THE UNIVERSITY OF NEW SOUTH WALES

Thesis/Project Report Sheet

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Title: MIGRATION MECHANISMS OF CORNEAL EPITHELIAL TISSUE AND CELLS

Abstract 350 words maximum:

The aim of the research described in this thesis was to understand the underlying mechanisms for the migration of stratified epithelial tissue over synthetic surfaces. The hypothesis tested was that there are differences between the mechanisms that operate when intact epithelial tissue migrates and when individual cells migrate. The experimental approach taken was to compare the structure and functioning of cytoskeletal components within bovine corneal epithelial tissue and cells migrating over synthetic substrata *in vitro*. Tissue and cell migration in response to two classes of surface topography were also considered.

The component cells within migrating tissue remained attached to each other by means of inter-digitations of plasma membranes and by desmosomes. This permitted coordination between the tissue cells and restricted their independent movement. Basal cells throughout the tissue maintained contact with the substratum by focal adhesions and both marginal and submarginal basal cells were implicated as active participants in migration. The tissue cells situated anteriorly were transported by the basal cells and appeared not to actively participate in migration. Optimal rates of tissue and cell migration were dependent upon intact actin filament and microtubule systems. Migrating tissue maintained its morphological structure and integrity when cytoskeletal elements were disrupted, unlike migrating cells, which retracted when the actin filaments were disassembled.

The basic sequence of mechanistic events that occurred during epithelial cell migration also occurred in relation to the cells within the tissue that were in contact with the substratum. The persistence of adhesion between the component cells within the migrating tissue added an element of complexity that was not encountered during cell migration and was manifested by the ability of cells to migrate over specific surface topography that inhibited tissue migration.

CERTIFICATE OF ORIGINALITY

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I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

(Signed) .....

MIGRATION MECHANISMS OF CORNEAL EPITHELIAL TISSUE AND CELLS

A Thesis submitted to the University of New South Wales

Beatrice Ann Dalton

For the Degree of Doctor of Philosophy

University of New South Wales

May, 2002

ABSTRACT

The aim of the research described in this thesis was to understand the underlying mechanisms for the migration of stratified epithelial tissue over synthetic surfaces. The hypothesis tested was that there are differences between the mechanisms that operate when intact epithelial tissue migrates and when individual cells migrate. The experimental approach taken was to compare the structure and functioning of cytoskeletal components within bovine corneal epithelial tissue and cells migrating over synthetic substrata *in vitro*. Tissue and cell migration in response to two classes of surface topography were also considered.

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This Thesis is dedicated to Joan Graham

PUBLICATIONS DURING THE PhD CANDIDATURE

The research for this thesis was conduced on a part-time basis between 1994 and 2001 at CSIRO, Molecular Science, North Ryde. Strategic research, related to the effect of surface topography on corneal epithelial tissue and cell migration, was conducted in parallel with the PhD research between 1995 and 2000 as part of the "Artificial Cornea" team. Relevant aspects of that published research are included in Chapter 5. Following is a list of journal articles and conference abstracts published during the PhD candidature detailing the relationship to the thesis (where applicable) and statements as to the extent of my contribution to the studies described in Chapter 5.

Published journal articles

(1) Dalton BA and Steele JG (2001) Migration mechanisms: Corneal epithelial tissue and dissociated cells. *Exp Eye Res* 73 (6):797-814.

The research described in this paper showed that there were both differences and similarities between the mechanisms that enable corneal epithelial tissue and cells to migrate over synthetic surfaces.

This research is covered in Chapters 3 and 4 and a copy of the paper is in Appendix B.

(2) Fitton HJ, Dalton BA, Beumer G, Johnson G, Griesser HJ and Steele JG (1998) Surface topography can interfere with epithelial tissue migration. *J Biomed Mater Res* 42 (2):245-257.

The authors showed that bovine corneal epithelial tissue migration over porous surfaces was reduced when the pores reached a critical size. I identified that cell migration was less inhibited by increase in pore diameter than was tissue migration, and assisted with the collation and interpretation of the data and writing of the manuscript.

Parts of this research are presented in Chapter 5 and a reprint of the paper is in Appendix B.

(3) Dalton BA, Evans MDM, McFarland GA and Steele JG (1999) Modulation of epithelial stratification by polymer topography. *J Biomed Mater Res* 45 (4):384-394.

The research described in this paper showed that the topography of a porous polymer affected the stratification of corneal epithelial tissue cultured on the surface of the polymer. The structure of corneal epithelial tissue that was cultured on surfaces which contained pores that were 0.1 or 0.4 μm in diameter was superior to that of tissue cultured on surfaces that contained larger diameter pores.

Some data from this paper was used in Chapter 5 and the methodology described in Chapter 2 was used in the development of the assay system used in the study. A reprint of the paper is presented in Appendix B.

(4) Dalton BA, McFarland GA, Steele JG (2001a) Stimulation of epithelial tissue migration by certain porous topographies is independent of fluid flux. *J Biomed Mater Res* 56 (1):83-92.

This publication demonstrated that there was no significant difference between the extent of epithelial tissue (and cell) migration over a porous surface in which the pores were "blind-ended", as compared with migration over that surface when fluid through-flux was maintained. This showed that it was topography and not porosity that was the factor that enhanced tissue migration over surfaces containing small diameter pores in comparison with migration over a planar surface of the same material.

This research is fully presented in Chapter 5 and a reprint of the paper is in Appendix B.

(5) Dalton BA, Walboomers XF, Dziegielewski M, Evans MDM, Taylor S, Jansen JA and Steele JG (2001b) Modulation of epithelial tissue and cell migration by microgrooves. *J Biomed Mater Res* 56 (2):195-207.

The studies described in this paper showed that corneal epithelial tissue and cell migration could be directed by surface microgrooves and that the depth of the grooves had a greater influence on migration than did the width of the grooves and ridges. Dr Frank Walboomers provided the microgrooved templates and supplied the expertise for casting the polymer replicates.

Aspects of this study are presented in Chapter 5 and a copy of the paper is included in Appendix B.

Patent Application

Patent Application Number(s):

US2001047203-A1; WO200009042-A1; AU9957326-A; NO200100694-A; EP1105070-A1.

Priority Application Information and Date:

EP-0115161 12 Aug 1998

Title:

Corneal onlay for synthetic epikeratoplasty or as an implanted contact lens, has a topography comprising surface indentations

Inventor Name(s):

DALTON BA, EVANS MD M, FITTON JH, GIPSON IK, JOHNSON G, STEELE JG, MACREA EVANS MD

Abstract:

NOVELTY - A corneal onlay or corneal implant to be placed within or onto the surface of the cornea has a surface topography comprising indentations. It is biocompatible, optically transparent, synthetic and biostable polymeric material. This material comprises surface that supports the attachment and growth of tissue cells.

USE - The corneal onlay is used as a synthetic epikeratoplasty or as an implanted contact lens.

ADVANTAGE - The invention provides a polymer surface that inherently supports tissue overgrowth without the need for an additional surface modification or biological coating. It also provides a polymer that combines this property with good biostability, optical properties, and mechanical properties that make the material suitable for the fabrication of epikeratoprostheses.

This patent was based on some of the results presented in Chapter 5.

Published journal articles that used methodology developed in the thesis

(6) Evans MDM, Dalton BA and Steele JG (1999) Persistent adhesion of epithelial tissue is sensitive to polymer topography. *J Biomed Mater Res* 46 (4):485-493.

(7) Walboomers XF, Dalton BA, Evans MDM, Steele JG and Jansen JA (2002) Transforming growth factor-beta 1, 2, and 3 can inhibit epithelial tissue migration on smooth and microgrooved substrates. *Biomed Mater Res* 60 (3):445-451.

Conference abstracts directly related to the thesis

(1) Dalton BA and Steele JG (1997) The effect of surface chemistry on the migration of bovine corneal epithelial cells. 7th Annual Conference Aust Soc Biomaterials, Nelson Bay, NSW.

This abstract was based on the studies described in Chapter 2.

(2) Dalton BA, Evans MDM and Steele JG (1998) Polymer surface topography and epithelial tissue structure. 8th Annual Conference Aust Soc Biomaterials, Marysville, Victoria.

Methodology that was developed from the research presented in Chapters 2 and 3 was used in the study presented here.

(3) McFarland GA, Dalton BA and Steele JG (1998) Polymer surface topography and the kinetics of epithelial tissue outgrowth. 8th Annual Conference Aust Soc Biomaterials, Marysville, Victoria.

Data used in Chapter 5 was the basis for this abstract.

(4) Dalton BA, Evans MDM, McFarland GA, Bojarski B and Steele JG (1999) Epithelialisation of a porous polymer. 9th Annual Conference Aust Soc Biomaterials, Canberra, ATC.

Methodology that was developed from the research presented in Chapters 2 and 3 was used in the study presented here.

(5) Dalton BA and Steele JG (1999) Migration mechanisms in corneal epithelium: Differences between cells and stratified tissue. 15th European Conference on Biomaterials, Arcachon, France.

Data from Chapters 3 and 4 were used in this abstract.

(6) Dalton BA, Walboomers XF, Dziegielewski M, Evans MDM, Taylor S, Jansen JA and Steele JG (2000) Modulation of epithelial migration by microgrooves. Smith and Nephew Tissue Engineering Workshop and Conference in York, UK.

Research described in Chapter 5 was presented at this conference.

(7) Dalton BA, Evans MDM, Dziegielewski M, Walboomers XF, Jansen JA, and Steele JG (2000) Guided migration of epithelial tissue by microgrooved substrata. 10th Annual Meeting Aust Soc Biomaterials. Melbourne, Victoria.

Research described in Chapter 5 was presented in this abstract.

(8) Dalton BA, McFarland CD, Dziegielewski M and Steele JG (2001) The cytoskeleton and epithelial tissue migration over synthetic materials. 11th Annual Conference Aust Soc Biomaterials. Beechworth, Victoria

Data from Chapter 4 was used in this abstract.

Journal articles published during the candidature period from areas outside the focus of the thesis

(1) Steele JG, Dalton BA, Johnson G and Underwood PA (1995) Adsorption of fibronectin and vitronectin onto Primaria and tissue culture polystyrene and relationship to the mechanism of initial attachment of human vein endothelial cells and BHK-21 fibroblasts. *Biomaterials* 16 (14):1057-67.

(2) Dalton BA, McFarland CD, Underwood PA and Steele JG (1995). Role of the heparin binding domain of fibronectin in attachment and spreading of human bone derived cells. *J Cell Sci* 108:2083-2092.

(3) Dalton BA, Dziegielewski M, Johnson G, Underwood PA and Steele JG (1996). Measurement of cell adhesion and migration using phosphor-screen autoradiography. *BioTechniques* 21:298-303.

(4) Dalton BA, McFarland CD, Gengenbach TR, Griesser HJ and Steele JG (1998). Polymer surface chemistry and bone cell migration. *J Biomater Sci Poym Ed* 9 (8):781-99.

(5) McFarland CD, Mayer S, Scotchford C, Dalton BA, Steele JG and Downes S (1999) Attachment of cultured human bone cells to novel polymers. *J Biomed Mater Res* 44 (1):1-11.

(6) Steele JG, Dalton BA, Thomas CH, Healy KE, Gengenbach TR and McFarland CD (2000) Underlying mechanisms of cellular adhesion during colonisation of synthetic surfaces by bone derived cells studied *in vitro*. Invited Book Chapter, Davies JE ed. Bone Engineering, em squared incorporated, Toronto, Canada. pp225-231.

ACKNOWLEDGEMENTS

I thank the following people who provided valuable help, advice and encouragement throughout the course of this project.

I am very grateful to my CSIRO supervisor, Dr Jack Steele for his advice, guidance and patience during the preparation of this thesis. Many thanks are also due to my university supervisor, Professor D. Sweeney and the supervisory panel at CRCERT.

I thank numerous friends and colleagues at CSIRO Molecular Science for help, advice and encouragement at all stages of the project. In particular Dr Meg Evans, Gail McFarland and Graham Johnson who have also been conducting research for the Artificial Cornea Project; Sarah Taylor, Barbara Barjarski and Joanne Cuomo for expert assistance with histology; Dr. Lloyd Graham for advice concerning the correct use of English; Jenny Young for explaining aspects of word processing; Mark Allan for solving all computer related problems; Laurie Morrison for the use of his printer and Debbie Lock for spreading good humour while taking care of all the washing-up and sterilising that was required for this project. I also acknowledge the valuable contribution made by a previous PhD student at Molecular Science, Dr Mark Dziegielewski for development of the time-lapse video microscopy system and Dr Clive McFarland for help with modification of the program.

I appreciate the support given by CRCERT, which enabled me to present my research at the 1999 European Society for Biomaterial Conference in Arcachon France and also for providing many useful short courses.

A special thank you to Brian for not complaining about the number of extra hours spent at work and the many hours spent in front of the computer at home.

ABBREVIATIONS

Brdu	5-bromo-2-Deoxyuridine
BSA	bovine serum albumin
DD	serum depleted of fibronectin and vitronectin
DMSO	dimethylsulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetracetate
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm (mouse tumour)
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
Fn	fibronectin
HGF	hepatocyte growth factor
hr	hours
IL-6	Interleukin 6
kDa	kilo Daltons
M	molar
min	minutes
MDCK	Madden-Derby canine kidney cells
PBS	phosphate buffered saline
PRI	Primaria™ tissue culture polystyrene
PS	untreated polystyrene
SCA	sessile contact angle
Sec	seconds
SEM	scanning electron microscopy
SF	serum free
SFM	serum free medium
TCPS	tissue culture polystyrene
TEM	transmission electron microscopy
TGF	transforming growth factor
THER	Thermanox™
TV	trypsin/versine, ie. trypsin/ PBS solution containing EDTA
Vit	Vitrogen® (collagen I)
Vn	vitronectin
% v/v	percentage by volume (ie. ml/100 ml)
% w/v	percentage by weight (ie. g/100 ml)

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CHAPTER 5

SUBSTRATUM TOPOGRAPHY AND CORNEAL EPITHELIAL TISSUE AND CELL MIGRATION

CHAPTER 1

1.1 INTRODUCTION TO THE ARTIFICIAL CORNEA PROJECT

The Cooperative Research Centre for Eye Research and Technology is involved in a project to produce a synthetic lenticule that may be incorporated into the cornea and correct refractive error. A review of the progress towards this endeavour has recently been published (Evans et al 2001). In effect, the device would be equivalent to a permanent contact lens that could be replaced if a change in the degree of correction was required. The development of this lenticule may also facilitate the future development of an artificial cornea that would alleviate the high demand for donor material in corneal transplant procedures.

The optimum method envisaged for the incorporation of the lenticule into the cornea is the use of a corneal "onlay", sitting immediately beneath the corneal epithelium and above the corneal stroma. This would entail the debridement of a small area of the epithelium and the attachment of the lenticule onto the underlying basement membrane, in a manner that would permit the migration of epithelial tissue over the anterior surface of the lenticule. The use of an "onlay" approach for incorporation of a lenticule into the cornea has major advantages over the placement of the lenticule deeper within the corneal tissue. It would permit easier removal and replacement of the lenticule, if required, so making the process completely reversible. Additionally, the basement membrane would be left intact so that the full "wound healing cascade" may not be activated so minimising the inflammatory response. The lesser disruption of corneal tissue would also reduce trauma to the recipient.

There are a number of specific requirements that need to be fulfilled in a material for use as a corneal onlay. The material would need to have suitable refractive properties, not induce a sustained inflammatory response in the recipient and not be degraded in any way by the ocular environment. The posterior surface of the lenticule would be required to attach to the debrided corneal surface, but not to cause the underlying tissue to become integrated into the lenticule. Other requirements include an anterior surface that would support epithelialisation. Initially, the surface would need to support the migration of epithelial tissue then the persistent adhesion of the tissue to the lenticule. Finally, the material would need to have the ability to sustain a

functional stratified tissue and to permit nutritional flux between the epithelium and the stroma.

1.2 INTRODUCTION TO THE THESIS AREA

Migration of the host's corneal epithelial tissue over the anterior surface of the lenticule is a critical first step in the required response to the attached lenticule. Therefore, the aim of this thesis was to better understand the mechanisms underlying the migration a stratified corneal epithelial tissue over a synthetic surface.

In an adult cornea there is a fully stratified epithelium, which in humans has approximately five layers of cells (Hogan et al 1971). The corneal epithelium functions as a barrier to fluid loss and to the entrance of pathogens. If the barrier function of the corneal epithelium is breached by a wound, there is expansion and migration of the surrounding epithelial tissue to cover the wound and to restore the integrity of the epithelium (Thoft and Friend 1975). In this situation, the epithelial tissue migrates over native biological surfaces. Similarly, the success of the corneal onlay device requires that the anterior surface becomes colonised by epithelial tissue. However, this would require the migration of epithelial tissue over a synthetic material. The mechanisms that are involved when an intact tissue sheet of adult corneal epithelium colonises a synthetic material have not been extensively studied. The elucidation of mechanisms that enable cells to migrate over synthetic surfaces have generally occurred as a result of studies concerning individual cells. The majority of mechanistic studies focused on fibroblasts or fish keratocytes rather than epithelial cells. However, some of the fundamental principles also apply to epithelial cells.

Epithelial cells have been shown to exhibit different modes of migration in culture (Bellairs et al 1982). They may move over a surface in culture as individual isolated cells (Vaughan and Trinkaus 1966); alternatively, as islands consisting of various numbers of epithelial cells attached together to form monolayer sheets (Liao and Gundersen 1998); or as a cohesive sheet forming an intact tissue structure which may in parts consist of several layers of epithelial cells (DiPasquale 1975a). By comparing the migration of the intact tissue with the locomotion of epithelial cells moving as a

monolayer or as individual cells, the aim was to further the understanding of the mechanisms that are operational when corneal epithelial tissue migrates over synthetic surfaces.

The hypothesis tested was that the mechanisms involved when intact corneal epithelial tissue migrates over synthetic surfaces had differences from the mechanisms that underlie the migration of individual epithelial cells. The experimental approach was to compare the structure and functioning of cytoskeletal components within bovine corneal epithelial tissue and cells migrating over synthetic substrata *in vitro*. Tissue structure depends upon cell-cell adhesion, therefore migrating tissue was investigated for the persistence and location of cell-cell adhesion.

1.3 LITERATURE REVIEW

1.3.1 Structure and function of the cornea

This overview considers literature related to the structure and function of ocular components in the human eye. While there are some differences between the human eye and those of the animal models used in parts of this project, the basic structure and physiology are comparable (Prince et al 1960). The differences between human and bovine cornea are discussed in section 1.3.4.

1.3.1.1 Relationship of the cornea to other ocular tissue

The cornea and sclera are collagenous tissues that form the outermost coat of the eyeball. Within this slightly elastic envelope are the anterior and posterior chambers that are partially separated by the iris and the lens. The transparent, gel-like vitreous humour, which has a volume of about four ml in humans, is separated from the aqueous chambers by the lens and its zonular attachments (Hogan et al 1971). The other fluid component of the eye, the aqueous humour, is produced by the ciliary body and flows from the posterior chamber through the pupil into the anterior chamber (Hogan et al 1971). These structures are illustrated in Figure 1.1.

At the anterior ocular surface are the tissues of the cornea and the conjunctiva. The transition zone between these two tissues is known as the limbus. These regions have some features in common but also differ in structural and functional aspects (Gipson and Sugrue 1994). A stratified, squamous epithelium forms a continuous layer over the region with the apical cell membranes supporting a filamentous glycocalyx that is associated with the tear film (Pfister 1973).

1.3.1.2 The cornea

Transparency is a requirement of the cornea and the refractive index of this tissue changes little with increasing age. Transparency is aided by the precise organisation of structural components (Hogan et al 1971). However, the regulation of cellular metabolism and the hydration state of the tissue are critical for the maintenance of this corneal transparency (Dikstein and Maurice 1972; Maurice 1957). The avascular nature of the normal cornea also contributes to corneal transparency. Branches of

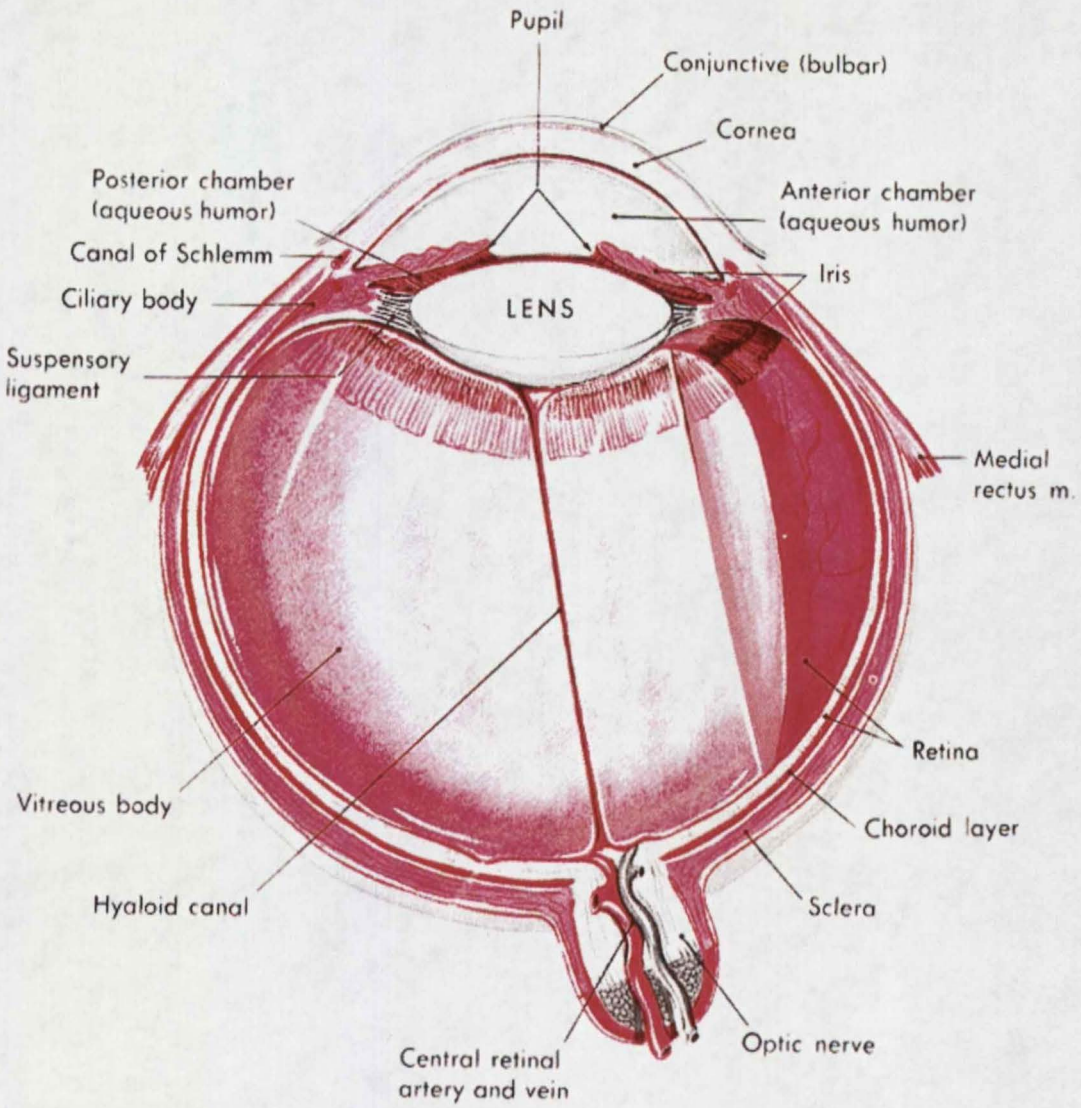


Figure 1.1 Horizontal section through a human eye showing the relationship between the cornea and the structure of the eye.

From Anthony, CP and Kolthoff, NJ (1975) Anatomy and Physiology, Ninth edition, The CV Mosby Co, St. Louis, USA.

circumcorneal blood vessels terminate at different levels of the limbus and play a small part in corneal nutrition and metabolism (Hogan et al 1971).

The human cornea can be divided into discrete layers: the epithelium; Bowman's layer; stroma with resident keratocytes; Descemet's membrane and the endothelium (Hogan et al 1971) {see Figure 1.2A(a) and 1.2B}. The layers of the cornea are innervated to varying extents, making the cornea a region that is rich in sensory nerve endings (Kaufman et al 1988; Maurice 1985; Maurice 1988). The density of nerve endings per area of the cornea has been estimated to be 300-400 times that of the epidermis (Rozsa and Beuerman 1982).

1.3.1.2.1 The corneal epithelium

A three dimensional view of the structure of the human corneal epithelium is illustrated in Figure 1.2(B). The corneal epithelium is characterised by regular thickness, which in humans is 50 to 52 μm (Hogan et al 1971). It is made up of between four and six layers of cells. A single layer of columnar basal cells is attached to the basement membrane by the hemidesmosomes, which are part of adhesion complexes linking cytoskeletal components of the epithelial cell with anchoring fibrils that emanate into the stroma. These specific cell-extracellular matrix (ECM) adhesion complexes are discussed later in this Chapter (section 1.3.2.8). Anterior to the basal cell layer are the wing cells, which have a rounded body and thin wing-like extensions. Two-to-three layers of flattened squamous cells occur on the anterior surface of the cornea (Hogan et al 1971). These are the terminally differentiated cells of the epithelium and are committed to desquamation. Mitosis occurs in the basal cell layer and the cells differentiate as they progress towards the anterior ocular surface to be desquamated from the apical surface into the tear film. Evidence suggests that the corneal epithelium is maintained by a population of slowly proliferating stem cells, which are located in the basal layer cells of the limbus (Cotsarelis et al 1989; Schermer et al 1986). It has been suggested that centripetal migration of corneal epithelia occurs (Hanna and O'Brien 1960). Thoft and Friend proposed a hypothesis, known as the "X, Y, Z" hypothesis, to explain the relationship between proliferation, migration and desquamation (Thoft and Friend 1983). Such that if X represents proliferation of basal epithelial cells, Y represents proliferation and migration of the

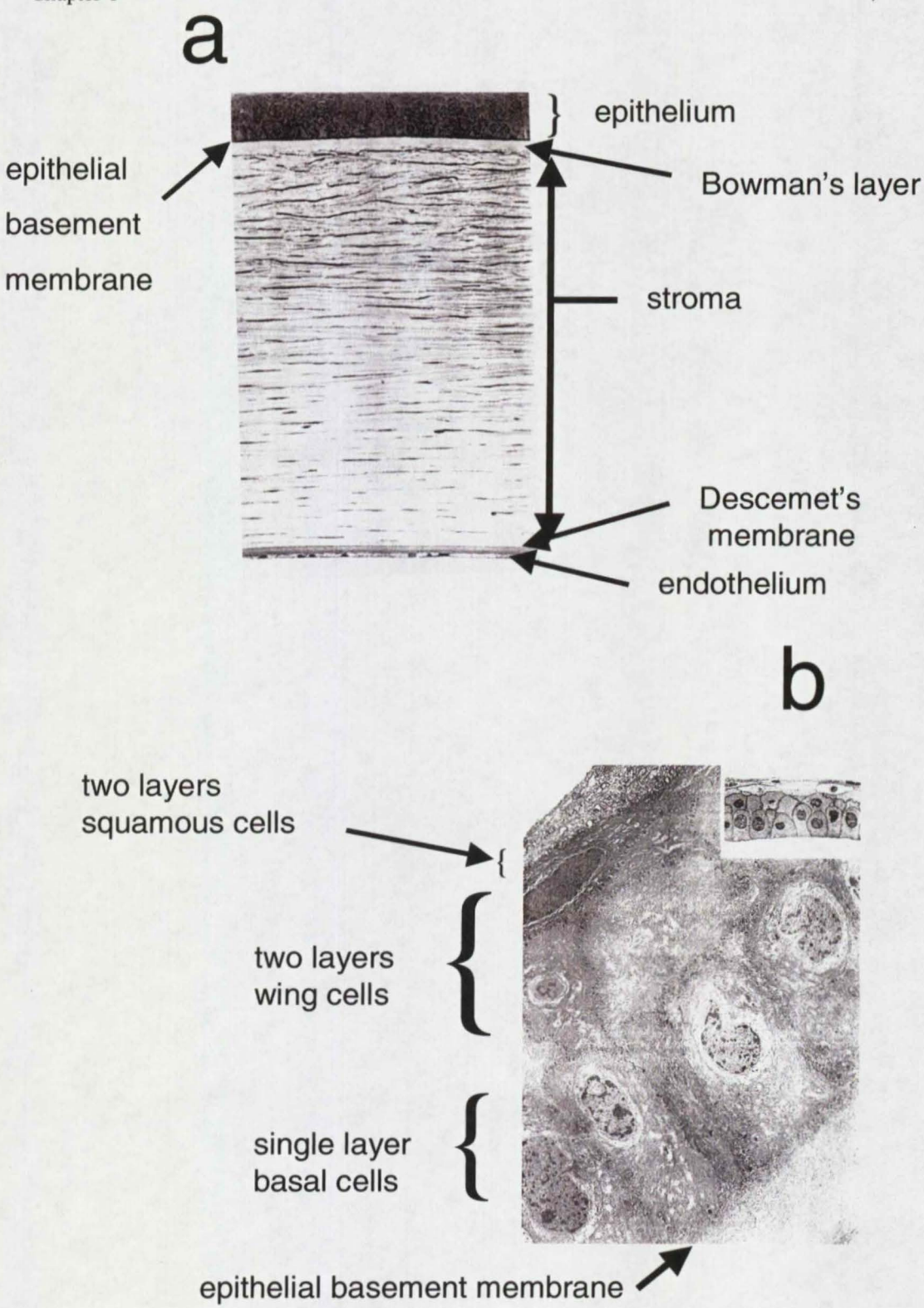


Figure 1.2A Tissue structure of the human cornea. The overall structure of corneal tissue is shown in (a) and the stratified epithelium is illustrated in (b) with a lower power view included in the inset.

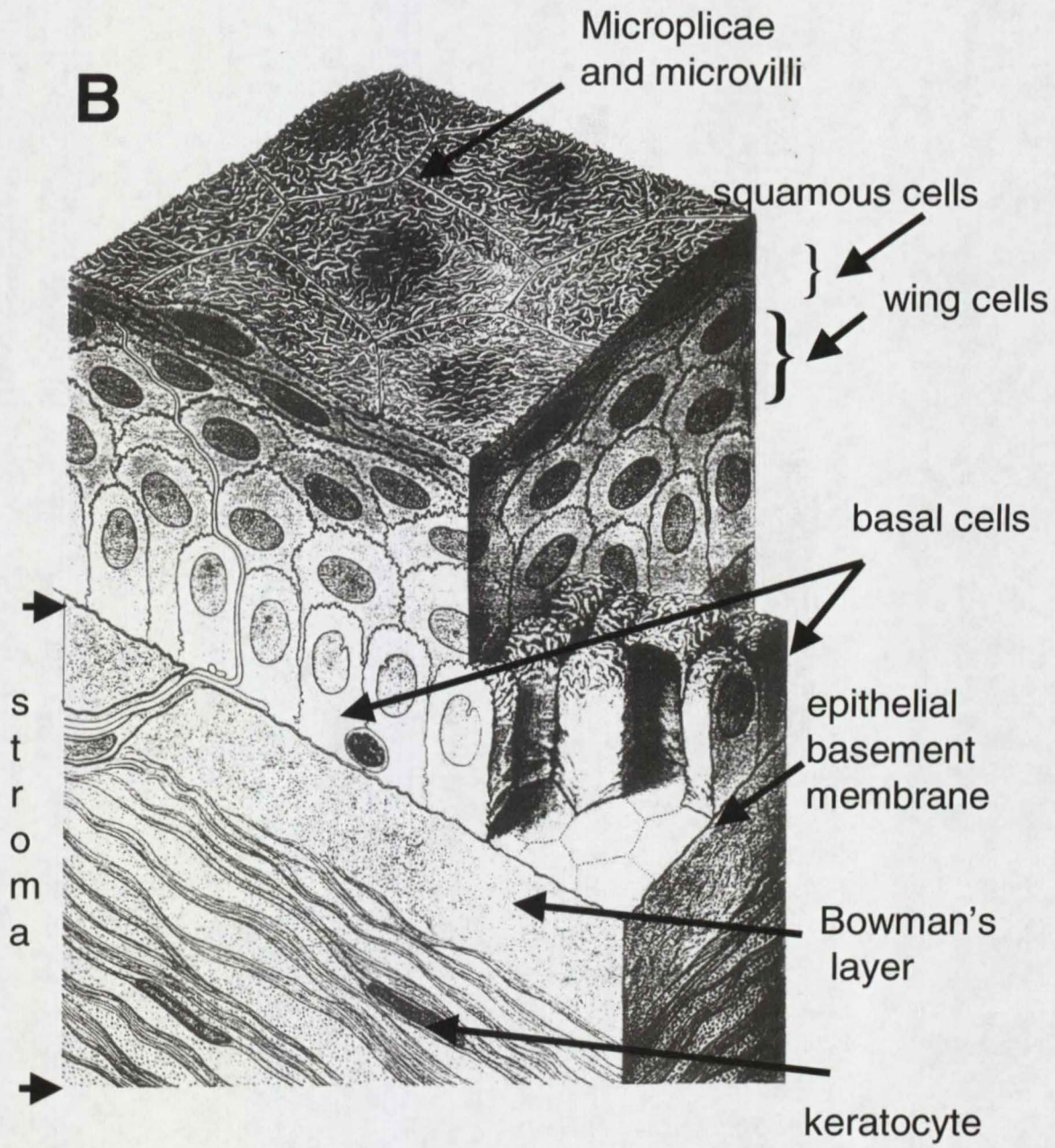


Figure 1.2B Three dimensional drawing of corneal epithelium from a human eye. The polygonal shape of the basal and surface cells is illustrated.

Figure 1.2A From Gipson IK and Sugrue, SP (1994) Cell Biology of the Corneal Epithelium, In: Principles and Practice of Ophthalmology, Albert, DM and Jackobiec FA eds Saunders and Co, Philadelphia, USA.

Figure 1.2B Adapted from Hogan M.J, Alvarado JA and Weddell JE (1971), Histology of the human eye, WB Saunders Co, Philadelphia, USA.

limbal cells and Z is epithelial desquamation, then for equilibrium X + Y must = Z. Gipson and Sugrue reviewed evidence for the "stem cell hypothesis" which suggests that corneal epithelial stem cells located in the crypts of the palisades of Vogt undergo asymmetrical division (Gipson and Sugrue 1994). It was proposed that one cell becomes a transient amplifying cell, which is able to undergo a limited number of divisions but will eventually become terminally differentiated. The other cell of the division remains in the pool of stem cells and maintains a capacity for unlimited self-renewal. Beebe and Masters challenged this hypothesis with a study that showed that a high percentage of daughter cells differentiated at the same time (Beebe and Masters 1996). However, it was not directly obvious from their work if they had specifically included the limbus in their confocal microscopy study. Presumably the slow cycling nature of the stem cell population would render them a minor population of the 5-bromo-deoxyuridine labelled cells that Beebe and Masters tracked in their study. The normal rate of corneal epithelial cell replacement was originally thought to be approximately every five to seven days (Hanna and O'Brien 1960). A more recent study of the rat cornea suggested that the turnover time was two weeks (Cenedella and Fleschner 1990). However when a model that used cell-shedding, as opposed to the metabolic labelling methodology, was used a turnover period of several months was predicted for rabbit corneal epithelium (Ren and Wilson 1996).

1.3.1.2.2 The epithelial basement membrane

Posterior to the basal cells of the corneal epithelium there is a basement membrane that provides mechanical support, divides tissues into compartments and also influences cell behaviour (Aumailley 1995; Schittny et al 1988; Yurchenco and Schittny 1990). Contact of epithelial cells with molecules of the basement membrane is one of the key events that determine their apical-basal polarity (Paulsson 1992). Interaction of basal epithelial cells with specific molecular constituents of the basement membrane may also play a role in production of ECM molecules by corneal epithelial cells. For example, enhanced collagen production was found in epithelial sheets *in vitro* when laminin, collagen or fibronectin (Fn) was added to the culture medium (Sugrue and Hay 1986).

Basement membranes are a dense network of complex macromolecules and can include collagens, proteoglycans, laminins, vitronectin (Vn), Fn and hyaluronic acid

(Kleinman et al 1993; Kuhn 1995; Uitto and Pulkkinen 1996). Tissue specific isoforms of these molecules confer heterogeneity amongst basement membranes from different locations (Paulsson 1992) and immunoelectron microscopy studies also suggest that a different topological orientation of these molecules may exist in corneal epithelial basement membranes (Schittny et al 1988). Epithelial cells are able to synthesise basement membrane components, but fibroblasts have been shown to stimulate the production of these molecules by epithelial cells *in vitro* (Ohji et al 1993). Marinkovich and colleagues demonstrated that dermal fibroblasts participate in the deposition and assembly of matrix molecules into basement membrane structures by keratinocytes (Marinkovich et al 1993), but of greater relevance here is the work of Zieske. This research, which used a culture system involving three cell types, suggested that there was an influence from endothelial cells (in addition to that of the corneal fibroblasts) in the assembly of the epithelial basement membrane by the epithelial cells (Zieske et al 1994).

1.3.1.2.3 Bowman's layer

Adjacent to the epithelial basement membrane is an acellular zone, known as Bowman's layer, which is made up of randomly dispersed collagen fibrils (Jacobsen et al 1984) and associated proteoglycans (Gipson and Sugrue 1994). This region is not present in all species and its specific role has not yet been positively defined, although various hypotheses as to its function have been suggested (Gipson and Sugrue 1994). However, the ability of a cornea to function in species where this region is totally absent tends to discredit these hypotheses. Additionally, if this region is destroyed in an adult eye it does not reform (Gipson and Sugrue 1994).

1.3.1.2.4 The lamellar stroma

The bulk of the stroma is made up of 200-250 flattened bundles of parallel collagen fibrils each extending the width of the cornea (Hogan et al 1971). Collagen fibrils within the bundles of the lamellar stroma have a larger diameter than those from Bowman's layer, however fibril diameters in both regions are more uniform than in other connective tissues (Jacobsen et al 1984). The distances between collagen fibrils are also equivalent, and a lattice theory was proposed to explain how this regularity maintains the transparency of the cornea (Maurice 1985; Maurice 1988). Between the

lamellae are very flat fibroblasts called keratocytes, which are linked to each other by gap junctions at the tips of processes that extend from the cell body (Nishida et al 1988; Ueda et al 1987). These cells are involved in metabolic activities that participate in the maintenance of the stroma (Nishida et al 1988). Epithelial cells may contribute to the production of collagen fibrils in Bowman's layer (Tisdale 1988), but additionally the primary collagenous stroma appears to be the product of corneal epithelial cells (Dodson and Hay 1971). It seems probable that both cell types may be involved in stromal maintenance, because if communication between the epithelium and the stroma is blocked there is a deterioration of stromal tissue (McCarey 1991). It has also been postulated that endothelial cells may produce some of the chondroitin and keratan sulfates of the stromal proteoglycans (Somolin and Thoft 1994).

1.3.1.2.5 Descemet's Membrane

This membrane, like the epithelial basement membrane, is composed of collagens, laminin-isoforms and proteoglycans. However, it differs from typical basement membranes both by overall thickness and by regional variations in thickness (Hogan et al 1971). Corneal endothelial cells are believed to secrete this membrane (Nevo et al 1984).

1.3.1.2.6 The endothelium

The corneal endothelium is a single layer of hexagonal shaped cells that are attached to Descemet's membrane and form the posterior surface of the cornea. The main function of the endothelium is corneal hydration, to counteract the forces that draw water into the corneal stroma (Dikstein and Maurice 1972). The endothelium also forms a barrier between the cornea and the aqueous humour. This barrier is maintained despite the decline in cell numbers, which occurs throughout life because natural cell loss is accommodated by expansion of the remaining cells (Laule et al 1978).

1.3.1.3 Conclusion of general review of corneal tissue

This brief summary of the overall structure and function of the cornea demonstrates some of the complexity and interrelationships between the major cell types and acellular components of the cornea. The design of an onlay lens would need to take

account of this complexity.

1.3.2 The integrity of corneal epithelium

The corneal epithelium functions as a barrier to fluid loss and to the entrance of pathogens. Additionally, the abrasive pressure of eye-lid movement over this tissue requires that the cells adhere tightly to one another and to their underlying basement membrane (Gipson and Sugrue 1994). The cell-cell adhesion and cell-ECM adhesion is accomplished by a range of specialised cell junctions in which the interactions between the various molecular components are gradually being understood. While the majority of the analytical research on components, structure and mechanism of action of these junctions has been conducted on epithelia other than that from the cornea, it provides some basis on which to predict the possible presence and role of these junction components in the cornea.

1.3.2.1 Cell-cell junctions

As cell-cell junctions are specialised features that enable groups of cells to make up a tissue structure, a consideration of these features is necessary for a study of tissue migration. Within the corneal epithelium, as with other epithelial tissue, cell-cell junctions perform critical roles in the maintenance of the barrier function.

1.3.2.2 Tight junction

Tight junctions (also called zonula occludens) are a feature of all polarised epithelia and they usually form part of a junctional complex that includes an adherence junction (Kendrew and Lawrence 1988). In polarised epithelial tissue, tight junctions form a continuous zone of cell-cell contacts at the boundary of apical and basolateral plasma membrane domains (Anderson et al 1993; Farquhar and Palade 1963). This creates both a permeable barrier between adjacent cells, which can control the flux of molecules through the paracellular space, and a boundary in the plasma membrane bilayer that separates the apical and basal membrane domains (Gumbiner 1993; Simons and Fuller 1985). The two barrier functions appear to be separately controlled in some epithelial cells. It was shown that sucrase-isomaltase retained its apical polarity in the absence of cell-cell contacts at the free edge of "wounds" in a cultured Caco-2 cell monolayer (Bement et al 1993).

There are several molecules known to be associated with tight junctions, including ZO-1, cingulin and ZO-2. The first component of tight junctions to be cloned and sequenced was ZO-1 (Willott et al 1993) which is a 210-220 kD peripheral membrane polypeptide that is tightly associated with the cytoplasmic surface of the tight junction (Anderson et al 1993). Cingulin is a 140 kD protein that is localised to the region of the tight junction, but is not tightly associated with the membrane (Stevenson et al 1989). ZO-2 is a 160 kD protein that was first identified as a molecule which bound to ZO-1, although it also was not tightly associated with the membrane (Gumbiner et al 1991). It has been suggested that it is the molecule occludins that forms the seal between epithelial cells in the paracellular space (Furuse et al 1994). Additional proteins associated with these junctions and classed as peripheral proteins of epithelial tight junctions include 7H6, a 155 kD protein and p130 (Zhong et al 1993). Some of the various components associated with the tight junction in epithelial cells play both a structural role and also take part in a cascade of cell signalling events which involve membrane associated guanylate kinases (Anderson et al 1993; Kim 1995). Functional control of the tight junction can involve actin filaments and a signalling cascade triggered by extracellular Ca^{2+} (Anderson et al 1993). A link has been demonstrated between ZO-1, occludin and F-actin that supports this finding (Fanning et al 1998).

Tight junctions are an important component of the corneal epithelium, as they provide a barrier by sealing neighbouring cells together so the water-soluble molecules cannot leak between the cells (Huang et al 1989). In the resting stratified corneal epithelium, tight junctions are located exclusively between the squamous cells that occupy a superficial position (McCartney and Cantu-Crouch 1992). This raises the interesting question as to the mechanisms which preserve the integrity of the epithelial barrier during the continuous natural loss of epithelial cells from the apical surface. One study suggests that the sloughing of superficial cells (containing tight junctions) from the anterior surface of the cornea is compensated for by the assembly of tight junctions in cells that occupy a more basal position (Wang et al 1993). This was demonstrated by showing the up-regulation of ZO-1 in wing cells when the removal of squamous cells was accelerated by digetoxin (Wang et al 1993). It was suggested that ZO-2 or cingulin might be more specific markers for tight junctions, because ZO-1 had been detected in filtration slits of glomerules cells and cadherin based contacts

of non-epithelial cells (Anderson et al 1993). However, from the work of Wang it appears that antibodies to ZO-1 may be used to identify tight junctions in corneal epithelial tissue (Wang et al 1993).

1.3.2.3 Intermediate junctions

In simple epithelia, the intermediate junction (also known as adherens junction, belt desmosome or zonula adherens) forms a band of close cell-cell associations around the apical end of each cell (Gipson and Sugrue 1994) and the junctions are frequently associated with a tight junction and a desmosome in a junctional complex (Farquhar and Palade 1963). However, these well-formed junctional complexes are not typically found in corneal epithelium (Gipson and Sugrue 1994).

Intermediate junctions may be divided into three structural domains: cytoskeletal, plaque structure and integral membrane components (Geiger and Ayalon 1992). Actin and associated components such as α-actinin and vinculin can be present in the cell-cell intermediate junction. Vinculin has been detected in a weak punctate pattern along the membranes of superficial cells in cryostat sections of rat cornea (Zieske et al 1989). Vinculin staining has also been found to delineate the lateral cell membranes of the basal cells in intact rabbit cornea (Wu et al 1995).

The integral membrane components of intermediate junctions are integrins and cadherins, each with specificities that depend on cell type (Geiger and Ayalon 1992). The calcium-dependent, cadherin-mediated interactions affect not only the integrity of intermediate junctions but also neighbouring tight junction (Gumbiner et al 1988). Gibson and Sugrue showed, by immunolocalisation, the presence of E-cadherin along the cell membranes of all layers of corneal epithelium apart from the basal and apical surfaces (Gipson and Sugrue 1994). It has also been stated that E-cadherin is a key molecule in the establishment and stabilisation of cellular junctions in epithelial tissue (Aberle et al 1996). E-cadherin is a member of a large family of calcium-dependent glycoproteins that are involved in cell-cell adhesion (reviewed by Geiger and Ayalon, 1992). In order to function effectively in cell-cell adhesion, cadherins need to be associated with actin filaments and the activity of the small GTPases RHO family of proteins (Braga 2000). A schematic representation of cadherin-mediated cell adhesion suggests that pairs of complementary cadherin residues on the extracellular domains

of adjacent cells participate in homophilic binding (Mareel et al 1996). The cytoplasmic domain of cadherin then interacts with either ß - or δ-catenin, which in turn interact with α-catenin. It is interesting to note that α-catenin is related to vinculin, which is one of the molecules involved in the connection of membrane proteins to the cortical actin cytoskeleton at sites of cell-substratum contact (Ranscht 1994). The domains of α-catenin which show homology for vinculin have been found to contain binding sites for talin, α-actinin and paxillin-which are all components of focal adhesions (Aberle et al 1996) (focal adhesions are discussed further in section 1.3.2.9). This suggests that these components may bind to α-catenin and be present in cell-cell junctions. Additionally δ-catenin is either identical to, or closely related to, a plakoglobin (a desmosomal protein) (Ranscht 1994).

1.3.2.4 Desmosomes

Desmosomes (also known as maculae adhaerentes or macula adhaerens) are homophilic, disc-shaped intercellular junctions that were first identified by electron microscopy and their morphology was described in a survey of the epithelia from several sources (Farquhar and Palade 1963). In cross section, the membranes of the two interconnected cells appear to be joined by a structure called the desmoglea (Gorbsky and Steinberg 1981), with the cytoplasmic region of the membrane coated by a dense plaque-like structure. Currently immunocytochemistry is used as a means to confirm the presence of desmosomes (Franke et al 1981), as there can be a wide variation from the normal 0.1 - 0.5 μm diameter structure (Koch and Franke 1994) with junctions that may vary both in size and shape (Schwarz et al 1990). Additional to their occurrence in epithelial tissues, the presence of desmosomes has been found in several other sites including mesingeal cells, the follicular dendritic reticulum cells of lymph nodes and in the Purkinje fibre cells of the heart (Schwarz et al 1990). In corneal epithelial tissue, large numbers of desmosomes line the lateral surfaces of the cells, particularly the wing cells. It is assumed that this, together with the presence of many interdigitations, aids the lateral tissue stability (Gipson and Sugrue 1994). One structural feature that identifies the desmosome from other intercellular junctions is the link with the intermediate filaments of the cytoskeleton. In the corneal epithelium, cytokeratin filaments form a loop close to the dense plaque of the junction, possibly linking them to the lateral epithelial membrane (Schwarz et al 1990).

Desmoglein and desmocollin are two subfamilies of transmembrane glycoproteins that are present in desmosomes. Molecules from these families are very closely related to the cadherins, sharing their calcium dependence and sometimes being referred to as desmosomal cadherins (Koch and Franke 1994). Desmocollins I and II are glycopeptides with molecular weight values of about 115 kD and 130 kD and which differ in their carbohydrate patterns (Kapprell et al 1985). The desmogleins are distinguished from the democollins (and E-cadherin) by the presence of a large cytoplasmic domain (Koch and Franke 1994). Three variations of this cytoplasmic domain of desmoglein have been identified and *in situ* hybridization studies of human epidermis suggest differential expression patterns in the various stratal layers of this tissue (Arnemann et al 1993). Similarly, three isoforms of desmocollin have also been identified and are expressed differentially throughout stratal layers of bovine and human epithelial tissue (Theis et al 1993).

The major plaque proteins present in desmosomes are the non-glycosylated cytoplasmic proteins plakoglobin and the desmoplakins I and II (Schwarz et al 1990). The desmoplakins have not been detected in the plaques of any junctional complex apart from desmosomes, so immunolocalisation of these proteins in the cell membrane may be taken as evidence of the presence of desmosomal junctions (Schwarz et al 1990). This is unlike plakoglobin, which has been demonstrated in cells devoid of desmosomes and shown to exist in all cell cultures and tissues tested. An equilibrium exists between the plaque bound form and the soluble molecules, which represents 21-31% of the total protein (Kapprell et al 1987).

1.3.2.5 Gap junctions

Gap junctions are communicating junctions between adjacent cells, that permit the passage of electrical signals and molecules that have molecular weights of up to 2 kD (Paul 1995). The channels are formed by connexins, which are a family of molecules having transmembrane and extracellular domains and molecular weights that can vary from 26 to 70 kD - with the greater variability being in the carboxy terminus (Beyer et al 1989). These molecules form a hexagonal connexon containing a central pore in which the extracellular domains of the connexin on one cell forms a "hemi-channel" which is in line with an appropriate "hemi-channel" in an adjacent cell (Paul 1995). In addition to homologous pairing, connectins are also able to participate in

heterologous pairing in which some of the specificity is under the control of phosphorylation of the cytoplasmic residues (Kendrew and Lawrence 1988). Larger gap junctions have been demonstrated on basal cells than on the more superficial cells in the rabbit epithelium (Gipson and Sugrue 1994; McLaughlin 1985), which may indicate greater communication between the basal cells than between the superficial cells.

1.3.2.6 Integrins in cell-cell adhesion

The integrins are a family of trans-membrane receptors that consist of one α and one β subunit. Many cell-cell and cell-matrix interactions depend upon the interaction of an integrin with a specific ligand (Haas and Plow 1994; Hynes 1992). The most documented integrin-ligand events involve components of the ECM, where binding by specific integrins may result in a response by cytoskeletal elements in the cell (Hynes 1992). The role of integrins in epithelial cell-cell adhesion is less well defined. For example, it was suggested that since the integrins α2β1 and α3β1 were localised mostly along the lateral cell borders of keratinocytes in adult skin, they might be involved in cell-cell adhesion (Kaufmann et al 1989; Larjarva et al 1990; Marchisio et al 1991). Others suggest that these integrins do not play a major role in keratinocyte intercellular adhesion (Jensen and Wheelock 1995), however these findings were based on *in vitro* studies.

In corneal epithelia, some integrins are dispersed within cell membranes. There appears to be a reduction in the number and types of integrins, particularly β1, present as cells differentiate into the apical layer (Gipson and Sugrue 1994). The integrin subunits α2, α3, αv, β1 and β5 may be involved in cell-cell adhesion since their presence in an intracellular location has been demonstrated within the epithelium of the rat cornea (Stepp et al 1993). The role of integrins in cell-ECM interaction will be considered in the following section of this review.

1.3.2.7 Cell-extracellular matrix junctions

Cellular interactions with the ECM are complex events that can initiate a range of cellular responses including attachment, spreading, mitosis and migration. The two major types of junction that link basal epithelial cells with the ECM are

hemidesmosomes and focal adhesions, with integrins being involved with both junctions.

1.3.2.8 Hemidesmosomes

The hemidesmosome is part of the cell-ECM junctional complex that plays the central role in the persistent adhesion of epithelial cells. On the cytoplasmic side of the hemidesmosome, keratin filaments insert into an electron dense plaque that contains "bullous pemphigoid antigen". Bullous phenphigoid antigen is comprised of an intracellular 230 kD protein BPAg1 (Gipson and Sugrue 1994) and a transmembraneous 180 kD protein BPAg2 (Jones et al 1994). The integrin $\alpha 6\beta 4$ forms the trans-membrane component of these junctions and binds to the basement membrane component laminin-5 (Neissen et al 1994) at the large globular "G domain" (Baker et al 1996; Mizushima et al 1997). The structure and assembly of hemidesmosomes is well described in a review article (Jones et al 1998).

When hemidesmosomes are viewed by electron microscopy they appear as punctate, electron-dense regions along the inside of the basal cell membrane adjacent to the epithelial basement membrane. Opposite the plaque of the hemidesmosomes, a thin, electron dense line with fine anchoring filaments stretching from it and extending into the basement membrane may be seen running parallel to and on the outside of the cell membrane (Gipson 1994). Subjacent in the stromal side of the basement membrane from the hemidesmosome are anchoring fibrils that contain type VII collagen (Gipson 1994). These fibrils span the region from the basement membrane to the anterior 1-2 μm of Bowman's layer, where they are associated with small patches of basement membrane-like material forming anchoring plaques (Gipson 1994). This extension of the type VII collagen - containing anchoring fibrils through the basement membrane and the anterior region of Bowman's membrane, where an interweaving with type I collagen occurs, acts to hold both the epithelium and the basement membrane to the stroma (Gipson 1994). Up to 28 percent of the basal cell membrane is occupied by hemidesmosomes in the central cornea (Gipson 1989).

During the natural renewal of corneal epithelial cells, squamous cells are lost from the anterior ocular surface. Homeostasis is maintained by a stem cell population and by mitosis in the basal cell layer limbus (Lavker et al 1991; Schermer et al 1986). As basal cells change their position relative to the basement membrane, hemidesmosomal

junctions are severed and replacement basal cells have been found to reattach to pre-existing collagen VII containing fibrils in the anterior stroma (Gipson 1994).

1.3.2.9 Focal adhesions

On synthetic materials, focal adhesions (also called focal contacts and adhesion plaque) are the points where cells make their closest contacts with the substratum and provide a link between the actin cytoskeleton and components of the ECM bound to the substratum. Weaker, more transient cell-ECM interactions called close contacts can occur. Close contacts are able to initiate cell attachment and spreading or migration, but the formation of focal adhesions is necessary for the development of actin containing stress fibres within a cell (Woods et al 1993).

Focal adhesions have been most extensively studied on fibroblasts, where they are found primarily at cell borders where bundles of actin filaments terminate (Sastry and Horwitz 1993). The transmembrane elements of focal adhesions are the integrins and the association of an α and a β subunit into a dimer creates a specific moiety with an affinity for a particular region of an ECM component (Hitt and Luna 1994; Hynes 1992). A number of cytoplasmic proteins are known to exist in focal adhesions and some have been implicated in the linkages between integrin and the cytoskeleton. These include the 82 kD protein, zyxin and α-actinin which is a 90 kD actin-filament crosslinking protein which is also able to bind both to the cytoplasmic domain of integrin and to the 116 kD protein vinculin (Hitt and Luna 1994). A 220 kD protein known as talin may act as a direct link between the membrane and the cytoskeleton, since it has been shown to bind to both vinculin and the β1 integrin subunit *in vitro* (Hitt and Luna 1994). Paxillin is another cytoskeletal protein which co-localises with integrin and has been shown in association with the signal transducing molecule pp 125^{FAK} (Schaller and Parsons 1994).

The transmission of signals generated by the binding of an integrin to a ligand or the clustering of integrins is dependent upon regulatory proteins such as the tyrosine kinases pp125^{FAK} and pp60v-src, protein kinase C or phosphoproteins (Sastry and Horwitz 1993). Interestingly, it seems that particular signalling pathways may be activated by interactions between several different ligands and different integins (Erickson 1993; Hynes 1994). Further, pp125^{FAK} may be activated by a number of

non-integrin receptors (Burridge et al 1992). This may in part explain why different integrins have been identified by various groups as being implicated in epithelial wound healing. It has additionally been suggested that signalling pathways may be similarly complementary or redundant, so that the inactivation of one pathway may not completely prevent a particular cellular response (Roskelley et al 1995). Finer control of the transduction of some signals is introduced by the requirement for a second message in addition to the binding of integrins to a ligand. This may be by way of the requirement for cellular contact with another polypeptide, as described by Woods and Couchman (Woods and Couchman 1992); alternatively by molecules that modulate the activity of integrins, for example the small lipid, lysophosphatidic acid (Kornberg et al 1991); or by membrane associated protein (Pullman and Bodmer 1992).

1.3.3 Migration of corneal epithelium *in vivo*

In normal unwounded cornea, centripetal migration of epithelial cells occurs (Thoft and Friend 1983). Response to a corneal epithelial wound is therefore an exaggerated response of a normal physiological process. It involves both cellular and subcellular events and is influenced by ECM components and growth factors. Some aspects of the migration of corneal epithelial cells over an onlay lenticule may be expected to be analogous to a wound healing situation. However, some of the "cues" which are present when epithelial cells migrate over a corneal wound would not be encountered in the same way.

Corneal wound healing has been extensively studied *in vivo* and in organ culture using animal models {see recent reviews (Lu et al 2001; Zieske 2001)}. Additionally, much of the research involving the healing of other epithelial wounds also has relevance to the migration of corneal epithelial tissue. The migration of tissue or cells over polymer surfaces *in vitro* presents a useful tool with which to study various factors that may promote or retard epithelial migration. A brief survey of epithelial wound healing and migration is therefore relevant to this study.

