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Erik Suuronen

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DEDICATION

I would like to dedicate this work to my wife, Angela and to my daughter, Ava.

Thank you for everything you have given me. I have been blessed with two angels.

Co-author Contributions to Manuscripts

Carlsson, David

Dr. Carlsson is a polymer chemist at NRC-ICPET and was the project leader for the chemistry and responsible, in part, for the development of the biosynthetic polymer matrices for implantation into pigs.

Griffith, May

Dr. Griffith was my supervisor and project leader for all three manuscripts. She was involved in the overall design and strategy for all the work performed.

Kobuch, Karin

Dr. Kobuch, who works in Dr. C. Lohmann's laboratory in Germany, was responsible for some of the in vivo confocal imaging and for some of the immunohistochemistry performed on the implants after removal from the host pigs.

Li, Fengfu

Dr. Li was a post-doctoral fellow in the laboratory who designed and synthesized the synthetic terpolymer and fabricated the bio-synthetic corneal implants that were transplanted into pigs.

Lohmann, Chris P.

Dr. Lohmann is an ophthalmic surgeon and researcher from Germany, and was the project leader for the surgical implantation of the biosynthetic polymer matrices.

Müller, Linda J.

From the Netherlands, Dr. Muller, specializes in electron microscopical study of the cornea and corneal nerves. She was responsible for providing the majority of the electron microscopic imaging of the structures within the tissue-engineered (TE) cornea.

Munger, Rejean

Dr. Munger, an optical physicist at the Univ. of Ottawa Eye Inst., helped me with the confocal microscopy for the visualization of structures in the TE cornea, performed the optical characterization of the bio-synthetic cornea implants and assisted with the statistical analysis of results.

Nakamura, Masatsugu

Dr. Nakamura, now General Manager of Cornea R&D, Santen Pharmaceuticals Inc., performed the immunoassays for Substance P. He also contributed suggestions of safety and efficacy tests with the in vitro model, as well as clinical testing of implants in animals.

Sheardown, Heather

Dr. Sheardown, Dept. of Chemical Engineering, McMaster University, performed the permeability assays and 125-iodine labeling for determination of YIGSR grafting in the development of the biosynthetic polymer matrices for implantation purposes.

Shinozaki, Naoshi

Dr. Shinozaki, Director, Ichikawa General Hospital Cornea Centre, Chiba, Japan, provided some electron microscopic images of the wounded TE corneas.

Stys, Peter K.

Dr. Stys is a neuroscience professor at the Univ. of Ottawa and a member of my supervisory committee. Dr. Stys performed the electrophysiological experiments on the TE corneal nerves and provided guidance for the use of his 2-photon confocal microscope. He is also the developer of the ImageTrak Software used for some of the data analysis.

Vascotto, Sandy

Dr. Vascotto is a post-doctoral fellow in the laboratory who performed some of the immunohistochemical work and analysis of the implants after removal from the host pigs.

Watsky, Mitchell A.

Dr. Watsky is a cornea physiologist. He provided guidance for the analysis of the in vitro TE cornea data and helped with preparation of the FASEB Journal manuscripts.

Abstract

A sensory nerve supply is crucial for optimal function of the cornea. However, the mechanisms for successful innervation and the signalling pathways between nerves and their target tissue are not fully understood. Engineered tissue substitutes can provide controllable environments in which to study tissue innervation. I have therefore engineered human corneal substitutes that promote nerve in-growth in a pattern similar to *in vivo* re-innervation. The methodology developed for the fabrication of such an innervated model cornea and for subsequent investigation of the function of these nerves is discussed in this thesis. Briefly, nerve in-growth into the tissue-engineered cornea is enhanced by the addition of laminin and nerve growth factor, but not retinoic acid. I demonstrated that these nerves are morphologically equivalent to natural corneal nerves and make appropriate contact with their target cells, which consequently, were found to be required for their survival. The nerves had functional sodium channels and generated action potentials similar to those of native nerve endings. I also demonstrated that the nerves could respond appropriately to chemical and physical stimuli and play an important role in the overall functioning of the bioengineered tissue. The presence of nerves conferred some protection to the epithelium from chemical insult and differential retention of sodium was observed within the nerve fibres themselves. As such, this model could be further developed for use as an *in vitro* alternative to animals for safety and efficacy testing of chemicals and drugs. Based on the concepts developed for these *in vitro* innervated corneas, hybrid biosynthetic matrices with the proper dimensions, transparency and biomechanical properties for use as corneal replacements in transplantation were also developed. These matrices were successfully implanted into corneas of pigs. Regeneration of corneal tissue and nerves was observed, along with restoration of sensory

function. The basic model developed therefore can be used for studying corneal wound healing, nerve-corneal cell interactions and provides a basis for developing corneal replacements for transplantation.

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List of Abbreviations

3-D – three-dimensional

aCSF – artificial cerebral spinal fluid

AM – acetoxymethyl

AP – action potential

BrdU – bromodeoxyuridine

CGRP – calcitonin gene-related peptide

CNS – central nervous system

CS – chondroitin-6-sulfate

DMSO – dimethyl sulfoxide

DRG – dorsal root ganglion

E8.0 – embryonic day eight

ECM – extracellular matrix

EGF – epidermal growth factor

H & E – haematoxylin and eosin

IGF-1 – insulin-like growth factor-1

LASIK – laser-assisted in situ keratomileusis

LKP – lamellar keratoplasty

Na^+ – sodium

NGF – nerve growth factor

NK1 – neurokinin-1

NTIs – nerve terminal impulses

PBS – phosphate-buffered solution

PFA – paraformaldehyde

RA – retinoic acid

RT – room temperature

SBFI – sodium-binding benzofuran-isophthalate

SE – standard error

SP – substance P

TBS – Tris-buffered saline

TCT – TBS containing carrageenan and Triton-X 100

TE – tissue-engineered

TERP - poly(*N*-isopropylacrylamide-coacrylic acid-coacryloxysuccinimide)

TERP5 – TERP/YIGSR copolymer

TTX – tetrodotoxin

v/v – volume to volume ratio

w/v – weight to volume ratio

w/w – weight to weight ratio

YIGSR – laminin pentapeptide

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CHAPTER 1

INTRODUCTION

1.1 The Cornea

1.1.1 Functional Overview

Over 80 % of the information from the external world processed by the brain is obtained by means of visual function, probably making vision the most important of all the senses (Nishida, 1997). Several structures within the eye are responsible for this phenomenon. The cornea is the most anterior surface and performs the majority of the refraction (bending and focusing) of light that enters the eye (Nishida, 1997). This coarse, non-variable focus is then fine-tuned by the lens. The fine-focused light then reaches the retina, the receptor system, which converts this information into a neural signal. The signal is carried along the optic nerve to the visual cortex of the brain for processing. Essential to the cornea's refractive function is the maintenance of its shape and transparency. The unique fibrous collagen infrastructure of the interior stroma ensures clarity and provides the mechanical strength required to maintain corneal shape (Freegard, 1997). Small changes in shape or smoothness of the surface can result in refractive error and cause visual distortion.

In addition to its role as a refractive interface, the cornea also serves as a protective barrier from the external environment. Although only about 1mm thick, the cornea is very tough due to the collagen scaffold of the interior stroma. This is essential for the protection of the delicate internal structures of the eye. The integrity of the ocular surface must be maintained for the defence against microbial, inflammatory and physical assault (Bron and Seal, 1986), without compromising the transparency of the cornea. In addition, the cornea has a role as part of the biodefence system of the eye. The corneal epithelium is equipped

with a relatively thick, insoluble mucous blanket through which microorganisms must pass. This constantly renewing, highly hydrated gel is resistant to enzymatic degradation and is supplied with numerous anti-inflammatory and anti-microbial factors (Sack et al., 2001). There is a need to understand the properties and underlying mechanisms responsible for the maintenance and healing of the cornea because of its critical function in both vision and the protection of the eye from physical injury and pathogenic invasion.

1.1.2 Anatomy and Physiology

The outer surface of the eye is comprised of three regions: the central cornea, a transitional zone called the limbus, and the surrounding conjunctiva (Fig. 1). The cornea is also surrounded by the sclera, which sits below the conjunctival layer. The epithelium of the

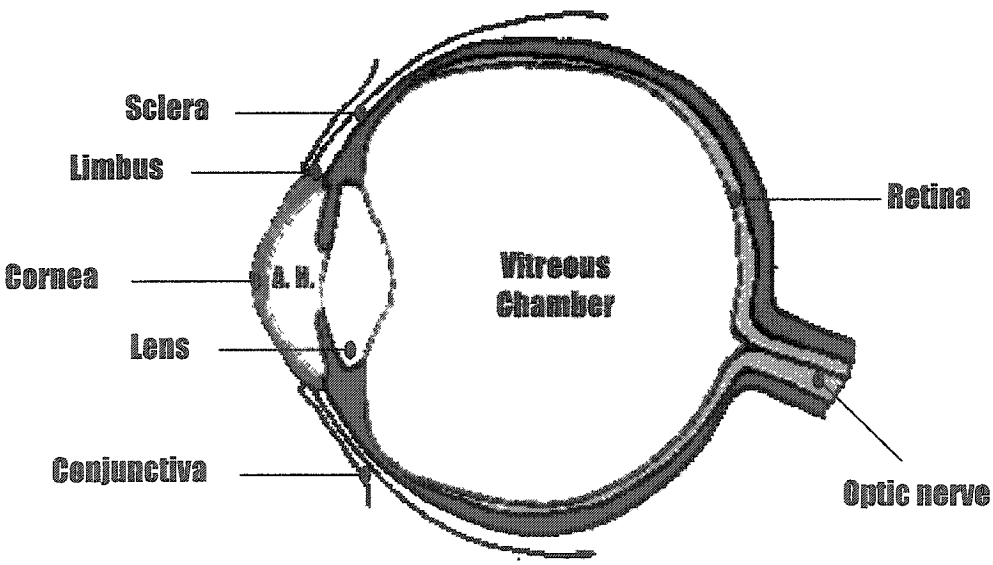

Fig. 1. Schematic of the eye in longitudinal section. Aqueous Humor (A.H.).

outer surface is continuous, although while the cornea is transparent, the surrounding sclera and conjunctiva are opaque. The cornea is avascular but the adjacent limbus is highly vascularized. It is believed that the intense vascularization of the limbus serves to nourish the

supply of stem cells that reside in the basal limbal region and repopulate the constantly renewing corneal epithelium (Dua and Azuara-Blanco, 2000). The conjunctival epithelium is intermixed with goblet cells, Langerhans cells and possesses blood vessels and lymphatic channels (Niederkorn, 1990; Rocha et al., 1998), not found in the cornea.

The corneal anatomy is relatively simple, consisting of three cell types within three main cellular layers. These are the epithelial cells of the anterior epithelium, the keratocytes of the central stroma, and the endothelial cells of the inner endothelium, which are separated by two interfaces (Fig. 2). The organization of the cornea therefore is as follows: epithelium, basement membrane, Bowman's layer, stroma, Descemet's membrane, and endothelium. In

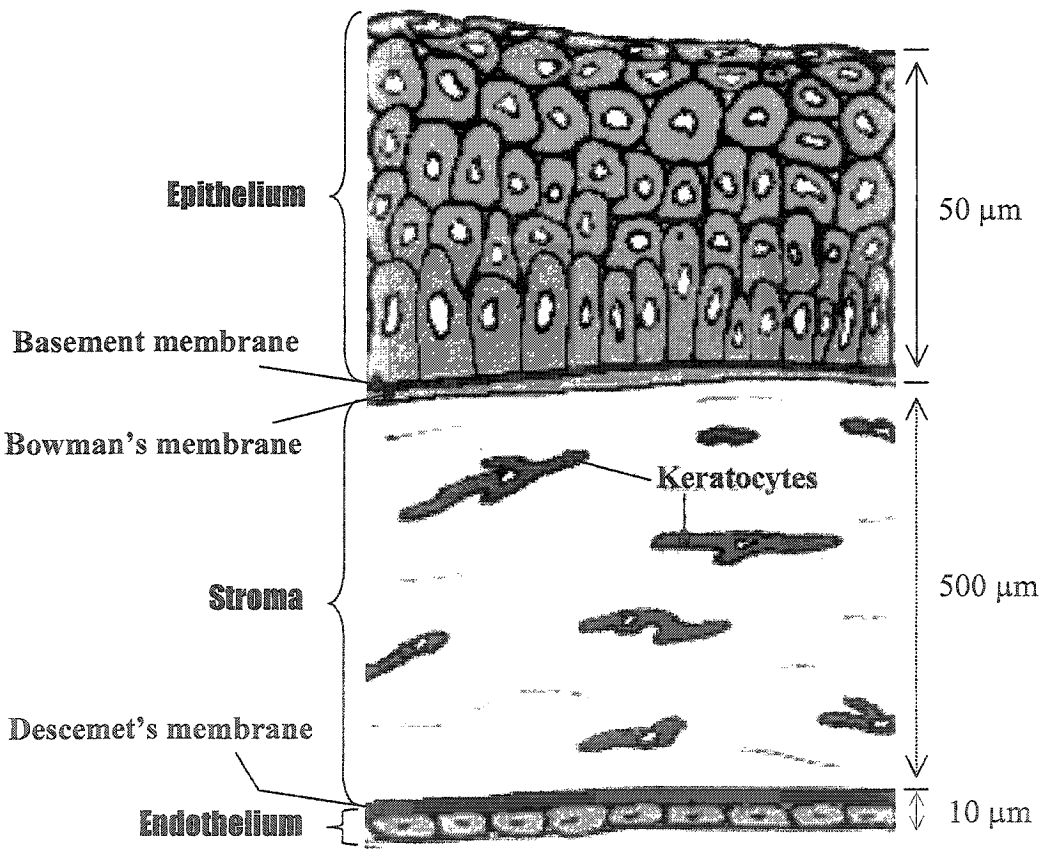

Fig. 2. Diagram of the layers of the cornea (not to scale)

addition, a tear film covers the epithelium of the cornea. This protects the tissue from dehydration, maintains the smooth epithelial surface and serves as a nutritional source to the epithelium. It contains several biologically active substances including interleukins and growth factors (Ohashi et al., 1989; Van Setten and Schultz, 1994). Therefore, the tear film also serves as a source of regulatory factors for the maintenance and repair of the epithelium, and some of these factors have been shown to modulate epithelial cell migration, proliferation, and differentiation (Nishida et al., 1990; Tsutsumi et al., 1988).

The corneal epithelium is non-keratinizing and stratified. It consists of a single layer of columnar basal cells, 2-3 layers of wing cells, and 2-3 layers of flattened superficial cells. The superficial cells are extremely flat and their surface is covered by microvilli, which allow the active exchange of oxygen and nutrients between the epithelium and the tear fluid. The epithelium is in a state of dynamic equilibrium. The terminally differentiated, superficial epithelial layers are constantly shed into the tear fluid (by desquamation), aided by eyelid blinking (Ren and Wilson, 1996). Proliferating cells from the basal layer migrate into the suprabasal and superficial layers and differentiate to replace these lost cells. Only the basal epithelial layer has proliferative potential and it is repopulated by the proliferation and differentiation of cells residing in the sclero-corneal limbus; cells that are considered to be stem cells (Schermer et al., 1986). As mentioned, one role of the cornea is to serve as a protective barrier. The multiple layers of the epithelium and the presence of desmosomes and tight junctions between epithelial cells, and hemidesmosomes between the epithelium and its underlying basement membrane in part accomplish this.

The cornea is separated from the underlying stroma by both the basement membrane and Bowman's layer. The basement membrane is secreted and formed by the overlying

epithelium. The establishment of epithelial cell polarity depends on the presence of the basement membrane (Schuger et al., 1996). The basement membrane is also believed to be important in the maintenance of a stratified and well-organized epithelium and plays a role in its wound healing process (Cao et al., 2002; Hahn et al., 1990; Phan et al., 1989). Bowman's layer is an acellular layer produced by stromal keratocytes that is composed of a condensation of some components of the extracellular matrix (ECM).

The stroma composes the majority of the cornea (approximately 90 %). It is the stroma that provides the cornea with its characteristic physical strength and shape. It consists of a collagen-based ECM (primarily collagen I) with cellular components occupying only 2-3 % of the volume of the stroma (Komai and Ushiki, 1991; Otori, 1967). The cells of the corneal stroma are specialized fibroblasts called keratocytes. These form a network within the stroma with cell-cell communication occurring at gap junctions between the long processes of neighbouring keratocytes (Ueda et al., 1987). Therefore, they function as a single cellular system. Upon injury, network connections can be lost and these separated cells function independently to perform roles in wound healing. The keratocytes are responsible for the maintenance of the ECM and actively remodel the stromal environment during wound healing.

Epithelial-stromal interactions are highly involved in the regulation of corneal function. Communication between the epithelium and the keratocytes is bi-directional and is important during development, homeostasis and wound healing of the cornea (Wilson et al., 1999). Signals from the stromal keratocytes play a role in the growth and proliferation responses of the epithelium. The presence of keratocytes improves the stratification of a squamous epithelium (Bell et al., 1983); and in the wounded epithelium, keratocytes induce

stromal contraction, which is required for proper recovery of the stratified epithelium (Taliana et al., 2001). In addition, the release of cytokines and other growth factors by keratocytes can trigger the production of growth factors and components of the ECM involved in the wound healing process (Wilson, 1998).

Descemet's membrane separates the stroma and the endothelium. This membrane is produced by the endothelial cells and is a condensation of collagenous protein, primarily collagen type IV (Fitch et al., 1990). The endothelium is a single layer of uniform, hexagonal-shaped cells. The main function of these cells is the to regulate the hydration level of the corneal stroma. This is accomplished by the active secretion of fluid from the stroma into the aqueous humor.

1.2 Anatomy of Corneal Innervation

The cornea is very highly innervated; its network of nerves is denser than any other organ in the human body. In the rabbit, sensitivity of the cornea is 300-600 times that of skin (Rozsa and Beuerman, 1982). The majority of the nerve fibres are of sensory origin, derived from the ophthalmic division of the trigeminal nerve (ten Tusscher et al., 1989). Despite the tremendous density of corneal nerves, the sensory network develops from a modest number (50-450) of trigeminal neurons, depending on the mammalian species (LaVail et al., 1993; Marfurt et al., 1989; Morgan et al., 1978). The cornea also receives sympathetic and parasympathetic innervation, from the superior cervical ganglion and ciliary ganglion, respectively. The density of sympathetic innervation varies from up to 10-15% of total innervation in the rabbit and cat cornea (Laties and Jacobowitz, 1966; Marfurt et al., 1989; Morgan et al., 1987) to extreme scarcity in primate corneas, including humans (Ehinger, 1971; Toivanen et al., 1987). While the corneas of both the rat and the cat receive modest

parasympathetic innervation (Marfurt et al., 1998; Morgan et al., 1987), however evidence for its innervation of human corneas remains unclear.

Human corneal nerve architecture (Fig. 3) has been well studied and documented (Müller et al., 1996; Müller et al., 1997). Sensory nerve fibres enter the cornea as bundles through the surrounding sclera, in a radial fashion parallel to the corneal surface. These bundles target mainly the stroma, just below its anterior third, due to the organization of the collagen lamellae (Radner and Mallinger, 2002). Corneal nerve fibres are mostly unmyelinated, losing their myelin sheath just outside the limbal region, a necessity for the maintenance of corneal transparency. Keratocytes within the stroma are often located in close proximity to the nerve fibres, with occasional physical contact by cytoplasmic extensions (Müller et al., 1996).

According to Müller et al. (1996), throughout the central and peripheral cornea, a 90° turn sends the stromal nerve bundles towards the corneal surface, first penetrating Bowman's layer. After crossing Bowman's layer, the nerve bundles divide into several smaller bundles. These smaller bundles again make a 90° turn and migrate parallel to the corneal surface between Bowman's layer and the basal layer of the epithelium, forming a network of nerves termed the sub-epithelial nerve plexus. These nerve plexus bundles contain both beaded and smooth nerve fibres. The bundles ramify into individual nerve fibres and small nerve bundles that penetrate the basal epithelium. From the basal epithelium, only the individual beaded fibres migrate and terminate in the more superficial epithelial layers. Within the epithelium, numerous invaginations of individual basal and wing epithelial cells by nerve fibres are observed, suggesting direct innervation. This may function to release or take up cellular or extracellular substances from adjacent cells.

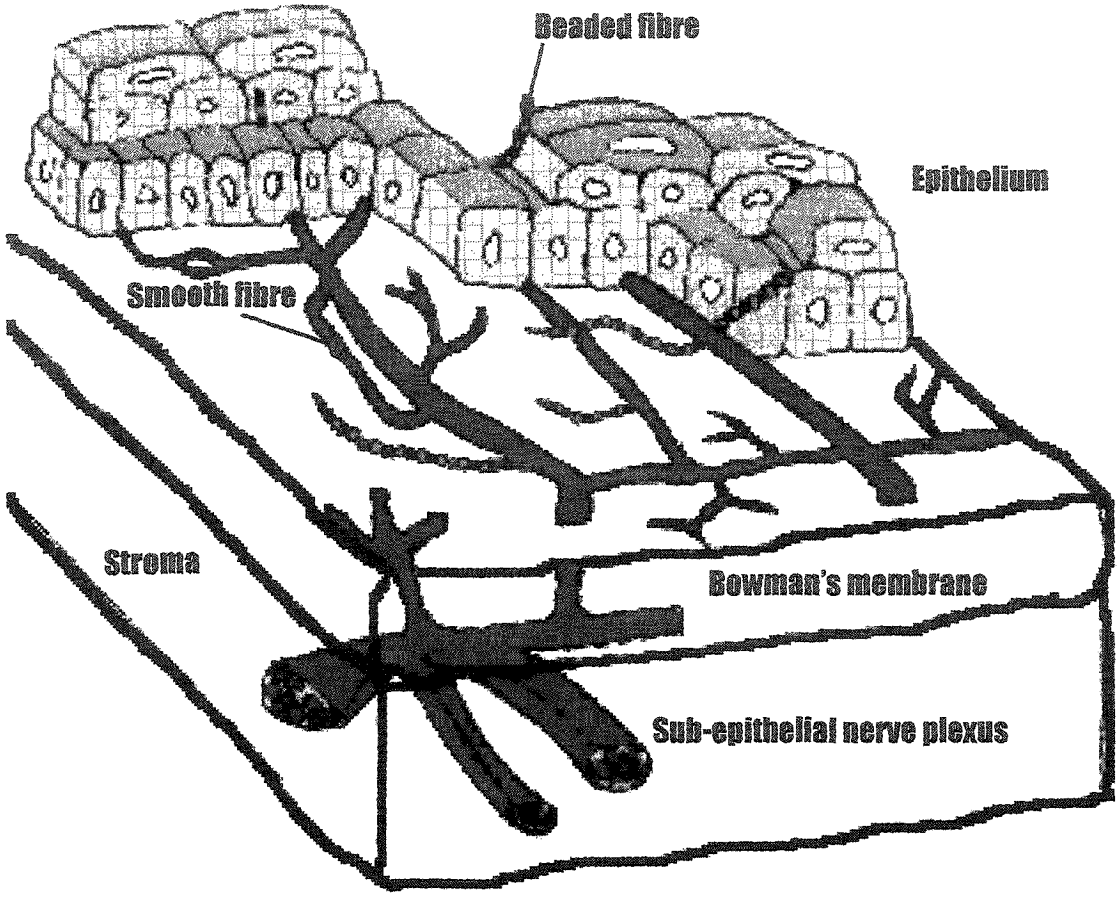

Fig. 3. Diagram of corneal nerve morphology.
Adapted from Müller et al. (1997) IOVS **38** p. 992

Examination of corneal nerve fibres by transmission electron microscopy reveals that the nerve fibre terminals in the TE cornea contain numerous mitochondria, glycogen particles, ribosomes and the presence of both small clear and dense core vesicles (Beckers et al., 1992; Müller et al., 1996). From tracing studies in rat (Marfurt et al., 1989; ten Tusscher et al., 1989) and immuno-cytochemical studies in man (Tervo et al., 1982; Uusitalo et al., 1989), it is known that the different vesicles contain different types of neurotransmitter (see below). Both vesicle types have been observed previously in all human corneal sensory nerves (Müller et al., 1996).

Both peptidergic and non-peptidergic fibres have been observed in nerves of the human corneal epithelium (Ueda et al., 1989). To date, a total of 17 biologically active neuropeptides and neurotransmitters have been identified in corneal nerves (Müller et al., 2003). Nerves of different origin contain different types and quantities of these neurochemicals, and there exist fibre subtypes within each corneal neural system (sensory, sympathetic and parasympathetic) that also differ in their neurochemical composition. Of particular interest are the calcitonin gene-related peptide (CGRP) and substance P (SP) neuropeptides, found in most sensory nerve fibres (Jones and Marfurt, 1991; Tervo et al., 1982; Uusitalo et al., 1989). These neuropeptides are involved in the regulation of growth and repair processes of the cornea (see below). As described above, different types of nerve fibres can be classified on the basis of their fine structures, including the composition of their vesicles (Beckers et al., 1993; Müller et al., 1996). In the rat cornea, CGRP has been localized in terminals with clear vesicles (Beckers et al., 1992) and SP has been co-localized with CGRP in the guinea pig cornea (Kuwayama and Stone, 1987).

1.3 Mechanisms of Corneal Development and Innervation

Descriptions of the embryonic development of the eye are readily available (Gilbert, 1994; Hay, 1980). Essentially, the eye develops from interactions of the neural tube with epidermal thickenings, called cranial ectodermal placodes. During gastrulation, the involuting endoderm and mesoderm contact the prospective head ectoderm. This initiates the formation of the lens (Saha et al., 1989). The proper formation of the eye depends on proper positioning with the retina, and this is accomplished by the optic vesicle (a bulge that grows laterally from the neural tube). When the optic vesicle contacts the head ectoderm, the ectoderm thickens into the lens placode. It has been shown that if the vesicles fail to contact

the surface, then eye formation ceases (Webster et al., 1984). The lens, as it develops, contacts the new overlying ectoderm and induces formation of the transparent cornea. For proper curvature of the cornea, the intraocular pressure must be maintained during development.

