4). Of the three human serum cultures studied, the first (plate 8.4A) most closely resembled normal human cornea in terms of stratification, differentiation and intercellular spacing however since both sera and limbal cells were derived from different donors it is difficult to accurately compare them. As encountered with any

clinical study, it is impossible to obtain large quantities of identical living tissue for experimentation and since FBS varies in composition, it is likely that human serum also differs slightly between individuals. The potential benefits of using autologous serum in creating an epithelial sheet for grafting should however outweigh any distinctions in serum condition. Of all cultured epithelial sheets in this study, the FBS-cultivated cells demonstrated the largest intercellular spaces, as well as the lowest numbers of desmosomes and hemidesmosomes and as such, least resembled the control human cornea and would be less-well equipped to survive transplantation onto the ocular surface.

A thorough review of the literature revealed that human serum has seldom been used in cell culture. There have been several reports on the serum-free cultivation of corneal, conjunctival and retinal pigmented epithelial cells (Hackworth *et al.*, 1990; Kruse and Tseng, 1991; Castro-Munozledo *et al.*, 1997; Tezel and Del Priore, 1998; Ang *et al.*, 2003; Tan *et al.*, 2004; Ang *et al.*, 2004) yet only one that documents the use of human serum in culture media. In a study on corneal endothelial cell culture, Amano (2003) compared serum-free with human serum and FBS-containing media and found that cell morphology and growth were enhanced when either of the sera was used, confirming that inclusion of serum in the culture media produces better results yet offering no direct comparison between the serum cultures. Amano's findings do however corroborate the results of the study in this thesis, confirming that the culture of corneal cells is possible using adult serum. Although not related to the ocular surface, Chachques and co-workers (2004) have investigated cell culture using human serum in studies on cell-based myocardial regenerative therapy, with similarly encouraging results. Having observed that injection of bovine serum-

cultivated myoblasts corresponded with an increased incidence of malignant ventricular arrhythmias and sudden death in patients, they hypothesized that the contact of human cells with bovine serum results in the fixation of animal proteins on the cell surface, forming an antigenic substrate for immunological and inflammatory attack. In an effort to improve cardiac function, they transplanted autologous muscle cells cultivated in human serum directly into 20 infarcted left ventricles and reported improvements in condition and no cases of arrhythmia or mortality in the post-operative year long observation period (Chachques *et al.*, 2004). While there have been no reports of bovine proteins being detected on the surface of FBS-cultivated limbal cells, it is conceivable that such factors could contribute to postoperative inflammation when the sheets are used as ocular surface grafts.

Given the encouraging findings of the morphological examination of the human serum-cultivated corneal epithelial cells, these sheets were also used in clinical trials and subsequently grafted onto ocular surfaces by colleagues in Japan. The photo montage overleaf (figure 9.7) was kindly provided by Dr. Nakamura of the Kyoto Prefectural University of Medicine for inclusion in this thesis and illustrates a series of slit lamp images which chart the successful restoration of a cornea using a human serum-cultivated limbal epithelial cell sheet. Although further relevant clinical details are unavailable at this time, it is encouraging that eight months post-transplant the grafted cornea remains clear and free from epithelial defects. This would imply that the graft continues to maintain a supply of stem cells and offers additional support for a suspension culture system using human serum and a 3T3 fibroblast layer.