1.3.3.1 Corneal epithelial wound healing in vivo

In a review of corneal wound healing, Dua and colleagues identified the following components of the corneal wound healing process: (1) cell migration; (2) cell

adhesion; (3) cell proliferation and (4) cell spreading. The contribution of each part is dependent upon the type of wound (Dua et al 1994). During the initial four to six hours following wounding of the corneal epithelium, no decrease in the wound size is observed. Video microscopy has been used to show an initial retraction of epithelial cells at the edge of a wound. Crosson and colleagues refer to this stage as "the latent phase" (Crosson et al 1986). The intracellular synthesis of structural proteins is increased and actin filaments are polymerised and reorganised during this stage (Gipson and Anderson 1977). Within two hours of wounding, hemidesmosomes which are responsible for the attachment of epithelial cells through the basement membrane and to the underlying connective tissue become disassembled both at the wound edge and from cells up to 70 μm from the wound (Crosson et al 1986). The tight inter-digitations between suprabasal cells are also lost, but some desmosome attachments remain (Kuwabara et al 1976).

It had previously been reported that sliding of suprabasal cells was responsible for healing of epithelial wounds (Kuwabara et al 1976). Other data suggested that migration of basal cells was responsible for wound closure, because lectin binding to the migrating cell layer was characteristic of lectin binding to basal cells and unlike lectin binding to apical epithelial cells (Gipson et al 1983). Cell spreading appears to be an early response to an epithelial wound. Small areas of basement membrane that have been denuded of epithelial cells can initially be covered by flattening of epithelial cells, accompanied by a rise in water content which increases the cell volume (Cintron et al 1981).

The glycoprotein Fn plays a relevant role in corneal wound healing. Fn has been detected at the wound interface of rabbit corneas within eight hours after a wound was made either by scraping off the epithelium, or by superficial keratectomy that also removed the epithelial basement membrane (Fujikawa et al 1984). These deposits of Fn do not persist after wound healing is complete (Fujikawa et al 1984; Nishida et al 1982). Expression of β1 integrin on the surface of epithelial cells has also been shown to correlate with Fn appearance during corneal wound healing (Murakami et al 1992). This suggests that the Fn receptor $\alpha5\beta1$ may be involved in epithelial attachment and migration over the wound area. However, it has also been suggested that the $\alpha6$ integrin subunit may also complex with β1 (Paallysaho et al 1992). Up regulation of

messenger (m) RNA for Fn in wounded rat corneas was demonstrated. Additionally, it was found that there was an increase in mRNA for a Fn isoform that contains the EIIIA segment that is highly expressed in embryonic tissue, suggesting that this isoform may have a specific function in corneal wound healing (Cai et al 1993). The keratocytes in the stroma were shown to be responsible for the production of the alternatively spliced Fn and it was concluded that different Fn isoforms were involved both in normal corneal maintenance and in wound healing (Vitale et al 1994). Vinculin has been shown to have an involvement with wound healing. This protein, was present at levels 27-fold higher in migratory epithelium than in stationary epithelium (Zieske et al 1989). This increased synthesis of vinculin and the location of vinculin at the leading edge of migratory epithelium suggest that vinculin may be involved in cell-cell or cell-substrate adhesion during the migration of a sheet of epithelium in wound coverage (Zieske et al 1989). It has been proposed that calreticulin, which is a 60 kD Ca^{2+}-binding protein, may be involved in regulation of vinculin expression (Opas et al 1996). This view was based on data which showed that expression of vinculin was upregulated in L-fibroblasts which overexpressed calreticulin (Opas et al 1996). However, the involvement of reticulin in the migration of corneal epithelial cells has not been documented. Experiments using an embryonic carcinoma cell line that lacked vinculin suggested that the role for vinculin was in the stabilisation of focal adhesions and transferring mechanical stresses that drive cytoskeletal remodelling (Ezzell et al 1997). So while it is clear that vinculin is implicated in the migration of corneal epithelial cells, the mechanisms of this interaction have not yet been fully documented.

A study that used rabbit epithelial cells in both organ and cell culture indicated that synthesis of neutral glycolipids is increased in migrating corneal epithelial cells (Panjwani et al 1990). Asparagine-linked glycoproteins are also involved with epithelial migration, because the presence of tunicamycin, which inhibits synthesis of asparagine-linked glycoproteins retards wound healing (Gipson et al 1984).

These studies have demonstrated that in the wounded cornea, cues are received by the epithelial cells that produce a change in gene expression as compared with gene expression in non-migrating cells in an unwounded cornea.

1.3.4 The animal model

Tissue and cells of bovine origin were used for the research conducted for this thesis. Bovine eyes are structurally and functionally similar to other mammalian species (Prince et al 1960) and the experiments undertaken in the course of this study, required access to a quantity of tissue that would not have been available from other animal models. The other advantages of this animal model were that additional animal sacrifice was not necessary and a consistent supply was readily available.

The difference between human and bovine corneal tissue that was most relevant for this study was related to the epithelium. In human tissue, the cornea is approximately 550 μm in thickness (Kaufman et al 1988) and the epithelium contains five to six layers of cells (Hogan et al 1971). The bovine cornea varies from approximately one mm in the centre to 400-600 μm in the bulbar conjunctiva and the epithelium comprises 14-17 layers of cells (Evans 1995). These differences were unlikely to impact upon migratory mechanisms and bovine corneas have been used as an effective organ culture model to test polymers for the ability to support epithelial tissue migration (Evans et al 2002). The similarity between epithelial cells from different species was demonstrated in a novel study that showed desmosome formation between epithelial cells from different species (Mattey and Garrod 1985).

Several animal models have been used to study various aspects of corneal epithelial cell and tissue migration over synthetic surfaces. Development of an onlay lenticule depends upon the migration of human corneal epithelial cells. However, animal models can provide valuable insight into factors that may influence tissue and cell migration, provided that care is taken with the extrapolation of data between species. Rabbit corneal epithelial cells and tissue have been widely used in culture systems (Melles and Binder 1992; Panjwani et al 1990; Pettit et al 1992; Watanabe et al 1987; Zieske et al 1994). Chick embryos have provided a source of corneal epithelial tissue for some *in vitro* migration studies (Takeuchi 1979; Takeuchi 1987), as have human tissues from eye banks (Zagorski et al 1990). One reported difference between the migration over synthetic surfaces of epithelial cells from different species concerns calmodulin, which is a multifunctional, calcium binding regulatory protein. This protein was found to play a role in the migration of rat epithelial cells, but the migration of rabbit epithelial cells was not dependent upon an intact calmodulin

pathway (Soong and Cintron 1985a; Soong and Cintron 1985b).

1.3.5 Factors influencing epithelial migration over synthetic surfaces

Some of the factors that may affect the migration of corneal epithelium over synthetic substrata include:-

(1) the surface chemistry properties of the substratum;

(2) the presence of, and orientation of, ECM molecules adsorbed onto the substratum;

(3) the presence of growth factors or motility factors (motogens);

(4) the presence and state of activation of proteases;

(5) the topography of the substratum; and

(6) the porosity of the substratum.

These factors are not mutually exclusive and one factor may modify the effect of other factors. For example, the surface chemistry of a polymer may have an effect on the adsorption and/or the orientation of ECM molecules (Grinnell and Feld 1981; Pettit et al 1992; Steele et al 1993; Underwood et al 1990). Also, the presence of particular ECM molecules may enhance the activity of a specific growth factor (Ruoslahti and Yamaguchi 1991). Additional factors involved may include growth/motility factor inhibitors and protease inhibitors and activators.

1.3.5.1 Surface chemistry

In some cases *in vitro,* the relationship between tissue or cell migration and the chemistry of a surface is related to the adsorption onto the surfaces of cell adhesive factors from the serum component of the culture medium. For example, enhanced migration of bone-derived cells in response to acid treatment of a polystyrene surface was due to increased adsorption of serum Vn onto that surface in comparison with adsorption onto the untreated polystyrene (Dalton et al 1998). In the absence of serum derived cell adhesive molecules, it is likely that modulation of migration by surface chemistry is related to adsorption of endogenous cell adhesive molecules. Polymers that exhibited a mid range of wettability were identified as providing the optimum surface chemistry for the migration of epithelial tissue from a corneal explant when various surfaces were compared (Pettit et al 1990). The study further showed that migration was influenced by both the chemistry of the substratum and by protein-substratum interactions concerned with the addition of exogenous proteins

(Pettit et al 1990). A proposal was made that the binding strength between the substratum and adsorbed Fn may influence the outgrowth of corneal epithelial cells, due to the contractile forces exerted by the cell during migration and mitosis (Pettit et al 1992). Other studies showed correlation between surface chemistry, the binding of a monoclonal antibody that recognises the RGD cell-binding domain of Fn, and tissue migration (Pettit et al 1994). From these findings and a study that examined the effects of the hydrophilicity of porous polymers on epithelial tissue migration (Steele et al 2000), it would appear that the relationship between tissue migration and hydrophobicity correlates with the relationship that has been demonstrated between cell adhesion and hydrophobicity (Grinnell and Feld 1981; Grinnell and Feld 1982; Horbett et al 1988; van Wachem et al 1985; van Wachem et al 1987).

Additional to the effects of the chemical characteristics of substrata, the physical topography can also influence tissue and cell migration. Studies concerning the response of tissue and cells towards two defined types of topographical features were conducted as part of the research present in this thesis and are discussed fully in Chapter 5.

1.3.5.2 Extracellular matrix molecules

In an *in vivo* scrape wound situation, corneal epithelial cells migrate over ECM components. Therefore pre-coating of synthetic surfaces with purified versions of these components seems a logical method by which to stimulate migration. An early study tested laminin, Fn and type IV collagen for their effect on the adhesion and the chemotactic and haptotactic migration of dissociated rabbit corneal epithelial cells (Cameron et al 1988). Fn was shown to be the more effective molecule for promoting cell adhesion but collagen IV was more effective for promoting chemotactic and haptotactic migration. Later it was demonstrated that specific peptides from collagen were implicated in the adhesion and migration of corneal epithelial cells (Cameron et al 1991). Fragments (Mooradian et al 1992) and peptides (Mooradian et al 1993) of the Fn molecule have also been shown to be involved in the attachment, spreading and motility of rabbit corneal epithelial cells. However, specific attachment of either bovine or rabbit corneal epithelial cells to the heparin binding fragment (Hep II) of Fn to promote cell adhesion could not be demonstrated by research conducted in our laboratory (Dalton et al 1995).

Some studies have used combinations of ECM components, in order to increase epithelial cell migration. For example, it was shown that the addition of both Fn and sodium hyaluronate to cultured explants of rabbit cornea enhanced epithelial migration down the stroma (Miyauchi et al 1990). The stimulatory effect of hualuronan on this type of migration was later shown to be independent of epidermal growth factor (EGF) and Fn (Nishida et al 1991). Hyaluronan has also been shown to enhance the adhesion of rabbit corneal epithelial cells to Fn-coated surfaces (Nakamura et al 1994).

1.3.5.3 Growth and motility factors

The effects of growth factors on corneal epithelial cell migration have been more extensively studied *in vivo* and in organ culture models, than in cell culture. These studies have provided valuable data for practical applications during wound healing, but identification of the specific mechanisms involved are more difficult to interpret than with simple mono-culture systems. A brief overview of some of these studies, while not directly applicable to epithelial migration over a synthetic surface, will provide insight into possible factors that may influence this type of migration.

1.3.5.3.1 Epidermal growth factor

EGF is a peptide growth factor which was originally isolated from mouse submaxillary gland (Cohen 1962), but appears to be present in several tissues and biological fluids (Stoker and Gherardi 1991). EGF was implicated in corneal wound healing as early as 1973 (Savage and Cohen 1973). More recent studies have investigated an interaction between and EGF and Fn in corneal wound healing and EGF was shown to enhance the migration of rabbit corneal epithelial cells down the cut surface of stroma in organ cultures (Watanabe et al 1987). These authors demonstrated that such enhanced migration in response to EGF was due to increased cell proliferation. In the same study, Fn was shown to promote migration by facilitating the "sliding" of epithelium down the surface of the cut stroma. Nishida and colleagues extended the investigation of the interactions between Fn and EGF in epithelial cell migration. They used a similar model system to Watanabe and showed that exposure to EGF for over six hours was necessary for a stimulatory effect to be observed (Nishida et al 1990). Furthermore, they also demonstrated that the effects of

EGF were dependent upon Fn because the presence of anti-Fn antiserum reversed the EGF stimulated increase in cell migration. These authors suggested that EGF increased the expression of Fn receptors on rabbit corneal epithelial cells. The EGF stimulated attachment of these cells to Fn was inhibited both by antibodies to Fn and by a synthetic peptide GRGDSP, which mimicked the amino acid sequence of the cell binding domain of Fn (Nishida et al 1992). Fn was found not to increase the rate of epithelial cell migration in a wound closure model (Soong et al 1989), as it had on a cut stromal surface (Watanabe et al 1987). This suggests that the epithelial basement membrane provides a more suitable substratum for epithelial migration than does the collagen of the stroma. Fn binding to the stroma could enhance epithelial migration, whereas Fn binding to the basement membrane did not create a matrix that enhanced migration. Rabbit and rat corneal epithelial cells in cultures were used to demonstrate that EGF promoted mitosis and not motility (Soong et al 1990). They confirmed this by showing that the EGF accelerated rate of epithelial wound closure in an organ culture model was negated by the presence of colchicine, which arrests mitosis (Soong et al 1990). These studies indicated that the direct effect of EGF on corneal epithelial cells was to promote mitosis rather than motility. However, another research group, using a Boyden chamber assay, showed that EGF actually did enhance epithelial cell motility in their system (Grant et al 1992). The results indicate that different assay systems may be needed to demonstrate effects that may be subtle and may not be apparent in the presence of more powerful stimulators of migration.

1.3.5.3.2 Transforming Growth Factor-α

In common with EGF, the transforming growth factors TGFs-α are a group of polypeptide growth factors. Human corneal epithelial cells synthesise TGF-α and it is suggested it that may act through an autocrine pathway to influence these cells (Schultz et al 1992).

1.3.5.3.3 Transforming Growth Factor-β

TGF-β was originally purified as a homodimer from platelets and three different forms have been isolated (Stoker and Gherardi 1991). TGF-β acts as a growth inhibitor on most cell types (Stoker and Gherardi 1991) and EGF stimulated migration of rabbit

corneal epithelial cells down the side of a cut stromal surface was inhibited by TGF-β (Mishima et al 1992). The apparent effects of TGF-β, like the effects of EGF, depend upon the type of the assay in which it is used. For example TGF-β was found to be a potent inducer of lysyl oxidase mRNA levels in human scleral fibroblasts, which may have induced upregulation of ECM components (Schultz et al 1992). This may, in turn, have follow-on effects upon events such as migration.

An interesting series of experiments that used Boyden chambers and "checker board" analysis to study the migration of pure populations of corneal epithelial cells demonstrated that TGF- β stimulates migration of epithelial cells (Grant et al 1992). This analysis also demonstrated that chemotaxis only occurred in a response to a specific dose range. In a recent study we found that pre-coating of microgrooved or planar surfaces with either TGF-β 1, TGF-β 2 or TGF-β 3 reduced the extent of migration of bovine corneal epithelial tissue (Walboomers et al 2002). The reduction in migration was directly related to the coating concentration and TGF-β 1 and TGF-β 2 caused greater a reduction in migration than did TGF-β 3. However, even though the extent of migration was reduced by TGF-β, the migration remained directed by the microgrooves (Walboomers et al 2002). The responses of epithelial tissue or cells towards TGF-β in these various studies reiterates how the style of an assay can effect the cellular response towards growth factors.

1.3.5.3.4 Hepatocyte growth factor/scatter factor

Hepatocyte growth factor (HGF), also known as 'scatter factor', was discovered as a protein secreted by fibroblasts that could induce scattering of epithelial cell colonies (Stoker and Gherardi 1991). HGF was separately identified as a serum mitogen that acted upon hepatocytes in culture (Stoker and Gherardi 1991). HGF and scatter factor have since been shown to be identical molecules (Weidner et al 1991; Naldini et al 1992) comprised of disulfide-linked heterodimer of a 55-65 kD α and a 32-36 kD β subunit (Weidner et al 1990).

HGF has been shown to promote the proliferation of human corneal epithelial and endothelial cells, but not keratocytes (Wilson et al 1993). Corneal keratocytes and endothelial cells produce greater levels of mRNA encoding for HGF than do epithelial cells (Wilson et al 1993). In this way, HGF may play a role in epithelial growth from

corneal explant cultures that is not played in cultures of pure epithelial cells. HGF has been clearly demonstrated to be a "motogen" (Stoker and Gherardi 1991) and this cytokine may have the ability to promote epithelial migration over a synthetic substratum. Such migration may rely upon a pathway involving TGF-β1, because anti-TGF-β1 antibodies negate the stimulator effects of HGF on the repair of gastrointestinal "wounds" in an *in vitro* model (Dignass et al 1994).

1.3.5.3.5 Interleukin 6

Interleukin 6 (IL-6) is a 26 kD cytokine that is secreted by a number of cells, including fibroblasts (Stoker and Gherardi 1991). Nishida and colleagues demonstrated an increase in the migration of epithelial cells down the cut edge of corneal blocks in response to IL-6, which can be abrogated by the presence of anti-Fn antibodies or a peptide that contains RGD (Nishida et al 1992). The study also showed that IL-6 increased the number of epithelial cells that attached to Fn, collagen IV or laminin coated surfaces after 45 minutes incubation. These authors suggested that the increased migration was due to increased expression of Fn receptors on the epithelial cells. It was surmised that the enhanced attachment to collagen and laminin might indicate an additional mechanism of migration. This study did not differentiate in any way between migration and mitosis of epithelial cells. Furthermore, the presence of both endothelial cells and keratocytes in the corneal explants leaves open the possibility that IL-6 stimulated those cells to produce factor/s that may influence the migration or mitosis of epithelial cells.

1.3.5.4 Proteases

The involvement of proteases in migration of corneal epithelial tissue over a synthetic surface is not directly addressed in the literature, but it is reasonable to assume that proteases may play a role in coverage of a lenticule *in vivo*. Tear fluid, epithelial cells, keratocytes and/or macrophages could all be *in vivo* sources of proteases. The action of the proteases may be advantageous or detrimental to obtaining epithelialisation of a lenticule. Remodelling of ECM components that may have been pre-coated onto the surface, or secreted by the epithelial cells, may promote or retard migration. In addition to direct effects on the ECM, proteases may also influence growth factors. For example, the full biological activity of HGF is reported to depend

upon proteolytic conversion to the heterodimeric form (Naka et al 1993). Serum serine proteases may produce this conversion in assay systems in which serum is present (Naldini et al 1992), or in the absence of serum the conversion could be due to serine protease production by cells (Naka et al 1993; Hernandez et al 1992).

The use of rats deficient in vitamin-A demonstrated the involvement of plasminogen activator in the healing of corneal epithelia wounds (Hayashi et al 1988). Further studies clarified this involvement to show a dependence upon urokinase type plasminogen activator in the migration of a sheet of epithelium down a cut stromal block (Morimoto et al 1993)". It was suggested that "the migration of corneal epithelial cells requires not only cell attachment to the extracellular matrix through fibronectin but also degradation of the fibronectin by the release of cellular urokinase plasminogen activator (Morimoto et al 1993). A separate investigation concluded that serine proteases were involved directly in epithelial migration, because the protease inhibitors aprotinin and PMSF reduced migration to a greater extent than was caused by inhibition of protein synthesis alone (Zieske and Bukusoglu 1991).

1.3.6 MECHANICS OF TISSUE AND CELL MIGRATION

In this section of the literature review, the focus is on the current understanding of the mechanisms underlying the ability of tissue and cells to migrate over synthetic surfaces. The cytoskeleton is an integral component of these mechanisms, therefore an overview of elements of the cytoskeletal is presented first. Where appropriate, this will be related to the corneal epithelium. Secondly, aspects of cell migration are considered and finally, the literature that has addressed the migration of an intact epithelial tissue sheet is reviewed.

1.3.6.1 The cytoskeleton

The cytoskeleton acts as the "bones" of a cell and besides the critical role in migration the cytoskeleton also mediates organelle transport, cell polarity, mitosis, cytokinesis, secretion and the maintenance of cell integrity. Three major cytoskeletal fibres have been identified, each having different mechanisms of assembly and being associated with different proteins. These structures do not act alone and various interactions between them have been identified (Chou et al 2001; Goldman et al 1999; Liao and Gundersen 1998).

1.3.6.1.1 Microtubules

The microtubules have the greatest diameter of the cytoskeletal components. They are fibres, which are composed mainly of α and β tubulin subunits that each have a molecular weight of about 55 kD. The subunits are organised in a helical array. In cross section, the microtubules appear as hollow, rigid tubes of about 24 nm in diameter with walls made up of 13 globular subunits composed of the α and β heterodimers, each 4-5 nm in diameter (Darnell et al 1984). Microtubules are dynamic, polarised organelles in which assembly and disassembly occurs by the addition to the "plus" end or the removal from the "minus" end of tubulin subunits. In cultured cells, microtubule turnover is normally very rapid, with a half-life averaging 5-10 minutes (Schultz and Kirschner 1986). However, a small subset of more stable microtubule may be formed as cells begin to migrate (Gundersen and Bulinski 1988). These longer lasting tubules were identified using a monoclonal antibody that recognised a post-translational modification of α-tubulin that was detyrosinated (Gundersen et al 1984). The detyrosinated tubules that were generated in response to the wounding of a cellular monolayer were shown to have greater resistant to the microtubule depolymerising drug nocadazole (Gundersen and Bulinski 1988).

Within corneal epithelial cells, microtubules function in mitosis, as they do in other cell types, by forming the mitotic spindle which organises the chromosomes and cytoplasm (Darnell et al 1984). Additionally, a specific microtubule distribution has been demonstrated in interphase polarised epithelial cells. This includes a dense mat of short microtubules between the apical membrane and the Golgi apparatus and bundles of microtubules oriented longitudinally with their minus ends pointed towards the apical membrane and the plus end towards the base of the cell (Bacallao et al 1989). Disruption of microtubules in the MDCK and Caco-2 epithelial cell lines, and mouse intestinal epithelial cells using nocodozol or colchicine showed that microtubules facilitate delivery of transport vesicles from the Golgi complex to the apical membrane domain (Mays et al 1994). It is likely that microtubules play a similar role in corneal epithelial cells. Supporting this view, intact microtubules were necessary to enable corneal epithelial cells to attach to a synthetic substratum in the absence of exogenous cell-adhesive glycoproteins (Evans and Steele 1997). This

suggested that the microtubules were required to deliver ECM molecules to the substratum so that sites were available for epithelial cell attachment.

Early studies suggested that microtubles may not have been *essential* for cell movement but played a role in *directed* migration (Schliwa and Honer 1993). Supporting this supposition were two findings: firstly, that disruption of microtubules over a 30 minute period caused no visible effect on the ultrastructure of migrating corneal epithelial cells in an organ culture wound (Gipson and Keezer 1982); and secondly, that stimulation of corneal epithelial migration by Fn was independent of microtubules unlike EGF stimulation of migration, which proved to be dependent upon microtubule function (Nakamura et al 1991). Microtubules have been implicated in the polarisation of migrating endothelial cells responding to an *in vitro* wound (Schliwa and Honer 1993). Schliwa also suggests that cultured cells which are not in primary culture, and larger cells, may have a greater requirement for microtubules for migration than do cells in primary culture and smaller cells (Schliwa and Honer 1993). Insight into a mechanism by which microtubular dynamics exert an influence on cell migration has been obtained from research that examined the effect of low doses of microtubule inhibitors on lamellipodia formation. The study supported a view that microtubules contributed to migration by controlling the size of lamellipodia, possibly by the delivery of diffusible factors to the base of lamellipodia (Mikhailov and Gundersen 1998); and see review (Small et al 1999).

1.3.6.1.2 Actin filaments

Actin filaments (F-actin) are assembled from globular subunits of G-actin, which have a molecular weight of 42 kD, to form a double -helical fibre about 7 nm in diameter (Darnell et al 1984). Both the polymerised actin filaments and the monomer subunits are polarised, with one end classed as the "barbed end". Polymerisation and depolymerisation of F-actin is under the control of "capping" proteins that prevent the addition or removal of subunits and "fragmenting" proteins which can break up long actin filaments (Darnell et al 1984). Additional control of F-actin is imparted by proteins that bind to the sides of the actin filaments. Tropomyosin, which binds to multiple subunits, decreases the rate of subunit loss from the ends of actin filaments and conversely ADF/cofilin increases the rate of subunit loss (Cooper and Schafer 2000). About half the total actin in animal cells is polymerised into filaments at any

one time and these are organised into at least three types of structure (Sheterline 1993). Within cultured cells, the stress fibres are the most prominent structure and contain several hundred loosely packed actin filaments that are often organised to be predominantly parallel to the major axis of locomotion (Sheterline 1993). Parallel bundles of actin filaments also form a second type of actin structure. Within these structures, the actin filaments are tightly packed and are uniformly polarised with their barbed ends towards the plasma membrane (Sheterline 1993). The filament bundles are found within the filopodia of migrating cells. The third arrangement of F-actin is the cortical web of filaments that is located over the cytoplasmic surface of the plasma membrane (Sheterline 1993). The cortical web contributes to the mechanical properties of the cell surface (Bray et al 1986), is implicated with membrane solute transport (Bennett 1985), is involved with the signal transduction apparatus at the plasma membrane (Goldschmidt-Clermont et al 1991) and possibly acts as the precursor for actin filament bundles in the leading lamellipodium (Sheterline 1993).

The role of actin in cell motility is complex. Aspects of the mechanism underlying the extension of lamellipodia, that have been elucidated using the bacteria *Listeria monocytogenes* and the yeast *Saccharomyces cerevisiae* as model systems, have been reviewed by Welch and colleagues (Welch et al 1997). Actin filaments at the leading edge undergo a continuous cycle of assembly at the surface of the inner-membrane, transport away from the membrane and depolymerisation (Heath and Holifield 1991).

Links between actin and microtubules were identified in research that suggested that actin dynamics may cause microtubule polarisation (Waterman-Storer and Salmon 1999). The results indicated that growth of microtubules at the leading edge, and shortening at the rear of the cell, may activate signals which in turn feed back to the actin to reinforce polarised actin assembly and breakdown (Waterman-Storer and Salmon 1999).

It is clear that F-actin plays a major role in the motility of corneal epithelial sheets in organ culture (Gipson et al 1982) and in tissue culture (Soong and Cintron 1985a), since the prevention of actin polymerisation using cytochalasin inhibits cellular migration.

1.3.6.1.3 Intermediate filaments

Intermediate filaments are approximately 10 nm in diameter, putting their size in between that of microtubules and actin filaments. They contribute to the maintenance of cellular integrity by their interaction with the cell surface and with other elements of the cytoskeletal system. There is great diversity within intermediate filaments, there being over 50 different gene products divided into six classes (Chou et al 1997). The roles of intermediate filaments were defined later than were the roles of actin filaments and microtubules, principally because there were no drugs that could perturb their function. From genetic studies of mice and humans that had defects in particular intermediate filament genes, the relevance of intermediate filaments in cell-cell and cell-ECM adhesion has been demonstrated (Fuchs and Cleveland 1998; Herrmann and Aebi 2000). In corneal epithelial cells, as with other epithelia, the principle intermediate filaments are the keratins (Cooper et al 1985). In general, a specific basic keratin is co-expressed and paired with a particular acidic keratin (Cooper and Sun 1986). The particular keratins expressed are dependent upon the differentiation state of the cells and expression of specific keratins occurs in differentiated corneal epithelial cells (Kurpakus et al 1990). A change in keratin expression has been found during epithelial migration over a corneal wound (Yu et al 1995). Also vimentin, which is a type of intermediate filament not normally found in resting corneal epithelium, is expressed in tissue culture and migrating corneal epithelia (SundarRaj et al 1992).

1.3.7 Mechanisms of cell migration

Some of the most detailed examinations of the migratory mechanisms of eucaryotic cells have been directed towards understanding the locomotion of fibroblast over synthetic surfaces. However the basic principles underlying fibroblast migration also have relevance for epithelial migration. At the leading edge of migrating fibroblasts, broad flattened lamellipodia and narrower filipodia are formed. Within these structures are actin filaments, where polymerisation occurs with the "plus" ends orientated towards the advancing cell membrane (Lauffenburger and Horwitz 1996; Welch et al 1997). The transmission of signals that initiate the actin polymerisation can occur by means of GTPases, which mobilise actin binding proteins that expose the actin filament plus ends (Schwarzbauer 1997). Adhesion receptors diffusing in the

cell membrane in the extended lamillipodia or filopodia can bind to molecules on the substratum (Sheetz et al 1998). Focal adhesions may be formed where integrins in the cell membrane bind to ligands on the underlying substratum, bringing the cell membrane in close association with the underlying surface. Vinculin, α-actinin, tensin, talin and paxillin are structural proteins which may also be present in focal adhesions and be involved in the linkage between integin and the actin cytoskeleton (Burridge et al 1988; Turner and Burridge 1991). The cytoplasmic domain of the integrin β1 subunit is needed in the linkage of the actin cytoskeleton to other components of focal adhesions (Sheetz et al 1998). It is believed that organised linkage that connects the cell membrane and cytoskeleton is necessary for cell migration (Huttenlocher et al 1995) and the role of integrins in the promotion of the intracellular signals that stimulate cell movement is covered in a review (Holly et al 2000). An inverse correlation between the number of focal adhesions and the rate migration in fibroblasts has been demonstrated (Dunlevy and Couchman 1993). This study identified the more transient "close contacts" as being the necessary sites of adhesion between the leading edge of the cell and the substratum during migration (Dunlevy and Couchman 1993). Close contacts are the major type of cell-substratum adhesion sites in more rapidly moving cells such as fish keratocytes (Lee and Jacobson 1997). Close contacts may be closely related structures to focal adhesions because the newest regions of close contact at the leading edge of a cell contain some of the components in common with focal adhesions such as β1 integrin and talin (Lee and Jacobson 1997). Older regions further back from the leading edge which have been in contact with the substratum for a longer time additionally contain vinculin and α-actinin (Lee and Jacobson 1997).

One hypothesis to explain the mechanism of cell migration was proposed by Sheetz and colleagues (Sheetz et al 1998). They suggested that after lamellipodial extension, actin polymerisation and the formation of the focal adhesions linking the actin cytoskeleton to the substratum bound molecules, there was contraction of actin, probably by means of myosin II (Luna and Hitt 1992). When release of adhesion receptors at the rear of the cell occurred during contraction, forward displacement of the cell would also occur. Within this model, the release of receptors at the rear of the cell is critical for cell migration and two major mechanisms have been identified that facilitate receptor release (Sheetz et al 1998). These are mechanical release and

enzymic release (Crowley and Horwitz 1995). In mechanical release, up to 80% of β1 integrin may be left behind on the substratum within tracks from a migrating fibroblast (Palecek et al 1996). Enzymic release may be due to dephosphorolation of integrin breaking the linkage between integrin, actin and substratum-bound molecules (Lawson and Maxfield 1995). Adhesion receptors are then recycled to the front of the cell by endocytosis and vesicular transport or forward-directed movement on the cell surface (Sheetz et al 1998). This model is illustrated in Figure 1.3. Within the model there is a requirement for the coordination of adhesion, both temporally and spatially (Cox and Huttenlocher 1998). While various possible mechanisms have been identified for the regulation of adhesion and adhesion release, the means for the integration of these events are not yet clarified (Cox and Huttenlocher 1998).

A related hypothesis for fibroblast migration was reviewed by Mitchison and Cramer (Mitchison and Cramer 1996). Within this model, which was originally proposed by Bray and White (Bray and White 1988), the actin contraction that was generated occurred in a polarised manner. Thus actin filaments attached to the substratum acted as tracks over which the rest of the cell was pulled (Mitchison and Cramer 1996). Basically, the major difference between these two proposed models is the direction of contraction.

It is likely that mechanisms of migration similar to the Sheetz (Sheetz et al 1998) or Bray and White models operate when close contacts rather than focal contacts are the major cell-substratum adhesions (Bray and White 1988). Migrating fish keratocytes extend a semicircular shaped lamellar in which close contacts form at the outer rim (Lee and Jacobson 1997). These cells move with a persistent gliding motion and a graded distribution of extension and retraction rates along the cell margin maintains the cell size and shape during movement (Lee et al 1993). This suggests efficient, graded, regulation of adhesion, cell contraction and adhesion release throughout the ventral cell surface.

1.3.8 Mechanisms of tissue migration

The mechanisms underlying the movement of intact sheets of epithelial tissue over synthetic surfaces have not been as extensively studied as have the migration mechanisms of individual cells. However, some aspects of the basic mechanistic

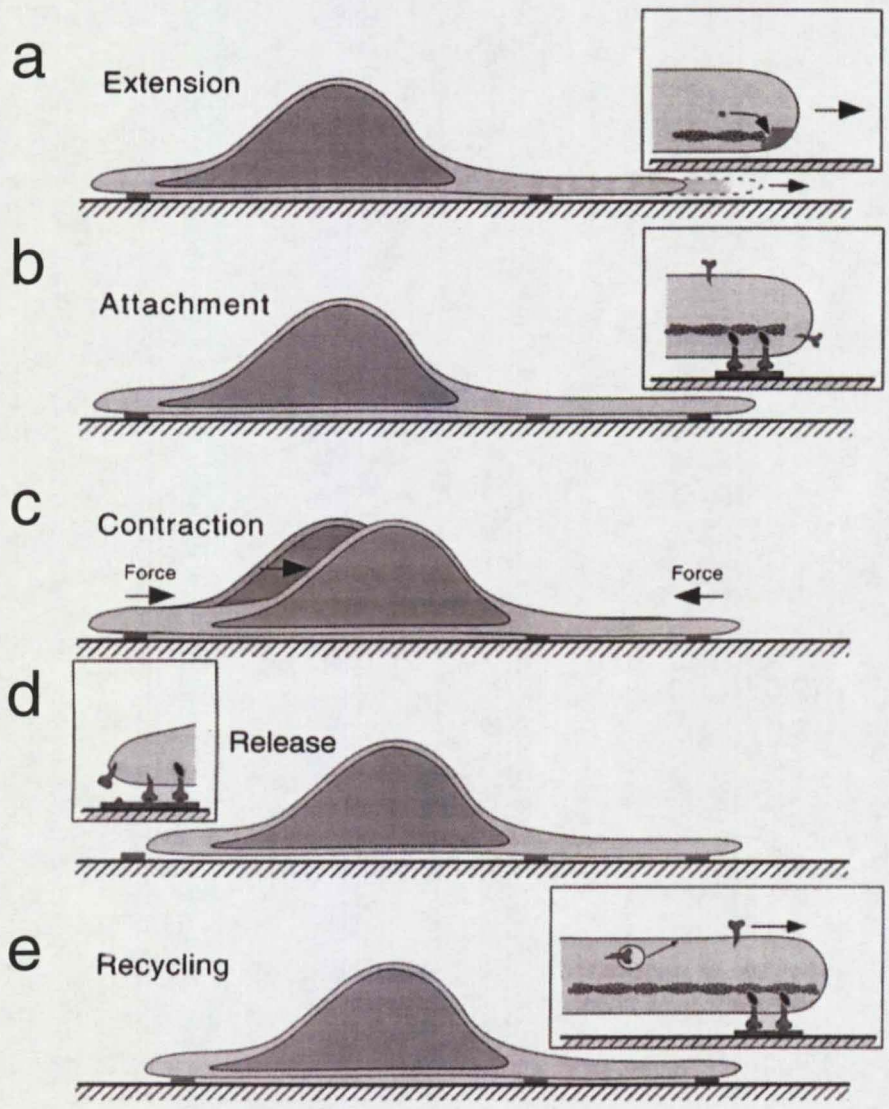


Figure 1.3 Steps of cell migration:

(a) Extension by assembly of actin filaments;

(b) attachment of integrins to substratum bound ligands;

(c) contraction of the cell towards the nucleus;

(d) release of adhesion receptors at the rear of the cell, with cell displacement in a forward direction;

(e) recyling of adhesion receptors to the front of the cell.

From Sheetz et al (1997) Cell migration as a five-step cycle. *Biochem Soc Symp* 65:233-243.

events which occur in cell migration are clearly applicable in tissue migration. Early *in vitro* studies concerning the migration of epithelial sheets over synthetic surfaces indicated that there was cooperation between component cells (Vaughan and Trinkaus 1966). Migration of epithelial sheets of embryonic chick corneal epithelium over glass surfaces was proposed to be caused by the marginal cells (meaning cells at the "leading edge"). This proposition was based on the location of cell adhesions to the substratum (DiPasquale 1975a; DiPasquale 1975b). The submarginal cells (those not at the leading edge) were thought to be dragged passively, because of their limited adhesion to the substratum (DiPasquale 1975a). The evidence for this limited adhesion to the substratum included the migration of fibroblasts under the submarginal epithelial cells, the presence of 2000A gaps between the basal cell membrane and the substratum, and rapid retraction of submarginal cells when a microneedle was used to break the adhesion of the marginal cells to the substratum (DiPasquale 1975a). However, an alternative migratory mechanism for tissue sheets was proposed by Mahan and Donaldson who examined the *in vivo* migration of newt epidermal tissue over several surfaces that had been coated with either fibrinogen or collagen I (Mahan and Donaldson 1986). They found that the basal cells contributed to the movement of the tissue sheets and that basal cells in all positions of the tissue sheet exhibited a similar migratory behaviour to that of the leading edge cells. Basal cells extended lamellipodia in the direction of the migration, and lamellipodia of the submarginal cells maintained contact with the substratum (which would be necessary to generate forward movement) by underlapping the cells in front. The interaction of the submarginal cells with the substratum was demonstrated by the adhesion of these cells to the substratum that was observed when regions of the tissue sheet were pulled from the substratum (Mahan and Donaldson 1986).

An alternative mechanism for tissue migration was proposed by Takeuchi, based on studies of corneal epithelial tissue from chick embryos that were migrating over Millipore™ filters (Takeuchi 1987). In this model, the epithelial tissue consisted of two layers of cells, the basal cells that were in contact with the substratum and the peridermal cells that constituted the anterior surface. The proposal was that the peridermal marginal cells migrated over the basal cells and reached the substratum, focal adhesions were formed and the leading cells exerted tension on the adjacent

peridermal cells which followed and also made contact with the substratum (Figure 1.4) (Takeuchi 1987).

For other information concerning the mechanisms of migration of epithelial tissue it was necessary to reconsider aspects of wound closure (Section 1.3.3.1). One of the major differences between these studies and tissue migration over synthetic surfaces was that migration during wound healing was taking place over native biological surfaces. Therefore the response of the tissue may have been to various cues that are not available on a synthetic surface.

A series of options for the mechanism underlying the migration of corneal epithelial tissue during wound healing have been examined (Gipson and Keezer 1982). In one study, an intact actin filament system was shown to be necessary for sheet movement in a situation where murine corneal epithelial tissue migrated over a debrided basement membrane (Gipson and Keezer 1982). This actin network was present only within cells at the leading edge and it was proposed that the leading edge (marginal) cells provided the force for sheet migration (Gipson and Anderson 1977).

A specific mechanism known as a "purse sting" has been identified as being involved in the movement of tissue in the closure of embryonic skin wounds (Martin and Lewis 1992). In these wounds an actin filament cable assembles in the basal layer of epidermis at the wound edge and myosin II is involved in contraction of the cable (Brock et al 1996). A similar mechanism has been observed in the closure of debridement wounds in mouse cornea *in vivo* (Danjo and Gipson 1998). The cell-to cell adhesion molecule E-cadherin is implicated in the linkage of the actin cable between adjacent cells (Danjo and Gipson 1998). This suggested that the cell-cell junction associated with the actin filament cable was an adherence junction (Danjo and Gipson 1998. Another mechanism implicated in corneal wound closure was described by Dua and colleagues. They described the production of lamellipodial extension by the leading edge epithelial cells at the wound margin, but the mechanisms of migration the following tissue sheet were less well defined (Dua et al 1994).

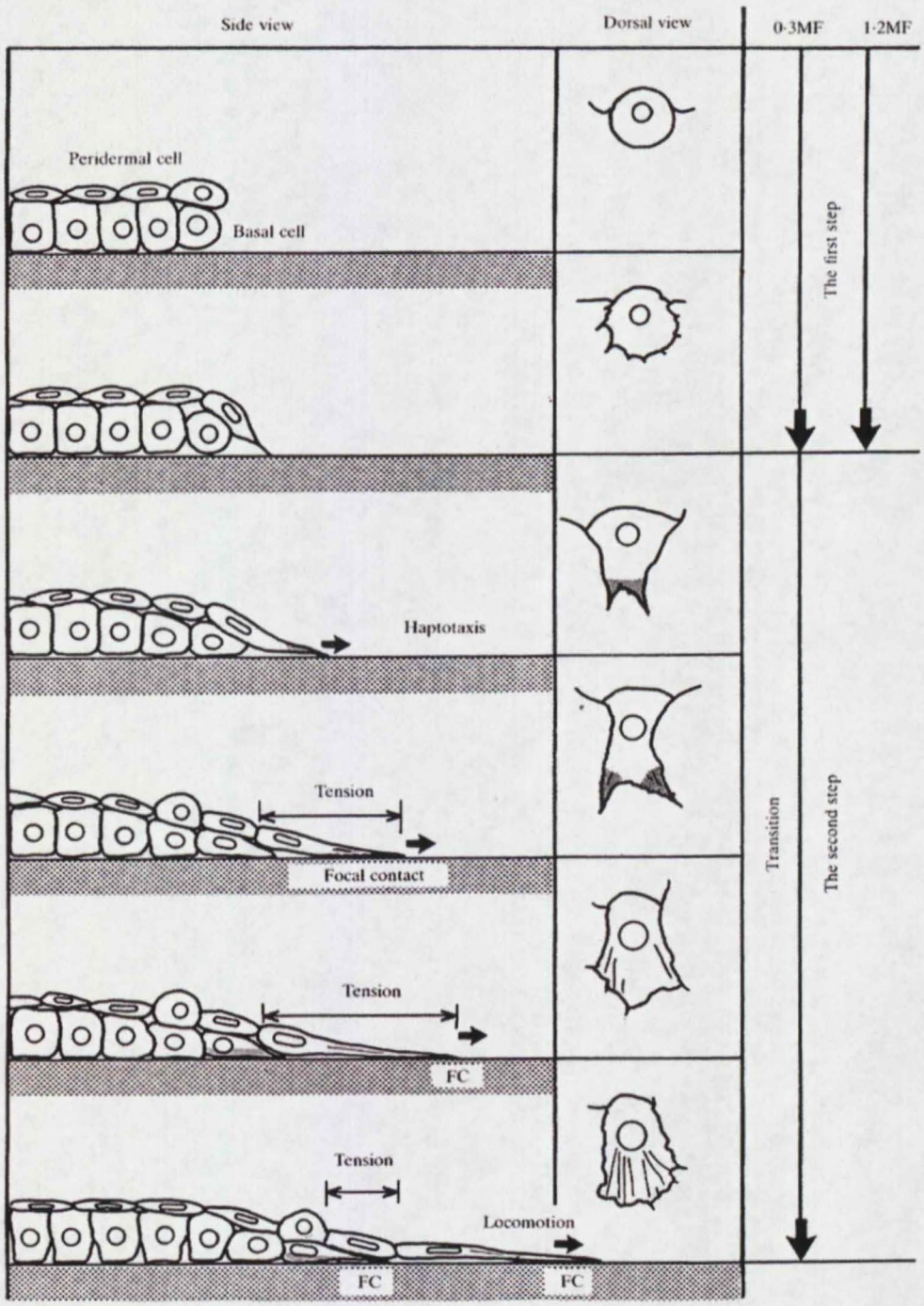


Figure 1.4 Illustration of the epithelial tissue migration model proposed by Takeuchi. The alignment of F-actin in the marginal cells is shown in the right hand panel. FC, focal contacts.

From: Takeuchi, S. (1987) The rearrangement of cytoskeletal systems in epithelial cells accompanying the transition from a stationary to a motile state at the start of epithelial spreading. *J Cell Sci* 88:109-119.

1.3.9 Possible migratory mechanisms of intact epithelial tissue over a synthetic surface

From the literature the possible modes of migration over a synthetic polymer for an intact epithelial tissue include:-

(1) cells at the leading edge provide the force which pulls the following cells along (as proposed by Vaughan and later by Dipasquale (DiPasquale 1975a; DiPasquale 1975b; Vaughan and Trinkaus 1966);

(2) proliferation or expansion of cells within the tissue sheet push the leading edge cells forward;

(3) within the tissue, each basal cell migrates as a separate entity as proposed by Mahan and co-workers (Mahan and Donaldson 1986);

(4) cells situated anterior to the basal cells migrate over the basal cells and over the substratum (Takeuchi 1987);

(5) a combination of any of the above, such as the leading edge cells pull but are also pushed by forces further back, or the leading edge cells pull but some cells migrate as separate entities.

These possibilities will be investigated in the course of this thesis.

CHAPTER 2

MIGRATION OF CORNEAL EPITHELIAL TISSUE AND CELLS *IN VITRO:* DEVELOPMENT AND TESTING OF MODEL SYSTEMS

2.1 INTRODUCTION

A prerequisite for the study of migratory mechanisms in intact corneal epithelial tissue and dissociated corneal epithelial cells was the development of models. One requirement for the models was that the extent of migration could be readily measured, after specific culture periods. This would show whether or not migration had occurred and would also provide a means to identify any difference between tissue and cells in their response towards specific surfaces. A different response between tissue and cells would suggest the operation of different migratory mechanisms and could be further investigated.

The method chosen to study the migration of intact epithelial tissue was to use a corneal explant which consisted of the intact stratified epithelium and a thin layer of stromal tissue. The intact epithelial tissue migrated down the cut side of the stroma and over the underlying culture surface in a radial direction. The refinements of this model for bovine corneal epithelial tissue migration were developed by Mr. Graham Johnson {Commonwealth Scientific and Industrial Research Organisation (CSIRO), Molecular Science} from previously described models using rabbit (Pettit et al 1992) and chick embryo (Takeuchi 1979) corneal tissue. The cell migration model was similar to the tissue migration model, in that the cells migrated radially outwards from a central source. These cells were seeded into the lumen of a stainless steel and silicon fence so that they initially formed a confluent monolayer of a defined size. Removal of the fence enabled the cells to migrate outwards from the circular area. This method was adapted by Dr PA Underwood from an assay that was developed as a means to measure the extent of migration of endothelial cells (Fischer et al 1990). The migration of a murine lung epithelial cell strain (Dalton et al 1996) and human bone derived cells (Dalton et al 1998) have also been measured using this type of assay. The rational of this technique for the measurement of cell migration has been investigated in a methodology review article (Manske and Bade 1994).

In addition to the migration or translocation of tissue and cells in these model systems, proliferation and cell spreading are also likely to contribute to the measurements made in this study. This will also be the case in the coverage of the corneal onlay device by host epithelial tissue, that is the basis of the Artificial Cornea project supporting this research. Here, while the phenomenon measured is referred to as "migration", it is acknowledge that the terms "outgrowth" "expansion" and "coverage" have also been used in connection with the model systems described in this thesis.

In vivo, movement or migration of corneal epithelial cells over the epithelial basement membrane is an ongoing phenomenon. Desquamated cells from the anterior surface of the cornea are replaced by a population of slowly proliferating stem cells which are located in the basal layer cells of the limbus (Cotsarelis et al 1989; Lavker et al 1991; Schermer et al 1986). Centripetal migration of corneal epithelial cells occurs (Buck 1985) and movement of basal cells from the periphery towards the centre of the cornea has been observed *in vivo* (Koester et al 1993). In wound healing situations, the movement of corneal epithelium is more rapid than the centripetal migration which maintains homeostasis. In the case of a debridement wound, the epithelium migrates over the denuded basement membrane (Fujikawa et al 1984). With a deeper corneal wound, which involves damage to the stroma, the epithelium migrates over stromal tissue in which the major constituent is collagen I (Fujikawa et al 1984). In addition there is an influx of Fn (Murakami et al 1992) {reviewed by (Gipson et al 1993)} which together with fibrin/fibrinogen provides a matrix for epithelial migration (Fujikawa et al 1984). From cell culture (Cameron et al 1988; Cameron et al 1991; Nishida et al 1990; Watanabe et al 1987) and *in vivo* wound healing studies (Fujikawa et al 1984; Gipson et al 1993), it appears that epithelial tissue or cells have the capacity to migrate over various ECM components and the response may be to a range of cell recognition sites within these matrices.

In order to study epithelial migration over synthetic surfaces a logical first step was therefore to pre-coat the surface with specific ECM molecules. The choice of surface coatings was directed towards molecules that the epithelium is known to migrate over *in vivo*. Laminin (Ln) and collagen IV were selected as molecules for pre-coating the culture surface because they are characteristic components of basement membranes. Fn and collagen I were used for pre-coating because of their significance when corneal

epithelial tissue migrates over deeper corneal wounds. The medium that was routinely used for the culture of corneal epithelial cells was usually supplemented with serum. Fn and vitronectin (Vn) are glycoproteins that are present in serum and can become coated onto culture surfaces (Grinnell and Feld 1981) and provide attachment sites for cells. When the culture surface is tissue culture polystyrene (TCPS), Vn in particular becomes coated onto the culture surface (Steele et al 1993a). In developing the model systems for use in this study, the aim was to identify ECM molecules that created a changed response by the tissue or the cells. Therefore, the migration assays were conducted either in the absence of serum or in the presence of serum from which Fn and Vn had been removed (Underwood and Bennett 1989).

Uncoated TCPS was used as the control surface for the migration assays conducted over the various ECM components. When it became apparent that migration occurred over this control surface it was of interest to determine if corneal epithelial tissue would be able to migrate over uncoated surfaces that had surface chemistries that were different from that of TCPS. There was also the possibility that dissociated epithelial cells would show a different response from the tissue, towards particular surfaces. If this occurred it may point to the operation of a difference in migratory mechanisms that could be further investigated.

A simple way to characterise one aspect of the chemistry of a surface is by measurement of the sessile contact angle (SCA) of a drop of water on the surface. This enables a comparison of the hydrophobicity/hydrophilicity or "wettability" of different surfaces. The degree of hydrophobicity being related to the air-water SCA. The hydrophobicity of a surface is known to have an effect on initial cell attachment *in vitro* (Amstein and Hartman 1975; Curtis et al 1983; Ertel et al 1990; Steele et al 1993b; Underwood et al 1993; van Wachem et al 1985; van Wachem et al 1987). One mechanism underlying this phenomenon involved the preferential adsorption of cell adhesive glycoproteins onto the culture surface (Chinn et al 1989). In the case of native polystyrene and polystyrene that has been rendered hydrophilic by using glow discharge, the enhanced cell adhesion on this modified surface in comparison with the native polystyrene is due to the hydrophilic surface being better able to adsorb Vn from the serum component of culture medium (Steele et al 1993a). In addition to effects on cell adhesion, cell-adhesive glycoproteins that are adsorbed onto a culture

substratum also affect cell migration. Bone-derived cells migrate more readily over a hydrophilic, acid-etched polystyrene surface than over a native polystyrene surface that is more hydrophobic (Callen et al 1993). The mechanism responsible for the enhanced cell migration over the acid-etched surface is due to the greater adsorption of Vn from serum onto that surface, as compared with the hydrophobic surface (Dalton et al 1998). Because of these known effects of polymer surface chemistry on the preferential adsorption of cell adhesive glycoproteins from serum, migration was again measured in the absence of exogenous Vn and Fn in this part of the study. In this way it was possible to compare cell migration over surfaces that were not already coated by undefined amounts of exogenous molecules that are known to facilitate corneal epithelial cell migration (Cameron et al 1988; Cameron et al 1991; Nishida et al 1990; Watanabe et al 1987).

In order to directly compare epithelial cell migration over different surfaces it was necessary to have equivalent cell populations adhere to the surfaces prior to the commencement of migration. Bovine corneal epithelial cells are able to attach to several uncoated surfaces in the absence of cell adhesive glycoproteins (Evans and Steele 1998). However, a greater proportion of cells attach to TCPS (compared with untreated PS) in the initial 24 hours after seeding (Evans and Steele 1997a; Evans and Steele 1998) (and personal observations). In the current study, equivalent attachment on each surface was achieved by pre-coating the restricted seeding area with Fn. A sufficiently high Fn coating concentration was selected so as to eliminate the effect of a less favourable Fn orientation on the hydrophobic surface that would reduce cell attachment (Pettit et al 1994; Underwood et al 1993).

There were several phases to the work described in this Chapter: firstly the extent of tissue and cell migration was compared over four ECM components. Secondly, the extent of migration was measured over polystyrene-based surfaces that differed in SCA. A glass surface was included as a test surface because glass would be a preferred material for immunohistochemistry, which would be required subsequently in order to examine other aspects of migration. Within these studies, the migratory response was measured both in the presence and absence of serum, but entirely in the absence of serum-derived Fn and Vn. Finally, the proliferation of cells within the model systems was considered.

2.1.1 Aim

The aim of the research described in this chapter was to develop defined model systems that would enable comparisons to be made between the extent of migration of corneal epithelial tissue and corneal epithelial cells *in vitro*.

2.1.2 Hypotheses to be tested

(1) The presence of ECM components pre-coated onto polystyrene can stimulate the migration of corneal epithelial tissue and cells.

(2) ECM components that promote the migration of intact epithelial tissue will also stimulate the migration of dissociated corneal epithelial cells. (If this is not the case, it may indicate a difference in the migratory mechanisms between cells and tissue).

(3) A surface with a moderate level of hydrophobicity would more effectively support the migration of epithelial tissue and cells than a highly hydrophilic or a highly hydrophobic surface.

2.2 MATERIALS AND METHODS

2.2.1 Migration of bovine corneal epithelial tissue

Freshly enucleated bovine eyes from animals aged between 9 and 14 months were immersed in Dulbecco's Modified Eagle's Medium/Ham's F12 cell culture medium (SFM) supplemented with 5 μg/ml amphotericin B, 120 μg/ml penicillin and 200 μg/ml streptomycin (all from ICN Biomedicals, Sydney, Australia) for a minimum of one hr. The corneas were excised from the globes using a scalpel and placed endothelial side down on a Petrie dish (Figure 2.1A contains a diagrammatic representation of these procedures). The limbal region was trimmed from the cornea so as to avoid the stem cells which are present in the limbus (Cotsarelis et al 1989; Lavker et al 1991; Schermer et al 1986). A tissue slice which comprised intact epithelial tissue, epithelial basement membrane and a thin layer of stroma was peeled from the bulk of the stroma and the endothelium using curved jeweller's forceps. After this slice had been separated from the remaining stroma and the endothelium, buttons were cut from the periphery using a six mm diameter skin biopsy punch (Stiefel Laboratories Pty Ltd, Castle Hill, NSW, Australia) producing a corneal button in which the area of epithelial tissue was 28.3 mm^2. The periphery of the cornea was selected because cells of the central cornea are less capable of continued doubling in culture (Lavker et al 1991). The buttons were placed on the centre of the test surfaces, with the stromal side down. Explants from three different eyes were used for each test surface and each experiment was repeated at least twice. The explants were allowed to adhere to the surface for 15 min before the gentle addition of sufficient medium to cover the explant. The culture medium was SFM supplemented with 2 mM glutamine (ICN Biomedicals), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenious acid (ITS™ Premix, from Collaborative Biomedical Products, Bedford, USA), 0.1% (v/v) non-essential amino acids (ICN Biomedicals) and 60 μg/ml penicillin/100 μg/ml streptomycin ("SFM+add"). The explants were cultured at 37° C in a humidified atmosphere containing 5% (v/v) CO_2. The culture medium was replaced after an interval of three days. (An illustration of corneal epithelial tissue migration is shown in Figure 2.1B).

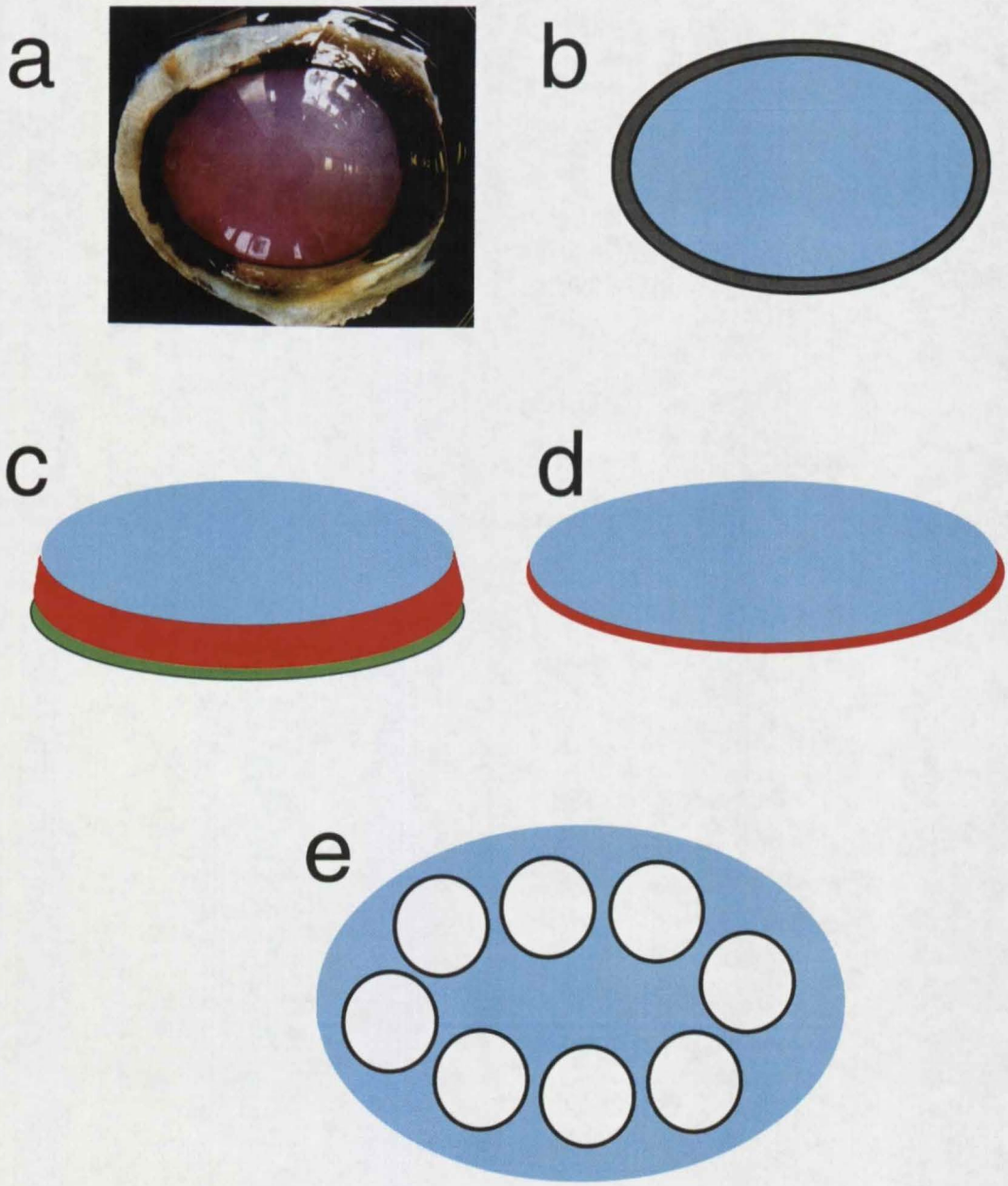


Figure 2.1A Dissection of the cornea to produce explants for epithelial tissue migration. (a) The cornea was detached from the excised bovine eye. (b) The corneal/scleral rim (grey area) was removed. (c) The epithelium (blue) and a thin layer of stroma (red) were separated from the bulk of the stroma and the endothelium (green). (d) The epithelium supported by a thin layer of stroma was placed stromal side down. (e) Explants were cut from the periphery of the this tissue.

