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# Tissue Engineering a Human Conjunctiva-Scleral Model for *In Vitro* Testing

by

**Léa Muzakare**

Thesis submitted to the department of Cellular and Molecular Medicine in partial fulfillment of the requirements for the degree of Master of Science

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# Abstract

My objective was to tissue engineer a human conjunctiva-scleral equivalent with very basic inflammatory components, using a combinatorial approach. The complete model would comprise an innervated, vascularised stroma, overlaid by a stratified epithelium within a bio-synthetic matrix. Matrices fabricated from fibrin and either poly (N-isopropylacrylamide) or poly (N-isopropylacrylamide)-co-acrylic acid, supported differentiation of a human vascular endothelial cell line I immortalized into vessel-like structures. Human neutrophils and a granulocytic cell line, HL60 were able to migrate through these matrices and produce matrix metalloproteinases, in response to chemotactic stimuli. Innervation was introduced by embedding dorsal root ganglia as nerve sources within the matrices, while epithelial cells were seeded on top of the matrix. Contributions of this thesis include: 1) methodology for tissue engineering a conjunctiva-scleral tissue substitute, and 2) demonstrating basic functionality. This model may be further developed for use as an alternative to animals for *in vitro* toxicology testing.

# Dedication

I would like to dedicate this work to my family.

## Acknowledgments

I would like to thank my supervisor, Dr. May Griffith for her guidance, endless supply of patience and for providing me with the opportunity to pursue research in an exciting and growing field.

I would like to extend my thanks to Dr. Charles Doillon and his technician Edith Gagnon, for the primary human umbilical vein endothelial cells (HUVECs), and use of their laboratory facilities and training.

I would also like to thank Dr. Frank Li for synthesis of the p(NIPAAm) and p(NIPAAm)-co-AAc polymers used in this work.

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Finally, a special thank you to my family for their lifetime of understanding, love, unconditional support and constant encouragement throughout all this. Most important of all is the support I received from Lise, my younger sister. I have been overwhelmed; I hope I will have the opportunity to reciprocate.

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## List of abbreviations

bFGF or FGF-2: basic fibroblast growth factor

Dil-Ac-LDL: di-acetylated low density lipoprotein

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimethyl sulfoxide

DRG: dorsal root ganglia

ECGS: endothelial cell growth supplement

ECM: extracellular matrix

EDTA: ethylenediaminetetra acetic acid

EGF: epidermal growth factor

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

FMLP: formyl-methionine-leucyl-phenylalanine

FpA: fibrinopeptide A

FpB: fibrinopeptide B

GAGs: glycosaminoglycans

H&E: haematoxylin and eosin

Hepes: N-2-hyodroxyethyl-piperazine-N-2-ethane sulphonic acid

HCEC: human corneal epithelial cell line

HBSS: hank's balanced salt solution

HL 60: human leukemic cells

HPV: human papillomavirus

hTERT: human telomerase reverse transcriptase

HUVEC: human umbilical vein endothelial cells

ICAM: intercellular adhesion molecule

KSFM: Keratinocyte Serum-Free Medium

LCST: lower critical solution temperature

MMPs: matrix metalloproteinases

MT-MMPs: membrane-type MMPs

NaOH: sodium hydroxide

NF: neurofilament

PBS: phosphate buffered solution

PFA: paraformaldehyde

PMN: polymorphonuclear

p(NIPAAm): poly (N-isopropylacrylamide)

p(NIPAAm)-co-AAC: poly (N-isopropylacrylamide)-co-acrylic acid

RA: retinoic acid

RAc: retinal acetate

Rb: retinoblastoma

RBCs: red blood cells

RPMI 1640: Rosewell Park Memorial Institute 1640

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TERT: telomerase reverse transcriptase

TIMPs: tissue inhibitor of metalloproteinases

TPA: phorbol ester

tPA: tissue-type plasminogen activator

UEA: *ulex europaeus* lectin

VEGFR: vascular endothelial growth factor receptor

VCAM: vascular cell adhesion molecule

VE-cadherin: vascular endothelial cadherin

vWF: von willebrand factor

# I Introduction and Objectives

## I.1. The eye

### I.1.1. Structure of the eye

The eye is generally divided into the anterior and posterior segments, separated by the lens and iris (Fig.1). The anterior segment comprises the cornea, conjunctiva, sclera and anterior chamber with aqueous humour, while the posterior segment includes the retina, choroid, posterior chamber with vitreous humour and optic nerve.

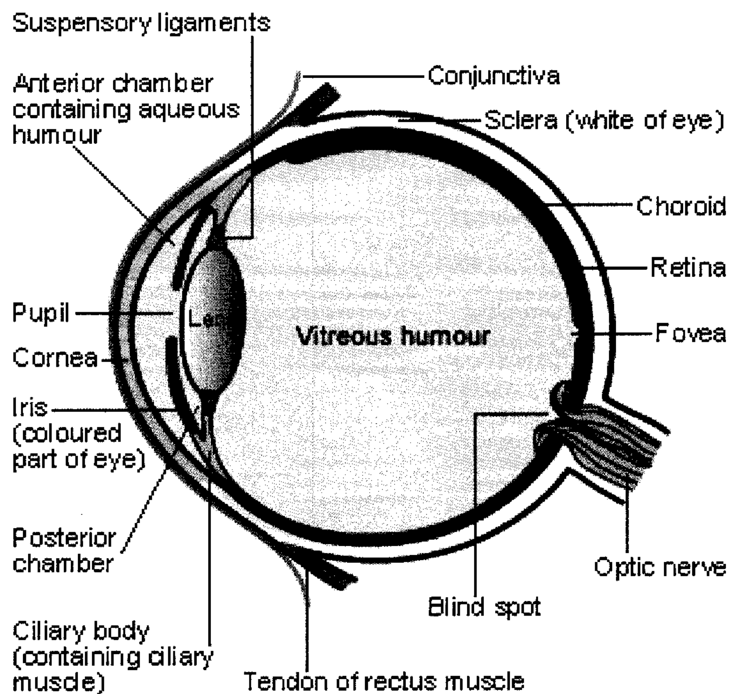


Fig. 1: Section through an eye showing various structures (reproduced from: www.mydr.com.au. With copyright permission from MediMedia Australia)

### I.1.2. Conjunctiva

The conjunctiva is a thin vascularised mucous membrane that lines the eyelids and the anterior surface of the eyeball except for the region that is occupied by the cornea. Its superficial layer, the conjunctival epithelium, is a non-keratinising stratified squamous epithelium that is continuous with that of the cornea. Along with the cornea, the conjunctiva is an area that is prone to accidental exposure to substances that are potentially irritating or toxic to the eye. The sensory nerve supply of the conjunctiva is almost entirely from branches of the ophthalmic division of the trigeminal.

### I.1.3. Sclera

The sclera, or the white part of the eye, sits underneath the conjunctiva and forms the principal part of the tough, outer fibrous coat of the eye. Its functions are to protect the intraocular contents and maintain the shape of the globe. In the front of the eye, it becomes continuous with the optically clear cornea. In the back of the eye, it meets the optic nerve. The sclera is separated from the overlying conjunctiva by a thin elastic connective tissue membrane known as "Tenon's capsule" that encases the eye. Both the cornea and the sclera are made of the same materials including collagen and fibroblasts. However, differences in the arrangement of the collagenous matrix and the water content of the tissue differentiate a clear cornea from the white sclera (Maurice, 1984). The sclera is traversed at a number of sites by blood vessels and nerves, which supply the middle and inner layer of the eye.

## I.2. Angiogenesis and *in vitro* models of angiogenesis

### I.2.1. Angiogenesis and vasculogenesis: blood vessel development in the sclera and conjunctiva

Vasculogenesis and angiogenesis are responsible for the formation of new blood vessels in the embryo (Gilbert, 1997). Hertig in 1935, coined the term "angiogenesis" to describe the formation of new blood vessels in the placenta and, later in 1971, Folkman used it to describe neovascularization (Folkman et al., 1971). During the early stages of embryogenesis, the vascular network develops from endothelial progenitor cells or angioblasts by a process termed vasculogenesis (Flamme et al., 1997). In contrast, angiogenesis is a fundamental process, which occurs during adult life in reproduction (Hyder and Stancel, 1999) and wound healing by which new blood vessels are formed from preexisting vessels. Blood vessels supply tissues with oxygen and nutrients necessary for survival and growth. Endothelial cells represent a large population of quiescent cells lining the walls of the blood vessels.

Angiogenesis plays an important physiological and pathological role in the eye. Pathological angiogenesis is a major contributing factor in vision loss and blindness due to macular degeneration, neovascular glaucoma and diabetic retinopathy (Adamis et al., 1999; Folkman, 1985; Folkman and Klagsburn, 1987; Lee, Wang and Adamis, 1998). Angiogenesis is a dynamic, multistep process that involves extensive interplay among cells, soluble factors and extracellular matrix (ECM) components. It occurs as an orderly series of events (illustrated in Fig. 2) that comprise:

- Release of proteases from activated endothelial cells deprived of oxygen
- Degradation of the ECM surrounding the pre-existing vessel
- Migration of endothelial cells toward an angiogenic stimulus and their proliferation
- Lumen formation
- Generation of new basement membrane with the recruitment of pericytes
- Connection of individual blood vessel tubes to form blood vessel loops
- Initiation of blood flow.

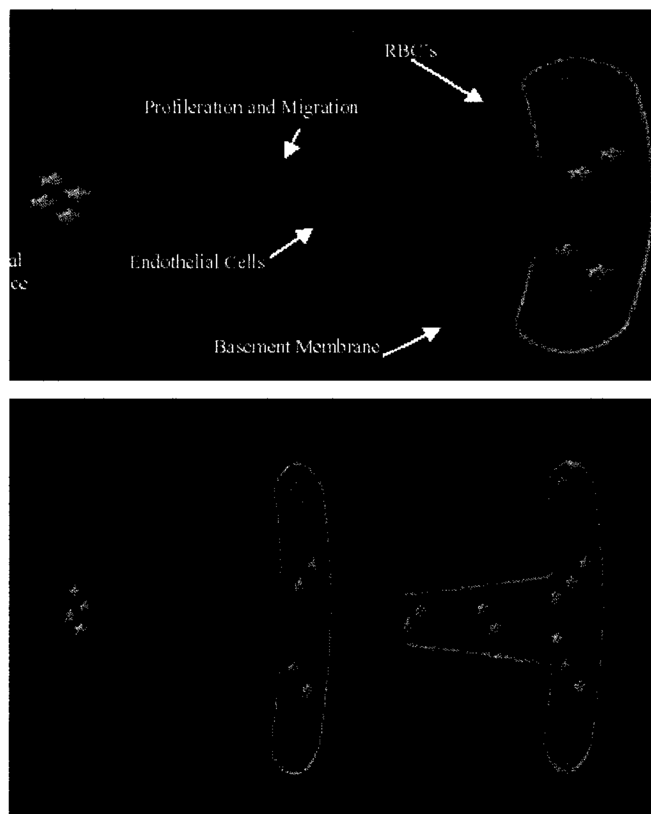


Fig. 2: Angiogenesis process (From: www.hepatitiscfree.com, with copyright permission).

### I.2.2. Regulation of angiogenesis: inducers and inhibitors

Angiogenesis is controlled by a finely tuned balance between levels of angiogenesis inducers and inhibitors (Glaser, 1998). When this balance is destroyed, it usually results in pathological angiogenesis, which causes increased blood vessel formation in diseases mediated by angiogenesis.

A variety of substances that promote angiogenesis have been described and can be divided in two categories (Klagsbrun and Moses, 1999) – those that directly influence the migration or proliferation of endothelial and other cells, particularly inflammatory cells (e.g. macrophages), and those that presumably act indirectly on the capillary cells via their intrinsic mitogenic activity.

Many endogenous, positive regulators of angiogenesis that promote endothelial cell growth have been described (Casey and Li, 1997). These include a variety of growth factors, matrix metalloproteinases, cytokines, and integrins (Table 1). In this study, three well-documented angiogenic factors known to act directly on cultured endothelial cells were examined. These are basic fibroblast growth factor (bFGF; also known as FGF-2), epidermal growth factor (EGF) and retinoic acid (RA).

bFGF is a peptide growth factor that has been shown to stimulate the proliferation, migration of endothelial cells and formation of tubes *in vivo* and *in vitro* (Presta et al., 1986; Bikfalvi et al., 1997). This factor has long been considered as the principal tumour angiogenic factor (Bikfalvi et al., 1997). Also, bFGF was found to induce tube formation in a three-dimensional collagen matrix

and to modulate gap junction intercellular communication (Bussolino et al., 1996; Montesano et al., 1986) in human umbilical vein endothelial cells (HUVECs).