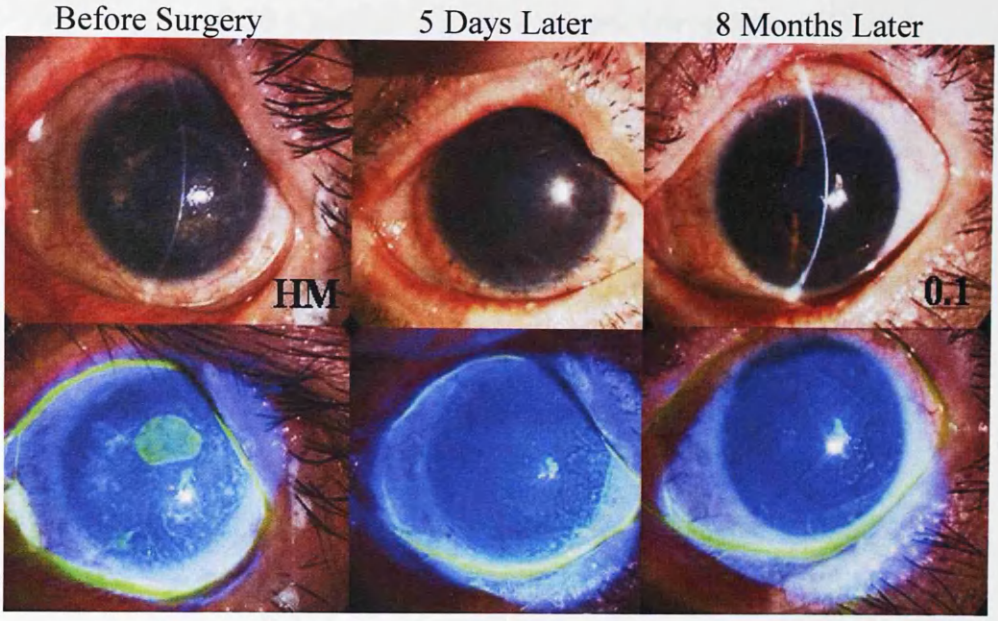

Figure 9.7: These slit-lamp images (with [lower] and without [upper] fluorescein staining) show the clinical course of human serum-cultivated corneal epithelial cell transplantation. Prior to surgery the eye manifested conjunctivalization with scarring and persistent epithelial defects; the patient could only recognize hand movements [HM]. Five days later, the ocular surface was completely re-covered by the cultivated corneal cells and free from epithelial defects. At the last follow-up visit (8 months), the corneal surface remained clear and visual acuity had improved to 0.1 (10/100).

9.9.3 Conclusions and Future Directions

This investigation confirmed quite comprehensively that confluent sheets of healthy-looking limbal epithelium can be cultivated using media containing human serum and that they more closely resemble *in vivo* cornea than FBS-grown cells. The major benefit of autologous serum cell culture is that it can be performed without risk of prion, viral or zoonose contamination. While very encouraging, further clinical studies are needed to verify the longevity of these grafts. Future challenges include incorporating the replacement of mouse 3T3 fibroblasts with those of human origin to eliminate the risks both of transmission of zoonotic infection and xenograft rejection during transplantation, though there has never been any confirmation that these components have resulted in adverse clinical consequences.

9.10 Conclusions and Future Directions

This PhD thesis primarily employed microscopic techniques to evaluate potential improvements to various components of the suspension culture system for corneal stem cells and has documented a number of original findings which have contributed significantly to the refinement of the culture technique and the development of safer epithelial sheets for use in ocular surface reconstruction.

In terms of the amniotic membrane carrier, with morphology more closely resembling control cornea, smaller intercellular spaces and superior mechanical strength, denuded AM appears to be the more practical substrate for human limbal epithelial cell culture. Additionally, freeze-drying the membrane was found to be a viable alternative to the conventional technique. Sterilized FD-AM retained all the valuable characteristics of the cryopreserved tissue (in terms of physical properties, levels and distributions of ECM proteins, biocompatibility and the ability to act as an stem cell culture substrate) while having the exciting potential to become more universally available. Though the results are preliminary in nature, it was determined that protein-coated gelatin hydrogels also seem to possess the desired qualities of a carrier. With the theoretical risk of prion/viral transmission via the transplantation of human tissue, this is an alternative which warrants further investigation.

A number of investigations involved evaluating alternative sources and types of cells for use in culture. Amniotic membrane was found to support the growth of quiescent human corneal endothelial cells and has very promising implications with regard to the creation of a complete artificial cornea. Incidentally, since the commencement of this thesis, two of the techniques described herein have been successfully combined

to produce viable cultivated human corneal endothelial cell sheets on FD-AM (Ishino *et al.*, 2005). Furthermore, oral mucosal stem cells were successfully used to recover the ocular surface and have been grown in conjunction with corneal stem cells to great effect. The replacement of donor tissue with that of host origin ought to revolutionize ocular surgery and remove any risks of allogeneic rejection. Human serum was additionally found to be a good substitute for bovine serum in culture media, removing a further potential source of prion, viral or zoonose infection. Future challenges include the replacement of mouse 3T3 fibroblasts with human feeder cells so as to totally eliminate any risks associated with zoonotic infection or xenograft rejection during transplantation.

These findings have made considerable contributions to an improved culture system which is more flexible, safer and potentially applicable to a greater population. Many of these improvements have been applied practically and are currently undergoing clinical trials; the resultant cell sheets having been grafted onto the ocular surface and undergoing regular examination. So to conclude, the results reported in this thesis have led to significant advances in stem cell culture for ocular surface reconstruction, some of which will produce immediate effects and others which may help in the long-term. The application of denuded and/or freeze-dried AM will have instant benefits. In the more medium-term, there is the potential to create a working culture system with no animal components. It will be more of a challenge to replace AM completely, though preliminary findings with gelatin hydrogels are encouraging. Perhaps the future of tissue engineering of the cornea lies in the creation of an entire artificial organ, combining cultured endothelial and epithelial cells with a suitable stromal matrix. Though there remain substantial obstacles to overcome before this dream becomes a reality, it is not beyond the realms of possibility.