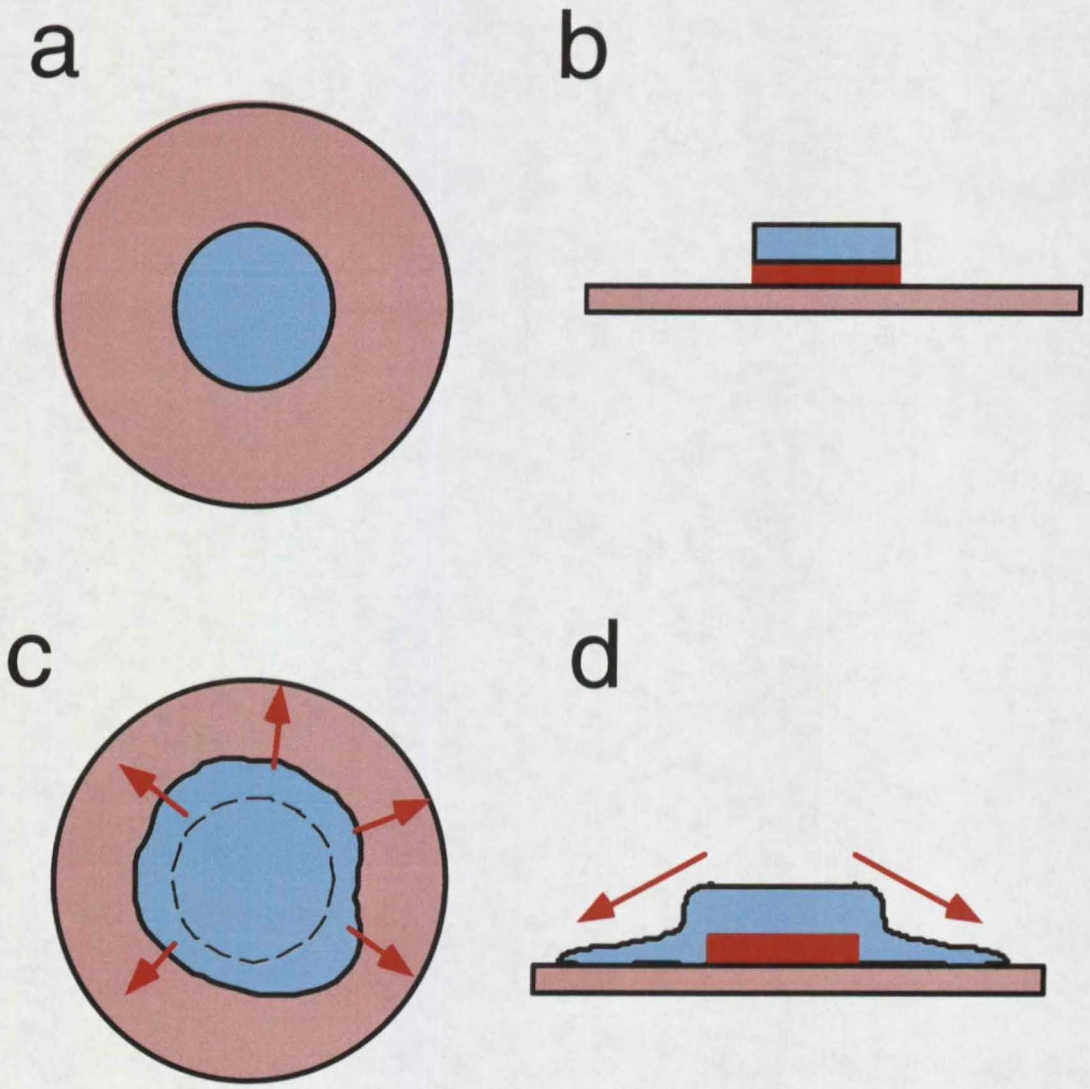


Figure 2.1B Diagram to illustrate the migration of corneal epithelial tissue. The corneal explant was placed on the culture surface with the epithelium (blue) uppermost. The culture surface and the explant are viewed from above in (a) and (c) and from the side in (b) and (d). The epithelial tissue migrated down the cut side of the stroma (red) and over the substratum in a radial fashion, as indicated by the arrows.

2.2.2 Bovine corneal epithelial cell cultures

In order to obtain dissociated corneal epithelial cells the bovine eyes were treated in the same way as for the preparation of buttons for tissue migration. Pieces of tissue, approximately 9 mm by 2 mm that consisted of the epithelium and a thin layer of stroma, were cut from the periphery of the corneal tissue. The cut tissue was incubated, epithelial side down, in a solution of 0.05% (w/v) dispase II (Boehringer Mannheim, Germany), in SFM at 37°C for 1.5 hr. The tissue was washed in phosphate buffered saline pH 7.2 (PBS) then the epithelial layer was peeled from the underlying stroma using curved jeweller's forceps. The epithelial tissue was incubated in a solution of 0.25% (w/v) trypsin (Serva, Feinbochemica, GmbH & Co, Heidelberg, Germany) 0.2 mM EDTA (BDH Chemicals Australia Pty Ltd, Vic., Australia) in PBS (TV) for 15 min at 37°C. The epithelial sheets were then dissociated by trituration, washed in SFM and seeded into 25 cm^2 flasks (Corning, MA, USA) in SFM+add containing 20% (v/v) foetal bovine serum (P. A. Biologicals, Sydney, Australia). Each flask contained epithelial cells isolated from two corneas. The cells were cultured at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. The culture medium was replaced at intervals of three days until the cells reached confluence. At this point the cells were either used in assays and designated as passage one (p1), or dissociated with TV, reseeded at a 1:3 ratio and cultured as before until they were again confluent. At this point they were used in assays and designated as passage two (p2) cells.

2.2.3 Migration of corneal epithelial cells

A stainless steel and silicon "fence" (Fischer et al 1990) was centred on each test surface (Figure 2.2). Within each experiment the culture surface that was isolated by the fence was pre-coated with a cell attachment factor, in order to ensure a confluent cell population on the test surface (see Section 2.2.4 *Surface coatings*). One hundred μl of SFM+add supplemented with 20% v/v serum from which Fn and Vn had been removed (DD serum) and containing $6X10^5$ epithelial cells per ml was then loaded into this fenced area *ie.* the lumen of the fence, (depletion of serum Fn and Vn is described in Appendix A3). The cells were cultured, restrained by the fence, for 20-24 hr at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium

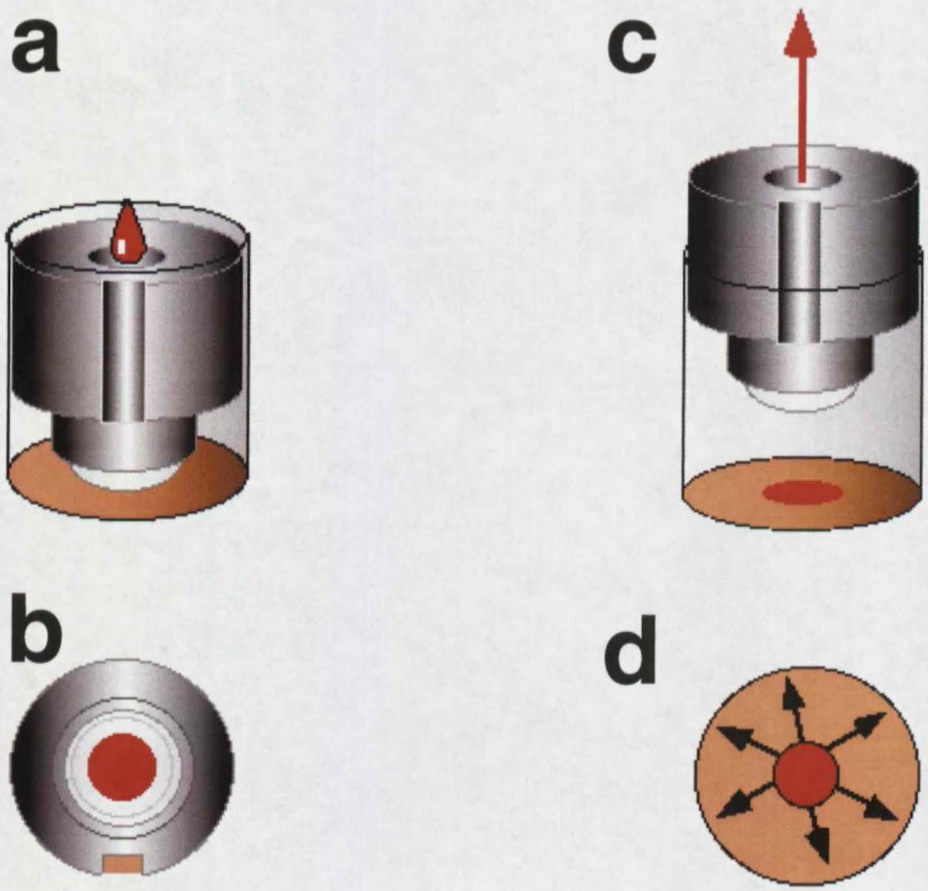


Figure 2.2. Illustration of the assay to measure the migration of cells from a confluent culture. A suspension of bovine corneal epithelial cells was seeded into the lumen of the fence (a). After the cells had attached to the underlying substratum (b) the fence was removed (c) and the cells were able to migrate in a radial fashion from the confluent culture (d).

From a diagram produced by Mr Graham Johnson CSIRO Moleular Science.

was then removed carefully from the adherent cells, without disturbing the fence. This region was washed twice with 200 μl of SFM before the removal of the fence. Any residual medium was then removed from the cells before each well was washed twice with 2 ml SFM. When the fence was removed the resultant circular island of cells covered approximately 38 mm^2. The cells were cultured in 1.8 ml of SFM+add or 1.8 ml of SFM+add containing 10% (v/v) DD serum. The medium was replaced after an interval of three days. After six days, the cells were washed with PBS and fixed and stained (see Section 2.2.6 Fixation and Staining). One set of cells for each surface coating was fixed and stained after the initial 20-24 hr attachment period at the time when the fences were removed. In each experiment, triplicate samples were used for each surface and condition tested. Each experiment was repeated at least twice.

2.2.4 Surface coatings

ECM molecules were coated onto the TCPS test surfaces prior to tissue and cell migration. The procedures for conducting these tissue and cell migration assays are summarised in Flow diagrams 2.1 and 2.2 respectively.

2.2.4.1 Fibronectin

The surfaces of six-well TCPS cluster trays (Costar) were incubated with a range of concentrations of bovine Fn (Sigma) in PBS. For each assay, six wells were used for each of the concentrations of Fn, which were 10, 4, 2, 1 and 0.1 μg/ml. To each well 1.5 ml of Fn solution was added and the trays were incubated for 1.5 hr at 37°C. The solution was removed and each well was washed once with PBS. For tissue migration, a corneal button was placed in the centre of each of the coated wells and six uncoated wells. The tissue was cultured as described in Section 2.2.1.

For cell migration, a fence was centred on each of the Fn pre-coated wells and also on each of nine uncoated wells. Six of these uncoated wells served as the uncoated TCPS controls and the other three wells were used to determine the initial cell areas at 20-24 hr after the cells were seeded and prior to cell migration. To the lumen of each of the fences was added 100 μl of a 10 μg/ml solution of Fn in PBS. The trays were incubated for a further 1.5 hr, before the Fn solution was carefully removed without disturbing the fence. The lumen of the fence was washed with 100 μl of SFM then the

Flow diagram: 2.1 Tissue migration over ECM components

ECM coating of surfaces for migration to occur	TCPS surfaces coated with ECM component (6 surfaces for each coating concentration)
	Wells washed with SFM
Tissue culture	An explant 6 mm in diameter placed in centre of each coated well and 6 uncoated TCPS wells
	Tissue cultured in medium specified (3 samples in SFM+add and 3 in medium containing 10% (v/v) DD serum, for each coating concentration and uncoated surface
Migration assay	Wells washed with PBS and tissue fixed with formol saline
	Wells washed with water then tissue stained with Crystal violet
	Wells washed with water and tissue areas measured using image analyser
	Results expressed a migration index (final tissue area/original tissue area)

Note: each ECM component was assayed separately for logistical reasons.

Flow diagram: 2.2 Cell migration over ECM components

ECM coating of surfaces for migration to occur	TCPS surfaces coated with ECM component (6 surfaces for each coating concentration)	
	Wells washed with SFM	
Preparation of initial cell seeding area, to ensure consistent cell population	Fence centred on each of the coated wells and on 9 uncoated wells	
	Lumen of each fence coated with highest concentration of matrix molecule used (except for collagen surfaces, where 10 μg/ml Fn was used)	
	Lumen of fence washed with SFM	
Cell culture and migration	Cells seeded into lumen of each fence and cultured 20-24 hr	
	20 24 hr controls Fences removed from 3 uncoated wells	**Cells for migration** Culture medium removed from lumen of fence
	Wells washed with PBS and adherent cells fixed (20-24 hr cultures)*	Lumen washed 2X with SFM and fences removed
		Residual SFM removed from adherent cells
		Wells washed 2X with SFM
	Cells cultured in medium as specified (3 samples in SFM+add and 3 in medium containing 10% (v/v) DD serum) for each ECM coating concentration and for uncoated TCPS	
	Culture medium replaced after 3 days	
	At day 6 wells washed with PBS	
	Adherent cells fixed with formol saline	
Migration assay	Cells stained with Crystal violet (including 20-24 hr cultures)*	
	Wells washed with water and the area covered by the cells measured using image analyser	
	Results expressed a migration index (final area covered by cells /original area covered by cells)	

Note: each ECM component was assayed separately for logistical reasons.

cells were seeded as described above. For each coating concentration (and uncoated control), tissue and cells were cultured in three wells in the absence of serum and in three wells in the presence of 10% (v/v) DD serum. The assays were conducted over six days as described in Section 2.2.3.

2.2.4.2 Laminin

Mouse Ln (Gibco BRL, Life Technologies, NY, USA) was pre-coated onto the culture surface of the six-well trays. The coating concentrations of Ln were 50, 25, 12.5 and 6 μg/ml in PBS and for each dilution 1.5 ml of each Ln dilution was added to six wells and incubated at 37°C for 1.5 hr. The wells were washed once with PBS. For tissue migration, an explant button was placed in the centre of each coated well and six uncoated wells. For cell migration, a fence was centred in each coated well and also in nine uncoated wells. To the lumen of each fence, 100 μl of a 50 μg/ml solution of Ln was added and the trays were incubated for an further 1.5 hr at 37°C. The solution in the lumen of the fence was removed without disturbing the fence and the lumen was washed with 100 μl of SFM. Tissue and cells were cultured as described above and in Flow diagrams 2.1 and 2.2.

2.2.4.3 Collagen IV

Collagen IV from human placenta (Becton Dickinson, MA, USA) at concentrations of 40, 20, and 10 μg/ml in 10 mM acetic acid (BDH) was incubated in six-well cluster trays at 37°C for 1.5 hr using 1.5 ml for each well. Six wells were used for each concentration. After incubation, the coating solution was removed and the wells were washed with PBS. Two ml of SFM was added to each well for five min and then removed. This ensured that any residual acetic acid was neutralised before a corneal button was placed in each well and six uncoated wells for tissue migration. For cell migration, a fence was centred in each well and in nine uncoated wells. Of these, six served as uncoated TCPS controls and three were fixed after the initial cell attachment and used to determine the original cell area. To the lumen of each fence, was added 100 μl of a 10 μg/ml solution of Fn in PBS and the trays were incubated for a further 1.5 hr at 37°C. The Fn solution was carefully removed without disturbing the fence before the lumen of the fence was washed once with 100 μl of SFM. The tissue and

cells were cultured as described above and the extent of migration was measured after six days.

2.2.4.4 Collagen I (Vitrogen™)

Twenty μl of 2.9 mg/ml solution of collagen I (Vitrogen 100 ™, Palo Alto, CA, USA) was added to wells of a six-well cluster tray. The Vitrogen™ was spread over the culture surface using a sterile glass spreader, that had been made by sealing and bending at 90° the end of a Pasteur pipette. The coated Vitrogen™ was air dried in a laminar flow cabinet (approximately two hr) and the wells were washed once with PBS and once with SFM. For tissue migration, a corneal button was attached in the centre of each coated well and to six uncoated wells. For cell migration, a fence was centred on each of six coated wells and nine additional uncoated wells. Of these six served as uncoated TCPS controls and three were fixed after the initial cell attachment and used to determine the original cell area. One hundred μl of a 10 μg/ml Fn solution in PBS was added to the lumen of each fence and the trays were incubated for a further 1.5 hr at 37°C. The Fn solution was carefully removed without disturbing the fence, before the lumen was washed once with 100 μm SFM. The tissue and cells were cultured as described above and the extent of migration was measured after six days.

2.2.5 Migration of tissue and cells over uncoated surfaces

Tissue migration studies over uncoated surfaces were conducted in the absence of any exogenous ECM components. For the cell migration studies over the uncoated surfaces, only the initial cell attachment area of each culture surface was pre-coated with a solution of 10 μg/ml of Fn. This was the area isolated by the fence (ie the lumen of the fence). This ensured that there was an equivalent cell population on each of the surfaces at the commencement of migration. The procedures for measuring cell migration and proliferation on the uncoated surfaces are outlined in Flow Diagram 2.3.

Flow diagram: 2.3 Cell migration and proliferation on uncoated surfaces

Preparation of surfaces	Fences placed in centre of TCPS, PS and PRI wells and on THER and glass coverslips placed within wells (12 replicates for each surface)	
Preparation of initial cell seeding area, to ensure consistent cell population	Fence centred on each surface	
	Lumen of each fence coated with 10 μg/ml Fn then washed with SFM	
Cell culture and migration	Cells seeded into lumen of each fence and cultured 20-24 hr (3 wells for each surface treated as cell free controls)	
	20-24 hr controls Fences removed from 3 wells on each surface	**Cells for migration** Culture medium removed from lumen of fences
	Wells washed with PBS and adherent cells fixed (20-24 hr cultures)*	Residual SFM removed from adherent cells
		Wells washed 2X with SFM
	Cells cultured in medium as specified (for each surface, 3 samples in SFM and 3 in medium containing 10% (v/v) DD serum)	
	Culture medium replaced after 3 days	
	At day 6 wells washed with PBS	
Migration assay	Adherent cells fixed with formol saline	
	Wells stained with Crystal violet (including 20-24 hr cultures)*and cell free controls	
	Wells washed with water and area covered by cells measured using image analyser	
	Results expressed a migration index (final area covered by cells /original area covered by cells)	
Measurement of cell proliferation	1 ml 10% (v/v) acetic acid added to each well	
	Stain eluted from cells by 5 min agitation on a shaker	
	100 μl aliquots of eluted stain transferred to a 96-well Dynatec tray	
	Absorbance of stain read on a microplate reader at a test wave of length 595 nm and reference wave length of 405 nm.	

The surfaces tested were:

1) Untreated polystyrene (PS) (35 mm diameter Petrie dishes from Falcon, Beckton Dickinson, New Jersey, USA),
SCA 89°-90° (Baier et al 1968; Ertel et al 1991; Ratner et al 1987; Steele et al 1994; van der Valk et al 1983);
2) Primaria™ (PRI) (six-well cluster trays from Falcon),
SCA approximately 48° (Steele et al 1995b);
3) TCPS six-well cluster trays (Corning),
SCA 60°-70° (Ertel et al 1991; Steele et al 1995b; Steele et al 1994; van der Valk et al 1983);
4) Thermanox™ (THER) coverslips with 25 mm diameter (Nunc, cat no.174985),
SCA 70±4 (Personal communication Marcella Brown);
5) Alcohol washed air dried glass coverslips with 25 mm diameter (Chance),
SCA 32±4 (Personal communication Marcella Brown).
When migration studies were carried out on Thermanox™ or glass coverslips, these were placed in six-well cluster trays.

2.2.6 Fixation and staining

At the conclusion of the migration period, the culture medium was removed from the tissue or cells and after one wash with PBS, the cultures were fixed by incubation for 30 min at room temperature with 4% (v/v) formol saline {1:10 solution of formaldehyde (BDH, Merch Pty. Ltd., Kilsyth, Victoria, Australia) in PBS}. After fixation the cultures were washed once with distilled water, air-dried and stained by incubation for 30 min at room temperature with 2 ml of a 0.1% (w/v) solution of Crystal violet (Edward Gurr Ltd, London, UK) in 0.02 M phosphate buffer at pH 7. Each well was then washed three times, using 2 ml of distilled water for each wash.

2.2.7 Measurement of migration

After staining, the cultures of tissue and cells were air-dried. Images of the tissue and cells were captured using a video camera (Panasonic, Japan) with the culture trays being illuminated from the base by a light-box. The areas covered by tissue or cells were measured using an image analyser (Cambridge instruments Quantimet Q570,

Cambridge, UK) and the results were expressed as a migration index, which was calculated as final tissue or cell area/initial tissue or cell area. Raw data from replicate experiments were analysed by Student-Newman-Keuls multiple comparisons test. Examples of stained tissue and cells are shown in Figure 2.3. The Flow Diagram 2.3 summarises the assay used to measure cell migration and proliferation on uncoated surfaces.

2.2.8 Cell proliferation on uncoated surfaces

After the cell migration area had been measured, the Crystal violet that had been taken up by the cells was solubilised with 1 ml of 10% (v/v) acetic acid by agitation on a shaker for five min. Control (blank) wells of each substratum which did not contain cells were subjected to identical fixation, staining and elution procedures as the test wells. Aliquots of the solubilised dye solution were then transferred to wells of a 96-well microtitre tray (Dynatec Laboratories inc, Virginia, USA) and the absorbances were read on a microplate reader (Biorad model 3550) at a test wave length of 595 nm and a reference wave length of 405 nm. The absorbancy values for blank wells were subtracted from the appropriate experimental wells. Cell numbers were compared by expressing the mean absorbancy of stain eluted from cells on each test surface as a percentage of the mean absorbancy of stain eluted from cells that had migrated on TCPS in the presence of DD serum. The results were analysed by the Student-Newman-Keuls Multiple Comparisons Test and are expressed as means and standard deviation from two separate experiments, each containing three replicates.

2.2.9 Localisation of proliferation within the migrating tissue and cells

Tissue and cells that had migrated over glass coverslips were cultured for 5.5 hr or 18 hr in the presence of $1x10^{-4}$ M 5-Bromo-2-Deoxyuridine (Brdu) (Sigma Chemical Co. MO, USA). Following a two hr incubation in culture medium without Brdu the tissue and cells were washed in PBS, fixed for 10 min in 4% (v/v) formol saline and washed again in PBS. The cultures were permeabilised with 0.5% (v/v) Triton X100 (Calbiochem, La Jolla, CA, USA) in PBS for 10 min, then incubated for 45 min in 95% (v/v) formamide (Aldrich, Milwakie, USA) in 0.15 M trisodium citrate (Sigma) at 60°C. This treatment partially denatured the double stranded DNA and made the Brdu more accessible to the antibody. All further steps were conducted at room

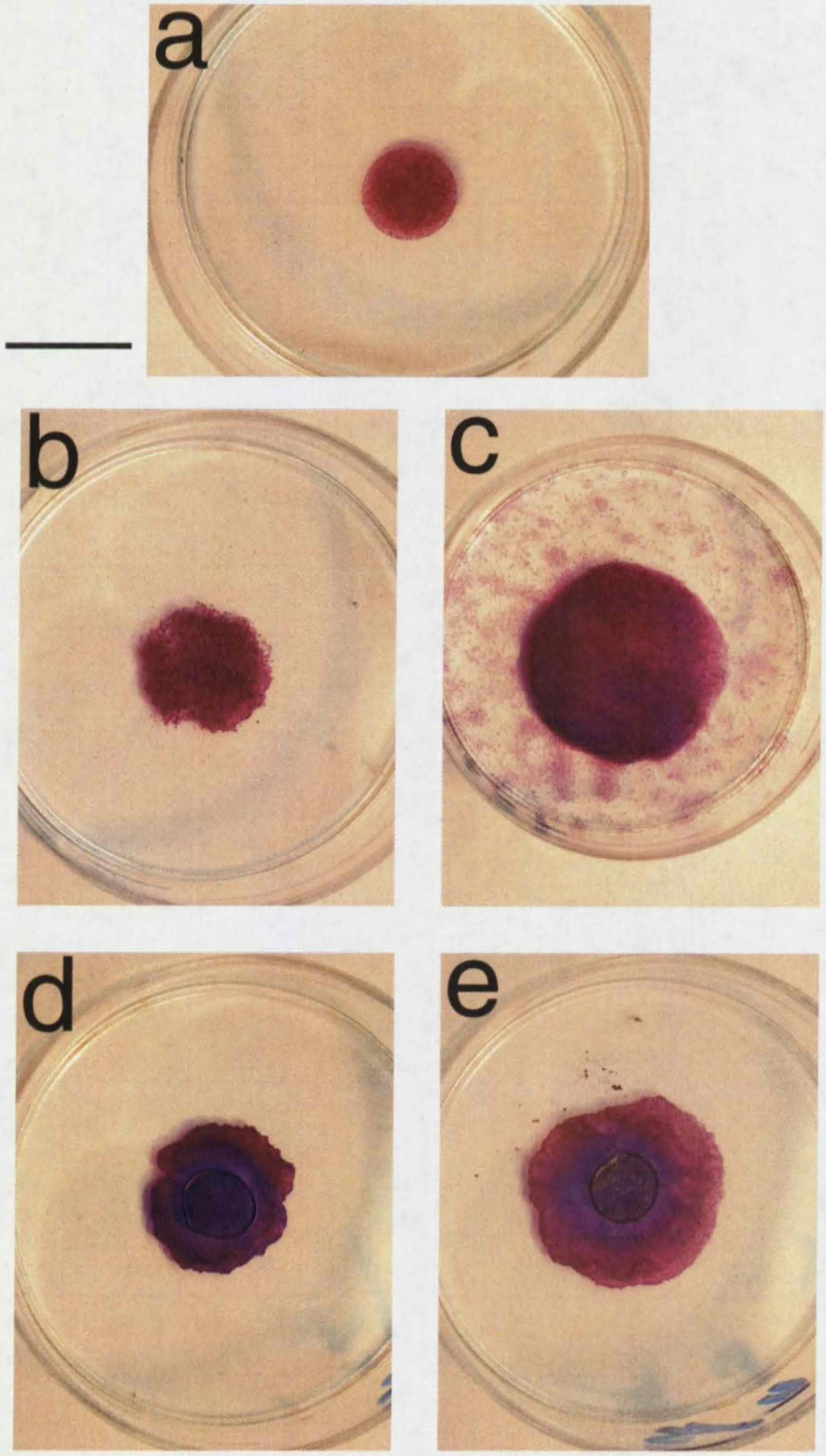


Figure 2.3 Examples of bovine corneal epithelial cells (a-c) and tissue (d-e) as were used in the migration assays. The cells in (a) were seeded in a fence and cultured for 20 hr. In (b) and (d), the samples were cultured for six days in SFM. The samples in (c) and (e) were cultured in medium containing 10% (v/v) DD serum. All samples were fixed and stained before they were photographed. Bar = 10 mm.

temperature. Following three washes with PBS, non-specific binding sites were blocked by incubation for 30 min in 2% (w/v) bovine serum albumin (BSA) (Boehringer Mannheim, IN, USA) in PBS. The corneal explants were carefully dissected from the coverslips, without disturbing the epithelial tissue that had migrated over the coverslip. The base of the coverslips were dried and they were placed on parafilm that had been pressed firmly on the base of a 100 mm diameter dish (tissue or cells uppermost). Damp filter paper was placed around the parafilm so that a humid atmosphere was maintained within the dish to protect the samples from drying. Antibodies to Brdu (DAKO Corporation, CA, USA) were diluted 1:50 in 2% (w/v) BSA/PBS and sufficient antibody solution was added to each coverslip to cover the sample. The dishes were sealed and the samples were incubated in the presence of the antibody for 1.5 hr. The coverslips were returned to the six-well trays and washed in PBS for 10 min (on a rocker) three times. The bases of the coverslips were again dried before they were returned to the parafilm in the humidified dishes. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (Dakopatts, F232) was diluted 1:20 in PBS and sufficient solution was added to cover each sample. The dishes were sealed, protected from light and incubated for 1 hr. The samples were washed in PBS on a rocker for 10 min, four times. The base of each coverslip was rinsed with distilled water before the sample was sandwiched between the coverslip and a glass microscope slide using Fluorosave™ (Calbiochem, CA, USA). The samples were examined using a laser confocal microscopy equipped with an argon/krypton laser (Leica, Heidelberg, Germany). Images in one focal plane were collected using a wave-length of 488 nm and a pinhole of 90. Samples that had not been pre-incubated with Brdu were subjected to the immunostaining protocol and used as negative controls.

In order to determine if proliferation had occurred in the epithelium above the stromal explant, tissue was cultured for six days on track-etched, porous polycarbonate (Poretics Corporation, Livermore CA, USA) and the cultures were similarly exposed to Brdu. These samples were fixed in formol saline and processed for paraffin embedding and sectioning as described fully in Materials and Methods, Chapter 3 and Appendix A4. Transverse sections of the explant and migrated tissue were cut 5 μm in thickness and collected onto gelatine coated glass slides. The slides were dewaxed

and rehydrated (see Chapter 3 and Appendix A5) before they were incubated for 20 min in 95% (v/v) formamide in 0.15 M trisodium citrate at 60°C. After three washes with PBS the slides were immunostained using the same protocol as was used for the tissue and cells cultured on coverslips. However, the slides were supported by two plastic rulers placed inside a shallow rectangular glass dish. Damp filter paper in the base of the dish was used to maintain a humidified atmosphere during incubation with the antibodies when the dishes were sealed with plastic wrap. The washing steps were carried out by placing the slides in Coplin jars.

The location of proliferating cells was examined by counting Brdu labelled nuclei at three locations within the migrating tissue and cells (as illustrated in the Results section Tables 2.1 and 2.2). Migration for these experiments took place in SFM.

2.3 RESULTS

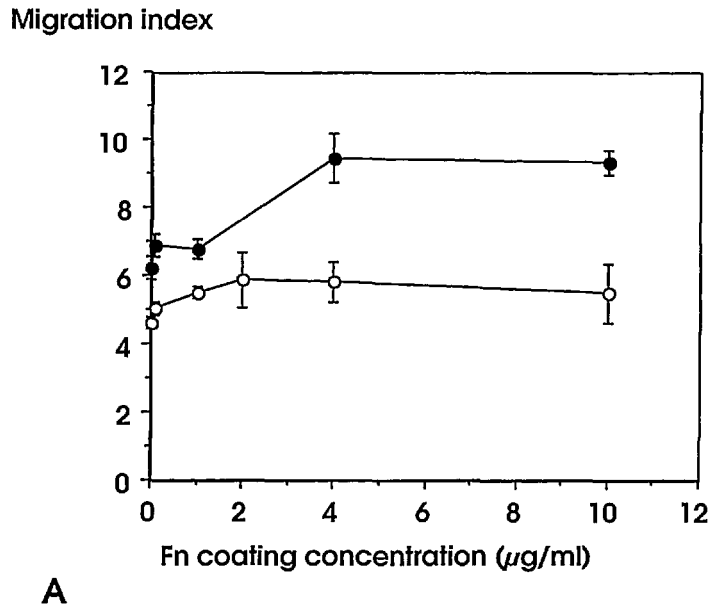
2.3.1 The effect of fibronectin on the migration of corneal epithelial tissue

Intact corneal epithelial tissue migrated over all coating concentrations of Fn tested and also over uncoated TCPS in SFM and in the presence of DD serum (Figure 2.4A). In SFM, the minimum coating concentration that created a significant increase in tissue migration, as compared with uncoated TCPS, was 1 μg/ml ($p<0.001$), where there was a small increase in the migration index (approximately 1). Coating concentrations of 2, 4 or 10 μg/ml of Fn did not cause any further increase in the migration index compared with 1 μg/ml. When DD serum was present there was a modest increase in the migration index over Fn coating concentrations of 0.1 and 1 μg/ml as compared with the uncoated TCPS surface. However, the greatest increase in migration compared with uncoated TCPS occurred over a coating concentration of 4 μg/ml where a plateau was reached. There was no further increase in the extent of migration at a coating concentration of 10 μg/ml (Figure 2.4A).

There was a significant increase ($p<0001$) in migration in the presence of DD serum in comparison with SFM. This was seen both on uncoated TCPS and on each of the Fn coating concentrations tested (Figure 2.4A).

2.3.2 The effect fibronectin on the migration of corneal epithelial cells

Bovine corneal epithelial cells migrated from the defined circular confluent culture, in both SFM and medium containing DD serum, over the entire range of Fn concentrations tested (Figure 2.4B). Migration also occurred over the uncoated TCPS surface in the absence of any exogenous Fn. In SFM there was no significant difference between the extent of migration on the uncoated TCPS surface and on the surfaces exposed to coating concentrations of 0.1 or 1 μg/ml of Fn ($p>0.05$). A significant increase in the extent of migration in SFM was found between the coating concentrations of 1 and 2 μg/ml ($p<0.05$), where the migration index increased from 2.7 to 3.6. Further increase in coating concentrations to 4 or 10 μg/ml provided no further increase in the extent of migration. In the presence of DD serum there was an increase in the migration index from 5.6 to 6.3 for migration over Fn coating concentrations of between 2 and 10 μg/ml. However, these were found not to be



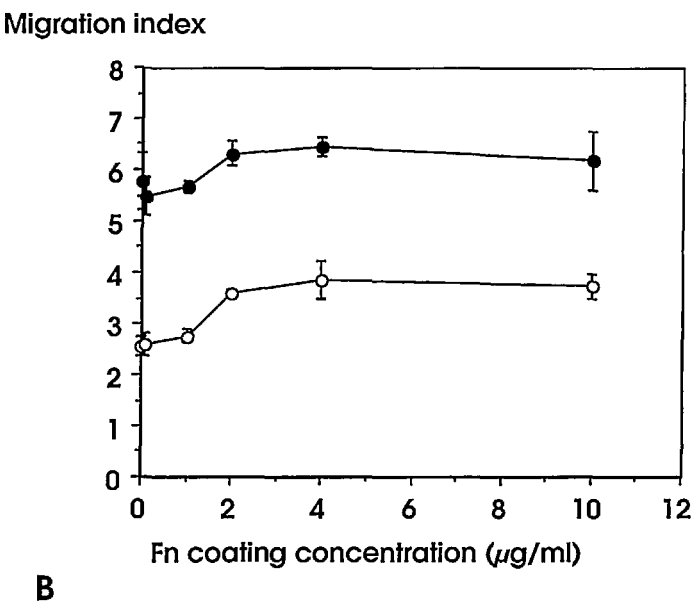


Figure 2.4 Migration of bovine corneal epithelial tissue (A) and epithelial cells (B) over fibronectin pre-coated onto TCPS. Open circles indicate cell migration in SFM and solid circles indicate migration in culture medium that contained DD serum. The results are the mean and standard deviation of three experiments each containing three replicates.

statistically significant ($p<0.05$) compared with migration over uncoated TCPS in the presence of DD serum (Figure 2.4B).

When the extent of cell migration in SFM was compared with migration in the presence of DD serum there was an approximate 50% increase at all coating concentrations and on the uncoated TCPS surfaces ($p<0.001$) (Figure 2.4B). In SFM, a Fn coating concentration of 2 μg/ml or greater provided a more stimulatorary surface for cell migration than the uncoated TCPS. In the presence of DD serum, where migration was already greatly stimulated compared with migration in SFM, there was minimal further enhancement of migration due to the Fn pre-coating.

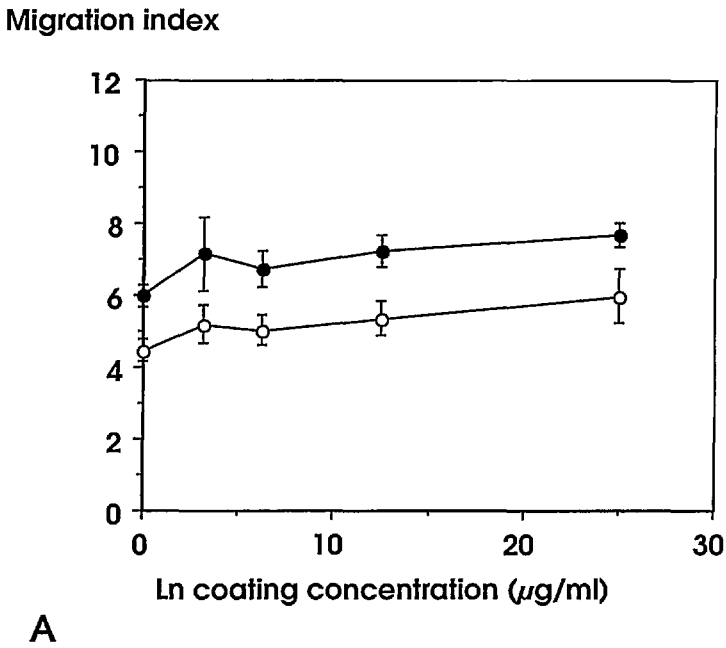
2.3.3 The effect of laminin on the migration of corneal epithelial tissue

Pre-coating TCPS with Ln did not stimulate tissue migration in SFM and DD culture in comparison with migration over the uncoated surface. There was no statistically significant difference in the extent of migration over uncoated TCPS and migration over each of the Ln treated surfaces ($p>0.05$). There was an increase in the extent of migration in the presence of DD serum in comparison with culture in SFM (Figure 2.5A).

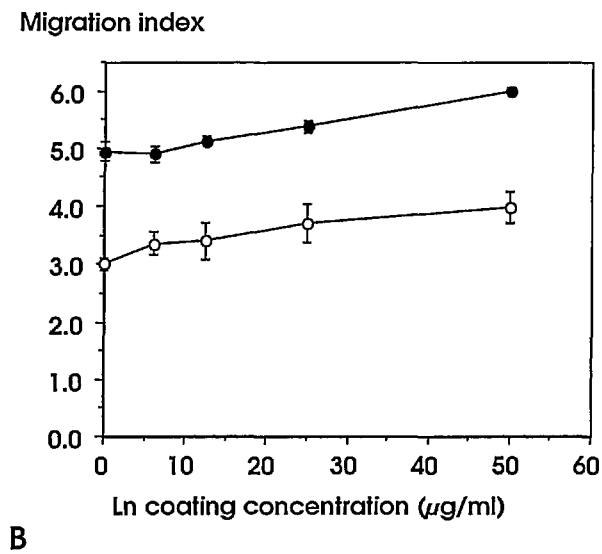
2.3.4 The effect of laminin on the migration of corneal epithelial cells

In SFM there was no significant increase in the extent of cell migration over Ln that had been pre-coated onto TCPS at coating concentrations up to 50 μm/ml as compared with uncoated TCPS ($p>0.05$) (Figure 2.5B). In the presence of DD serum there was no significant increase in cell migration on surfaces subjected to coating concentrations of 6.25, 12.5 or 25 μg/ml of laminin compared with migration over uncoated TCPS ($p>0.05$). A coating concentration of 50 μg/ml provided a significant increase in migration as compared with the uncoated surface ($p<0.05$) (Figure 2.5B).

As with Fn coating, there was a significant increase in the extent of cell migration in the presence of DD serum in comparison with SFM conditions but in this case it was less than two fold.



A



B

Figure 2.5 Migration of bovine corneal epithelial tissue (A) and epithelial cells (B) over laminin pre-coated onto TCPS. Open circles indicate cell migration in SFM and solid circles indicate migration in culture medium that contained DD serum. The results are the mean and standard deviation of two experiments each containing three replicates.

2.3.5 The effect of collagen IV on the migration of corneal epithelial tissue

There was no significant increase in the extent of tissue migration on TCPS that had been pre-coated with solutions of 10-40 μg/ml of collagen IV, in comparison with migration over uncoated TCPS ($p>0.05$). This effect was seen both in SFM conditions and in medium containing DD serum (Figure 2.6A). The increase in the migration index in response to the presence of DD serum was approximately the same on the collagen IV coated surface as it was on uncoated TCPS.

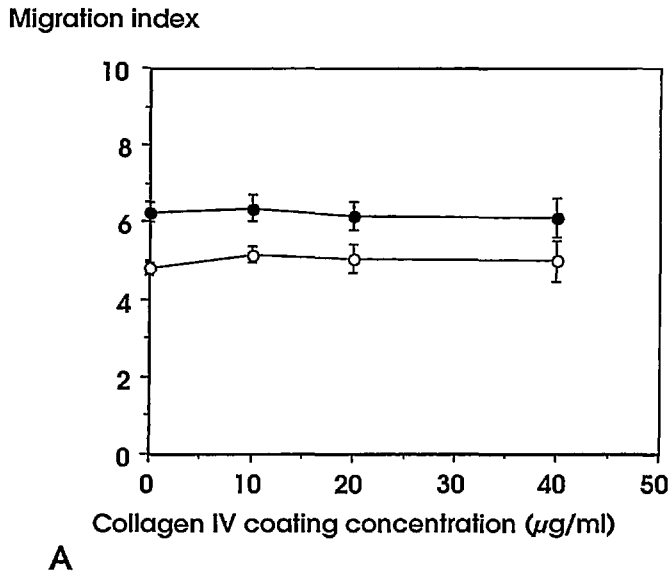
2.3.6 The effect of collagen IV on the migration of corneal epithelial cells

There was no significant increase in the extent of migration of the epithelial cells on TCPS that had been pre-coated with solutions of 10-40 μg/ml of collagen IV, in comparison with uncoated TCPS (Figure 2.6B). At the highest collagen coating concentration there appeared to be a slight decrease in cell migration in SFM. However, this was not significantly different from the extent of migration over uncoated TCPS ($p<0.05$). In the presence of DD serum there was a small but significant ($p>0.05$) increase in cell migration on collagen IV coating concentrations of 10 and 20 μg/ml, as compared with uncoated TCPS. There was an approximate two-fold increase in migration in the presence of DD serum as compared with SFM (Figure 2.6B).

In general, pre-coating TCPS with Collagen IV provided only a minimal stimulation of epithelial cell migration in comparison with uncoated TCPS. This increase was only observed in the presence of DD serum at coating concentrations of 10 or 20 μg/ml. The higher coating concentration of 40 μg/ml appeared to reduce the extent of migration slightly in comparison with the lower coating concentrations.

2.3.7 The effect of collagen I on the migration of corneal epithelial tissue

In SFM the extent of tissue migration over a collagen I coated surface was significantly increased ($p<0.001$) as compared with migration over uncoated TCPS (Figure 2.7A). When tissue was cultured in the presence of DD serum, there was also a significant increase ($p<0.001$) in migration in response to the collagen I coating. Additionally, the migration of tissue over collagen I in the presence of DD serum was



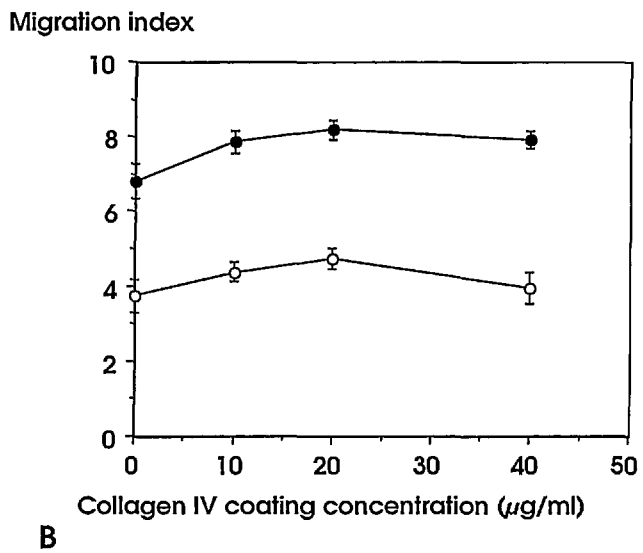


Figure 2.6 Migration of bovine corneal epithelial tissue (A) and epithelial cells (B) over collagen IV pre-coated onto TCPS. Open circles indicate cell migration in SFM and solid circles indicate migration in culture medium which contained DD serum. The results are the mean and standard deviation of two experiments each containing three replicates.

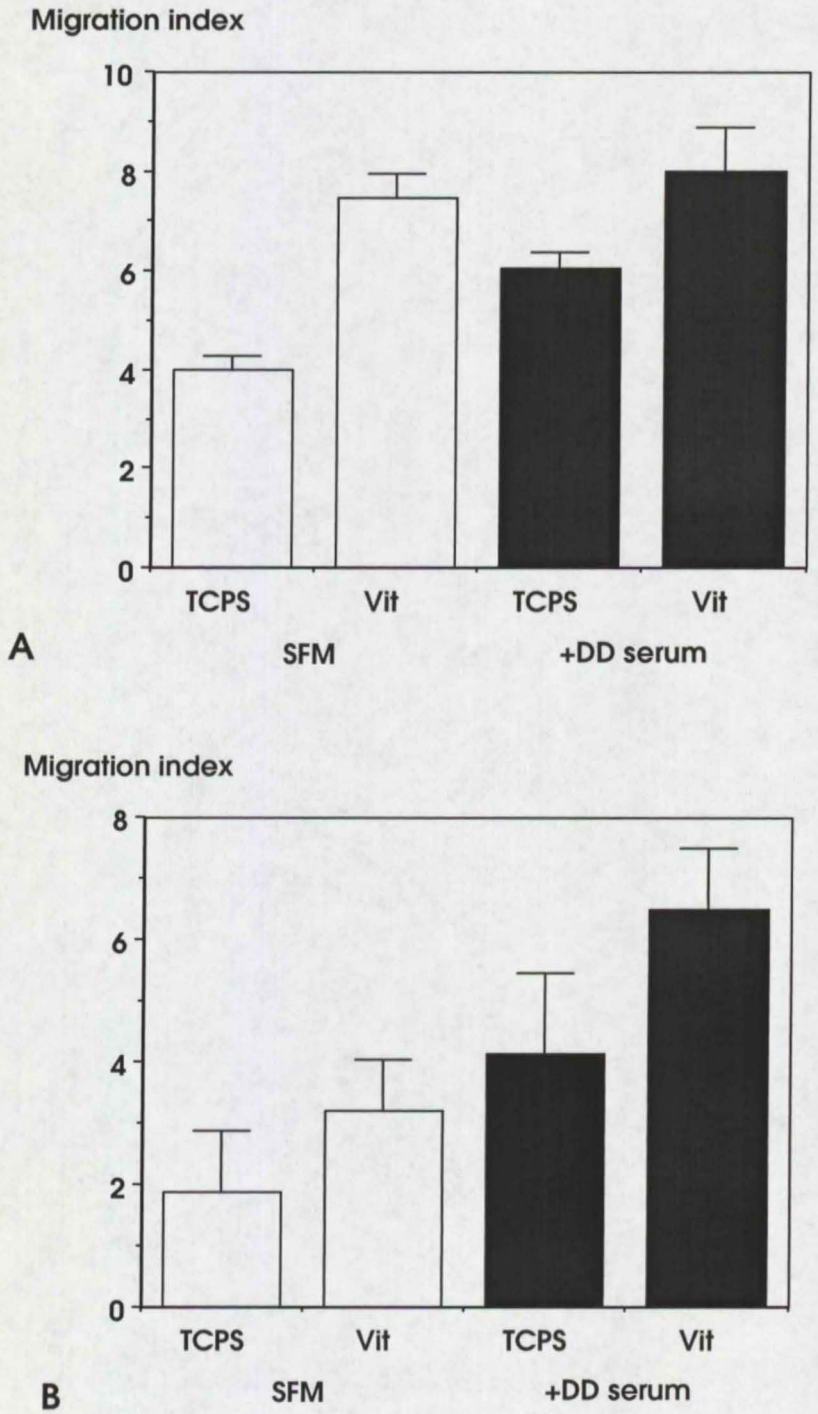


Figure 2.7 Migration of bovine corneal epithelial tissue (A) and cells (B) over collagen I (Vit) spread onto TCPS and air-dried. Tissue and cells migrated in SFM or in medium that contained DD serum. The results are the mean and standard deviation of three experiments each containing three replicates.

increased by a small but statistically significant ($p<0.05$) amount, in comparison with migration in SFM (Figure 2.7A).

2.3.8 The effect of collagen I on the migration of corneal epithelial cells

There was a significant increase in the extent of migration of the epithelial cells over collagen I coated surfaces in comparison with uncoated TCPS, both in SFM ($p<0.001$) and in the presence of DD serum ($p<0.001$) (Figure 2.7B). The presence of serum created an approximate two-fold increase in the extent of migration in comparison with migration in SFM culture, both on uncoated TCPS and on the collagen I coated surfaces (Figure 2.7B).

2.3.9 Migration of corneal epithelial tissue over uncoated synthetic surfaces

Epithelial tissue from corneal explants migrated over all the test surfaces during six days in culture. In SFM, the surface that promoted the most extensive migration was the moderately hydrophilic TCPS with less migration observed on the hydrophobic PS. PRI and THER supported marginally less extensive migration than did TCPS. The glass surface supported the least migration (Figure 2.8A). On each surface there was a significant increase ($p<0.001$) in the extent of tissue migration in the presence of DD serum in comparison with migration in SFM (Figure 2.8A, open bars compared with solid bars).

2.3.10 Migration of corneal epithelial cells over uncoated synthetic surfaces

Corneal epithelial cells migrated, to some extent, in SFM over each of the surfaces tested (Figure 2.8B, open bars). However the glass surface was the least supportive substratum for cell migration (Figure 2.8B). Additionally there was poor perseverance of adhesion of the cells to this substratum. This was seen from the staining pattern of the migrated cells, which showed discontinuity in the cell layer that was unlike cells on TCPS or PRI surfaces. The most hydrophobic of the polystyrene-based surfaces was untreated PS and this surface was significantly ($p<0.05$) less supportive of cell migration than the more hydrophilic TCPS (Figure 2.8B). On the PRI and THER surfaces, the extent of migration was marginally less than that on TCPS, however the differences were not statistically significant ($p>0.05$) (Figure 2.8B).

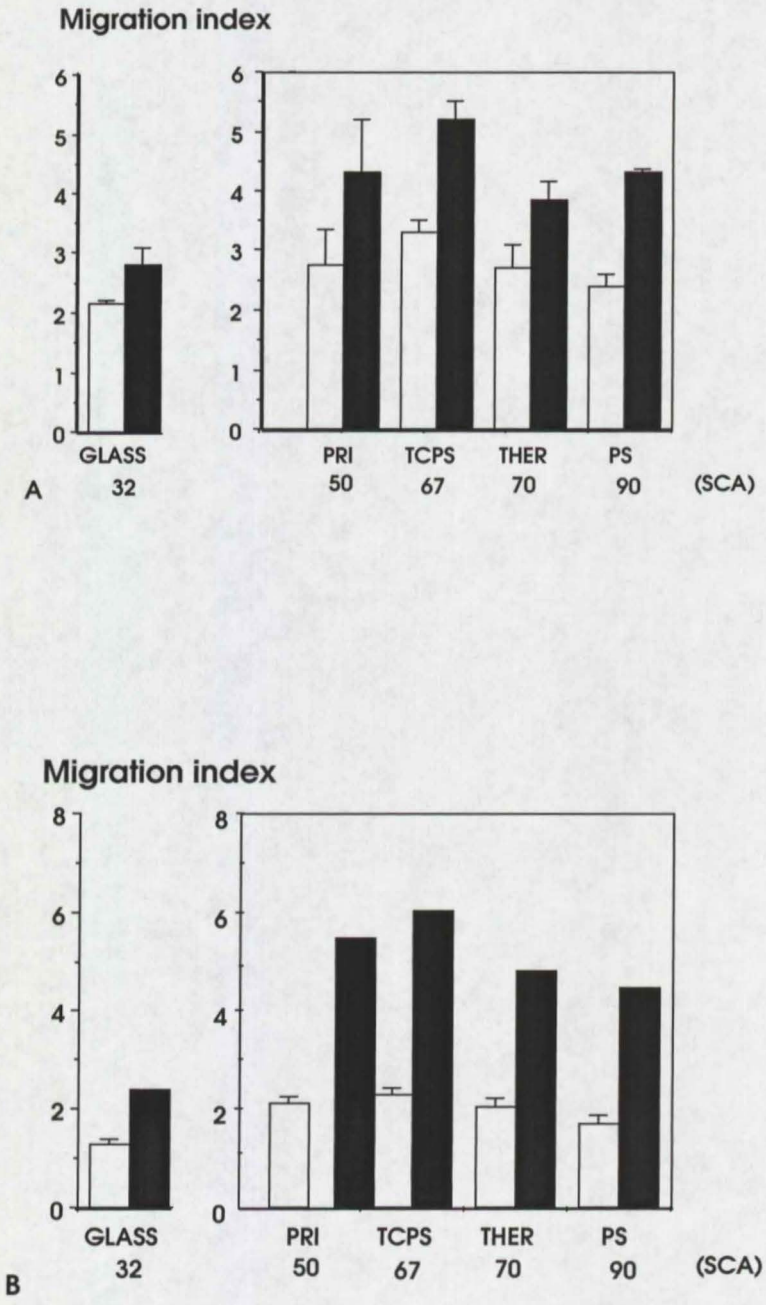


Figure 2.8 Migration of corneal epithelal tissue (A) and cells (B) over uncoated surfaces. Migration in SFM (open bars) or the presence of DD serum (solid bars). The surfaces are arranged in order of sessile contact angle (SCA). The results are the mean and standard deviation of three experiments each containing three replicates.

The presence of DD serum increased the extent of cell migration over each of the surfaces (Figure 2.8B, open bars compared with solid bars, $p<0.001$). The presence of DD serum in the culture medium also improved the perseverance of epithelial cell adhesion to glass over the six-day migration period of these assays. The order of substrata that promoted the most extensive cell migration was the same in SFM and in the presence of DD serum.

The initial cell island prior to migration was greater than the initial tissue area (approximately 38 mm^2 as compared with 28.3 mm^2). However, the geometry of each system was very similar as in each case migration was radially outwards from a circular source. Direct comparisons between calculated values for tissue and cell migration need to be viewed with consideration of these facts. However, for the range of migration indices obtained the differences in the actual distance travelled by the leading edge of the tissue or a leading cell were within experimental error for a biological system. For example; a migration index of 5 would mean that the maximum, average distance that tissue had migrated over the substratum was 3.9 mm. For the same migration index, the average, maximum distance travelled by a cell from the edge of the confluent culture would be 4.1 mm. However, before the tissue migrated over the substratum it would have migrated approximately 0.5 mm down the cut side of the stroma. The differences between the two systems being approximately 0.3 mm.