EGF is also a peptide growth factor. It has a normal physiological role in many species in stimulating the growth and differentiation of a variety of mammalian cells, including epithelial cells and is also involved in mechanisms such as oncogenesis, and wound healing (Cohen, 1965). *In vivo*, it induces the production of angiogenic mediators from another cell type, for example, fibroblasts, in addition to the endothelial cells.

RA is a metabolic product of retinol or Vitamin A, and putative morphogen. It is part of a complex signalling system that triggers key steps during development, control maintenance of homeostasis (Gudas et al., 1994), and induces or inhibits cellular proliferation, differentiation and death of a variety of normal and transformed cells (Sporn et al., 1994; Kastner, Mark and Chambon, 1995).

Various factors that inhibit angiogenesis have also been identified. Several of the best known ones are shown in Table 1.

Table 1: Factors regulating angiogenesis

| **Inducers of angiogenesis** | **References** |
| --- | --- |
| Angiogenin | Casey and Li, 1997 |
| Angiopoietin 1 | Black, 1998; Peters, 1998 |
| Epidermal growth factor | Cohen, 1965 |
| Fibroblast growth factors | Relf et al., 1997 |

| | |
|---|---|
| Granulocyte colony-stimulating factor | Casey and Li, 1997 |
| Hepatocyte growth factor or Scatter factor | Bussolino et al., 1992; Casey and Li, 1997 |
| Interleukin-8 | Casey and Li, 1997; Koch et al., 1992 |
| Placental growth factor | Casey and Li, 1997; Relf et al., 1997 |
| Platelet-derived endothelial cell growth factor | Jampol et al., 1994; Miyazono and Takaku, 1991; Relf et al., 1997; Ishikawa et al., 1989 |
| Retinoids | Gudas et al., 1994; Sporn et al., 1994 |
| Transforming growth factors | Jampol et al., 1994; Pepper et al. 1990; Schreider et al., 1986 |
| Tumour necrosis factor alpha | Frater-Schroder et al., 1987; Folkman et al., 1971 ;Jampol et al., 1994; Schereiber et al., 1986 |
| Vascular endothelial growth factor | Breir et al., 1992; Relf et al., 1997; Shweiki et al. 1992; Peters, 1998; Tischer et al. 1991 |
| **Inhibitors of angiogenesis** | |
| Angiostatin | Casey and Li, 1997; Folkman, 1994; O'Reilly and Folkman, 1995; O'Reilly et al., 1996; |
| Endostatin | Casey and Li, 1997; O'Reilly et al., 1997 |
| Interferon alpha | Sidky and Borden, 1987 |
| Metalloproteinase inhibitors | Johnson et al., 1994 |
| Platelet factor 4 | Casey and Li, 1997; Sato, Abe and Takaki, 1990 |
| Prolactin 16-kd fragment | Casey and Li, 1997 |
| Thrombospondin-1 | Casey and Li, 1997 |
| Transforming growth factor beta | Frater-Schorder et al., 1986; Merwin et al., 1990; Jampol et al., 1994; Roberts et al., 1986 |

### I.2.3 *In vitro* models of angiogenesis

Numerous forms of *in vitro* assays have been developed to study the molecular and cellular biology of angiogenesis by using endothelial cells (Jain et al., 1997). *In vitro* models comprising isolated endothelial cells are useful but have been limited by the availability of their suitable sources, the difficulty in obtaining adequate numbers of primary isolates, and changes during prolonged culture, including alterations in activation state, expression of cell surface antigens and growth properties (Jaffe, 1984).

Most steps in the angiogenic cascade can be analyzed in *in vitro* assays, including endothelial cell proliferation, migration and differentiation (Montesano et al., 1992) in response to angiogenic stimulation. The *in vitro* models that best reproduce the angiogenesis process operate on the principle that endothelial cells form three-dimensional tube-like structures when cultured on supportive matrices such as collagen, fibrin or matrigel (Madri et al., 1988). The presence of exogenous growth factors as well as extracellular matrix molecules is usually required for tube formation (Vailhé et al., 1996).

Methods are therefore available for recreating angiogenesis *in vitro*. These can be used as a guide for introducing vascular components into a conjunctiva-scleral model such as the one for this thesis project. Angiogenesis rather than straight vasculogenesis was mimicked since it is a more accurate re-capitulation of the in-growth of vessels into the anterior of the eye during development than spontaneous development vessels within the tissue (Ashton and Cook, 1953; Kenyon et al., 1996; Klintworth, 1991; Langham, 1953; Gimbrone et al., 1974).

## I.3. Vascular endothelial cell lines

### I.3.1. Endothelial cells *in vitro*

A major part of our knowledge on endothelial cells physiology and biochemistry functions comes from *in vitro* experiments with HUVEC, the most commonly used source of human endothelial cells (Jaffe et al., 1973). A significant problem using these cells is that they have a limited lifespan *in vitro*. Endothelial cells require significant concentrations of serum for growth, and display characteristics that differ in functional assays depending on their vascular origin (Denekamp, 1999). Experimental results obtained with different endothelial cells cannot easily be compared to each other because of their different donor origin.

Primary endothelial cells have an average lifespan of 10 serial passages and can be kept in culture up to 5 months (Jaffe et al., 1973) before they stop proliferating and die. The first attempts to keep endothelial cells in long-term culture used tumour-conditioned medium, gelatin-coated plates and endothelial cells enrichment in the primary culture (Folkman et al., 1979). Both culture and native endothelial cells share the following characteristics:

- Von Willebrand Factor (vWF) secretion, also known as Factor VIII-related antigen. It can be constitutive or regulated. vWF is a large adhesive glycoprotein synthesized in endothelial cells. In the blood it serves as a stabilizing carrier for Factor VIII with which it circulates as a complex (Ruoslahti and Rajotte, 2000)

- Intercellular adhesion molecule (ICAM), Vascular cell adhesion molecule (VCAM) and E-selectin-expression which are up-regulated upon activation (Pober et al., 1986)
- Vascular endothelial cadherin (VE-cadherin) expression in the cellular junctions (Dejana, 1986)
- Binding of the *Ulex europaeus* lectin agglutinin 1 (UEA) (Holthöfer et al., 1982)
- Uptake of acetylated low density lipoprotein (acLDL) (Voyta et al., 1984)

### I.3.2. Immortalization and telomerase

The use of primary endothelial cells for research, biotechnology, and therapeutic purposes has been restricted by their limited lifespan, the inability to generate large amounts of cells, and phenotypical instability from batch to batch due to their multidonor origin (Hayflick and Moorhead, 1961). These facts make it difficult to compare results from experiments with different cell preparations or passages. For many applications, there is very clear need for characterized cells with the desired phenotype that will not undergo senescence, and that can be expanded to give unlimited numbers of cells as needed. In this study, therefore, it was necessary to establish a homogenous endothelial cell population with an expanded lifespan.

In humans and other vertebrates, the distal ends of chromosomes consist of sequences known as telomeres (Dahse et al., 1997). Telomeres consist of hundreds to thousands of hexanucleotide repeats of the sequence TTAGGG

(Rhyu, 1995) that serve a critical function in protecting the ends of chromosomes. Normal cells demonstrate a strictly limited growth potential and senescence after a defined number of cell divisions, where telomeres undergo progressive shortening (Watson, 1972). The telomere is therefore the "clock of replicative senescence by which cells count their divisions in normal cells and can reset gene expression, cell morphology, and the replicative lifespan" (Fossel, 1998). The telomeric shortening is suppressed by the presence of telomerase, which contains an integral RNA with a short template element that directs the synthesis and extension of telomeric repeats at chromosome ends (Feng et al., 1995; Fu et al., 2000; Greider et al., 1989).

Telomerases are specialized structures that maintain chromosome stability by protecting from DNA degradation, end-to-end fusions, rearrangements, chromosome loss and also ensure complete chromosome replication and proper segregation (Rhyu, 1995). Telomerase consists of several proteins including a 120 kDa catalytic subunit called telomerase reverse transcriptase (TERT). This enzyme is expressed in many tissues during embryonic and early postnatal development, but decreases dramatically in association with growth arrest and cell differentiation. A variety of cell lines and malignant tumours are known to express telomerase activity (Kim et al., 1994) whereas most normal somatic cells do not. This suggests that telomerase plays an important role in cellular immortalization and tumourigenesis (Counter et al., 1992). By reactivating telomerase activity, which maintains telomere length, the lifespan of cells can therefore be extended.

### I.3.3. Human papillomavirus (HPVs)

Common methods used to extend the lifespan of cell populations of interest include the introduction of viral oncogenes to block the natural senescence process. Viral genes used to immortalize human endothelial cells include simian virus 40 (SV40) large T antigen, human papillomavirus (HPV) E6 E7 region, and Epstein-Barr virus (EBV) (Katakuran et al., 1998).

HPVs are small DNA viruses. The HPVs can be classified as either "low risk" HPVs (HPV 6 and HPV 11), which cause benign squamous epithelial tumours (warts and papillomas); or "high risk" HPVs (HPV 16, HPV 18, HPV 31, and HPV 54), that are associated with malignant tumours (Zur Hausen, 1996). The transforming activities of the high risk HPVs have been shown to reside primarily in the E6 and E7 genes (Vousden, 1994).

The E6 and E7 proteins have been shown to target and interfere with the normal function of key cellular proteins that physiologically regulate cell proliferation and differentiation (White et al., 1994). E6 and E7 from high-risk HPV16 (Nakamura et al., 1997) bind with high affinity the p53 and retinoblastoma tumour suppressor (Rb) proteins, respectively (Zur Hausen, 1999; Solinas et al., 1997; Dyson et al., 1989), causing loss of control of cellular growth, and thereby assisting the immortalization and maintenance of a fully transformed phenotype in a broad spectrum of cell types (Reznikoff et al., 1994; Heselmeyer et al., 1996; Song et al., 2000).

Portions of the HVP E6 E7 genetic material, however, have been shown to extend the lifespans of primary human cells by reactivation of the telomerase gene without transforming the cells into a malignant phenotype (Rhim et al., 1998).

## I.4. Extracellular matrix (ECM)

### I.4.1. Brief introduction to ECM

The ECM is a hydrated, interconnected molecular network surrounding the cells of the body and is found in nearly all multicellular animal species. It is produced by epithelial cells, endothelial cells, and many mesenchymal cells. The ECM serves not only as an inert scaffold for cell attachment, but interacts with the cells that have secreted these molecules. Many studies show that cellular-ECM interaction plays an important role in biochemical and cellular processes such as adhesion, migration, gene expression, differentiation, and remodelling of blood vessels during angiogenesis (Xu et al., 2001; Lin and Bissell, 1993; Hay, 1981; Yamada and Akiyama, 1984). These functions are dependent on the binding of cell receptors to matrix determinants.

The ECM is made up of resident and transient components. The resident components include structural proteins such as collagen, fibrin (that provide the necessary architecture and scaffold for the cells), glycoproteins like laminin, fibronectin and elastin (that mediate cell adhesion and induce a variety of cellular responses), as well as complex polysaccharides or glycosaminoglycans (GAGs; that act to hydrate the scaffold) (Hakomori and al., 1981). The transient components include diffusible molecules such as the angiogenic FGFs, which act

in a time- and site-specific manner on target cells (Sasisekharan et al., 1997). Each component binds to and interacts with several of the other components to form a complete matrix, which have an even greater activity than the individual components (Timpl et al. 1984; Charnt et al., 1981; Kleinman et al., 1986).

The exogenous ECMs for tissue engineering can be fabricated from two classes of biomaterial: naturally derived materials and synthetic materials. Naturally occurring materials isolated from human or animal tissue are typically not available in large quantities and suffer from batch-to-batch variations, but have the potential advantage of specific cell interactions. Synthetic materials, by contrast, can be manufactured reproducibly on a large scale, and their properties can be controlled and manipulated (Kim and Mooney, 1998).

### I.4.2. Polymers

Tissue engineering research often uses the approach of making three-dimensional polymeric structures to recreate *in vitro* microenvironments that closely mimic those naturally provided by the ECM. During development of an organ, the ECM provides the scaffolding and environmental cues necessary for differentiation. The polymers of the ECM therefore act as scaffolding to bring the desired cell types into contact in an appropriate three-dimensional environment, and also provide mechanical support until the newly formed tissues or organs are fully functional (Kim and Mooney, 1988). The polymer material may be either a homopolymer or a copolymer, cross-linked or not with a cross-linking agent. Both natural and synthetic polymers have been used to recreate biological matrices as

tissue or organ scaffolds, or drug delivery vehicles for biomedical applications, and therefore, have been referred to as biomaterials.