As mentioned, the lens placode induces cornea formation. The overlying layers of ectoderm become columnar and fill with secretory granules. These migrate to the basal layer and secrete the stroma (about 20 layers of type I and II collagen). Capillary endothelial cells migrate into this stromal region and secrete hyaluronic acid (HA). The HA causes the matrix to swell, rendering it a good substrate for the migration of mesenchymal cells from the neural crest. Upon the arrival of these cells, they secrete collagen I and hyaluronidase that causes the stroma to shrink. Under the influence of thyroxine (from the thyroid), the stroma is dehydrated and the collagen-rich matrix of epithelial and mesenchymal tissue becomes the cornea (Hay, 1980).

Little is known regarding the specific mediators involved in directing the development of the cornea during embryogenesis, and even less about the mechanisms of innervation. However it has been speculated that cytokine- and growth factor-mediated communications are likely involved in directing the neural crest cells to the appropriate location and regulating their proliferation, differentiation and function. This is based on the cytokine and growth factor relationships that are known in the adult cornea (Wilson et al., 1999). The developmental study of corneal innervation has been restricted to the investigation of animal models. In the avian cornea, it is at the dehydration stage that innervation of the cornea begins (Bee, 1982). In vitro, it has been demonstrated that innervation promotes the process of dehydration and transparency, although not exclusively

(Clarke and Bee, 1996). The formation of the pericorneal nerves, which later penetrate the stroma, is inhibited by 6-diazo-5-oxo-L-norleucine (Bee et al., 1982). This glutamine analog inhibits the formation of sulfated glycosaminoglycans and consequently affects the architecture of the stroma. Therefore, there appears to be a role for sulfated glycosaminoglycans in the formation of the nerves surrounding the cornea.

The attractive cues and mechanisms responsible for the penetrance and guidance of the corneal nerves within the stroma and epithelium have only been alluded to. In work using trigeminal neurons and the different cell types of the cornea (Chan and Haschke, 1982), it was established that when neurons were grown in contact with each of the corneal cell types, neurites were extended in every case. However, when neurons were not in contact with the corneal cells in the co-culture, only epithelial cells permitted neurite outgrowth. The data suggested two types of cellular interactions between corneal cells and sensory neurons, one of which is contact-mediated and the other which may be the specific release of a neurotrophic factor by epithelial cells. It is most probable that one of these factors is nerve growth factor (NGF). NGF is produced by the corneal epithelium (You et al., 2000) and plays a significant role in the development of sensory neurons, including those of the trigeminal system (Davies, 1993; Davies et al., 1987). NGF is a member of the neurotrophin family, which signal through specific receptors of the Trk family of tyrosine kinases (Snider, 1994). Nociceptive sensory neurons, like the type expressed in the trigeminal ganglia, consist of NGF-dependent neurons expressing TrkA (Carroll et al., 1992; Mu et al., 1993). Therefore, one can speculate that NGF-mediated nerve guidance in the cornea acts via the TrkA receptor on the trigeminal nerves. This would result in the activation of a MAP kinase pathway (Kaplan and Miller, 2000), which is essential for axonal growth in other sensory

systems (Schaefer et al., 1999). Another pathway activated via Trk receptors involves the GTPase Ras, Raf, phosphatidylinositol-3'-kinase (PI3K), and the protein kinase Akt, which also has been shown to be essential in NGF-dependent survival of sensory neurons (Borasio et al., 1993; Klesse and Parada, 1998; Wiese et al., 2001). These different Trk effector pathways have been shown to mediate distinct morphological features of developing neurons (Markus et al., 2002). Other work suggests that corneal epithelial cells secrete factors other than NGF that promote neurite extension via the p75 NGF receptor, however they did not establish what the factors were (Pavlidis et al., 1994). Possible candidates include brain-derived neurotrophic factor (BDNF) and neurotrophin-3, both known to act through this receptor (Rodriguez-Tebar et al., 1991; Rodriguez-Tebar et al., 1990) and which are expressed within the cornea (You et al., 2000). Interaction with the p75 receptor modulates axonal outgrowth by regulating the activity of the small GTPase Rho and its effectors (Yamashita et al., 1999). Therefore release of neurotrophic factors by the cornea and their interactions with receptors on developing neurons likely plays a significant role in the guidance, differentiation and survival of corneal nerves.

Another possibility for the guidance of corneal innervation is the interaction of the nerves with their ECM environment. One study has suggested that collagen IV secretion is involved in the guidance of the nerves through the stroma (Bee et al., 1988). During development, the nerves are associated with this ECM molecule, but following their extension through the matrix, its expression diminishes. In addition, it has been demonstrated that axon outgrowth can be mediated by ligand-induced alterations in the expression of integrins (Condic and Letourneau, 1997), the ECM receptors. Integrins control nerve extension over ECM proteins and have been shown to signal to MAP kinase (Holly et al.,

2000). Because of the relationship of neurotrophins to the MAP kinase pathway (described above), it is possible that neurotrophins can mediate nerve growth responses to ECM proteins in their environment.

It is likely that the guidance and survival of nerves within the cornea during development is modulated, in part, by a combination of responses to both neurotrophin release by the corneal cells and to the changes in ECM that occur during corneal maturation.

1.4 The Role of Corneal Nerves

Peripheral sensory nerves play an important role in the homeostasis of the tissue that they innervate. This nerve-target tissue relationship has been observed in multiple tissue types. For example, denervation often results in thin skin that is prone to ulceration (Head and Sherren, 1905). Also, several experimental sensory denervation models have demonstrated trophic changes in their respective target cells. These include epithelial cells in the corneas of monkeys and rabbits (Beuerman and Schimmelpfennig, 1980; Lim, 1976); lamellar and epithelial cells from rat foot pads (Del Valle et al., 1993; Li et al., 1997); and the tracheal mucosa, muscle, liver, and adipose tissues of rats (Bowden et al., 1996; Koopmans et al., 1998). A compromised nerve supply has also been shown to significantly impair wound healing (Araki et al., 1994; Carr et al., 1993). Following injury, sensory nerves are stimulated to provide the site of injury with signals that promote tissue repair (Harsum et al., 2001; Westerman et al., 1993). In addition, sensory nerves respond to signals from the wound site by extending axons into the injured area (Reynolds and Fitzgerald, 1995). Axonal growth into the wound is important for healing, and factors that control early axonal outgrowth influence the final outcome of recovery (Krarup et al., 2002). Molecular changes within the axon include an increase in NGF mRNA levels within hours of axonal injury, along with that

of its receptors (Sebert and Shooter, 1993). Vascular endothelial growth factor, a potent angiogenesis growth factor, and insulin-like growth factor-1 have also been shown to have an important role in nerve regeneration (Meirer et al., 2001; Rabinovsky, 2004).

1.4.1 Sensory role of corneal nerves

In the cornea, the nerve endings within the epithelium act as nociceptors to detect mechanical, thermal and chemical signals (Belmonte et al., 1991). Acute ocular pain is an immediate response of the stimulation of these nociceptors, acting at or near injurious levels. The heightened sensitivity of this system is fundamental to the subsequent activation of responses to minimizing damage and promoting tissue repair. These responses include the blinking of the eye, which dilutes potential irritants and physically shields the ocular surface. The action potential (AP) is the neuronal message carrier that relays the signal from the nerve terminals to the central nervous system (CNS) for the perception of pain (Belmonte, 1997). The opening of sodium (Na^+) channels, and the subsequent Na^+ current and depolarization of the axonal membrane generates the AP in the sensory nerve. Propagation of the AP occurs by conduction of the electrical current along the axon. The uniform distribution of Na^+ channels in corneal sensory nerves supports continuous (as opposed to saltatory, found in most other axons) conduction along these axons (Black et al., 2002).

Electrophysiological knowledge of corneal sensory nerves is relatively difficult to obtain, in part due to their small diameter and inaccessibility in intact tissues, like skin (Belmonte, 1996). By nature of the experiments, the recording of electrical activity in corneal fibres has been performed exclusively in animals. In the majority of the nervous system, tetrodotoxin (TTX)-sensitive Na^+ channels are responsible for action potential generation and propagation. However, sensory nerves of the cornea express TTX-resistant Na^+ channels in

addition to the TTX-sensitive type (Akopian et al., 1996). The TTX-resistant Na^+ channels are present in sufficient density for action potential generation in the cornea, a characteristic exclusive to these channels of neurons in the trigeminal ganglia, and in dorsal root ganglia (Brock et al., 1998). It is generally assumed that sensory nerve terminals are incapable of supporting APs and that their generation results from generator potentials that spread to a proximal area of the axon that is excitable (Block, 1992). In the cornea, this is the mechanism by which it is believed thermo-sensitive receptors function, but in addition, APs can propagate actively in the sensory nerve endings of polymodal receptors (Brock et al., 2001). These make for some fairly unique physiological properties of corneal sensory nerves.

The three functional classes of corneal nociceptors can be distinguished by the characteristics of their nerve impulses. Nerve impulses can occur spontaneously in the cornea and can be evoked by electrical stimulation. The nerve terminal impulses (NTIs) of the corneal neurons are diphasic (positive-negative) with a prominent positive component and their estimated conduction velocities of range from 0.3 to 2.7 m/s (Brock et al., 1998). The spontaneous NTIs of polymodal neurons appear at low amplitude (0-4.5 Hz) with irregular frequencies that increase upon chemical or mechanical stimulation, properties similar to those found in skin (Brock et al., 1998; Kumazama, 1996). The polymodal nociceptor is the most abundant class of neuron found in the cornea (Belmonte et al., 1991). Corneal mechano-receptor NTIs occur at 0-2.7 Hz and frequency increases only upon mechanical stimulation, while thermo-sensitive receptor NTIs (1.4-17 Hz) are discharged at higher frequencies, sometimes in bursts and increase in frequency upon heating or cooling of the environment (Brock et al., 1998). In addition, there are a significant number of nociceptors that produce NTIs only when evoked by electrical stimuli and may represent a population of

fibres that are activated in response to inflammation (Brock et al., 1998; Schmidt et al., 1995). Overall, the different types of nociceptors within the cornea can be identified by the characteristics of its NTI and the type of stimuli that generates it.

1.4.2 Neurotrophic influence on the cornea

Corneal nerves have been shown to affect corneal health, in particular, by modulating epithelial cell properties (Baker et al., 1993). Within hours of corneal nerve loss, the normally squamous surface epithelial cells become rounded, lose their microvilli (essential for interaction with the tear film) and shed at an accelerated rate into the tear film (Gilbard and Rossi, 1990). This leads to an increase in the permeability of the epithelium and a decrease in epithelial cell migration and proliferation (Beuerman and Schimmelpfennig, 1980; Garcia-Hirschfeld et al., 1994; Holden et al., 1982). Previous work has also demonstrated thinning of the epithelium in denervated corneas (Araki et al., 1992). This may be due to changes in the adhesive properties of the epithelium in the absence of innervation. Indeed, widened intercellular spaces between epithelial cells and fewer desmosomes at the epithelial-basement membrane interface and disorganization of the epithelial layer were observed in these corneas (Araki et al., 1994). A loss of innervation has also been shown to slow corneal wound healing. It has been observed that denervation by surgical destruction or chemical disruption significantly inhibits the ability of the epithelium to heal itself after corneal injuries (Araki et al., 1994; Beuerman and Schimmelpfennig, 1980; Gallar et al., 1990). The lack of corneal nerves renders the healed epithelium susceptible to recurrent, spontaneous epithelial erosions.

There is significant evidence to suggest that neuropeptides of sensory nerves are involved in the trophic effects of innervation on the epithelium of the cornea. During cornea

wound healing, neuropeptides are released from nerve terminals and are believed to promote healing effects associated with corneal innervation (Gallar et al., 1990). As mentioned above, neuropeptides present in nerves of the corneal epithelium include SP and CGRP (Palkama et al., 1986; Unger et al., 1988). SP is expressed in physiologically relevant quantities in the normal cornea and its receptor, the neurokinin-1 (NK1) receptor is expressed abundantly on corneal epithelial cells (Nakamura et al., 1997c). SP has been shown to exert a stimulatory effect on corneal epithelial cell proliferation and migration (Garcia-Hirschfeld et al., 1994; Nishida et al., 1996) and to play a role in epithelial cell adhesion (Araki-Sasaki et al., 2000). In order to exert some of its effects, it has been shown that SP acts synergistically with insulin-like growth factor-1 (IGF-1) and/or epidermal growth factor (EGF). Together, SP with IGF-1 and/or EGF promote corneal epithelial wound healing (via migration and proliferation of epithelial cells) and epithelial cell attachment to its basement membrane proteins (Nakamura et al., 1998; Nakamura et al., 1997a; Nakamura et al., 1997b; Nishida et al., 1996). SP can affect epithelial cell adhesion through effects on E-cadherin, a protein that mediates cell-cell interaction (Araki-Sasaki et al., 2000), and through effects on the expression of receptors for ECM proteins such as fibronectin (Chikama et al., 1999). Fibronectin is deposited on the wound bed of corneal injuries to provide a temporary matrix for epithelial cell migration. Therefore, SP can contribute indirectly to epithelial wound healing by the regulation of interactions between epithelial cells and wound healing matrix proteins, such as fibronectin.

Another neuropeptide, CGRP, often co-localizes with SP (Kuwayama and Stone, 1987) and is also involved in the regulation of corneal epithelial cell renewal and wound repair. CGRP-positive nerves are abundant in the human cornea (Jones and Marfurt, 1991)

and its receptor is expressed by the epithelial cells (Heino et al., 1995). This neuropeptide is also present in the tear fluid and its content increases in the event of injury (Mertaniemi et al., 1995). CGRP is expressed at sites of corneal injury (Murphy et al., 2001) and has been shown to enhance epithelial cell migration and wound closure in an organ-cultured wound healing model (Mikulec and Tanelian, 1996). In addition, CGRP appears to inhibit corneal epithelial cell DNA synthesis and proliferation, and may promote epithelial cell differentiation (Garcia-Hirschfeld et al., 1994; Reid et al., 1993). There are other neuropeptides expressed within the nerve fibres of the cornea including galanin and pituitary adenylate cyclase-activating peptide, and it is likely that some fibres express some unidentified neuropeptides, possibly excitatory amino acids such as glutamate and aspartate (Jones and Marfurt, 1998; Silverman and Kruger, 1988; Wang et al., 1995). These neuropeptides are not widely expressed, as are SP and CGRP, less is known about their function, and they will not be discussed here.

1.4.3 Effects of the Cornea on Innervation

While innervation affects the properties of the epithelium, the epithelium, in return affects the growth and survival of the corneal nerves. Corneal epithelial cells are known to produce and release soluble factors, which may contribute to axonal extension and survival (Chan and Haschke, 1982; Emoto and Beuerman, 1987). One of these factors is nerve growth factor (NGF). The corneas of mutant mice in which TrkA, the high affinity receptor for NGF, has been inactivated (trkA (-/-) mice) have a drastic reduction in the number of stromal nerve branches and thin nerve terminals in the epithelium, with a resulting loss of corneal sensitivity (de Castro et al., 1998). In addition to NGF, the production of some to be determined additional factors by corneal epithelial cells results in enhanced extension of

neurites from trigeminal neurons (Chan et al., 1987; Pavlidis et al., 1994). Some of these additional factors may include components of the ECM, which are produced and maintained by an interaction between the different corneal cells (Wilson et al., 1999; Zieske et al., 1994). During development of the avian cornea, the appearance of collagen IV within the stroma (possibly produced by both epithelial and stromal cells) coincides with the invasion of the nerve fibres, and expression of collagen IV diminishes following innervation and the end of development (Bee et al., 1988). The distribution of another ECM protein, laminin, has also been suggested as a cue for the guidance of trigeminal nerves to their peripheral target (Riggott and Moody, 1987). In addition to promoting neurite extension and survival, it has been suggested that epithelial cells affect neural targeting in the cornea, likely by the release of the soluble growth factors discussed above (Chan et al., 1987).

Keratocytes have been observed to be directly targeted by SP-containing corneal nerve fibers in the human cornea (Müller et al., 1996). This suggests that, like the epithelium, the cells within the stroma may also play a role in corneal nerve growth. In fact, the corneal stromal keratocytes produce neurotrophic factors, including NGF, that are likely to affect neurite extension and growth (Lambiase et al., 2000; You et al., 2000). In addition, these cells are involved in the maintenance of the stromal and basement membrane ECM, and as noted above, this could prove to be important for innervation. Overall, there has been little characterization of the interactions between the corneal nerves and their targets, and in particular the factors regulating nerve growth.

1.5 Corneal Nerve Injury and Disease

The superficial location of the cornea makes it susceptible to chemical and physical injury. Severe injuries or disease may result in a loss of corneal transparency and when

irreversible, blindness may occur. Corneal blindness affects more than 10 million patients worldwide (estimates from The Vision Share Consortium of Eye Banks, USA). As the focus of the thesis is on corneal innervation, this section will cover the role of nerves in corneal disease and injury.

The causes of corneal nerve loss are many, and include both injury and disease: viral infection, photorefractive surgery, keratoplasty surgery, chemical/thermal burns, topical medication, chronic ocular surface injury or inflammation, contact lens wear, and corneal dystrophies. Most common of the diseased states is neurotrophic keratitis. The pathogenesis of this condition includes impaired sensitivity leading to diminished protective blink reflexes, desiccation of the cornea resulting from decreased lacrimal secretions, abnormal epithelial cell metabolism with a reduced wound healing ability, and the loss of trophic effects supplied by the corneal nerves (Heigle and Pflugfelder, 1996; Wilson and Ambrosio, 2001). Specific details on the morphological and metabolic epithelial disturbances resulting from denervation were reviewed in section **1.4.2**. The loss of corneal innervation by any cause can result in "dry eye" syndrome, a pathological condition involving loss of proper tear formation that can result in desiccation of the corneal surface (Stern et al., 1998). This can lead to corneal epithelial erosions, and/or loss of corneal sensitivity. When sensitivity is lost, the cornea becomes vulnerable to irreparable injury and ulceration. A progressive neurotrophic ulcer can lead to perforation of the cornea and in severe cases, blindness (Gilbard and Rossi, 1990).

Current treatments for neurotrophic keratitis depend on the stage of the disease and focus on the prevention of the progression of corneal damage and the promotion of epithelial wound healing (Lambiase et al., 1999). In the early stages of the disease (before epithelial defect), therapies aim to improve epithelial quality and transparency. This is commonly

accomplished by the topical application of artificial tears. Once the epithelial defect develops, the goal is to promote epithelial healing to prevent ulceration. Therapy strategies include the use of therapeutic contact lenses (Pfister, 1992), transplantation with amniotic membrane (Prabhasawat et al., 2001; Solomon et al., 2002) and treatment with topical steroids (Cavanagh and Colley, 1989). Once the ulcer develops, therapy is aimed at the promotion of epithelial wound healing and the prevention of corneal melting (enzymatic digestion of the stroma) and perforation. To impede the progress of corneal melting, collagenase inhibitors are sometimes used (Davis and Dohlman, 2001). To promote corneal healing of later stage neurotrophic keratitis there are several surgical procedures. Conjunctival flap, cyanoacrylate glue with accompanying soft bandage contact lens, plug therapy, and lamellar or penetrating keratoplasty procedures have all been employed (Hirst et al., 1982; Lugo and Arentsen, 1987; Tai et al., 2002). The use of neuropeptides and growth factors, such as NGF, SP, and EGF at various stages of disease progression has also been suggested (Araki-Sasaki et al., 2000; Bonini et al., 2000; Daniele et al., 1992). While these therapeutic strategies have some strong points, they typically are inadequate in restoring full function to the cornea (Bonini et al., 2003).

Following laser surgical procedures for vision correction such as radial keratectomy, photorefractive keratectomy and laser in situ keratomileusis (LASIK), complications such as dry eye and loss of sensitivity affect some individuals and can be attributed to the effects of inadequate innervation (Heinz et al., 1996). These effects include damage to the stromal nerves and sub-basal plexus, and varying degrees of epithelial alterations (Tervo and Moilanen, 2003; Wilson and Ambrosio, 2001). The occurrence of these complications will rise in the future as the number of individuals undergoing corneal refractive surgery increases

(Eye Bank Association of America, 1999). It is important to study wound healing and the molecular interactions between the cornea and its nerve supply to gain a better understanding of corneal diseases and post-injury complications that are a result of improper innervation. But, due to the fine structural nature of corneal sensory nerves and their inaccessibility for study in the human eye, investigation of these interactions is difficult. A representative in vitro model of the human cornea would facilitate research of corneal innervation.

1.6 The Development of a Tissue Engineered Cornea Model

1.6.1 Current Needs

At present, the Draize or LVET rabbit ocular irritancy tests (Daston and Freeberg, 1991) are the standard tests for measuring potential ocular toxicity of manufactured consumer products. Rabbits are the preferred test animals for several reasons including their large eyes, well described physiology, and their eyes are generally more susceptible to irritation than human eyes (Gershbein and McDonald, 1977; Roggeband et al., 2000). To describe briefly, in the Draize test, a material is instilled into one eye of albino rabbits and the ocular response of the animals is monitored. The effect of the material is scored using a standardized system for the cornea, conjunctiva and iris, for up to 21 days. Among the factors that are considered in the test application are the exposure, the volume and dilution of material and the time length of monitoring. The LVET test uses the same animals and scoring system as the Draize test, but uses a lower volume of test material that better reflects the human eye's volume capacity (Mishima et al., 1966). The LVET test gives results that are linearly correlated to the Draize test and has proven to be a more reproducible test method than the Draize test (Cormier et al., 1996). In addition, it has been shown that the LVET test is more accurate in predicting degrees of irritancy among materials that are moderately

irritating and in discriminating between severe eye irritants and those that will cause permanent damage (Griffith et al., 1980). The use of rabbit models for ocular toxicity testing has been widely criticized. Rabbit eyes are anatomically and physiologically different from humans (Wilkie and Wyman, 1991), and do not reliably predict human eye responses (Freeberg et al., 1986a; Freeberg et al., 1986b). In addition, there are animal welfare concerns over the use of animals for all testing and research purposes. A recent ban in Europe on animal testing for the development of consumer products is expected to expand from Europe into North America, and the need for alternative test models will grow. Despite these facts, a validated alternative has not yet been developed to replace the current standard rabbit eye tests. However, recent advances in the tissue engineering of corneas present great strides towards the reconstruction of a complete and functional cornea.

There is also a need for the development of tissue-engineered (TE) corneas for transplantation. As mentioned, corneal blindness affects more than 10 million patients worldwide and the only widely accepted treatment is transplantation with human donor tissue. Currently, the United States is the only country that is self-sufficient in the supply and demand of corneas for transplantation (Eye Bank Association of America, 1999). Furthermore, for patients afflicted with specific disorders such as autoimmune conditions, alkali burns or recurrent graft failures, this procedure has a poor success rate (Hicks et al., 2000). The demand for corneas for transplantation will escalate with an aging population and the increased use of corrective laser eye surgery (Eye Bank Association of America, 1999). An alternative for these patients is the replacement of the damaged cornea with an artificial substitute. At present, widely accepted corneal substitutes (prostheses or tissue equivalents) are not available (Hicks et al., 2000). In addition, none of the substitutes proposed to date

have addressed the re-innervation issue. This is an important issue since loss of corneal innervation can lead to loss of vision (Lambiase et al., 1998). Tissue engineering of corneas represents a means of overcoming the present disadvantages of corneal transplantation, including immune rejection, shortages of donor tissue and the failure to address innervation.

1.6.2 Current tissue engineered cornea models

There have been attempts made at constructing a fully functional corneal equivalent using various strategies. Several models have been developed from animal corneal cells. Cells from all three layers of the rabbit, porcine or bovine cornea have been used to successfully reconstruct a cornea in a three-dimensional collagen gel (Minami et al., 1993; Schneider et al., 1999; Zieske et al., 1994). The disadvantage of these animal models is the question of validity for their use in predicting human corneal responses, as already discussed. Human corneal equivalents have also been developed using similar construction methods. One model was developed using a collagen-based matrix to reconstruct the stroma, but lacked an endothelium and the membranes (Descemet's and Bowman's) to separate the tissue layers (Germain et al., 1999). The expression patterns of various integrins in the basement membrane of this TE cornea were similar to the human cornea. However, the characterization of other corneal properties was not addressed: the physiological properties of the different cell lines, biochemical marker expression, transparency, ion and fluid transport, and gene expression in response to external stimuli, such as wounding or chemical application. These properties were addressed in a TE cornea developed in our lab (Griffith et al., 1999). This TE cornea comprised all three main layers of the cornea (epithelium, stroma, and endothelium), including the separating membranes. The cellular layers were constructed using immortalized human corneal cells. These corneas gave quantifiable changes in transparency and expression

of wound healing genes indicating different degrees of cellular damage in response to different chemicals; but provide no indications of sensitivity and pain. The addition of nerves to the model may further enhance the similarity of the TE cornea to the human cornea.

1.7 Research Summary

1.7.1 Rationale

It is known that a sensory nerve supply is crucial for optimal tissue function. However, several issues of innervation in the cornea are yet to be fully addressed: 1) the mechanisms responsible for successful nerve growth and the interaction between nerves and their target tissues are not yet fully understood; 2) a validated innervated cornea model has not yet been developed for in vitro toxicology testing; and 3) acceptable corneal substitutes for transplantation are not available and fail to address innervation. The recently developed human corneal equivalent (Griffith et al., 1999) may provide a suitable system for the study of innervation in the cornea. This tissue equivalent is superior to other models developed in that it is derived from human cells, it comprises all cellular layers and their membranes and it mimics the human cornea in key physical and physiological functions.

1.7.2 Objectives

1. To innervate the TE cornea and to determine if it is representative of the native human cornea in terms of both morphology and nerve function
2. To investigate the potential for the use of such a model in toxicology testing
3. To use this technology to provide insight for the development of corneal substitutes for transplantation

If these objectives can be successfully met, this model could address all of the above-mentioned issues about innervation in the cornea.

1.7.3 Research Approach

This summary of the research approach is given to direct the progression through the following chapters (collection of manuscripts).

Manuscript 1: Innervated human corneal equivalents as in vitro models for nerve-target cell interactions

Dorsal root ganglia (DRG), isolated from chick ganglia, served as the nerve source for introduction to the previous model of the TE cornea (Griffith et al., 1999). The DRG is a suitable substitute for trigeminal ganglia because the sensory neurons of both share the same morphological and physiological properties and are unique from the majority of other nerve cells (Elliott and Elliott, 1993; Yoshida and Matsuda, 1979). The DRG were embedded within the stromal matrix or within a surrounding scleral ring. The matrix and culture conditions were modified from the previous model to promote the ingrowth of nerves from the DRG. The addition of a laminin gradient and NGF to the matrix allowed DRG substitutes for trigeminal ganglia to produce nerve growth patterns in the TE cornea similar to the natural human cornea. This TE cornea had functional innervation with demonstrated nerve-target cell interactions including: a) the generation of action potentials; b) a neurotrophic effect on the epithelium during wound healing; c) a stimulatory effect on proliferation and/or stratification of epithelial and stromal cells; and d) the appropriate release of SP neurotransmitter following exposure to chemicals. This model provides a useful tool for studying corneal innervation and cell-cell interactions, with the advantage of a controlled

environment and natural transparency for imaging. The next step was to assess the potential of this model for use in toxicology testing.