2.3.11 Proliferation of corneal epithelial cells on synthetic surfaces

The numbers of cells on each of the surfaces were compared by measuring the absorbancies of Crystal violet stain eluted from the cells. The adsorbance of stain eluted from cells after six days culture on TCPS in the presence of DD serum was taken as 100%. The absorbance of the stain from cells initially seeded onto each surface inside the fence was approximately 30% in each case (Figure 2.9 grey bars). This confirmed that there was a similar number of cells in the confluent culture on each surface at the start of migration. On the glass surface there was no increase in absorbance after six days migration in SFM (Figure 2.9 open bar compared with grey bar). This suggested that the increase in area occupied by the cells (Figure 2.8B) was

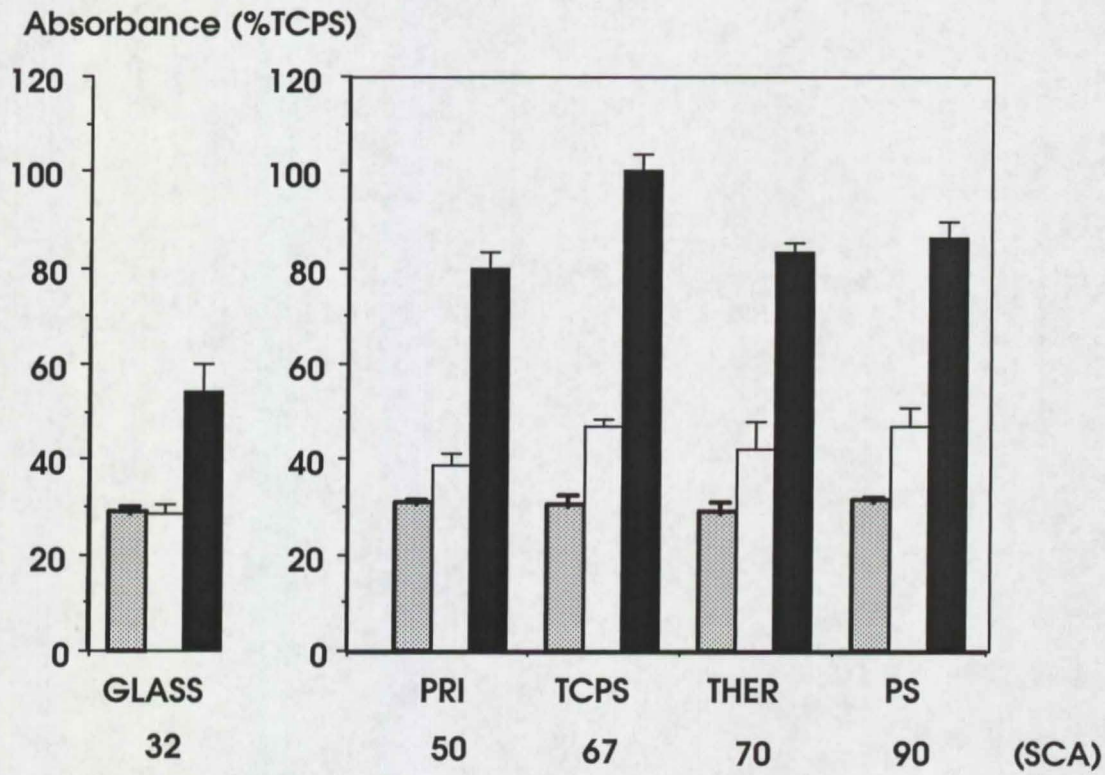


Figure 2.9 Proliferation of epithelial cells on uncoated surfaces. The cells migrated from a confluent culture over four polystyrene-based surfaces, PRI, TCPS, THER and PS. A glass surface was also tested. The culture surfaces are arranged in order of their SCA. The absorbancy of stain eluted from cells that migrated over TCPS in the presence of DD serum was taken as 100% and the results are expressed as a percentage of that value. The percentages are relative to the number of cells present on that surface. The percentage of the absorbancy of stain from the original confluent culture is indicated by grey bars, after migration in SFM by open bars and in the presence of DD serum by black bars. The results are the mean and standard deviation from two separate experiments each containing three replicates for each surface and each treatment.

entirely due to migration or spreading of the original cell population or that some cells had detached during migration. The appearance of the migrated cells on the glass surface in the absence or serum supported the latter supposition. On the other surfaces, there were 10-20% increases in absorbance in SFM after the six days of migration, indicating that some cell proliferation occurred (Figure 2.9) open bars compared with grey bars). However, the greatest increase in adsorbance occurred in the presence of DD serum (Figure 2.9 black bars).

The increases in absorbance in comparison with SFM ranged from 30% on the glass surface to 70% on TCPS (Figure 2.9 black bars compared with grey bars). The proliferation that occurred in the absence of serum (open bars compared with the grey bars) was at a much lower level than was observed in presence of DD serum. This suggested that in the absence of DD serum, migration rather than proliferation, was the principle mediator for the observed increase in the migration index (Figure 2.9 solid bars). Whereas in the presence of DD serum, cell proliferation made a greater contribution to the observed increase in the area covered by the cells.

2.3.12 Localisation of proliferating cells within the migrating tissue and cells

Immunostained nuclei were observed in all regions of the tissue that had migrated for six days prior to exposure to Brdu for 5.5 hr (Figure 2.10A). However in the region adjacent to the advancing edge of the tissue, there was less proliferation than in the region close to the explant {Figure 2.10A, (a) as compared with (b) and (c)}. This trend was also apparent in the tissue that had been exposed to Brdu for 18 hr and in tissue that had been migrating for 14 days {Table 2.1 and Figure 2.10B, (a) as compared with (b), (c) and (d)}. Transverse sections of migrating epithelial tissue are illustrated in Figure 2.10C where (a) and (b) show Brdu labelled nuclei in the basal cell layer of the epithelium above the stroma. There were no labelled nuclei visible in the migrating tissue close to the leading edge (c), but several labelled nuclei were observed closer to the explant (d). These proliferating cells also appeared to be in the basal layer.

In cells that had been migrating for three days, similar numbers of Brdu labelled nuclei were observed at the leading edge, in the mid-region and in the original cell

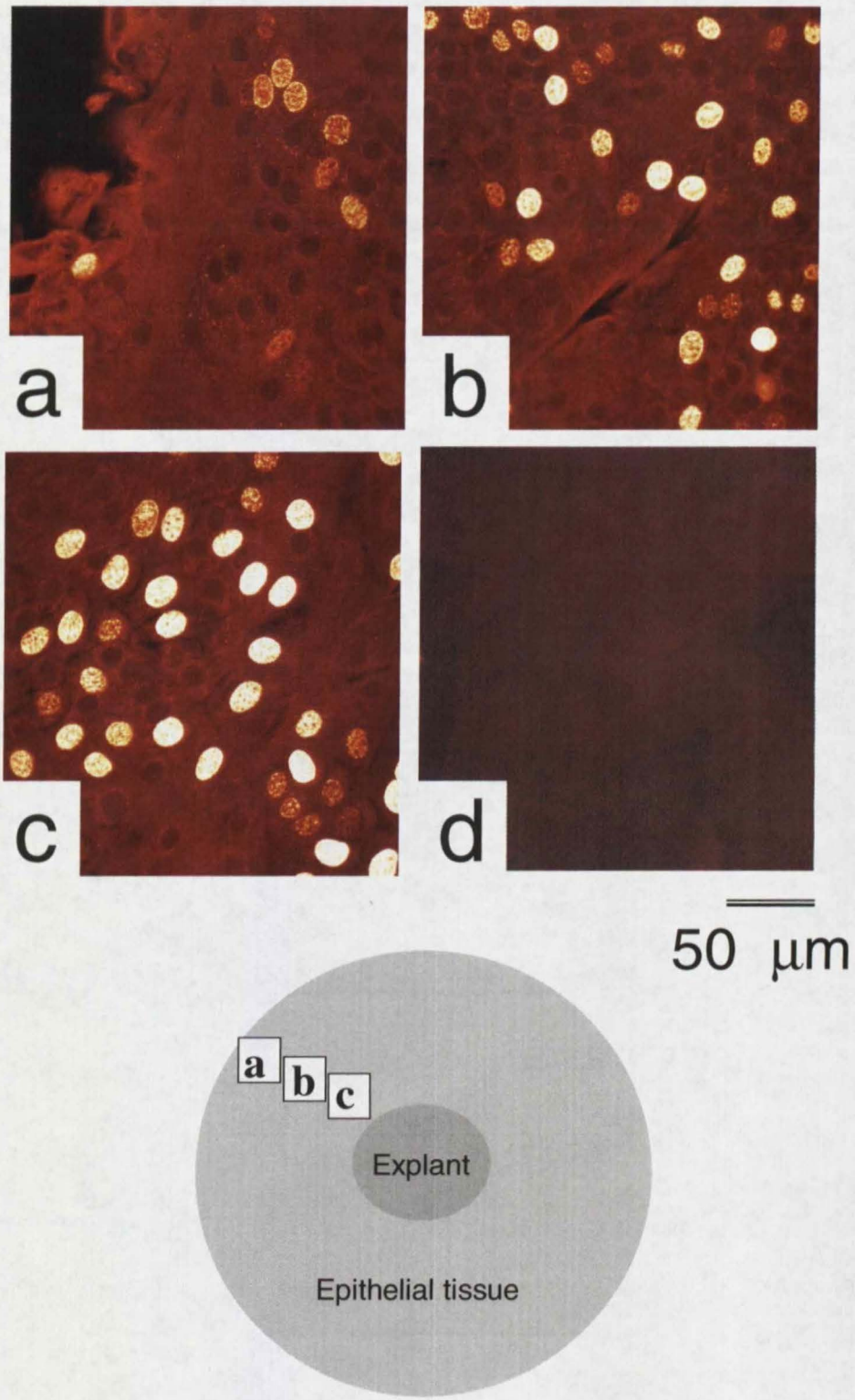


Figure 2.10A Brdu-labelled cells in epithelial tissue after six days migration (5.5 hr exposure to Brdu). The tissue was photographed at 400X magnification to better illustrate the labelled nuclei. The region of tissue (not to scale) that was photographed is shown in the diagram under the micrographs (a-c). The negative control was immunostained tissue that was not exposed to Brdu (d).

Table 2.1 Cell proliferation in migrating tissue

	Leading edge A	Mid B	Close to explant C
Day 6 5.5 hr Brdu exposure	6±5	35±15	52±18
Day 6 18 hr Brdu exposure	15±11	61±20	113±
Day 14 5.5 hr Brdu exposure	2±3	15±9	190±84
Day 14 18 hr Brdu exposure	9±6	40±15	260±97

The results are the mean and standard deviation of the number proliferating cells counted from four microscope fields, selected at random, within each of the designated areas as indicated in the diagram. Each experiment was repeated at least twice.

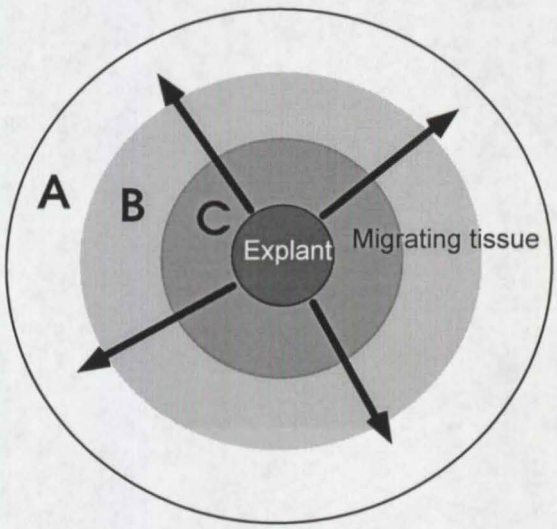


Diagram of corneal epithelial tissue migrating from a stromal explant. The areas from which samples of Brdu labelled cells were counted are illustrated by shading. The leading edge region (A) is shown as a clear area, the mid region (B) is light grey and the area close to the explant is indicated by a darker grey. (Not to scale).

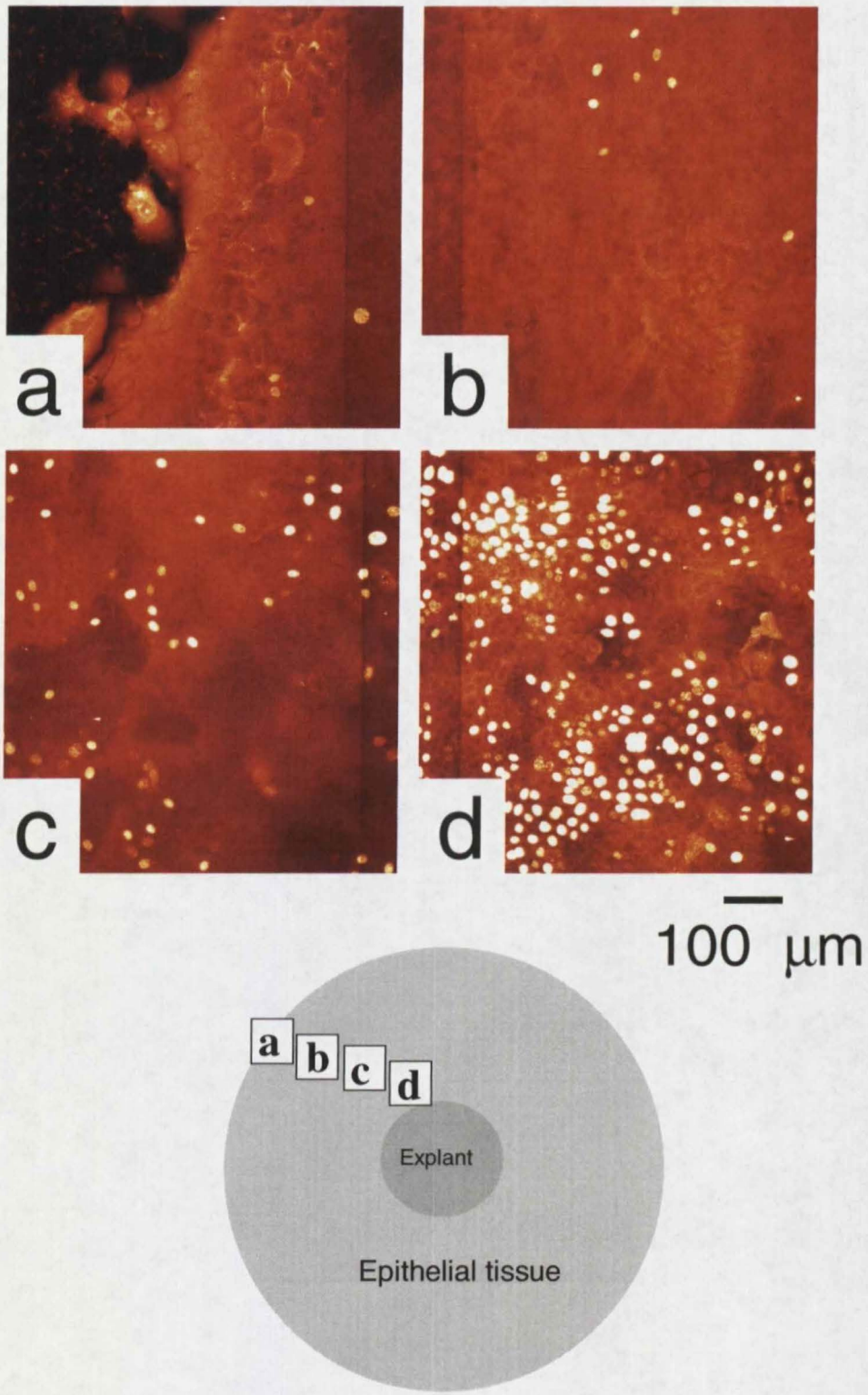


Figure 2.10B Brdu-labelled cells in epithelial tissue that had migrated for 14 days (18 hr exposure to Brdu). The region of tissue (not to scale) that was photographed is shown in the diagram under the micrographs (a-d). The micrographs were selected from a sequence of images captured using a laser confocal microscope and lines near the edge of images are areas that were previously exposed to the laser.

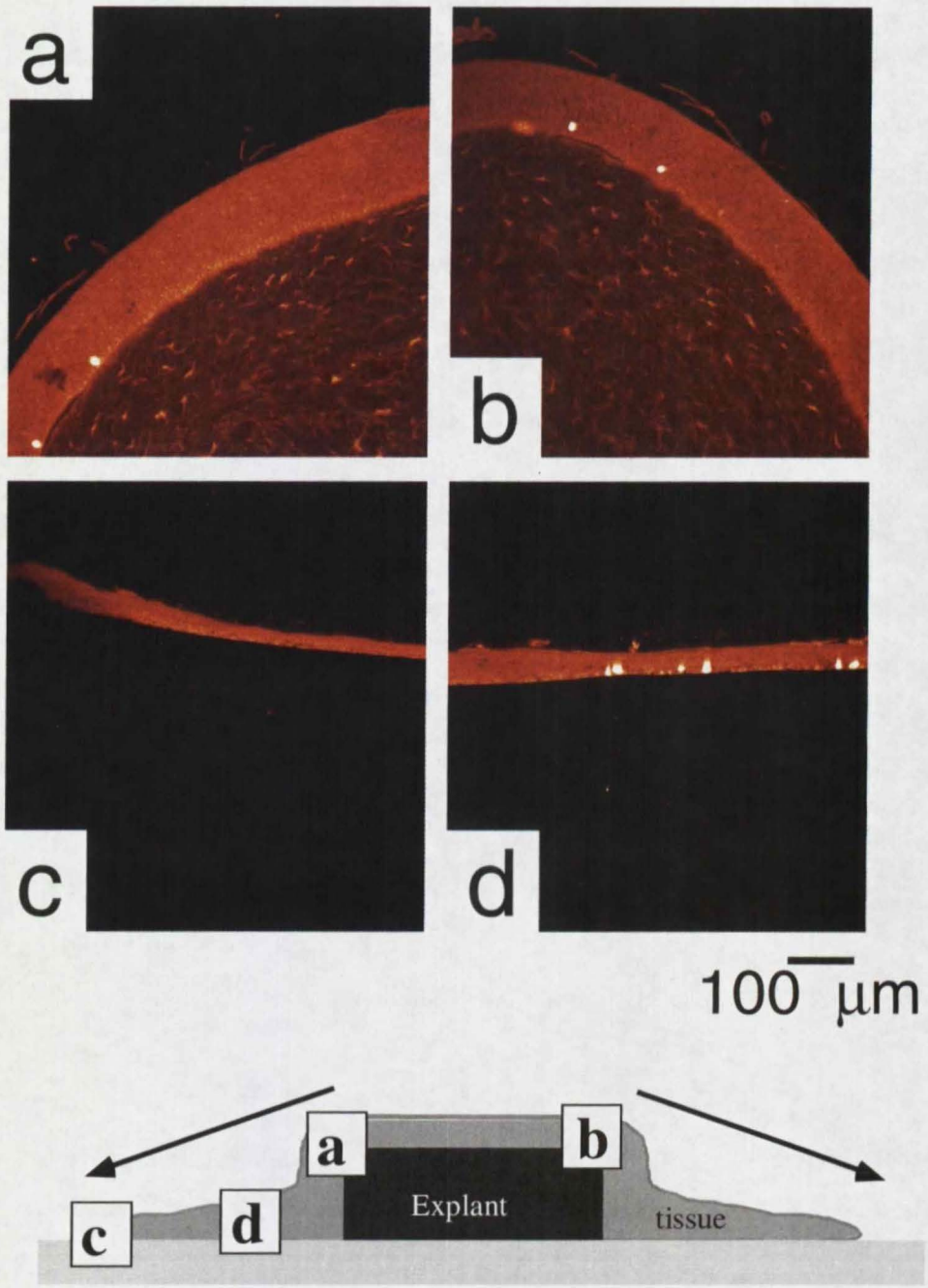


Figure 2.10C Brdu-labelled cells in epithelial tissue that had migrated for six days (5.5 hr exposure to Brdu). The tissue was fixed and embedded and transverse sections were cut and stained to illustrate cell proliferation on the explant. The regions of the sections that were photographed are illustrated in the diagram under the micrographs (a-d). The diagram is not drawn to scale.

Table 2.2 Proliferation in migrating cells

	Leading edge A	Mid B	Original cell seeding area C
Day 3 5.5 hr Brdu exposure	20±11	15±10	20±6
Day 3 18 hr Brdu exposure	48±20	32±18	43±16
Day 6 5.5 hr Brdu exposure	16±13	19±6	6±4
Day 6 18 hr Brdu exposure	26±10	24±11	11±9

The results are the mean and standard deviation of the number of Brdu-labelled cells counted from four microscope fields selected at random within each of the designated areas indicated in the diagram. Each experiment was repeated at least twice.

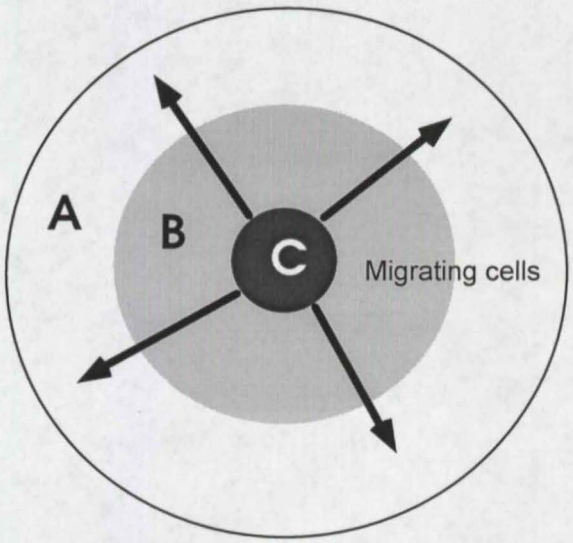


Diagram of corneal epithelial cells migrating from a cell island (C). The areas from which samples of Brdu labelled cells were counted are illustrated by shading. The leading edge region (A) is shown as a clear area, the mid region (B) is light grey and the original seeded area by dark grey. (Not to scale).

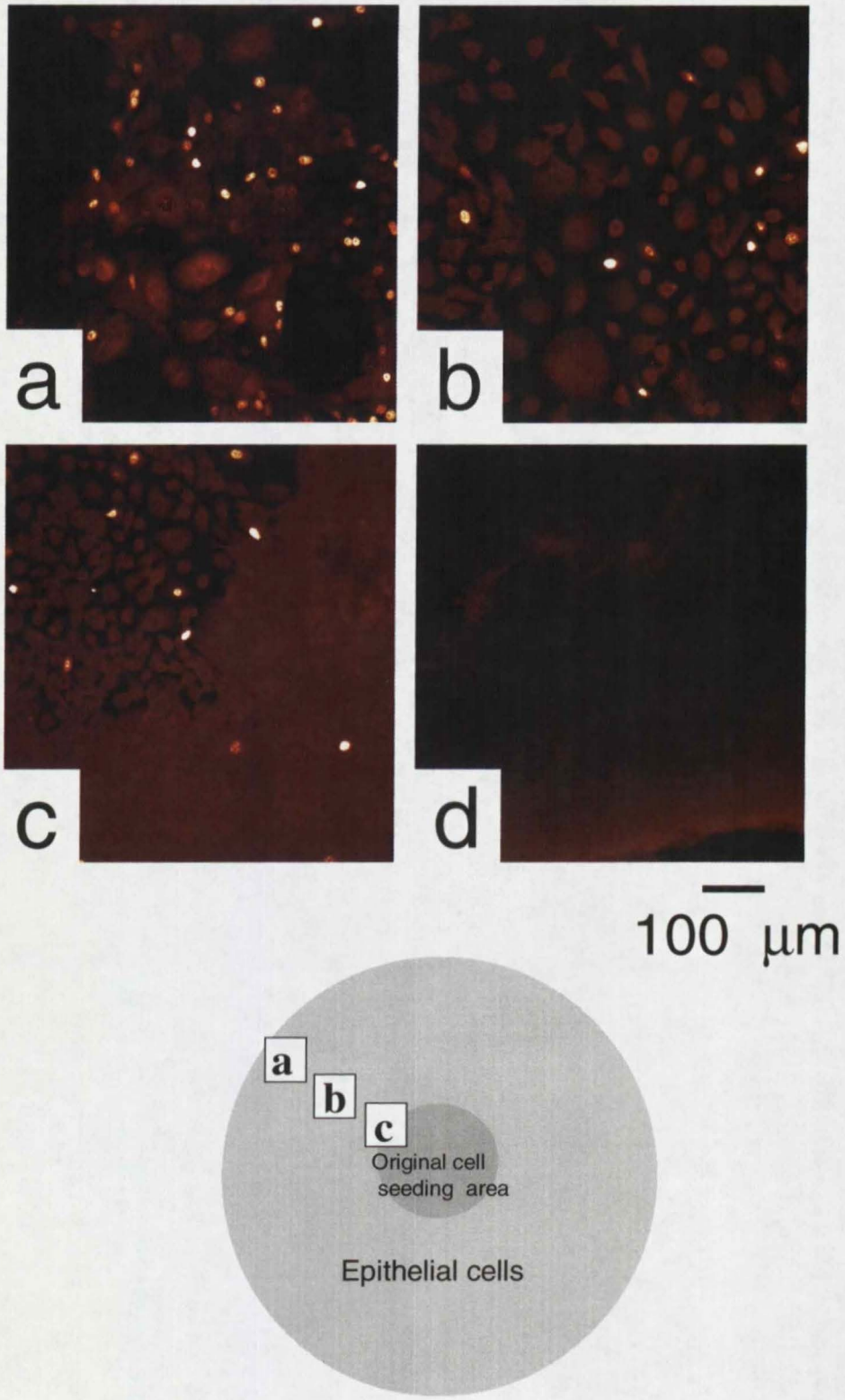


Figure 2.10D Brdu-labelled nuclei in epithelial cells that had migrated for six days (5.5 hr exposure to Brdu). The region of the cells that were photographed is shown in the diagram under the micrographs (a-c). The diagram is not drawn to scale. The negative control was immunostained cells that were not exposed to Brdu (d).

seeding area, but at the leading edge and in the mid region similar numbers of labelled nuclei were seen (Figure 2.10D and Table 2.2).

For the cell migration assays the lower numbers of dividing cells, as compared with migrating tissue, was probably related to the reduced proliferation that occurred when serum was excluded from culture medium. Figure 2.9 showed that cell proliferation under these conditions was less than when DD serum was included in the culture medium.

2.4 DISCUSSION

This study established defined model systems that would provide a means to compare the migration of intact epithelial tissue with the migration of a population of dissociated epithelial cells. There were several findings concerning the migration of epithelial tissue and cells that were of relevance to the aim of comparing migratory mechanisms. First, tissue and cells were able to migrate over uncoated surfaces in the absence of exogenous ECM components. Second, the migration of cells was increased to a greater extent by the presence of DD serum than by any of the ECM matrix molecules tested. Third, the exogenous ECM components that provided the greatest increase in the extent of tissue migration (Fn and Collagen I) also increased the extent of cell migration.

When a comparison of the effects of ECM components on tissue and cell migration was made, the coating concentrations of the ECM component were used as indicators of the amount of protein bound. The migration assays were conducted over six days and during that time there would have been some changes in the initial amount of protein bound, therefore it was not possible to specify amounts of protein bound.

The demonstration that the epithelial cells were able to migrate over a synthetic surface in the absence of any exogenous ECM components illustrated a novel ability of these cells that is not shared by the majority of cultured cells. Usually the style of cell migration studies performed here are conducted using culture medium that is supplemented with serum which contains the cell adhesive glycoproteins Fn and Vn (Hayman et al 1985). Serum Fn and/or Vn can become coated onto the culture surface to provide a matrix that will support cell attachment and migration. The chemistry of the culture surface has an effect on which serum glycoprotein will be adsorbed (Steele et al 1995a; Steele et al 1993a) and also on the quantity (Steele et al 1995a) and orientation (Underwood et al 1993) of the glycoprotein. In the case of a TCPS substratum, as was used in the initial part of this study, Vn would have been the principal cell adhesive molecule that would have adsorbed onto the surface if normal intact serum had been used in place of the DD serum (Steele et al 1993a). That situation would have confused the interpretation of the migration results, because it would not have been clear whether the tissue and cells were responding to the Vn (or

Fn) from the serum or to the ECM component being tested. In view of this, the current study was conducted either in serum free culture conditions or in the presence of serum from which the Fn and Vn had been removed. Bovine corneal epithelial cells have a novel attachment mechanism, in common with a transformed lung epithelial cell line (Steele et al 1991), that enables their adherence to a polystyrene substratum in the absence of any known exogenous ECM components (Evans and Steele 1997). The present study demonstrated that bovine corneal epithelial cells were also able to migrate over TCPS and other polymeric surfaces in the absence of exogenous ECM components. This ability is likely to depend upon endogenous secretion of ECM components. Stationary bovine corneal epithelial cells deposit Fn, Ln, perlecan and collagen IV onto the underlying polymer (Evans and Steele 1998) and laminin-5 has been identified in the trails left by corneal epithelial cells that were stimulated to migrate by TGF-α or by EGF (Qin and Kurpakus 1998). Additionally, endogenous Fn and Ln were implicated in the attachment of human corneal epithelial cells to TCPS because attachment was reduced in the presence of antibodies to Fn and Ln. (Ohji et al 1993). In view of these previous findings concerning the endogenous secretions of cultured epithelial cells, there are several ECM components that could conceivably have provided a matrix which supported the epithelial cell migration.

The presence of serum produced a greater increase in the extent of epithelial tissue and cell migration than any of the ECM coatings tested. This enhanced migration may have been due to the presence of cytokines or trophic factors in the serum acting directly to stimulate migration or proliferation. Alternatively, or additionally, serum components may have stimulated the endogenous secretion of ECM components. One of the ECM components that corneal epithelial cells are able to secrete is laminin-5 (Qin and Kurpakus 1998) and secretion of laminin-5 by human keratinocyte cultures was found to be increased by over 400% in the presence of serum (Amano et al 1999). Therefore, it is possible that serum may similarly increase the production of laminin-5 or other ECM components by bovine corneal epithelial cells, which in turn may provide a matrix over which the epithelial tissue and cells could migrate.

The attachment of bovine corneal epithelial cells to uncoated synthetic polymer surfaces in the presence of DD serum is dependent upon an intact microtubule system (Evans and Steele 1997) and proceeds at a relatively slow pace in comparison with cell

attachment to exogenously supplied cell-adhesive proteins (Evans and Steele 1998). If a similar mechanism is operational during tissue and cell migration this may account for the relatively slower rate of migration on uncoated TCPS compared with migration over Fn or collagen I coated substrata. In the current study, the relatively slower rate of cell attachment that occurs in the absence of exogenous ECM coatings, was compensated for by pre-coating the central (seeding) region of the substrata with an ECM component. In each case a high ECM coating concentration was used for this isolated region so that there would be equivalent cell populations on each of the surfaces at the start of the migration period. Fn in particular is known to adopt a less favourable conformation for cell attachment when adsorbed onto a hydrophobic surfaces as compared with adsorption onto a hydrophilic surface (Underwood et al 1993). However, this effect is not seen with higher Fn coating concentrations (Underwood et al 1993). Additionally, the cells were seeded into the fences in medium which contained DD serum because reduced cell attachment occured in SFM. Other studies have also shown serum to have a positive effect on corneal epithelial cells. The presence of serum was shown to enhance the "healing" of epithelial "wounds" created by means of freezing regions of cells in confluent corneal epithelial cultures (Jumblatt and Neufeld 1986). However, in these studies the serum had not been depleted of Fn or Vn so the adsorption of either of these molecules onto the underlying substratum could also have contributed to the enhanced migration.

In the style of migration assay used in the current study, cell proliferation was also a factor that could contribute to the final area covered by the tissue or cells. The presence of cytokines in the serum probably stimulated cell proliferation and so contributed to the final tissue and cell areas that were measured in this study. The presence of serum did not cause as great a stimulation of tissue migration as it did for cell migration. This may be because the keratocytes that were resident in the stromal tissue of the explant may have provided a separate source of cytokines. The occurrence of a greater number of cell divisions within tissue that was in closer proximity to the corneal explant, as compared with the leading edge, also provides support for this idea.

The enhanced migration of the epithelial tissue and cells observed when the substratum was coated with Fn is consistent with various reports in the literature

concerning the response of corneal epithelial cells to Fn (Fujikawa et al 1984; Nishida et al 1982) {reviewed by (Gipson et al 1993)}. The migration of tissue and cells on a substratum coated with Fn may be analogous to the epithelial response to Fn during wound healing *in vivo*. Fn is detected at the wound interface within eight hours of either a scrape wound or a keratectomy wound to rabbit corneas (Fujikawa et al 1984). Expression of the β1 integrin on the surface of epithelial cells also correlates with Fn appearance during corneal wound healing (Murakami et al 1992). This suggests that the integrin α5β1, which recognises the RGD cell-binding region of Fn may be involved in epithelial tissue migration over Fn. When wound healing is complete and rapid epithelial migration is no longer required, the deposits of Fn do not persist (Fujikawa et al 1984; Nishida et al 1982).

In the series of experiments concerning tissue and cell migration over a Fn coated substratum, the maximum migration occurred over Fn coating concentration of two to four μg/ml. Increasing the coating concentration to ten μg/ml caused no further increase or decrease in the extent of migration. The phenomenon described by Palecek and colleagues was not observed: they found that maximum migration speeds by Chinese Hamster Ovary cells were recorded on intermediate Fn coating concentrations, with a reduction in speed occurring at higher coating concentrations (Palecek et al 1996). The coating concentration of Fn at which migration was reduced was dependent upon cell integrin expression level and integrin ligand binding affinity (Palecek et al 1996). Therefore either the integrin expression level or integrin binding affinity of the corneal epithelial tissue and cells may have been less than that required to impeded migration on a coating concentration of 10 μg/ml of Fn.

Collagen I was the other matrix molecule that caused an increase in the migration of corneal epithelial tissue and cells in comparison with uncoated TCPS. The coating methodology for collagen I differed from that used to coat the other molecules tested. Only one coating concentration was used and the collagen was spread over the surface of the TCPS and air-dried. The air- drying method of application may additionally have given rise to a changed surface topography that may also have contributed to the enhanced tissue and cell migration. Certain topographic features stimulate the migration of epithelial tissue. The effects of surface topography on tissue and cell migration are considered in greater depth in Chapter 5.

Pre-coating TCPS with Ln provided very little increase in the extent of migration as compared with migration over uncoated TCPS. Ln has been shown to enhance the migration of corneal epithelial cells (Qin and Kurpakus 1998). However, in that study the enhanced migration was noted for cells that had been stimulated to migrate by the presence of TGB-β or EGF. The Ln used in Qin's studies had been isolated from human placenta and was therefore a different isoform from the commercial preparation of Ln (from EHS murine sarcoma) that was used in the current study. The lack of an increase in the extent of migration found in response to murine EHS sarcoma laminin in the current study is in agreement with the findings of Cameron and colleagues (Cameron et al 1988). The assays used by Cameron were the more traditional Boyden chamber cell migration assays. This type of assay is usually conducted over 24 hours and evaluates the numbers of cells that penetrate the pores of polycarbonate membranes. In the Boyden chamber style of assay it was found that collagen IV stimulated epithelial migration at coating concentrations of between 10^{-9} M and 10^{-7} M and migration did not occur at the higher coating concentration of 10^{-6} M (Cameron et al 1988; Cameron et al 1991). The present results did not show the same stimulation of epithelial migration in response to collagen IV that was found by Cameron. This may be because a completely different style of assay was used from the Cameron assay; with the current assay system the epithelial cells were still able to migrate effectively in the absence of an ECM coating, whereas in the Boyden assays no cells migrated in the absence of exogenous ECM coatings (Cameron et al 1988).

The two ECM molecules (Fn and collagen 1) which enhanced tissue and cell migration in comparison with uncoated TCPS are both molecules that would be encountered by the epithelium in wound healing situations where migration is stimulated. Whereas Ln and collagen IV, which did not increase the extent of migration in comparison with uncoated TCPS, are present in basement membranes. Epithelial cells in an unwounded cornea would interact with Ln and collagen IV during homeostasis, where stimulation of migration was not a requirement. Therefore it appears that there may be a logical basis for the results of tissue and cell migration over ECM components presented here.

The model systems that have been tested here could provide versatile systems that may be used by others to study various aspects of the cellular response. For example, the

confluent cell island that had been seeded in a fence could be used in a serum free assay to examine the effects of growth factors on cell migration and proliferation. The subtle effect of some growth factor is often overshadowed by the presence of serum or by the stromal keratocytes present in the corneal explant assay. The model systems have been used to determine the effect of surface topography on tissue and cell migration (Dalton et al 2001a; Dalton et al 2001b) and see Chapter 5. The migration of bovine corneal epithelial tissue from a corneal explant in serum-free culture has proved to be a versatile model. It has been used to examine the response of epithelial tissue towards specific substrata in terms of stratification and formation of stable adhesion (Dalton et al 1999; Evans et al 1999).

2.5 CONCLUSIONS

Epithelial tissue and cells were able to migrate over all the surfaces tested in the absence of exogenous ECM components. In view of this novel ability it would be possible to use uncoated surfaces in subsequent studies into migratory mechanisms. However, because of the comparatively poor migration of cells in the absence of serum it would be preferable to use serum when considering cell migration. The migration response of tissue and cells towards each of the ECM molecules and each surface chemistry tested was very similar and did not provide a basis for further investigation of differences in between the mechanisms of migration.

CHAPTER 3

THE STRUCTURE OF MIGRATING EPITHELIAL TISSUE AND CELLS

3.1 INTRODUCTION

Although cell migration has frequently been studied using individual cultured cells that have been derived from tissue, an obvious potential difference between migrating tissue and cells is that tissue structure may constrain cellular mechanisms, possibly in different ways depending on individual tissue. In order to test the hypothesis that migration of corneal epithelial tissue differs from the migration of individual epithelial cells, in this chapter the following questions were addressed: (1) do the constituent cells in epithelial tissue remain fully attached to each other or do they migrate as separate cells, albeit in very close proximity? (2) are several layers of cells transported when epithelial tissue migrates? and (3) are the submarginal cells dragged passively over the substratum by the force generated by the marginal cells? Examination of the superior surface of the migrating tissue using light microscopy during the studies described in the previous chapter suggested that migrating tissue does remain as an integrated unit. In this chapter experiments are described that are directed towards substantiating this, by testing whether intercellular connections between component cells within the tissue persist during migration. Several different strategies were used to investigate aspects of tissue structure during migration.

The structure of the tissue was studied using histological techniques. In order to use these techniques to examine the migrating tissue, it was necessary to culture migrating tissue on substrata that were compatible with histological processing and sectioning. The results from Chapter 2 and other studies (Dalton et al 1999; Dalton et al 2001a; Dalton et al 2001b) showed that bovine corneal epithelial tissue and cells were able to migrate over a range of surfaces. Consequently a range of materials were available that could be processed for light and electron microscopy after serving as the substrata for these experiments. This enabled transverse sections through the entire migrating tissue to be examined. The examination of the ultrastructure of transverse sections of migrating tissue would provide insight into the persistence and location of adhesion complexes between adjacent cells within migrating tissue.

Desmosomes are one of the cell-cell adhesion complexes that contribute to the maintenance of the integrated tissue structure within a stratified corneal epithelium (Gipson and Sugrue 1994). In a human unwounded corneal epithelium, desmosomes are most numerous between the wing cells. The normal bovine corneal epithelium has approximately 12 layers of cells, which is approximately double the number of layers present in a human corneal epithelium. A transverse section showing the cell layers within the bovine corneal epithelium is presented in Figure 3.1(a) and shows that, in common with the human eye, desmosomes are numerous between adjacent wing cells. Desmosomes can be identified as precisely aligned, electron-dense regions on adjacent cell membranes, some of which are marked by arrows on the electron micrographs in Figure 3.1(b-d). Desmoplakins I and II are components of desmosomes. The desmoplakins have not been detected in the plaques of any junctional complex apart from desmosomes and so immunolocalisation of these proteins in the cell membrane may be taken as evidence of the presence of desmosomal junctions (Schwarz et al 1990). The persistence of desmoplakin within migrating tissue would indicate that desmosomes were contributing to intercellular adhesion.

In addition to the relationship between adjacent cells within migrating tissue, an interaction between the tissue and the underlying substrata is required for migration to occur. The migration of individual cells relies upon an ability to adhere to the surface with sufficient strength to allow for subsequent spreading of the cell margin (Huttenlocher et al 1995). One part of the margin must be able to extend outwards, while the opposite side retracts inwards (Lee et al 1993). The adherence to a synthetic surface may involve the formation of transient focal adhesions involving a series of molecules that link elements of the cytoskeleton to the ECM molecules bound to the substratum (Huttenlocher et al 1995). Talin, vinculin, and alpha-actinin are components of focal adhesions (Crowley and Horwitz 1995) together with syndican-4, which appears to act as a regulator of focal adhesion formation (Longley et al 1999). The location of focal adhesions can be identified by immunostaining of permeabilised cells with anti-vinculin antibodies. Within migrating cells, focal adhesions that have been located by immunostaining generally appear as small, punctate, arrow-shaped regions at the periphery of extending lamellae. If, as suggested by DiPasquale (DiPasquale 1975(a); DiPasquale 1975(b)), the marginal cells are responsible for the

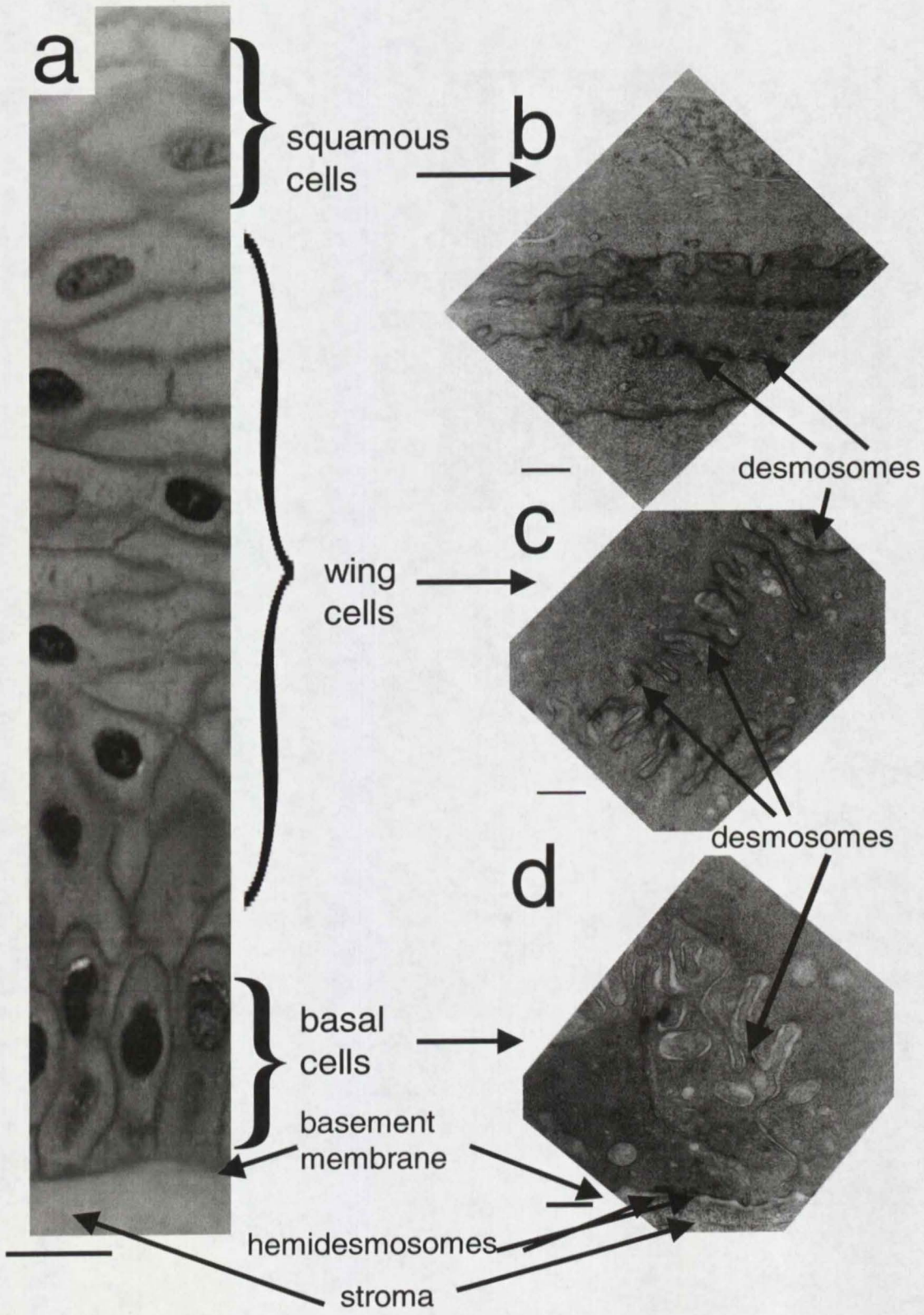


Figure 3.1 Light micrograph (a) and electron micrographs (b-d) showing transverse sections of normal bovine corneal epithelial tissue. The light micrograph on the left shows the general structure of the tissue and the electron micrographs on the right are placed opposite appropriate regions. Bar in (a) = 5 μm; bars in (c-d) = 500 nm.

force which drags epithelial tissue forwards, then focal adhesions would not be found at the base of submarginal cells within the tissue. However, if both marginal cells and submarginal cells are active participants in tissue migration {as suggested by Mahan (Mahan and Donaldson 1986)} then focal adhesions would be located on both the marginal and the submarginal cells.

In this Chapter, aspects of the relationship between the component cells within migrating tissue were considered and compared with epithelial cells migrating from a confluent culture. Firstly, the anterior surfaces of the migrating tissue and cells were examined using scanning electron microscopy. Secondly, the structure of tissue was viewed in transverse section using histological methods. Light microscopy enabled the overall structure of the migrating tissue to be viewed, and the ultrastructure of the intercellular junctions within the tissue sheet were examined using transmission electron microscopy. Thirdly, immunocytochemistry was used to investigate the persistence of desmosomes during migration, through probing for the presence of desmoplakin. Finally, the relationship between the migrating tissue and cells with the substratum was examined by investigating the location of focal adhesions.

3.1.1 Aim

The aims of the research described in this chapter were to determine if the component cells within corneal epithelial tissue remained attached to each other during migration and to establish if the submarginal cells actively participated in migration.

3.1.2 Hypotheses to be tested

(1) Within the migrating tissue, the component cells maintain some form of cell-cell adhesion, possibly desmosomes, during migration.

(2) More than one layer of cells is present within the migrating tissue.

(3) Basal cells located both at the leading edge (marginal cells) and following basal cells (submarginal cells) are active participants in the transport of anteriorly located cells. Therefore focal adhesion will be present at the interface between basal cells and the substratum in all regions of the tissue sheet.

3.2 MATERIALS AND METHODS

3.2.1 Tissue and cells

Corneal epithelial tissue and individual corneal epithelial cells were cultured (as described in Chapter 2) on glass coverslips, polycarbonate disks (Poretics Corporation, Livermore, California) and perfluropolyethylene disks (Molecular Science, CSIRO) that were placed within culture wells of a six-well tray (Corning). The tissue and cells were fixed after three to seven days culture while they were still actively migrating, then they were either processed for observation by light or electron microscopy or immunostained.

3.2.2 Tissue and cell processing for scanning electron microscopy

Epithelial tissue that had migrated over the polycarbonate discs, was fixed in 3% (v/v) glutaraldehyde (ProSciTec, Thuringow, Queensland, Australia) in 0.1M cacodylate buffer (pH 7.3) for a minimum of two hr. After washing, the tissue was fixed in 1% (w/v) osmium tetroxide (ProSciTec) for one hr, washed and post-fixed in 1% (w/v) tannic acid (Sigma Chemical Company, St Louis, MO, USA). The samples were dehydrated through a graded series of ethanol, soaked in hexamethyldisilazane (Sigma) for ten min and air-dried. The samples were sputter-coated with a thin layer of gold using established standard coating proceedures (Fitton et al 1998) and viewed using a scanning electron microscope (Cambridge Stereoscan) at magnifications of up to 5000x.

3.2.3 Tissue and cell processing for light microscopy

Tissue and cells that had migrated over the polymer disks were washed in PBS and fixed by incubation in 4% (v/v) formol saline for a minimum of two hr. The samples were dehydrated and infiltrated with paraffin wax using a tissue processor (Leica, RRI050, Germany) programmed with the schedule outlined in Appendix A 4.

The samples were embedded in 56°C melting point paraffin wax (Paraplast, Oxford Labwork, St. Louis, MO, USA) on a tissue embedding station (Leica, EG 1160) and oriented so that transverse sections could be cut to include the original explant (or confluent culture) and the migrated tissue (or cells). Ribbon sections, 5 μm thick, were cut on a Leitz rotary microtome using stainless steel disposable blades (Feather

A35, HD Scientific, Sydney, NSW, Australia) and expanded on a waterbath maintained at 45°C. The sections were collected onto gelatine-coated glass slides, placed horizontally in racks and dried by incubation at 60°C for a minimum of 1 hr. The slides were stained with Haematoxylin and Eosin using the protocol described in Appendix A5, before they were mounted using Ultramount (Fronine, Riverstone, NSW, Australia). Colour images were captured on Kodak Gold ASA 100 print film and black and white images were obtained using a digital camera. A Leica upright microscope was used in each case.

3.2.4 Tissue and cell processing for transmission electron microscopy

The tissue that had migrated over polymer surfaces was fixed for two to three days at 4°C in 1.25% (v/v) glutaraldehyde, 1% (w/v) paraformaldehyde (Sigma) freshly made in 0.2M sodium cacodylate buffer (pH 7.3). After washing with cacodylate buffer the tissue was post-fixed with 1% (w/v) osmium tetroxide (ProSciTec) reduced with 2% (w/v) potassium ferrocyanide (Sigma) for two hr, followed by overnight *en bloc* staining with 2% (w/v) aqueous Uranyl acetate (Ajax Chemical Ltd., Sydney, Australia) at 4°C. The samples were dehydrated through a graded series of ethanol for 15 min at each step, infiltrated over three days with increasing concentrations of Epon-Araldite (ProSciTec) mixed with dehydrated absolute ethanol. Samples were sandwiched between overhead transparency sheets to reduce curling during the embedding procedure that was done in flat moulds. Curing took place at 60°C for 60 hr. Semi-thin sections (500 nm) were cut using a glass knife, collected onto glass slides and stained with 1% (w/v) Toluidine blue (Sigma Chemicals) in 0.02M boric acid (Ajax Chemicals) to determine the correct angle. Ultrathin sections (100 nm) through the tissue and the underlying polymer were cut using a diamond knife (Diatome US, Fort Washington, PA, USA), collected onto uncoated 200 mesh copper/palladium grids (ProSciTec), stained with 4% (w/v) aqueous Uranyl acetate for one hr and Renold's lead citrate (Appendix A6) for one min then viewed using a Philips CM 100 transmission electron microscope. Images were photographed using Electron Microscope cut film (Kodak). The negatives were scanned at 300 ppi (ArtixScan 1100, Microtek). The digitised images were rotated so that the base of the tissue in contact with the polymer was at the lower edge of the image.

3.2.5 Immunostaining for desmoplakin

Corneal epithelial tissue and cells that had migrated over the glass coverslips were washed in PBS, fixed and permeabilised by incubation in ice-cold methanol for 10 min. All subsequent procedures were done at room temperature. The tissue was further permeabilised by incubation with 0.5% (v/v) Triton X100 (Calbiochem, CA, USA) in PBS for 10 min. Non-specific binding sites on the tissue and cells were blocked by incubation for 30 min in PBS containing 2% (w/v) BSA. The corneal explants were carefully dissected from the coverslips, without disturbing the epithelial tissue that had migrated over the coverslip. The bases of the coverslips were dried and they were placed on parafilm (tissue or cells uppermost) in 60 mm diameter dishes. Damp filter paper was placed around the parafilm so that a humid atmosphere was maintained within the sealed dish to protect the samples from drying. Antibodies to desmoplakins I and II (Boehringer Mannheim, Germany) supplied as ascites fluid were diluted 1:50 in 2% (w/v) BSA/PBS and sufficient antibody solution was added to each coverslip to cover the sample. The dishes were sealed and the samples were incubated in the presence of the antibody for 90 min. The coverslips were returned to the six-well trays and washed in PBS for 10 min (on a rocker) three times. The base of the coverslips were again dried, before they were returned to the parafilm in the humidified dishes. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (Dakopatts, F232) was diluted 1:20 in PBS and sufficient solution was added to cover each sample. The dishes were sealed, protected from light and incubated for 60 min. The samples were washed in PBS on a rocker for 10 min, four times. The base of each coverslip was rinsed with distilled water before the sample was sandwiched between the coverslip and a glass microscope slide using Fluorosave™ (Calbiochem). The samples were examined using a laser confocal microscopy equipped with an argon/krypton laser (Leica, Heidelberg, Germany). Images in one focal plane were collected using a wavelength of 488 nm and a pinhole of 90.

3.2.6 Immunostaining for vinculin

The protocol used for vinculin detection was the same as was used to probe for desmoplakin, except that after fixation the tissue and cells were permeabilised for 20 min, with 0.5% (v/v) Triton-X100 in PBS. The primary antibody was a mouse

monoclonal antibody against human vinculin (Sigma-Aldrich, Missouri, USA). Images were collected in one focal plane adjacent to the substratum.

3.2.7 Negative controls

The negative controls for desmoplakin and vinculin staining comprised samples of tissue and cells in which normal mouse serum was used in place of the primary antibody.

3.2.8 Reflection microscopy

An image of the same field as was used for the vinculin localisation was captured using the laser confocal microscope in reflection mode. Tissue at the leading edge and individual cells were examined by reflection microscopy. It was not possible to use the reflection mode on regions of tissue apart from the leading edge, due to the depth of the tissue.

3.3 RESULTS

During the seven-day culture period, epithelial tissue migrated radially outwards from the corneal explants for approximately three millimetres onto the glass coverslips or the synthetic polymers. In the cell model where dissociated epithelial cells were seeded to form a monolayer, the cells also migrated in a radial direction and started to separate from each other after leaving the confluent culture.

3.3.1 The anterior surface of migrating tissue and cells

Scanning electron microscopy was used to closely examine the anterior surface of the migrating tissue and cells. Scanning electron microscopy was an effective method with which to view migrating tissue because of the depth of the field of focus. At the (lower) magnification of 500x, the migrating tissue generally presented as a unified structure in which it was often difficult to identify boundaries between the component cells. In some regions, desquamating squamous cells were observed {Figure 3.2A(a) small arrow}. Along the leading edge, some areas were comparatively smooth and did not exhibit any ruffling activity {Figure 3.2A(a) tissue front between two * markers}, whereas other areas appeared to be uneven (see lower left region of Figure 3.2A(a). When the "uneven" areas were viewed at higher magnification, thin flat lamellipodia extended forwards and microspikes were present on some of these lamellipodia {Figure 3.2A(b) arrow-heads}. Other regions of the advancing tissue-front had extensive ruffling activity with lamellipodia extending in the direction of migration {Figure 3.2A(c)}. Close to the corneal explant, the anterior surface of the tissue was composed of large squamous cells covered with microplicae and microvilli {Figure 3.2A(d)}.

The cells that had migrated from a confluent island of cultured cells ("confluent culture") appeared flatter and more spread than component cells in the migrating tissue and it was difficult to differentiate between the lamella or lamellipodia and the cell body {(Figure 3.2B(a-c)}. In some areas, the cells appeared to be in very close contact with each other and cells from the original confluent culture had developed what looked like interdigitations between adjacent cells {(Figure 3.2B(d) arrow}.

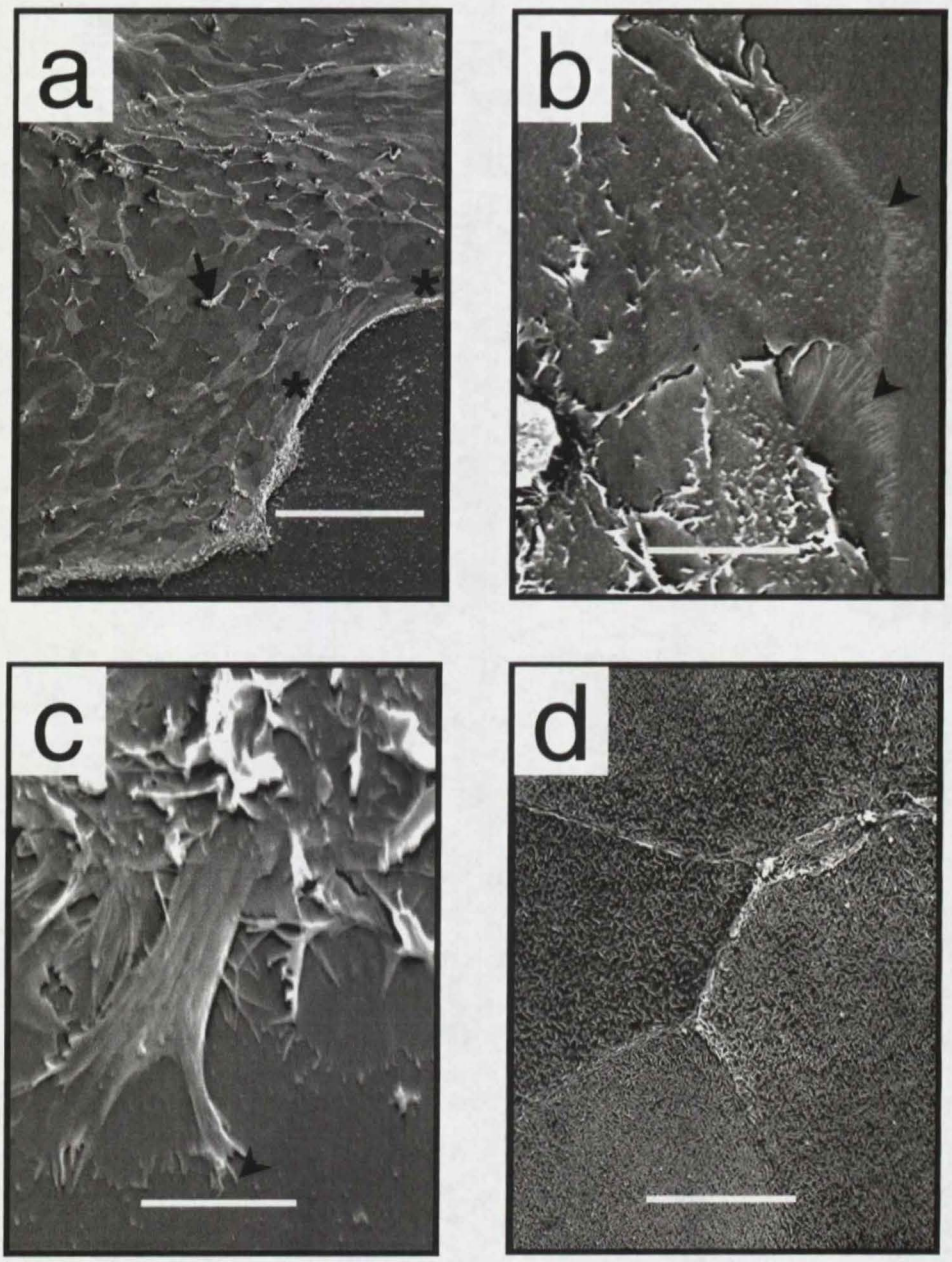


Figure 3.2A Scanning electron micrographs of bovine corneal epithelial tissue migrating from a corneal explant. The advancing edge of the tissue is shown in (a-c) and the anterior squamous cells on tissue close to the explant is shown in (d). The scale bar in (a) is 500 µm, the bar in (b) is 100 µm, the bar in (c) is 10 µm and the bar in (d) is 200 µm.