The polymers used in this thesis project are hydrogels. The term "polymer hydrogel" refers to a polymeric material that is characterized by its ability to absorb water and retain it even under considerable pressure. Hydrogels are sensitive to the surrounding environmental conditions such as pH, temperature, ionic strength, solvent composition, pressure and the application of electric fields. Most hydrogels used in our experiments are temperature-sensitive. A temperature-sensitive hydrogel is a porous polymeric matrix that swells below and collapses above its lower critical solution temperature (LCST), resulting in dramatic changes in average pore size and volume of the matrix.

Polymer hydrogels are interesting in that they exhibit both liquid and solid-like properties. When a hydrogel is made up of greater than 80% of water, it exhibits liquid-like properties. However, due to the network formed by the crosslinking reaction within the polymer, it shows solid-like properties.

Polymer hydrogels have been around for a while. Since the mid-1980s, synthetic hydrogel applications have grown rapidly. Currently, they are widely used throughout the industry and manufacture of personal hygiene products like contact lenses. They have shown to be useful in pharmaceutical applications such as in controlled drug release.

In this project, I studied hydrogels made from combinations of natural polymers (fibrin and collagen) and synthetic polymers (such as poly (N-

isopropylacrylamide) (p(NIPAAm)) and poly (N-isopropylacrylamide)-co-acrylic acid ( p(NIPAAm)-co-AAC)).

### I.4.2.1. Fibrin

Fibrinogen, one of the more abundant proteins in human blood, is synthesized by the liver and secreted into plasma. It represents about two to three percent of the total plasma protein. Fibrinogen is an acute phase response protein whose synthesis is increased during injury, inflammation, cigarette smoking and other environmental stimuli (Dvorak, 1986). Circulating plasma fibrinogen is a complex polypeptide chain, composed of two pairs of A$\alpha$, the B$\beta$ and the $\gamma$ chains linked by disulfide bonds and forming a dimer (Gorkun et al., 1997).

Fibrinogen is implicated in a number of physiological and pathological processes including haemostasis, thrombotic diseases, and blood vessel injury (Dvorak, 1986). Fibrinogen has a double function: it participates in both the cellular phase and the fluid phase of coagulation. In the cellular phase, fibrinogen acts to promote platelet aggregation and fibrin supports clot retraction, which may lead to abnormal arterial blood clots and diminished delivery of oxygen to the body. In the fluid phase, fibrinogen is converted to fibrin in a reaction catalyzed by thrombin, which releases two small amino-terminal peptides, fibrinopeptides A (FpA) and B (FpB) and produces insoluble fibrin monomers (Gorkun et al., 1997). During coagulation, fibrin along with platelets forms the meshwork in which blood cells and proteins are trapped (Matsuda and Moghaddam, 1993). The clot is stabilized by plasma Factor XIIIa, a transglutaminase that forms

intermolecular amide bonds and crosslinks the fibrin monomers (Schense and Hubbell, 1999).

Fibrin-based polymers represent a natural, biodegradable, and biocompatible matrix (Doillon, 2002). Reputed to be involved in haemostasis and wound healing, fibrin has been popular in tissue engineering and other biomedical applications (Kerényi, 1984). In addition to being non-toxic (as monomer, polymerized gels, or degraded gel forms), fibrin has been used for temporary tissue replacement and as a scaffold for new adhesion sites of cell-matrix interactions during cell migration (Thiagarajan et al, 1996) and formation of new tubular structures during wound healing (Dvorak et al., 1987). It has been shown *in vivo* and *in vitro* that fibrinogen and fibrin interact with endothelial cells, macrophages, and fibroblasts.

#### I.4.2.2. Collagen

Collagens are a large family of proteins. Many members are fibrous proteins of very high tensile strength involved in the formation of connective tissue in all multicellular animals. Collagen is the major component of cartilage, tendon, skin, bone and blood vessels and serves to hold cells together in discrete tissues. In addition to providing structural support in tissues, collagen interacts with other extracellular matrix components.

The characteristic feature of a typical collagen molecule is its triple-helical structure formed by three extended protein chains that wrap around one another. The helix forming region contains peptide chains with repeating Gly-X-Y triplets,

and hydroxyproline and hydroxylysine, which are relatively specific to collagens (Piez and Reddi, 1984).

Collagen occurs throughout the body in different forms known as types. Each type has structural features that make it suitable for a particular function in a tissue. The following represent the most abundant and important types:

- Type I collagen is the principal component of the human body. It is present in bone, skin, and tendon, and is the predominant type of collagen in a scar tissue.
- Type II collagen is the major type found in articular cartilage.
- Type III collagen is abundant in embryonic tissues. It is produced quickly by young fibroblasts before the tougher type I collagen is synthesised. In the adult, it predominates in pliable organs such as blood vessels, the uterus, and the GI tract.
- Type IV collagen is a long chain molecule of non-fibril origin, found exclusively in basement membranes (Odioso and al., 1995).

Blends of collagen and synthetic polymers have been prepared in order to obtain collagen-based biomaterials with good mechanical properties (Joos and Ries, 1978). Water-soluble polymers (Molymeux, 1985) have been used to achieve good miscibility with collagen. Crosslinking was carried out using fairly traditional techniques already described in the literature (Eye, Paz and Gallop, 1984), for example, by heat treatment or by reaction with glutaraldehyde. Collagen-based biomaterials are used for a variety of biomedical applications

including dialysis membranes, wound dressings and artificial skin (Parkany, 1984).

### I.4.2.3. Poly (N-isopropylacrylamide acid)

Poly (N-isopropylacrylamide acid) (p(NIPAAm)) is a typical temperature-sensitive hydrogel. It exhibits LCST behaviour at 32-33°C in aqueous solution (Matsuda and Moghaddam, 1993). Below that temperature, PNIPAAm chains hydrate to form expanded structures in water and become hydrophilic, but above the LCST will shrink to form a compact structure with more hydrophobic component on the outside of polymer chains (Stile and Healy, 2002). The conversion between the water-soluble (hydrophilic) and water-insoluble (hydrophobic) states is thermally reversible (Schild, 1992; Bae et al., 1990). The degree of solubility is a sequence of the interplay between hydrophilic and hydrophobic forces in solution.

p(NIPAAm)'s phase transition behavior can be controlled by incorporating more hydrophilic or hydrophobic monomers (Yoo et al., 2000). Copolymerization of NIPAAm with a more hydrophilic monomer increases the LCST of p(NIPAAm) copolymers hydrogels and incorporation of a more hydrophobic monomer tends to have the opposite effect (Stile and Healy, 2002).

### I.4.2.4. Poly (N-isopropylacrylamide-co-acrylic acid)

Poly (N-isopropylacrylamide-co-acrylic acid) (abbreviated p(NIPAAm)-co-AAc) is synthesized by copolymerization and cross-linking NIPAAm and

acrylic acid (AAc). The AAc co-monomer is more hydrophilic than NIPAAm and therefore increases the LCST of p(NIPAAm)-co-AAc hydrogels, which is around 34°C to higher temperatures (more than 50°C). In my experiments, the use of the modified monomer allowed composite polymers containing p(NIPAAm)-co-AAc to remain in gel form at 37°C.

p(NIPAAm) and p(NIPAAm)-co-AAC hydrogels are both thermo-responsive water-soluble synthetic polymers and demonstrate stability at 37°C. These hydrogels have been developed for tissue engineering applications for the regeneration of tissues and organs.

### I.4.3. Matrix metalloproteinases system (MMPs)

The MMPs are a family of structurally related secreted zinc-containing enzymes that have the potential to turnover and degrade structural ECM components, a function that is clearly performed by several family members (Cimpean and Kaoliang, 1997; Starlight et al., 2000). These are $Ca^{2+}$- and $Zn^{2+}$-dependent end peptidases and are active at neutral pH. These enzymes are present in normal healthy individuals and have been shown to have an important role in processes such as wound healing, angiogenesis, and embryogenesis. They also play a part in pathological processes such as rheumatoid arthritis, tumour invasion and metastasis. All MMPs are synthesized in the latent form (Thiennu and Zena, 2000). These enzymes are secreted as proenzymes by a variety of cells, including infiltrating inflammatory cells. They require extracellular activation by

proteinases *in vitro,* and tissue or plasma proteinases or opportunistic bacterial proteinases *in vivo* (Nagase and Woessner, 1999).

Over 20 members of this family (Westermarck and Kahari, 1999) have been described. They are distributed into five groups depending upon their substrate specificities or similar structural domains: 1) collagenases that are active against fibrillar collagen, 2) gelatinases or type IV collagenases that have high activity against denatured collagen, 3) stromelysins that degrade noncollagen components of the ECM, 4) membrane-type MMPs (MT-MMPs) that are transmembrane molecules, and 5) other less characterized members (Thiennu and Zena, 2000).

The matrix degrading metalloproteinases have been implicated in the matrix remodelling process of a number of normal physiological processes (e.g. embryogenesis, morphogenesis, angiogenesis, cell migration, proliferation, reproduction and wound healing, apoptosis, etc.) as well as the pathological development of a wide variety of proliferative inflammatory conditions (e.g. arthritis, cancer, cardiovascular disease, nephritis, neurological disease, breakdown of blood brain barrier, skin ulceration, gastric ulcer, corneal ulceration) (Nagase and Woessner, 1999; Cimpean and Caloianu, 1997; Mignatti and Rifkin, 1996; Parks and Shapiro, 2001). Several studies have shown that excessive levels of many MMPs are present in chronically inflamed tissues throughout the body (Parks and Shapiro, 2001). The timely ECM degradation provides the maintenance of a dynamic structural and functional balance of the ECM as well as the ECM-cell relationships (Cimpean and Caloianu, 1997).

McQuibban et al. (2000) suggested that matrix metalloproteinases are both effectors and regulators of the inflammatory response.

MMPs are secreted with their inhibitors, ensuring a stringent control of local proteolytic activity, in order to preserve normal tissue structure. At least four specific inhibitors of metalloproteinases called Tissue Inhibitors of Metalloproteinases (TIMPs, e.g., TIMP-1, TIMP-2, TIMP-3, and TIMP-4) exist and form inactive complexes with MMPs. These complexes prevent MMP action by binding at the active site of MMPs and blocking access to substrate (Thiennu and Zena, 2000). TIMPs have proven efficacy in animal models of diseases in which inappropriate or excessive proteinase expression constitutes part of the pathogenic process and several are now being tested in clinical trials (Cimpean and Caloianu, 1997). MMP inhibition, therefore, is recognized as a good target for therapeutic intervention.

The gelatinase family contains two members, 72 kDa type IV collagenase or gelatinase-A (MMP-2) and 92 kDa type IV collagenase or gelatinase-B (MMP-9) (Collier et al., 1988). These enzymes are capable of degrading basement membrane components including type IV collagen, laminin, and denatured collagens or gelatin (Liotta et al., 1981). They are not expressed in normal, healthy, resting tissues or their production and activity are maintained at nearly undetectable levels.

### I.4.3.1. <u>MMP-2</u>

MMP-2 is the most widely distributed enzyme of the MMP family and it was originally described and purified as a basement membrane collagen degrading enzyme activity from a metastatic murine tumour (Liotta et al., 1981; Eble et al., 1996). MMP-2 is secreted from skin fibroblasts, keratinocytes, epithelial cells, and monocytes. Tissue degradation by the matrix metalloproteinase gelatinase A is pivotal to inflammation and metastasis.

### I.4.3.2. <u>MMP-9</u>

Neutrophils synthesize type IV collagenase (gelatinase B) and store it intracellularly (Senior et al., 1991). Gelatinase B is released in response to variable stimuli including most neutrophil chemoattractants (Vu and Werb, 1998). The movement of polymorphonuclear (PMNs) from the circulation to extravascular sites (emigration) is essential for host defense and a key event in inflammatory disease (Brown, 1997). The capacity of gelatinase B to degrade basement membrane components, and its inhibition activity in blocking PMN migration through basement membrane *in vitro*, has suggested an important role for gelatinase B in PMN emigration (Delclaux et al., 1996). MMP-9 is also induced and found localized in the neurites, suggesting that it participates in neurite growth (Chambaut-Guérin et al., 2000).

## I.5. Inflammation

### I.5.1. Inflammation in the eye

Inflammation in the eye is the result of the body's standard defence mechanism against toxic foreign material. Just as in the rest of the body, when there is an inflammation, the eye responds by increasing its blood flow and permeability of its blood vessels, resulting in the escape of inflammatory cells from the blood into the tissues. There are numerous blood vessels in the sclera and conjunctiva. In cases of scleritis or conjunctivitis (inflammation of the sclera and conjunctiva respectively), small blood vessels become more prominent, resulting in a redness of the eye. Conjunctivitis develops when the conjunctiva is exposed to bacteria and other irritants (parasites, foreign bodies, allergies, and viruses) resulting in damage injuries. The ruptured blood vessels in the sclera during scleretis attacks are due to various factors that temporarily raise blood pressure (such as sneezing or coughing).