Manuscript 2: Innervation in the development of tissue engineered models for in vitro study and testing purposes

The innervated TE cornea described above was further characterized to assess some of the properties that would be relevant for its use in the testing of potential ocular irritants. These include epithelial thickness and the production of the protective mucin layer by the epithelium. It was demonstrated that the presence of nerves promoted greater epithelial thickness and formation of the mucin layer. Innervation also conferred protection to the epithelium from chemical insult, as determined by the level of post-treatment epithelial cell death. Combined with the results from the first manuscript, it can be concluded that innervation is important in the maintenance of the TE cornea and its protective function. In addition, differential responses of the nerve fibres to chemical stimuli were demonstrated. These were recorded by observation of changes in the concentration of intracellular sodium (Na^+) within the nerve fibres, as measured by 2-photon microscopy. Therefore, the degree of cell death, the levels of neurotransmitter release (as demonstrated in manuscript 1) and the response of the nerves themselves (Na^+ concentration changes) could all be used to ascertain the toxicity of potential ocular irritants. Overall, this work demonstrates a role for innervation in the protective quality and function of the engineered tissue, and the potential to use the nerves themselves as indicators of the severity of an insult. These results are important to consider for the development of any optimized TE models for *in vitro* study and ocular toxicology testing purposes.

Manuscript 3: Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation

The development of *in vitro* models of the cornea, as described above, can also enhance the development of suitable cornea replacements for transplantation. Dr. Frank Li, a post-doctoral fellow within the lab, translated the *in vitro* cornea formulation into chemically cross-linked collagen-copolymer matrices (also termed hydrogels) with the potential to serve as such replacements. At the time, these had not been designed with the issue of innervation in mind. Based on the in vitro work described above, it was determined that the addition of laminin, or a peptide thereof, would enhance the ability of copolymer matrices to support nerve growth. Therefore, several laminin peptides were tested for their ability to promote nerve extension in the in vitro model. It was determined that laminin pentapeptide (YIGSR) and laminin A chain were the best candidates based on their ability to promote the extension of the greatest number of neurites from DRG. Because of its smaller size, the YIGSR motif was selected for grafting to the polymer, to deter interference with the chemistry of collagen copolymerization. Different collagen matrices, including one with YIGSR, were then tested in vitro for their ability to promote epithelial stratification and the ingrowth of nerves. It was determined that the collagen-copolymer with YIGSR was superior in the assessment of these criteria and was used for subsequent implantation into the corneas of pigs. Implants showed successful in vivo regeneration of host corneal epithelium, stroma and nerves. In fact, nerve regeneration and sensitivity of the tissue was superior in these co-polymer cornea substitutes than in allograft controls, which are the current standard for transplantation procedures. Therefore, this implantable matrix can perform as a physiologically functional tissue substitute and is the first cornea substitute to address the issue of innervation.

1.8 Summary

The topic of corneal innervation is important and the results presented demonstrate significant advances in the understanding of this issue. The innervated TE cornea model provides a useful tool for studying corneal innervation and wound healing, and has the advantage of a controlled environment. In addition, it could be further developed for use as an alternative to animal ocular toxicity testing and it provides insight for the development of cornea substitutes for use in transplantation.

CHAPTER 2

2.1 Manuscript 1

Innervated human corneal equivalents as in vitro models for nerve-target cell interactions

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Innervated human corneal equivalents as in vitro models for nerve-target cell interactions

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ABSTRACT

A sensory nerve supply is crucial for optimal tissue function. However, the mechanisms for successful innervation and the signaling pathways between nerves and their target tissue are not fully understood. Engineered tissue substitutes can provide controllable environments in which to study tissue innervation. We have therefore engineered human corneal substitutes that promote nerve in-growth in a pattern similar to in vivo re-innervation. We demonstrate that these nerves (a) are morphologically equivalent to natural corneal nerves; (b) make appropriate contact with target cells; (c) can generate action potentials; (d) respond to chemical and physical stimuli; and (e) play an important role in the overall functioning of the bioengineered tissue. This model can be used for studying the more general topics of nerve ingrowth or regeneration and the interaction between nerves and their target cells and, more specifically, the role of nerves in corneal function. This model could also be used as an in vitro alternative to animals for safety and efficacy testing of chemicals and drugs.

Key words: extracellular matrix • innervation • cornea • nerve function • tissue engineering

Peripheral sensory nerves play an important role in the homeostasis of the tissues that they innervate (1). This nerve-target tissue relationship is observed in multiple tissue types. For example, denervation often results in thin skin that is prone to ulceration (2). Also, several experimental sensory denervation models have demonstrated trophic changes in their respective target cells. These include epithelial cells in the corneas of monkeys and rabbits (3, 4); lamellar and epithelial cells from rat foot pads (5, 6); and the tracheal mucosa, muscle, liver, and adipose tissues of rats (7, 8). A compromised nerve supply has also been shown to significantly impair wound healing (9, 10). Axonal growth into the wound is important for healing, and factors that control early axonal outgrowth influence the final outcome of recovery (11). Although it is known that a sensory nerve supply is crucial for optimal tissue function, the interaction between nerves and their target tissues and the mechanisms responsible for successful nerve restoration are not fully understood.

The cornea is an ideal tissue for modeling nerve in-growth and studying nerve-target cell interactions. Its transparency allows observation of nerve fiber growth, and its structural simplicity and the importance of innervation for optimal corneal function make it a mechanistically relevant tissue model. The cornea is one of the most highly innervated tissues in the body, and in rabbits, it has a sensitivity that is 300–600 times that of skin (12). Corneal nerves are important for overall corneal health and, in particular, the health of the epithelial cells that they innervate (13). Loss of corneal innervation has been shown to decrease epithelial cell proliferation and migration, to lead to a loss of epithelial barrier function and to slow corneal wound healing (4, 14).

Nerve terminals within the epithelium contain and release neuropeptides, including substance P (SP) and calcitonin gene-related peptide (CGRP) (15, 16). SP, in particular, has

been shown to exert a stimulatory effect on corneal epithelial cell proliferation and migration (14, 17) and to play a role in wound healing (18). It is important to study the molecular interactions between the cornea and its nerve supply to gain a better understanding of corneal diseases and post-injury complications that are a result of improper innervation or re-innervation.

Axonal outgrowth is supported and guided by environmental cues, including numerous growth factors, extracellular matrix (ECM) components, chemotactic factors, and small molecules. Of particular interest to our cornea model are laminin, nerve growth factor (NGF), and retinoic acid (RA). Laminin is a multifunctional ECM glycoprotein implicated in nerve growth and is present in the basement membrane of the human corneal epithelium (19). Studies in chick embryos demonstrate laminin's role as a guidance molecule for in-growing nerve bundles from the trigeminal ganglia that innervate the cornea (20). Neurotrophic factors secreted by the corneal epithelial cells, including NGF, have also been implicated in the guidance and survival of corneal nerves (21, 22). RA, which has morphogenetic properties, is necessary for peripheral neurite growth during quail embryonic development (23) and induces neurite outgrowth from isolated embryonic mouse dorsal root ganglia (DRG) in culture (24). Therefore, laminin, NGF, and RA are excellent candidate exogenous macromolecules for promoting axonal growth and guidance within an in vitro system.

Our objective was to test the effects of exogenous macromolecules on nerve in-growth in order to obtain a morphologically and physiologically accurate innervated in vitro corneal model that can be used to study the mechanism of nerve in-growth (or re-growth) and the interaction between nerves and their cellular targets. We report that nerve growth patterns into our tissue engineered (TE) in vitro cornea are similar to those in natural human corneas.

The generation of action potentials (APs) and the release of SP neurotransmitter in response to stimulation confirm nerve function, and the axons properly target corneal epithelial cells and exert a neurotrophic influence on the epithelium.

MATERIALS AND METHODS

Effects of exogenous factors and target cells on nerve growth

Stromal matrix was prepared within a 4-μm-pore-sized, 12-mm-diameter insert (Corning, Acton, MA) by blending neutralized, type I rat tail collagen (0.3% [w/v]; Becton Dickinson, Oakville, Canada) with chondroitin-6-sulfate (CS; Sigma, Oakville, Canada) (1:5 w/w ratio collagen: CS) on ice. The blended matrix was cross-linked with glutaraldehyde (0.02% [v/v]), and residual aldehyde groups were inactivated by reaction with 0.8% aqueous glycine (w/v) solution. This collagen-CS mixture is liquid at 4°C but forms a hydrated gel at 37°C. DRGs, isolated from chick embryos (E8.0) for the neural source, were embedded within the stromal matrix before being thermogelled at 37°C for 2 h.

To determine optimal concentrations of exogenous factors that would promote nerve growth within the constructs, we added a range of doses of laminin, NGF, or RA to the matrices. For laminin, concentrations of 0, 10, 20, or 40 μg/ml were added to the matrix. For NGF, concentrations of 0, 1, 10, 100, or 200 ng/ml were added to the upper layer of a three-layered matrix. For RA, concentrations of 0, 1, or 2 nmol/ml were added to the culture medium. To address the possibility that an RA gradient may be necessary for its effect, we added increasing concentrations from bottom to top of 0 and 1 nmol/ml RA (2-step) or 0, 1, and 2 nmol/ml RA (3-step) to the matrix and compared them with uniform concentrations of 0, 1, or 2 nmol/ml also added to the matrix. To protect RA from degradation, constructs were protected from light during media changes using amber filters, and culture plates were

wrapped in aluminum foil. In all experiments, neurites sprouting from the DRG were counted at 2, 4, and 6 days.

The numbers of neurites observed was decreased at 4 days post-culture in the above experiments (see Results). To determine whether this decrease was due to the lack of a target tissue, we seeded some constructs with cells from an established corneal epithelial cell line (25) to provide a target for the in-growing nerves.

Construction of optimized TE corneas

Human corneal cell lines with extended life spans (25, 26) were used. Optimized corneal constructs, designed using results from the previous section, were prepared with a laminin (Becton Dickinson) gradient of increasing concentrations from bottom to top of 0, 10, and 20 μg/ml. Stromal cells (keratocytes) were added to all three layers of the collagen mix, and NGF (100 ng/ml; Sigma) was added to the top layer of collagen mix. After thermogelling, epithelial cells were seeded onto the construct, which was supplemented with modified SHEM medium (27) containing 2% B27, 1% N2 nerve growth supplements (Life Technologies, Burlington, Canada), and 1 nmol/ml retinal acetate (Sigma). At epithelial confluence, the constructs were maintained at an air-liquid interface for up to 10 days until used.

To simulate nerve in-growth through the sclera into the cornea, we fabricated a pseudo-scleral ring within a 24-mm-diameter culture insert (Corning) from a 2:5 ratio of collagen to CS, cross-linked and post-treated as above. Isolated DRG were embedded within this pseudo-scleral ring. TE corneas were placed inside the scleral ring. The pseudo-sclera and TE cornea were thermogelled, epithelial cells were seeded on top, and the entire scleral/TE cornea construct was cultured as described above.

Immunohistochemistry

For sodium channel labeling, 4% paraformaldehyde (PFA)-fixed constructs were rinsed in 0.05 M Tris buffer, pH 7.4, and permeabilized in Tris buffer containing 0.3% Triton X-100. After blocking with 10% normal goat serum in buffer, tissues were incubated overnight at 4°C with monoclonal anti-PAN sodium channel antibody (Sigma; diluted 1:250 in Tris buffer containing 2% normal goat serum). Constructs were then rinsed in Tris buffer and reacted with goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR; 1:100 in Tris buffer) for 90 min.

For neurofilament staining, PFA-fixed constructs were permeabilized with a detergent mix (150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 50 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) for 20 min (28). They were then rinsed in Tris-buffered saline (TBS) and incubated with a monoclonal anti-neurofilament 200 (Sigma; diluted 1:40 in TBS containing 0.6% carrageenan and 0.3% Triton-X 100 [TCT]) overnight at 4°C. The constructs were then rinsed in TBS and incubated with a Cy3-conjugated goat anti-mouse antibody (1:200 in TCT; Amersham, Baie D'Urfé, Canada) for 150 min. Negative controls lacked the primary antibody. Positive controls included staining for DRG and neural tube explants. Nerves were visualized under fluorescence confocal microscopy.

Transmission electron microscopy

Samples were fixed overnight at 4°C in 1.25% glutaraldehyde/1% paraformaldehyde in 0.08 M cacodylate buffer (pH 7.4) and washed in 0.1 M sodium cacodylate. After post-fixation in 1% osmium tetroxide supplemented with 1.5% ferrocyanide in 0.1 M sodium cacodylate buffer (pH 7.4), pieces were dehydrated in a graded series of ethanol and

embedded in epoxy resin. The embedded samples were then heat-cured in resin and thin sectioned for observation using Philips EM 201 and CM 12 electron microscopes (Philips, Eidenhoven, The Netherlands).

Electrophysiology

TE corneas were transferred into interface recording chambers and perfused with artificial cerebral spinal fluid (aCSF; in mM): NaCl, 126; KCl, 3.0; $MgSO_4$, 2.0; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2.0; and dextrose, 10) oxygenated with 95% O_2/ % CO_2 at room temperature. Cathodal stimulation of ganglia was performed using silver wires pressed lightly against the ganglion surface and applying square wave stimulus pulses of 50 μs duration and typically 60–80 V in amplitude. Differential recordings of electrical responses from nerve fiber bundles were recorded with glass micropipettes (≈50 μm tips) filled with 150 mM NaCl. Because of the close proximity of the stimulation to the recording electrodes, a large stimulus artifact was generated that obscured the very small APs. To observe APs in isolation, evoked responses were recorded before and after addition of 50 mM lidocaine HCl, a sodium channel blocker that abolishes APs (29). Subtracting the responses in lidocaine from control responses yielded isolated APs with the stimulus artifacts largely removed. TE corneas without DRG served as controls. In these controls, cathodal stimulation was performed at sites where DRG would have been placed, and electrical responses were recorded from the epithelial surface as above.

Substance P assay

To elicit SP release from the nerve terminals, innervated TE corneas were treated with either capsaicin or veratridine, both shown previously to initiate this functional response

in corneal nerves (30, 31). A time series examining the effects of capsaicin was obtained by incubating innervated TE corneas with SHEM containing 8.5% Tween 80 and 1.5% ethanol alone (drug vehicle control) or with 1% (w/v) capsaicin for 0, 1, 3, 6, 12, and 24 h. After treatment, culture supernatants were collected, flash frozen in liquid nitrogen, and stored at –80°C. SP content of the medium was measured using a SP-specific competitive peptide enzyme immunoassay kit (Peninsula Laboratories, San Carlos, CA).

To investigate differential responses to different drugs, constructs were incubated with 1% (w/v) capsaicin, 50 μM veratridine, or drug vehicle alone. Culture medium was collected at 0, 6, and 24 h post-treatment, and SP content was measured as above. Data from two experimental sets of $n = 3$ was normalized (relative increase for each test group over the control at t=0). There were no significant differences between experimental sets (three-way ANOVA, P=0.27); therefore, results are based on the means of $n = 6$ from the two experiments.

Effects of innervation on epithelial and stromal cells

To investigate the effects of innervation on epithelial stratification and the growth and spread of keratocytes, we cultured constructs with and without DRG. TE corneas were fixed in 4% PFA at 2, 4, 6, and 10 days. To examine epithelial stratification, we sectioned samples and processed them for routine hematoxylin and eosin (H&E) staining (27). The number of cell layers was counted from six random sections (three sections from two separate slides) for each sample, and the mean was calculated for each time point. Stromal cell growth was measured by counting keratocytes in two random fields per whole mount sample and calculating the mean number of keratocytes per mm^2 for each time point. Only cells that had spread were counted; those that remained spherical were omitted. Initial spherical keratocyte

numbers (mean ± SE) of innervated (16.92±0.68/mm^2) and non-innervated constructs (17.33±0.28/mm^2) were not significantly different (n=6; t test, P=0.58).

Wound healing

Epithelial wounds were made by applying a filter paper disc to the epithelium and lifting off to remove underlying epithelial cells. Scanning electron microscopy confirmed the effectiveness of this process. Wound closure (re-epithelialization) of TE corneas was determined morphometrically (BioRad Quantity One software, Mississauga, Canada). Initial wound areas (mean ± SE) of innervated (6.68±0.21 mm^2) and non-innervated constructs (7.02±0.17 mm^2) were not significantly different (n=16; t test, P=0.21). The wound healing rate at each time point was calculated as area of re-epithelialized wound/mm of original wound circumference.

Bromodeoxyuridine (BrdU) assay

BrdU labeling was performed as described previously (32), with modifications. In brief, TE corneas were incubated with BrdU labeling reagent (Roche, Laval, Canada) for 1 h at 0, 6, and 24 h after epithelial wounding (as described above) and then fixed in PFA. Constructs were then digested with 0.3 mg/ml pepsin in 0.1 M HCl, rinsed in TBS, treated with 2 M HCl, and rinsed again. Constructs were then incubated with an anti-BrdU monoclonal antibody (Calbiochem, San Diego, CA; 2 μg/ml) overnight at 4°C and treated for 1 h each with biotinylated goat anti-mouse antibody (Amersham; 1:100 in TCT) and ABC reagent (Vector laboratories, Burlington, Canada). Staining was visualized by incubation in a 0.1% solution of 3,3′-diaminobenzidine for 8 min. Controls omitted the primary antibody. Counting the number of epithelial cells with BrdU-labeled nuclei within five different areas

per construct and dividing by the total number of cells, then multiplying by 100, determined the percentage of proliferating epithelial cells.

RESULTS

Exogenous factors and neurite outgrowth

Three exogenous factors, laminin, NGF and RA, were tested independently for their effects on neurite outgrowth from the DRG into the stromal matrix of the TE cornea. A laminin concentration of 20 μg/ml was superior in promoting neurite outgrowth over a 6-day culture period (Fig. 1A). Following this, the creation of a three-stepped laminin gradient within the stroma—from 0 μg/ml in the basal, 10 μg/ml in the middle, and 20 μg/ml in the uppermost layer—was successful in guiding the growth of nerve processes upward through the stroma into the overlying epithelium. Tested separately, the addition of exogenous NGF to the corneal matrix produced an increase in neurite outgrowth (Fig. 1B). The greatest number of neurites extended from the DRG into the cornea when at least 100 ng/ml of NGF was added.

Also tested independently, the addition of RA to the culture medium had no significant effect on neurite extension from the DRG into the TE cornea (data not shown). Within the matrix, 1 nmol/ml RA and both the two- and three-step concentration gradients promoted greater neurite extension at 2 days compared with 2 nmol/ml RA, but not compared with 0 nmol/ml RA (Fig. 1C). There were no differences in the ability to promote nerve growth between the two RA concentration gradients and 1 nmol/ml RA. There was a trend for 1nmol/ml of RA to increase the number of neurites extending into the matrix in both experiments. Also, the number of neurites in constructs with 1 nmol/ml RA added to the

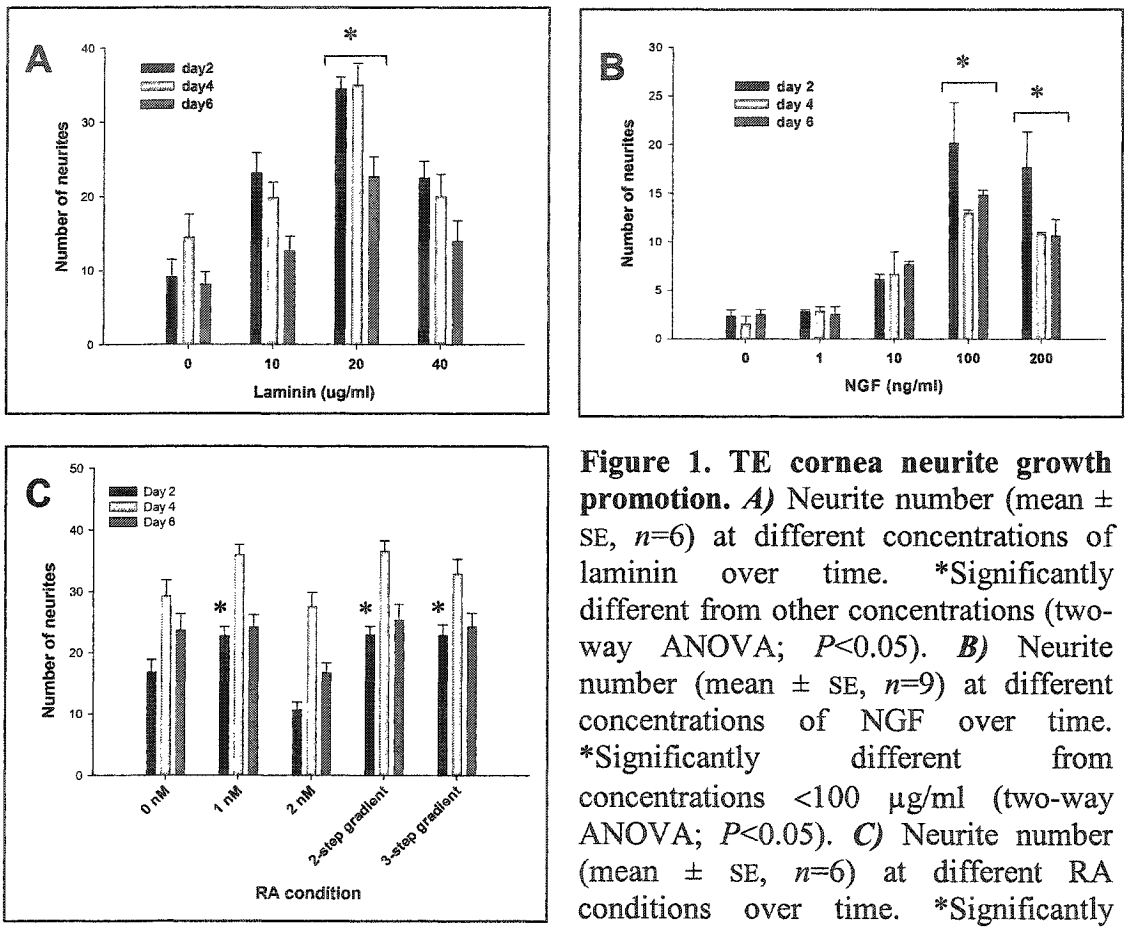

Figure 1. TE cornea neurite growth promotion. ***A)*** Neurite number (mean ± SE, n=6) at different concentrations of laminin over time. *Significantly different from other concentrations (two-way ANOVA; P<0.05). ***B)*** Neurite number (mean ± SE, n=9) at different concentrations of NGF over time. *Significantly different from concentrations <100 µg/ml (two-way ANOVA; P<0.05). ***C)*** Neurite number (mean ± SE, n=6) at different RA conditions over time. *Significantly different from 2 nmol/ml concentration at 2 days (two-way ANOVA; P<0.05).

medium (n=18) or the matrix (n=6) was not statistically different at any time point (t test, P=0.70, 0.27, and 0.59 for day 2, 4, and 6, respectively). Therefore, based on these results, a combination of the three-stepped laminin gradient, 100 ng/ml of NGF, and 1 nmol/ml of RA (in the culture medium) was used in all subsequent experiments.

Nerve morphology and epithelial innervation

Whole mounts of anti-neurofilament antibody-stained TE corneas (Fig. 2A) showed nerve bundles originating from encapsulated DRG within the pseudo-sclera, penetrating the cornea, and progressing upward into the overlying epithelium (Fig. 2B). Stromal nerve bundles (Fig. 2C) bifurcated with successively finer branches to form a plexus (Fig. 2D) below the basal epithelial cells. Many bundles of this nerve plexus ran parallel to each other with bifurcations occurring at near right angles. Both beaded (Fig. 2E) and smooth (Fig. 2F) nerve fibers from the sub-epithelial network proceeded to target and migrate within the epithelium. Transmission electron microscopy (TEM) showed that nerve fibers within the epithelium contained many small clear and large dense core secretory vesicles (Fig. 2G). These fibers were also observed to invaginate individual epithelial cells (Fig. 2H).

Generation of nerve action potentials

Antibodies against sodium channels and neurofilaments showed co-localization of these structures within nerve fibers of TE corneas (Fig. 3A, 3B, and 3C). Electrical stimulation of DRG resulted in the generation of action potentials (APs) (Fig. 3D, 3E). Because of the close proximity of the stimulating to the recording electrodes, a large stimulus artifact was generated that obscured the small APs (Fig. 3D). Addition of 50 mM lidocaine HCl and subtracting lidocaine from control responses yielded isolated APs (Fig. 3E).