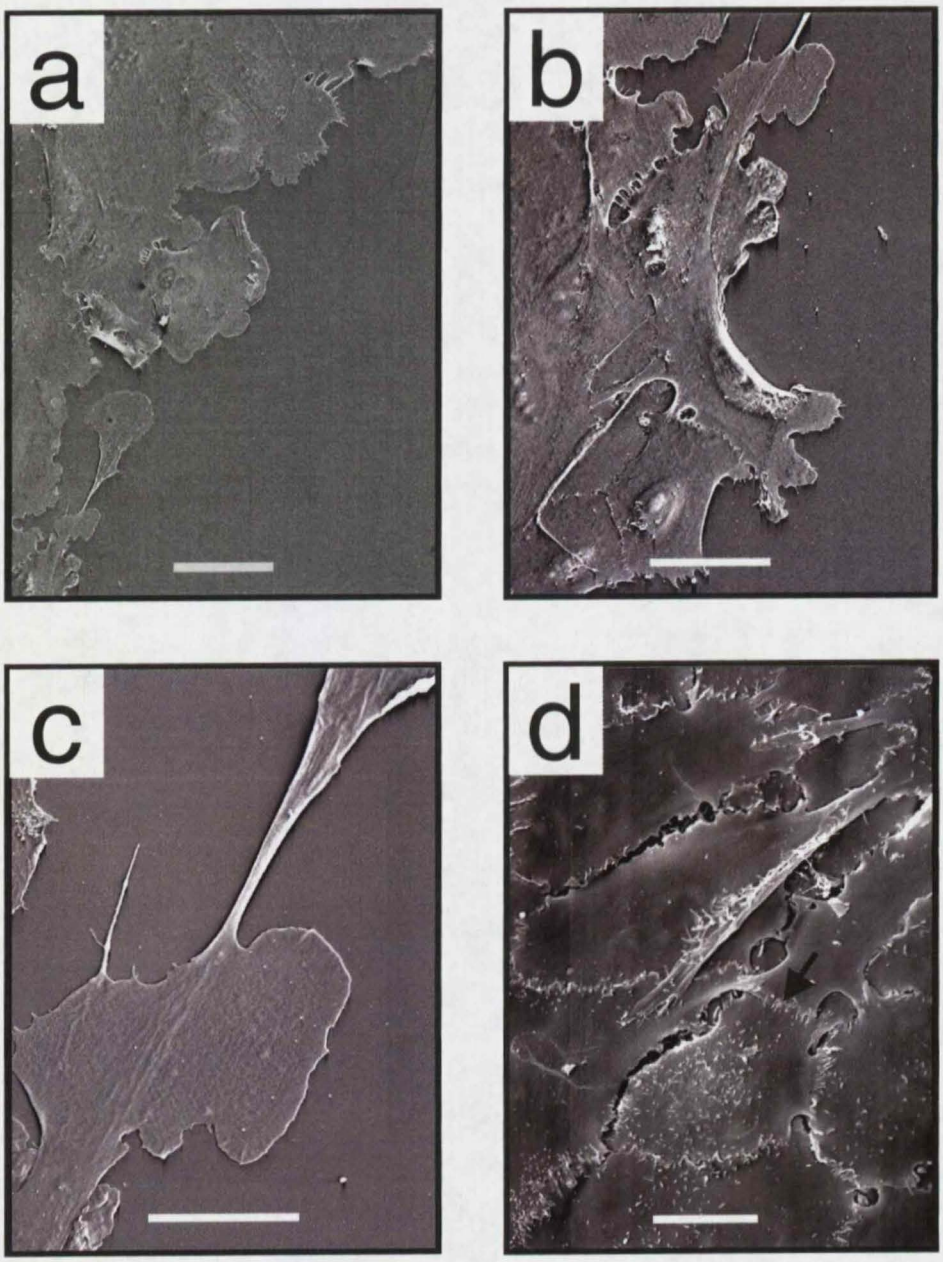


Figure 3.2B Scanning electron micrographs of bovine corneal epithelial cells migrating from a confluent culture. Cells at the advancing edge are shown in (a-c) and cells in the center of the culture in (d). The scale bar in (a) is 100 µm, the bar in (b) is 50 µm and the bars in (c) and (d) are 20 µm.

3.3.2 The structure of migrating tissue and cells

Transverse sections were obtained to enable the vertical structure of the migrating tissue and cells to be examined. The sections transected the explant (or confluent cell cultures) and the tissue (or cells) that had migrated over a synthetic polymer. This was achieved by embedding fixed, dehydrated samples in paraffin wax or resin and cutting vertical sections through the sample and underlying polymer. The multilayed nature of the migrating corneal epithelial tissue was clearly demonstrated by examination of stained, transverse sections of the tissue. Figure 3.3A(a) shows epithelial tissue, which was composed of several layers of cells, migrating down the cut side of the stroma of the corneal explant {far left in the image in Figure 3.3A(a)} and over the surface of the polymer. The epithelial tissue that remained on the corneal explant maintained similar morphology to the stationary stratified epithelium, with columnar basal cells, diamond shaped wing cells and flattened squamous cells {Figure 3.3A(a)}. The extent of migration over the substratum is illustrated by the continuing series of micrographs show in Figure 3.3A(b-d). Adjacent to the explant and along the majority of the migrated tissue, the tissue comprised several layers of cells {Figure 3.3A(a-c) and Figure 3.3B(a)}. Within the leading edge, there were fewer layers of cells but the integrity of the tissue was still maintained {Figure 3.3A(c) and Figure 3.3B(b)}. Ahead of the migrating tissue in Figure 3.3B(b) there appeared to be a faint band of blue-staining material that may be ECM.

The micrographs of migrating cells showed that the cells which were originally seeded as the confluent culture were in very close proximity to each other {Figure 3.3C(a)}. The migrating cells at the leading edge were spread and very flat and became difficult to identify using light microscopy {Figure 3.3C(b)}. However, their shape could be more easily identified by electron microscopy {Figure 3.3C(c)}.

Electron microscopy was also used to examine the interactions between component cells within the migrating tissue. The electron micrographs in Figure 3.4A show tissue from approximately the middle region of tissue that had migrated over the synthetic polymer (see diagram under the micrograph). The tissue in this region comprised about five layers of cells, which were very elongated in comparison with cells in the resting stratified epithelium (Figure 3.1). In particular, the basal cells had

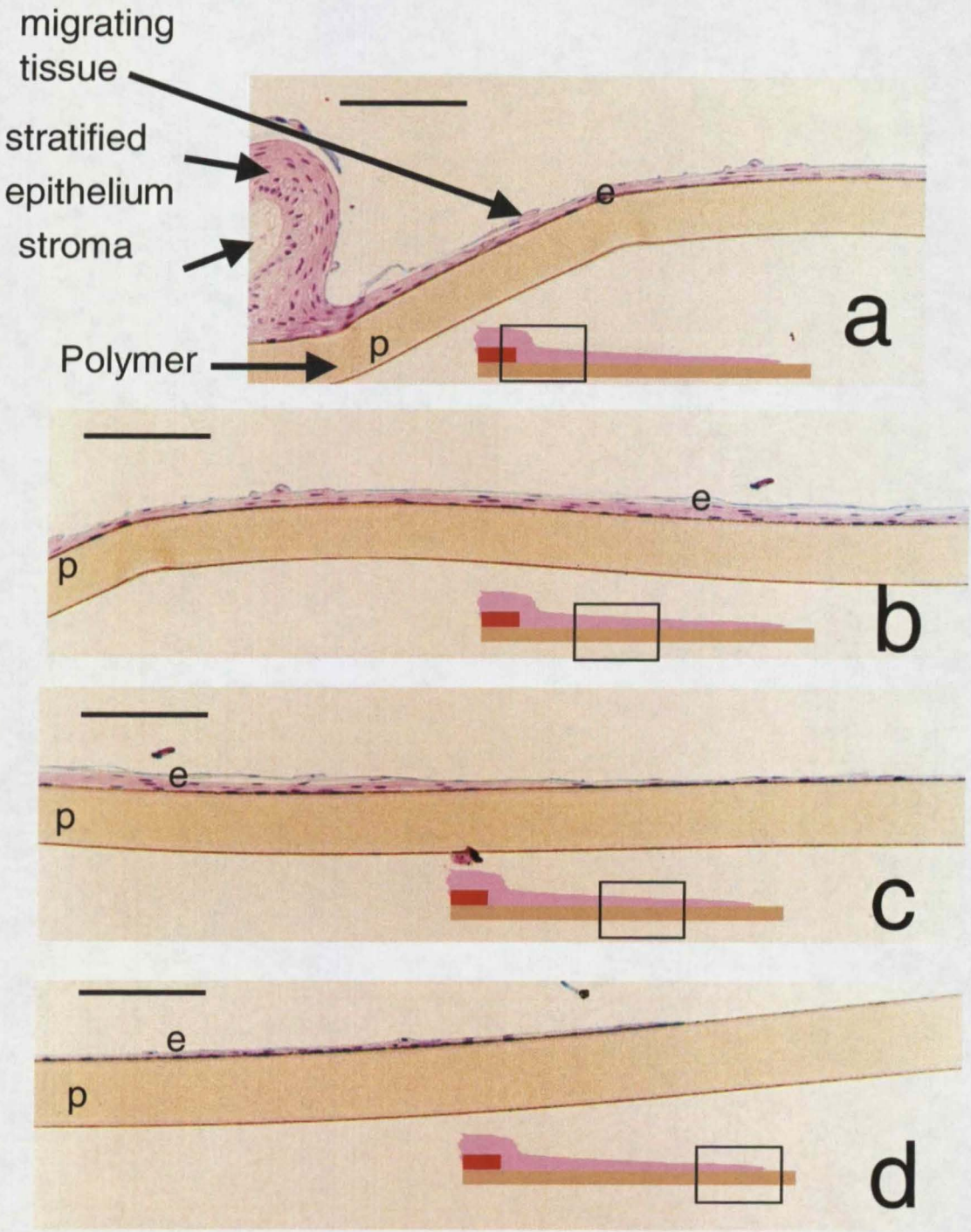


Figure 3.3A Transverse sections of bovine corneal epithelial tissue (e) that had migrated for six days over a perfluropolyethylene disk (p). The region of tissue from which the micrographs were photographed is illustrated by the rectangle in the inset diagrams. Epithelium migrating down the cut side of the stroma is visible in (a), tissue migrating along the polymer in (b) and (c) and the leading edge of the tissue in (d). The scale bars are 150 μm.

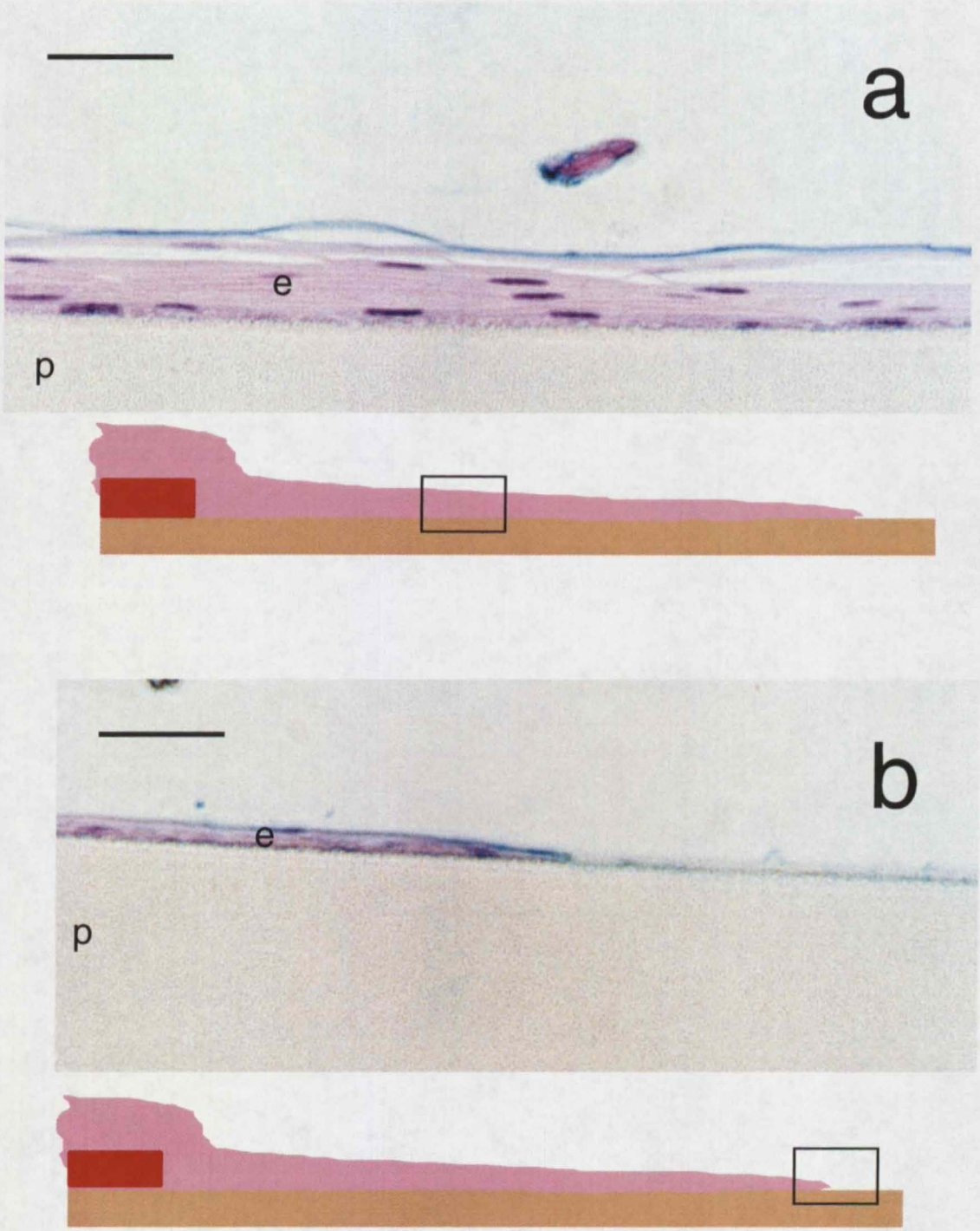


Figure 3.3B Transverse sections of bovine corneal epithelial tissue (e) that had migrated for six days over a perfluropolyethylene disk (p). The region of tissue from which the micrographs were obtained is illustrated by the rectangle in the inset diagrams. These micrographs are enlargements of the tissue shown in Fig.3.3 A (b) and (d). The scale bars are 50 μm.

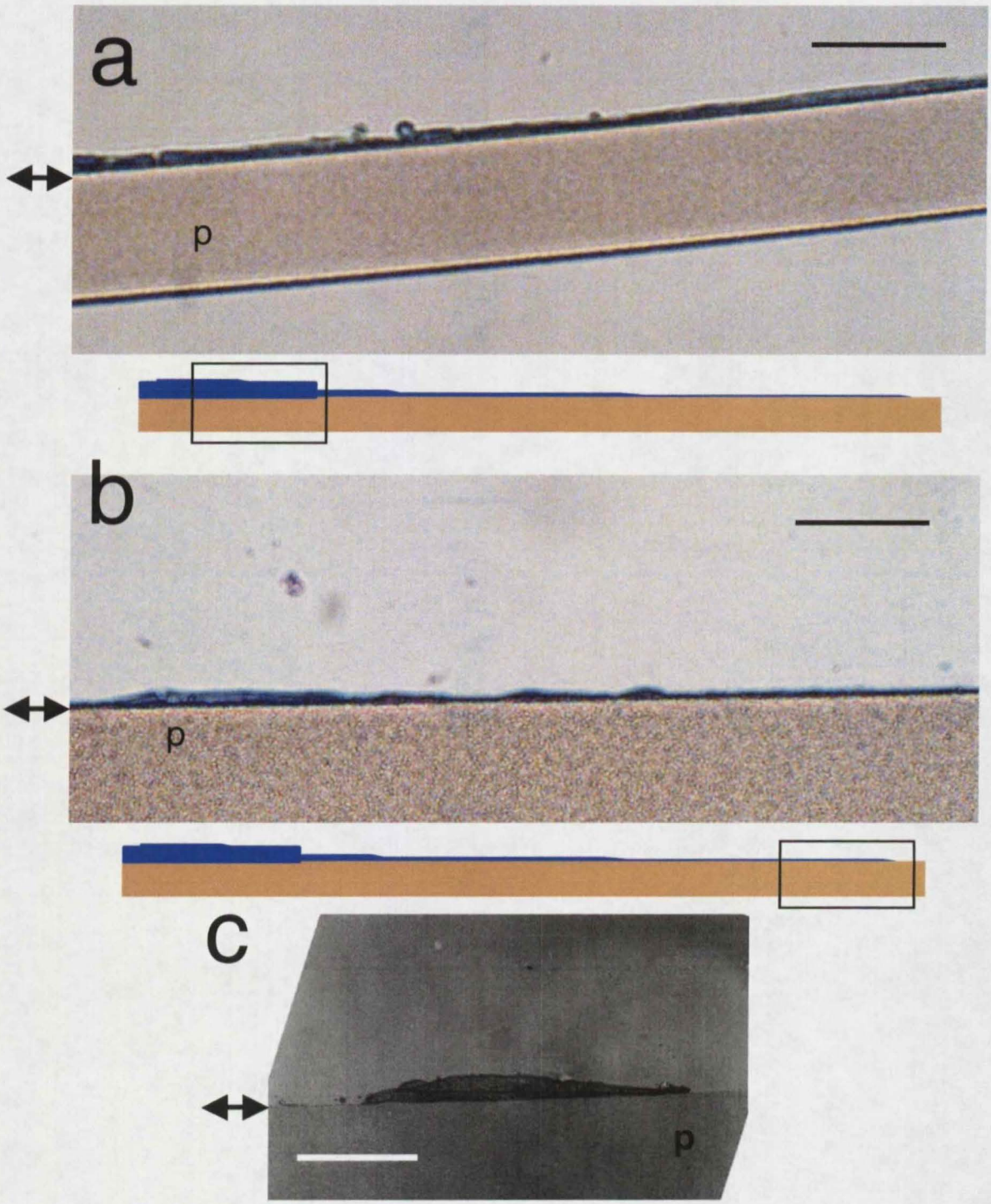


Figure 3.3C Transverse sections of bovine corneal epithelial cells migrating from a confluent culture over a perfluropolyethylene disk. The interface between the cell and the polymer is indicated by a double-headed arrow. The region of cells from which the micrographs were obtained is illustrated by the rectangle in the diagrams under the images. The cell in the electron micrograph (c) was moving towards the right. The scale bars in (a-b) are 50 µm and in (c) the bar is 10 µm.

lost the columnar appearance of basal cells in the non-migrating stratified epithelium. Punctate electron dense regions, characteristic of desmosomes, could be identified between adjacent membranes of some cells and these were more numerous in the anterior layers {see (Figure 3.4A(a) and viewed at a higher magnification in micrograph (b)}. Close to the advancing edge of the migrating tissue, the basal cells became more elongated but the electron dense regions between adjacent cells could still be observed between the numerous interdigitation that persisted between the cells {(Figure 3.4B(a) and (b)}. In the same figure, protrusions from following cells can be observed extending under the cells travelling ahead. In some regions of the advancing edge, the tissue was reduced to one layer of cells. Figure 3.4C(a) shows the persistence of interdigitations and an electron dense plaque between a leading edge cell and a following cell. The micrograph (b) in the same figure shows a region of a leading cell in which the interdigitation between the leading cell and the second cell have loosened. An electron dense region at the interface between the underlying polymer and the leading cytoplasm (see arrow) is indicative of a focal adhesion. It appeared that the leading cell show in the micrograph {Figure 3.4C(b)} has separated from the following cell. However, because an electron micrograph only shows a transverse section through one plane of a cell it may have been possible that connections between the first and second cell were maintained in a different region. Between the membranes of the adjacent cells visible in Figure 3.4C(b) there appeared to be a material that was probably ECM. Material with a similar electron-microscopic appearance is also visible at the interface between the basal cell membrane and the underlying substratum {Figure 3.4C(a) and (b)}.

3.3.3 Immunolocalisation of desmoplakin

Desmoplakin, which is indicative of desmosomes was detected on immunostained tissue and cells that had migrated over glass coverslips. The tissue that was close to the explant showed extensive staining for desmoplakin on the cell membranes (Figure 3.5a). At the advancing edge of the tissue there was no desmoplakin at the "free" side of the leading cells, but desmoplakin was visible on membranes between all adjacent cells (Figure 3.5b). In the cell model, desmoplakin staining was localised to the membranes between adjacent cells located in the original seeding zone where the

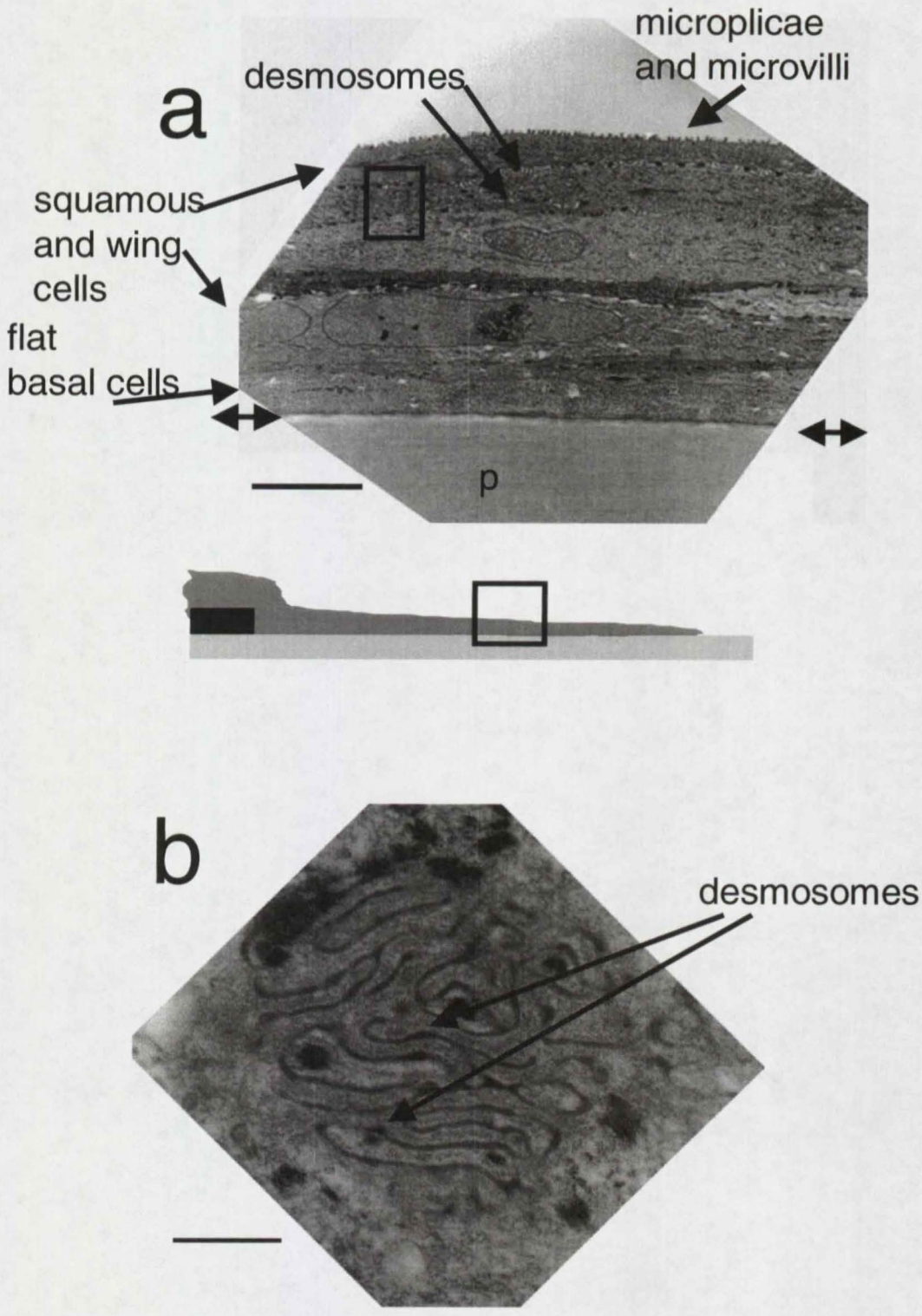


Figure 3.4A Electron micrographs of transverse sections of bovine corneal epithelial tissue that had migrated for six days over a perfluropolyethylene disk (p). Double headed arrows mark the interface between the tissue and the polymer. The approximate location of the tissue in (a) is indicated by the rectangle in the diagram under the micrograph. The rectangle on micrograph (a) indicates the approximate region of the tissue in micrograph (b), which shows the interdigitation between adjacent cells. The scale bar in (a) is 5 μm and in (b) is 500 nm.

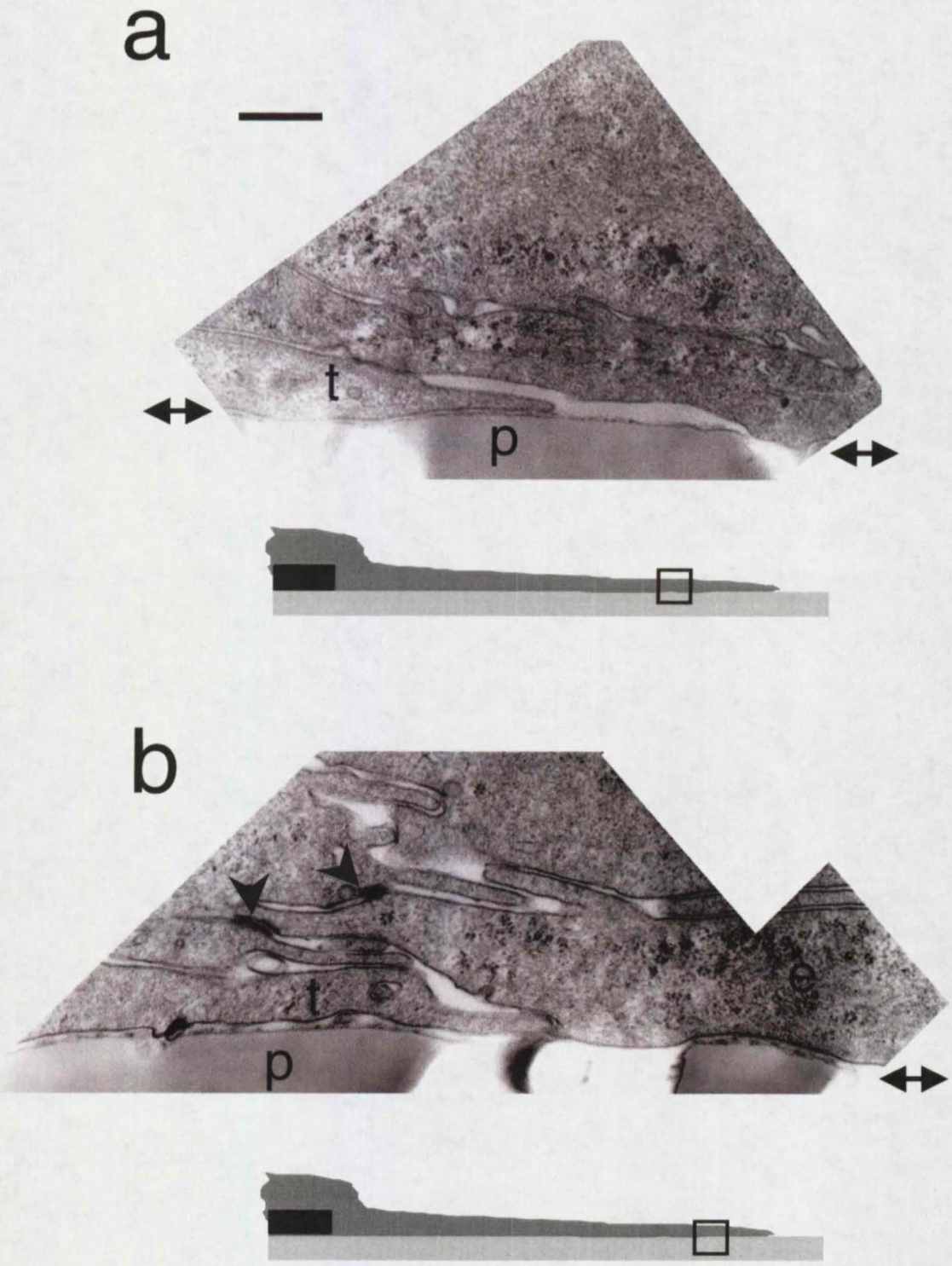


Figure 3.4B Electron micrographs of bovine corneal epithelial tissue migrating over a porous polycarbonate disk (p). Desmosomes are indicated by arrow-heads and the interface between the tissue and the polymer is marked with double-headed arrows. The rectangle on the diagram under each micrograph indicates the approximate location of the tissue. Cytoplasm from a following cell (t) is extending under a cell ahead. The scale bar is 500 nm and both images are at the same magnification.

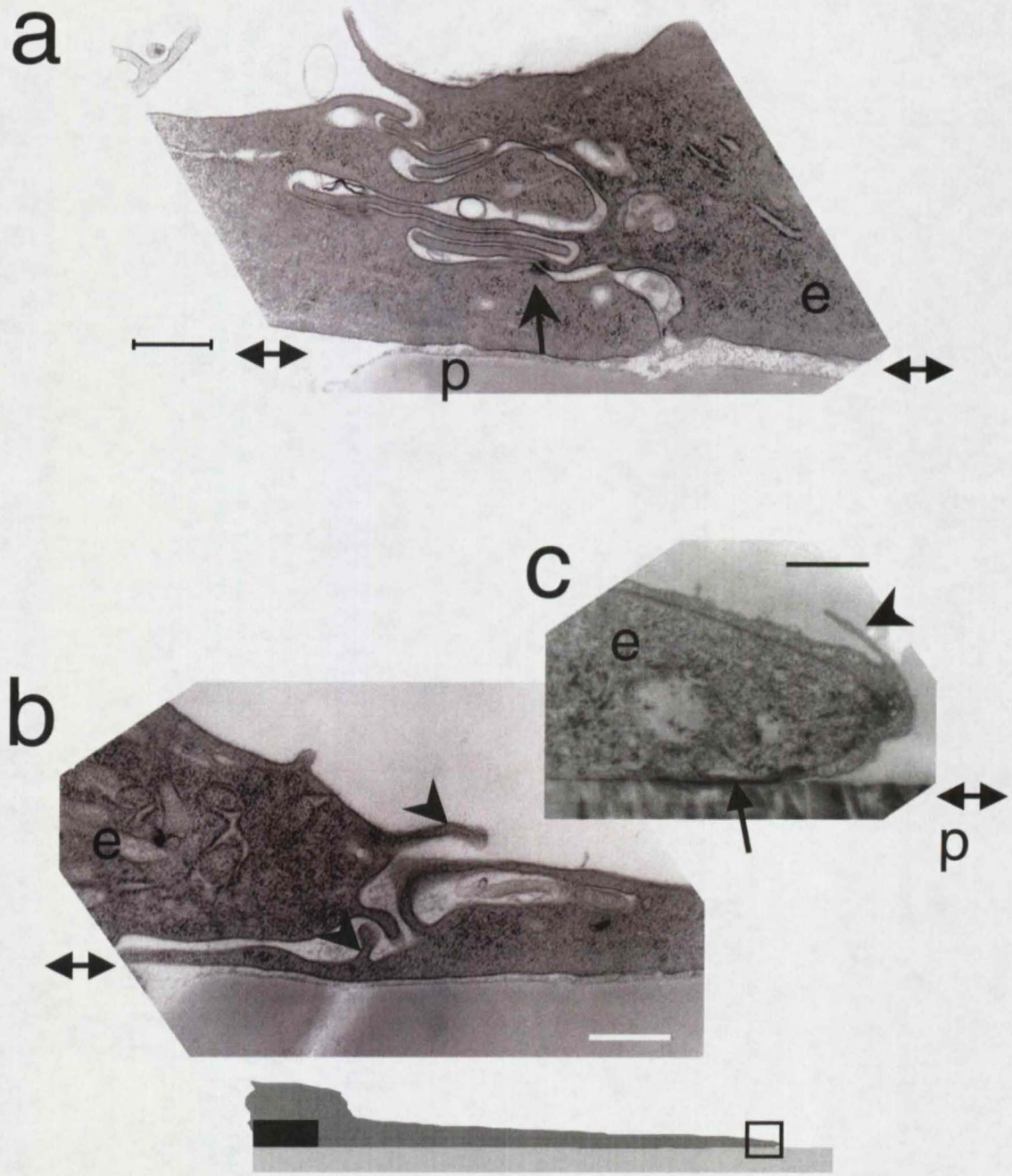


Figure 3.4C Electron micrographs of migrating bovine corneal tissue showing the junctions between the leading and the second cell. The position of the tissue in the micrographs is indicated by a rectangle on the diagram. Desmosomes are indicated by an arrow in (a). Arrow-heads mark the remains of interdigitated cytoplasm and double-headed arrows the polymer-tissue interface. The arrow in (c) marks a focal adhesion. The substratum is labelled (p) and the epithelial tissue (e). The scale bars in (a and b) are 500 nm and in (c) the bar is 2 μm.

culture was confluent. The cells within this region (Figure 3.5c) had a larger surface area in the plane of the culture substratum than the constituent cells in the migrating tissue (Figure 3.5a). Cells that had migrated out from the confluent region separated from each other and did not stain for desmoplakin (Figure 3.5d).

3.3.4 Immunolocalisation of focal adhesions

Focal adhesions and regions identified by reflection microscopy indicate the areas of closest contact between cellular membranes and the underlying substrata. The use of reflection microscopy was only possible in regions where the overlying cellular material was thin and the gradations in shade represent the distance between the basal cell membrane and the underlying substratum. The areas of membrane that are in closest contact with the substratum appear as the darkest regions. At the advancing edge of the migrating tissue, focal adhesions were formed around some extremities of extending cytoplasm (Figure 3.6a). However, there was more extensive formation of focal adhesions in submarginal basal cells directly behind the leading cell. From the reflection microscopy image (Figure 3.6b), the location of the junctions between component cells can be seen as a light coloured area. Also visible are some dark areas which correspond to the focal adhesions identified by the immunostaining for vinculin. At the leading edge of the migrating cells, a comparison of focal adhesions (Figure 3.6c) and the dark regions in closest contact with the substratum (Figure 3.6d) suggest that associated with focal adhesions there were longitudinal regions of the plasma membrane also in close contact with the substratum. Extensive formation of focal adhesions was apparent at the base of migrating tissue that was several microns behind the advancing edge (Figure 3.6e). This indicated an interaction between the submarginal basal cells within the tissue and the underlying substratum.

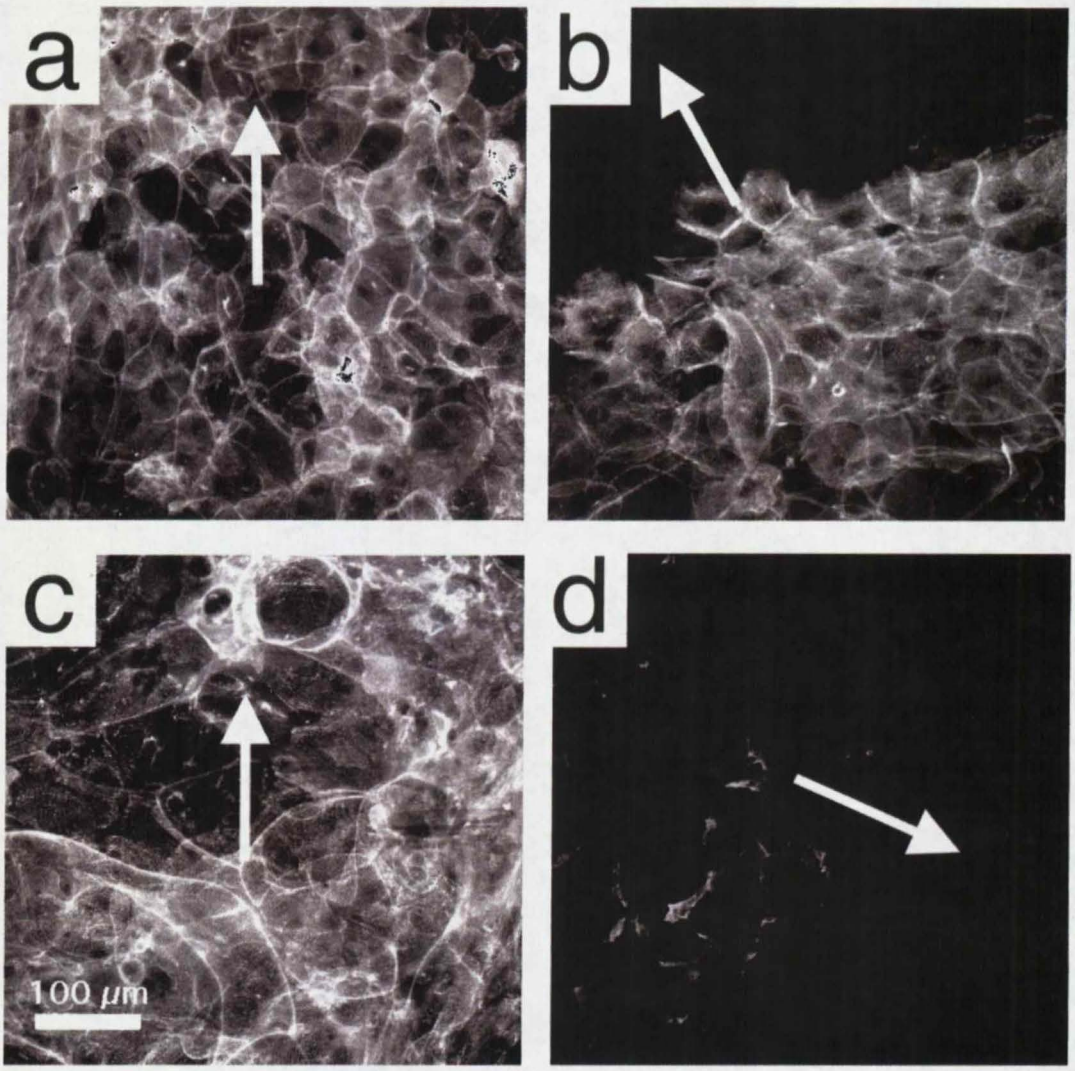


Figure 3.5 The presence of desmosomal intercellular adhesion junctions is shown by immunostaining for desmoplakins. The bovine corneal epithelial tissue and cells had migrated for seven days over glass cover slips. Junctions between component cells in the tissue sheets are shown in (a) and (b) and between separate cells (c) and (d); the leading edge of migration is shown in (b) and (d). Tissue close to the explant is shown in (a) and cells that were seeded as the confluent island in (c). The arrows indicate the direction of migration.

Bar = 100 µm; all images are at the same magnification.

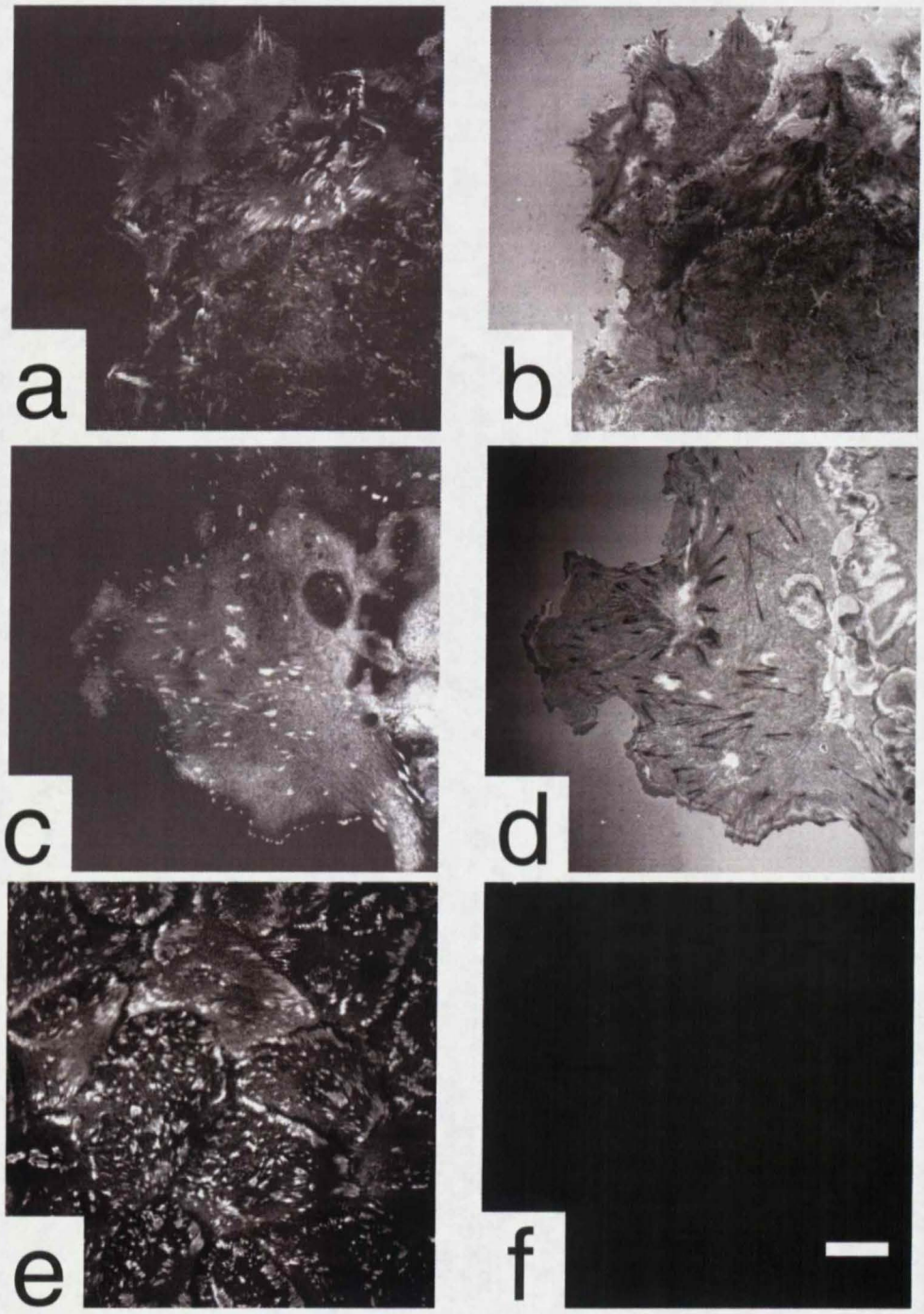


Figure 3.6 Immunolocalization of vinculin, a component of focal adhesions, at the advancing edge of migrating bovine corneal epithelial tissue (a) and migrating cells (c). Reflection images of the same field are shown in (b) and (d) respectively. In the reflection images the darker areas are indicative of the closest contact between cellular membranes and the substratum. Vinculin present at the tissue substratum interface of submarginal cells is shown in micrograph (e) and the tissue substratum interface of control sample in (f). The direction of migration in each micrograph is towards the left of the field.

Bar = 20 μm; all images are at the same magnification.

3.4 DISCUSSION

The research described in this Chapter examined the structure of migrating tissue and the relationship between component cells within the tissue. Additionally the localisation of focal adhesions within the tissue was examined, in order to determine which cells were maintaining contact with the substratum. A comparison with migrating cells was made between each facet of migrated tissue that was examined.

When bovine corneal epithelial tissue migrated, the component cells were integrated into a structure that was several microns in depth. This was apparent from the scanning electron micrographs of the migrating tissue, but was confirmed by examination of histological section that transected the tissue. Interdigitation was apparent, both horizontally and vertically, between the adjacent cells within the tissue when electron micrographs of the migrating tissue were studied. Interestingly, electron dense plaque typical of desmosomes, was also observed between adjacent cells throughout the tissue. The pattern of immunostaining for desmoplakin supported the concept that desmosomes persisted during tissue migration. The interdigitations and the presence of desmosomes demonstrated a means by which component cells within the tissue could remain as constituents of an integrated structure during migration. The finding that desmosomes persisted within the migrating tissue *in vitro* may differ from the wound healing situations *in vivo*. A study, which used immunostaining for desmoplakin and plakoglobin to predict the persistence of desmosomes in rat eye during the healing of an ablated epithelium concluded that these desmosomal components were degraded prior to epithelial migration and reconstructed during healing (Okada et al 2001). This was different from the present findings in which immunostaining for desmoplakin persisted between adjacent cell membranes throughout migration.

Basal cells situated at the leading edge of the migrating tissue (marginal basal cells) and also the basal cells that were not situated directly at the leading edge (submarginal basal cells) actively participated in tissue migration. Two lines of evidence supported this conjecture. Firstly, the basal cells in contact with the substratum in all parts of the migrating tissue sheet showed positive immunostaining for vinculin at the interface between the basal plasma membrane of the basal cells and the underlying polymer. Vinculin is one of the proteins present in focal adhesions and is involved in the

linkage between integrin and the actin cytoskeleton (Burridge et al 1988). Interaction of the cytoskeleton with the underlying substratum occurs by means of focal adhesions that are formed when integrins in the cell membrane bind to ligands on the substratum. The observation of the typical immunostaining pattern for focal adhesions indicated that there was uniform contact between the basal cells of the tissue and the substratum. If the tissue was dragged by the marginal cells, as proposed by DiPasquale, (DiPasquale 1975(a); DiPasquale 1975(b)) adhesions between substratum and the submarginal basal would not have persisted. The second line of evidence to support active participation of the submarginal basal cells in tissue migration was observed using electron microscopy. Trailing cells were seen to extend lamellae under preceding cells. Mahan (Mahan and Donaldson 1986) observed similar projections in newt epidermal tissue that was migrating *in vivo* over fibrinogen or collagen I coated glass coverslips.

The more anterior cells within the tissue were probably transported by the basal cells because the anterior tissue cells remained attached to the basal cells by means of desmosomes and interdigitation of cell membranes. Additionally, when the superior surface of the anterior tissue cells was observed using scanning electron microscopy, the cells did not appear to be polarised in the direction of migration. In particular, the cells that were close to the corneal explant presented a superior surface that resembled that of squamous cells on normal, non-migrating corneal epithelial tissue.

Scanning electron microscopy also established that there was heterogeneity along the advancing edge of the tissue. At some regions of the advancing edge, there appeared to be extensive activity with the generation of lamellipodia, filopodia and microspikes. In contrast, in other regions there was no evidence to suggest active migration. This may mean that migratory activity occurs sequentially in different parts of the advancing edge or that an alternative migratory mechanism is able to operate in specific circumstances.

In contrast to the tissue, the migrating cells were much thinner and appeared to be more extensively spread. This was apparent from the scanning electron micrographs of the migrating cells and also from the transverse sections. In the absence of the restrictions that were imposed by extensive cell-cell adhesions within the tissue, a greater area of the peripheral cell membrane would be available to enable the cells to

spread. One area of the cells that had some similarity to the tissue, was the region where the cells were originally seeded as a confluent culture. There appeared to be an interaction between component cells and some evidence of interdigitation between adjacent cells could be seen {Figure 3.2B(d)}. When the cells were first seeded, they were restrained from migrating for 20-24 hr by the presence of a fence and in this time they were held in close proximity to each other as a confluent culture. The localisation of desmoplakin by immunostaining also indicated that desmosomes were present between adjacent cells in this original confluent culture (although these were lost when the cells migrated away from this area). These data suggested that the epithelial cells formed aggregates before migration commenced. Corneal epithelial cells from chick embryo have also been shown to be capable of forming aggregates in which desmosomes form between adjacent cells (Overton and DeSalle 1980) so it would be reasonable to expect that bovine corneal epithelial cells were also able to do this.

3.5 CONCLUSIONS

The research described in this Chapter demonstrated that migrating bovine corneal epithelial tissue maintains an integrated structure by means of desmosomes and interdigitation of adjacent cell membranes. Parts of the tissue comprised several layers of cells in which it appeared that the basal cells were providing the force required to transport anterior cells. This was the first demonstration of the distribution of focal adhesions at the interface between migrating tissue and the underlying polymer. It provided clear evidence to support the view that the submarginal basal cells, in addition to the marginal basal cells, contributed the transportation of cells located in a more anterior position during migration of tissue. The cytoskeleton is known to play a major role in cell migration and the research described in the following Chapter therefore examined the effects that the integrated tissue structure imposes on the organisation and function of cytotoskeletal elements.

CHAPTER 4

THE ORGANISATION AND FUNCTION OF THE CYTOSKELETON IN MIGRATING EPITHELIAL TISSUE AND CELLS

4.1 INTRODUCTION

In Chapter 3, studies of the structure of migrating tissue were described and compared with the structure of migrating cells. Within the migrating tissue, interdigitations between adjacent cell membranes and the presence of desmosomes indicated cooperation between component cells. Several layers of cells were present in the tissue apart from the extreme leading edge. Focal adhesions were identified throughout the tissue, at the interface between the tissue and the underlying substratum. This, together with evidence of cytoplasmic extensions from following cells under cells ahead, supported the hypothesis that submarginal basal cells contributed to migration and were not dragged along passively by the marginal cells. In comparison, the migrating cells adopted a much flatter morphology than did the component cells within the tissue. There was some evidence of formation of interdigitations between adjacent cells within the original restrained cell culture but this did not persist when the cells migrated. In comparison, in this chapter the roles of the cytoskeletal components in tissue and cell migration are described.

The three major cytoskeletal fibres present in the corneal epithelium are microfilaments, microtubules and intermediate filaments. In combination with various associated proteins, the cytoskeleton is implicated in a diverse range of cellular functions including: polarization and protein sorting (Mays et al 1994), locomotion or migration (Schliwa and Honer 1993) and mitosis, (Ault and Rieder 1994). Of the components of the cytoskeleton, the actin filaments have most clearly been identified as playing a major role in cell migration (Mitchison and Cramer 1996). Cell migration is halted if the normal turnover of actin filaments is disrupted with drugs (Mitchison and Cramer 1996). The dependence on functional actin filaments has also been demonstrated for the migration of intact epithelial tissue sheets (Gipson and Anderson 1977).

There are diverse views as to the importance of microtubules in cell migration {reviewed by (Schliwa and Honer 1993)}. While microtubules were thought not to be essential for the migration of some cell types (Euteneuer and Schliwa 1986), other studies have shown a linear relationship between microtubule dynamics and the formation of lamellipodia (Mikhailov and Gundersen 1998) and identified a clear link with the ability of a cell to migrate. The later research complemented an earlier investigation, which showed that concentrations of microtubule disruptive agents that were insufficient to depolymerise microtubules were also capable of reducing migration rates (Liao et al 1995). It was thereby demonstrated that microtubule dynamics were critical for the maximum speed of cell locomotion (Liao et al 1995). These studies that have demonstrated aspects of microtubule function have been conducted using cells and little research has been directed towards microtubule function within tissue migrating over synthetic surfaces. The present study directly compared the structure and function of microtubules in migrating corneal epithelial tissue and migrating corneal epithelial cells.

Drugs are available that affect the dynamics of actin or microtubules. Therefore these were used to compare tissue and cells for the involvement of these cytoskeletal elements during migration. No such tools are available to demonstrate the role for intermediate filaments in migration so the focus of this study has necessarily been limited to actin filaments and microtubules. First, the organisation of actin filaments and microtubules within migrating epithelial tissue was compared with the organisation of these structures in epithelial cells that were migrating from a confluent culture. Second, the migrating tissue and cells were exposed to drugs that either disrupted actin filament or microtubule assembly and the effects were monitored over time using immunofluorescent staining. Finally, the effects of disruption of the actin cytoskeleton or the microtubule system were compared between migrating tissue and cells by using time-lapse video microscopy, to measure changes in migration rates. This enabled direct comparisons to be made between the roles of actin filaments and microtubules in migrating tissue and cells.

4.1.1 Aim

The aim of the research described in this chapter was to identify differences between the organisation and the function of cytoskeletal components in migrating tissue and in a migrating population of cells. If the migration of an intact sheet of epithelial tissue was due to the migration of each of the component cells acting independently, then the arrangement of the cytoskeleton of each cell would resemble that of an individual cell in the process of migration. However, if tissue migration proceeded by means of a different mechanism from the migration of cells, then this may be reflected in the organisation of the cytoskeletal elements within the cells in each system. Additionally, if the migrating tissue showed a different sensitivity from cells to the presence of drugs that disrupt elements of the cytoskeleton, this could be indicative of the operation of different mechanism of migration that was less dependent upon either actin filaments or microtubules.

4.1.2 Hypotheses to be tested

In order to identify differences between the mechanisms of migration of tissue and cells the specific hypotheses tested were: (1) the arrangement of the actin filaments and the microtubules in migrating epithelial tissue differ from these in migrating cells; and (2) the disruption of cytoskeletal components in migrating tissue has a lesser effect on the migration than would occur if these components were disrupted in cells.

4.2 MATERIALS AND METHODS

4.2.1 Tissue and cells

The model systems for corneal epithelial tissue and corneal epithelial cells in the process of migrating were prepared in six-well tissue culture trays (TCPS) (Costar, Corning Incorporated Corning, NY) or on glass coverslips placed in six-well culture trays (as described in Chapter 2, Sections 2.2.1, 2.2.3 and 2.2.5). Briefly, for tissue immunostaining studies, explants were placed onto the glass coverslips in the cluster trays and cultured for seven days. For cell migration, a fence was centred on each of the coverslips that had been placed in the wells of the culture tray. Cells were seeded into the lumen of the fence and cultured for 20-24 hours then the fence was removed. The cells were cultured for a further seven days. For the time-lapse video microscopy studies the tissue and the cells were cultured directly on the TCPS base of the six-well cluster trays.

4.2.2 Histochemistry

All procedures were carried out on tissue and cells that had migrated for seven days (using the culture conditions described in Chapter 2, Sections 2.2.1 and 2.2.3). In order to detect filamentous actin, the cultures were washed in PBS and fixed with formol saline for 10 min. After one wash with PBS, cultures growing on glass coverslips were permeabilised with ice-cold acetone for 10 min and air dried. Cultures grown on TCPS were permeabilised by incubation for 20 min with 0.5% (v/v) Triton X-100. Non-specific binding sites were blocked by incubation in 2% (w/v) BSA in PBS for 30 min, as recommended in the protocol for actin filament staining supplied by Molecular Probes (Oregon, USA). The cultures were protected from the light and incubated with Texas Red phalloidin (Molecular Probes) (20U/ml in PBS) for 90 min. The coverslips were washed four times with PBS, once with distilled water and inverted onto microscope slides with FluroSave™ (Calbiochem). The slides were viewed at magnifications up to 1000x using a confocal microscope equipped with an argon/krypton laser (Leica). Images were collected in one focal plane using a wavelength of 568 nm and a pinhole of 97. Electronic noise was filtered using line filtration.

4.2.3 Immunohistochemistry

All procedures were carried out in the same way as for the immunohistochemistry described in Chapter 3, Section 3.2.5, except that in this case the primary antibody used was a monoclonal antibody against β-tubulin (Chemicon International Inc, Temecula, CA. Cat No MA065). The antibody was supplied as ascites fluid and this was used at a dilution of 1:50 in PBS containing 2% (w/v) BSA. Laser confocal microscopy was conducted as for the observation of filamentous actin, except that the wavelength used was 488 nm and the pinhole was 90.

4.2.4 Evaluation of the ability of drugs to inhibit actin polymerisation

In order to assess the degree of actin filament disruption tissue and cells were fixed and stained after exposure, for various times, to the actin filament-disrupting drug cytochalasin-B (Sigma Chemicals, C-6762). The cytochalasin-B was used at a final concentration of 25 μg/ml and the samples were cultured for 0, 5, 10, 20, 30 and 60 min prior to fixation. The cytochalasin-B stock was dissolved in dimethylesulphoxide (DMSO) (Sigma), therefore an equivalent volume of DMSO was added to control cultures. A minimum of two samples were evaluated for each time point in three separate experiments. Grey scale digital images of stained tissue and cells were inverted and contrast-enhanced, to better illustrate actin filament disruption.

4.2.5 Evaluation of the ability of drugs to disrupt microtubules

In order to assess the degree of microtubule depolymerisation after exposure for various times to microtubule disrupting drugs, the migrating epithelial tissue and cells were fixed and immunostained using an antibody against tubulin. Demicolcine (Sigma Chemicals, D-6279) was added to the migrating cultures at a final concentration of 1 μg/ml. Tissue and cells were cultured in the presence of the drug for intervals of 0, 5, 10, 20, 30 or 60 min, prior to fixation and immunostaining with anti β-tubulin antibodies. A minimum of two samples from each time were evaluated in three separate experiments. Grey scale digital images of immunostained tissue and cells were inverted and contrast-enhanced to better illustrate microtubule disruption.

4.2.6 Evaluation of the contribution of cytoskeletal components to tissue and cell migration

Time-lapse video microscopy was used to monitor the effects of disruption of actin microfilaments and microtubules on the rate of migration of tissue and cells. Short-term studies were used because extended use of cytoskeletally active drugs may affect other aspects of tissue and cell function, apart from migration. The culture medium of the migrating tissue or cells was replaced with two ml of CO_2 Independent Medium (GibcoBRL, Life Technologies, Grand Island, NY) containing the appropriate additives (Chapter 2, Section 2.2.1 and 2.2.3). The culture tray was transferred to a Leitz motorised microscope stage attached to an Olympus inverted microscope. The culture tray, stage and microscope optics were contained within a purpose-built perspex chamber. The temperature within the chamber was maintained at 37°C by circulation of air warmed by a heat exchanger through which flowed the output of a thermostatically controlled waterbath and heat pump. The microscope stage, focus mechanism and lamp were connected to an image analyser (Cambridge Instruments) and associated IBM-PC compatible computer. After permitting the culture to stabilise for 30 min, migration was observed under phase-contrast optics at 200x total magnification and captured by a video camera (JVC-138). A computer program, written in QBASIC language by Dr M. Dziegielewski, automatically recorded an image every two minutes. The program only turned on the lamp during the recording interval to avoid phototoxicity. Drugs were added by replacement of 50% of the original culture medium with medium containing a drug at double the required final concentration. The replacement medium containing the drugs was equilibrated to 37°C before it was added to the cultures. The tissue or cell area within the image frame was measured from the collected images and the increase in area was plotted as a function of time. Initial migration, and migration after the addition of drugs, were measured for a minimum of two hours each. Migration was also monitored after the replacement of 50% of the medium with medium that contained an equivalent concentration of DMSO as was used as a diluent for cytochalasin-B.