### I.5.2. Inflammatory cells

All the inflammatory cells are derived from common bone marrow multipotential stem cells by a process called hematopoeisis. During hematopoiesis, bone marrow-derived stem cells differentiate under the influence of cytokines into either mature cells or into pluripotent precursors of cells that migrate out of the bone marrow to continue their maturation elsewhere. Pluripotent stem cells can then diverge into two main families of cells: lymphoid stem cells or myeloid stem cells. The lymphoid stem cells develop into B cells

and T cells. The myeloid stem cells give rise to both erythrocytes (red blood cells) and leukocytes (white blood cells).

Leukocytes are classified into granulocytes and agranulocytes based on whether or not cytoplasmic granules are present. Granulocytes include neutrophils, eosinophils, and basophils. Agranulocytes are represented by monocytes and lymphocytes. Neutrophils, along with other granulocytes, are an essential component of the body's cellular defences against inflammation or infection. Neutrophils constitute the "first line of defence" in the immune system, meaning that it is the first defensive cell type to be recruited to sites of inflammation or injury; however, there are other inflammatory cells already present (such as the mast cell).

Since neutrophils are designed to function as the first line of defence against invading microorganisms, they are faced with the problem of leaving the blood, finding their targets, and lastly, killing their targets. Neutrophils are released into the circulation from where they can enter the tissues in response to chemotactic signals released locally during infection or tissue damage.

The activated neutrophils collect in the infected area, find the foreign organisms and anything which does not belong in the body, engulf them, release enzymes, hydrogen peroxide and other chemicals from its granules to kill the bacteria and ingest them by phagocytosis. Their targets include bacteria, fungi, protozoa, viruses and virally-infected cells. After these phagocytic cells engulf and digest the bacteria, some of them die, forming pus within an infected area.

Migration of neutrophils out of the bloodstream is a critical component of the host inflammatory response, which is considered to be one of the most crucial functional characteristics of neutrophils. The movement of neutrophils from the vessel lumen into a damaged area is mainly dependent upon chemotaxis. Some mediators act as chemoattractants directing the movement of neutrophils by signalling cells through specific surface receptors. Mediators include a diverse array of molecules, including proteins, peptides, and lipids. One mediator, N-formyl-methionine-leucyl-phenylalanine (FMLP) is a synthetic analog of bacterial products that induce a variety of neutrophil responses and have been extensively employed as activating stimuli *in vitro* (Israel et al., 1998). In this project, neutrophils served as the basic immune component, and their migration through matrices in response to FMLP was examined.

**I.5.2.1. <u>Established inflammatory cell lines</u>**

Although neutrophils may play a key role in the inflammatory response of the eye, they have a short lifespan that imposes severe experimental limitations in an *in vitro* model. The need to get stable inflammatory cell lines with the phenotypic properties of granulocytes has increased the demand of immortalization. To overcome this problem, a human leukemic cell line HL 60 was studied. This line provides a continuous source of human cells for studying the inflammatory response.

Leukemic cells can be induced to differentiate by several agents (Collins et al., 1978; Koeffler et al., 1979). Under the influence of retinoids, such as all-trans-retinoic acid (all-trans RA), HL 60 myelocytic leukemia cells differentiate

into granulocytes (Apfel et al., 1995; Collins, 1987), whereas the presence of phorbol ester (TPA) results in differentiation to macrophages (Lotem and Sachs, 1979; Rovera, Santoli and Damsky, 1979). The induction of granulopoiesis in immature leukemic cells is associated with the acquisition of functional properties that are well characterized in neutrophils including chemotaxis (Sham et al., 1996). Antibodies for differentiated HL60 and granulocytes are shown in Table 2.

Table 2: Specific antibodies for differentiated HL60

| **Antibody** | **References** | **Function** |
|---|---|---|
| CD 11b | Barbe et al., 2000<br>Charrad et al., 2002<br>Sham et al., 1996<br>Taetle et al., 1991 | Cell migration |
| CD 15 | Charrad et al., 2002<br>Chen et al., 1994 | Cell adhesion |
| CD 33 | Andrews et al.,1983<br>Scheinberg et al., 1989,<br>Prin-Mathieu et al., 2002 | Adhesion and cell migration |
| CD 38 | Nagatsuka, 2003 | Cell adhesion |

## I.6. Objectives

The objective of my thesis, therefore is to develop a very basic, prototypical model of the human conjunctiva with adjoining underlying sclera, using the information available as discussed above. This basic model contains a stratified epithelium that is non-keratinizing and a stroma that is vascularized and innervated (Fig. 3).

A basic inflammatory component was added to the system. This was done by developing a system whereby granulocytic cells, in this case, human neutrophils isolated from peripheral blood and an established human granulocytic cell line, HL60, were tested for their ability to migrate across the matrix, and produce matrix metalloproteinases in response to a chemotactic agent.

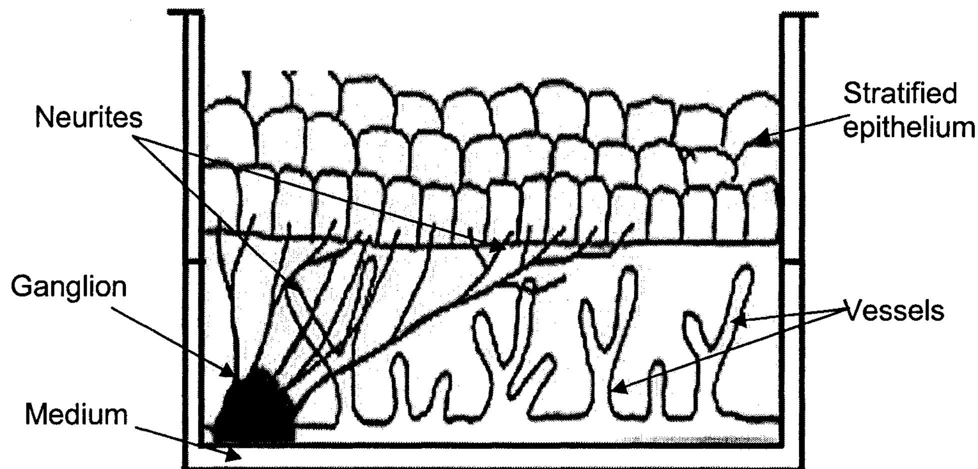


Fig.3: Diagrammatic representation of the basic conjunctiva-scleral model showing various components.

# II. Material and Methods

## II.1. Cell culture and media

Primary HUVECs were provided by Dr. C. Doillon from Laval University. The cells were plated on gelatin-coated tissue-culture dishes and fed with HUVEC growth medium that consisted of M199 medium (Invitrogen, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 90 mg/l heparin (Sigma, Missouri, USA), 2mM L-glutamine (Invitrogen, Ontario, Canada) and 50 μg/ml Endothelial Cell Growth Supplement (ECGS; Sigma, Missouri, USA), 5 mg/ml gentamicin (Invitrogen, Ontario, Canada). They were maintained in a humidified tissue culture incubator at 37°C, in an atmosphere of 95% air and 5% $CO_2$ until 80% confluence. The culture medium was replaced every other day. Once the monolayer approximated 80% confluence, the cells were harvested by incubation for 2 min with 0.05% trypsin-ethylenediaminetetra acetic acid (trypsin/EDTA; Invitrogen, Ontario, Canada) and stopping the reaction with serum-containing HUVEC media. The dislodged cells were centrifuged for 5 min, adjusted to the appropriate concentration of $10^6$ cells/ml and split in different dishes.

HL60 cells were obtained from the American Type Culture Collection. They were maintained in RPMI 1640 (Invitrogen, Ontario, Canada) with 10% heat inactivated FBS, 2 mMol/100 ml glutamax (Invitrogen, Ontario, Canada) and 5 mg/ml gentamicin at 37° C in a humidified incubator with 5% $CO_2$.

Fresh epithelial progenitor cells were extracted from the limbal rims of eye bank corneas or whole globes (obtained from the Eye Bank of Canada,

Toronto) by dissection and subsequent culturing. The resulting cells were maintained in Keratinocyte Serum-Free Medium (KSFM) (Invitrogen, Ontario, Canada) and passaged as per normal procedure at confluence until use.

**II.2. Cell immortalisation**

Primary HUVECs were cultured to 50% confluence on 60 mm tissue culture dishes. They were immortalized by replacement of the tissue culture medium with a viral supernatant containing human papilloma virus PA17 LXSN 16 E6E7 (ATCC; Yeager and Reddel; 1999; Rhim et al, 1998) . Serial dilutions of the viral supernatant at 1/6 (vol/vol), 1/8 (vol/vol), 1/32 (vol/vol) and 1/64 (vol/vol were tried, with a total volume of 5 mls. After 48 hours, the viral supernatants were removed and the medium replaced with HUVEC medium. Cells were then allowed to multiply again to 80% confluence.

After splitting the cells, selection medium ([1]HUVEC medium with 400 μg/ml antibiotic-G418 (Invitrogen, Ontario, Canada)) was added until non-infected control cells were all killed, and neomycin-resistant clones were obtained. In general, selective pressure was maintained for 7 days. The G418-selected cells with extended lifespans were then grown in HUVEC medium and further expanded. A portion of the selected cells was cryopreserved for storage while the rest were used for long-term propagation.

[1] Tittering the antibiotic with HUVECs and obtaining a killing curve predetermined the concentration of G418. The dose chosen was one that killed off HUVECs over a period of 7 days.

## II.3. Telomerase activity

Immortalized HUVECs were assayed for telomerase activity by immunocytochemistry. Cells were cultured on Lab-Tek (Nalge Nunc International, Illinois, USA) chamber slides for tissue culture. At near confluence, the culture chambers were peeled off and cells on the slides were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered solution (PBS) for 1h at room temperature, and then washed with PBS for 20 minutes. A polyclonal antibody to human telomerase reverse transcriptase (hTERT) (Calbiochem, California, USA) was then added to a final dilution of 1:50 and cells were then incubated overnight at 4° C. After a PBS wash to remove unbound primary antibody, the cells were incubated for 3 hours with a fluoresceinated goat anti-rabbit antibody (Molecular Probes, Inc., Oregon, USA) at a ratio of 1:500. The slides were further rinsed off, coverslipped with PBS/glycerol and examined under an Axioskop-2 microscope. Controls consisted of 1) non-immortalized primary cells, 2) immortalized cells stained without primary antibody.

## II.4. Demonstration of vascular endothelial cell specific markers

Endothelial cell phenotype was verified by uptake of di-acetylated low-density lipoprotein (Dil-Ac-LDL) and positive staining for factor VIII-related antigen by immunocytochemistry.

- **Factor VIII-related antigen**

Immunostaining for Factor VIII-related antigen was carried out using mouse anti-human Factor VIII-related antigen and immunocytochemistry, as

described previously (Gagnon et al, 2002). Briefly, HUVECs grown on Lab-Tek chamber slides were fixed with 4% PFA in PBS for 1h at room temperature and then washed with PBS for 20 minutes. The fixed cells were subsequently stained with factor VIII-related monoclonal antibody (Cedarlane, Ontario, Canada) and visualized using a fluoresceinated cy3 secondary antibody.

- **Uptake of acetylated low density lipoprotein (Dil-Ac-LDL)**

Living HUVEC grown on Lab-Tek chamber slides were incubated for 4h at 37°C in the HUVEC medium containing 10 μg/ml Dil-Ac-LDL (Molecular Probes, Inc., Oregon, USA). To visualize the Dil-Ac-LDL uptake, the cells were washed six times (10 minutes/each) with the HUVEC medium at 37°C, and incubated in the HUVEC medium for overnight.

The cells were examined by fluorescence and confocal microscopy. Images of all immunostained preparations were captured microscopically with a Nikon CoolPix 990 camera mounted on an Olympus inverted microscope.

### II.5. Effects of growth factors on Angiogenesis

bFGF (Sigma, Missouri, USA) at 50 ng/ml and EGF (Sigma, Missouri, USA) at 10 ng/ml were added to the culture medium. These were previously optimized concentrations for induction of angiogenesis (Gagnon et al., 2000). Each one was introduced separately or combined.

One of a range of different concentrations of water-soluble retinal acetate (RAc): 2, 4, 6, 8, 10, 12 and 14 μM/ml were introduced into the medium of each culture. Their effects on angiogenesis were examined.