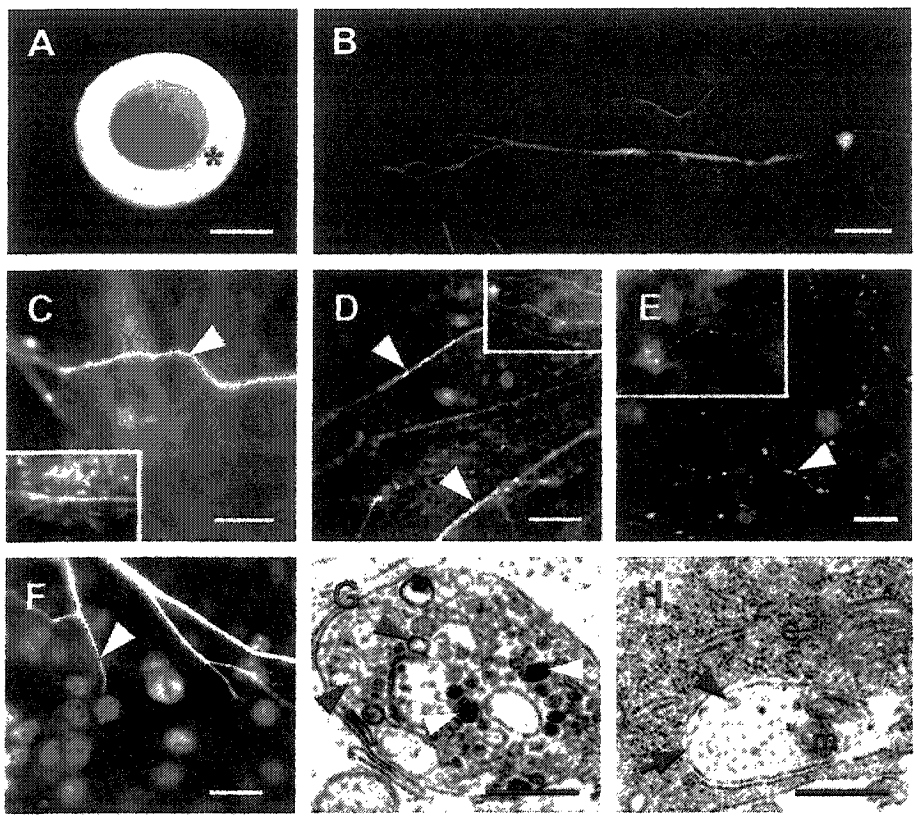

Figure 2. Morphology of nerve growth in the TE cornea. ***A)*** Transparent TE cornea with surrounding ring of opaque collagen (*). ***B)*** Top view shows progression of a nerve bundle from the plexus toward the epithelial surface, with bifurcations within the epithelium: red, 60 μm; green, 40 μm; and blue, 20 μm from the surface. ***C)*** Nerves (arrowhead) extending in the TE cornea stroma resemble those of the human corneal matrix (inset). ***D)*** Nerves (arrowheads) within the nerve plexus below the basal epithelial cells. Note the parallel growth pattern, which resembles that of the native human cornea (inset). ***E)*** Beaded nerve fibers (arrowhead) were observed within the epithelium similar to those of the human cornea (inset). ***F)*** Smooth nerve fibers (arrowhead) in the epithelium. ***G)*** A single nerve fiber terminal within the epithelium showing dense (white arrowheads) and clear (dark arrowheads) vesicles. ***H)*** An individual nerve fiber (arrow) invaginating an epithelial cell (e). Mitochondria (m), vesicles (arrowhead), and microtubules can be seen. Scale bar, 1 cm (*A*); 10 μm (*B*, *C*); 20 μm (*D*); 10 μm (*E*, *F*); 0.3 μm (*G*); 0.5 μm (*H*).

Controls consisted of recordings from TE corneas without DRG, before and after the application of aCSF perfusion medium. Subtraction of pre- and post-aCSF treatment responses abolished the stimulus artifact recordings (Fig. 3F), which demonstrated that the addition of fluid did not generate a response that would mistakenly be interpreted as an AP.

Substance P neurotransmitter release

Innervated corneas show unstimulated, baseline SP release (Fig. 4A). To test the ability of the nerves within the TE cornea to respond to chemical stimuli by releasing SP, we treated constructs with capsaicin and veratridine. Capsaicin is a neurotoxin that depletes SP from sensory nerves (30), and veratridine is a specific activator of voltage-gated sodium channels. Capsaicin treatment significantly increased SP release over baseline levels at all time points post-treatment (Fig. 4A). Differential SP release was seen when levels of the neuropeptide were compared among constructs treated with capsaicin, veratridine, or drug vehicle only (Fig. 4B). At 6 h post-treatment, only capsaicin elicited an increase in SP release, whereas at 24 h, both capsaicin and veratridine elicited increases compared with controls.

Interaction between nerves and their target tissue

At 6 days, there was a decrease in the number of neurites in the laminin experiments (Fig. 1A) compared with earlier days. A decrease in the number of neurites was also observed in the NGF experiments at 4 days (Fig. 1B). When epithelial cells were added to TE corneas with laminin (20 μg/ml) or NGF (100 ng/ml), no reduction in neuronal outgrowth was observed through 6 days of culture (Fig. 5A, 5B).

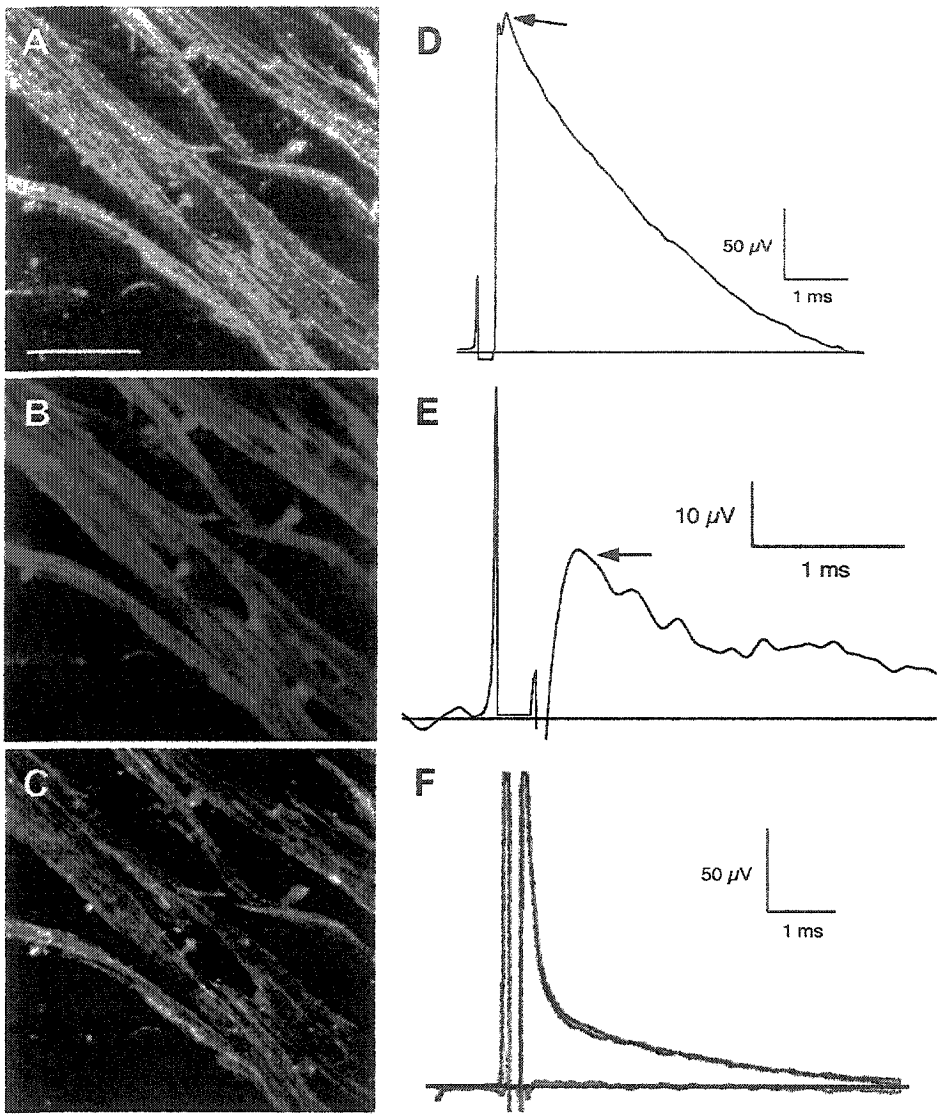

Figure 3. TE corneal nerve sodium channels and action potentials. *A)* Sodium channels (green) are present in the nerve fibers. *B)* The sodium channel staining colocalized with neurofilament staining (red). *C)* Composite image of *A* and *B*. *D)* Example of a raw, unsubtracted trace evoked by a constant voltage stimulus pulse delivered to the ganglion cell cluster, illustrating a large stimulus artifact, with a small AP occurring near the top (arrow). *E)* Subtraction of the response obtained after lidocaine application reveals a compound AP in isolation (arrow), with short latency and an amplitude of ~26 μV. *F)* Subtraction of responses (black) before and after artificial cerebrospinal fluid application from constructs without DRG results in the abolition of the response (red). Scale bar, 10 μm (*A–C*).

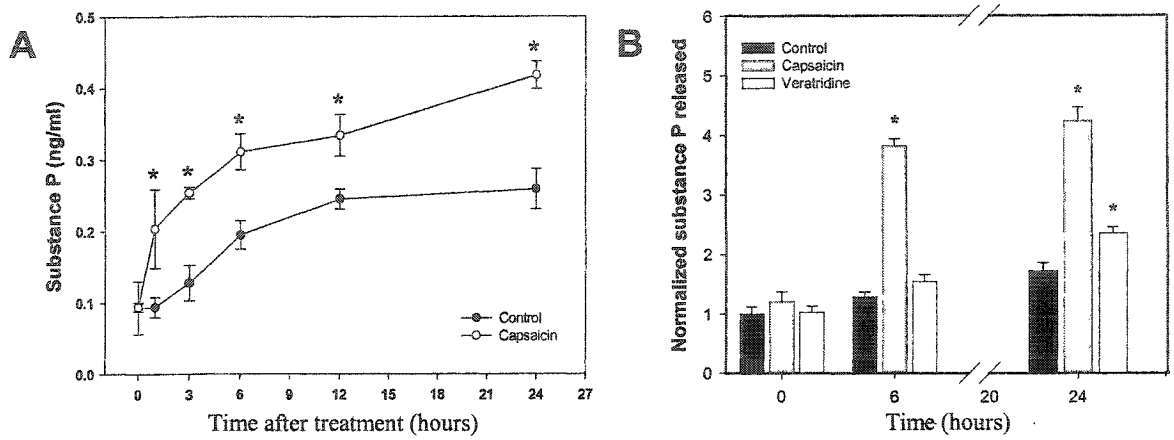

Figure 4. Substance P release. *A)* SP release (mean ± SE, n=3) from innervated constructs in response to capsaicin. *Significantly different from control at all time points after capsaicin (1%) treatment (two-way ANOVA; P<0.001). *B)* Substance P release (mean ± SE, n=6) from innervated corneas, shown as relative increase over control, after treatment with 1% capsaicin or 50 μM veratridine. *Significantly different from control at that time point (three-way ANOVA; P<0.05).

Innervation stimulated epithelial and keratocyte growth in TE corneas. After 2 days of culture, there was no difference in the number of epithelial cell layers between innervated and non-innervated TE corneas. However, at 4, 6, and 10 days of culture, a greater number of cell layers (≈twofold greater after 10 days) was observed in the epithelium of innervated TE corneas compared with non-innervated controls (Fig. 5C). The number of keratocytes in innervated constructs was greater than in non-innervated controls at all time points (Fig. 5D). After 10 days of culture, the number of keratocytes in innervated TE corneas was almost twofold greater.

In TE corneas with epithelial wounds, wound closure during the first 18 h was faster in innervated constructs than in non-innervated controls (Fig. 5E). By 24 h, however, wound closure rates had equalized. Incorporation of BrdU (a mitotic indicator) at 0, 6, and 24 h after wounding revealed an overall higher percentage of labeled, dividing epithelial cells (≈2.5-fold higher) in innervated constructs (Fig. 5F). Although there is a difference in proliferation

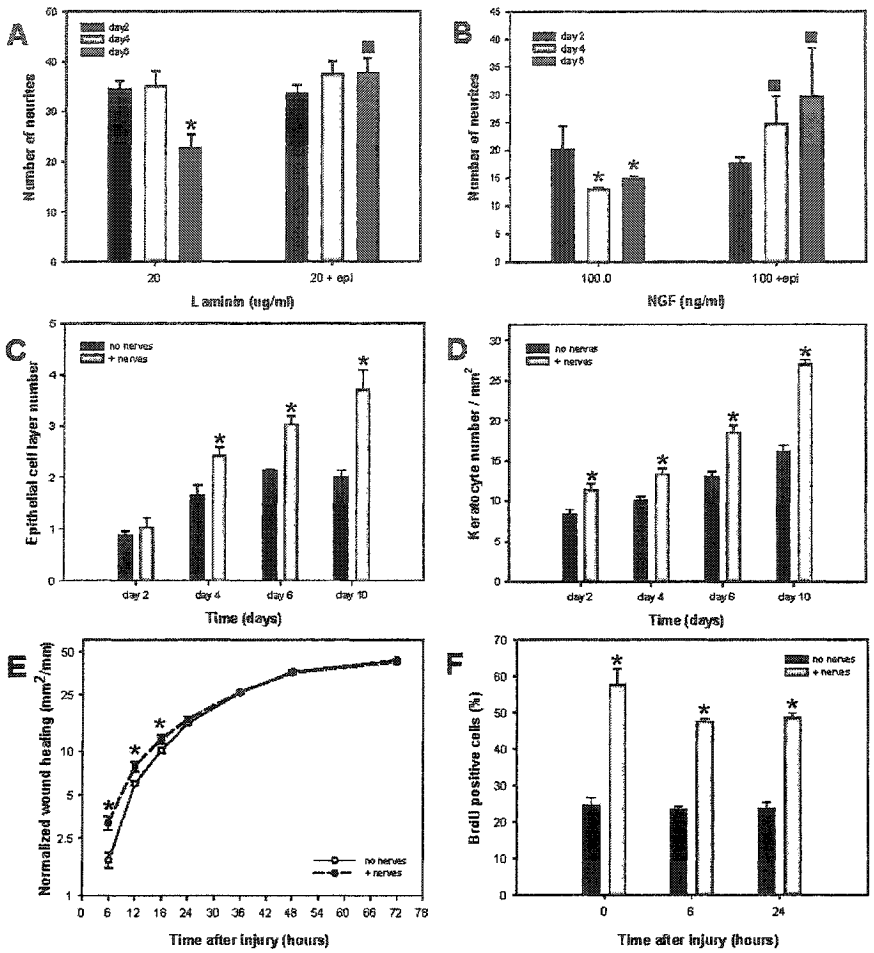

Figure 5. Nerve-target cell interaction. ***A)*** Neurite number (mean ± SE, n=6) over time in constructs with 20 μg of laminin with and without the addition of epithelial cells. *Significantly different from control at day 2 and 4 (two-way ANOVA; P<0.05). ▪Significantly different from control without epithelial cells at day 6 (two-way ANOVA; P<0.05). ***B)*** Neurite number (mean ± SE, n=9) over time in constructs with 100 ng/ml of NGF with and without epithelial cells. *Significantly different from control at day 2 (two-way ANOVA; P<0.05). ▪Significantly different from control without epithelial cells at day 4 and 6 (two-way ANOVA; P<0.05). ***C)*** The number of epithelial cell layers (mean ± SE, n=3) for innervated and non-innervated TE corneas over time. *Significantly different from non-innervated control at day 4, 6, and 10 (two-way ANOVA; P<0.05). ***D)*** The number of keratocytes that had spread within the matrix (mean ± SE, n=6) for innervated and non-innervated TE corneas over time. *Significantly different from non-innervated control at all time points (two-way ANOVA; P<0.05). ***E)*** Normalized wound healing (mean change in wound area (mm^2)/original wound circumference (mm) ± SE, n=16) for innervated and non-innervated TE corneas. *Significantly different from non-innervated control at 6, 12, and 18 h (two-way ANOVA; P<0.05). ***F)*** The percentage of epithelial cells that are dividing (BrdU positive; mean ± SE, n=3) in innervated vs. non-innervated TE corneas. *Significantly different from non-innervated control at all time points (t test; P=0.001).

between the innervated and non-innervated groups, there were no changes in cell proliferation within either group over the first 24 h after wounding.

DISCUSSION

We have developed an in vitro cornea model for studying innervation and nerve-target cell interactions and have demonstrated accurate, key morphological and functional aspects of the nerves within this model. Innervation of the TE cornea was achieved by enhancing the matrix environment to support the in-growth of nerves. Laminin has been used previously in three-dimensional agarose gels and polymer substrates to enhance neurite outgrowth and extension from chick and rat DRGs (33, 34). The addition of laminin to the present collagen-based matrix promoted extension of nerves from the DRG within the scleral scaffold into the TE cornea. Addition of NGF further enhanced nerve extension into the matrix.

The addition of RA showed a trend toward enhancing neurite extension, but this was not statistically significant, although it has previously been shown to induce neurite outgrowth from embryonic mouse DRG cells cultured in the presence of NGF (24). Also, in addition to its role in stimulating neurite number and length (35), RA has been shown to induce the differentiation of neurons (36) and increase neurite responsiveness to laminin (37). So, although the addition of RA alone did not significantly enhance neurite outgrowth, it is possible that RA in combination with NGF and/or laminin may play a role in neurite growth into the complete TE cornea. On the other hand, a combination of only laminin and NGF has been shown to be sufficient to promote the bridging of peripheral sciatic nerve gaps in adult rats (38).

In the cornea, sensory nerve bundles originating from the trigeminal ganglia penetrate the anterior stroma from the surrounding sclera (39). These bundles bifurcate before penetrating Bowman's layer at various locations to form a dense network below the basal epithelial cells and establish terminals or nociceptors in the outer layers of the epithelium (40–42). DRG substitutes for trigeminal ganglia produced a similar morphology in the TE cornea. Nerves within the epithelium of human corneas consist of both beaded and smooth fibers, and nerve growth below the epithelium has been described as parallel running nerve fibers that connect and bifurcate (41). Parallel nerve growth patterns were observed below the epithelium, and both smooth and beaded nerve fibers were seen within the epithelium of the TE cornea.

Characteristic of sensory nerves in the cornea, TEM revealed that the nerve fiber terminals in the TE cornea contained numerous mitochondria, glycogen particles, ribosomes, and the presence of both small clear and dense core vesicles (42). The TEM results also demonstrate that nerve fibers invaginated individual epithelial cells in the TE cornea as previously demonstrated in human corneas (42), suggesting that some epithelial cells are directly innervated. Direct epithelial innervation might help modulate corneal homeostasis and responses to insult or injury. The present TE cornea model may be used to elucidate molecular and cellular signal mechanisms between sensory nerves and their directly innervated target cells. To our knowledge, a model for studying such a relationship has not previously been described.

Upon stimulation of the nociceptive nerve endings supplying the corneal epithelium, action potentials (APs) are generated, which propagate to the central nervous system to cause the sensation of pain (43), and to the nerve terminals within the epithelium to trigger

neuropeptide release (44). Sodium channels are integral to the generation of nerve APs. Using specific immunofluorescence, we demonstrated the co-localization of sodium channels and neurofilaments in the nerve fibers of the TE corneas. This indicated that the axons possess some of the machinery necessary to be excitable and functional. APs can trigger the release of neuropeptides, such as SP, from sensory nerve terminals, which are necessary for axon reflexes (45). Further data supporting the notion that the sodium channels are functional was obtained from the analysis of SP release in constructs exposed to veratridine, a specific activator of voltage-gated sodium channels. Veratridine induced SP release exceeding baseline leakage, indicating that fibers possess functional sodium channels in sufficient densities to trigger the release of this neuropeptide. Moreover, direct electrophysiological recording from the surface of the corneas confirmed that ingrown nerve bundles were able to conduct lidocaine-sensitive APs evoked by stimulation of the ganglion cell cluster. These APs exhibited a configuration and amplitude similar to those recorded from native guinea-pig cornea polymodal nociceptors (43), the most abundant class of neuron found in the cornea (46).

The neurotoxin capsaicin selectively depletes SP from peripheral nerve terminal stores of thin fiber sensory nerves (30). These are the types of sensory nerves found within the corneal epithelium (46, 47). In the present study, capsaicin treatment elicited a marked increase in SP release from nerves in TE corneas. Capsaicin causes SP release by an AP-independent mechanism that is not fully understood (48). Veratridine, described above, causes SP release from nerve terminals by opening sodium channels and depolarizing the membrane (31). Therefore, both sodium channel-dependent and -independent mechanisms of SP release were likely observed in our innervated cornea model. SP has been shown to exert

trophic effects on corneal epithelial cells (14, 17, 49). Therefore, the ability of nerves in the TE cornea to differentially release SP suggests that these nerves may influence epithelial properties by the release of this neuropeptide.

We demonstrate that this model could provide an excellent in vitro system for investigating the complex pathways of communication between nerves and their target tissues. While higher laminin and NGF concentrations promoted increased initial neurite outgrowth, the number or extending axons decreased with time. This loss of nerve outgrowth could be attributed to a lack of target tissue (22) because the addition of epithelial cells to the surface of the TE cornea reversed this trend. This suggests that the target epithelial cells were essential for neuronal survival. This contention is further supported by reports that corneal epithelial cells produce and release neurotrophic factors, including NGF (21, 50) that may contribute to axonal survival.

While target tissues promote the survival of nerves, the sensory nerve supply, in return, influences phenotypic characteristics important to the maintenance of a healthy tissue. Decreased stratification of the epithelium was observed in non-innervated TE corneas. This is similar to in vivo effects of trigeminal denervation, where thinning of the corneal epithelium and the exfoliation of epithelial cells is observed in denervated eyes (51). These phenotypic changes of the corneal epithelium associated with interruption of the sensory nerves are attributed to decreased proliferation, decreased migration, and anatomic disorganization of the tissue (13).

In addition to their effects on the epithelium, sensory nerves have been observed in close association with fibroblasts (52), and their neuropeptides, including SP, have been shown to promote fibroblast proliferation in several tissues (53–55). Keratocytes, which are

specialized corneal fibroblasts, have also been observed to be directly targeted by SP-containing corneal nerve fibers (42) in the human cornea. Consistent with these observations, the number of keratocytes was greater in innervated TE corneas in the present study. Therefore, as expected, the presence of nerves had an effect on the properties of both the epithelial and stromal cells in the TE cornea.

The presence of nerves in TE corneas promoted the proliferation of epithelial cells, a result consistent with previous in vitro rabbit studies (14). In addition, the presence of nerves allowed for higher wound closure rates during the first 24 h of healing. This early-enhanced resurfacing of the wound could be attributable to increased pressure exerted as a result of the increased epithelial cell proliferation in the innervated TE corneas, a phenomenon described previously in mouse corneal epithelium (56). Therefore, the initial higher rate of wound closure appears to result from an overall more actively migrating or sliding population of epithelial cells rather than from an actual increase in post-wounding proliferation, in keeping with previous reports that increased epithelial cell proliferation begins in the wound area only 24 h after wounding (57). Previous in vivo rabbit studies have also shown that denervation slows down wound healing of the corneal epithelium, with an overall delay to full wound closure (4, 9). This wound closure delay was not observed in our model, likely due to the differential stratification of the epithelium. As described earlier, the stratification of the epithelium of innervated TE corneas was greater than in non-innervated controls. It has been shown that as epidermal epithelial cells stratify and differentiate, their ability to migrate can decrease by as much as 50% (58) due to changes in their adhesive properties (59). Therefore, in the present study, cells from the less stratified epithelium of non-innervated constructs may have an increased ability to migrate, thus explaining the equal time for wound closure

between innervated and non-innervated constructs, despite the initial enhanced wound closure of the innervated TE corneas.

At present, the Draize test and the low volume eye test are rabbit ocular irritancy tests (60) used for measuring the potential ocular toxicity of manufactured consumer products. Our model of an innervated, functional cornea could provide an alternative to animal ocular toxicity testing. Differences in ocular irritancy are related to differences in the extent of initial injury (61). We observed differential responses of TE corneas to capsaicin, veratridine, and drug-vehicle-only treatment, as measured by SP release. With further refinement of our model, the degree of cell death, the levels of neurotransmitter release, and the AP response may in the near future be used to ascertain the toxicity of potential ocular irritants.

In summary, we report the development of an in vitro TE cornea complete with functional innervation and demonstrate nerve-target cell interaction. This model provides a useful tool for studying corneal innervation and cell-cell interactions, with the advantage of a controlled environment and natural transparency for imaging. Although the focus of this work was on the cornea, these results may have broader implications for the field of tissue engineering by providing new insight into nerve regeneration and the innervation of other engineered organ or tissue systems designed for transplantation, both major goals in tissue engineering.

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2.2 Summary

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INNERVATED HUMAN CORNEAL EQUIVALENTS AS *IN VITRO* MODELS FOR NERVE-TARGET CELL INTERACTIONS

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SPECIFIC AIMS

A sensory nerve supply is crucial for optimal tissue function. Since the mechanisms for successful innervation and the signaling pathways between nerves and their target tissue are not fully understood, we developed engineered tissue substitutes in which to study tissue innervation and the associated interactions between nerves and their targets.

PRINCIPLE FINDINGS

1. Exogenous factors and in-growth of nerve fibers

Innervated tissue engineered (TE) corneas (Fig. 1*A*) were fabricated by making modifications to a corneal tissue equivalent we previously developed. Optimal concentrations of exogenous laminin and nerve growth factor (NGF) were added to the construct after being tested individually without corneal cells for their ability to promote axonal growth from chick embryo dorsal root ganglia (DRG). The creation of a 3-stepped laminin gradient within the stroma, and the addition of NGF to the uppermost layer of corneal matrix were successful in guiding and promoting the growth of nerve processes.