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Figure 1.2: *Future of Optometry* (last updated October 2004). Berkeley Optometry, University of California, Berkeley, California 94720 USA.
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Figure 1.6: *Anatomy and Physiology of the Eye*, Version 2.0 (last modified March 2001) by Dr Thomas Caceci, Virginia/Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute & State University, Virginia 24061 USA.
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Figure 1.10: *Basal Lamina Histology* (last revised January 2001) Faculty of Medicine, Kagoshima University, 35-1 Sakuragaoka 8-Chome, 890 Kagoshima, Japan. URL: http://www.kufm.kagoshima-u.ac.jp/~anatomy2/EPITH/EF09.jpg

Figure 1.15A: *Ocular Disease* (last updated April 2003) Advanced Eye Care, 2145 Office Park Drive, San Angelo, Texas 76904 USA.
URL: http://www.aeceyedocs.com/OcularDisease.htm

Figure 1.15B: *Ocular Cicatricial Pemphigoid: Patient Education Monograph for the American Uveitis Society* (last revised January 2003), by C.S. Foster and Saadia Rashid, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, USA. URL: http://www.uveitissociety.org/pages/diseases/ocp.pdf

Figure 1.15C: *Ocular Disease* (last updated April 2003) Advanced Eye Care, 2145 Office Park Drive, San Angelo, Texas 76904 USA.
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Figure 1.16 [left]: *The Multidimensional Human Embryo* (last modified February 2003) by Bradley R. Smith, Biomedical Visualization School of Art and Design, University of Michigan, Ann Arbor, Michigan USA.
URL: http://embryo.soad.umich.edu/carnStages/stage19/stage19.html

Figure 1.16 [top]: *Amniotic Membrane Transplantation* (last modified December 2002) by Dr Scheffer Tseng, Ocular Surface Centre, Suite 213, 97th Avenue, Florida 33173, USA. URL: http://www.ocularsurface.com/clinicalservices_oss.html#3eamt

Figure 1.18 [before and after]: *New Efforts for Ocular Surface Reconstruction* (last updated March 2004) by Shigeru Kinoshita, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto, Japan.
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Figure 1.18 [during]: *Amniotic Membrane Transplantation in Ophthalmology* (last revised April 2002) Royal College of Ophthalmologists Guidelines, 17 Cornwall Terrace, London, England, NW1 4QW.
URL: http://www.site4sight.org.uk/Quality/RGov/Guidelines/Amniotic.htm

PUBLICATIONS AND PRESENTATIONS

Published Papers

Takahiro Nakamura, Makoto Yoshitani, **Helen Rigby**, Nigel J. Fullwood, Wakana Ito, Tsutomi Inatomi, Chie Sotozono, Tatsuo Nakamura, Yukiko Shimazu and Shigeru Kinoshita (2004) Sterilized, freeze-dried amniotic membrane: A useful substrate for ocular surface reconstruction. *Invest Ophthalmol Vis Sci* **45**: 93-99.

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Papers in Press

Noriko Koizumi, **Helen Rigby**, Nigel J. Fullwood, Satoshi Kawasaki, Hidetoshi Tanioka, Kan Koizumi, Antonia M. Joussen, Norbert Kociok and Shigeru Kinoshita (2005) Comparison of intact and denuded amniotic membrane as a substrate for human limbal epithelial cell culture. *Graefes Arch Clin Exp Ophthalmol.*

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Papers Submitted for Publication

Helen Rigby, Takahiro Nakamura, Noriko Koizumi, Shigeru Kinoshita and Nigel J. Fullwood. Freeze-dried and frozen amniotic membranes – A comparison of extracellular matrix molecule distribution. *Cornea.*

Takahiro Nakamura, Eiichi Sekiyama, **Helen Rigby**, Leonard P.K. Ang, Maho Takaoka, Tsutomu Inatomi, Chie Sotozono, Nigel J. Fullwood and Shigeru Kinoshita An evaluation of autologous serum for stem cell cultivation in patients with Stevens-Johnson Syndrome. *Invest Ophthalmol Vis Sci.*

Papers in Preparation

Takahiro Nakamura, **Helen Rigby**, Nigel J. Fullwood and Shigeru Kinoshita (2005) Evaluation of hybrid corneal epithelial and oral mucosal stem cell co-cultures for potential use in ocular surface reconstruction.

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Published Abstracts

H. Rigby, N. Koizumi, S. Kawasaki, H. Tanioka, K. Koizumi, A.M. Joussen, N. Kociok, S. Kinoshita and N.J. Fullwood (2004) Ultrastructural and quantitative comparison of cellular and denuded amniotic membranes in human limbal epithelial cell culture. *Ophthalmic Research* **36**: 253

N. Koizumi, **H. Rigby**, N.J. Fullwood, K. Koizumi, A.M. Joussen, N. Kociok and S.Kinoshita (2004) Comparison of intact and denuded amniotic membrane as a substrate for human limbal epithelial cell culture. *Invest Ophthalmol Vis Sci* **45**: E-Abstract 3923

Presentations

Characterizing corneal epithelium and basal lamina. Presented at the 8th Corneal Conference, Cardiff University, 9th July 2004.

Ex vivo expansion of corneal epithelial cells on amniotic membrane. Presented at a postgraduate seminar, Royal Manchester Eye Hospital, 10th November 2004.