## II.6. Three dimensional endothelial cell culture system

- **Fibrin matrix**

Three mg/ml Fibrinogen (Sigma, Missouri, USA), dissolved in Hank's balanced salt solution containing $Ca^{2+}$ and $Mg^{2+}$ (HBSS, Sigma, Missouri, USA), was mixed with thrombin (1.75 mg/ml; Parke-Davis, Ontario, Canada) at a ratio of 1:0.03 v/v. The thrombin was added to catalyze polymerization of fibrinogen. To simulate angiogenesis within the fibrin matrix, endothelial cells were first seeded on the bottom of gelatin-coated wells at high density to form a confluent monolayer within 48 hours (Fig. 4). Then, 5 x $10^4$ endothelial cells/ml were mixed into the fibrinogen solution prior to polymerization to form a stromal matrix. Fibrin gels were obtained by gentle agitation. The gels were then incubated at 37°C in a humidified $CO_2$ incubator until required for analysis. Cells from the monolayer were then allowed to grow out and join up with the pre-seeded cells within the stroma matrix to form tubular, vessel-like structures.

- **Combination of fibrin with other polymers:**

**Fibrin + p(NIPAAm) or Fibrin + p(NIPAAm)-co-AAc**

Fibrinogen (3 mg/ml) was dissolved in HBSS with $Ca^{++}$ and $Mg^{++}$ (as described above) and combined with a synthetic crosslinker, either p(NIPAAm; Polyscience, Inc., Pennsylvania, USA) or p(NIPAAm)-co-AAc (provided by Dr. F. Li, OHRI-Vision Research), prior to polymerization with thrombin (1.75 mg/ml). Both p(NIPAAm) and p(NIPAAm)-co-AAc were tried at different ratios

in combination with fibrinogen. Endothelial cells were incorporated into the matrix as described above.

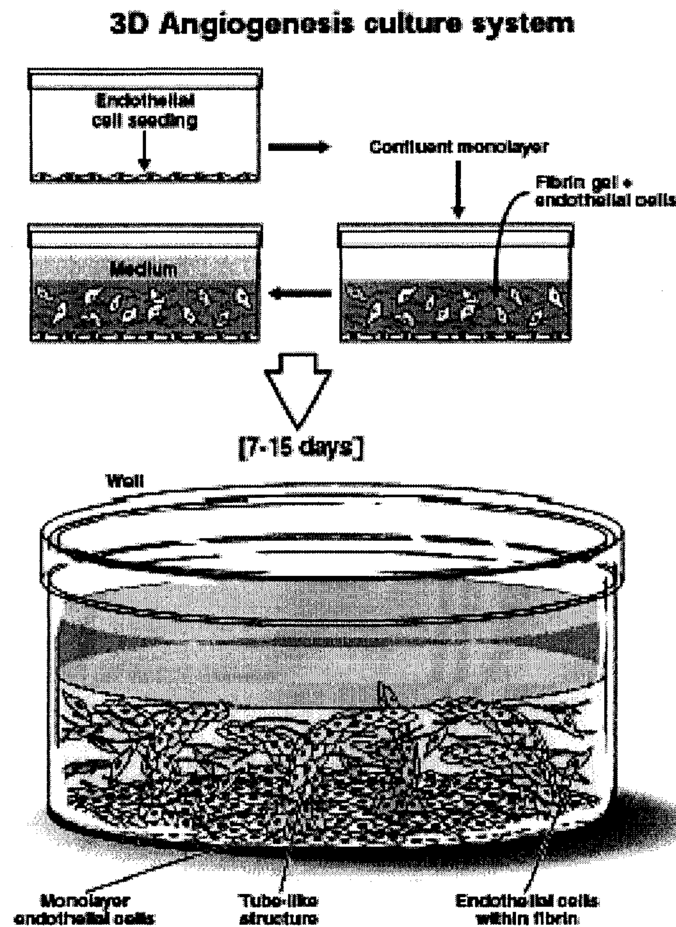


Fig. 4: Diagram showing the three-dimensional angiogenesis culture system used as the basis for developing a conjunctiva-scleral model (From: Gagnon et al., 2000, with kind permission of Dr. Charles Doillon).

- **Collagen matrix**

A precooled (4°C) 50 ml glass tube was used to mix 3.3 ml of 0.4% w/v type I collagen from rat tail in acetic acid (Becton-Dickinson, Ontario, Canada) with 1 ml of collagen buffer (consisting of 9ml of 10X N-2-hydroxyethyl-

piperazine-N-2-ethane sulphonic acid (Hepes) (200 mM Hepes and 100 mM sodium hydroxide (NaOH), 9 ml of 10X Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), 10 ml FBS and few drops of gentamicin). The collagen solution was then neutralized (pH 7.5) by addition of sterile 1 N NaOH by titrating 10 μl at a time. Chondroitin sulfate C 1:5 (w/w ratio of collagen : chondroitin sulphate) (Sigma, Missouri, USA) and 6 μl of glutaraldehyde in 94 μl of 10 % w/v dextran (Sigma, Missouri, USA) were subsequently added to the mixture. After 1hour of cross-linking on ice, 500 μl of 20 % glycine in DMEM was added to terminate the reaction by binding up free, unreacted glutaraldehyde. 500 μl of matrix was added to each insert and incubated at 37°C to form a thermogel.

## II.7. Cell culture conditions

Cells were grown in 10% v/v FBS supplemented-Medium 199 (Invitrogen) containing heparin, L-glutamine and antibiotics at the concentration as described above, with ECGS (50 μg/ml), bFGF (50 ng/ml) and EGF (10 ng/ml).

Cell behaviour was observed periodically under phase contrast microscope; pictures were taken at 7 days of culture using phase contrast, inverted microscope and confocal microscope.

**II.8. Nerve growth**

To stimulate nerve in-growth into the sclera and cornea, conjuntiva-scleral constructs were fabricated around a central cornea comprising the collagen matrix previously described that was seeded with corneal cells as in Suuronen et al. (2004) (Fig. 5). Cultures were established using both human corneal epithelial cell line (HCEC) and epithelial progenitor cells isolated as per section II.1.Dorsal root ganglia (DRG) were dissected from 8 day old chicken embryos and stored in DMEM (Invitrogen) until used. Fibrin + P (NIPAAm)-co-AAC gels together with 10 μl/ml of laminin (Sigma) and 10 μl/ml of nerve growth factor (NGF; Sigma) were mixed and aliquoted into 6-well culture plates. Dorsal root ganglia were placed into the above mixture, which was then thermogelled. The construct was supplemented with a modified SHEM medium: 1:1 mixture of DMEM and Ham's F-12 nutrient (Invitrogen), 15% FBS, 10ng/ml EGF, 5 μg/ml insulin, 0.1 μg/ml cholera toxin (Sigma), α subunit, 5 mM L-glutamine, 0.5% dimethyl sulfoxide (DMSO), and antibiotics containing 2% B27 and 1% N2 supplements (Invitrogen).

The constructs were fixed with 4 % PFA in PBS for 1h at room temperature and washed with PBS for 20 min. The fixed constructs were stained with neurofilament 200 (NF 200) monoclonal antibody (Sigma) using a fluorescence cy3 linked secondary antibody for visualization.

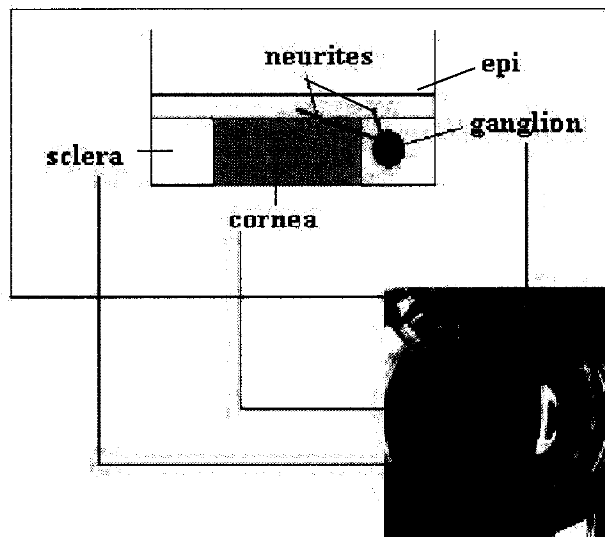


Fig. 5: Diagrammatic representation of 3-D system for innervation of a complete cornea and pseudo-conjunctiva-scleral model.

## II.9. Induction of differentiation in HL60 cells

HL60 cells are known to take up RA and subsequently undergo differentiation. Uptake of the all-trans form of RA was significantly higher in differentiated cells than other isomers (13-cis-RA and 9-cis-RA) (Lanvers et al, 1998). Due to its better uptake and correlation with differentiation, all-trans RA was the isomer used in most *in vitro* experiments and also in this study.

All-trans RA (Sigma) was dissolved in 100% ethanol at concentrations of $10^{-5}$, $10^{-7}$, $10^{-9}$ Mol. To avoid photoisomerization, all flasks containing retinoids were covered with aluminium foil. HL60 at 5 X $10^{4}$ cells/ml were exposed to one of these concentrations for 5 days at 37°C in a humidified air. The cells were

harvested at 1, 3 or 5 days following treatment. After harvesting, cells were washed twice in the culture medium and pelleted by centrifugation. A Live/Dead solution (comprising 100 μl/ml acridine orange and 100 μl/ml ethidium bromide) was applied to the cell pellets. The pellets were then smeared onto a microscope slide and observations were made using a fluorescence microscope to determine the proportion of live cells (visualized as green cells) and dead cells (red cells) after the activation process.

### II.10. Neutrophil isolation

Whole peripheral blood samples (20 ml each) obtained from healthy volunteers with Ottawa Hospital Research Ethics Board consent (copy of consent form is in the Appendix), were collected in heparinized tubes and processed within 24h of collection. The heparinized whole blood was diluted 1:1 with HBSS without $Ca^{2+}$ and $Mg^{2+}$ and centrifuged at 1,500 rpm for 15 min. The buffy coat was collected and diluted 1:1 with HBSS and laid on top of Polymorphprep™, (Axis-shield, Oslo, Norway) a medium used for the isolation of human PMN granulocytes from whole blood; and centrifuged at 1,500 rpm for 30 min. After centrifugation, two cell bands and a pellet were visible (Fig. 6). The top band at the sample medium interface consisted of mononucleated cells (monocytes and lymphocytes) and the lower of PMNs; the red blood cells (RBCs) were pelleted at the bottom. The PMN band was harvested, diluted in 40ml of HBSS and centrifuge at 1,500 rpm for 20 min. The supernatant was aspirated and discarded, leaving the PMN pellet. Sterile $H_2O$ was added to the PMN pellet and agitated for 10 sec to lyse any residual RBCs. HBSS was added immediately thereafter to stop

the reaction. This was centrifuged at 1,500 rpm for 10 min. The supernatant was aspirated and discarded. This process of RBC lysis, followed by HBSS, was repeated until the PMN pellet was without visual evidence of RBC contamination. A total of PMN count was obtained using the hemocytometer and cell viability was assessed by Live/Dead staining. PMNs were then resuspended in RPMI 1640 medium serum-free at a concentration of $10^4$/ml for use.

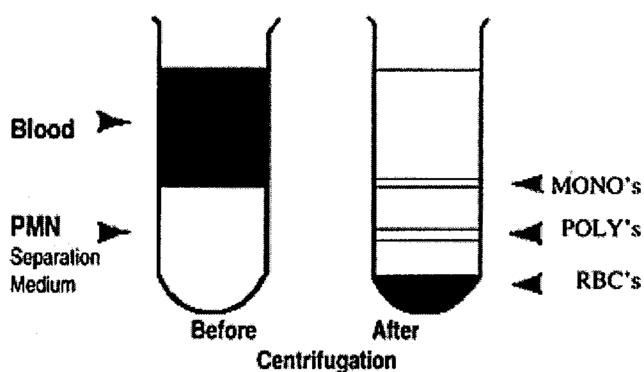


Fig. 6: Neutrophil isolation using the Polymorphprep™ kit (Axis-shield, polymorphprep product information sheet, Sept. 2000, reproduced with copyright permission).

**II.11. <u>Expression of granulocyte marker</u>**

The expression pattern of CD15; a specific marker for granulocytes (Lanotte et al, 1991) was evaluated by immunohistochemistry analysis on human PMNs and HL60 cells before and after granulocytic differentiation. Isolated PMNs served as positive controls. Negative controls consisted of omitting the primary antibody.

For labelling, cells were suspended in the culture medium at $10^6$ cells/ml and incubated at 4°C for 30 min with 2 $\mu$g/ml of a fluorescein isothiocyanate

(FITC)-conjugated monoclonal antibody to CD15 (Becton-Dickinson, Ontario, Canada).