2. Nerve morphology and epithelial innervation

Immunofluorescence with a nerve-specific neurofilament antibody revealed nerve morphology that was similar to that of the natural human cornea. Nerve bundles originating from DRG within the scleral scaffold penetrated the cornea. These then progressed through the corneal stroma and into the overlying epithelium (Fig. 1*B*). Stromal nerve bundles (Fig. 1*C*) bifurcated to form a plexus (Fig. 1*D*) below the basal epithelial cells. Both beaded (Fig. 1*E*) and smooth (Fig. 1*F*) nerve fibers from the sub-epithelial network proceeded to target the epithelium. Transmission electron microscopy (TEM) revealed small fiber bundles within the basal epithelial layer. From these bundles, single nerve fibers, containing secretory vesicles

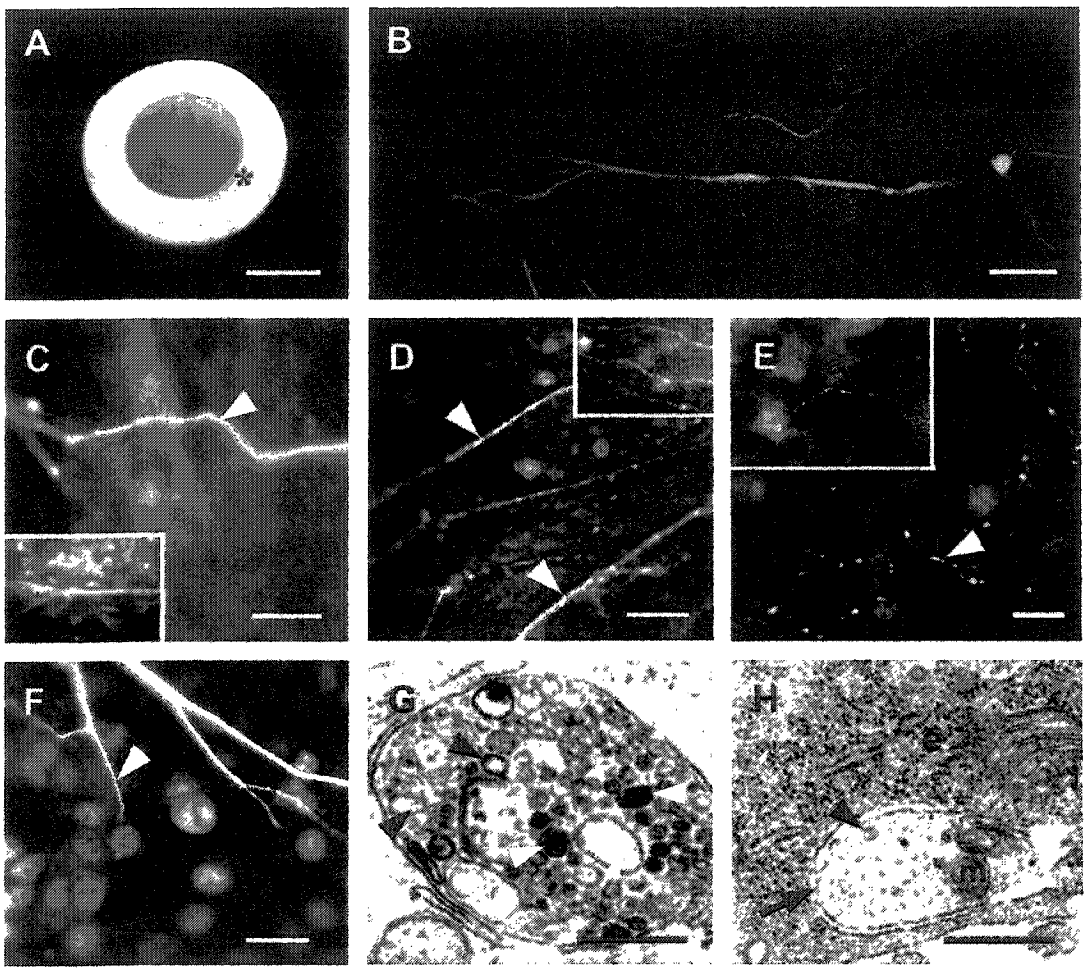

Figure 1. Morphology of nerve growth in the TE cornea. *A)* Transparent TE cornea with surrounding ring of opaque collagen (*). *B)* Top view shows progression of a nerve bundle from the plexus toward the epithelial surface, with bifurcations within the epithelium: red, 60 μm; green, 40 μm; and blue, 20 μm from the surface. *C)* Nerves (arrowhead) extending in the TE cornea stroma resemble those of the human corneal matrix (inset). *D)* Nerves (arrowheads) within the nerve plexus below the basal epithelial cells. Note the parallel growth pattern, which resembles that of the native human cornea (inset). *E)* Beaded nerve fibers (arrowhead) were observed within the epithelium similar to those of the human cornea (inset). *F)* Smooth nerve fibers (arrowhead) in the epithelium. *G)* A single nerve fiber terminal within the epithelium showing dense (white arrowheads) and clear (dark arrowheads) vesicles. *H)* An individual nerve fiber (arrow) invaginating an epithelial cell (e). Mitochondria (m), vesicles (arrowhead), and microtubules can be seen. Scale bar, 1 cm (*A*); 10 μm (*B*, *C*); 20 μm (*D*); 10 μm (*E*, *F*); 0.3 μm (*G*); 0.5 μm (*H*).

(Fig. 1*G*), branched and established terminals in the epithelium. As described for natural human corneas, terminal nerve fibers were observed invaginating individual epithelial cells (Fig. 1*H*).

3. Generation of nerve action potentials

Antibodies against sodium channels and neurofilaments showed co-localization within nerve fibers of the TE corneas. To determine if these sodium channels could carry action potentials (APs), DRG were electrically stimulated. This electrical stimulation resulted in the generation of APs, which were recorded from nerve terminals at the surface of the epithelium.

4. Substance P neurotransmitter release

Upon axonal stimulation, the neuropeptide SP is released from sensory nerve terminals in the epithelium of the cornea. To elicit a functional response such as SP release, innervated TE cornea constructs were treated with capsaicin or veratridine, both shown previously to cause SP release from corneal nerves. At all time points post-treatment, capsaicin elicited an increase in SP release over baseline leakage observed in controls (Fig. 2*A*). Differential SP release was observed between innervated corneal constructs treated with capsaicin, veratridine or drug vehicle only (Fig. 2*B*). At 6 hours post-treatment, only capsaicin elicited an increase in SP release, whereas at 24 hours, both capsaicin and veratridine elicited increases compared to controls.

5. Interaction among nerves and their target tissue

In testing the effects of laminin and NGF, a significant decrease in the number of neurites was observed over time. To test the possibility that this loss may be associated with the lack of a target tissue, epithelial cells were seeded onto the surface of the corneal matrix.

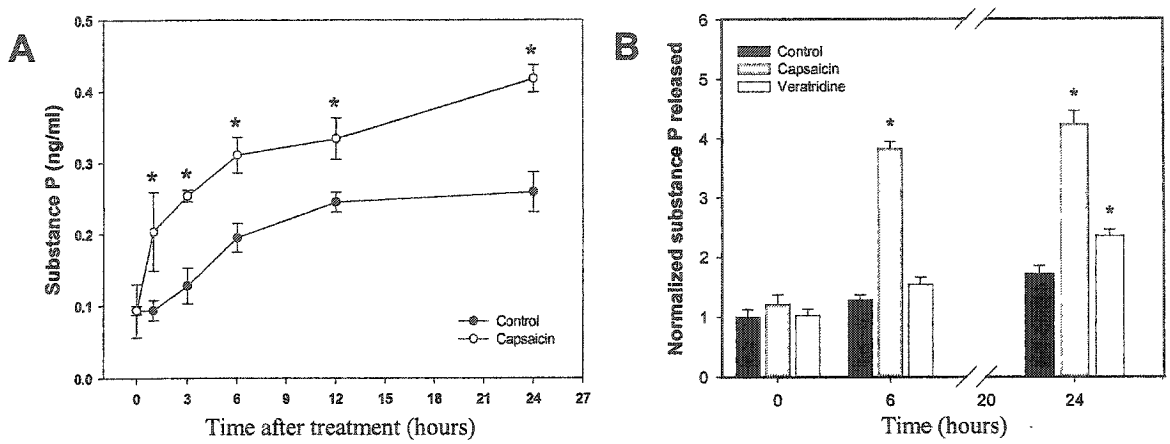

Figure 2. Substance P release. ***A)*** SP release (mean ± SE, n=3) from innervated constructs in response to capsaicin. *Significantly different from control at all time points after capsaicin (1%) treatment (two-way ANOVA; P<0.001). ***B)*** Substance P release (mean ± SE, n=6) from innervated corneas, shown as relative increase over control, after treatment with 1% capsaicin or 50 μM veratridine. *Significantly different from control at that time point (three-way ANOVA; P<0.05).

With the addition of epithelial cells, no reduction in neuronal outgrowth was observed over the same culture period.

Sensory innervation is important for optimal tissue function and is known to promote proliferation and to enhance wound healing of its target tissue. The presence of nerves in the TE cornea promoted greater epithelial stratification (up to 2-fold) and increased the number of keratocytes present within the stroma (approximately 2-fold) compared to non-innervated controls. To test the effect of nerves on wound healing in our system, epithelial wounds were created in TE corneas constructed with and without nerves, and wound closure rates were measured. During the first 18 hours, wound closure was faster in innervated corneal constructs, but had equalized by 24 hours. Incorporation of bromodeoxyuridine (BrdU, a mitotic indicator) at 0, 6 and 24 hours after wounding revealed that proliferation in innervated constructs was approximately 2.5 fold greater in innervated constructs at all time points. This demonstrated that the presence of nerves in the TE cornea had a positive influence on the proliferation of epithelial cells and early epithelial wound healing.

Conclusions

We have developed an *in vitro* model for studying innervation and nerve-target cell interactions; and have demonstrated accurate key morphological and functional aspects of the nerves within this model. While the addition of laminin and NGF promoted increased initial neurite outgrowth from the DRG into the TE cornea, the number of extending axons decreased over time. This loss of nerve outgrowth could be attributed to a lack of target tissue since the addition of epithelial cells to the TE cornea reversed this trend. This suggests that the target epithelial cells were essential for neuronal survival.

While target tissues promote the survival of nerves, the sensory nerve supply, in return, influences phenotypic characteristics important to the maintenance of a healthy tissue. As in the human cornea, TEM results demonstrate that nerve fibers invaginated individual epithelial cells in the TE cornea. Direct innervation might help modulate corneal responses to insult or injury. Also, the presence of nerves in TE corneas promoted proliferation and enhanced the stratification of epithelial cells, and resulted in a greater number of keratocytes within the stroma. In wounded TE corneas, innervation allowed for significantly higher wound closure rates during the first 24 hours of healing compared to non-innervated controls. However, there was no difference in the time for complete wound closure. The early-enhanced resurfacing of the wound in the innervated TE corneas could be attributable to increased pressure exerted as a result of the increased epithelial cell proliferation, a phenomenon also observed in mouse corneal epithelium. The lack of a difference in the time for complete wound closure was likely due to the differential stratification of the epithelium. It has been shown that as epithelial cells stratify, their ability to migrate can decrease by as much as 50%. Therefore, cells from the less stratified epithelium of non-innervated

constructs may have an increased ability to migrate, allowing for equal time for full wound closure, despite the initial enhanced wound closure of innervated TE corneas. To our knowledge, a 3-D model for studying the relationship between nerves and their target cells has not been developed. This model may provide an excellent *in vitro* system for investigating the complex pathways of communication between nerves and their target tissues.

Direct electrophysiological recording confirmed that in-grown nerve bundles were able to conduct APs. These APs exhibited a configuration and amplitude similar to those recorded from native guinea-pig cornea polymodal nociceptors, the most abundant class of neuron found in the cornea. The generation of APs, which can trigger the release of SP, is important to corneal nerve function in the epithelium. SP stimulates both corneal epithelial cell proliferation and migration and enhances wound healing. We triggered SP release in our innervated TE corneas by treatment with capsaicin and veratridine. Capsaicin is a neurotoxin that depletes SP from nerve terminal stores by an action potential-independent mechanism. Veratridine, however, causes SP release from nerve terminals by opening sodium channels and depolarizing the membrane. Both sodium channel-dependent and independent mechanisms of SP release were observed in our model. Furthermore, differential levels of neuropeptide release in response to treatment with capsaicin, veratridine or drug vehicle may suggest that the nerves are able to distinguish among different chemicals. Similar to native nerve processes, the present results show that nerves growing into our TE corneas respond to chemical stimuli and contribute to the maintenance of the tissue.

In the short term, these functional, innervated corneas could be used as research models, or as *in vitro* alternatives to animal-based eye tests for chemical, or drug safety and

efficacy testing. At present, the Draize, and the low volume eye test are rabbit ocular irritancy tests used for measuring potential ocular toxicity. Differences in ocular irritancy are related to differences in the extent of initial injury. The degree of cell death, the levels of neurotransmitter release and the AP response could all be used to ascertain the toxicity of potential ocular irritants in our model.

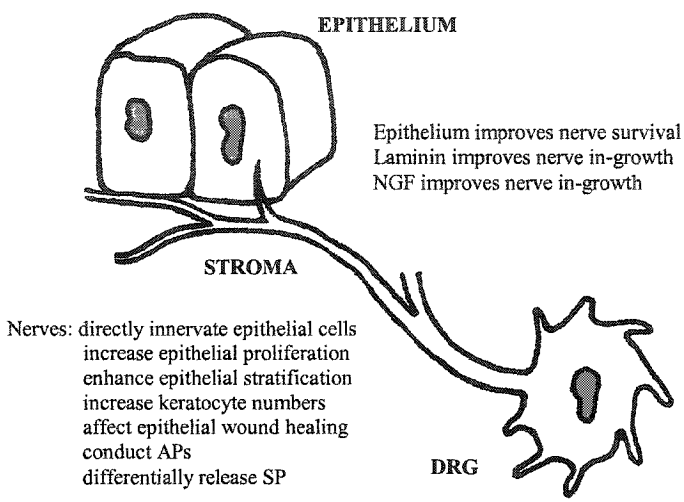

Figure 3. Schematic Diagram

In summary, we report the development of an *in vitro* tissue engineered cornea complete with functional innervation, and demonstrated nerve-target cell interaction. This model provides a useful tool for studying corneal innervation and cell-cell interactions, with the advantage of a controlled environment and natural transparency for imaging. Although the focus of this work was on the cornea, these results may have broader implications for the field of tissue engineering by providing some insight into nerve regeneration and the innervation of other engineered organ or tissue systems designed for transplantation, both major goals in tissue engineering.

CHAPTER 3

3.1 Manuscript 2

Innervation in the development of tissue engineered models for in vitro study and testing purposes

Prepared for future submission

While the previous manuscript described a three-dimensional cornea model for the investigation of nerve-target cell interactions, its potential for use in testing ocular irritancy was only alluded to. Therefore, after the development of the model and its functionality confirmed, the potential for its use in sensitivity testing was assessed. Additional properties of the TE cornea that would be important for its use as a model for the toxicology testing of drugs and consumer products were investigated. In addition, the responses of the nerves themselves to treatment with different chemicals were also examined within the TE cornea using 2-photon microscopy techniques. These results are presented in the following manuscript, providing evidence that this model, and specifically the inclusion of innervation, significantly advances the mission to develop an in vitro alternative to animals for toxicological assessment.

FUNCTIONAL INNERVATION IN TISSUE ENGINEERED MODELS FOR IN VITRO STUDY AND TESTING PURPOSES

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Running title: Innervation in Tissue Engineering

Summary

The biotech industry is rapidly expanding and the emerging field of tissue engineering is projected to have a high impact in the near future. Recently the field of cellular, drug, and prosthetic delivery has melded with the field of tissue engineering (TE) to make simulated tissues. In addition to their roles as tissue substitutes for transplantation, these simulated tissues may provide more accurate models and environments for toxicology testing and the study of nerves. The current study demonstrates the importance of innervation, in general, for the function of engineered tissues. We observe that the presence of nerves in a TE human cornea model enhances the growth of the epithelium and the formation of its protective mucin layer. Innervation also confers protection to the epithelium from chemical insult, as determined by the level of post-treatment epithelial cell death. We demonstrate differential responses of the nerves to chemical stimuli by changes in intracellular sodium as measured by 2-photon microscopy. Two-photon imaging techniques also allow for the visualization and study of the fine sensory axon fibers within the 3-dimensional tissue. This work demonstrates a role for innervation in the protective quality and function of the engineered tissue, and the potential to use the nerves themselves as indicators of the severity of an insult. These results are important to consider for the development of any optimized TE models for *in vitro* study and testing purposes.

KEYWORDS

innervation / tissue engineering / toxicology / 2-photon microscopy / cornea

Introduction

Tissue engineering can be described as the fabrication of biological or semi-synthetic living tissue in the laboratory for use as replacement tissues for damaged or diseased body parts. It is a rapidly growing area and the development of tissue engineered (TE) substitutes for several organs including liver, heart, skin, kidney and cornea is being pursued by a variety of approaches (Atala and Lanza, 2002). Although optimal function, homeostasis and wound healing of many tissues is dependent in part on proper peripheral sensory innervation (Anand et al., 1996; Gover et al., 2003; Holzer, 1988), the restoration of innervation to engrafted engineered tissue replacements is often overlooked.

In addition to their proposed use for transplantation, TE substitutes also have the potential to provide new toxicology models. While tissues need to confer function as a replacement to a tissue or organ in transplantation, for use as an in vitro model for testing, TE tissues need to mimic key morphological, physiological and biochemical properties of the natural tissue as closely as possible. Like TE constructs for transplantation, however, in vitro models may also require a sensory nerve supply to be mechanistically accurate. With the ban on animal testing for development of consumer products expected to expand from Europe into North America, in vitro methods for safety and efficacy testing (e.g. toxicology and drug testing) is expected to grow. Currently, the response of many TE constructs to external stimuli is overly sensitive, possibly due to a lack of innervation (Griffith et al., 1999).

We recently described the fabrication of a fully innervated in vitro human corneal model that demonstrated basic anatomical and physiological similarities to the natural tissue in situ (Suuronen et al., 2004). In this paper we demonstrated the importance of nerve-target cell interactions in the overall functioning of the engineered tissue. In the present study, we

examined in greater detail the contributions of sensory innervation to the function of an engineered tissue. In particular, 2-photon confocal microscopy was used to examine in detail the reaction of fine, terminal sensory nerves to external stimuli.

The innervated TE cornea demonstrates the potential of TE substitutes to serve as replacements to animals for *in vitro* ocular toxicology testing or as models for the study of fine sensory nerves. The cornea's transparency, structural simplicity and the importance of innervation for optimal corneal function make it an ideal tissue model for studying sensory nerve function. We observe that innervation affects the characteristics of the TE cornea and confers protection to its epithelium from chemical insult. Two-photon imaging techniques allow for the visualization and study of the fine sensory axon fibers within the 3-dimensional tissue. We demonstrate differential responses of the nerves to chemical stimuli by changes in intracellular sodium as measured by 2-photon microscopy. This work demonstrates a role for innervation in the protective quality and function of the engineered tissue, and the potential to use the nerves themselves as indicators of the severity of an insult. These results are important to consider for the development of any optimized, TE substitutes for *in vitro* use, either as toxicology models or for the study of innervation.

Materials and Methods

Corneal Construction

Corneal cell lines with extended life spans (Araki-Sasaki et al., 2000; Griffith et al., 1999) were used. Innervated corneas were constructed as described previously (Suuronen et al., 2004), with modifications. Briefly, collagen matrix was prepared using blended neutralized, type I rat tail collagen (0.3% (w/v), Becton-Dickinson, Oakville, Canada) and chondroitin-6-sulfate (CS) (1:5 w/w of these biopolymers; Sigma, Oakville, Canada) and cross-linked with

glutaraldehyde (0.02% (v/v)). Residual aldehyde groups were inactivated by reaction with 0.8% aqueous glycine (w/v) solution. This collagen-CS mixture is liquid at 4 °C, but forms a hydrated gel at 37°C. Corneal stromal matrix was constructed with a laminin (Becton-Dickinson) gradient of increasing concentrations from bottom to top of 0, 10 and 20 μg/ml. Cornea stromal cells (3 X 10^4 cells/ml) were added to all three layers of the collagen mix, nerve growth factor (NGF; 100 ng/ml; Sigma) was added to the top layer of collagen mix, and the stromal matrix was layered within 0.4 μm pore culture inserts (Costar, Fisher Scientific, Ottawa, Canada). Dorsal root ganglia (DRG), isolated from chick embryos (E8.0) were used for the neural source. These were embedded within the collagen matrix (Fig. 1) after an overnight pre-incubation in M199 at 37°C to remove contaminating fibroblasts. The construct was then thermo-gelled by incubation at 37°C for 2 hours. Corneal epithelial cells at a density of 70 cells/mm^2 were then seeded onto the construct, which was supplemented with a modified SHEM medium (Jumblatt and Neufeld, 1983) containing 2% B27 and 1% N2 nerve growth supplements (Life Technologies, Burlington, Canada) with 1 nM of retinal acetate (RA; Sigma). At epithelial confluence, the constructs were maintained at an air-liquid interface for up to 10 days until used.

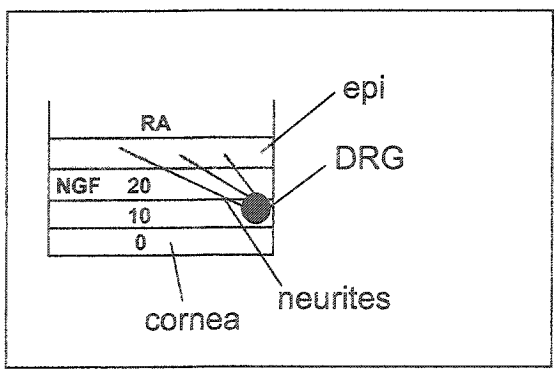

Fig. 1. TE cornea construction and neurite growth promotion. The schematic shows the TE cornea with DRG (black circle) placed in the stromal matrix and neurites extending into the corneal epithelium (epi). Note the concentration gradient of laminin (0, 10, 20), NGF in the uppermost stromal layer and RA in the medium.

Immunohistochemistry

For Na^+ channel labeling, 4% paraformaldehyde (PFA)-fixed constructs were rinsed in 0.05 M Tris buffer, pH 7.4 and permeabilized in Tris buffer containing 0.3% Triton X-100. After blocking with 10% normal goat serum in buffer, tissues were incubated overnight at 4°C with the primary antibody, monoclonal anti-PAN sodium channel antibody (Sigma), at a dilution of 1:250 in Tris buffer containing 2% normal goat serum. The tissues were rinsed thoroughly in Tris buffer and reacted with a 1:100 dilution of goat anti-mouse Alexa 488 secondary antibody (Molecular Probes, Eugene, OR), in Tris buffer for 90 minutes prior to visualization.

For neurofilament staining, PFA-fixed constructs were permeabilized by treatment with a detergent mix (150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 50 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate (Brugge and Erikson, 1977)) for 20 minutes. Constructs were rinsed in Tris buffered saline (TBS), and incubated with the primary antibody, anti-neurofilament 200 (Sigma; diluted 1:40 in TBS containing 0.6% carrageenan and 0.3% Triton-X 100 (TCT)) overnight at 4°C. The constructs were then rinsed in TBS and incubated with a Cy3-conjugated secondary antibody (1:200 in TCT; Amersham, Baie D'Urfé, Canada) for 150 minutes at room temperature (RT). Negative controls were incubated without the primary antibody. Positive controls included staining for DRG and neural tube explants. Nerves were visualized under fluorescence confocal microscopy.

For visualization of live nerve fibers, acetoxymethyl (AM) ester (Calcein AM, Molecular Probes) was used. Calcein AM, provided at a concentration of 1 mg/ml in dimethylsulfoxide (DMSO), was diluted into 0.1 M phosphate-buffered solution (PBS) to a 1

μM working concentration. The dye was loaded for 30 minutes at RT, and then the constructs were rinsed several times in PBS prior to visualization with the 2-photon laser-scanning microscope. Imaging was performed on a Nikon C1 laser scanning microscope custom-modified for 2-photon operation (Risdale et al., 2004). Fluorophores were excited by a 800 nm pulsed beam from a Ti:sapphire laser (Tsunami, Spectra Physics, Mountainview, CA), and fluorescence collected by a 60x 1.0NA immersion objective. Images were analyzed using ImageTrak software written by PKS (http://www.ohri.ca/stys/imagetrak).

Effects of innervation on the epithelium

To investigate the effects of innervation on stratification of the epithelium and the production of its protective mucin layer, constructs with and without DRG were cultured. TE corneas were fixed in 4% PFA at 2, 4, 6, and 10 days, and subsequently sectioned. For examination of epithelial thickness, sections were processed for routine hematoxylin and eosin (H&E) staining. The thickness of the epithelium was measured in six random sections for each sample, and the mean was calculated for each time point. For mucin staining, sections were rinsed in TBS and incubated with the primary antibody, anti-MUC16 (CA125; Dako) at a dilution of 1:50 in TCT overnight at 4°C. The constructs were then rinsed in TBS and incubated with a Cy2-conjugated secondary antibody (1:200 in TCT; Amersham) for 150 minutes at RT. Negative controls were incubated without the primary antibody. Nerves were visualized under fluorescence confocal microscopy.

Live/dead staining

Innervated and non-innervated constructs were exposed to a mixture of 8.5% Tween-80 surfactant and 1.5% ethanol in SHEM medium for 1 hour. Controls consisted of untreated constructs with and without nerves. Quantification of the viability of epithelial cells was done

using the acridine orange/ethidium bromide method. The dye mix consisted of 100 μg/ml of acridine orange and 100 μg/ml of ethidium bromide in 0.1 M PBS. Following the surfactant treatment, a volume of 20 μl of the dye mix was added to the inside of each insert, which contained 500 μl of medium. The constructs were stained for 5 minutes and images were captured for epithelial cell counts. Dead cells (apoptotic or necrotic) stained red and live cells stained green. The numbers of live and dead epithelial cells were counted within 5 different areas per construct. Dividing the number of dead cells by the total number of cells, and multiplying by 100 determined the percentage of epithelial cells that were dead. A One-way ANOVA was performed to determine statistical significance set at $P < 0.05$.

Sodium visualization

For these experiments, some modifications were made to the methods for cornea construction. In the experiments described above, the constructs were removed from their inserts for visualization by the 2-photon system. Due to the soft nature of the stromal matrix, there was a slow expansion of the tissue over time, which did not affect single image capture. However, for time series experiments, there is a requirement for tissue stability over time. The dimensions of the inserts described above do not allow for observation by the 2-photon system, and for this reason, constructs were transferred to organ tissue culture dishes (Becton-Dickinson) and surrounded by a ring of collagen-CS mixture, prepared as described above. This insert has dimensions suitable for use with the 2-photon system apparatus and its use ensures that the field of interest is maintained over time. However, it cannot be used for long-term culture due to its lack of a membrane, essential for proper nutrient delivery within the corneal stroma.

Constructs within organ culture dishes were loaded with the Na^+-indicator dye, sodium-binding benzofuran-isophthalate (SBFI; 10 μM) and the solubilization facilitator Pluronic F-127 (both from Molecular Probes), at a ratio of 1:1, prepared just before use. Loading time was 4 hours and the tissues were rinsed in PBS several times after loading. The construct was transferred to a custom-built chamber and immersed in artificial cerebral spinal fluid (aCSF; in mM): NaCl, 126; KCl, 3.0; $MgSO_4$, 2.0; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2.0; and dextrose, 10). Axons within the construct were imaged before and 10 minutes after application of the following treatments: aCSF, 14 μM dimethyl sulphoxide (DMSO), 50 μM veratridine, 500 μM ouabain or 50 μM veratridine + 500 μM ouabain at RT. Because DMSO is used at a final concentration of 14 μM to reconstitute both veratridine and ouabain, its effect on Na^+ retention was also tested to examine the possibility that it contributes to any observed response in the treatment groups. The signal from the Na^+-insensitive background was subtracted from the Na^+-dependent signal to give the Na^+ fluorescence intensity. The subtraction of fluorescence intensity at time = 0 from the intensity at time = 10 minutes, and multiplication by 100, yielded the percent change of Na^+ fluorescence intensity over time. Data were then normalized (relative percentage increase for each test group over the aCSF control).