4.2.6.1 Inhibition of actin polymerisation during migration

One ml of culture medium was replaced with 1 ml of medium containing 50 μg/ml of cytochalasin-B (to give a final concentration of 25 μg/ml).

4.2.6.2 Microtubue depolymerisation during migration

One ml of medium was replaced with 1ml of medium containing 2 μm/ml of demecolcine (to give a final concentration 1 μg/ml).

4.3 RESULTS

Epithelial tissue from corneal explants migrated radially outwards for approximately three mm over the glass coverslips during the seven-day culture period. In the cell model, where dissociated cells were seeded to form a monolayer, the cells migrated as individual cells separate from each other when they left the original confluent zone.

4.3.1 Actin filament organisation in migrating tissue and cells

Bundles of actin filaments were localised at the cell periphery in both the tissue and the cells that were not directly adjacent to the leading edge {Figure 4.1(a) and Figure 4.1(c)}. At the leading edge of the migrating tissue there were concave shaped regions in which actin filaments parallel to the leading edge appeared to span several adjacent cells, forming cables {(Figure 4.1(b)}. These concave regions constituted about 30% of the leading tissue front that had migrated from the explant. Adjacent to the concave regions of the leading edge, there were leading edge cells, which had extended lamellipodia. Within these lamellipodia, actin filaments were visible radiating towards the advancing edge. In the cell model, some cells at the leading edge had actin filaments radiating towards the direction of their migration {(Figure 4.1(d)}. Ruffling and formation of lamellipodia were seen at the leading edge of the cells {(Figure 4.1(d)}.

4.3.2 Microtubule organisation in migrating tissue and cells

The microtubules in the cells forming the epithelial tissue close to the corneal explant appeared to be extensively intermeshed {Figure 4.2(a)}. In the cell model, there was no intermeshing of microtubules between adjacent cells and it was possible to distinguish separate cells, even in the central confluent region where the cells were originally seeded {Figure 4.2(c)}. The positions of cell nuclei in this confluent region were visible as areas devoid of microtubules {Figure 4.2(c)}. At the leading edge of the migrating tissue, there were areas of apparent intermeshing between microtubules of some adjacent cells, but in other areas there were gaps between the microtubules of adjacent cells {Figure 4.2(b)}. When cells migrated from a confluent culture the microtubules within individual cells close to the leading edge were clearly separated {Figure 4.2(d)}.

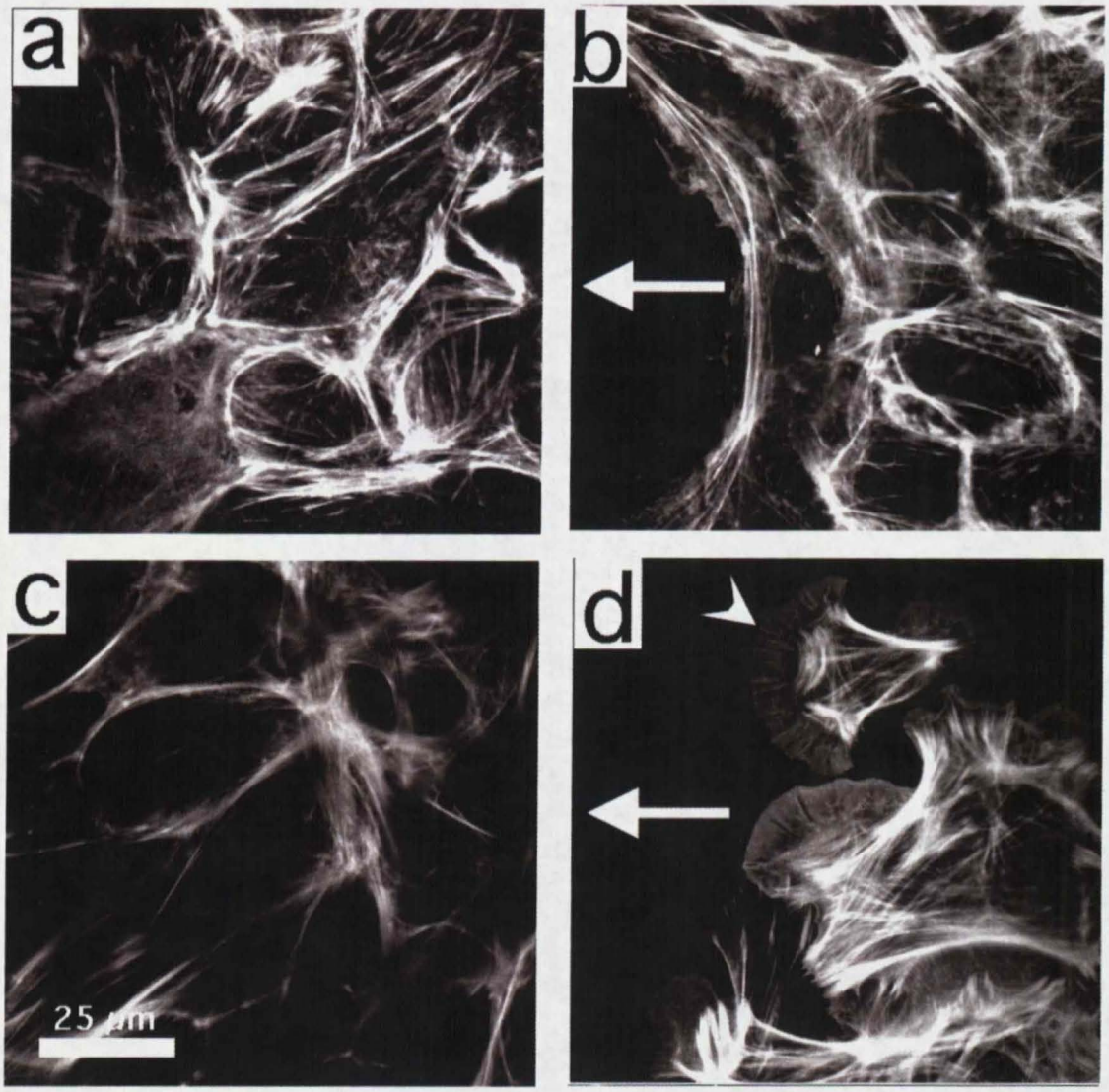


Figure 4.1 Actin filaments (delineated by Texas Red phalloidin) in bovine corneal epithelial tissue and cells. The tissue and cells had migrated for seven days over glass coverslips. Tissue is shown in (a) and (b) and cells in (c) and (d); the leading edge of migration is seen in (b) and (d). The actin filament arrangement in tissue close to the corneal explant is shown in (a) and in cells that were seeded as the confluent island in (c). The large arrows indicate the direction of migration. The small arrowhead points to ruffled lamellipodia.

The scale bar is 25 µm.

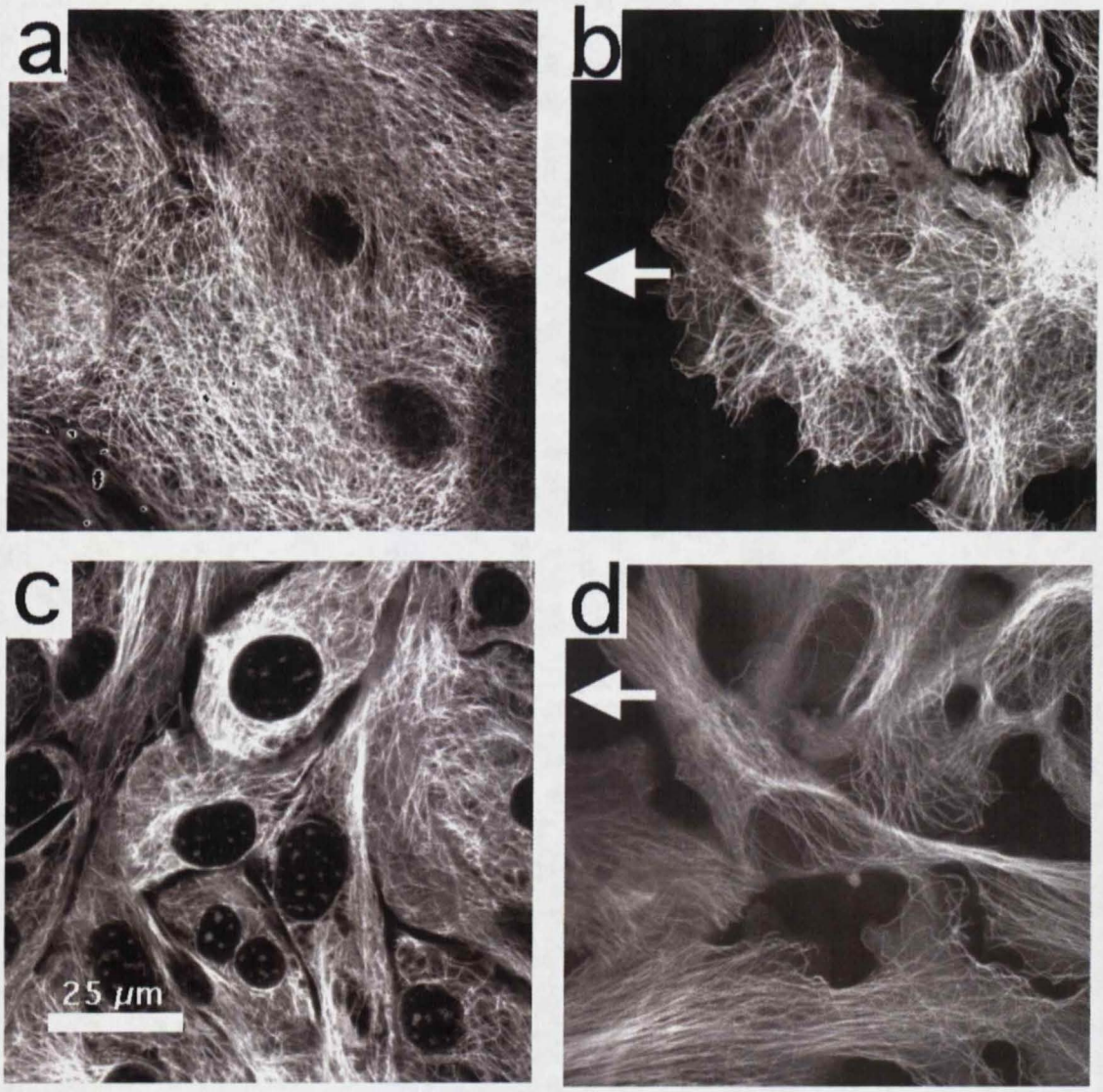


Figure 4.2 Microtubules, identified by immunostaining for tubulin. The bovine corneal epithelial tissue and cells had migrated for seven days over glass cover slips. Tissue is shown in (a) and (b) and cells in (c) and (d); the leading edge of migration is shown in (b) and (d). The microtubule arrangement close to the corneal explant is shown in (a) and in the cells that were seeded as a confluent culture in (c). The arrows indicate the direction of migration.

The scale bar is 25 µm.

4.3.3 Monitoring the disruption of cytoskeletal components

The relative importance of actin microfilaments and microtubules was investigated, in order to determine if the requirement for either of these cytoskeletal components was less within an intact tissue structure than it was within separate cells. First, a time course study was conducted to chart the rate of disruption of microfilaments and microtubules within tissue and cells after exposure to appropriate drugs.

4.3.3.1 Evaluation of cytochalasin-B on actin filaments in migrating tissue and cells

Prior to the addition of cytochalasin B, intact actin filaments were visible throughout the migrating tissue and cells. Within lamellipodia at the advancing edge of the tissue {Figure 4.3(a)} and cells {Figure 4.4(a)}, actin filaments radiated towards the direction of movement. In concave regions of the tissue front actin filament cables appeared to span several cells {the start of such a filament is visible in the upper left hand corner of Figure 4.3(a)}. Closer to the original explant, particularly at the anterior surface of the migrating tissue, actin was visible at the periphery of component cells {Figure 4.3(d)}. (See also the appearance of undisrupted actin filaments in Figure 4.1, which shows images that have not been inverted.) After five min exposure to cytochalasin-B some evidence of disruption of actin filament bundles was observed in both the tissue {Figure 4.3(b) and (e)} and cells {Figure 4.4(b) and (e)}. While there were some linear regions of filamentous actin within tissue at the leading edge, punctate staining was also present. Actin filament bundles within lamellipodia could no longer be found in either the tissue or the cells, but some actin remained at the periphery of cells within the tissue, that was not adjacent to the leading edge {Figure 4.3(e)}. Ten min exposure to cytochalasin-B had reduced the majority of actin staining, in both the tissue and the cells, to a series of punctate spots {Figure 4.3(c) and (f) and Figure 4.4(c) and (f)}. Component cells at the advancing edge of the tissue lost intercellular connection {Figure 4.3(c)}, but cell-to-cell contacts within the tissue distant from the leading edge appear to have been maintained {Figure 4.3(f)}. Ten min exposure to Cytochalasin-B effectively disrupted actin filaments within both the tissue and cells, therefore images of later time points were not included.

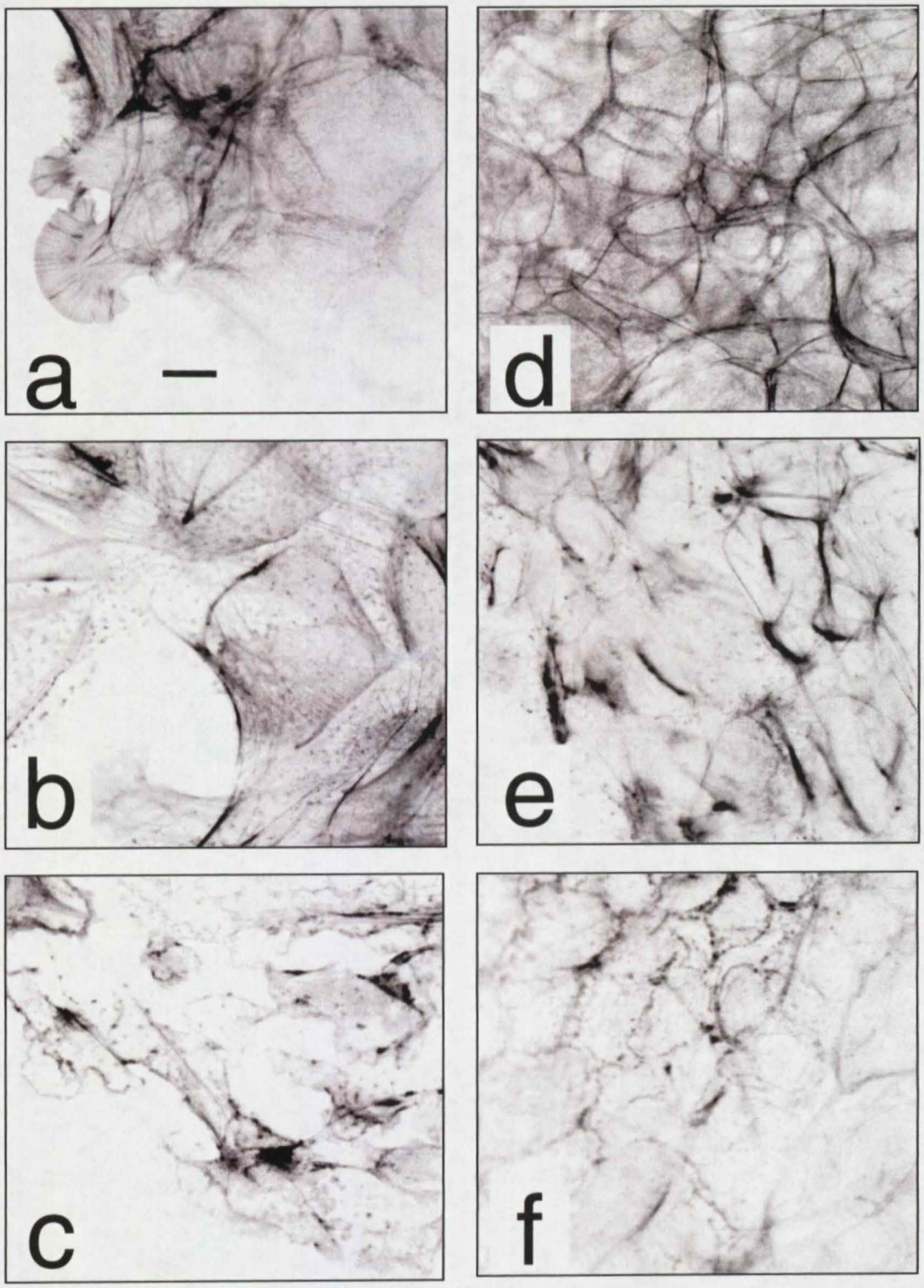


Figure 4.3 The effect of cytochalasin-B on filamentous actin in migrating bovine corneal epithelial tissue. The tissue at the advancing edge is shown in micrographs (a), (b) and (c) and more central tissue in micrographs (d), (e) and (f). Tissue prior to exposure with cytochalasin-B is shown in (a) and (d), after 5 minutes exposure (b) and (e) and 10 minutes exposure (c) and (f). Digital images of fluorescent labelled actin have been inverted and contrast enhanced. The scale bar is 20 μm.

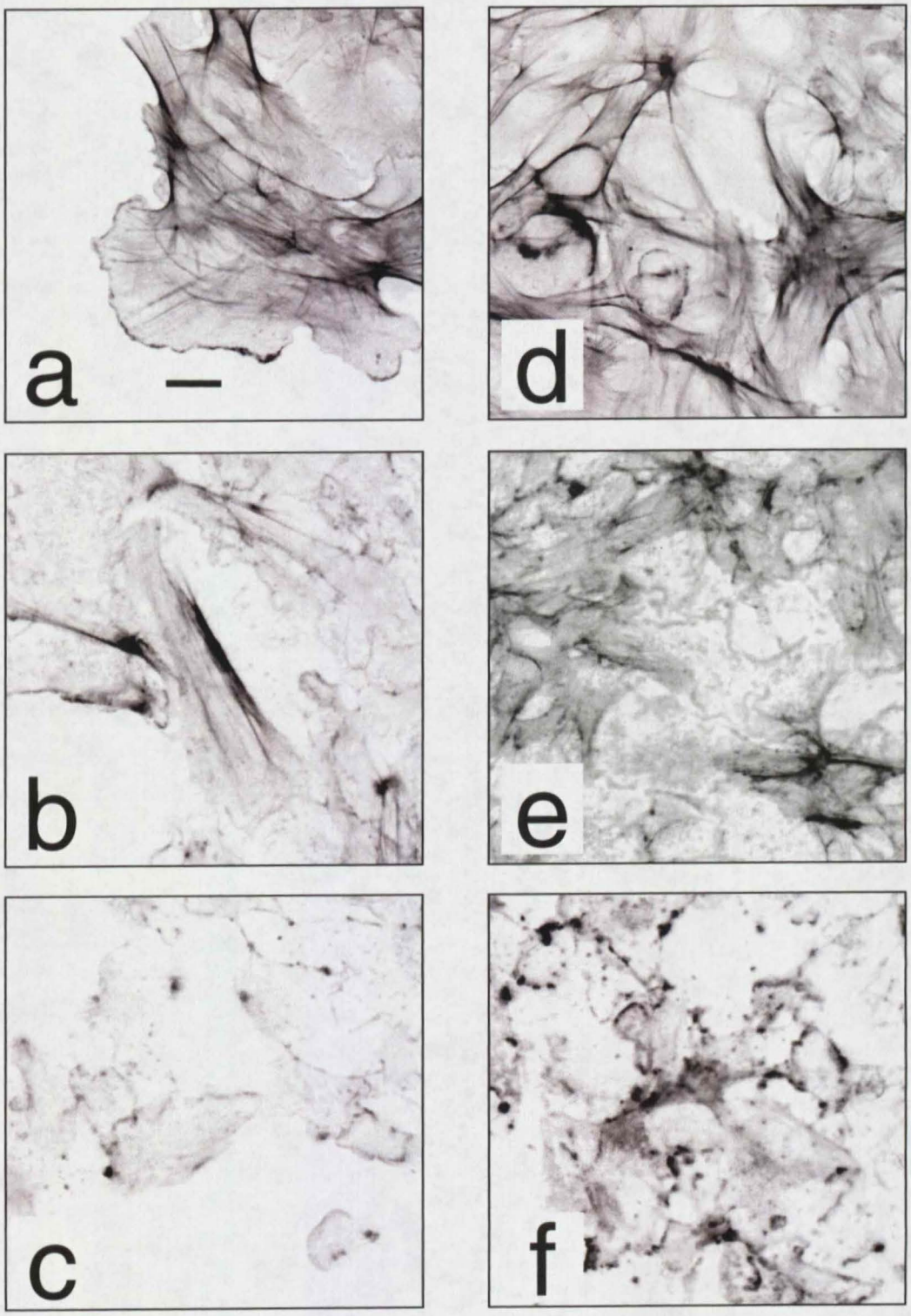


Figure 4.4 The effect of cytochalasin-B on filamentous actin in migrating bovine corneal epithelial cells. The cells at the advancing edge are shown in micrographs (a), (b) and (c) and more central cells in micrographs (d), (e) and (f). Cells prior to exposure to cytochalasin-B are shown in (a) and (d), after 5 minutes exposure (b) and (e) and 10 minutes exposure (c) and (f). Digital images of fluorescent labelled actin were inverted and contrast enhanced. The scale bar is 20 μm.

4.3.3.2 Evaluation of demecolcine on microtubules in migrating tissue and cells

Intact microtubules were still visible in the migrating tissue after five min exposure to demecolcine {Figure 4.5, compare (a) and (b)}. After ten min exposure to this drug there was a change in the appearance of the microtubules, which became slightly distorted {Figure 4.5(c)}; this was more obvious after 15 min exposure {Figure 4.5(d)}. After 30 min exposure to demecolcine, microtubules could not be identified. Within the migrating cells, depolymerisation of microtubules occurred faster than in the intact tissue. While microtubules could still be identified after five min exposure to demecolcine {Figure 4.6, compare (a) and (b)}, after ten min or longer microtubules were no longer discernible {Figure 4.6(c) - (f)}.

4.3.3.3 Time-lapse video microscopy to monitor the effect of cytochalasin-B on tissue and cells

In order to determine if there were any differences between the rates of migration of tissue and cells in response to disruption of either actin filaments or microtubules, tissue and cell migration were monitored over six hr, using time-lapse video microscopy. The graphs in Figures 4.7A and 4.8A show that the addition of cytochalasin-B to the migrating tissue and cells halted the steady increase in the areas covered by tissue or cells that had occurred for the preceding three hr. The morphology of the tissue at various time points is shown in Figure 4.7B(a) - (d). After ten min exposure to cytochalasin-B component cells at the advancing edge of the tissue front became rounded, with minimal change to the overall morphology of the tissue {Figure 4.7B(c)}. The tissue sheet maintained its general shape and form after three hr exposure to cytochalasin-B, while component cells at the advancing edge were distended in appearance {Figure 4.7B (d)}. The greater susceptibility of cells to cytochalasin-B, as compared with tissue, is apparent from the graph in Figure 4.8A, which shows that the addition of cytochalasin-B to the migrating cells caused the cells to retract within ten min. Further retraction occurred during the three hr that the cells were monitored. The change in the morphology of the cells in response to cytochalasin-B can be seen by comparing the micrographs in Figure 4.8B(c) and (d), with Figure 4.8B(a) and (b). After ten min exposure, cells at the advancing edge rounded up {Figure 4.8B, (c)}. However, at this time point cells further back from the

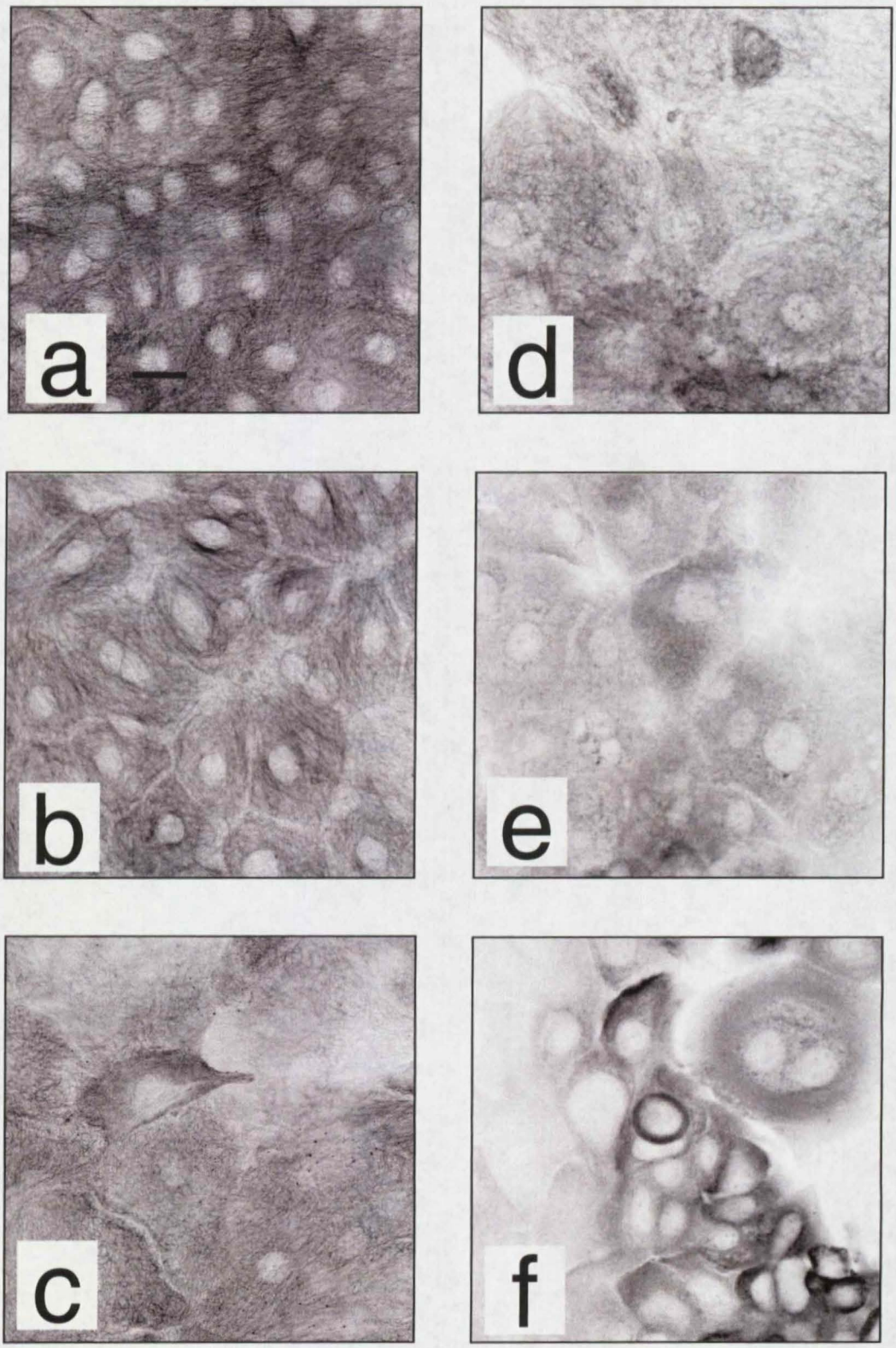


Figure 4.5 The effect of demecolcine on microtubule organisation in migrating bovine corneal epithelial tissue. Tissue is shown after incubation with demecolcine for various times: prior to exposure (a), after 5 minutes (b), 10 minutes (c), 20 minutes (d), 30 minutes (e), and 60 minutes (f) exposure. Digital images of fluorescent-labelled tubulin were inverted and contrast-enhanced. The scale bar is 20 μm.

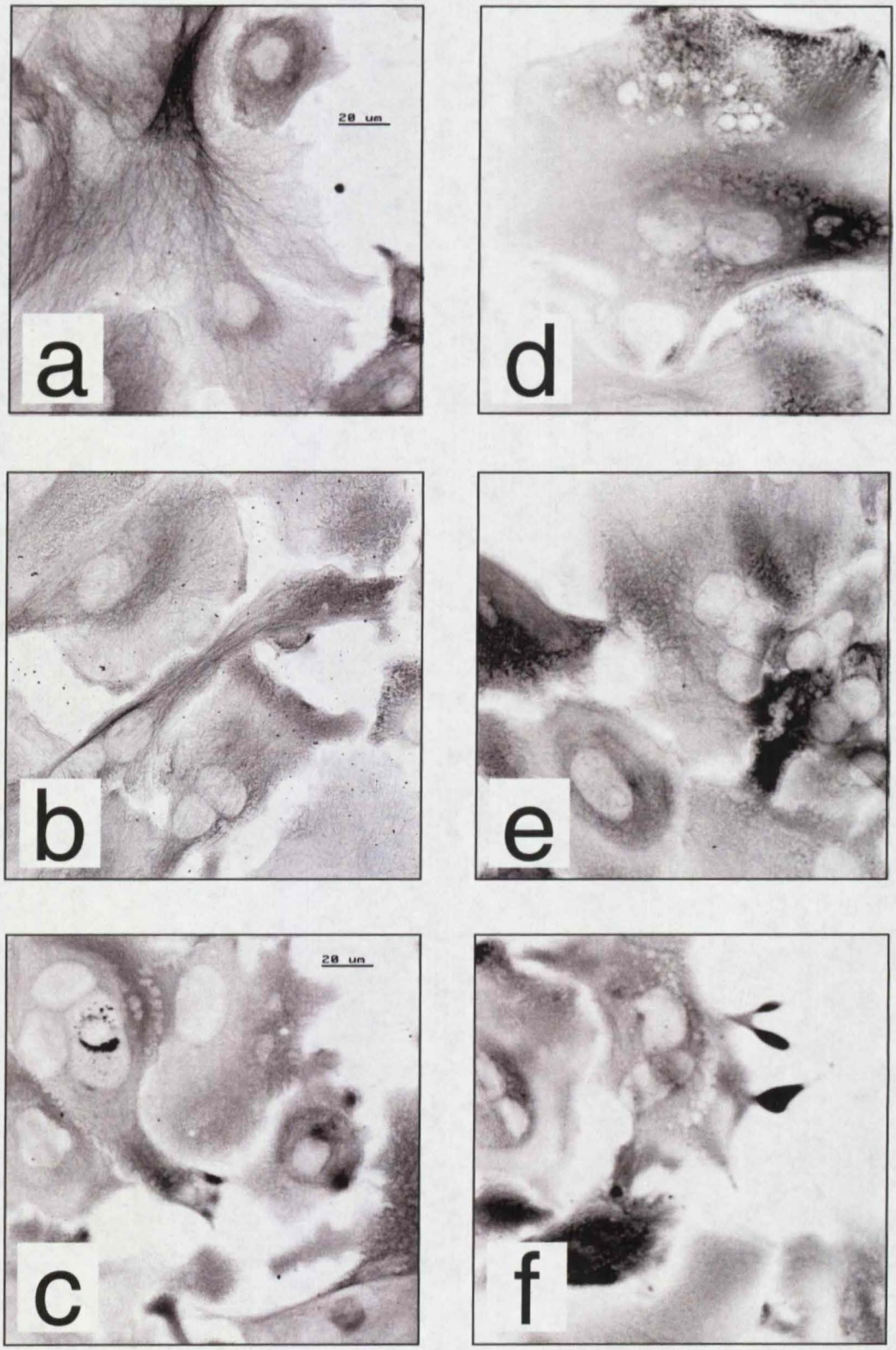


Figure 4.6 The effect of demecolcine on microtubule organisation in migrating bovine corneal epithelial cells. Cells are shown after incubation with demecolcine for various times: prior to exposure (a), after 5 minutes (b), 10 minutes (c), 20 minutes (d), 30 minutes (e), and 60 minutes (f) exposure. Digital images of fluorescent-labelled tubulin were inverted and contrast-enhanced. The scale bar is 20 μm.

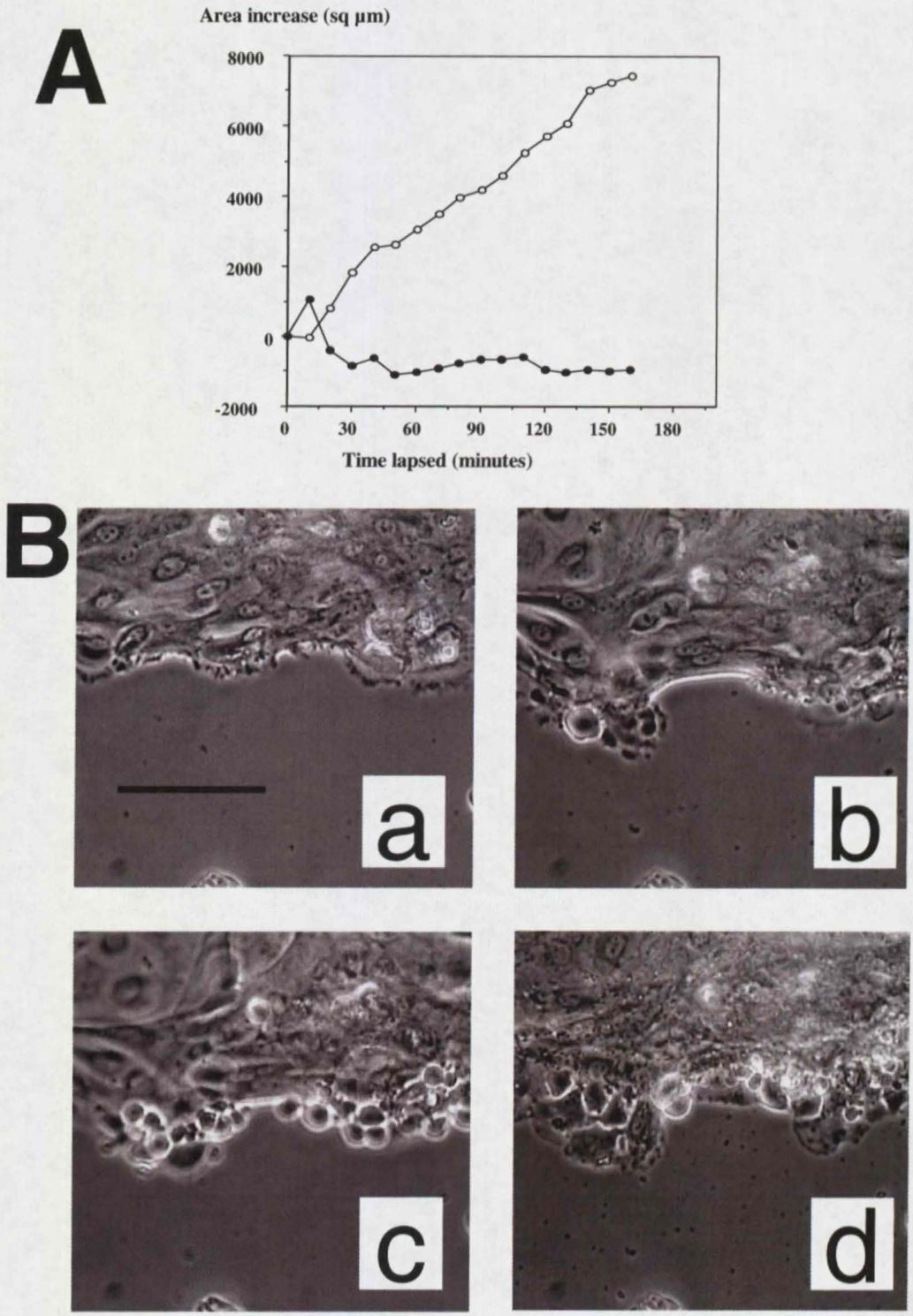


Figure 4.7 The effect of cytochalasin-B on the rate of migration (A) and morphology of migrating bovine corneal epithelial tissue (B). The graph in panel (A) shows the area covered with tissue in the absence of drugs (open circles) and after exposure to cytochalasin-B (solid circles). In panel (B), tissue at zero time point is shown in (a), tissue that had migrated for three hours in the absence of drugs in (b), after 10 minutes exposure to cytochalasin-B in (c), and after 3 hours exposure to cytochalasin-B in (d). The scale bar is 100 µm.

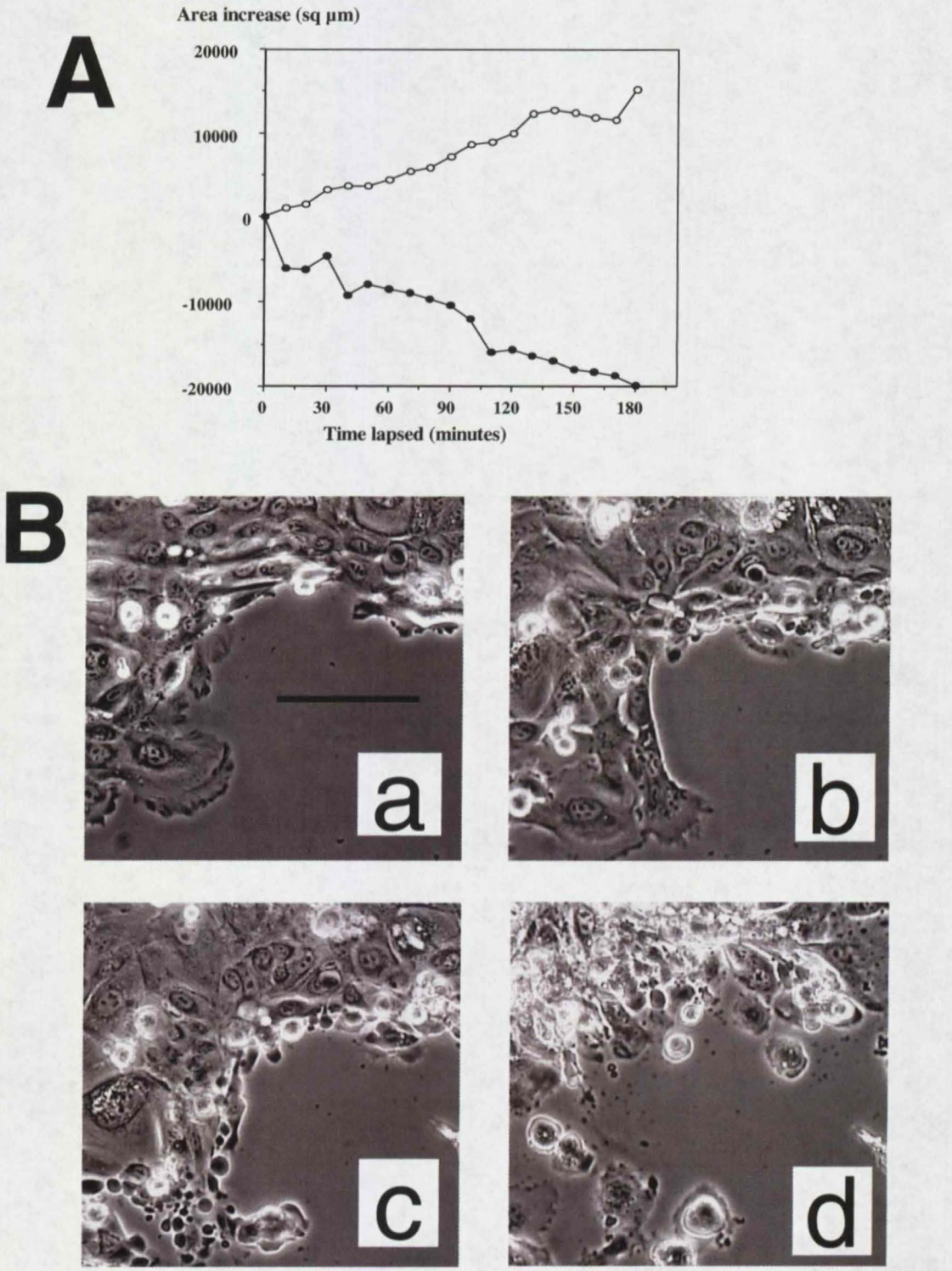
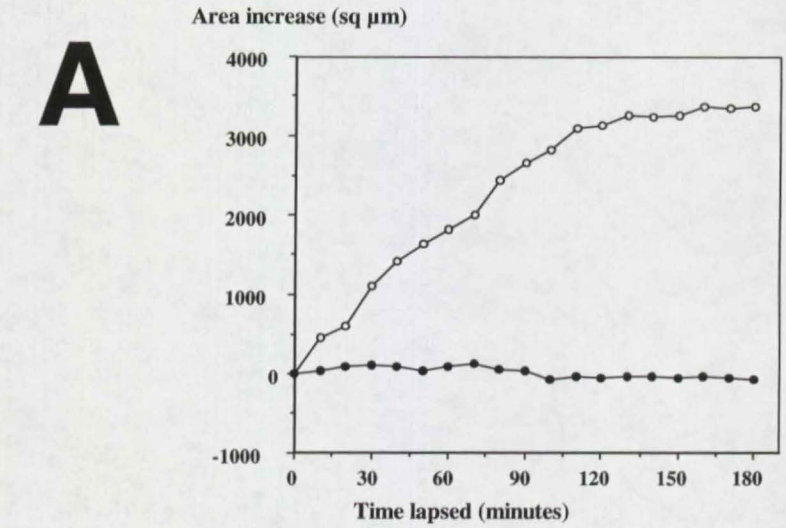


Figure 4.8 The effect of cytochalasin-B on the rate of migration (A) and morphology of migrating bovine corneal epithelial cells (B). The graph in panel (A) shows the increase in the area occupied by the cells in the absence of drugs (open circles) and after exposure to cytochalasin-B (solid circles). In panel (B), cells at zero time point are shown in (a), cells that had migrated for three hours in the absence of drugs in (b), after 10 minutes exposure to cytochalasin-B in (c), and after 3 hours exposure to cytochalasin-B in (d). The scale bar is 100 µm.

leading edge maintained a spread morphology, which was lost after three hr exposure to the drug. After three hr exposure, the submarginal cells also rounded up and the cell-front retracted {Figure 4.8B(d)}.

4.3.3.4 Time-lapse video microscopy to monitor the effect of demecolcine on tissue and cells

Demecolcine had much more subtle effects on both tissue and cells, in comparison with cytochalasin-B. The graphs recording the tissue (Figure 4.9A) and the cell (Figure 4.10A) response to demecolcine, show that the migration of both the tissue and cells was halted, but there was minimal retraction. In the presence of the drug, the original morphology of the tissue and cell was maintained {Figures 4.9B and 10B, (a) and (b) compared with (c) and (d)}.



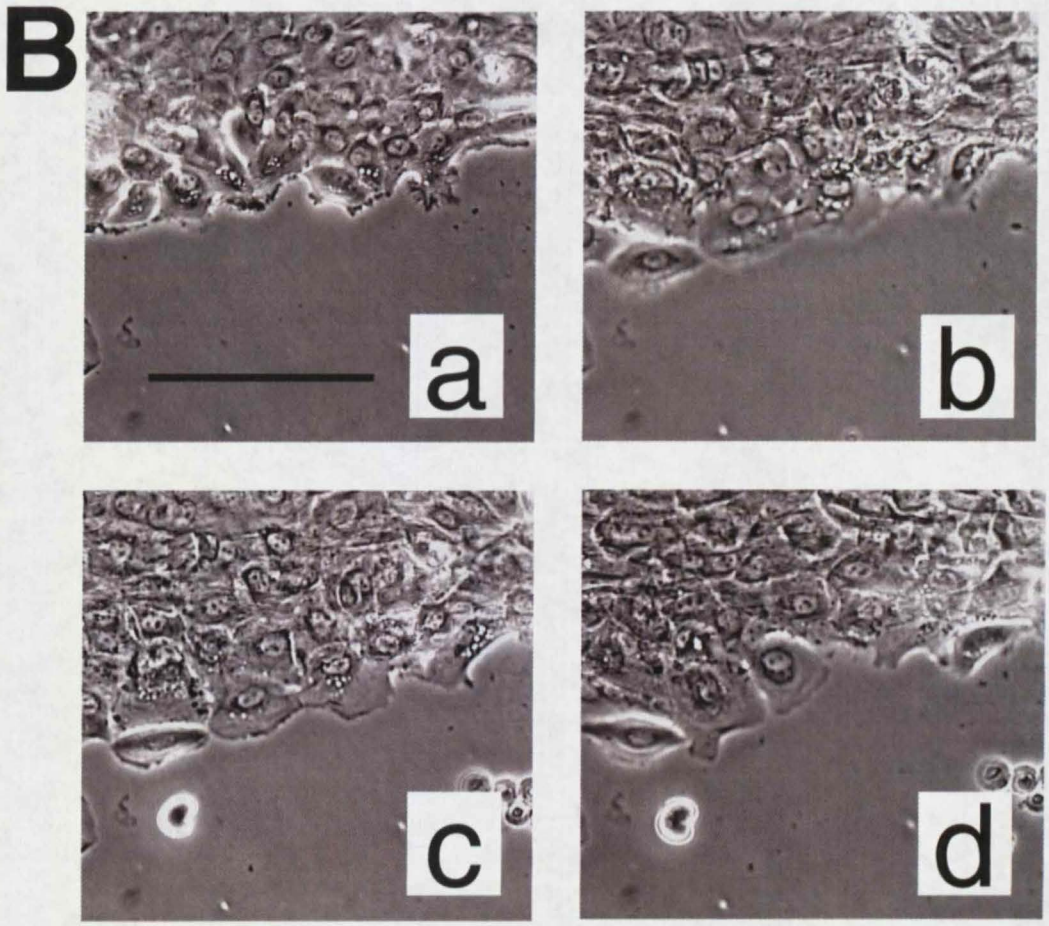
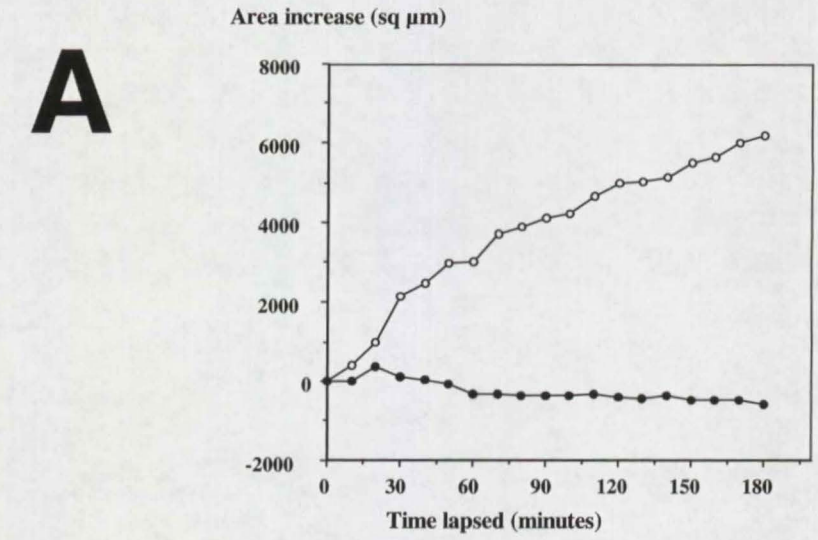


Figure 4.9 The effect of demecolcine on the rate of migration (A) and morphology of migrating bovine corneal epithelial tissue (B). The graph in panel (A) shows the increase in area covered by cells in the absence of drugs (open circles) and after exposure to demecolcine (solid circles). In panel (B), tissue at zero time point is shown in (a), tissue that had migrated for three hours in the absence of drugs in (b), after 10 minutes exposure to demecolcine in (c), and after 3 hours exposure to demecolcine in (d). The scale bar is 100 µm.



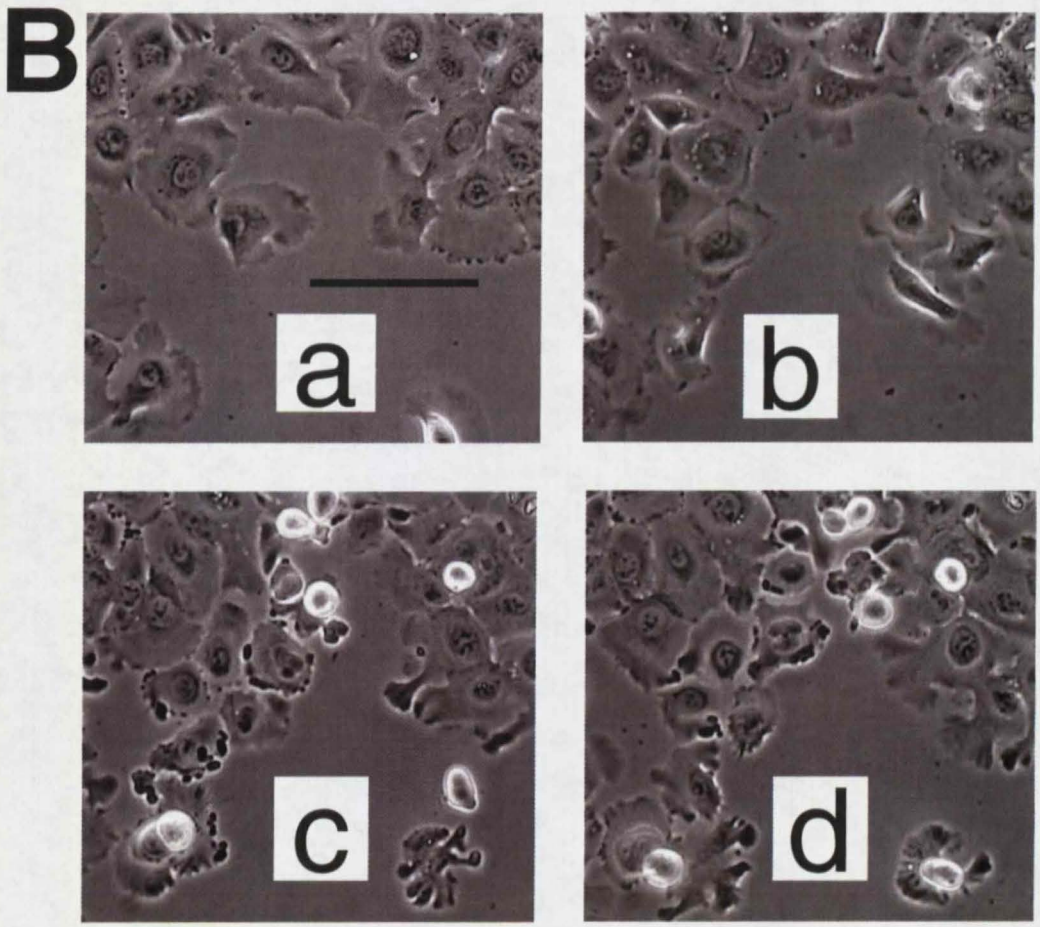


Figure 4.10 The effect of demecolcine on the rate of migration (A) and morphology of migrating bovine corneal epithelial cells (B). The graph in panel (A) shows the increase in area covered by the cells in the absence of drugs (open circles) and after exposure to demecolcine(solid circles).

In panel (B), cells at zero time point are shown in (a), cells that had migrated for three hours in the absence of drugs in (b), after 10 minutes exposure to demecolcine in (c), and after 3 hours exposure to demecolcine in (d). The scale bar is 100 µm.

4.4 DISCUSSION

The importance of the actin cytoskeleton has been established in both tissue and cell migration (Gipson and Anderson 1977; Mitchison and Cramer 1996). However the role microtubules, particularly in tissue migration, is less clearly defined. Here aspects of cytoskeletal structure and function in actively migrating epithelial tissue and cells were examined. The hypothesis that the migration of intact epithelial tissue differs from the migration of epithelial cells was tested by comparing both the organisation and the function of cytoskeletal components in migrating tissue and cells. When the cytoskeletal organisation within migrating tissue and the cells were compared, the most striking difference was at the leading edge of the migration. In the tissue, some areas of the leading edge had cables of actin filaments that appeared to span several cells. These regions were concave with respect to the profile of the leading edge. Similar actin cables have been observed in embryonic epidermal wounds and it was proposed that this arrangement of actin acted as a contractile "purse string" to close up the wound (Martin and Lewis 1992). Actin filament cables have also been observed in the healing processes that followed circular debridement corneal wounds in mice (Danjo and Gipson 1998), but could not be identified in organ culture models (Danjo and Gipson 1998). The *in vivo* mouse study showed that an adherens junction was the cell-cell junction associated with the linking of the actin filament between adjacent cells. The operation of the actin cable depended on the function of the E-cadherin acting as a cell-to-cell adhesion molecule, because the inactivation of E-cadherin with blocking antibodies disrupted the actin filament cable (Danjo and Gipson 1998). On the basis of the actin staining patterns observed in the present study, it is proposed that the "purse string" closure method may be one of the mechanisms that occur in tissue migration over synthetic surfaces.

This is the first demonstration of actin cables that appeared to span cells at the leading edge of adult corneal epithelial tissue that is migrating over a synthetic surface from an explant in culture. The finding was surprising, since the tissue was migrating radially outwards from a central explant. Within this type of model system, the concave regions that formed when tongues of tissue extended slightly ahead of the leading edge were the only areas where a purse string mechanism could function. The leading edge of narrow regions of the tissue sheet that had extended forwards when

the 'concave' areas were formed had ruffled membranes at their tips and actin filaments parallel to the direction of movement. The cells following showed no ruffled membrane activity and actin filaments were arranged at the circumference of the cells. This suggested that leading cells with ruffled membranes were providing the force to advance these parts of the tissue sheet. There has been a previous *in vitro* demonstration of "purse string" wound closure; Bement and colleagues showed that small stab wounds in monolayer cultures of an intestinal epithelial cell line healed by this means (Bement et al 1993). In that study it was also proposed that the actin filament cable additionally acted to maintain the apical/basolateral polarity of the cells bordering the wound edges. Sucrase-isomaltase, which is an apically-localised integral membrane protein, maintained an apical localisation in cells where the actin filament cable formed, but was present in lamellipodia that extended into the wound in areas lacking the actin cable (Bement et al 1993). The present study supports the proposal made by Bement and colleagues, that purse-string driven cellular motility may represent a wide-spread biological phenomenon (Bement et al 1993). Clearly within this model system the "purse sting" mechanism could only be marginally functional at specific regions of the leading edge. However epithelialisation of a corneal implant will entail the closure of a circular wound, which is the natural situation for this mechanism. These results demonstrate a potential for purse string wound closure over a synthetic material. It will be of interest to see if this mechanism can be encouraged to operate, possibly by manipulation of surface characteristics of the implant. This may be a desirable mechanism of epithelialisation of a synthetic polymer, because embryonic skin wounds closed by this mechanism and do not generate scars (Nodder and Martin 1997).

Microtubule were the other cytoskeletal component to be compared between migrating tissue and cells. The apparent dense intermeshing of microtubules within tissue that was not directly adjacent to the leading edge, indicated a very close association of the component cells within the tissue sheet. At the leading edge of the migrating tissue, the apparent intermeshing of microtubules was less evident. The preferential orientation of microtubule-organising centres towards the leading edge of migration was not detected, as was found in endothelial cell migration in culture (Gotlieb et al 1983).

The results showed that intact actin filaments and microtubules were both necessary to maintain the rate of migration of corneal epithelial tissue and cells over a synthetic surface. Disruption of actin halted the migration of both epithelial tissue and cells, as would have been predicted from other migration studies concerning corneal epithelial tissue (Gipson and Anderson 1977; Gipson et al 1982). The morphology of the epithelial cells, particularly at the advancing edge, was changed to a greater extent than was tissue morphology in response to cytochalasin-B. This suggested that persisting cell-cell adhesions within the intact tissue conferred some protection against the contraction which occurred within the migrating cell population. Whereas in the absence of cell-cell adhesion the disruption of actin filaments had a significant effect on the cells. The actin cytoskeleton is an integral component of the cell-cell adherens junction complex (Krendel and Bonder 1999). Therefore it would appear that other mechanisms of cell-cell adhesion within the tissue could maintain the tissue structure.

Although it was found that intact microtubules were necessary for optimum migration of tissue and cells, nevertheless intact microtubles were not necessary to maintain the morphology of the tissue or the cells during three hours exposure to the drug. This extended the time frame of a previous study, which showed that depolymerisation of microtubules had no effect on the ultrastructure of migrating corneal epithelium in organ culture after 30 min (Gipson and Keezer 1982). In the present study, the intact tissue was found to be particularly refractive to morphological change and a comparison of images of migrating tissue and cells exposed to demecolcine, suggests that some microtubules may have persisted longer within the tissue than those within the migrating cells. One explanation for the difference may be extrapolated from the studies of Bulinski and Gunderson (Gundersen and Bulinski 1988; Gundersen et al 1987). A subset of stable microtubules in which α-tubulin is tyrosinated have been identified in fibroblasts (Gundersen et al 1987). At the onset of migration, the fibroblasts generated an asymmetric array of tyrosinated microtubules which were oriented towards the direction of migration (Gundersen and Bulinski 1988). These modified microtubules showed enhanced resistance to depolymerisation by microtubule-disruptive drugs (Gundersen and Bulinski 1988). It is possible that, within the component cells of the migrating epithelial tissue, a greater percentage of

microtubules may exhibit the tyrosinated phenotype in comparison with microtubules within the migrating cells.