## II.12. Chemotaxis studies

PMNs isolated as described above and HL60 cells were used for granulocytes-migration studies. Non-activated PMNs and HL60 cells served as controls and the cells subjected to a chemoattractant gradient as test cells. The synthetic peptide N-formyl-methionine-leucyl-phenylalanine (N-FMLP; Sigma, Missouri, USA) was used as a chemotactic agent.

Chemotaxis was assayed using 12 well plates with inserts of 8 μm pore size. The upper compartments of the wells were filled with 500μl of matrix (fibrin + p(NIPAAm)-co-AAC or collagen matrix) each and a cell suspension of $10^4$cells/ml on top of the matrix (Fig. 7). FMLP chemoattractant was then carefully added to the lower compartment of the well, allowing for some diffusion into the matrix and establishment of a concentration gradient. The assembled plate was incubated for 4, 6, 8 hours at 37°C in a humidified $CO_2$ incubator. Movement and responses of the PMNs and HL60 cells were analyzed for time-dependent behavioural characteristics. Cells orienting and moving toward the FMLP solution were scored to obtain a quantitative measure.

The matrix was fixed in 4% PFA in PBS and stained by haematoxylin and eosin (H&E) method, where the nuclei of cells were stained by the haematoxylin, the cytoplasm was coloured by the eosin. Matrices were embedded in 30% sucrose for overnight at 4°C and switched in 1:1 30% sucrose-OCT for 2 hours at

room temperature. Embedded matrices were kept at 80°C until used for frozen sections. Sections were done and analyzed under high power on a standard light microscope. A minimum of 10 sections was observed for each experimental condition. All experiments were performed in triplicate and the results averaged.

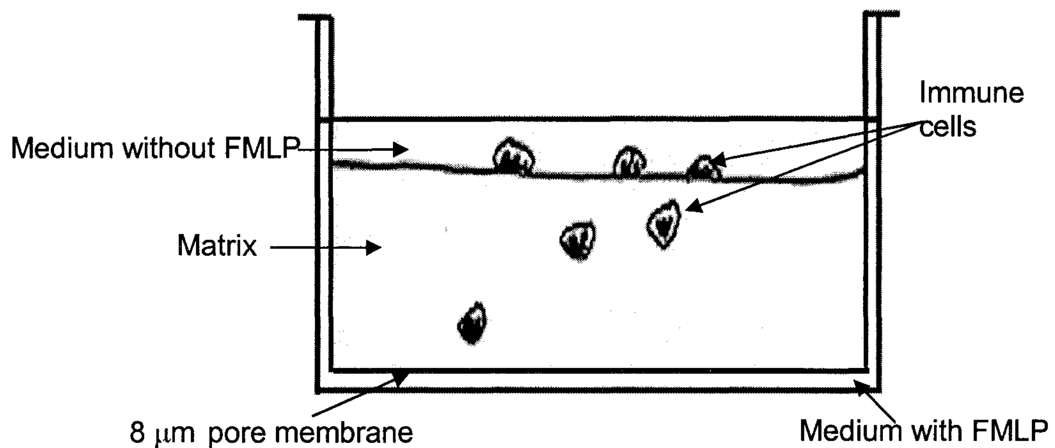


Fig. 7: Diagram of conjunctiva-scleral model used for studying migration and metalloproteinase production by human neutrophils or HL60

**II.13. <u>Detection of metalloproteinases</u>**

Zymography has been extensively used to establish the presence of MMPs (Zhao et al, 1996; Duncan et al, 1995). Non-denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate different enzymes in a protein mixture on the basis of their molecular weights and thereby to identify the MMP molecule of interest (Henssen and Dowdle, 1980). PMNs were added on top of fibrin + p(NIPAAm)-co-AAC or collagen matrices and induced to migrate toward FMLP chemoattractant in the culture system described

in II.12., culture supernatants were collected at different times after PMN addition: 4, 6 or 8 hours and examined by zymography, to detect the presence of gelatinases A and B (MMP-2 and -9 respectively). To achieve this, dissolved gelatin (0.5 mg/ml) was incorporated into 7.5% polyacrylamide gel; samples were added to the gel, separated by electrophoresis and the gel allowed to incubate for 5 hours to allow the enzymes to degrade the gelatin. When the gels were stained for proteins, clear bands of digested gelatin indicated the presence of metalloproteinases. Relative molecular masses were determined from MMP-2 and -9 standards.

# III. Results

## III.1. HUVECs with extended lifespans

Cells resulting from the transfections with HPV viral constructs showed either neomycin resistance or were killed off by addition of antibiotic. The resistant cells were of two groups. One group showed phenotypic transformation with fibroblastic morphology and lack of contact inhibition in cell culture. Others retained the morphology and contact inhibition properties of the primary cells. Immortalized HUVECs showed staining with a monoclonal anti-telomerase (hTERT) antibody (Fig. 8B), indicating the reactivation of telomerase activity while primary cells were unlabelled (Fig. 8D). Like primary cells, however, HUVECs with extended lifespans expressed Factor VIII-related antigen (Fig. 9) and took up Dil-Ac-LDL (Fig. 10), standard markers for cells of endothelial origin (Gagnon et al, 2002).

## III.2. Effects of Extracellular Matrix Macromolecular Scaffolds on Angiogenesis

- **Blood vessel formation within fibrin matrices**

Numerous cord-like structures were formed within the HUVECs seeded with fibrin co-polymerized with either P (NIPAAm) or P (NIPAAm)-co-AAc matrices. These cords then differentiated into vessel-like structures that were selectively stained with Dil-Ac-LDL, indicating that the cells were vascular endothelial in origin (Fig. 11). Tubular vessel-like structures reaching a diameter of 10 μm and 40 μm in length were seen by day 7 of culture (Fig. 12).

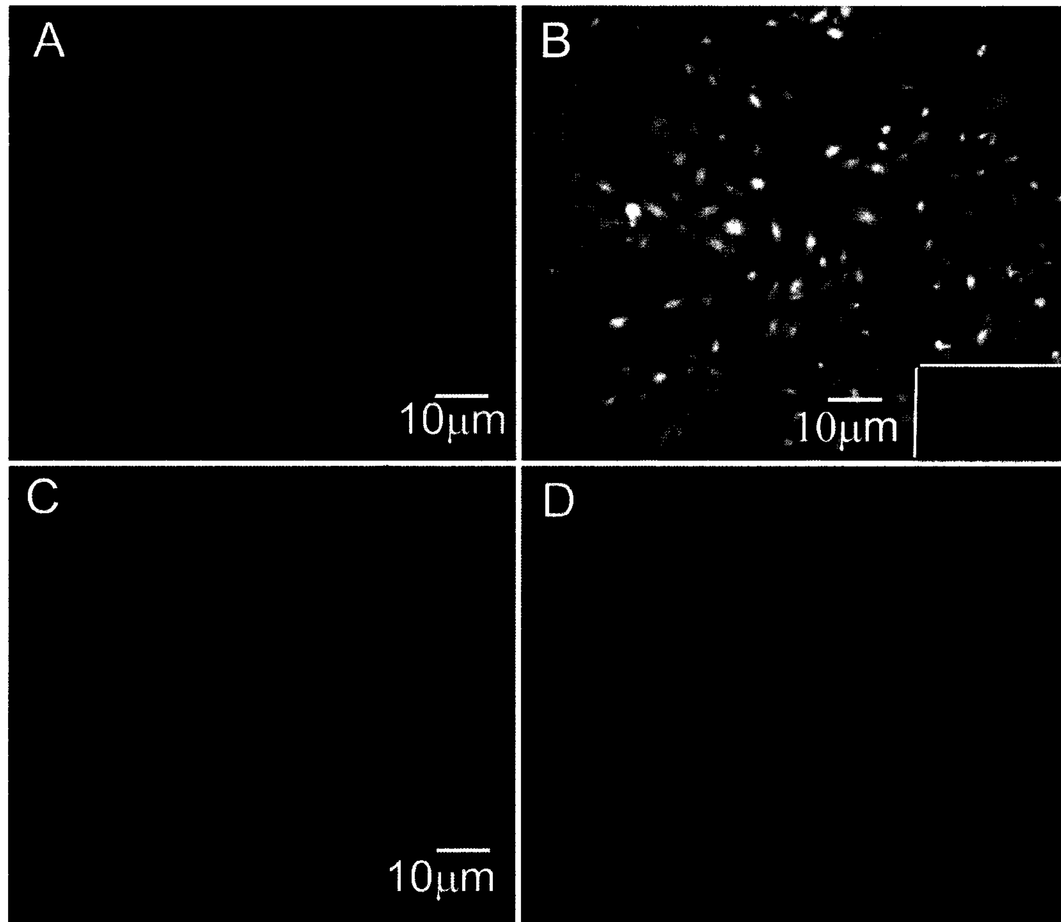


Fig. 8: Immunohistochemical staining for hTERT antibody. Telomerase activity was present in immortalized cell lines 1/8V P14. Phase contrast (A), and (B) hTERT staining, but absent in the PBS control (insert in B) and primary HUVEC ((C) phase contrast and (D) hTERT staining). From Gagnon et al., (2002) Angiogenesis 5 : 21-23 with permission.

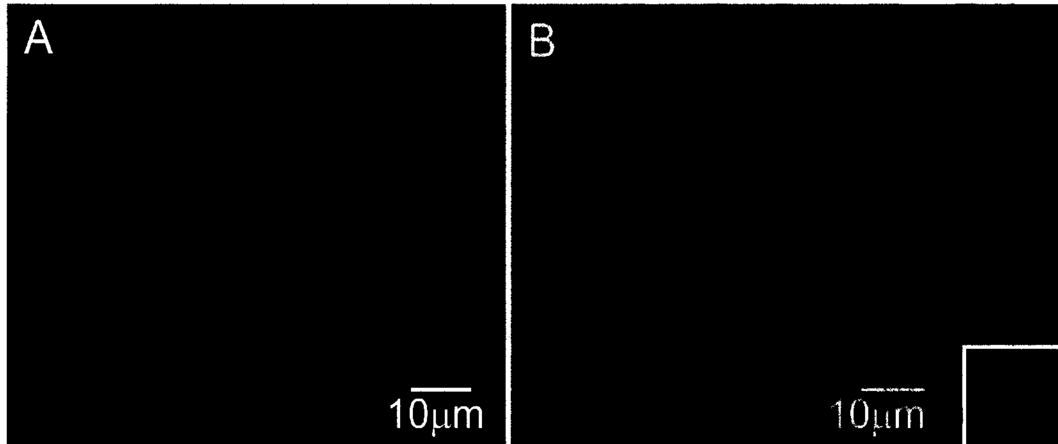


Fig. 9: Expression of factor VIII-related antigen following immortalization of HUVEC. (A) Phase contrast, and (B) positive factor VIII-related antigen staining by immortalized HUVEC. Insert: Control with primary antibody omitted.

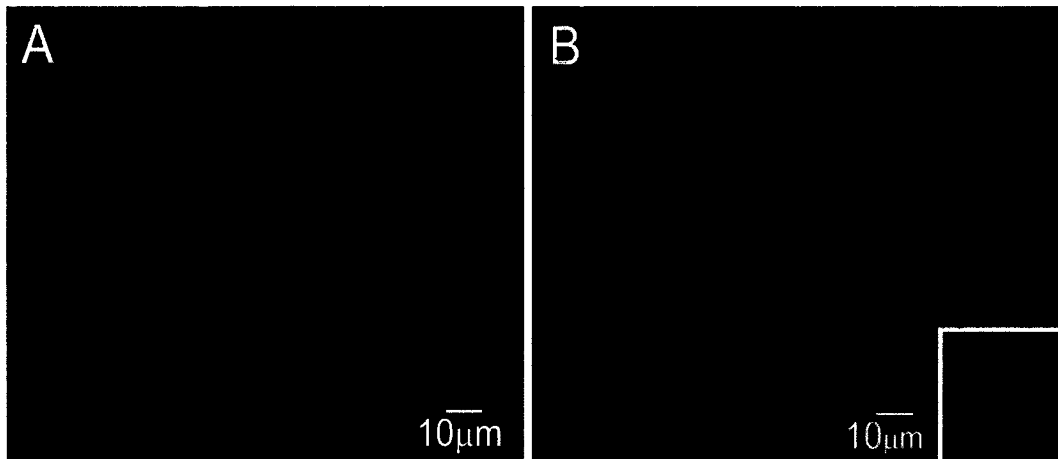


Fig. 10: Uptake of Dil-Ac-LDL following immortalization of HUVEC. (A) Phase contrast, and (B) uptake of Dil-Ac-LDL by immortalized HUVEC. Insert: Control where primary antibody was omitted.