Results

Effects of Innervation on TE Corneal Properties

Innervation stimulated epithelial growth in TE corneas. After 2 days of culture, there was no difference in the thickness of the epithelium between innervated and non-innervated TE corneas. However, at 4, 6, and 10 days of culture, greater epithelial thickness (~twofold greater after 10 days) was observed in the epithelium of innervated TE corneas compared

with non-innervated controls (Fig. 2A). The presence of the protective mucin layer, as determined by staining for MUC16, on the surface of the epithelium was greater in innervated TE corneas versus non-innervated controls. At 2 and 4 days of culture, mucin staining was not observed in either innervated or non-innervated constructs (data not shown). However, at 6 and 10 days, mucin staining was more intense on the surface of the epithelium of innervated, compared to non-innervated TE corneas (Fig. 2B-E). The mucin layer of the TE corneas from either group was not uniform, but instead displayed a patchy expression.

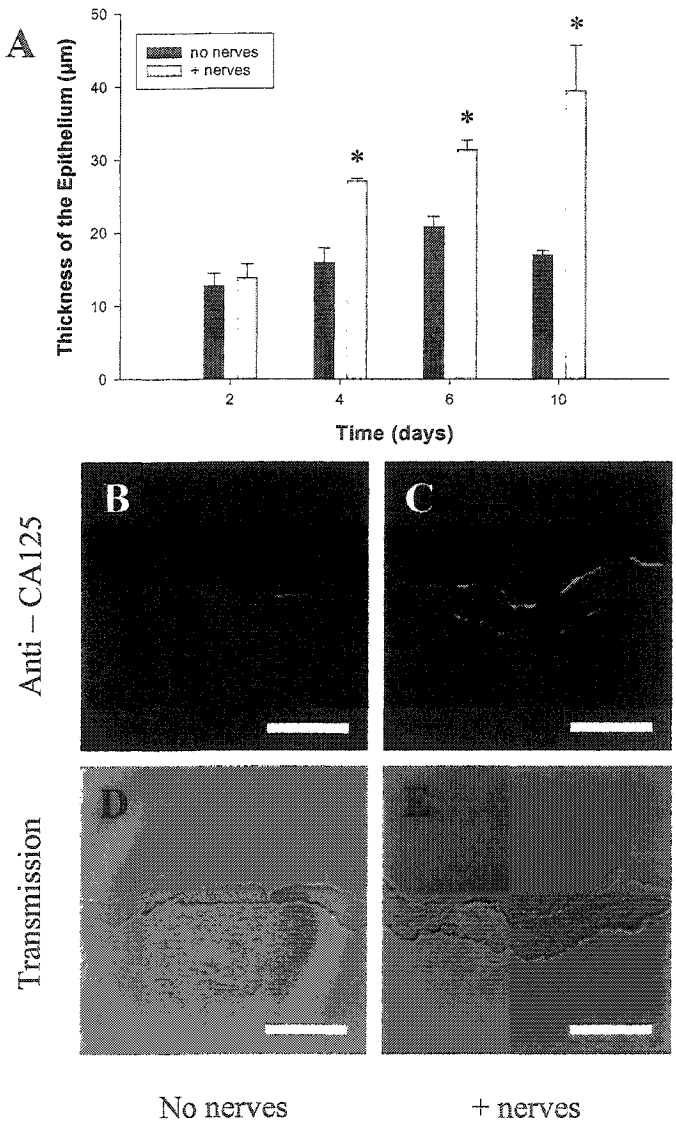

Fig. 2. Effects of innervation on properties of the epithelium. (**A**) The thickness of the epithelium (mean ± SE, n=3) for innervated and non-innervated TE corneas over time. *Significantly different from non-innervated control at day 4, 6, and 10 (two-way ANOVA; P<0.05). (**B**, **C**) Mucin staining at 6 days in non-innervated (**B**) versus innervated (**C**) TE corneas. (**D**, **E**) Respective transmission images of overlying images. Scale bar, 60 µm.

Effects of Innervation on Epithelial Cell Viability

In untreated control experiments, no difference in the percentage of dead cells was observed between innervated and non-innervated TE corneas (Fig. 3 A). However, when treated with the chemical irritant, significantly greater cell death was observed in the epithelium of non-innervated TE corneas (Fig. 3 A-C). The percentage of dead cells was approximately 3.5 fold greater in non-innervated constructs compared to their innervated counterparts.

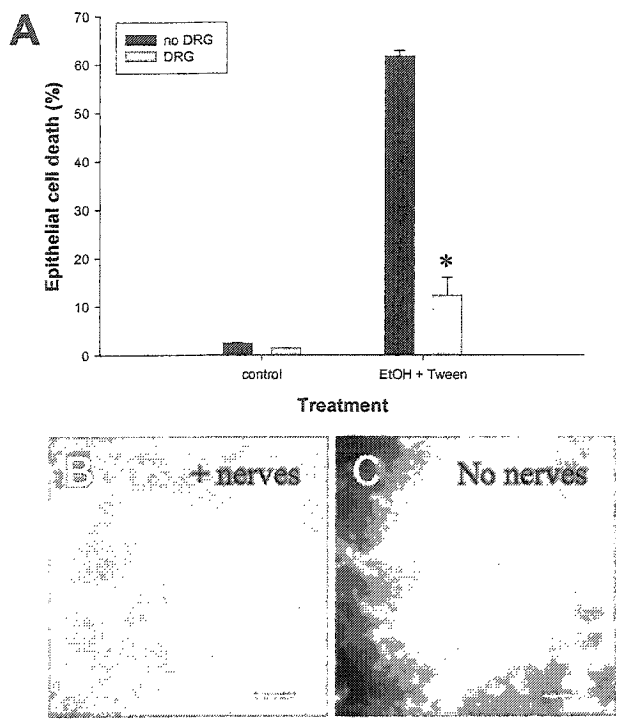

Fig. 3. Epithelial cell viability. (**A**) Epithelial cell death in innervated versus non-innervated TE corneas, with or without detergent treatment. *Significantly different from non-innervated control (two-way ANOVA; $P<0.05$). Epithelium of innervated (**B**) and non-innervated (**C**) TE cornea after treatment, stained with live/dead stain. Red indicates dead cells; green indicates live cells. Scale bar, 50 μm.

Nerve study using 2-photon confocal microscopy

Two-photon microscopy showed extensive nerve growth throughout the corneal construct (Fig. 4A), from the epithelium through to a depth of 200 μm into the stromal matrix of the tissue. Characteristic of human corneal nerves, both smooth and beaded nerve fibers were observed within the epithelial cell layers and the plexus network just below the epithelium (Fig. 4B). The feasibility of visualizing live TE corneas was also assessed. For this,

constructs were loaded with the cell viability indicator, Calcein AM. This dye allowed for the observation of nerves within both the epithelium and the stroma (Fig. 4 C and D), and its retention by both epithelial cells and nerve fibers indicated that these cells were alive and healthy.

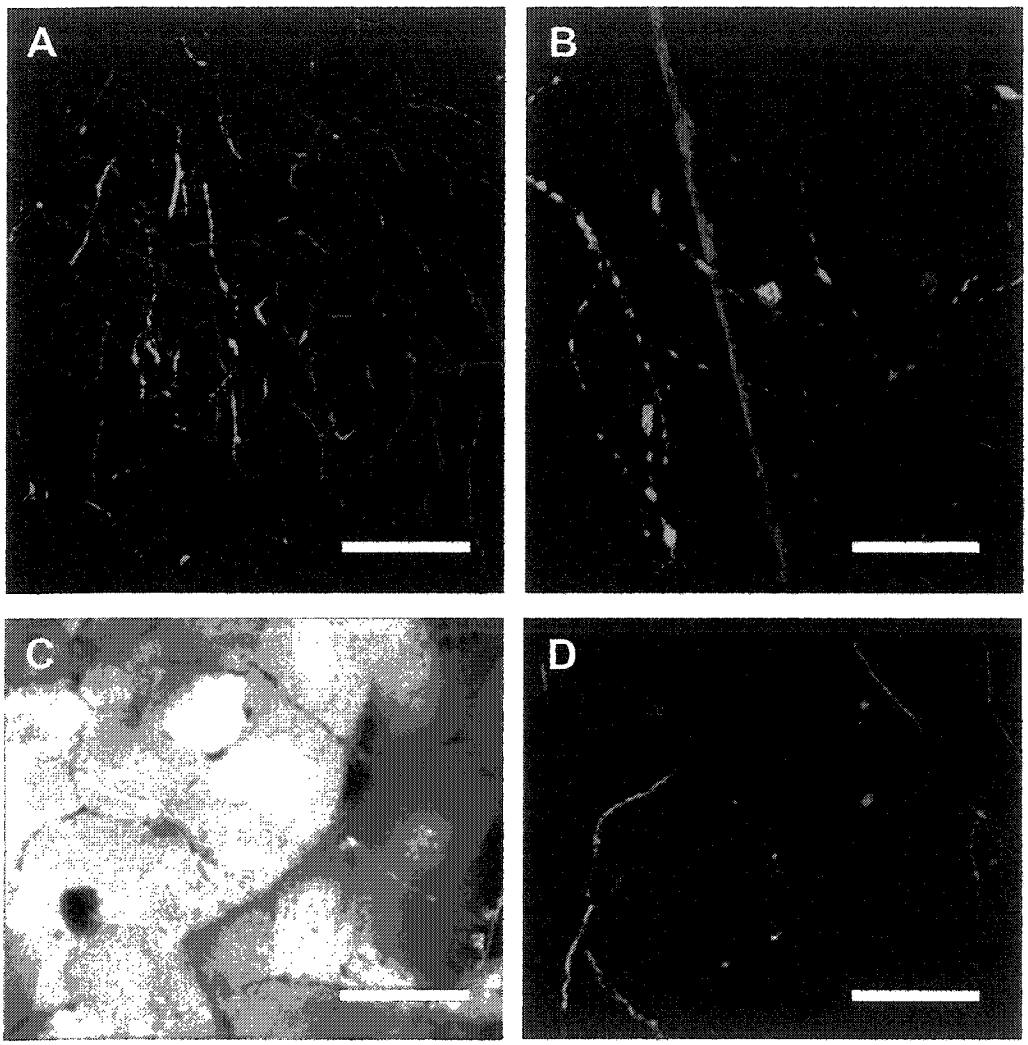

Fig. 4. Visualization with 2-photon microscopy. (**A**) NF-200 staining revealed extensive innervation throughout the TE cornea. (**B**) Both smooth and beaded fibres were observed in the anterior regions. (**C, D**) Calcein AM staining revealed healthy epithelial cells and nerve fibres within the epithelium (**C**) and stroma (**D**). All panels are composite images with the different colors representing different depths within the tissue. Scale bar, 200 μm (**A**); 50 μm (**B**); 20 μm (**C**); 150 μm (**D**).

Immunohistochemistry revealed the presence of Na^+ channels, co-localized with neurofilament staining and expressed ubiquitously along the length of the fibers in the TE cornea (Fig. 5A). This indicated that the nerves possessed the machinery necessary to mediate changes in $[Na^+]_i$. Differential changes in $[Na^+]_i$ were observed in TE corneas treated with DMSO, the Na channel opener veratridine, ouabain (an inhibitor of Na-K-ATPase), or veratridine and ouabain together (Fig. 5B). Changes in $[Na^+]_i$ were significantly different between veratridine and ouabain treatments and both elicited greater Na^+ accumulation within the fibers compared to DMSO treatment. In addition, simultaneous treatment with veratridine and ouabain resulted in a greater change in internal Na^+ than either treatment alone.

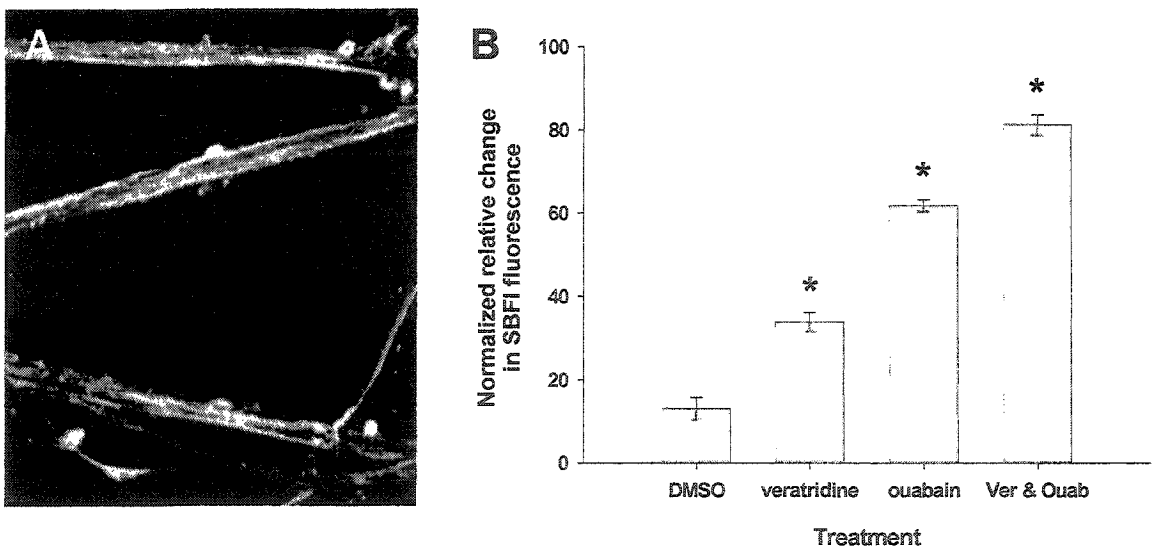

Fig. 5. Na^+ channels and internal Na^+ concentration in nerve fibres of the TE cornea. (**A**) Na^+ channels (green/yellow) were distributed uniformly along the length of the nerve fibres. (**B**) The normalized relative change in fluorescence of SBFI, a Na^+ indicator, in response to treatment with various chemicals. *Significantly different from all other treatment groups (one-way ANOVA; $P<0.05$), including the control (not shown; control normalized to 0).

Discussion

Currently, the standard for measuring the potential ocular toxicity of manufactured consumer products is the Draize or the low volume live rabbit ocular irritancy tests (Daston and Freeberg, 1991). However, the reliability and relevance of these methods obtained from non-human tissues remains questionable (Symposium Proceedings, technical committee on alternatives to animal testing, 1996). In addition, there is a push for a ban on the use of live animals for testing, which has already been implemented in Europe. This has resulted in the need to develop alternatives for the safety and efficacy testing of consumer products. While several promising models have been developed, including organ culture systems (Gautheron et al., 1992; Xu et al., 2000), and cultures or tissue equivalents using immortalized cell lines (Griffith et al., 1999; Kruszewski et al., 1997), there still is no validated alternative method. These models all share a common factor that may contribute to their failure as absolute, suitable replacements for the rabbit eye tests: lack of innervation.

The tissue engineered *in vitro* human cornea model we developed addresses the issue of innervation. This is important, considering that the optimal function, maintenance and wound healing of many tissues are dependent, in part, on proper peripheral sensory innervation (Anand et al., 1996; Gover et al., 2003; Holzer, 1988). The effect of nerves on the properties of the epithelial and stromal cells in our model was previously demonstrated (Suuronen et al., 2004). Here, we provide evidence that these effects contribute to the overall function of the model and its response to chemical irritation. We showed that the presence of nerves in the TE cornea was able to protect the epithelium from chemical irritation. After exposure to a surfactant/ethanol mixture, epithelial cell death was 3.5-fold greater in non-innervated corneas compared to innervated constructs. This was consistent with the

demonstrated role for nerves in the homeostasis of corneal epithelial cells in the human cornea (Araki-Sasaki et al., 2000). The nerves affected several properties of the epithelium, which in turn may affect the response of the TE cornea to chemical irritation.

One property of the epithelium that can play a role in its protective function is its thickness. Differences in ocular irritancy are related to differences in the extent of initial injury (Maurer et al., 1999) and a thicker epithelium will provide greater protection from insult. The thickness of the epithelium in innervated TE corneas was greater compared to non-innervated constructs over a culture period of 10 days. Trigeminal denervation of the rabbit cornea has been previously observed to cause thinning of the epithelium (Araki et al., 1992) and increased permeability (Beuerman and Schimmelpfennig, 1980). Therefore, it is possible that the significantly greater epithelial cell death observed in non-innervated corneas upon chemical treatment could be due to greater permeation of the toxicant through the epithelium as a result of its compromised thickness. The thinning of the epithelium in denervated corneas has been associated with impairment of epithelial cell adhesion (Araki et al., 1994). We observed compromised adhesion in non-innervated TE corneas after prolonged chemical assault (data not shown). These differential adhesive properties between innervated and non-innervated constructs may also have been a factor in the response of the cornea to chemical insult. Overall, the presence of nerves in the TE cornea model promoted an increase in epithelial thickness, which can potentially reduce permeability and enhance the resistance to chemical irritation.

The production of the mucin layer is another protective mechanism of the epithelium of several tissues including the cornea. Mucins are glycoproteins produced by the surface epithelia (Gipson et al., 1995) that lubricate the surface to provide a barrier for the cell

membrane and protect the cornea from desiccation (Argueso and Gipson, 2001). Mucin 16, in particular, is expressed by corneal surface epithelia (Argueso et al., 2003) and its expression is altered in the conjunctival epithelia of patients who suffer from dry eye syndrome (Danjo et al., 1998), a condition related to compromised innervation (Stern et al., 1998). In the TE cornea model, innervation promoted greater production of the mucin layer by the surface epithelia, as determined by MUC16 staining. This demonstrated that innervation plays a role in the expression of mucin in the cornea, as had already been observed in the surrounding conjunctiva (Danjo et al., 1998), and that this model could be used to study the pathology of dry eye syndrome. In addition, the lessened mucin production in non-innervated TE corneas may contribute to the observed increase in epithelial cell death. Without a protective surface layer of mucin, the permeation of the chemical assault may be deeper and cause greater damage to the epithelial tissue of the cornea. Although not investigated, deep chemical penetration could also affect stromal cell viability and alter its interaction with the epithelium, itself important for the health of the epithelial cells (Wilson et al., 1999).

Overall, the presence of nerves within the TE cornea promoted greater epithelial thickness, improved production of the protective mucin layer, and as was shown previously, increased epithelial and stromal cell proliferation, and had an effect on epithelial wound healing (Suuronen et al., 2004). These are important considerations in the development of tissue-engineered models for toxicology testing, as they have an effect on the resistance and protective qualities of the epithelial surface, and hence can alter sensitivity of the model. The lack of innervation may be responsible, in part, for the failure of the current alternative toxicology models to provide undisputed, suitable, replacements for rabbit eye tests.

While innervation affected the protective quality and function of the engineered tissue, we also investigated the effects of irritation on the nerves themselves. Upon stimulation, the nerve transmits its message by the generation of action potentials (APs), which propagate via the axon to the central nervous system to cause the sensation of pain (Brock et al., 1998), and to the nerve terminals to cause the release of neuropeptides (Unger, 1990). AP generation occurs by the opening of Na^+ channels, and the subsequent Na^+ current and depolarization of the membrane. Variability of the Na^+ current has been correlated with the amplitude of the nerve response (Johansson, 1994). In the TE cornea, differential changes in internal Na^+ concentration $[Na^+]_i$ were observed within the nerve fibers when treated with different chemicals, including veratridine and ouabain. While veratridine operates by a Na^+ channel-dependent mechanism, ouabain does not, yet both elicited responses that can be detected by changes in $[Na^+]_i$. These results indicate that this technique could be used to detect the effects of irritants that do and don't act via Na^+ channels. Differential $[Na^+]_i$ responses could be used as indicators of the severity of an insult in this model, by both the assessment of intensity and depth of permeation.

One mechanism by which sensory nerves exert their influence on their environment is by the release of neuropeptides. This may include the release of substance P (SP) neuropeptide (Maggi, 1991), which can be regulated by Na^+ channel-dependent mechanisms. The differential changes in $[Na^+]_i$ observed in nerves of the TE cornea could translate to differential responses of the nerve (Johansson, 1994), including release of SP. Therefore, the release of SP can be correlated to $[Na^+]_i$, which can be regulated by the influx of Na^+ into the fibers through the Na^+ channels of the axonal membrane. Unlike the highly focal distribution of Na^+ channels in myelinated axons, Na^+ channels present in the TE cornea were distributed

continuously along the membrane of the nerve. This pattern is typical of nonmyelinated sensory axons (Black et al., 2002), the type of axons found in the cornea, and supports continuous (as opposed to saltatory) conduction along these axons. We previously observed differential release of SP upon treatment with various chemicals, including veratridine (Suuronen et al., 2004). Veratridine causes the release of SP by opening Na^+ channels and depolarizing the membrane (Neubert et al., 2000). Therefore, it appeared as though the nerves within the TE corneal model behaved in a manner typical of native corneal nerves. Described simply, the application of a chemical irritant to the innervated TE cornea caused changes in $[Na^+]_i$, and through the depolarizing effects, SP neuropeptide is released, which then can affect epithelial properties (Gallar et al., 1990; Nishida et al., 1996). The magnitude of the response would depend on the level of irritation. As mentioned above, some chemicals operate by Na^+ channel-independent mechanisms, but can still result in the release of neuropeptides, including SP (Keen et al., 1982). This mechanism of SP release was also observed in the TE cornea in previous work (Suuronen et al., 2004). Therefore, our innervated TE cornea model could be developed for use as an alternative to animals in ocular toxicity testing. The degree of cell death, the levels of neurotransmitter release and the response of the nerves themselves could all be used to ascertain the toxicity of potential ocular irritants.

Knowledge of sensory nerve function has come largely from monolayer or thin tissue slice culture systems that do not fully replicate the *in vivo* environment. The emergence of tissue engineering is providing new, more relevant models for the study of tissues and the function of their constituent cells. The re-creation of both the natural environment and the appropriate interactions between nerves and their target tissues make tissue-engineered

systems superior to traditional cell culture, and more accessible and easier to manipulate than *in vivo* models for the study of innervation. However, the investigation of sensory nerve fibers, in particular, and their terminals and interactions with their target tissue is difficult in whole, 3-dimensional tissue due to their miniature size. As such, their study has been limited and many of their functional properties remain unclear. Two-photon microscopy has significantly enhanced the visualization and study of fine nerve fibers (Malone et al., 2002; Rose et al., 1999). Two-photon imaging has the advantage of high resolution imaging at substantial depths in intact tissue, despite the scattering effects of thick tissues (Denk and Svoboda, 1997), and it minimizes the effects of photobleaching and photodamage (Denk et al., 1995). Combining the technologies of tissue engineering and 2-photon microscopy presents methodologies that can improve our current knowledge of nerve behavior and function, using more accurate, 3-dimensional models.

Nerves within the TE cornea, stained with anti-NF 200 (fixed tissue) or Calcein AM (live tissue), were easily visualized in the epithelium and the stroma using the 2-photon system. Typical of corneal nerves (Müller et al., 1997), both smooth and beaded fibers migrated within the epithelium. The 2-photon system allowed observation of the nerves from just below the epithelial surface to a depth of 200 μm within the stroma. The retention of the Calcein AM dye, an indicator of cell viability, provided evidence that both the nerve and epithelial cells were alive and healthy. Therefore, 2-photon microscopy enables the study of the fine sensory structures within the TE cornea and their interactions with their target tissue. These results emphasize the potential of combining the technologies of tissue engineering and 2-photon microscopy to improve our current knowledge of nerve behavior and function.

In summary, we demonstrate the importance of nerves in an engineered tissue with respect to its function. The presence of nerves affects several properties of the TE cornea, including its epithelial thickness, its production of the protective mucin layer, and its proliferation of epithelial and stromal cells, all of which can affect both its maintenance and its sensitivity. These are important considerations for the development of any optimized, TE substitutes for transplantation that promote the growth of nerves from the host tissue, or for *in vitro* use, either as toxicology models or the study of innervation.

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CHAPTER 4

The previous two manuscripts focus on the *in vitro* TE cornea model and its application for the study of corneal innervation and for its use in sensitivity testing for ocular irritancy. The following manuscript details the use of the *in vitro* model for the development and testing of stronger and tougher biosynthetic polymer matrices for use in implantation surgeries. My contribution to this chapter/manuscript involved the determination of the best candidate molecules, for grafting into already developed matrices, for the promotion of neurite in-growth. I also conducted the in vitro tests for the selection of the substitute matrices that would best promote the infiltration and repair of the tissue by the host's corneal cells and nerves following implantation into pigs' corneas (for a more complete description of the methods involved, see the appendix). This work demonstrated nicely the successful translation of in vitro testing into the selection of a polymer implant ECM replacement that: a) allowed for cell-matrix interaction in the restoration of functional structures including the regeneration of the epithelium and nerve axon in-growth; and b) allowed for the recovery of sensitivity within the implant, which is a problem in both human donor tissue and other implant models.

4.1 Selection of laminin peptide

As discussed in the following manuscript, the formulation for the collagen-copolymer matrices developed for transplantation was derived from the *in vitro* model. As seen in **chapter 2**, the use of laminin in the *in vitro* TE cornea was successful in promoting the growth of nerve fibres from DRG into the collagen-based matrix. Therefore, it was predicted that the addition of a peptide of laminin would also enhance the ability of the collagen-copolymer matrices to support nerve growth. To determine the best suitable peptide for

grafting to the copolymer matrices, some preliminary *in vitro* testing was performed on the ability of various available peptides to promote the growth of neurites from DRG in culture. Acellular TE collagen gels, as used in the previous manuscripts, were coated with molar equivalents of solubilized laminin pentapeptide (YIGSR; Novabiochem), hexapeptide (VSLSPG; Calbiochem) and nonapeptide (CDPGYIGSR; Calbiochem), and laminin A chain (Sigma). The collagen gels were air-dried for 20 minutes and excess liquid was suctioned off. DRG were then cultured on these surfaces for 10 days and the number of extending neurites were counted at 2, 5, 7 and 10 days. The results are presented in Table 1.

Table 1. Neurite growth on different laminin peptides. The relative amount of nerve extension observed on different peptides of laminin over time. (-) 0-10 neurites; (+) 11-50 neurites; (++) 51-100 neurites; and (+++) > 100 neurites.