4.5 CONCLUSIONS

This study showed that there were differences between the arrangement of actin filaments and microtubules within migrating tissue and migrating cells. The differences were caused by the persistence of cell-cell adhesion in the migrating tissue. The presence of an actin filament cable in some regions of the leading edge of the tissue suggested a possible mechanism of migration that was not available to cells. The results pointed to the operation the "purse string" mechanism for advancing regions of the leading edge of the tissue. This was the first identification of this mechanism for advancing regions of adult corneal epithelial tissue that was migrating over a synthetic substratum.

The disruption of cytoskeletal components had the same effect in the inhibition of migration of tissue as it did in the cells. However disruption of cytoskeletal components had minimal effect on the morphology of the tissue, whereas the cells were found to retract. This suggests that cell-cell adhesion within the tissue provided some support for the morphology of the component cells.

CHAPTER 5

SUBSTRATUM TOPOGRAPHY AND CORNEAL EPITHELIAL TISSUE AND CELL MIGRATION

5.1 INTRODUCTION

The studies described in the previous Chapters compared migration of epithelial tissue and cells over substrata that were virtually planar. In addition to the effects that surface chemistry or pre-coated ECM molecules can have on tissue and cell migration (some of which were discussed in Chapter 2), the topography of surfaces can also exert an affect on migration. Early studies identified that cells became aligned in response to particular topographic features and this alignment was termed "contact guidance" (Weiss 1945). The specific cellular response was shown to be governed by the nature of topographic feature and the type of cell or tissue {see reviews (Brunette and Chehroudi 1999; Curtis and Wilkinson 1997)}. The rate of migration of some cells can be increased by the presence of microgrooves in a surface (Curtis et al 1995). On the other hand, increasing the rugosity of certain randomly arranged surface topographic feature may inhibit the migration of epithelial tissue (Takeuchi 1976). In other cases, it was shown that an increase in surface roughness had no effect upon cell migration but did increase tissue adhesion (Lampin et al 1997). At the start of the studies described in this Chapter it appeared that the rules governing the effects of surface topography on tissue and cell migration were not precisely defined, but clearly there were differences between migration over planar surfaces and over surfaces that had specific topographic features. Therefore, concurrent with the research reported in the previous Chapters, tissue and cell migration over two non-planar surfaces was studied.

Two different "classes" of topographic features were chosen for these studies. Firstly, the topography that was created by the presence of pores in the surface of a polymer; and secondly, the topography resulting from the creation of parallel microgrooves in a polymer surface. These two types of topography were chosen because they differed greatly from each other in that the pores formed a random non-directional topographic feature, whereas the topography created by microgrooves was directional and structured. Both classes of topography had the advantage that the size and the

distribution of the topographic features could be measured. Therefore, within each class of topography the effects of the size of topographical features (with the features having the same basic "shape") upon migration could be measured. The hypothesis tested was that increasing the size of topographic features would inhibit tissue migration to a greater extent than any inhibition of cell migration.

The first class of the topographical features was the random topography that resulted from making a polymer porous. In addition to providing a test material with which to compare tissue and cell migration, the selection of porous surfaces had relevance for the Artificial Cornea project. Research concerning the health of the epithelium and the anterior stroma when material was impanted below the epithelium identified a requirement for the porosity of the material to be greater than that afforded by membranes containing pores 0.05 μm in diameter (Sweeney et al 1998). One of the requirements for a corneal onlay was an ability to support epithelial tissue migration over the anterior surface of the device. Therefore it was necessary to identify which porosity-producing topographic features inhibited tissue migration. In relation to one type of topography that resulted from a porous surface, previous studies had shown that embryonic epithelial tissue from chick cornea was able to migrate over low porosity filter membranes (Millipore^{TM}) but migration over higher porosity filters was impeded (Takeuchi 1976). The pores in this type of filter are trabecular and the nature of the topography that is created by some of these filters with different levels of porosity is illustrated a publication in Appendix B (Fitton et al 1998). However, previous research had not considered the effect of topography produced by other methods of making a material porous. Track etching is able to produce defined columnar pores in materials and the effect of pores of this type on tissue or cell migration had not been studied. Therefore, the questions posed here were: what effect do columnar pores have on the extent of migration of adult corneal epithelial tissue, and is the effect the same for migration of a population of epithelial cells?

As the introduction of pores into a polymer surface creates a topography that is dependent upon the diameter of the pores, the extent of migration was measured over surfaces that contained pores of different diameters. In addition to creating a topographic effect, the introduction of pores into a polymer also creates porosity which enables fluid-flux between the culture medium and the basal surfaces of tissue

or cells migrating over the polymer. A system was devised that enabled migration to be measured over substrata in which fluid-flux was blocked in a manner that did not affect the surface topography. This enabled comparisons to be made between the cellular response towards the topography in the absence, or in the presence, of fluid through-flux.

The second "class" of topographic feature considered was that of parallel microgrooves. It is well documented that if cells are placed upon microgrooves of particular dimensions, many cell types align themselves in the direction of the grooves (Brunette and Chehroudi 1999; Clark et al 1987; Clark et al 1990; Curtis and Wilkinson 1997; Oakley and Brunette 1995; Walboomers et al 1998). Some studies have shown that in addition to directional constraints imposed by alignment to microgrooves, the migration of some cell types can be enhanced in response to the geometry of particular microgrooves (Curtis et al 1995). Other studies have considered the response of intact epithelial "tissue sheets" to microgrooved substrata (Brunette et al 1983; Chehroudi et al 1988; Chehroudi et al 1989). The differences between individual epithelial cells and clusters of epithelial cells towards grooves of various dimensions has been studied in regards to orientation and spreading (Brunette 1986). However, no previous quantitative studies comparing tissue and cell migration over a flat surface with migration over microgrooved surfaces of various dimensions existed. The questions posed were: what effect do microgrooves of various dimensions have on the extent and direction of corneal epithelial tissue migration; and secondly, how does this differ from the effect on cell migration?

5.1.1 Aim

The research described in this Chapter aimed to determine if effects on the extent of epithelial tissue migration in response to surface topography were the same as the effects on the extent of epithelial cellular migration.

5.1.2 Hypothesis to be tested

There is a difference between the migration of tissue and the migration of cells, this difference is evident in that when migration is occurring over different topographies, the migration over the non-planar surfaces is interfered with (impeded or enhanced) by topography (as compared with planar surfaces) and this interference is to a measurably different extent in the case of tissue as compared to the case of cells.

5.2 MATERIALS AND METHODS

5.2.1 Culture substrata

5.2.1.1 Columnar pore surfaces

Two sizes of polycarbonate discs (free from wetting agents) containing track-etched columnar pores were used. In one set, the discs were 13 mm in diameter and contained pores with nominal diameters of 0.1, 0.4, 0.8, 1 or 3 μm. In the second set, the disc were 25 mm in diameter and contained pores with nominal diameters of 0.1, 0.4, 0.8, 1, 2 or 3 μm, together with non-porous planar discs of the same base polymer (Poretics Corporation, Livermore California). The pores were created by the manufacturer using proprietary irradiation and etching techniques. Figure 5.1 shows the type of topography that resulted from these pores.

5.2.1.2 Microgrooved substrata

The microgrooved surfaces used in the study were made by casting polystyrene into templates. Each template had four different groove/ridge widths (1, 2, 5 and 10μm) arranged in the quadrants of the template. Two templates were used, one having groove depths of 1 μm and the other 5 μm. An example of polystyrene that was cast in a template with 5 μm deep microgrooves can be seen in Figure 5.2 and the dimensions of each surface are shown in the Results section (Table 5.3). The surfaces were formed from a solution made by dissolving 50 gm of polystyrene beads (Pierce, Rockford, IL, USA) in 300 ml of chloroform (APS Finechem, Seven Hills, NSW, Australia) by stirring gently for 24 hours (Chesmel and Black 1995). This solution was cast into templates and the chloroform was evaporated overnight. The templates were silicon wafers in which Photolithographic techniques had been used to make microgrooves (Twente Microproducts, The Netherlands). A flat glass surface was used as the template to produce the planar surface. The polystyrene replicas were removed from the templates by soaking in distilled water. Culture dishes were made by gluing the rims from 60 mm diameter tissue culture dishes (Corning) to the polystyrene replicas using the casting solution (Walboomers et al 1998; Walboomers et al 1999a; Walboomers et al 1999b; Walboomers et al 2000). The prepared dishes

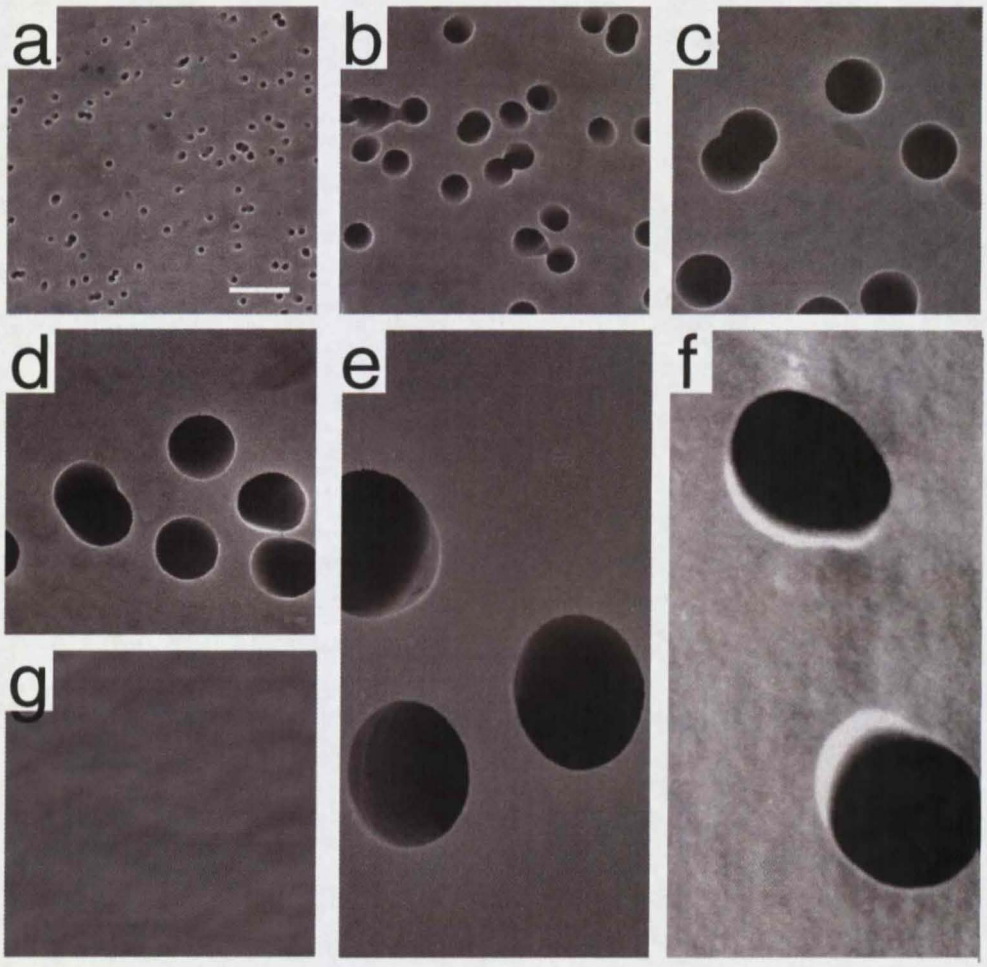


Figure 5.1 Scanning electron micrographs of the surfaces of the polycarbonate discs used in this study. The diameter of pores were 0.1 μm in (a), 0.4 μm in (b), 0.8 μm in (c), 1.0 μm in (d), 2.0 μm in (e), 3.0 μm in (f) and (g) was a non-porous surface. The scale bar is 1 μm and all images are of the same magnification.

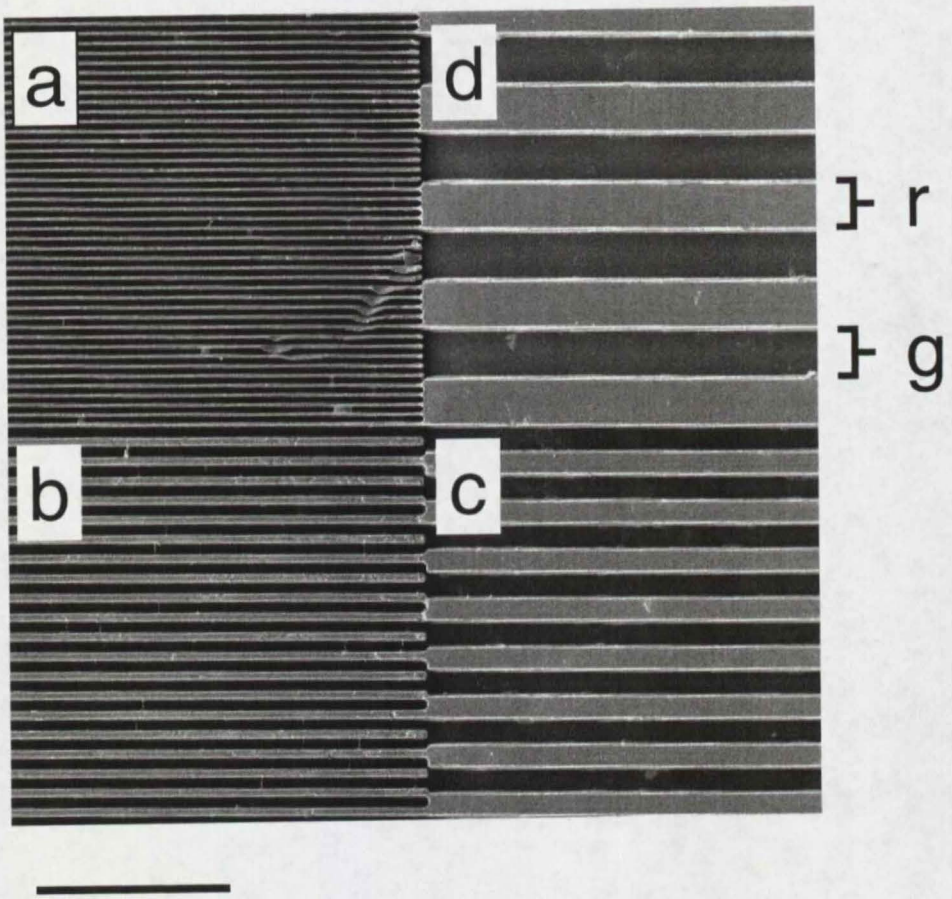


Figure 5.2 Scanning electron micrograph of a microgrooved polystyrene surface showing each groove/ridge dimensions tested. The replica was made using a silicon wafer template that had microgrooves 5 µm in depth. For each quadrant the microgroove width was equal to the ridge width and these were 1 µm (a), 2 µm (b), 5 µm (c) and 10 µm (d). The ridge width is indicated by (r) and the groove width by (g). The scale bar is 50 µm.

were sterilised by the addition of 70% (v/v) ethanol. The ethanol was replaced with several changes of sterile distilled water. Distilled water remained in the dishes until they were rinsed before use with serum-free culture medium.

5.2.1.3 Determining surface morphology

The polycarbonate discs and the polystyrene replicas were sputter-coated as previously described in Chapter 3 and viewed using a scanning electron microscope (Cambridge Stereoscan) at magnifications of up to 5000x.

5.2.2 Prevention of fluid-flux through pores

Modified Boyden chambers (see illustration in Figure 5.3) were used to examine tissue and cell migration over a surface in which the pores could be "blind-ended" to eliminate fluid-flux through the pores. The chambers were designed to accommodate 25 mm diameter discs, which separated the upper and lower chambers (the characteristics of the discs are summarised in Table 5.1). The polycarbonate discs were seated on a small rim in the base chamber and a silicone "o-ring" was placed on top of the discs. The top chamber was then screwed down to engage onto the "o-ring", ensuring tight apposition between the two polycarbonate discs and a watertight seal between the upper and lower compartments. The base chamber was "vented" to the outside by two narrow channels that were situated on either side of the compartment. These channels provided a means to replace the culture media in the lower chamber and were sealed with plugs during tissue culture.

In preliminary experiments, the exchange of fluid components through the discs was tested. The flux of culture medium constituents through the discs between the chambers was determined by measuring the diffusion of a BSA solution from the lower to the upper chamber. The diffusion of BSA through two porous discs was unimpeded as compared with a single porous disc. However a non-porous disc, whether clamped above or below a porous disc, prevented flux through the porous disc. These results demonstrated that there would be no fluid-flux to the basolateral membranes of migrating tissue or cells on surfaces designated (P) and (T), whereas there would be fluid-flux on surface (T+F) (see Table 5.2, for code). Even though it

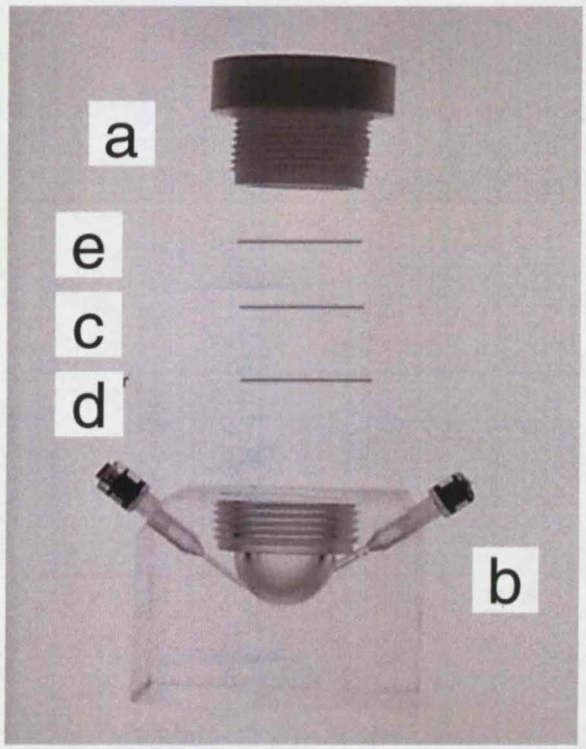


Figure 5.3 Illustration of a modified Boyden chamber. The upper chamber (a) has a threaded region which is screwed tightly into the base (b) which forms a lower chamber. The disc over which tissue or cells migrated (c) was placed directly above a second disc (d) and both were placed so that they separated the upper and lower chambers. The 25 mm diameter discs were supported at their circumference by a ridge (approximately two mm wide) within the lower chamber. A silicone washer (e) was fitted over the discs where they were supported on the ridge before the upper chamber was screwed into place.

would be unlikely that all the pores in the two discs "lined up", clearly sufficient overlap occurred to permit fluid-flux.

Table 5.1

Physical characteristics of the discs used in the Boyden chambers

Pore diameter[a]	Pore density (pores/cm^2)[a]	Surface area of disc covered by pores (%)[b]	Nominal disc thickness (μm)[a]
No pores	0	0	10
0.1	4X10^8	3.14	6
0.4	1X10^8	12.56	10
0.8	3X10^7	15.70	9

Table 5.2

Arrangement of discs to provide culture surfaces with specified characteristics

Code	Characteristics	Upper most disc[c]	Lower disc
(P)	Non-porous/no fluid-flux	Non-porous	Contains pores
(T+F)	Topographic features, permits fluid-flux	Contains pores	Contains pores
(T)	Topographic features, no fluid- flux	Contains pores	Non-porous

[a] Data published by the manufactures (Poretics catalogue 1997-1998).

[b] See reference (Evans et al 1999).

[c] Tissue and cells migrate over this disc.

5.2.3 Corneal epithelial tissue and cell migration over polycarbonate discs

Initial studies concerning tissue and cell migration over the porous polycarbonate surfaces were conducted using 13 mm diameter porous discs. The subsequent studies, which first examined the kinetics of tissue migration and then sought to separate the effects of porosity and topography, were conducted using 25 mm diameter discs.

The techniques for obtaining corneal tissue explants and epithelial cells are fully covered in Chapter 2, Materials and Methods as are the culture methods and methodology for measuring migration. Briefly, for tissue migration over the polycarbonate discs, corneal buttons were placed stromal side down in the centre of the discs that had been placed in the wells of cluster trays (Corning).

For cell migration the "fence" (as described in Chapter 2, section 2.2.3) was centred on the polycarbonate disc, the lumen was pre-coated with 10 μg/ml Fn and the bovine corneal epithelial cells were seeded into the lumen of the fence in 100 μl of culture medium that contained 20 % (v/v) DD serum.

In the initial migration studies over polycarbonate discs, tissue and cells were cultured for six days. In the kinetic study, explants were cultured for 3, 7, 10, 15, 18, 21 and 30 days.

5.2.4 Tissue and cell culture in Modified Boyden Chambers

Two 25 mm diameter polycarbonate disc were assembled together in the Boyden chamber (described in section 5.2.2) to provide surfaces with three different characteristics, as described in Table 5.2. A corneal explant was placed (stromal side down) on the centre of the upper disc in the chamber. The top chamber fitting was screwed down firmly without disrupting the tissue. After 25-30 min, when the explant had adhered to the disc surface, the base chamber was filled with SFM+add (Chapter 2, section 2.2.1). One ml of this medium was added to the top chamber to completely cover the explant. The explants were cultured under the conditions described in Chapter 2, section 2. . The culture medium was replaced in the top chamber only, after three and six days of culture. After nine days of culture, the discs containing the explants and migrated tissue were removed, fixed and stained and the migration area was measured as described in Chapter 2, section 2.2.6.

For cell migration in the Boyden chamber, each disc that would become an upper disc when assembled in the Boyden chamber was first placed in the base of a well in a six-well cluster tray and a fence was centred upon it. Prior to cell seeding, the lumen of the fence was pre-coated with a 10 μg/ml solution of Fn in PBS as described in Chapter 2, section 2.4.1. After the cells had attached and any non-adherent cells had been removed by washing with SFM, the disc was transferred to a chamber, placed above the appropriate disc (Table 5.2) and the assembly was clamped together. The lower chamber was filled with culture medium containing 10% (v/v) DD serum. One ml of this culture medium was also added to the upper chamber. The cells were cultured in the same way as the tissue, except that the culture medium in the upper chamber was replaced after three days. After seven days, the cells were fixed, stained and the area over which the cells had migrated was measured (as for the tissue). The area covered by cells after migration was divided by the original cell area after attachment in the fence. For this purpose one set of cells on each surface was fixed and stained after the initial 24-hr attachment period, while the cells were restricted by the fence. Each experiment consisted of four replicates for each treatment and the experiments were repeated at least three times.

5.2.5 Tissue and cell migration over microgrooved surfaces

For tissue migration, four explants one in each quadrant, (Figure 5.2 shows the centre of the quadrants) were cultured in each of the fabricated grooved dishes and on the flat surface. The tissue was cultured on the uncoated surfaces for nine days. Three replicates of each surface were used in each experiment and the experiment was repeated four times. Examples of explant cultures and the measured distances of migration are shown in Figure 5.4.

For cell migration, the dishes were pre-coated with 5 μg/ml Fn and the fence was centred on each quadrant. The cells were seeded and cultured as described above. Replicate cultures for each experimental surface were fixed and stained after the initial 24-hr culture period when the cells were restrained by the fence, to determine the initial seeding area. The cell migration studies were conducted over six days, because after that time leading cells on the 1 μm deep microgrooves reached the edge of the particular microgroove quadrant on which they were seeded and began to

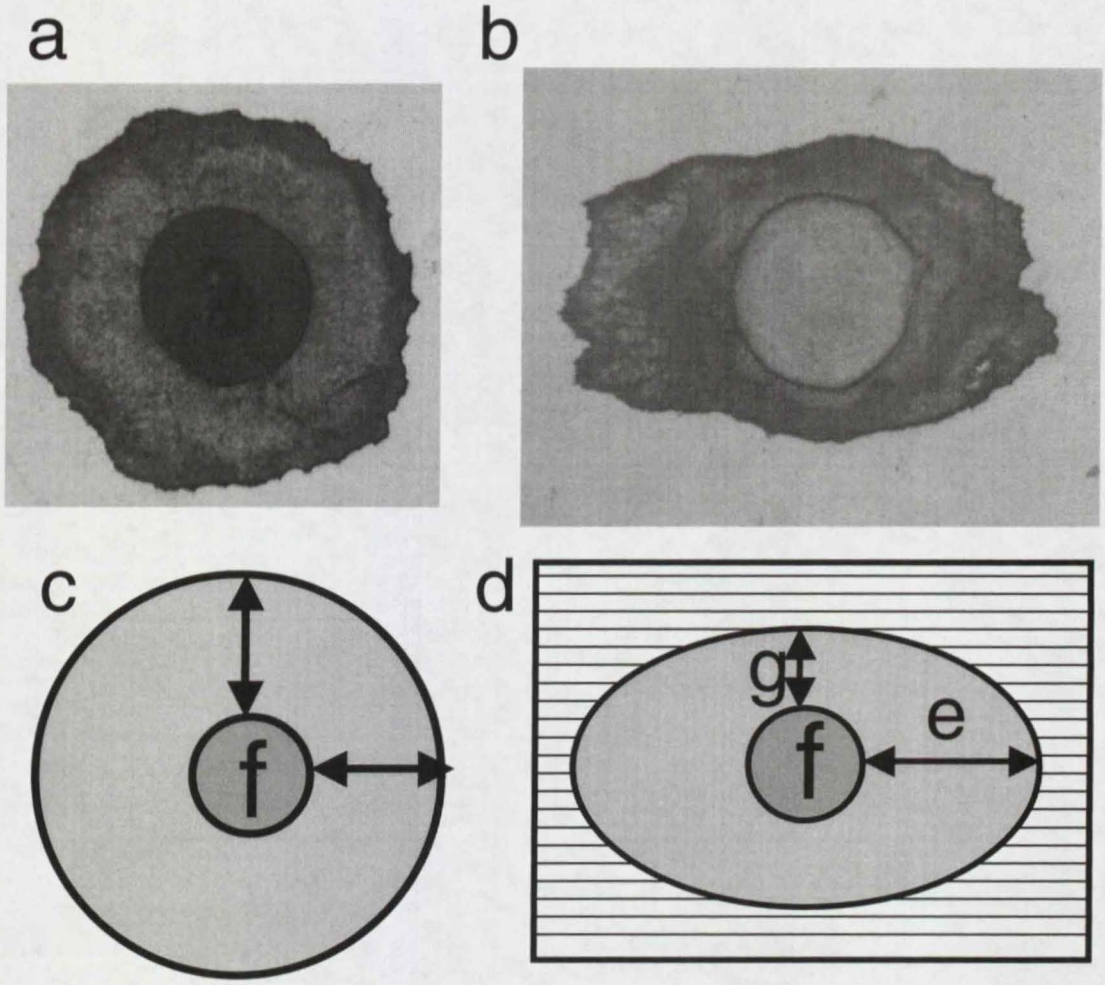


Figure 5.4 Light micrographs of epithelial tissue that had migrated from corneal explants over a flat-cast surface (a) and a surface with 1 μm deep microgrooves with groove/ridge widths of 1 μm (b). On the flat-cast surface {see diagram (c)} the migration distance from the explant (f) to the leading edge of the advancing tissue was measured in two directions perpendicular to each other (double headed arrows). On the microgrooved surfaces {see diagram (d)}he migration was measured *along* the microgrooves {double-headed arrow, (e)} and *across* the microgrooves {double-headed arrow, (g)}.

migrate into quadrants of different groove/ridge widths.

Following migration, the tissue and cells were fixed and stained with Crystal violet as described in Chapter 2, section 2.2.6. Examples of migrated tissue and diagrams to indicate the distances that were measured are shown in Figure 5.4). An image analyser (Quantimet Q570) was used to measure:

(1) the migration distance from the tissue explant (or original cell culture) to the "leading edge" of the epithelial tissue (or cells) *along* the microgrooves {'e' in Figure 5.4(d)};
(2) the migration distance to the leading edge *across* the microgrooves {'g' in Figure 5.4(d)}; note that this measurement did not take into account the vertical contribution of the groove walls;
(3) on the flat-cast surface, the migration distance to the leading edge, measured in two directions (chosen at random) perpendicular to each other {(Figure 5.4(c), double headed arrows};
(4) the area of the tissue or cells that had migrated from the explant or initial cell culture {Figures 5. 4(c) and (d), pale grey region in diagram}.
For tissue, the area covered by the migrated tissue was obtained by subtracting the initial explant area (f) from the final total area. For cells, the area was obtained by subtracting the initial area of the confluent cell culture (f) from the final area covered by the cells. Each experiment was repeated at least three times using four replicates for each treatment. Statistical significance for all data was determined using Analysis of Variance and the Student-Newman-Keuls multiple comparison test.

5.2.6 Transmission electron microscopy

Epithelial tissue that had migrated over the polycarbonate discs in the chambers, was fixed and processed for electron microscopy as described in Chapter 3, section 3. 2.4. Ultrathin sections through the tissue and the underlying polymer were collected onto uncoated copper grids, stained with 4% (w/v) aqueous Uranyl acetate and Renold's lead citrate and viewed using a Philips CM 100 transmission electron microscope.

5.2.7 Histochemistry

In order to detect actin filament bundles, the cultures on the polystyrene microgrooves and flat-cast surfaces were washed in PBS and fixed with formol saline for 30 min at room temperature. After one wash with PBS, the cultures were permeabilised with 0.5% (v/v) Triton X100 (Calbiochem) for 20 min. The cultures were incubated with Texas Red phalloidin (Molecular Probes) (20U/ml in PBS) for 90 min, protected from light. The samples were washed three times with PBS, cut out with a scalpel and secured between a coverslip and a microscope slide with FluorSave™ (Calbiochem). The samples were viewed using a confocal laser-scanning microscope (Leica Inc) at magnifications of up to 1000x. Images were collected in two focal planes within the tissue: close to the superficial surfaces; and adjacent to the microgrooves. Additionally, the confocal microscope was used in reflectance mode to capture an image of the underlying microgrooves.

5.3 RESULTS

In this section the results are presented in the following sequence: first, are the results of a comparison that was made between the effects of pore diameter on the extent of migration of corneal epithelial tissue and the migration of epithelial cells; secondly, are the results from the kinetic study that compared the extent of migration of tissue over surfaces containing pores of various diameters with the migration of tissue over a planar surface of the same material; thirdly, the results are presented from a technique that "blind-ended" the pores to separate the effects of the surface topography and the effects of the porosity of the polymer surfaces on tissue and cell migration. Finally, are the results from a study that compared the effects of parallel microgrooves on the extent and the direction of tissue and cell migration.

5.3.1 Tissue and cell migration over surfaces containing pores

In this study, tissue and cell migration was measured after six days migration over discs that contained pores ranging from 0.1 to 3 μm in diameter. The migration of tissue over surfaces containing pores 0.8 or 1 μm in diameter was reduced in comparison with migration over surfaces containing pores of 0.1 or 0.4 μm in diameter. On the surface with pores 3 μm in diameter, migration was totally inhibited {Figure 5.5(a) black bars}. In contrast, corneal epithelial cells migrated, to some extent, over each of the surfaces tested and there was no significant difference between the extent of migration over surfaces with pores 0.1, 0.4, 0.8 or 1 μm in diameter ($p<0.05$). Migration was reduced by 25% over the 3 μm diameter pore surface as compared with that for the 0.1-0.8 μm pore surfaces ($p<0.001$) {Figure 5.5(a) red bars}.

The reduction in tissue migration, in response to the larger diameter pores persisted over time {Figure 5.5(b)}. Minimal tissue migration was observed on surfaces that contained pores of either 2 or 3 μm in diameter, even after 21 days culture. Tissue migration over surfaces containing pores 0.1 or 0.4 μm in diameter was enhanced in comparison with migration over a non-porous surface of the same material {Figure 5.5(b), blue and red lines compared with the black line}.

The enhanced migration of tissue in response to the 0.1 and 0.4 μm pore surfaces in comparison with a non-porous surface of the same base polymer was further

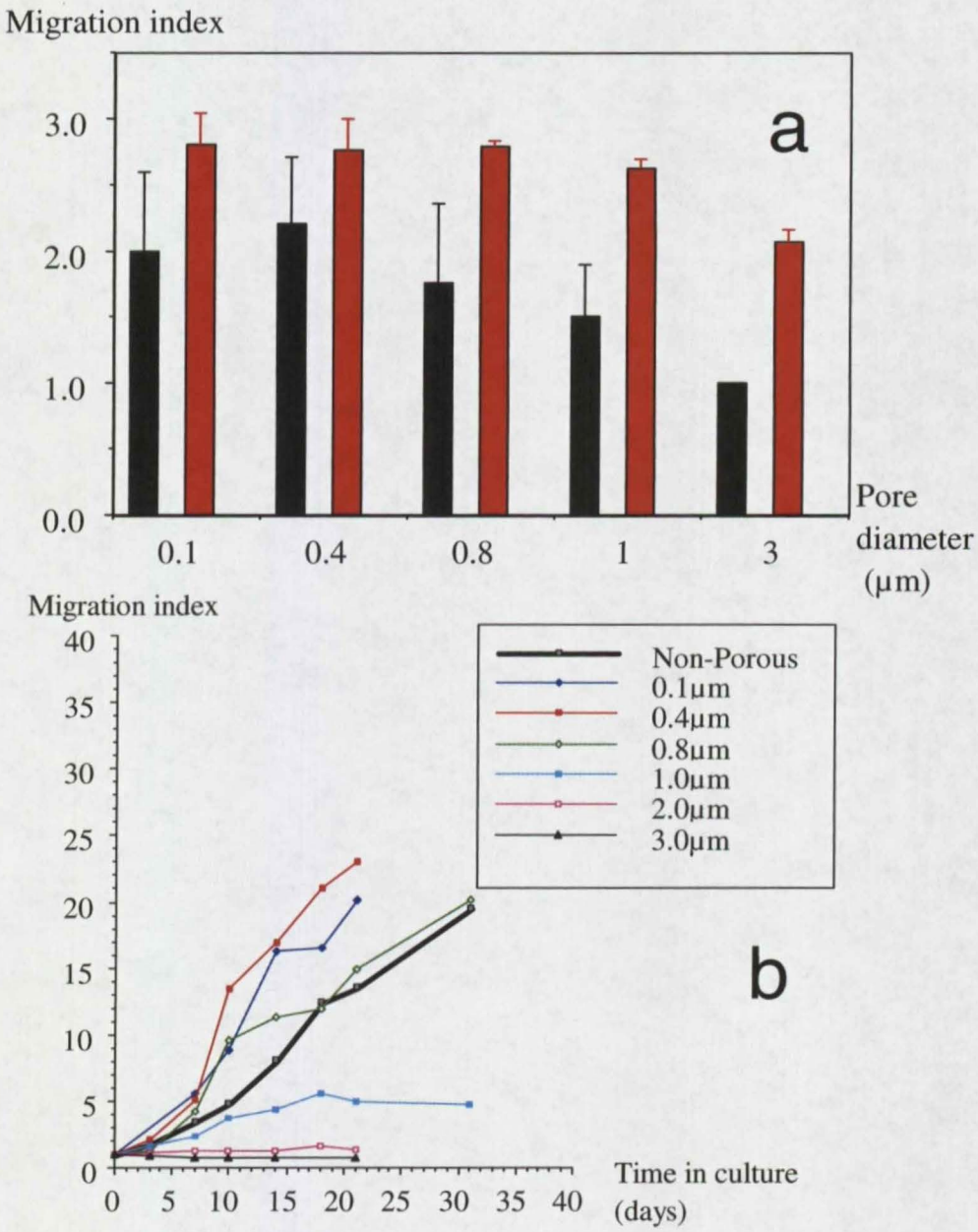


Figure 5.5 The effect of pore size on the migration of bovine corneal epithelial tissue and cells. Tissue migration {(a) black bars}and cell migration {(a) red bars} was measured after after six days culture on 13 mm diameter polycarbonate discs. Data are the mean and standard deviation, n = 6 for each surface.

The kinetics of tissue migration over 25 mm diameter discs is shown in (b). Tissue migration was measured by image analysis on a minimum of three replicates for each time point, a total of 192 explants. The average standard error was 13%. Migration over the 0.1 and 0.4 µm pore membranes was limited by the membrane diameter after 21 days.

investigated in order to determine if it occurred as a result of the porosity of the surface or because of the topography of the surface. Epithelial cells were included in the investigation to identify if it was a tissue specific phenomenon.

5.3.1.1 Separation of the effects of topography and fluid through-flux for tissue and cell migration

The technique of culturing tissue and cells in the modified Boyden chamber enabled comparisons to be made between the extent of migration over surfaces that had identical topography, but fluid through-flux was restricted on one surface and permitted on the other.

In the Boyden chambers epithelial tissue migrated radially outwards from corneal explants over each of the defined substrata tested. The area covered by the migrated tissue was approximately 80 mm^2 on the non-porous surface and approximately 170 mm^2 on both the 0.1 and 0.4 μm pore surfaces after nine days of culture. These areas represented a migration distance of 2 to 4.5 mm, from the edge of the 6 mm diameter corneal explant. It is a migration index value of approximately three on the planar, non-porous surface and six on the 0.1 and 0.4 μm pore surfaces. The migration index was approximately 1.5 to two fold ($p<0.05$, $p<0.001$) higher for migration over surfaces that contained pores of 0.1 or 0.4 μm diameter {Figures 5.6A(a) and (b), see T and T+F}, as compared with the planar, non-porous surface {Figures 5.6A(a) and (b), see P}.

The aim was to separate the effects of topography on the migration of epithelial tissue and cells, from the combined effects of topography and fluid through-flux. A porous disc placed above a non-porous disc provided a surface with the topographic features of pores, but with fluid through-flux being precluded (T). As the discs were 10 μm thick, this placement of a non-porous disk had the effect of producing 10 μm deep "blind-ended" cylindrical pits. On the other hand, two porous discs placed together created a substratum that had the defined topographic features of the pores, but also allowed fluid through-flux (T+F). The third experimental surface was a non-porous disc, which was used as a planar substratum that lacked both the topographic features of the pores and fluid through-flux (P) (See Material and Methods section,Table 5.2). A porous disc was placed under this surface in the chambers so that, in each case,

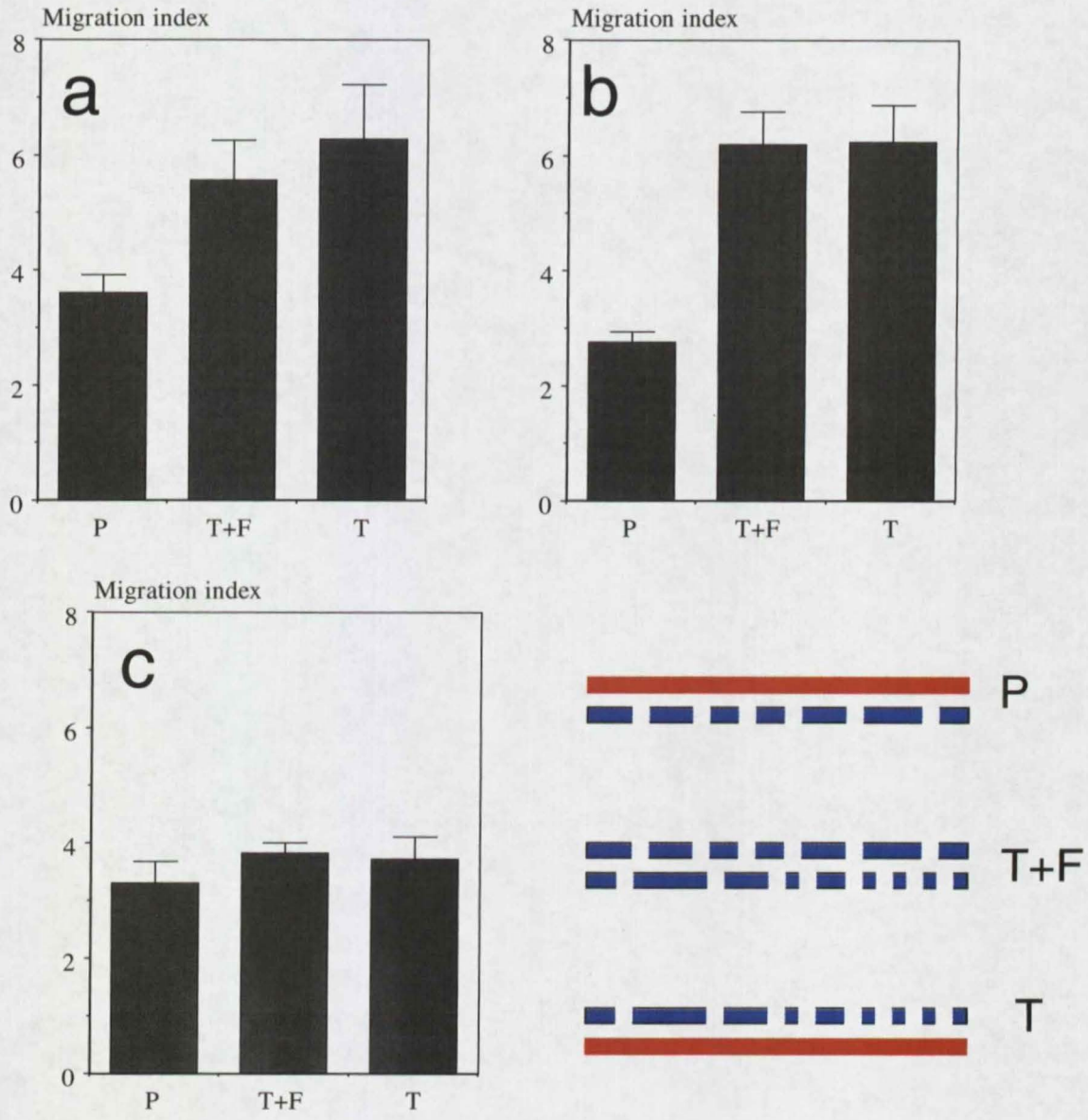


Figure 5.6A The effect of surface topography and fluid-flux on the migration of epithelial tissue. The diagram illustrates the arrangement of the discs to create surfaces with the desired characteristics. The non-porous disc is indicated by red and the porous disc by blue.
(a) Migration over a non-porous, planar surface (P); a porous surface with non-planar topography (T+F); and a surface with non-planar topography but without fluid through-flux (T). The mean diameter of the pores was 0.1 μm.
(b) Migration of epithelial tissue as described for (a), except that the mean diameter of the pores in the porous discs was 0.4 μm.
(c) Migration of epithelial tissue as described for Figure (a), except that the mean diameter of pores in the porous discs was 0.8 μm.
Tissue migration after nine days culture is expressed as a migration index (final tissue area/original explant area) and is the mean and standard error from three separate experiments, each containing four replicates.

there was equivalent mechanical support for the tissue. When the pores were "blind ended", the same level of stimulation of migration was seen as for the case of pores that permitted fluid-flux ($p>0.05$) {see Figures 5.6A(a) and (b), T compared with T+F}. Furthermore and consistent with this, the extent of tissue migration was not reduced for the case where two porous discs were present, as compared with a single porous disc being present. Therefore, reducing or abolishing fluid-flux through the substratum did not cause a reduction in tissue migration and had no impact on the stimulation of migration seen with porous (as compared with non-porous surfaces). Stimulation of epithelial tissue migration was not seen with larger diameter pores. There was no significant difference between the extent of tissue migration on the planar non-porous surface as compared with the 0.8 μm pore surfaces ($p>0.05$) {Figure 5.6A(c), P compared with T+F and T}. This was the case for both the 0.8 μm surface when it was porous {consistent with the results in Figure 5.5(b)}, but also when it was "blind-ended" with a non-porous disc (Figure 5.6A(c), see T compared with T+F). There was some variation in the extent of migration over the non-porous surface between various experiments, as is common with the use of a primary tissue source (abattoir supplied bovine eyes). However, in each case data followed the same trend, in that migration was enhanced by the small pores and "blind-ending" the pores did not reduce the level of enhancement.

A similar series of experiments to those in the tissue migration study was conducted with the cell source being epithelial cells, instead of the tissue sheets. The aim was to determine whether the extent of migration of bovine corneal epithelial cells from restricted confluent cultures was modified by topographic features, in the absence of fluid through-flux.

Cell migration was stimulated on the porous surfaces as compared with a non-porous substratum {Figures 5.6B(a) and (b)}. During seven days culture, the distance of cell migration was increased as compared with the planar, non-porous surface, by approximately 1.5 fold on the 0.1 μm pore surface and 1.3 fold on the 0.4 μm pore surface, (Figures 5.6B(a) and (b), see T and T+F compared with P). However, there was no significant difference ($p>0.05$) in the extent of migration on the 0.1 μm pore surface between the case where fluid through-flux was maintained and when it was blocked {Figure 5.6B(a), see T+F compared with T}. Similarly, there was no

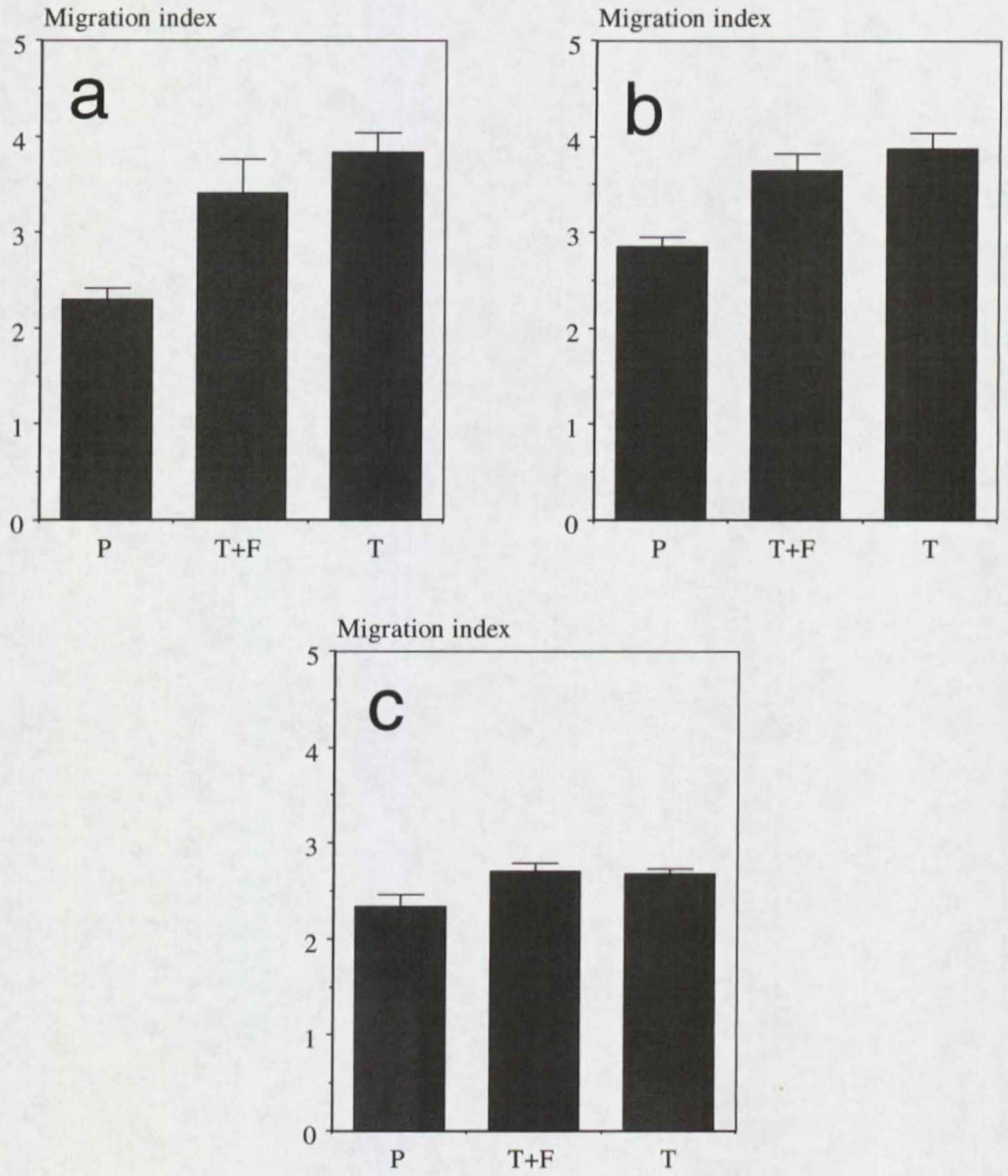


Figure 5.6B The effect of surface topography and fluid-flux on the migration of epithelial cells.

(a) Migration over a non-porous, planar surface (P); a porous surface with non-planar topography (T+F); and a surface with non-planar topography but without fluid through-flux (T). The mean diameter of the pores was 0.1 μm.

(b) Migration of epithelial cells as described for (a), except that the mean diameter of the pores in the porous discs was 0.4 μm.

(c) Migration of epithelial cells as described for (a), except that the mean diameter of pores in the porous discs was 0.8 μm.

Cell migration after seven days culture is expressed as a migration index (final cell area/original cell area) and is the mean and standard error from two separate experiments, each containing four replicates.

significant difference ($p>0.05$) between the extent of migration on the 0.4 μm pore surface when through-flux was maintained and when it was blocked {Figure 5.6B(b), see T+F compared with T}.

On the 0.8 μm pore surface, there was no significant difference between the extent of cell migration as compared with migration over the planar surface ($p>0.05$). This was the case for the 0.8 μm surface both when fluid through-flux was maintained and when it was blocked {Figure 5.6B(c), P compared with T+F and T}.

From the previous comparisons of the effect of pores on the extent of epithelial tissue and cell migration the findings showed that:
(1) migration of tissue was inhibited by large diameter pores to a greater extent than was migration of cells;
(2) both tissue and cell migration were stimulated by small diameter pores;
(3) the stimulation of tissue and cell migration in response to the small diameter pores was independent of fluid through-flux and occurred as a result of the topography of the surface.

5.3.1.2 Tissue morphology in response to pores

In order to determine the tissue response towards the pore channels, the interface between the migrated tissue and the polymer was examined using transmission electron microscopy. The examples shown are of tissue that migrated over a surface that contained "blind-ended", 0.4 μm diameter pores. The electron micrograph in Figure 5.7(a) shows a low magnification image of the ultrastructure of epithelial tissue (e) migrating over a 0.4 μm pore disc (T). The region shown is approximately mid-way between the explant and the advancing edge of the tissue sheet. The tissue consists of between three and four layers of closely apposed, elongated cells and is approximately 10 μm thick. The columnar pores in the disc underlying the tissue are visible in transverse section. Tissue closer to the advancing edge of the tissue sheet is shown at a higher magnification in Figure 5.7(b). The tissue has bridged over the 0.4 μm pores. The cells in direct contact with the polymer are squamous in shape and membranes of adjacent cells are interdigitating at a slope of approximately 45° {Figure 5.7(b)}. The tissue closer to the original explanted tissue button, which had been in contact with the polymer for a longer time than the tissue at the advancing

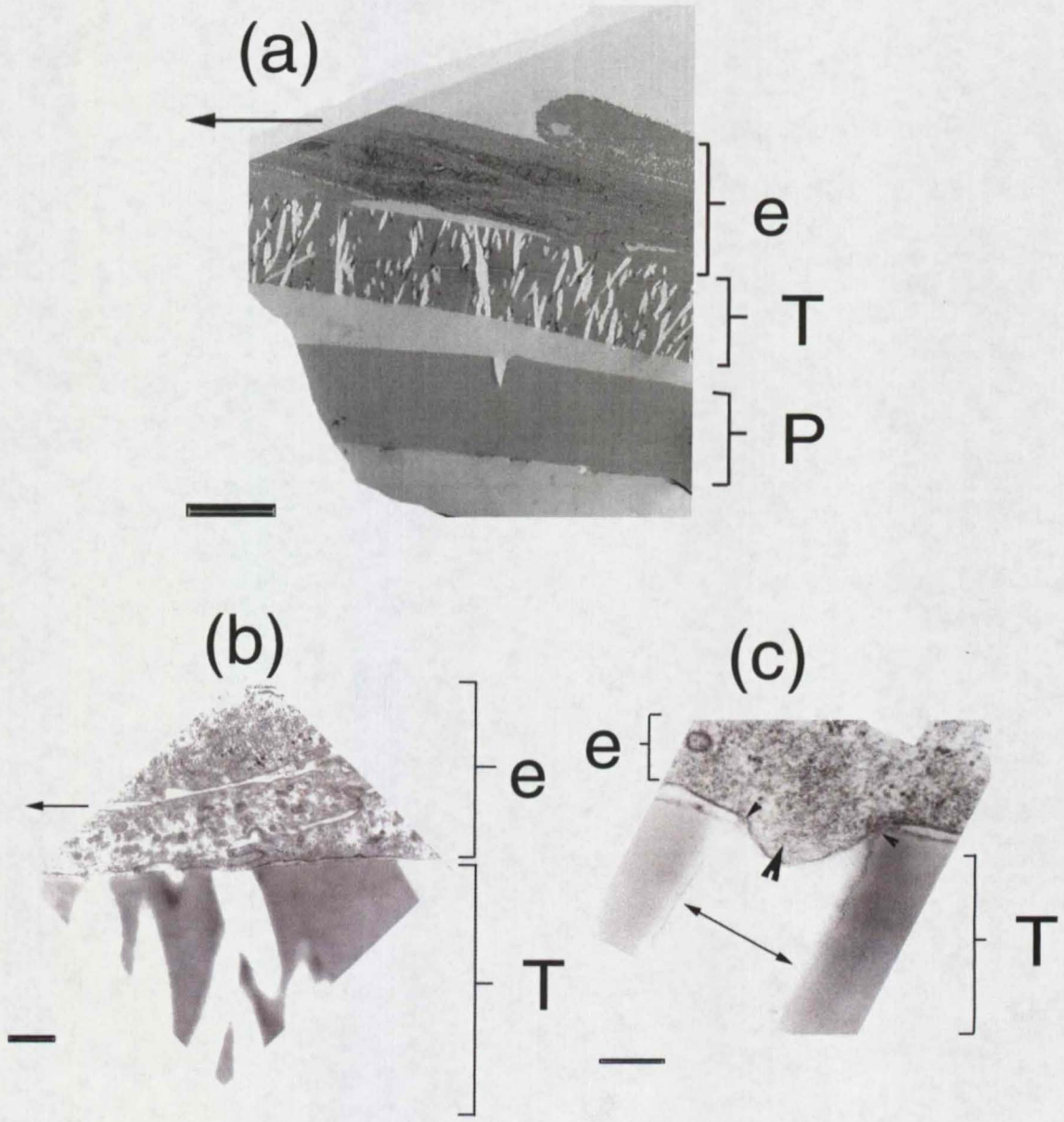


Figure 5.7 Ultrathin sections of bovine corneal epithelial tissue (e) that had migrated over a disc containing pores 0.4 μm in mean diameter (T). A nonporous disc (P) was clamped to the underside of the 0.4 μm disc during migration, but it separated during processing and is only visible in (a). Epithelial tissue close to the advancing edge and moving in the direction of the arrows is shown in (a) and (b). In (b) the tissue bridged the 0.4 μm pores. In (c) cytoplasm (marked by a large arrowhead) from epithelial tissue that was about 1 mm back from the advancing edge is starting to penetrate a pore channel (marked with a double headed-arrow), focal adhesions are marked by small arrow-heads. The scale bar in (a) is 10 μm, in (b) 500 nm and in (c) 200 nm.

edge, had extend cytoplasmic processes into some of the pores {Figure 5.7(c)}. Focal adhesions or close contacts formed at each edge of some pores {Figure 5.7(c), small arrowhead}, but were not seen at the edge of every pore examined.

5.3.2 The microgrooved surfaces

The microgrooved surfaces comprised quadrants of four different microgroove widths (the centre of the culture surface is illustrated in Figure 5.2). The surfaces of the dishes were examined using SEM, which showed that the polystyrene replicas resembled the silicon template. Figure 5.2 shows a SEM image of a microgrooved surface, with microgroove and ridge widths of 1, 2, 5 or 10 μm, where the template had 5 μm deep microgrooves.

5.3.2.1 Migration of epithelial tissue over microgrooved surfaces

For all substrata examined, epithelial tissue from the corneal explants migrated down the side of the underlying stroma and over the cast polystyrene surfaces. Tissue migrated further *along* than *across* the microgrooves on each of the microgroove widths {solid bars compared with open bars in Figure 5.8A(a)}. The extent of this effect was dependent upon the groove depth. There was approximately a twenty-fold increase in the distance travelled by the tissue *along* the microgroove, as compared with the distance travelled *across* the microgrooves, on the surfaces with microgrooves that were 5 μm deep ($p<0.001$). The increase in migration *along* (as compared with *across*) the grooves was approximately three-fold on the surfaces containing microgrooves with a depth of 1 μm ($p<0.001$).

The migration distance along the microgrooves was further, as compared with the flat-cast surface, for all microgrooves that were 1 μm in depth ($p<0.001$) and for microgrooves 5 μm in depth with ridge/groove widths of 2 μm ($p<0.05$), or 10 μm ($p<0.01$). These increases ranged from 1.4 to 1.6-fold {Figure 5.8A(a)}. As compared with migration over the flat-cast surface, migration *across* the microgrooves was reduced approximately 15-fold on the 5 μm deep microgrooves and approximately two-fold on the 1 μm deep microgrooves (p values variously $p<0.01$ - $p<0.001$).