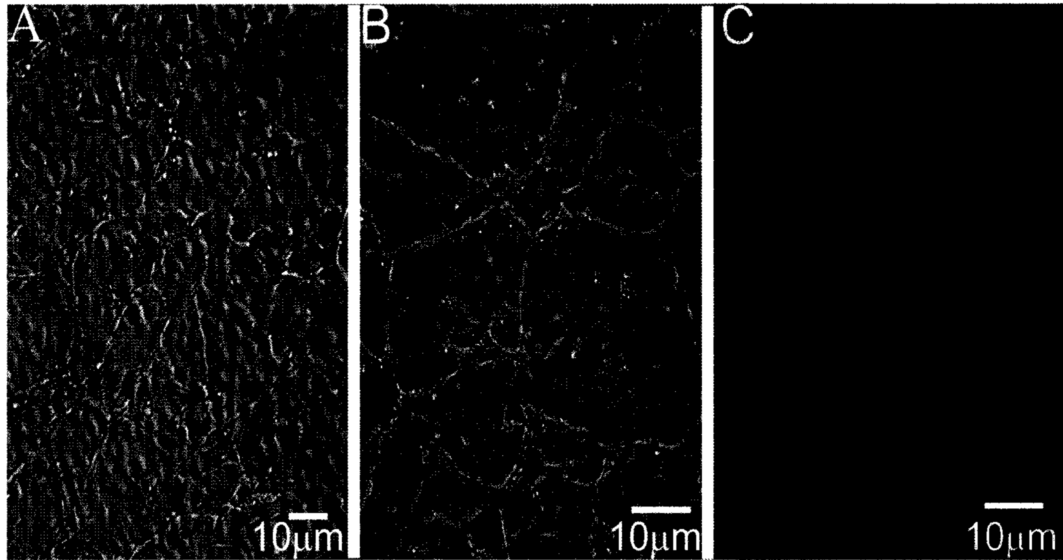


Fig. 11: Phase contrast images (A, B) of cord-like structures that have formed from confluent immortalized HUVEC within the fibrin. The cells forming the periphery of these structures took up the Dil-Ac-LDL endothelial marker (C).

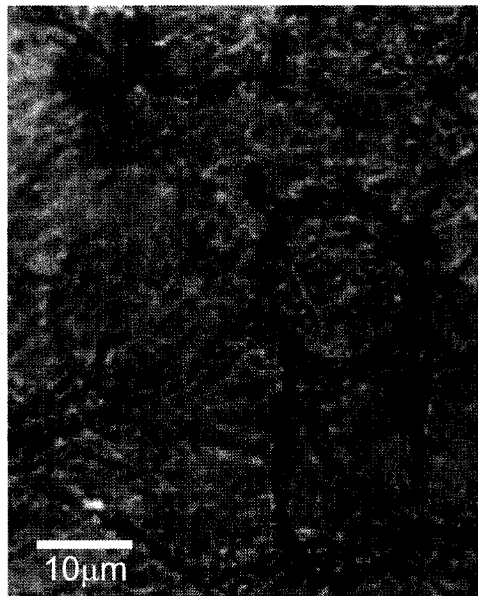


Fig. 12: Tube-like structure in three-dimensional fibrin matrix. Confocal shows a tube-like structure formed by HUVEC in fibrin + p(NIPAAm)-co-AAC.

- **Fibrin + p(NIPAAm) matrices**

Table 3 shows the effects of different fibrin to p(NIPAAm) ratios on blood vessel formation. In 0.3% fibrin alone and in fibrin + 0.5% p(NIPAAm) at 1: 0.1, the number of vessel-like tubes was similar and the matrix remained intact for over 10 days before fibrinolysis occurred. However, in 0.3% fibrin + 0.5% p(NIPAAm) at 1:0.2 and 1:0.3 ratios, the number of tubes was decreased but the fibrinolysis occurred at the same time as in other matrices. 0.5% p(NIPAAm) was found to be a good concentration for our matrix when combined with 0.3% fibrin at a ratio of 1:0.1 (Table 3). There were more blood vessels formed in the matrix and the fibrinolysis occurred after 10 days. In general, fibrin + p(NIPAAm) matrices were soft and weak, i.e. susceptible to fibrinolysis. Increasing the p(NIPAAm) concentration in order to increase the strength of the matrix, however, resulted in opaque composite matrices. p(NIPAAm) demonstrated a rapid and dramatic change of reversible hydrophilic-hydrophobic properties in response to the variation of environmental temperature.

Table 3: Effects of different ratios of fibrin + p(NIPAAm) on gel stability
+ +: many blood vessels; +: few blood vessels. Gel stability was measured by number of days before fibrinolysis of constructed days.

| | Presence of tubes | Gel stability (days) |
|---|---|---|
| Fibrin | + + | > 10 |
| Fibrin + 0.5% p(NIPAAm) (1:0.1) | + + | > 10 |
| Fibrin + 0.5% p(NIPAAm) (1:0.2) | + | > 10 |
| Fibrin + 0.5% p(NIPAAm) (1:0.3) | + | > 10 |

- **Fibrin + p(NIPAAm)-co-AAC matrices**

Table 4 shows the effects of different proportions of fibrin + p(NIPAAm)-co-AAC on formation of vessel-like structures. A combination of 0.5% fibrin and p(NIPAAm)-co-AAC, at a ratio of 1:1 gave the highest density of vessel formation. At high concentrations of p(NIPAAm)-co-AAC, there were fewer blood vessels and matrix degradation occurred around the 7$^{th}$ day (Table 4). Many tubes were formed when the fibrin was combined with p(NIPAAm)-co-AAc at the ratio of 1:1 and the fibrinolysis occurred after 10 days. At the ratios of 1:3 in all the combinations studied there was no blood vessels formation and the matrix did not last more than 3 days.

Table 4: Effects of different ratios of fibrin + p(NIPAAm)-co-AAc on gel stability. + +: many blood vessels; +: few blood vessels; -: no blood vessels. Gel stability was expressed in number of days prior to fibrinolysis.

| | Presence of tubes | Gel stability (days) |
|---|---|---|
| Fibrin + 0.5% p(NIPAAm)-co-AAc (1:0.1) | + + | >10 |
| Fibrin + 0.5% p(NIPAAm)-co-AAc (1:1) | + + + | >10 |
| Fibrin + 0.5% p(NIPAAm)-co-AAc (1:2) | + | < 7 |
| Fibrin + 0.5% p(NIPAAm)-co-AAc (1:3) | - | < 3 |
| Fibrin + 1% p(NIPAAm)-co-AAc (1:0.1) | + + | >10 |
| Fibrin + 1% p(NIPAAm)-co-AAc (1:1) | + | >10 |
| Fibrin + 1% p(NIPAAm)-co-AAc (1:2) | + | < 7 |
| Fibrin + 1% p(NIPAAm)-co-AAc (1:3) | - | < 3 |
| Fibrin + 2% p(NIPAAm)-co-AAc (1:0.1) | + | < 7 |
| Fibrin + 2% p(NIPAAm)-co-AAc (1:1) | + | < 7 |
| Fibrin + 2% p(NIPAAm)-co-AAc (1:2) | + | < 7 |
| Fibrin + 2% p(NIPAAm)-co-AAc (1:3) | - | < 3 |
| Fibrin + 3% p(NIPAAm)-co-AAc (1:0.1) | + | < 7 |
| Fibrin + 3% p(NIPAAm)-co-AAc (1:1) | + | < 7 |
| Fibrin + 3% p(NIPAAm)-co-AAc (1:2) | + | < 7 |
| Fibrin + 3% p(NIPAAm)-co-AAc (1:3) | - | < 3 |

- **Blood vessel formation in collagen matrices**

No vessel-like structures were seen in the collagen matrices.

**III.3. Effects of growth factors on tube formation (Total number of tubules)**

ECGS added to the culture medium did not support tube formation. However, addition of 50 ng/ml bFGF (at the start of co-cultures and with each medium change) resulted in formation of blood vessel-like tubes. Tube formation increased by 10-fold when 50 μg/ml ECGS was combined with 50 ng/ml bFGF (Fig. 13). Addition of 10 ng/ml EGF alone had no significant effect on vessel formation (10 blood vessels/565 $ml^3$ matrix). However, in combination of EGF with 50 μg/ml ECGS, the density of tubes formed increased by over 2-fold and when combined with 50 ng/ml bFGF it increases by 4-fold (Fig. 13).

The results therefore clearly indicate that the two growth factors worked synergistically when combined, and enhanced the angiogenesis activity. A combination of bFGF, EGF and ECGS was therefore used in subsequent experiments.

**III.4. Effect of retinoids on blood vessel formation**

Fig.14 shows the effects of increasing doses of retinal acetate (0 to 14 μM/ml) on promotion of angiogenesis. Addition of RAc at concentration of 2 μM/ml induced a significant increase ($P<0.05$) of tube formation compared to the control, where there was no evident morphologic differentiation of the cells into tubes (Fig. 14). A maximum of 20 tubes/565 $ml^3$ matrix was observed with a concentration of 4 μM/ml RAc. At the highest concentration of RAc tested (14 μM/ml), complete inhibition of tube formation was observed.

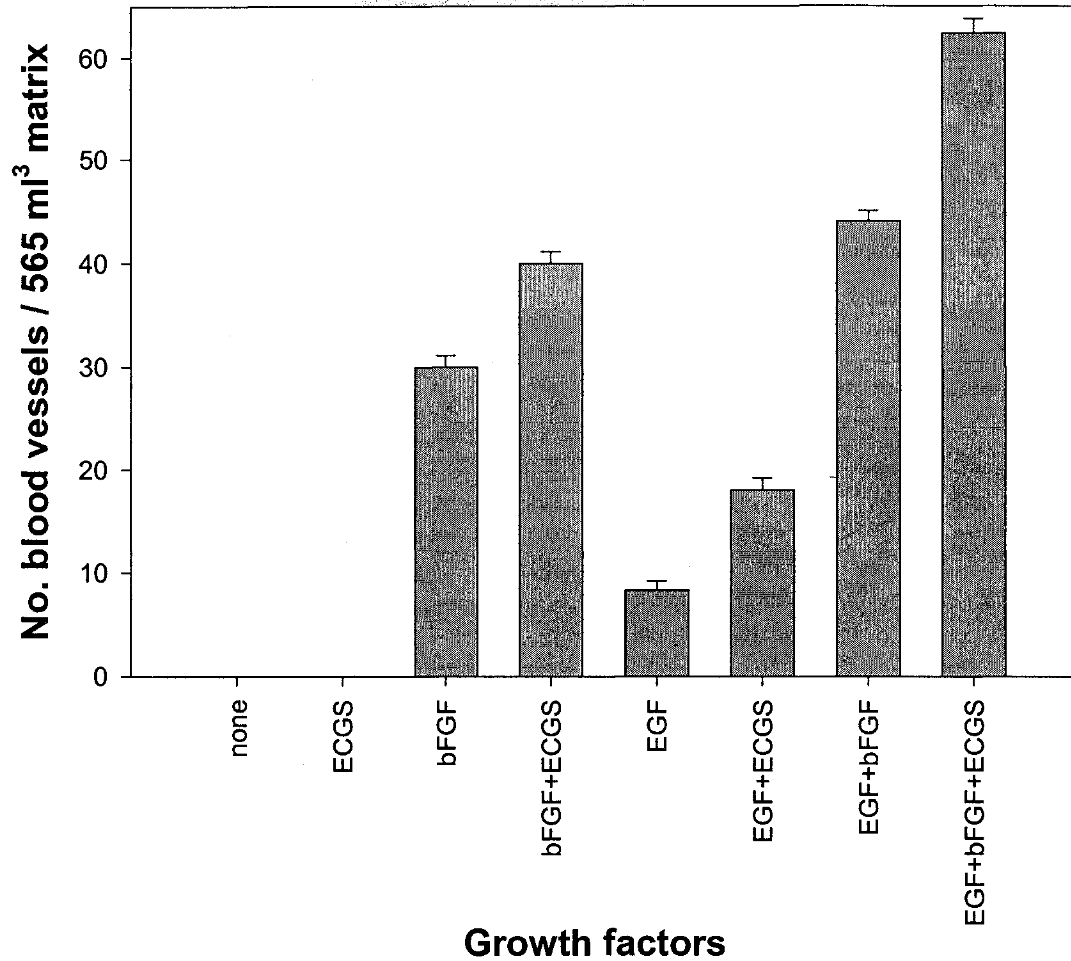


Fig 13: Effects of bFGF and EGF growth factors on angiogenesis in a three dimensional fibrin matrix. Angiogenesis was assayed following 7-day of incubation. Growth factors were used at the following concentrations: ECGS, 50 μg/ml; bFGF, 50 ng/ ml and EGF, 10 ng/ml. n=3 samples of each condition.