Time in culture (days)	Laminin fragment			
	VSLSPG	YIGSR	CDPGYIGSR	A chain
2	++	+++	++	+++
5	+++	+++	+++	+++
7	++	+++	++	+++
10	-	++	+	+++

Both the pentapeptide and the A chain were equally supportive of neurite extension over 10 days of culture. However, it was determined that the laminin pentapeptide (YIGSR) would be the best candidate for grafting into the collagen-copolymer due to its smaller size (5 amino acids). This would cause less steric hindrance with the chemistry of the copolymerization process. At 19 amino acids, the laminin A chain would have been too large for the inter-collagen fibre spaces of these particular hydrogels. Details and results for the grafting of YIGSR to the collagen-copolymer can been found below (section **4.2**).

4.2 Manuscript 3

Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation

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Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation

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ABSTRACT

Our objective was to determine whether key properties of extracellular matrix (ECM) macromolecules can be replicated within tissue engineered, biosynthetic matrices to influence cellular properties and behavior. To achieve this, hydrated collagen and N-isopropylacrylamide copolymer-based ECMs were fabricated and tested on a corneal model. The structural and immunological simplicity of the cornea and importance of its extensive innervation for optimal functioning makes it an ideal test model. In addition, corneal failure is a clinically significant problem. Matrices were therefore designed to have the optical clarity and the proper dimensions, curvature, and biomechanical properties for use as corneal tissue replacements in transplantation. *In vitro* studies demonstrated that grafting of the laminin adhesion pentapeptide motif, YIGSR, to the hydrogels promoted epithelial stratification and neurite in-growth. Implants into pigs' corneas demonstrated successful *in vivo* regeneration of host corneal epithelium, stroma, and nerves. In particular, functional nerves were observed to rapidly regenerate in implants. By comparison, nerve regeneration in allograft controls was too slow to be observed during the experimental period, consistent with the behavior of human cornea transplants. Other corneal substitutes have been produced and tested, but here we report an implantable matrix that performs as a physiologically functional tissue substitute and not simply as a prosthetic device. These biosynthetic ECM replacements should have applicability to many areas of tissue engineering and regenerative medicine, especially where nerve function is required.

Key words: regenerative medicine | tissue engineering | cornea | implantation | innervation

The macromolecules of the extracellular matrix (ECM) are elaborated by cells and create microenvironments that these and other cells will respond to, by differentiating or maintaining their differentiated state. Simulation of appropriate ECM environments within fabricated matrices or scaffolds therefore should encourage parenchymal cells and nerves to infiltrate the matrix, regenerate a structure, and restore key functions to damaged tissues and organs (1). This general tissue engineering (TE) strategy could potentially alleviate problems of organ failure and donor organ shortages for transplantation. To test this strategy, we chose the clinically important regeneration of damaged corneas.

Corneal diseases are a major cause of vision loss, second only to cataracts in overall importance (2), and affect >10 million individuals worldwide (estimates from Vision Share, Raleigh, North Carolina), with corneal scarring from measles being a major cause of childhood blindness (3). Currently, the only widely accepted treatment for corneal blindness is transplantation with human donor tissue. The worldwide demand for transplantation corneas exceeds the supply and this situation will worsen with an aging population and increased use of corrective laser surgery (4). For patients with disorders such as autoimmune conditions, alkali burns or recurrent graft failures, corneal transplantation has a poor success rate (5). An alternative for these patients is replacement of the damaged cornea with an artificial substitute. Successful corneal replacement by synthetics is yet to be achieved and existing prostheses neither integrate seamlessly into the host tissue (5), nor promote re-innervation, even though corneal innervation loss can lead to vision loss (6).

The cornea is an avascular, transparent, and immune privileged tissue comprising three main layers (outer stratified epithelium; stroma of cells networked within a hydrated, mainly collagen-proteoglycan matrix; and inner endothelial layer). The cornea is the main

optical element in the eye that refracts light onto the retina for vision, and a tough protective barrier for the delicate internal eye tissues. It is one of the most highly innervated tissues in the body (7) and is equipped with nociceptive nerve endings that terminate within the epithelium. These nerve endings are polymodal, mechano-sensory or cold-sensitive (8). Nerve activity is responsible for maintaining overall corneal health; and innervation loss can cause "dry eye" (9), a pathological condition that may result in decreased corneal sensitivity and/or corneal epithelial erosions. When sensitivity is lost, the cornea becomes vulnerable to irreparable injury, ulceration, eventual loss of vision (6), and in severe cases, blindness (10). The structural and immunological simplicity of the human cornea, and importance of innervation for optimal function, make it an ideal tissue for investigating TE requirements in general and induction of peripheral nerve regeneration in particular.

Our objective was to evaluate novel combinations of biological and synthetic mimics of ECM macromolecules that could be fabricated into TE matrix replacements or tissue templates. By using corneal tissue as the test system, we report here the successful growth of stratified epithelium, stromal fibroblasts and nerve infiltration with our optically clear ECM substitutes and the successful post-surgical integration of these substitutes into surrounding host tissues of micropig animal models with rapid nerve regeneration.

MATERIALS AND METHODS

Collagen-copolymer ECM replacements

Copolymer [poly(*N*-isopropylacrylamide-coacrylic acid-coacryloxysuccinimide), PNiPAAm-coAAc-coASI, designated TERP] was synthesized by free radical co-polymerization of its three monomers (PolySciences) in dioxane. The monomer molar equivalent ratio in the purified TERP was 84.2:9.8:6 by proton NMR in tetrahydrofuran-D_8.

The number and weight average molecular mass were 5.6 x 10^4 and 9.0 x 10^4 Da, respectively, as determined by gel permeation chromatography. After purification and solution in PBS, the cloud point was >55°C. Acryloxysuccinimide groups spontaneously crosslink proteins and anchor peptides through their primary amine groups (11). A second copolymer (designated TERP5) was synthesized from TERP by reacting some of its acryloxysuccinimide groups with the peptide YIGSR (YIGSR-NH_2, Nova Biochem, Laufelfingen, Switzerland) in *N,N*-dimethyl formamide (48 h at 21°C). The YIGSR peptide is a well-known cell adhesion mediator that promotes epithelial growth (12) and enhances neurite extension (13). Collagen-TERP and collagen-TERP5 hydrogels were prepared by mixing neutralized 4% (wt/wt) bovine atelocollagen [1.2 ml; concentrated from 0.3% (wt/vol) collagen; Becton Dickinson], with each purified copolymer (collagen/copolymer = 1.4:1, wt/wt) at 4°C. Each cold solution was injected into plastic contact lens moulds, where it reacted at 21°C and then 37°C at 100% humidity to give crosslinked hydrogels of controlled thicknesses. Unreacted acryloxysuccinimide groups in these hydrogels were terminated by immersion in 0.5% (wt/vol) glycine in PBS. Reaction products were extracted and hydrogels sterilized with 1% (wt/vol) chloroform in PBS. YIGSR-NH_2 content of extensively washed gels was 4.3×10^{-11} mol/ml of hydrated gel (2.6×10^{-8} g/ml), based upon ^{125}I labeled YIGSR-NH_2 using the Iodogen method (14). The final, total polymer concentration in each hydrated, PBS-equilibrated hydrogel was 3.4% (wt/vol) [collagen and TERP5 at 2 and 1.4% (wt/vol), respectively]. Collagen alone was also neutralized and cast in the same molds and then incubated, first for 24 h at 21°C and then at 37°C, to spontaneously form thermogel controls.

The permeability coefficient of glucose in PBS (pH 7.4) through collagen-copolymer hydrogels was calculated from permeability measurements after enzymatic conversion of glucose to glucose-6-phosphate followed by production of dinucleotide quantified by its UV absorption (15). Topographies of hydrogels in PBS were examined by atomic force microscopy (Molecular Imaging, Tempe, AZ) in the "contact" mode. Pore sizes from this technique were compared with average pore diameters calculated from PBS permeability (13). Implants recovered after 6 wk *in vivo* were examined by IR spectroscopy (Midac M, Fournier transform IR spectrometer, ZnSe beam condenser, and diamond cell).

In vitro characterization

Immortalized corneal epithelial cells (16) were used to evaluate epithelial coverage. Collagen, collagen–TERP, and collagen-TERP5 hydrogels (500 μm thick for easy handling) were separately embedded on top of a collagen-based matrix that consisted of a mixture of blended neutralized type I rat-tail tendon collagen (0.3% wt/vol; Becton Dickinson) and chondroitin 6-sulfate (1:5, wt/wt), crosslinked with 0.02% (vol/vol) glutaraldehyde (followed by glycine termination of unreacted aldehyde groups) and then thermo-gelled at 37°C. Epithelial cells were seeded on top, and constructs were supplemented with epidermal growth factor-containing keratinocyte serum-free medium (Life Technologies) until confluence. The medium was then switched to a serum-containing medium (modified supplemental hormone epithelial media; ref. 17) for 2 d, followed by maintenance at an air-liquid interface. At 2 wk, constructs were fixed in 4% paraformaldehyde in 0.1M PBS and were processed for routine hematoxylin and eosin staining. The number of cell layers and thickness of the epithelium were measured from six random areas for each of four samples within each of the three experimental groups of hydrogels.

Other constructs, as above, were used to examine early nerve in-growth. Dorsal root ganglia from 8-d-old chick embryos were embedded within the surrounding matrix adjacent to each implant. Cultures were supplemented with keratinocyte serum-free medium containing 2% B27 and 1% N2 supplements (Life Technologies) and 1 nM retinal acetate (Sigma). After 4 d, constructs were fixed as above for immunohistochemistry on whole mounts (details below) to visualize construct innervation. Nerve density (the number of nerves per μm^2) was calculated at distances of 75 and 100 μm from the edge of the dorsal root ganglia within a 90° pie-shaped wedge extending into the implant.

Implantation and clinical evaluation

Following the Association for Research in Vision and Ophthalmology animal use guidelines, six identical collagen-TERP5 matrices (5.5 mm in diameter, 200 ± 50 μm thick) were implanted into the right corneas of six Yucatan micropigs (Charles River Breeding Laboratories) by lamellar keratoplasty (LKP). Contralateral unoperated corneas served as controls. A further four pigs received allografts of fresh pig donor corneas with the same dimensions as TERP5 matrices, implanted by LKP.

Briefly, in LKP, under general anesthesia, a 250-μm-deep, 5-mm-diameter circular incision was made using a Barraquer trephine (Geuder, Heidelberg). A lamellar dissection was then performed using a microsurgical pocket blade (Geuder) along a natural uniform stratum in the corneal stroma to remove host epithelium and anterior stroma. Tissue removed was replaced with an implant 0.5 mm larger in diameter to allow adequate wound apposition between graft and host tissue. The host's posterior stroma, Descemet's membrane and endothelium remained. For three implants and four allografts, after surgery, an amniotic membrane was sutured over the entire corneal surface for 7 d to keep implants in place. In

another three animals, collagen-TERP5 implants were sutured into the host tissue using eight interrupted 10-0 nylon sutures. Post-operative medication consisted of dexamethasone and gentamycin four times daily for 21 d.

Pigs were examined daily for 7 d after the operation and then weekly. Examinations included slit-lamp assessment of corneal optical clarity, sodium fluorescein staining to assess epithelial integrity and barrier function (18), intraocular pressure measurements to ensure appropriate aqueous humor flow, and *in vivo* confocal microscopy with a ConfoScan microscope (Nidek, Erlangen, Germany) to assess cell and nerve in-growth. For *in vivo* confocal microscopy, light scattering at the graft-host tissue interphase marked the implant boundaries. Z scans were taken and nerves were qualified as stromal when they were embedded in the corneal stroma (keratocytes above and below nerves) and as subepithelial when nerves were directly under the epithelium. Corneal touch sensitivity was measured using a Cochet-Bonnet esthesiometer (Handaya, Tokyo) at five points within the implant area of each cornea as described (19). In brief, a fine filament was extended from this tool to contact the cornea and reflex responses of the pig were monitored. Initially very soft contact was made using a long filament extension, which was then shortened progressively (becoming stiffer and the touch firmer) until the pig clearly responded. This extension was recorded as the touch-sensitivity threshold.

Immunohistochemistry and histopathological examination

Tissues and constructs were fixed in 4% paraformaldehyde in 0.1M PBS. For nerve immunolocalization, flat mounts were permeabilized with a detergent mixture (20) (150 mM NaCl/1 mM ethylenediamine tetraacetic acid/50 mM Tris/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS), blocked for non-specific staining with 4% FCS in PBS and

incubated in antineurofilament 200 Ab (Sigma). They were then incubated with FITC or Cy3-conjugated secondary Abs (Sigma and Amersham Biosciences, respectively) and visualized by confocal microscopy.

For histology and further immunohistochemistry, samples were paraffin embedded and sectioned. Sections were haematoxylin- and eosin-stained for histopathological examination. Immunofluorescence was performed as described above on deparaffinized sections for expression of type VII collagen (Sigma), a hemidesmosome complex marker (in anchoring fibrils) (21). Immunohistochemical staining using peroxidase-diaminobenzidine visualization was performed with the following: AE1/AE3 Ab (Chemicon) for epithelial markers, anti-vimentin Ab (Roche) for stromal fibroblasts, anti-smooth muscle actin Ab, 1A4 (Cell Marque, Austin, TX) for activated stromal fibroblasts (myofibroblasts) and SP1-D8 Ab (Developmental Studies Hybridoma Bank, Iowa City, IA) for procollagen 1 synthesis (to localize sites of *de novo* collagen synthesis). CD15 and CD45 staining for immune cells (Becton Dickinson) was performed using the ARK peroxidase kit (DAKO) to preconjugate the primary Abs to their respective secondary Abs and peroxidase for visualization. For anti-vimentin, anti-smooth muscle actin and SP8-D1 Abs, antigen retrieval was preformed by pretreating with proteinase K (2 mg/ml) for 30 min at 37°C prior to incubation in primary Ab. *Ulex europaeus agglutinin* lectin staining was used to visualize tear film mucin deposition (22). Samples were incubated with biotinylated *Ulex europaeus agglutinin* (Sigma) and then reacted with avidin-horseradish peroxidase and visualized with diaminobenzidine. For transmission electron microscopy, all samples were treated in conventional fixative, stain and potting resin (Karnovsky's fixative / OsO_4 / uranyl acetate / epoxy).

RESULTS

Collagen-copolymer implants

Hydrogels were prepared from atelocollagen cross-linked with TERP or its YIGSR-modified analogue, TERP5. Based on ^{125}I labeling, water-insoluble collagen-TERP5 hydrogels contained 60% of the YIGSR-NH_2 expected from the TERP5 composition (Fig. 1*A*) after exhaustive aqueous extraction. IR spectroscopy showed progressive loss of oxysuccinimide groups during reaction of TERP and TERP5 with collagen, consistent with the expected cross-linking reaction (11). The composite collagen-copolymer matrices, molded to the curvature and dimensions of a cornea (Fig. 3*B*) were adequately robust and suturable and had refractive indices (1.343 ± 0.003) comparable to the human tear film (1.336-1.357; ref. 23). They showed high optical clarity compared to collagen only matrices (Fig. 1 *B* and *C*), with direct transmission and backscatter of visible light superior to human corneas. The collagen-copolymer matrices had pore diameters of 140–190 nm (from both atomic force microscopy and PBS permeability; ref. 13). They also had a glucose diffusion permeability coefficient of 2.7×10^{-6} cm^2/s, higher than the value for natural stroma [$\approx 0.7 \times 10^{-6}$ cm^2/s, from published diffusion (2.4×10^{-6} cm^2/s) and solubility (0.3) coefficients (24)].

In vitro tests matrices

The epithelium on collagen-TERP5 hydrogels had a significantly greater number of cell layers (Fig. 1*D*) and was thicker (Fig. 1 *E-G*) than collagen-TERP or collagen only hydrogels. The density of nerves growing into collagen-TERP5 matrices (Fig. 1*H*) was significantly higher than that into collagen matrices. Only nerves grown in collagen-TERP5 hydrogels reached 100 μm from the edge of the matrix, but this trend for collagen-TERP5

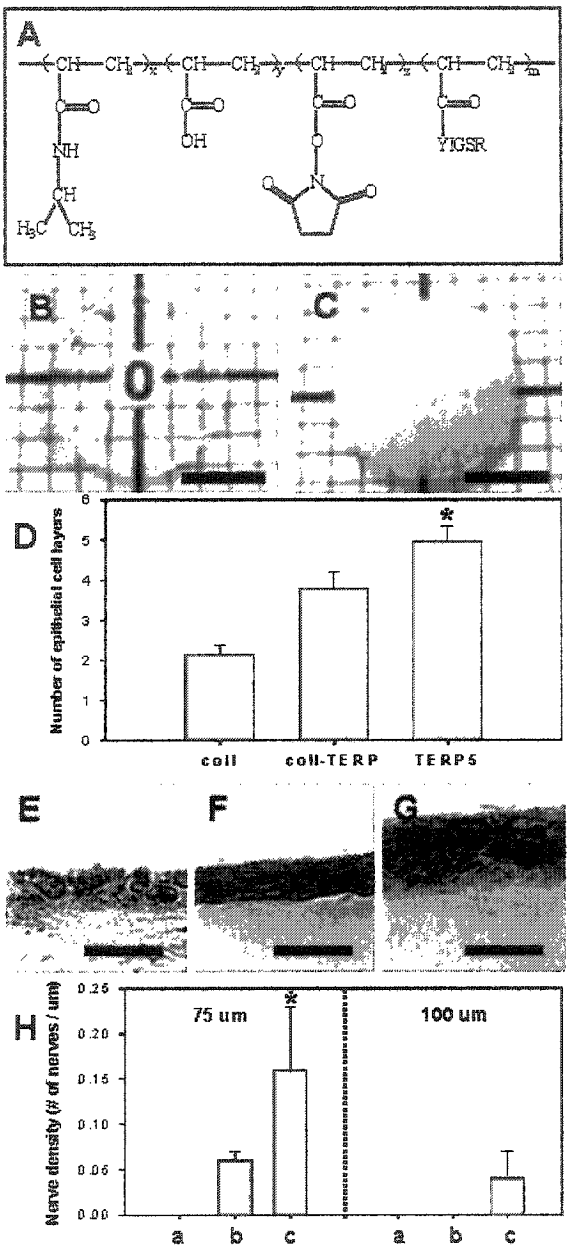

Fig. 1. Biosynthetic matrices *in vitro*. (*A*) Structure of TERP5 copolymer (x/y/z/m = 84.2:9.8:6:5 × 10^{-5}, all in molar equivalents). (*B*) Corneal implant comprising collagen-TERP5 hydrogel, showing optical clarity that was not achieved in matrices containing only collagen (*C*). (Bar = 5 mm, thickness = 500 μm in both cases.) (*D*) Number of cell layers within the stratified epithelium grown on different hydrogels, n = 4 samples each. *, $P < 0.05$ by ANOVA. (*E-G*) Thickness of the epithelium grown on different hydrogels: collagen only (*E*), collagen-TERP (*F*), and collagen-TERP5 (*G*). (Bar = 30 μm for *E-G*.) (*H*) Nerve density within different hydrogels (collagen, a; collagen-TERP, b; collagen-TERP5, c) at 75 and 100 μm from the hydrogel edge, n = three samples each. *, $P < 0.05$ vs. collagen by ANOVA.

polymer to support longer nerves was not statistically significant compared to collagen-TERP. Nevertheless, these results established collagen-TERP5 hydrogel as the superior candidate in supporting cell growth and only this hydrogel matrix was used as an implant for the *in vivo* studies. Collagen only gels were too weak to suture, opaque, and had poor epithelial overgrowth and nerve in-growth (Fig. 1).

Regeneration after biosynthetic matrix implantation

Collagen-TERP5 matrices were suturable with careful surgical manipulation (Fig. 2 *A-C*), with no indication of cracking or cutting by sutures. As with control allografted corneas, intraocular pressures were 10-14 mmHg (1 mmHg = 133 Pa) preoperatively, and 10-16 mmHg postoperatively over the 6-wk study period, showing that the implants did not block aqueous humor flow within the eye. Neither implants nor allografts gave adverse inflammatory or immune reaction, and both remained optically clear. By 4-5 d postoperative, both implants and allografted corneas were covered with epithelium. By 1 wk, sodium fluorescein dye was excluded, indicating that the epithelium was intact and had re-established barrier properties. Stratified epithelium remained intact (excluded fluorescein) and firmly attached (as indicated by wiping a Weck sponge over the epithelial surface during clinical examinations) over the entire 6 wk. Clinical *in vivo* confocal microscopy of implanted stromal matrices at 3 wk after surgery showed a regenerated epithelium (Fig. 2*D*), newly in-grown nerves (Fig. 2*G*), and stromal (Fig. 2*J*) and endothelial cells (Fig. 2*M*) with cellular morphology mimicking that of unoperated controls (Fig. 2 *F, I, L,* and *O*). Epithelial and endothelial cell morphology in the allografts (Fig. 2 *E* and *N*) resembled that of untreated controls. However, sub-epithelial and stromal nerves were not observed at 3 (Fig. 2 *H* and *K*) or 6 wk after surgery.

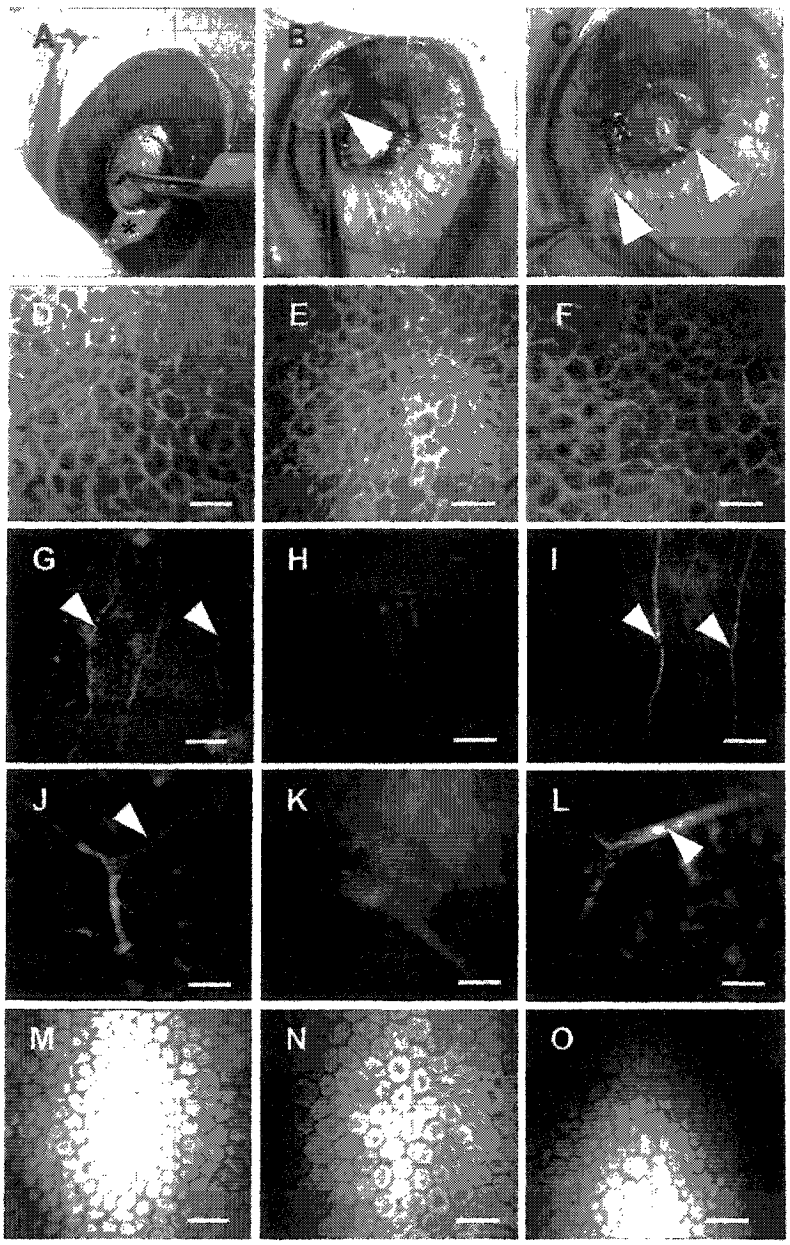

Fig. 2. Corneal implantation by LKP and *in vivo* confocal microscopic images of 6-wk implants. (*A-C*) LKP procedure on a Yucatan micropig. (*A*) A trephine is used to cut a circular incision of preset depth into the cornea. Anterior corneal layers are removed (*B*) and replaced with a biosynthetic implant (arrow), which is sutured in place (*C*). Arrowheads indicate sutures. (D) *In vivo* confocal image showing regenerated corneal epithelium on implant surface. Corresponding allograft control (*E*) contains donor epithelium, whereas the unoperated control (*F*) has intact epithelium. Regenerated nerves (arrowheads) at the interface between implant and overlying regenerated epithelium *(G)*, correspond to the subepithelial nerves in the unoperated control (I). (*H*) In the allograft, however, subepithelial nerves are absent. (*J-L*) Stromal cells and branching nerve bundle (arrowhead) deeper within the underlying stroma of corneas with implant (*J*), and allograft (*K*) and in a corresponding region in the control (*L*). (*M-O*) The endothelium in corneas with implant (*M*), allograft (*N*) and unoperated controls (*O*) are intact and show similar morphology. (Bar = 25 μm for *D-F*, and 5 μm for *G-O*.)

Implants recovered after 6 wk *in vivo* had IR spectra clearly indicating the presence of the copolymer. Histological sections through corneas with implants showed a distinct but smooth, implant-host tissue interface (Fig. 3*A*) resembling that of control corneas with allografts (Fig. 3*B*). In both corneas with implants or allografts, the regenerated epithelium was stratified with no significant difference in epithelial thickness between treatments ($P < 0.05$). Detailed examination showed a fully differentiated epithelium that was positively stained by AE1/AE3 Ab markers (Fig. 3 *D* and *E*), overlying a regenerated basement membrane that was positive for Type VII collagen, a marker for hemidesmosomes (21) at the basement membrane-epithelium interface (Fig. 3 *G* and *H*). Transmission electron microscopy observations indicated morphology consistent with the presence of hemidesmosomes (Fig. 3 *J* and *K*). In implants, neurofilament-positive in-growing nerves had begun to reestablish a subepithelial network and showed extension into the epithelial cells (Fig. 3*M*). No subepithelial nerves were located in the allografted corneas (Fig. 3*N*). The mucin layer of the tear film was restored in corneas with implants (Fig. 3*P*) as in the allograft (Fig. 3*Q*).