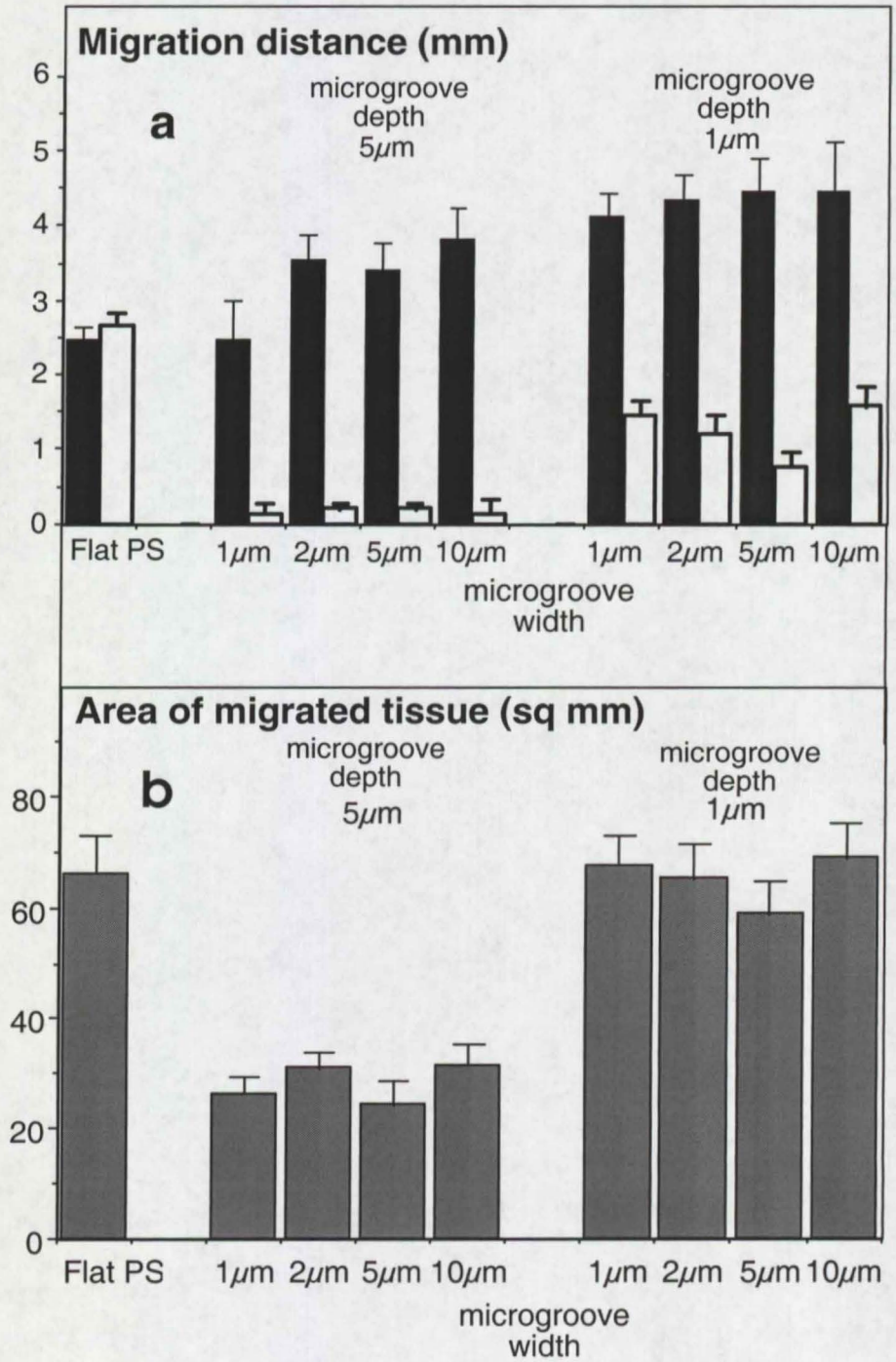


Figure 5.8A The effect of microgrooves on the extent of epithelial tissue migration. (a) Solid bars show the migration distance (in mm) *along* the microgrooves. Open bars show the migration distance (in mm) *across* the microgrooves. The microgroove depth and groove/ridge widths were as indicated on the graph. For the flat-cast surfaces, migration was measured in two directions perpendicular to each other. (b) The effect of microgrooves on the area of tissue migrated from a corneal explant. The results are the mean and standard error from four separate experiments, each containing three replicates.

The total areas covered by the migrated tissue on the surfaces with microgrooves 1 μm in depth were not significantly different from the areas on the flat-cast surface ($p>0.05$). On the surfaces with microgrooves 5 μm in depth, there was a two-fold decrease in tissue area ($p<0.001$) as compared with the tissue area on the flat-cast surface {Figure 5.8A(b)}. These results demonstrate tissue guidance by the microgrooves 1 μm in depth, and guidance coupled with some inhibition of tissue migration by microgrooves 5 μm in depth. When tissue migration was measured over Fn coated surfaces the results followed the same trend as migration over uncoated surfaces.

5.3.2.2 Migration of epithelial cells over microgrooves

The effect of the surface microgrooves on the migration of a confluent culture of previously dissociated epithelial cells was also examined. In terms of comparative distance moved *along* vs *across* the microgrooves, and over the microgrooved surface vs the flat-cast surface, the results were very similar to those observed for the intact tissue. That is, migration was further *along* than *across* microgrooves and further *along* microgrooves 1 μm in depth than over the flat-cast surface. The main difference between intact tissue and dissociated cells was that the latter moved proportionally about twice the distance of the former {Figure 5.8B(a)}. The greater distance that the cells migrated as compared with the tissue may have been due to the Fn coating that was used for the cell migration experiments. This coating was used for the cells because, unlike the intact tissue, the cells migrated poorly on the uncoated polystyrene surfaces. As with migration distance, the area covered by the migrated cell sheet was greater with all treatments when compared with intact tissue. There was a similar reduction in area seen with dissociated cells and intact tissue in response to 5 μm deep grooves as compared with the flat-cast surface. Unlike intact tissue, the migration area of cell sheets on the 1 μm deep microgrooves was less than that on the flat-cast surface.

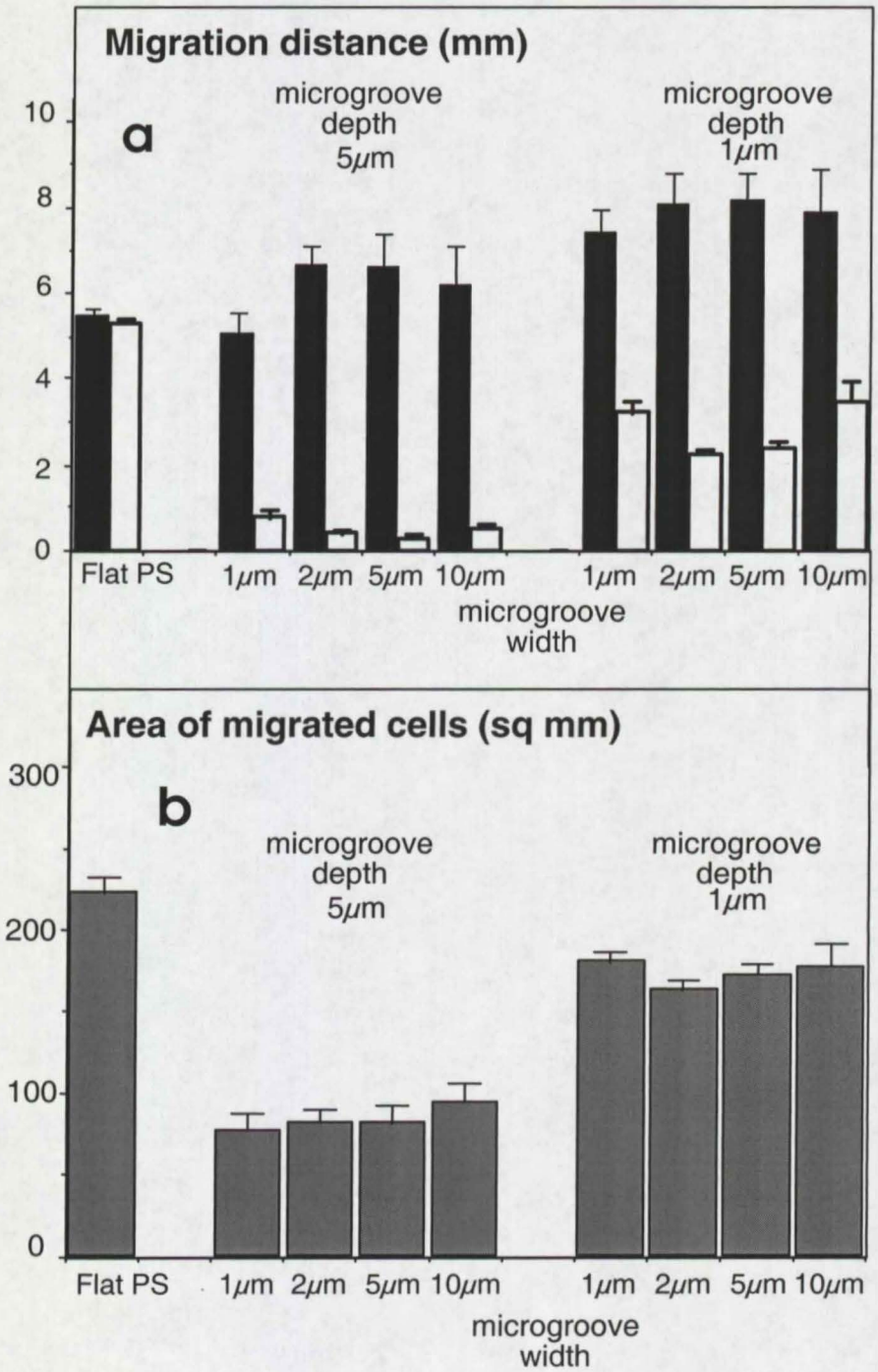


Figure 5.8B (a) The effect of microgrooves on the extent of epithelial cell migration. Solid bars show the migration distance (in mm) *along* the microgrooves. Open bars show the migration distance (in mm) *across* the microgrooves. The microgroove depth and groove/ridge widths were as indicated on the graph. For the flat-cast surfaces, migration was measured in two directions perpendicular to each other. (b) The effect of microgrooves on the area occupied by the migrated cells. The results are the mean and standard error from four separate experiments, each containing three replicates.

5.3.2.3 Surface area of microgrooved substrata

In the results presented above, the distance that the tissue or cells migrated is the planar distance (ie, in the horizontal plane for both the flat and the microgrooved substrata). However, when the direction of migration is across the grooves, the migration may also theoretically involve migration up and down the vertical faces of the grooves. Furthermore, on any grooved substrata the effective distance travelled may be altered by the angle between the migration track and the grooves: if the migration is along the groove (ie at an angle of 0°), then the migration distance is equivalent to that on a flat surface. As the angle increases, however, so may the effective distance, which will be maximal for cells migrating across the grooves (ie, at 90°) if they have traversed vertical and horizontal faces. If tissue migration involves traversing these vertical faces, estimating the actual distance of travel along the contours of the microgroove surface would be necessary to calculate the distance of migration. Therefore, it is possible that a simple measurement of migration may not take the actual distance covered into account.

In particular it might be important to calculate this to determine if the observed lesser rate of migration in the direction across the microgrooves (measured as the planar distance travelled) results from the migration being partly directed up and down the vertical surfaces whereas on the flat surfaces, including along the grooves, migration would be totally in the horizontal direction. However, such calculation is mathematically complex because the migration is radial. Therefore, a mathematical model approach was used to determine whether it was likely that the lesser (planar) distance of migration across the grooves reflected an equivalent total distance of migration when estimated using the actual contours of the microgrooved surface. The analysis of relative distances travelled in the most extreme cases of microgroove contours (ie 0 and 90°) indicated that the effect of microcontours was not significant. Based on the calculations of the relative distances presented in Table 5.3, no effect of incorporating the microgroove contours into the calculated distances travelled by migrating cells was found. The reasoning was that if the observed lesser rate of migration across the microgrooves (measured in the planar distance) resulted from equivalent total migration activity, which was directed partly up and down the vertical surfaces, then the following assumptions can be made:

* the migration distance on the microgrooved surface should be essentially the same (within experimental error) when calculated on the basis of the microgroove contours (although they would be expected to show a different planar distance of migration); *in particular, microgrooved surfaces that had precisely the same calculated distance across the contours of the microgrooves would be expected to show the same distance of migration (calculated across the contours). In two cases the calculated surface distances were the same for substrata with 1 or 5 μm deep microgrooves. For these examples (Table 5.3) it would be expected that migration on these paired surfaces would be equivalent.

Table 5.3

Dimensions of microfeatures on substrata used and calculated surface distance across microgrooves taking microgroove contours into consideration

Substratum	Microgroove depth (μm)	Microgroove width (μm)	Ridge width (μm)	Calculated surface distance across microgrooves (μm)
Microgrooved	1	1	1	*200
Microgrooved	1	2	2	**150
Microgrooved	1	5	5	120
Microgrooved	1	10	10	110
Microgrooved	5	1	1	500
Microgrooved	5	2	2	250
Microgrooved	5	5	5	*200
Microgrooved	5	10	10	**150
Smooth	0	0	0	100

Overall there was no concordance for the distances of migration on the different surfaces (when calculated with the vertical faces of the microgroove walls included). Furthermore, there was no correlation between surfaces with different groove dimensions but the same effective distance (Table 5.3) with respect to cell migration.

Taken together, these data indicated that the apparent decrease in migration across the grooves was not due to any increase in the effective distance resulting from the surface topography.

5.3.2.4 Alignment of actin microfilaments in migrating tissue

The arrangement of actin was examined at various levels within the migrating tissue, which generally comprised between two and four layers of cells. A comparison was made between the actin organisation in tissue that migrated over the flat-cast and the microgrooved surfaces, in order to determine the effect of microgrooves on the organisation of the actin filament bundles within the tissue. The actin filaments close to the superficial layers of the tissue on the flat-cast surface were arranged at the periphery of component cells {Figure 5.9(a)}. The actin filaments at the advancing edge of the tissue on the flat-cast surface extended radially in the direction of migration {Figure 5.9(b)}. The actin filament bundles close to the superficial surface of the tissue on the microgrooved surfaces were arranged at the periphery of the cells, as they were in tissue on the flat-cast surface {Figure 5.9(f)}. However, the actin filament bundles adjacent to the microgrooves were generally aligned in the groove direction {Figure 5.9(c), (d) and (e)}. The spacing between actin filament bundles appeared to be related to the groove and ridge width of the underlying substratum {see Figure 5.9 (c) and (d) in consideration of the underlying groove/ridge profile shown in the inset at the top right hand corner of each micrograph}. Thus, there was a very clear difference between the actin distribution in cells (within the tissue) that were adjacent to the microgrooves compared with the distribution in the more anterior, superficial cells {Figure 5.9(e) compared with (f)}.

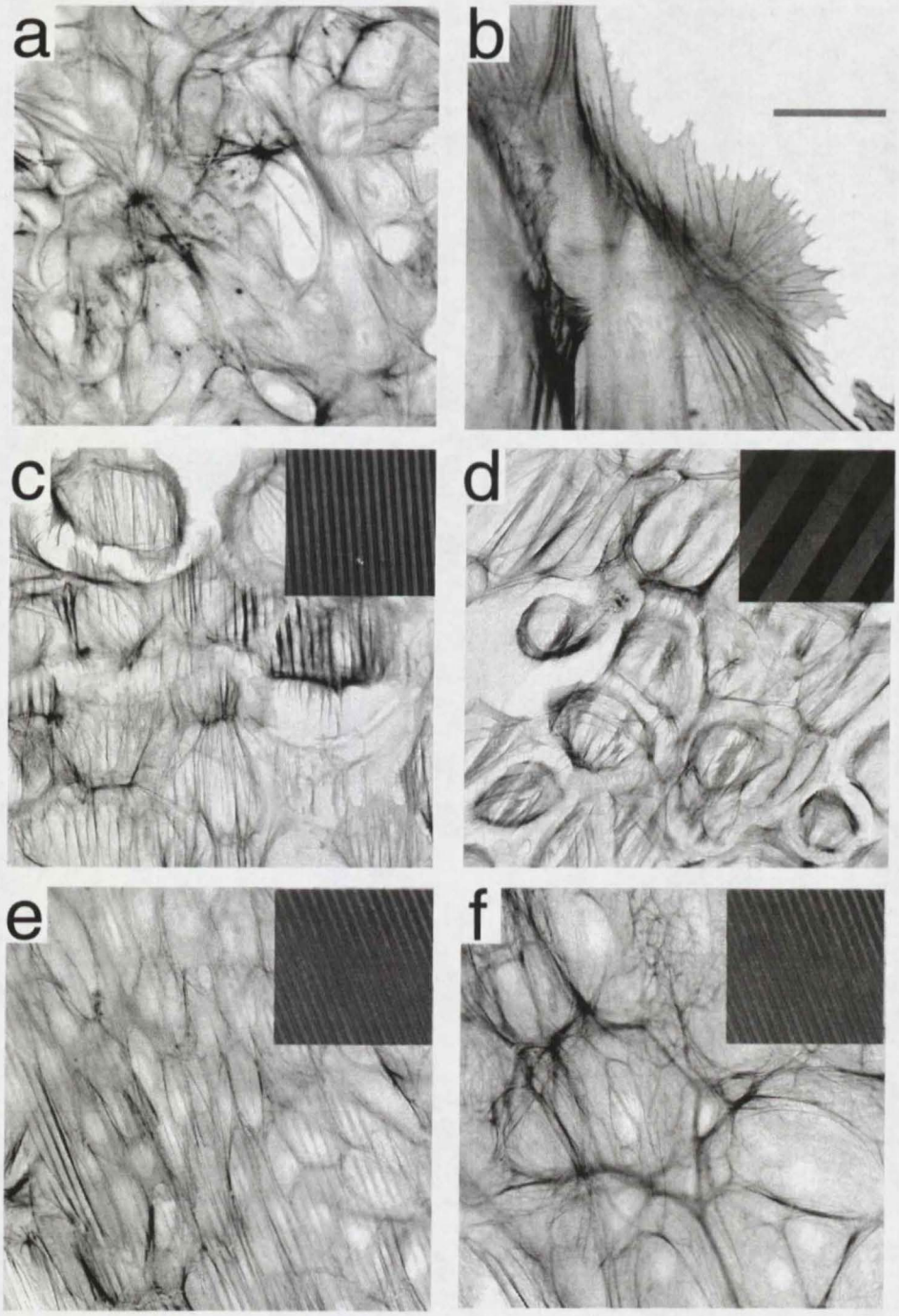


Figure 5.9 Filamentous actin in migrating epithelial tissue, stained with Texas Red phalloidin. The polystyrene surfaces were: (a and b) flat-cast; (c, e and f) 1µm deep/2µm wide; and (d) 1µm deep/5µm wide microgrooves. The alignment of the microgrooves under the tissue is shown in the inset, top right (c, d, e and f). Laser confocal images of the same field are shown in panels (e) and (f). The focus in (e) was of the actin on the lower surface of the basal cells and the focus in F was on actin in the tissue close to the superficial surface. The scale bar is 40 µm and all images are of the same magnification.

5.4 DISCUSSION

5.4.1 Porous surfaces

In the course of these experiments several effects of certain topographies were demonstrated on epithelial tissue and cell migration. The effect of pores on epithelial tissue and cell migration proved to be a complex phenomenon that was dependent upon pore diameter in a number of ways. Track-etched, columnar pores with diameters of 0.8 μm or greater were found to reduce the extent of epithelial tissue migration in comparison with smaller diameter pores. Corneal epithelial cell migration was reduced to a lesser extent by the presence of large pores than was migration of tissue. The migration of bovine corneal epithelial tissue has also been found to be reduced over membranes that contained trabecular pores, when porosity was increased (Fitton et al 1998). These results are consonant with the findings of Takeuchi who measured the migration of epithelial tissue from embryonic chick cornea (Takeuchi 1976). One explanation for the reduction in the extent of tissue migration in response to large diameter columnar pores is as follows. When the tissue encountered pores that had nominal diameters greater than 0.8 μm, the cytoskeletal structure was unable to sufficiently support the local tissue structure to move across the mouth of the pores. Because the tissue was an integrated structure, any distortion into the pores would inhibit forward movement. However, cells had the possibility of traversing the planar surface around the pores and so migration of cells was less sensitive to the topography than was that of tissue. Also, as was observed with the pores of 3 μm diameter (Fitton et al 1998), if a migrating cell descended into a pore this would not impede the progress of other cells. In contrast, if a cell or part of a cell within the tissue descended into a pore, then the forward movement of the tissue would be restricted due to cell-cell adhesion (Fitton et al 1998).

Surprisingly, a different effect occurred in response to smaller diameter pores. On surfaces that contain pores with diameters of 0.1 or 0.4 μm, tissue and cell migration were stimulated as compared with migration over a planar surface (Dalton et al 1999; Steele et al 2000). For tissue, this enhanced migration was apparent for over three weeks in culture {Figure 5.5(b)}, raising the question as to the influence of fluid through-flux on tissue and cell migration. In order to separate the effects of

topography and fluid-flux, the pores were blocked in a manner that did not affect the surface topography. The data in Figures 5.6A and and 5.6B showed that the enhanced tissue and cell migration in response to the small pores occurred as a direct result of the topography and not because of fluid-flux through the pores {see also Dalton et al 2001a). If the enhanced migration over the 0.1 and 0.4 μm pore surfaces had been due to fluid-flux then "blind ending" the pores would have negated the enhancement. This was clearly not the case, which showed that the enhanced migration was due to the topography of the surface.

One explanation for the enhanced migration over the small diameter pores could be extrapolated from a proposal of Curtis that cells respond to regions of discontinuity (Curtis and Wilkinson 1998). Curtis proposed that the reaction of cells towards a discontinuity progressed through the following sequence of events: (1) changes in adhesion, as the cell encountered a region of discontinuity; (2) cytoskeletal condensation in response to the formation of two or more adhesions; and (3) the development of asymmetrically contractile forces. The cell then elongates in an orientated way. The stretching may start the signal transduction that leads to tyrosine phosphorylation and related events (Curtis and Wilkinson 1998). In the case of porous surfaces, the edge of the pore channels could be perceived as the region of discontinuity and in support of this, the formation of adhesions at the mouth of small pores was observed using transmission electron microscopy {Figure 5.7(c)}. Another mechanism whereby small diameter pores could enhance tissue migration in comparison with migration over a planar surface was also proposed. If cytoplasmic extensions from basal cells in the migrating tissue partially decline into the mouth of the pore, then the wall of the pore may provide enhanced leverage for traction forces generated by motor proteins associated with the actin cytoskeleton (Steele et al 2000).

Pore diameter has another effect on epithelial tissue, in the enhancement of stratification. Epithelial tissue cultured on porous polycarbonate membranes with pores of 0.1 or 0.4 μm in diameter formed a superior stratified structure, as compared with epithelial tissue cultured on either porous membranes with greater pore diameters or on non-porous polycarbonate (Dalton et al 1999). The formation of stable adhesion complexes was also influenced by pore diameter. Continuous basement membrane and a regular pattern of hemidesmosomal plaque was only assembled at the interface

between epithelial tissue and the underlying substratum on the 0.1 μm pore surface (Evans et al 1999). Pores with diameters of 0.4 μm or greater precluded the continuity of this organisation (Evans et al 1999).

Overall, the greatest difference between epithelial tissue and cell migration over the surfaces containing pores was the ability of cells to migrate over a surface containing pores 3 μm in diameter, whereas tissue migration was completely inhibited. This, as discussed earlier, was a result of the integration between component cells within the tissue restricting the forward movement of the tissue when cytoplasm entered pores. In the case of enhanced migration in response to small diameter pores, tissue integration did not appear to be a factor involved, therefore there was similarity between tissue migration and cell migration.

5.4.2 Microgrooved surfaces

The non-random topography of parallel microgrooves had a different effect on tissue and cell migration from the topography created by the random distribution of pores. There was stimulation of migration *along* the microgrooves and inhibition of migration *across* the microgrooves. However, in comparison with migration over a flat surface, there was no stimulation of the overall area of migration as there was over the 0.1 and 0.4 μm diameter pores but rather the effect was of redirection of the tissue. It should be noted that the microgroove dimensions tested were an order of magnitude larger than the pore diameters considered.

The microgroove depth had a greater influence on migrating tissue and cells than did differences in the groove/ridge width. Similarly, other studies found cell orientation was more sensitive to groove depth than to ridge width. However, it should be noted that these studies were generally conducted using grooves with geometries different from the current study (Clark et al 1990; Clark et al 1991; Curtis and Clark 1990; Walboomers et al 1999b). Other studies identified ridge width as having a greater effect than groove depth on cell alignment. Therefore it appears that groove geometry, substratum and cell type may all be determinants in controlling the relative effects of depth and width (den Braber et al 1996; Wood 1988). Additionally, migration may be slightly more sensitive to groove depth than is the alignment of single cells. Epithelial cells were found to align better on surfaces with smaller

groove widths, but shallow grooves of 0.5 μm were found to be less effective at guiding migration than were deeper grooves (Brunette 1986).

Migration across the orientation of the microgrooves was reduced compared with that on a planar surface. One aspect considered in regard to migration *across* the microgrooves was that if tissue followed the surface contour across each microgroove, the distance travelled would be greater than the planar distance that was actually measured. However, mathematical modelling did not support the concept of tissue or cells following the contours of the grooves.

The response of dissociated epithelial cells towards the microgrooves was remarkably similar to the response of intact tissue, both in the extent of enhancement of migration along microgrooves and in reduction of migration across microgrooves. The similar response of intact tissue and cells towards microgrooves would not have been predicted from the findings concerning tissue and cell migration over pores greater than 0.8 μm or from another study (Brunette and Chehroudi 1999); but may be related to the groove dimensions selected for the current study. Clark and colleagues found that the MDCK epithelial cell line showed a slightly different response towards grooves, depending on whether the cells were isolated or part of a cell island (Clark et al 1990). On surfaces textured with ultrafine features (groove depths 100-400 nm, groove/ridge widths of 130 nm) individual MDCK epithelial cells showed some orientation, while cell islands were hardly affected by the grooves (Clark et al 1991). Brunette also examined the orientation of epithelial cell clusters in response to grooves and demonstrated some degree of orientation of epithelial cell clusters on grooved substrata (Brunette 1986). In this instance, the grooves were 'v'-shaped with depths of 0.5 or 22 μm and groove/ridge widths of between 2.6 and 110 μm. The smaller groove/ridge widths and the deeper grooves caused greater cell alignment (Brunette 1986). These previous studies generally considered the orientation of individual epithelial cells that had been seeded uniformly and sparsely onto the various grooved substrata. This is in contrast to the present study, in which tissue and cells migrated out over the substratum as a confluent sheet from a central island.

5.4.3 Hypotheses concerning cellular response to topography

Several hypotheses have been proposed to explain the reaction of tissue and cells towards surface topography. The first explanation was developed from findings that fibroblasts were very sensitive to curvature and became aligned on narrow glass fibres (Dunn and Heath 1976). It was proposed that the actin cables would be unable to assemble and exert traction if they were not straight. Therefore, a cell would be limited to spreading and movement lengthways on the glass fibre (Dunn and Heath 1976). After an extensive study of the response of fibroblasts to grooves and ridges of various widths, it appeared that the same hypothesis might be applied to grooved surfaces (Dunn and Brown 1986). Another hypothesis was that the elongated nature of focal adhesions would favour their lengthways formation on ridges. The attached actin filaments would then be oriented along the grooves (Ohara and Buck 1979). Several authors have found that focal adhesions can also be formed on the base and sides of grooves (Brunette 1986; Clark et al 1990; Dunn and Brown 1986).

In the present study, the similarity between the effect of parallel microgrooves on tissue and cell migration, suggests that the alignment of the cytoskeleton in response to groove geometry outweighs the effects of cellular integration within the tissue.

5.4.4 Implications for biomaterial surface design

The findings of the study concerning the response of corneal epithelial tissue and cells towards microgrooves led to several propositions for the design of biomaterials and certain implants. It is unlikely that microgrooves would adversely affect epithelial tissue phenotype. Deeper grooves would be preferable in situations where inhibition of epithelial migration in a specific direction was required. On the other hand, shallow grooves would enable epithelial tissue to migrate further in a specific direction. The explanations of the cellular response towards topographic features remain hypothetical to some extent. However, the results from the present study clearly demonstrate the capacity for microgrooves to guide and enhance epithelial tissue and cell migration along microgrooves, while inhibiting migration across the microgrooves. Optimisation of microgroove geometry may enable further modification of tissue or cell migration patterns.

In the case of the Artificial Cornea project, grooves would not be preferred as these could have adverse optical input. However, coverage of the implanted lenticule by epithelial tissue may be aided by the presence of small diameter pores or pits in the anterior surface of the lenticule. The research presented here predicted that pores 0.1 or 0.4 μm in diameter would enhance the rate of tissue migration. However, other studies have shown that pores 0.4 μm in diameter preclude the formation of a continuous basement membrane and adhesion complexes at the interface between the tissue and the underlying material (Evans et al 1999). Therefore, the recommendation for a surface compatible with eithelialisation would be for one that contained pores of 0.1 μm in diameter. Interestingly this topographic feature shows some similarity to the topography of the corneal epithelial basement membrane (Abrams et al 2000) as has been noted previously (Evans et al 1999; Steele et al 2000). The manufacture of such surfaces as hydrogel-like materials commonly used for contact lenses and the like, will be a significant technical challenge. Conversely, in biomaterial applications where epithelialisation needed to be discouraged, the judicious placement of surface microgrooves and/or pores with diameters of 2 μm or greater may be employed to impede or retard epithelialisation.

5.5 SUMMARY

The first question posed in this study concerning the effects of surface topography on epithelial tissue and cell migration was: what effect do columnar pores in a polymer surface have on the migration of adult corneal epithelial tissue, and is the effect the same for epithelial cells?

The migration of epithelial tissue was reduced when pore diameter increased above 0.4 μm and was completely inhibited by pores 3 μm in diameter. In contrast, the migration of epithelial cells was not impeded by pores with diameters of 0.8 or 1 μm. The cells were also able to migrate over surfaces that contained pores 3 μm in diameter, albeit at a reduced level as compared with migration over the smaller diameter pores.

A second question was: is there a difference between the extent of epithelial tissue migration over a porous surface and the extent of migration over a non-porous surface

of the same polymer? Do cells show the same response towards a porous surface as is shown by tissue?

The extent of tissue migration over surfaces that contained pores 0.1 or 0.4 μm in diameter was enhanced in comparison with migration over a non-porous surface. The same effect was observed when cell migration was considered.

In light of the enhancement of the extent of tissue and cell migration in response to the presence of small pores in a polymer surface, the next question posed was: was migration enhanced because of the porosity of the surface or was it a result of the topography that was created by the pores?

The enhanced extent of tissue and cell migration over surfaces that contained pores that were 0.1 or 0.4 μm in diameter, was found to be due to the topography of the surface. Tissue and cell migration over these surfaces was not reduced when fluid through-flux was prevented by rendering the pores "blind-ended".

The effect that the random topography resulting from pores in a polymer surface had on epithelial tissue and cell migration was dependent upon the diameter of the pores. Therefore when considering the tissue and cell response towards microgrooves, migration was measured over microgrooves of various dimensions. The questions posed were: what effect do parallel microgrooves have on the extent and the direction of tissue and cell migration? Which feature (groove depth or groove/ridge width) has the greater influence on migration? For each dimension of microgroove tested, tissue and cell migration both followed the same trend. Migration was enhanced *along* the microgrooves, while migration *across* the microgrooves was inhibited. Changing the depth of the microgrooves had a greater effect on migration than alteration of groove/ridge width.

These results are discussed further in Chapter 6, in the contex of their relevance to the design of an Artificial Cornea device.

5.6 CONCLUSIONS

These studies showed that there were both differences and similarities between the responses of migrating corneal epithelial tissue and cells towards surface topography. When the responses were governed by factors that controlled the alignment of cytoskeletal components, tissue and cells migration followed a similar pattern. In contrast, the random topography formed by pores of 1 μm or greater, inhibited tissue migration to a larger extent than cell migration. In these cases, the integrated nature of the tissue abrogated the greater flexibility inherent in the cells which otherwise enabled the navigation of such surfaces.

CHAPTER 6

CONCLUDING DISCUSSION

In this Chapter, the major findings of the thesis are summarised and a model of the mechanisms of bovine corneal epithelial tissue migration over a synthetic surface is presented. The proposed model is based on the investigations described in this thesis, together with information from the published literature. Additionally, the relevance of the studies to corneal epithelial tissue migration over the corneal onlay device being developed in the Artificial Cornea project is considered.

The aim of the research described in this thesis was to better understand the mechanisms that enable an intact stratified epithelial tissue to migrate over a synthetic surface. Towards this objective, the hypothesis that was tested was that there are differences between the mechanisms that operate when intact corneal epithelial tissue migrates over a synthetic surface and the mechanisms operational when individual cells migrate. This is important because for most biomaterial applications it is an intact tissue rather than individual cells that interact with the implant. While in some situations inhibition of epithelial tissue migration would be the desired outcome, in the Artificial Cornea project migration of host epithelial tissue over the anterior surface of the lenticule is mandatory for successful incorporation of the device into the cornea. The research strategy employed in this thesis to advance the knowledge of tissue migration used the fact that the migration of cells has been extensively studied; therefore, documented aspects of cell migration could be used as benchmarks against which to compare tissue migration. In some situations, aspects of cell migration had not previously been described in the literature. The ability of corneal epithelial cells to migrate over several synthetic polymer surfaces in the absence of exogenous cell adhesive molecules was a novel finding and acted as a basis for the model system that was used to compare tissue and cell migration. The experimental approach towards testing the hypothesis was to compare migrating tissue and cells for the following facets:

- The structure of migrating tissue was examined for evidence of persistence of cell-cell adhesion, in order to determine whether tissue migrated as an intact assemblage;

- The arrangements of actin filaments at the advancing edge of tissue and cells were examined to determine if cooperation between the leading edge cells was apparent;
- In order to test for differences between the functioning of cytoskeletal components within migrating tissue and cells, the effects of cytoskeletal-disruptive drugs upon actin filaments and microtubules were monitored using time-lapse video microscopy;
- The distribution of focal adhesions at the interface between the tissue and the underlying substratum was probed, in order to test the supposition that marginal cells provided the force to drag submarginal cells;
- In parallel with these studies, an investigation was undertaken into the effects of two classes of surface topography upon epithelial tissue and cell migration. The aim was to identify differences or similarities between the response of tissue and the response of cells towards specific topographic features and extrapolate that information to draw conclusions concerning tissue migration that are significant for the design characteristics of surface of biomaterials.

The first approach to testing the hypothesis was to determine whether component cells within the tissue remained attached to each other when migration occurred. This would suggest coordination between the tissue cells rather than independent movement. Several lines of evidence showed that they did remain attached. Firstly desmoplakin, which is a component of desmosomes that are the cell-cell adhesion complexes, was shown to persist during migration - even between adjacent cells at the advancing edge of migrating tissue. Secondly, desmosomes were observed in the membranes of adjacent cells throughout migrating tissue when transverse sections of tissue were examined. Thirdly, inter-digitations between component cells within the tissue sheet were seen. These inter-digitations were present between adjacent cells throughout the tissue, including between the basal cells that were in contact with the substratum and more anterior cells. They were also present between cells that were close to the leading edge. These findings suggested that the anterior cells were not separately participating in the migration, but were being transported by the basal cells that were in contact with the substratum. Considering the basal cells, active participation of both the marginal (cell at the leading edge) and submarginal (all cells that do not form part of the not leading edge) was supported by the presence of focal

adhesions (identified by immunostaining for vinculin) at the interface between the substratum and basal cell membranes throughout the tissue. The arrangement of focal adhesions in individual cells in culture is frequently demonstrated using vinculin detection (Ezzell et al 1997). However, this was the first demonstration of the distribution of focal adhesions at the interface between migrating multilayered tissue and the underlying polymer. It provided clear evidence to support the view that the submarginal basal cells, in addition to the marginal basal cells, contributed to tissue migration.

The tentative conclusion that both the marginal and submarginal basal cells are involved in tissue migration is different from the migration mechanism proposed by DiPasquale {DiPasquale 1975(a); DiPasquale 1975(b)}. Dipasquale concluded that, within a migrating tissue sheet, only the marginal cells actively participated in migration. However in the current study, the identification (by electron microscopy) of cellular protrusions by rear cells under the cells ahead supported the notion of active migration by submarginal basal cells and would not be expected if the marginal cells only were active. Mahan and Donaldson also reached this conclusion in their study of the migration of newt epithelial cells (Mahan and Donaldson 1986). Therefore, two findings from the current study have helped to clarify the controversy concerning the role of submarginal basal cells within a tissue migrating over a synthetic surface.

Aspects of cytoskeletal structure and function were compared between migrating tissue and cells, because the cytoskeleton is known to be implicated in cell migration. The first hypothesis tested was that there were differences between the organisation of actin and microtubules at the advancing edge of migrating tissue, in comparison with the organisation of these structures at the advancing edge of a migrating cell population. One major difference found was that in some concave regions of the advancing tissue there were actin filament cables that appeared to span several cells. This suggested that contraction of an actin cable, which previously has been demonstrated in healing foetal wounds, might be one mechanism available to advance an intact corneal epithelial tissue sheet over a synthetic surface. The operation of the "purse-string" phenomenon in epithelial tissue, migrating over a synthetic substratum *in vitro,* was a finding that illustrated a difference between the mechanisms of tissue

migration and cell migration. This was a novel discovery for tissue that was migrating radially outwards from a corneal explant and was also the first demonstration of the "purse string" mechanism in adult corneal epithelial tissue migrating over a synthetic surface. Tissue migration by means of contraction of an actin cable is suited to the closure of circular wounds. The centripetal migration of a host's epithelium over a lenticule implanted in the cornea would be conducive for the operation of the "purse string" mechanism. Coverage of a lenticule would have similarity to the healing of small, circular corneal wounds in mice that have been demonstrated to close by this mechanism (Danjo and Gipson 1998).

In addition to an examination of the organisation of the actin cytoskeleton, microtubule organisation was also considered. A major difference observed was that within the migrating tissue the microtubules between adjacent cells were extensively intermeshed, unlike the microtubules in a population of migrating cells. (The requirement for intact microtubules is discussed below). This is consonant with electron microscopic observations of the migrating tissue that showed extensive inter-digitation between the membranes of adjacent cells. It was also consistent with the view that there was "cooperation" between cells within the tissue and that migration was not simply a result of each cell behaving as a separate entity. Because of this, consideration of model systems based on the migration of intact tissue for evaluation of putative biomaterials would be preferable to the use of systems based on individual cells.

The second hypothesis concerning the cytoskeleton to be tested was: do cytoskeletal-disruptive drugs have the same effect on migrating tissue that they have on migrating cells? In one approach, migrating tissue and cells were exposed to the drugs and the effects on the distribution of actin filaments or microtubules were monitored over time using appropriate staining techniques. The study showed that actin filaments were rapidly disrupted by cytochalasin-B in both the tissue and the cells, whereas the microtubules in migrating tissue (particularly away from the leading edge) persisted longer after exposure to demecolcine than did microtubules within migrating cells. In a second approach, time-lapse video microscopy was used to measure rates of migration before and after drug exposure. Disruption of the actin filaments halted the migration of both the tissue and the cells. However, the morphology of the tissue was

maintained. This was in sharp contrast to the migrating cells, which retracted when the actin filaments were disrupted. Within the tissue, the adhesion between adjacent cells may have compensated for the loss of actin structure. When the microtubules were disrupted, there was a lesser effect of the drug on morphology in the tissue as compared with the cells (as was observed with the actin filaments). However, the time-lapse video microscopy showed that intact microtubules were necessary to sustain optimum rates of migration in both the tissue and the cells. This was a similar result to the finding of Liao and colleagues, who showed that the rate of cell migration was reduced even when the level of microtubule inhibitor was below that which caused complete microtubule breakdown (Liao et al 1995).

Prior to testing the hypothesis that there were differences between the migration mechanisms of tissue and cells, it was necessary to develop and test model systems that would enable intact epithelial tissue and individual corneal epithelial cells to migrate over synthetic surfaces. When the extent of migration of tissue and cells was compared over several ECM molecules that were pre-coated onto TCPS surfaces, collagen I and Fn enhanced tissue and cell migration as compared with migration over uncoated TCPS. However, a coating of laminin or collagen IV did not increase the extent of migration in comparison with the uncoated surface. These results suggested that ECM components that are encountered by corneal epithelium in wound healing situation *in vivo* might provide a stimulus for migration that is not provided by ECM components encountered in conditions of homeostasis. In addition, it was unexpectedly found that both the epithelial tissue and the cells were able to migrate over uncoated surfaces in the absence of exogenous ECM components. The migration of bovine corneal epithelial tissue from a corneal explant in serum-free culture has proved to be a versatile model. It has been used to examine the response of epithelial tissue towards specific substrata in terms of stratification and formation of stable adhesion (Dalton et al 1999; Evans et al 1999). Results from those studies showed that the optimum surface to support epithelial stratification and the formation of a continuous basement membrane and adhesion complexes would be porous and contain pores that were less than 0.2 μm in diameter. This was relevant for the design of the lenticule for the Artificial Cornea project as discussed in Chapter 5.

Migration over synthetic surfaces in the absence of exogenous cell adhesive molecules may have involved the secretion of molecules that can adhere to the substratum and provide adhesive sites for extending lamellipodia of filopodia. Corneal epithelial cells are known to secrete several ECM molecules that could potentially fill this role (Dodson and Hay 1971; Evans and Steele 1997; Qin and Kurpakus 1998). Also, Panjwani showed that secretion of neutral glycolipids was up-regulated in migrating epithelial tissue and cells as compared with stationary tissue and cells (Panjwani et al 1990), suggesting a role for these molecules in migration. Bovine epithelial tissue that migrated from a corneal explant (as used in the model system) secreted laminin and collagen VII and assemble a basement membrane-like structure when cultured for 21 days on a suitable substratum (Dalton et al 1999). This study showed that the tissue that migrated over a synthetic surface *in vitro* had the capacity to perform some of the functions executed by corneal epithelial tissue *in vivo*. These data suggested that it would be possible that an uncoated lenticule would become epithelialised *in vivo*. However in the feline model, surface modification of the anterior surface of the lenticule in the form of ECM based molecules was necessary to achieve rapid coverage. This may have been due to the more demanding *in vivo* environment that included eye lid-induced friction. Alternatively, feline epithelial tissue may have more stringent migration requirements than bovine epithelium.

In addition to comparisons between corneal epithelial tissue and cell migration over surfaces that were planar in nature, the use of surfaces that had defined topographic features showed both differences and similarities between the responses of tissue and cells. Hypotheses aimed at explaining the reason for the different responses can assist with development of models of mechanisms that operate during tissue migration. The finding that epithelial tissue, unlike cells, was unable to migrate over surfaces that contained pores of 2 μm or greater in diameter suggested integrity of cell-cell adhesion within the tissue. If the component cells within the tissue had been able to separate from each other when encountering pores, one would have expected migration to be only reduced and not to be fully blocked. However, tissue migration was practically halted so it appeared that the integrity of a migrating epithelium persisted even when obstacles were encountered that inhibited forward movement.

On the other hand, in the situations where both tissue and cells showed similar responses towards topography, one can assume that factors controlling the response are common to both tissue and cell migration. There was stimulation of the migration of both tissue and cells on surfaces containing small diameter pores in comparison with migration over a planar surface. Additionally, the same trends were followed for tissue and cell migration in response to microgrooves. A hypothesis that was proposed and developed by Curtis and Clark (Clark et al 1991; Curtis and Clark 1990) to explain the response of cells towards surface topography may be extrapolated to explain some aspects of the enhanced tissue migration. It was proposed that cells react to regions of discontinuity in a surface by forming focal adhesions, which act as nucleation points for actin filaments (Curtis and Clark 1990). This part of the hypothesis was supported by Wojciak-Stothard et al who found that vinculin- and actin-rich sites formed along groove-ridge boundaries in the first 10 min of cell attachment (Wojciak-Stothard et al 1995). This was accompanied by phosphorylation of tyrosine colocalised with the F-actin and vinculin. It is possible that stretch receptors are implicated in the signal transduction that leads to tyrosine phosphorylation and subsequent cellular responses (Wojciak-Stothard et al 1996) including migration. In the current studies, regions of discontinuity could be the edge of pore channels or the groove/ridge edges and it is possible that the response from the epithelial cells and the basal cells within the tissue were being influenced in this way.

From these studies we can start to develop a model concerning the mechanism of migration of an intact corneal epithelial tissue over synthetic surfaces. Firstly, ECM molecules secreted by the tissue adhere to the substratum. Some basal, marginal cells at the advancing edge of the tissue extend lamellipodia that contact these endogenous ECM molecules and form focal adhesions. Integrins are likely to be involved in this interaction. Actin filaments are present in the advancing lamellipodia, and filament bundles are also present within the cell body. Substratum contacts at the rear of these marginal cells are released, possibly aided by the insertion of lamellipodia from following cells under the leading cells and by contraction of the cell. In parts of the advancing tissue front, some cells are "overtaken by" the more actively moving cells. These regions are dragged forward by contraction of an actin filament cable that forms close to the leading edge of these cells. Adhesion between component cells within the tissue is maintained by the inter-digitation of the peripheral cell membranes and by

the presence of desmosomes linking some parts of these membranes. A study by Danjo and Gipson also demonstrated that another cell-cell adhesive molecule, E-cadherin, was associated with the adherence junctions between adjacent marginal cells in migrating corneal epithelium *in vivo* (Danjo and Gipson 1998). It is likely that E-cadherin performs a similar functional role in epithelial tissue *in vitro*. Clearly, because of the adhesion between cells within the migrating tissue, forward movement by marginal cells would exert a force on submarginal cells. The most probable occurrence is that submarginal basal cells extend cytoplasm in the direction of migration, in some cases by underlapping cells further ahead. If the basal cells were not adherent to the substratum, the force generated by the marginal cell may have been sufficient to drag along the submarginal cells, as suggested by DiPasquale (DiPasquale 1975(a); DiPasquale 1975(b)). However, the presence of focal adhesions throughout the basal cell membranes adjacent to the substratum precludes this premise. The extension of cytoplasm by marginal and submarginal basal cells would be accompanied by formation of new focal adhesions at the extremities of the extended cytoplasm and release of focal adhesions at the rear of the cell, as occurs in the migration of individual cells. Spatial and temporal control and coordination of the formation and release of focal adhesions throughout the basal cells within the tissue would be essential. The anterior cells remain attached to basal cells by inter-digitating membranes, by desmosomes and probably also by cadherins. The anterior surface of the migrating tissue sheet appeared to be actively participating in migration when observed by time-lapse video microscopy. However, since actin filaments were only visible at the periphery of these cells and the cells were attached to neighbouring cells on three sides, the anterior cells were probably totally dependent upon the basal cells for their translocation.

Turning now to cell migration: some of the following proposed events concerning the mechanism of corneal epithelial cell migration are extrapolated from migration studies, documented in the literature, that were conducted using various cell types. The epithelial cells, like the tissue, were able to migrate in the absence of exogenous ECM molecules; therefore it is likely that secretion of cell adhesive molecules also takes place during cell migration. In the model used in this study where the cells had been seeded as a confluent culture, migration was only able to proceed radially out from the original culture and therefore extension of lamellipodia of filopodia occurred

on the "free side" of the cells. The continuous growth of actin filaments towards the leading edge of the lamellipodia or filopodia is the mechanism by which this proceeds (Lauffenburger and Horwitz 1996). Close contacts and focal adhesions form at the periphery and other regions of the extending lamellipodia and actin filament bundles develop parallel to the direction of migration. It is possible that lamellipodial extension, adhesion and actin filament bundle formation are under the control of the small GTPases Rho and Rac (Nobes and Hall 1999; Waterman-Storer and Salmon 1999). Contraction of actin occurs, with the force generated being dependent upon myosin. Adhesions at the rear of the cell are released by a mechanism that appears to be dependent upon microtubules (Ballestrem et al 2000). The cytoplasm at the rear of the cell then moves forward. The receptors that participated in adhesion are recycled to the front of the cell by endocytosis and vesicular transport and/or by forward-directed movement on the cell surface (Sheetz et al 1999), and the cycle can recommence.

The basic sequence of mechanistic events proposed to occur during epithelial cell migration would also be part of tissue migration as related to the basal cells. The operation during migration of cognate cytoskeletal mechanisms in the basal cells of the tissue, and in individual epithelial cells, was supported by similarity of the responses that tissue and cells showed towards surface microgrooves (Chapter 5 and Dalton et al 2001b). Aside from the similarities in mechanisms as apply during migration of individual cells, additional complexity arises in tissue migration. The coordinated contraction of actin filament bundles within adjacent cells at concave regions of migrating tissue is an example of a mechanism that is specific to tissue, as is the ability of the basal cells within the tissue to transport anterior cells notwithstanding the persistence of lateral attachments on all sides. The cell-cell adhesion within tissue involves the greater part of the peripheral membrane, thereby certain restrictions are imposed on tissue migration that do not apply to cell migration. The inability of tissue to traverse surfaces with topographic features that are navigable by cells (Chapter 5 and Fitton et al 1998) illustrates a consequence of the restrictions.

Corneal epithelial tissue migration has greater complexity than cell migration, due to the persistence of adhesion between the component cells. As research continues to elucidate the molecular events that control and coordinate the spatially separated

sequence of steps that enable an individual cell to migrate, future studies could be directed towards explaining how these events apply when tissue migrates. For example, what mechanism is operational for recycling of receptors that are released at the rear of a tissue cell, given that the greater part of the cellular membrane is occupied by cell-cell adhesion? Is there a mechanism that integrates or coordinates the responses of cells within a tissue? The model systems and methodologies employed in the research described in this thesis, may facilitate answering these questions.

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APPENDIX A

SOLUTIONS AND METHODS

All chemicals were purchased from Ajax Chemicals (Auburn, NSW), unless otherwise specified.

A1: Phosphate buffered saline (PBS) (10x stock)

To 800 ml distilled water add, 80g NaCl
2g KCl
2g KH_2PO_4
11.5g Na_2HPO_4

Adjust pH to 7.2, make to volume of 1 litre

Filter using 0.2 μm filter.

For use dilute stock 1:10 with distilled water.

A2: Formol saline

10 ml formaldehyde solution (AnalaR BDH from Merck, Australia)

90 ml PBS.

A3: Depletion of Fn and Vn from FBS

This protocol was originally developed by Dr PA Underwood (Underwood et al 1989).

Reagents used

High salt solution

1.21g Tris

5.8g NaCl

150 ml H_2O

Adjust pH to 7.5 with HCl

Bring final volume to 200 ml with distilled H_2O

Store after filtration through a 0.22 μm filter.

3M $MgCl_2$ solution

40 ml made immediately before use

24.36g dry $MgCl_2$

Make up the volume to 40 ml with distilled water

Adjust pH to 7.5.

pH 8.5 buffer

12.1g Tris

29g NaCl

800 ml H_2O

10 ml of 8% (w/v) sodium azide (AnalaR BDH, UK)

Make up to 1 litre with distilled H_2O.

pH 4 buffer

8.5g NaCl

900 ml distilled water

Adjust pH to 4 with acetic acid

Make up to 1 litre with distilled water

Store at 4°C.

PBS containing azide

10 ml 8% (w/v) sodium azide

990 ml PBS.

4M urea

Prepared freshly for each use

120g urea

Make up volume to 500 ml with distilled water.

Method used

A column containing approximately 200 ml of gelatine sepharose (Pharmacia, Sweden) and a column containing anti-Vn monoclonal antibody (MAb62 CSIRO) coupled to Protein-A Sepharose (Pharmacia) were consecutively connected with plastic tubing leading to a pump system (Pharmacia). The columns were washed with 60 ml of serum free culture medium at 0.5 ml per min pump speed. Intact FBS (200

ml) was loaded at 0.1 ml per minute and followed by 50 ml of serum free medium (approx 17 hr). A fraction collector (LKB Bromma 7000 Ultrorac) was used to collect 10 ml fractions into centrifuge tubes.

Maintenance of the columns

Vn affinity column

After collection of the serum samples the two columns were disconnected. The Vn affinity column was washed by running 36 ml of High salt solution through the column at 10 ml/min.

25 ml of freshly made 3M $MgCl_2$ solution was run onto the column and left in contact with the gel overnight.

The column was washed with 30 ml of High salt solution and the 3M $MgCl_2$ eluate was collected (this contained the Vn).

The column washed with 50 ml of pH 8.6 buffer then 50 ml of pH 4 buffer and stored in PBS+azide.

Gelatine sepharose column

Fn was eluted using freshly made 4M urea after the depletion of 600 ml of serum. The column was stored in PBS +azide.

ELISA to check that the serum was completely depleted of Fn and Vn

Solutions for ELISA

Substrate buffer 10x stock

0.2 M Citric acid

0.2 M Sodium citrate

Mix to give pH 4-4.2

Store at 4°C

Substrate

2, 2-Azino-bis(3-ethylbenz-thiazoline-6sulfonic acid (ABTS) from Sigma Chemical Co

1 mg/ml of ABTS in substrate buffer + 2 μl H_2O_2 for each five ml. Mixed immediately before use.

ELISA Method

Duplicate 50 μl aliquots of serum samples, each diluted 1:20 in PBS, were loaded into 96-well cluster trays. The trays were: (1) TCPS (Corning); (2) Primaria™ (Falcon, Becton Dickinson); and (3) Microtiter 2000 tray (Dynatec Laboratories, Inc. Virginia, USA). The samples and controls, which included intact FBS and eluate (diluted 1:100) from the 3M $MgCl_2$ that was used to remove Vn from the affinity column, were incubated in the trays for 48 hr at 4°C. This enabled any Fn or Vn present in the serum to adsorb onto the substratum. All further steps were carried out at room temperature. After the samples were removed from the wells, non-specific binding sites were blocked by incubation with 1%(w/v) BSA in PBS for 1 hr. Primary antibodies were diluted to 2 μg/ml in PBS/BSA and 50 μl was incubated in each well for 90 min. The anti-Vn antibody MAb62 (CSIRO) was added to the TCPS tray and anti-Fn monoclonal antibody MAb35 (CSIRO) was added to the Primaria™ tray. No primary antibody was added to the microtiter tray because this tray was used as a precaution to detect any loss of antibody from the affinity column. The wells were washed 4x with PBS and 50 μl of the secondary antibody, Peroxidase Conjugated Rabbit Anti-Mouse (Dako) diluted 1:1000 in PBS was added to all wells. After 90 min incubation, the wells were washed 4x with PBS and 100 μl of freshly made ABTS substrate was added to each well. When colour development was observed in the wells containing the FBS samples (approx 10 min) the absorbance was read in a plate reader using a wavelength of 405 nm and a reference wavelength of 495 nm. Serum samples that had no detectable Fn or Vn were pooled, filtered and stored at - 20°C. TCPS trays were used for Vn detection and Primaria™ trays for Fn detection because each shows optimum adsorption of these molecules from serum (Steele et al 1993; Steele et al 1995).

A4: Tissue Processor programme for light microscopy of tissue and cells

50% ethanol	1 hr
70% ethanol	1 hr
95% ethanol	1 hr
95% ethanol	1 hr
100% ethanol	1 hr
100% ethanol	1 hr

100% ethanol	1 hr
Histochoice (clearing agent) *	1 hr
Histochoice	1 hr
Paraffin wax#	1.5 hr
Paraffin wax	1.5 hr
Paraffin wax	1.5 hr

*Histochoice (Amresco^{R,} Solon, Ohio, USA)

#Paraffin wax (Protoplast, Oxford Labware, St Louis, USA).

A5: Haematoxylin/Eosin staining schedule for paraffin sections

xylene	5 min
xylene	5 min
100% ethanol	30 sec
100% ethanol	30 sec
70% (v/v) ethanol	30 sec
tap water	rinse
Haematoxylin**	4 min
acid alcohol##	3 dips
tap water	rinse
blue	1 min
Eosin***	1 min
70% ethanol	rinse
70% ethanol	30 sec
100% ethanol	rinse
100% ethanol	30 sec
100% ethanol	30 sec
xylene	2 min
xylene	2 min

**Instant Haematoxylin from Shandon, Pittsburgh, PA

##1% (v/v) HCl in 70% ethanol

*** Gurrs, Poole, UK (1% (w/v) in 70% ethanol)

A6: Reynold's lead citrate

1.33g lead nitrate (BDH Chemicals, Poole, UK)

1.76g tri-sodium citrate

30 ml boiled deionised water

Mix vigorously over 30 min

Add 6 ml of 1N NaOH and make up to 50 ml with boiled deionised water

Check that the pH is between 11.9 and 12.1

Store at 4 °C

Filter using a 0.2 μm filter before use.

APPENDIX B

Dalton BA, Steele JG (2001): Migration mechanisms: corneal epithelial tissue and dissociated cells. *Exp Eye Res* 73:797-814.

Fitton HJ, Dalton BA, Beumer G, Johnson G, Griesser HJ, Steele JG (1998): Surface topography can interfere with epithelial migration. *J Biomed Mater Res* 42:245-257.

Dalton BA, Evans MDM, McFarland GA, Steele JG (1999): Modulation of corneal epithelial stratification by polymer surface topography. *J Biomed Mater Res* 45:384-394.

Dalton BA, McFarland GA, Steele JG (2001a): Stimulation of epithelial tissue migration by certain porous topographies is independent of fluid flux. *J Biomed Mater Res* 56:83-92.

Dalton BA, Walboomers XF, Dziegielewski M, Evans MDM, Taylor S, Jansen JA, Steele JG (2001b): Modulation of epithelial tissue and cell migration by microgrooves. *J Biomed Mater Res* 56:195-207.

The following articles have been removed from the digital copy of this thesis. Please see the print copy of the thesis for a complete manuscript.

Title: Migration Mechanisms: Corneal Epithelial Tissue and Dissociated Cells
Authors: B. A. Dalton and J. G. Steele
Journal: Experimental Eye Research

Title: Surface topography can interfere with epithelial tissue migration
Authors: J. H. Fitton, B. A. Dalton, G. Beumer, G. Johnson, H. J. Griesser, J. G. Steele.
Journal: Journal of Biomedical Materials Research

Title: Modulation of corneal epithelial stratification by polymer surface topography
Authors: B. A. Dalton, M. D. M. Evans, G. A. McFarland, J. G. Steele
Journal: Journal of Biomedical Materials Research

Title: Stimulation of epithelial tissue migration by certain porous topographies is independent of fluid flux
Authors: B. Ann Dalton, Gail A. McFarland, John G. Steele
Journal: Journal of Biomedical Materials Research

Title: Modulation of epithelial tissue and cell migration
by microgrooves
Authors: B. Ann Dalton, X. Frank Walboomers, Mark Dziegielewski, Margaret D. M. Evans, Sarah Taylor,
John A. Jansen, John G. Steele
Journal: Journal of Biomedical Materials Research

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