Immunohistochemistry indicated that cells within both implant and allograft were synthesizing procollagen I. However, more procollagen synthesis occurred in the allografts as indicated by more intense staining in allografts compared to implants (Fig. 4 *A* and *B*). Both allografts and implants had vimentin-positive stromal cells (no statistically significant difference between groups, $P < 0.05$; Fig. 4 *D* and *E*), indicating a fibroblastic phenotype. Both also showed smooth muscle actin staining and therefore the presence of activated stromal fibroblasts, with no statistically significant difference in positive cell counts ($P < 0.05$; Fig. 4 *G* and *H*).

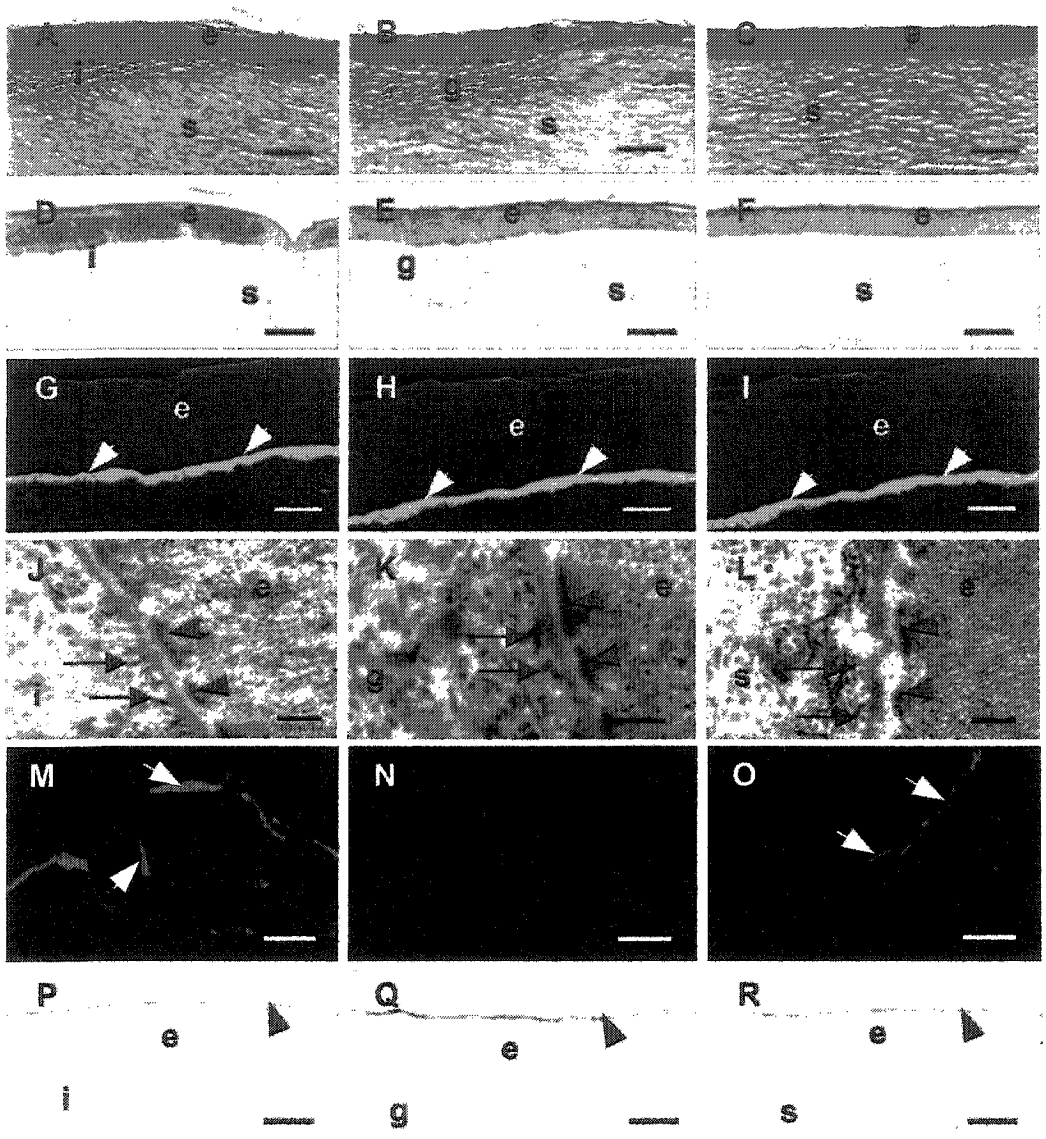

Fig. 3. Postsurgical corneal regeneration. (*A-C*) Hematoxylin- and eosin- stained sections showing stromal cells in implant (*A*) and allograft control (*B*). Both appear to be seamlessly integrated into the host. (*C*) Unoperated control. (D-F) Positive AE1/AE3 cytokeratin Ab staining in regenerated epithelium overlying implant (*D*), which is similar to epithelium of allograft *(E)* and unoperated control (*F*). (*G-I*) Immunolocalization of type VII collagen, a marker for hemidesmosomes, at the epithelium-implant interface (arrows) in the implant (*G*), allograft (*H*) and control (*I*). (*J-L*) Transmission electron microscopy of epithelium-implant interface. Hemidesmosome plaques (arrowheads) and anchoring fibrils (arrows) have formed within the ECM between the epithelial cells and underlying implant *(J)*, emulating the structures normally found at the epithelial-stromal interface as demonstrated in the allograft (*K*) and control (*L*). Flat mount of cornea showing nerve fibers (arrows) within the implant (*M*), and unoperated control (*O*) but not in the allograft (*N*), stained with an antineurofilament Ab. *Ulex europaeus agglutinin* binding (arrowheads) to the epithelial surface on the implant (*P*) and allograft (*Q*) indicate restoration of tear film mucin layer in all cases. (*R*) Unoperated control. e, epithelium; i, implant; g, allograft; s, stroma (Bar = 100 μm for *A-F*, 40 μm for *G-I*, 200 nm for *J-L*, 20 μm for *M-O*, and 30 μm for *P-R*.)

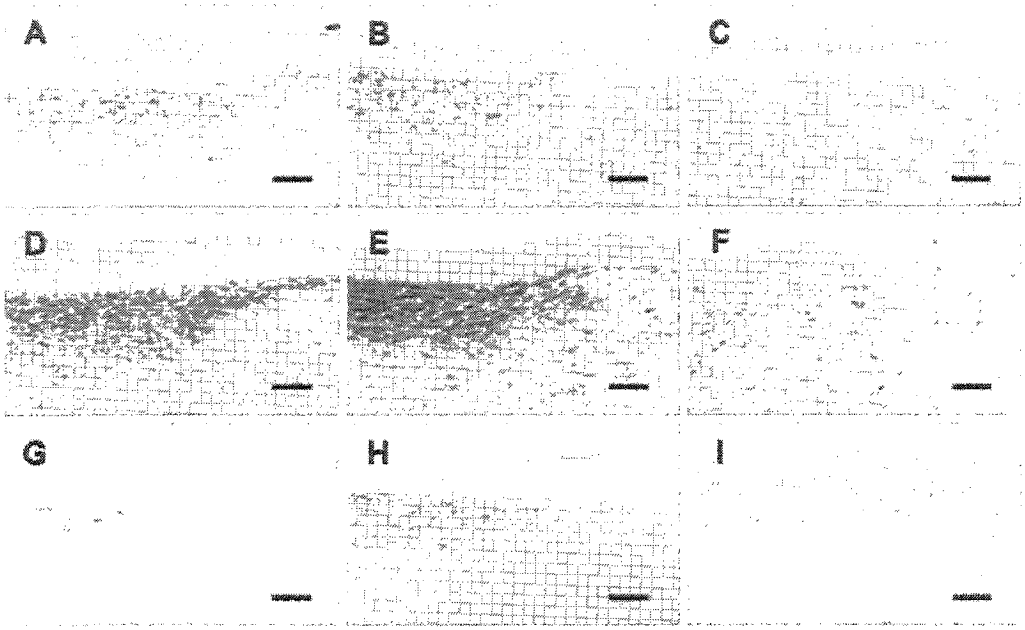

Fig. 4. Implant-host integration at 6 wk after surgery. (*A-C*) Staining for procollagen type I. Positive staining is observed in matrix of both the implanted hydrogel (*A*) and the allograft control (*B*) indicating sites of new collagen deposition. Unoperated control (*C*) has no new collagen synthesis. (*D-F*) Staining for vimentin throughout stroma identifies stromal fibroblasts. Staining throughout the implanted hydrogel (*D*) demonstrates cell invasion. Cells may also been seen within the implanted allograft (*E*) and throughout the unoperated control (*F*). (*G-I*) Smooth muscle actin staining indicates activated myofibroblasts and the potential for scarring. In the hydrogel implant (*G*), staining is occasionally present in the hydrogel, but is not found in the host stroma or the transition zone between host and implant. Positive staining in the allograft-implanted cornea (*H*) is identified both in the allograft and the transition zone but not in the intact host stroma. (*I*) Unoperated control. . e, epithelium; i, implant; g, allograft; s, stroma. (Bar = 100 μm in all cases.)

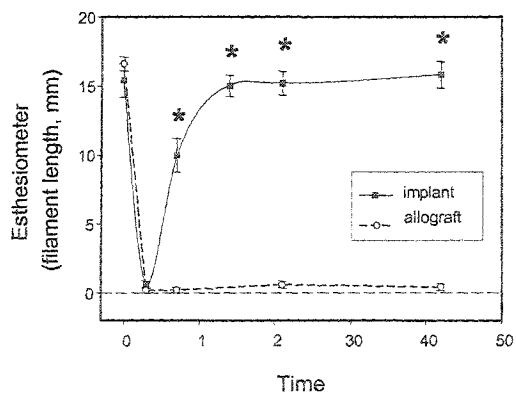

Fig. 5. Corneal touch sensitivity in implants vs. allografts by esthesiometry. All groups, $n = 3$. *, $P < 0.01$ by repeated-measures ANOVA with Tukey two-way comparisons.

Corneal touch sensitivity, measured pre- and postoperatively by esthesiometry (19), showed a dramatic drop in touch sensitivity after surgery. However, recovery of sensitivity occurred between 7 and 14 d, and by 21 d postoperative had returned to preoperative levels (Fig. 5). In control animals that had received donor corneal allografts, however, the corneas remained anesthetic over the 6-wk period (Fig. 5).

DISCUSSION

The human cornea comprises $\approx$70% dry weight type I collagen (25). Other ECM molecules present include primarily smaller keratin sulphate proteoglycans that form intercollagen linkages. During natural corneal development, cellular secretions and ECM sequesterization produces microenvironments that dictate later functions (26). Our corneal ECM replacement was designed to mimic natural ECM characteristics by containing collagen, and emulating the natural crosslinks with TERP5 copolymer synthesized from *N*-isopropylacrylamide. Such polymers are well known to allow cell growth on their surface (27) and, more importantly, when cells are completely encapsulated (28). TERP5 contained grafted YIGSR and also spontaneously reacted with ϵ-NH_2 groups of collagen at neutral pH to form gels at 21°C. This resulted in collagen becoming locked in a form with microfibril diameters well below the wavelengths of visible light. This is critical for optical clarity (29) and similar to the human cornea (30). The crosslinking reaction allowed us to take advantage of the cell growth promoting and chemotrophic properties inherent to collagen and the laminin YIGSR peptide, an established cell adhesion mediator and promoter of epithelial growth (12) and enhancer of neurite extension (13), as well as the propensity of the "rigid rod" collagen backbone to form robust hydrogels. Our hydrogel matrices were less robust

than the highly regular human corneal ECM (30), probably because they were random arrays of "rigid rod" collagen chains, but were adequate for suturing.

We had removed and replaced only the more superficial portion of the cornea (epithelium and anterior stroma) by LKP, a surgical procedure used to treat patients whose more superficial portions of the corneal are damaged. Nonpenetration of the anterior chamber keeps the inner contents of the eye intact and reduces the rate of rejection and postoperative complications (e.g. infection), thus improving long-term graft stability (31), and in this study, eliminates the potential confounding factor of post-surgical infection. Our collagen-TERP5 matrix replacements were designed with the goal of promoting host tissue repair and regeneration by allowing in-growth of host corneal cells and nerves. Nerve re-growth into these biosynthetic composites occurred within 3 wk of surgery in micro-pigs, as found in wounded rabbit corneas (32). Touch sensitivity was also restored within the same time frame, although no nerve re-growth or touch sensitivity was observed in corneas of pigs that received allografts. Here, traditional allografts containing donor cells were used, as is the case of human corneal transplantations that the implants are designed to replace. This corresponds to reports where nerve in-growth and touch sensitivity recovers slowly (>18 mo after laser refractive eye surgery; >10 yr after LKP) after human corneal wounding or transplantation (33, 34). Our result, therefore, represents a dramatic advance over the slow nerve regeneration after current human donor allograft therapy (34) and a demonstration of *in vivo* nerve regeneration in the cornea promoted by a fabricated material.

Implants recovered after 6 wk *in vivo* had IR spectra clearly indicating the presence of copolymer scaffold in regions showing cell and nerve in-growth and that were tested for touch sensitivity. While the haematoxylin and eosin demarcates a continuous transition

between polymer and host, this was most likely due to stromal cell in-growth (Fig. 4*D*). The low level of smooth muscle actin indicates a limited conversion of stromal cells into activated myofibroblast-like cells, which might produce opacity (by scarring, causing light scatter). Overall, the ECM replacements were able to emulate key properties of natural ECM macromolecules by (*i*) allowing for cell-matrix interaction in the restoration of functional structures including the generation of a basement membrane between the implant and overlying epithelium, stromal cell, and nerve axon in-growth; (*ii*) potentiating the differentiated cell state; and (*iii*) integrating into the host tissue. Finally, this concept of emulating a natural ECM to stabilize a damaged tissue and allowing for regeneration makes the TE polymer implant distinct from current prosthetic cornea strategies (5).

Overall, we can now control the strength and optical clarity of noncytotoxic, biosynthetic composites to the point that TE corneal replacements may, ultimately, address future world shortages of donor corneas. In addition, such corneal implants could circumvent potential problems resulting from the lack of nerve regeneration after surgery, found both in human donor tissue (34, 35) and in other, proposed, fully synthetic or acylated collagen implants (5, 36). These biosynthetic matrices with bound cell adhesion factors may have implications for the general field of tissue engineering, especially for the challenging problem of nerve regeneration and the innervation of other engineered organ and tissue systems.

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CHAPTER 5

General Discussion

It is known that a sensory nerve supply is crucial for optimal tissue function. For this reason, innervation is an issue in the development of organ or tissue substitutes in the field of tissue engineering. Major goals of tissue engineering with respect to innervation include the restoration of nerves damaged by disease, injury or surgery in patients, and to innervate engineered organ and tissue substitutes to confer control and to ensure their proper functioning. This is important for the cornea specifically, since the absence of innervation can lead to vision loss. Despite the availability of several cornea models (prosthetic or tissue-engineered), none are ideal for the study of corneal nerves.

Therefore, several issues of innervation in the cornea are yet to be fully addressed: 1) the mechanisms responsible for successful nerve growth and the interaction between nerves and their target tissues are not yet fully understood; 2) a validated innervated cornea model has not yet been developed as a replacement for animals for in vitro toxicology testing; and 3) acceptable corneal substitutes for transplantation are not available and fail to address innervation. In the present work, the human corneal equivalent developed in our lab (Griffith et al., 1999) provided a suitable system for the study of innervation in the cornea.

5.1 Significant Scientific Contributions

No ideal system for the study of human corneal innervation has been available. The use of ex vivo human corneas is not feasible due to a shortage of corneas for transplantation, and animal models are not accurate in assessing the human response. The results presented therefore demonstrate significant advances for the study of several issues that are yet to be fully addressed:

1. The development of the innervated TE cornea model provides a useful tool for studying corneal innervation and wound healing, and has the advantage of a controlled environment. The TE cornea also provides a model in which to study the mechanisms for successful innervation and the signaling pathways between nerves and their target tissue. In addition, the presence of nerves adds an important element of the natural human cornea, hence providing a more complete and accurate tissue.
2. To date, no validated alternative model has been produced to replace live animal toxicology assays. The models proposed have all neglected the issue of innervation, which plays an important role in the sensitivity response. The present innervated TE cornea demonstrated the potential for use in the assessment of irritancy; and with further assessment and validation with a panel of chemicals, it could provide an alternative to animals in ocular toxicity testing.
3. Successful corneal replacement by the cornea substitutes (prosthetic or tissue-engineered) developed to date is yet to be achieved. In addition, none have addressed the issue of innervation, despite the fact that loss of corneal innervation can lead to vision loss. The *in vitro* model and its tested functionality provided the basis for the development of stronger and tougher cornea substitutes for use in transplantation that promoted nerve in-growth. In addition, the culture system developed for the in vitro model was useful for the testing and selection of optimal fabricated matrices for future implantation.

5.2 Specific novel technologies, methods or results

1. The development of a three-dimensional (3-D) in vitro cornea model that incorporates innervation.

2. The fabrication of a TE cornea and its surrounding tissue-engineered sclera, each with distinct characteristics.
3. The development of a laminin gradient within a 3-D construct for the promotion and guidance of nerve growth.
4. The provided ECM components, growth factors and culture conditions were successful in reproducing the morphology of human corneal nerves within the TE cornea.
5. The development of a model in which to study interactions between nerves and their directly innervated targets.
6. The presence of nerves in the TE cornea affects the properties of the corneal cells. The result is an innervated model cornea that better represents the morphology and physiology of the natural human cornea.
7. The development of an in vitro assay system for testing prospective tissues for in vivo transplantation.
8. The discovery that Na^+ concentration changes within nerve fibres in an in vitro cornea model could be used for assessing ocular toxicity.
9. The development of 2-photon microscopic methods for the visualization and study of fine nerve structures within thick, whole TE tissues.

5.3 Tissue engineering and innervation

Although the focus of this research was on the innervation of the cornea, these results may have broader implications for the field of tissue engineering. Major goals of tissue engineering with respect to nerves include the restoration of nerves damaged by disease,

injury or surgery in patients, and to innervate engineered organ and tissue substitutes to confer control and to ensure their proper functioning.

Several systems have been developed to study neurite growth, including animal and cell culture models. These systems are useful for the discovery and understanding of many aspects of nerve growth and survival and provide models for the study of nerve regeneration following injury or disease, but are not ideal. Animal models are not accurate in predicting the human nerve response and cell culture systems cannot reliably demonstrate physiological changes of nerves that occur in vivo (Symposium Proceedings, Technical committee on alternatives to animal testing, 1996). The influence of cell-cell contacts has been studied in the CNS using tissue slices (Knoops et al., 1991; Ruscheweyh and Sandkuhler, 2001), but this method does not allow for complete monitoring of cells and molecules contained within the slice. Therefore, there is a need for three-dimensional culture systems that closely reproduce the natural environment for peripheral nerve growth.

Various tissue-engineering strategies have also been employed to develop 3-D biomaterials for the guidance of axonal growth in the peripheral nervous system, particularly for use in bridging peripheral nerve gaps caused by injury or deficiency. These matrices have included the use of agarose, polyglactin scaffolds, polysulfone tubes and hydrogels among others (Balgude et al., 2001; Borkenhagen et al., 1998; Woerly et al., 1996; Yu and Bellamkonda, 2003). However, none of these models truly reproduces the in vivo environment and the interactions between the nerves and their target tissue.

A recent TE model was developed for the study of neurite growth by the co-culture of murine dorsal root ganglion neurons within a chitosan/collagen biopolymer containing fibroblasts, endothelial cells and keratinocytes from the skin of the human breast (Gingras et

al., 2003). While these methods produced a model tissue with extensive innervation, the function of the nerves and their effects on the properties of the other cells was not assessed. In addition, the keratinocytes (epithelial cells) were seeded onto the construct 10 days after the seeding of the DRG neurons. The intent of this model was to recapitulate the peripheral nerve regeneration process in vitro, however the epithelium, the target tissue and perhaps the most important component of the regenerative process, was not present during the growth of the nerves. Because nerve function was not assessed and the target tissue was absent during the period of nerve growth, it cannot be concluded that this model truly mimics the nerve growth process. As such, its value as a tool for the study of innervation is yet to be validated. Therefore, our functional innervated model appears to be the most promising model developed to date for the study of nerve-target tissue interactions in any tissue system.

In conclusion, the topic of corneal innervation is important and the results presented demonstrate significant advances in the development of an in vitro model for the understanding of this issue. The three manuscripts focus on three important applications of the model. The innervated TE cornea provides a useful tool for studying corneal innervation and wound healing, and has the advantage of a controlled environment. In addition, it could be further developed for use as an alternative to animal ocular toxicity testing and it provides insight for the development of cornea substitutes for use in transplantation. The development of the present innervated TE cornea may also provide some insight into nerve regeneration and the innervation of other engineered organ or tissue systems designed for in vitro use or transplantation.

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Appendix

Expanded Materials and Methods

Dissection of Dorsal Root Ganglion

Dorsal root ganglia (DRG) were isolated from day 8 chick embryos. Only embryos that displayed a healthy colour, well-formed vascular network, and a constant heartbeat and were devoid of any defects or abnormalities were used; others were discarded. Dissections were performed in Hank's Basic Salt Solution (HBSS; Life Technologies, Burlington, Canada) with gentamycin. Essentially, spinal cords were isolated from the embryo by the removal of the limbs, visceral tissue and head. In earlier experiments, these were placed in 0.25% collagenase for ½ hour at 37°C to digest the extracellular matrix and make subsequent steps in dissection easier. However, with experience at the dissection, this step was no longer necessary. Spinal cords were then dissected with microneedles to isolate and remove the neural tube. The ganglia, which protrude off of the neural tube, were then removed using the microneedles and placed in M199 medium + 10% fetal bovine serum (FBS) + 1% insulin transferase selenium (ITS) + gentamycin. Medium applied to the surface in the culture dish caused the submergence of the DRG to the bottom of the dish. Prior to embedding in the constructs, the DRG were incubated for a minimum of 2 hours, and up to overnight, at 37°C to allow for the migration and partial removal of non-neuronal cells.

Optimized TE Corneas

Human corneal cell lines with extended life spans (Araki-Sasaki, 2000; Griffith, 1999) were used. Optimized corneal constructs, designed using results from the previous section, were prepared with a laminin (Becton-Dickinson) gradient of increasing concentrations from bottom to top of 0, 10 and 20 μg/ml. Stromal cells (keratocytes) were

added to all three layers of the collagen mix, and NGF (100 ng/ml; Sigma) was added to the top layer of collagen mix. After thermogelling, 1 X 10^4 epithelial cells were seeded onto the construct, which was supplemented with modified SHEM medium (Jumblatt, 1983) containing 2% B27, 1% N2 nerve growth supplements (Life Technologies, Burlington, Canada) and 1 nmol/ml RA (Sigma). At epithelial confluence, usually after 2 days, the constructs were maintained at an air-liquid interface for up to 10 days until used. This involved the removal of the medium inside the insert, leaving the epithelium exposed to air. The TE cornea continues to be supplemented with the modified SHEM medium from the outside of the insert only. This air-liquid interface allows for the stratification of the epithelium.

TE corneas with surrounding pseudo-scleral ring

To simulate nerve in-growth through the sclera into the cornea, a pseudo-scleral ring was fabricated within a 24 mm diameter culture insert (Corning). The scleral matrix was prepared as above, with modifications. A uniform laminin concentration of 10 μg/ml and a 2:5 ratio of collagen to CS were used. A circular plug was placed in the centre of the insert and the scleral matrix was poured into the area surrounding the plug. Isolated DRG were embedded within this pseudo-scleral ring. The pseudo-sclera was then thermogelled for 2 hours and incubated overnight at 37 °C. The following day, the centre plug was removed and TE corneas were constructed, as described above, inside this scleral ring. The TE cornea was thermogelled for 2 hours, 1 X 10^4 epithelial cells were seeded on top, and the entire scleral/TE cornea construct was cultured as described above. The epithelial cells were grown to confluence and then the constructs were cultured at an air-liquid interface to allow for epithelial stratification.

In vitro Characterization of Polymer Matrix Implants

Prior to their use in transplantation experiments, polymer matrix implants, which were developed by Dr. Fengfu Li (ICPET, National Research Council), were tested for biocompatibility. Immortalized corneal epithelial cells (Araki-Sasaki, 2000) were used to evaluate epithelial coverage on collagen–TERP, collagen-TERP5 and collagen only implants. Implants (500 μm thickness) were embedded on top of a collagen-based matrix that consisted of a mixture of blended neutralized, type I rat-tail tendon collagen (0.3% w/v, Becton-Dickinson, Oakville, Canada) and chondroitin 6-sulfate (1:5 w/w ratio), cross-linked with 0.02% v/v glutaraldehyde (followed by glycine termination of un-reacted aldehyde groups) and then thermo-gelled at 37 ˚C. Controls consisted of the collagen matrix alone. Epithelial cells were seeded on top, and constructs were supplemented with a serum-free medium containing epidermal growth factor (Keratinocyte Serum-Free Medium (KSFM; Life Technologies, Burlington, Canada)) until confluence. The medium was then switched to a serum-containing medium (modified SHEM medium (Jumblatt, 1983)) for 2 days, followed by maintenance at an air/liquid interface. At 2 weeks, constructs were fixed in 4% paraformaldehyde (PFA) in 0.1M PBS and were processed for routine haematoxylin and eosin (H&E) staining. The number of cell layers and the thickness of the epithelium were measured from 6 random areas for each of 4 samples within each of the 3 experimental groups: control and 2 implant types.

Other constructs, as above, were used to examine early nerve in-growth. Dorsal root ganglia (DRG) from chick embryos (E 8.0), were embedded within the surrounding matrix adjacent to the polymer. Cultures were supplemented with KSFM medium containing 2% B27 and 1% N2 supplements (Life Technologies) and 1 nM retinal acetate (Sigma, Oakville,

Canada) to support nerve growth. After 4 days, constructs were fixed as above for immunohistochemistry on whole mounts (see below for details) to visualize nerves within constructs. Nerve density (the number of nerves per μm^2) was calculated at distances of 75 and 100 μm from the edge of the DRG adjacent to the implant within a 90° pie-shaped wedge extending into the implant.