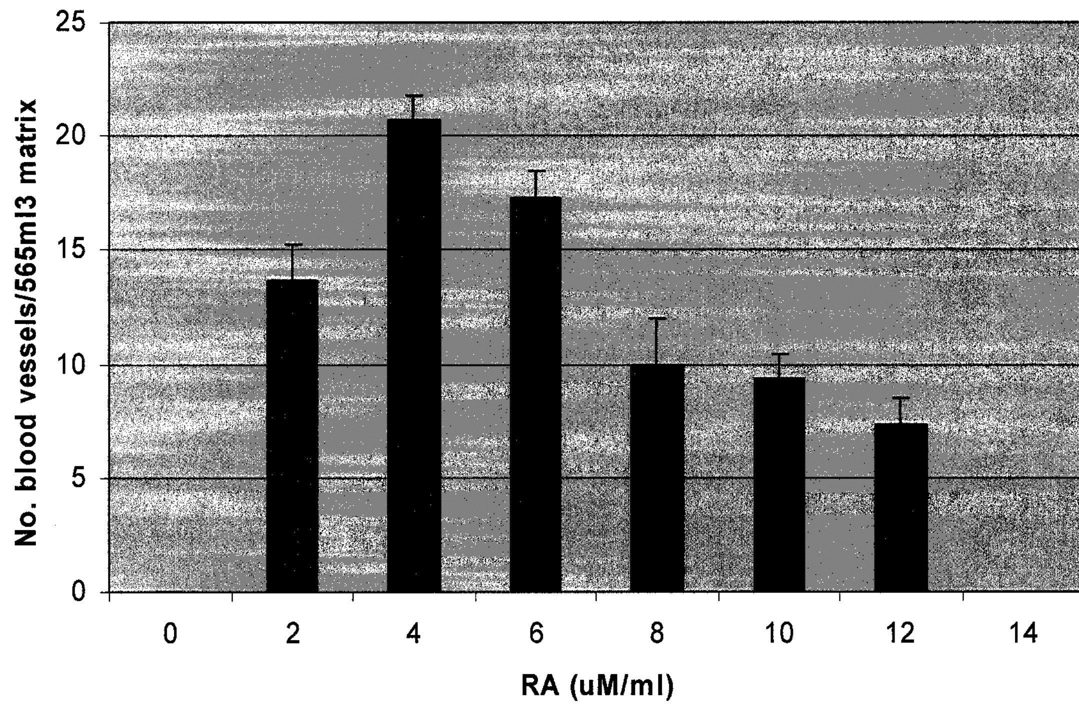


Fig. 14: Effect of RAc growth factor on angiogenesis in a three dimensional fibrin matrix. HUVEC were treated with different concentrations of RA. Control cultures were maintained in the absence of growth factor and have no blood vessels formation. The maximum of blood vessels was reached when 4 μM/ml was added to the medium. Increasing concentration of RAc causes progressive inhibition of angiogenesis. n=3 for each data point.

### III.5. <u>Nerve growth in cornea and pseudo-conjunctiva-sclera</u>

For this experiment, an engineered collagen-based cornea was surrounded by a pseudo-conjunctiva-sclera (the fibrin-polymer matrix) which has a DRG embedded within it. The entire construct was overlaid with either epithelial progenitors or an established corneal cell line (Fig.5). Both epithelial progenitors and the established corneal line gave complete coverage of the model. The progenitor cells differentiated into epithelium that was common to both cornea and conjunctiva (limbal cells), but were not able to differentiate into conjunctiva specific goblet cells.

When a matrix of 0.3% fibrin + 0.5% p(NIPAAm)-co-AAC pre-seeded with HUVECs was layered around a tissue engineered cornea, it served as a pseudo-sclera that supported neurite outgrowth from DRG embedded within it. Vessel formation was observed in the pseudo-sclera and but not in the cornea (collagen matrix). However, the outgrowing neurites extended into both pseudosclera and cornea (Fig. 15). These neurites were neurofilament positive.

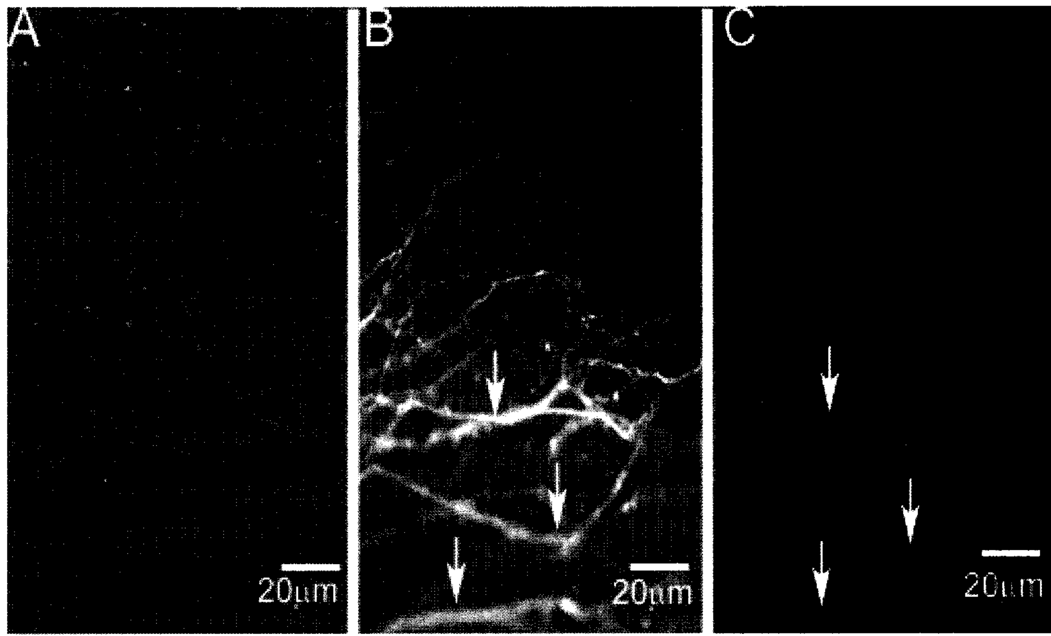


Fig. 15: Nerves and blood vessels in the conjunctiva-sclera. Blood vessels were formed in the three dimensional fibrin + p(NIPAAm)-co-AAc matrix pseudo-sclera but not in the cornea (collagen matrix). (A) Phase contrast, (B) anti-NF 200 staining, (C) anti-NF 200 (red) and anti-factor VIII (green) staining. White arrows show nerves and black arrows, blood vessels.

### III.6. <u>HL 60 differentiation</u>

HL 60 were induced to differentiate by different all-trans retinoic acid concentrations tried ($10^{-9}$, $10^{-7}$, and $10^{-5}$ Mol). At all concentrations and lengths of incubation chosen: 1, 3 or 5 days; HL 60 cells were alive and were differentiated in granulocytes (as shown by CD15 staining; a granulocytic differentiation antigen) (Fig. 16). Viability of samples after induction of differentiation was checked by Live/Dead staining and 90-95% of cells (n = 3 samples) were live cells (Fig. 22). The number of live cells did not change during the treatment. Both undifferentiated and differentiated HL 60 were CD 15 positive, showing intense staining after 5 days of differentiation with $10^{-5}$ Mol all-trans RA.

### III.7. Neutrophil migration

Neutrophils ($10^4$ cells/ml) added to fibrin + P (NIPAAm)-co-AAc showed migration into and through the matrix after 4 hours of $10^{-7}$ M FMLP activation. After 6 hours, more neutrophils (65%) migrated and >70% after 8 hours (Fig. 17). In controls that were not FMLP activated, only about 50% of cells had migrated into the matrix, and between 10-60 μm from the surface after 8 hours. On the collagen matrix, only 10% had adhered to the top of the matrix (Fig. 19, 21).

### III.8. HL 60 migration

Like neutrophils, activated HL 60 migrated into fibrin + P (NIPAAm)-co-AAc hydrogels when stimulated by $10^{-7}$ M FMLP. The matrix was degraded when RPMI 1640 medium was used. Without FMLP, only 30% of cells migrated into the matrix between 10-30 μm (Fig. 18). In collagen matrix, cells did not migrate (Fig. 19). Only a small proportion (10%) of cells seeded had adhered to the top of the matrix and was retained during subsequent sample processing (Fig. 20, 21).

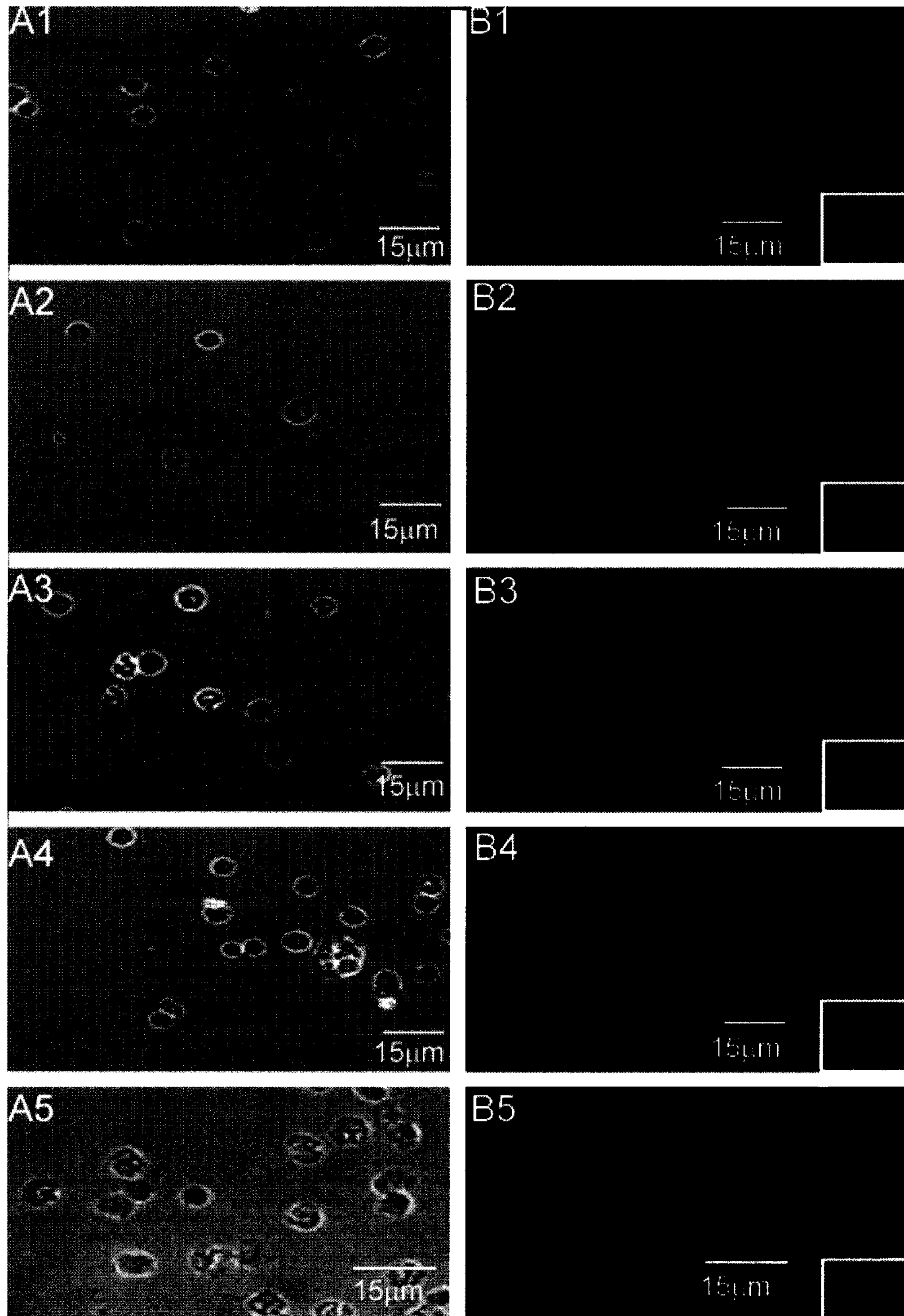


Fig. 16: Expression of CD15 in human PMNs and HL60 cell lines; A: Phase contrast B: CD15 staining after 5 days of differentiation by different concentrations of all-trans RA. (1): undifferentiated HL60; (2): $10^{-9}$ M all-trans RA; (3): $10^{-7}$ M all-trans RA; (4): $10^{-5}$ M all-trans RA; (5): human PMNs. Inserts show negative controls where the primary antibody was omitted in each case.

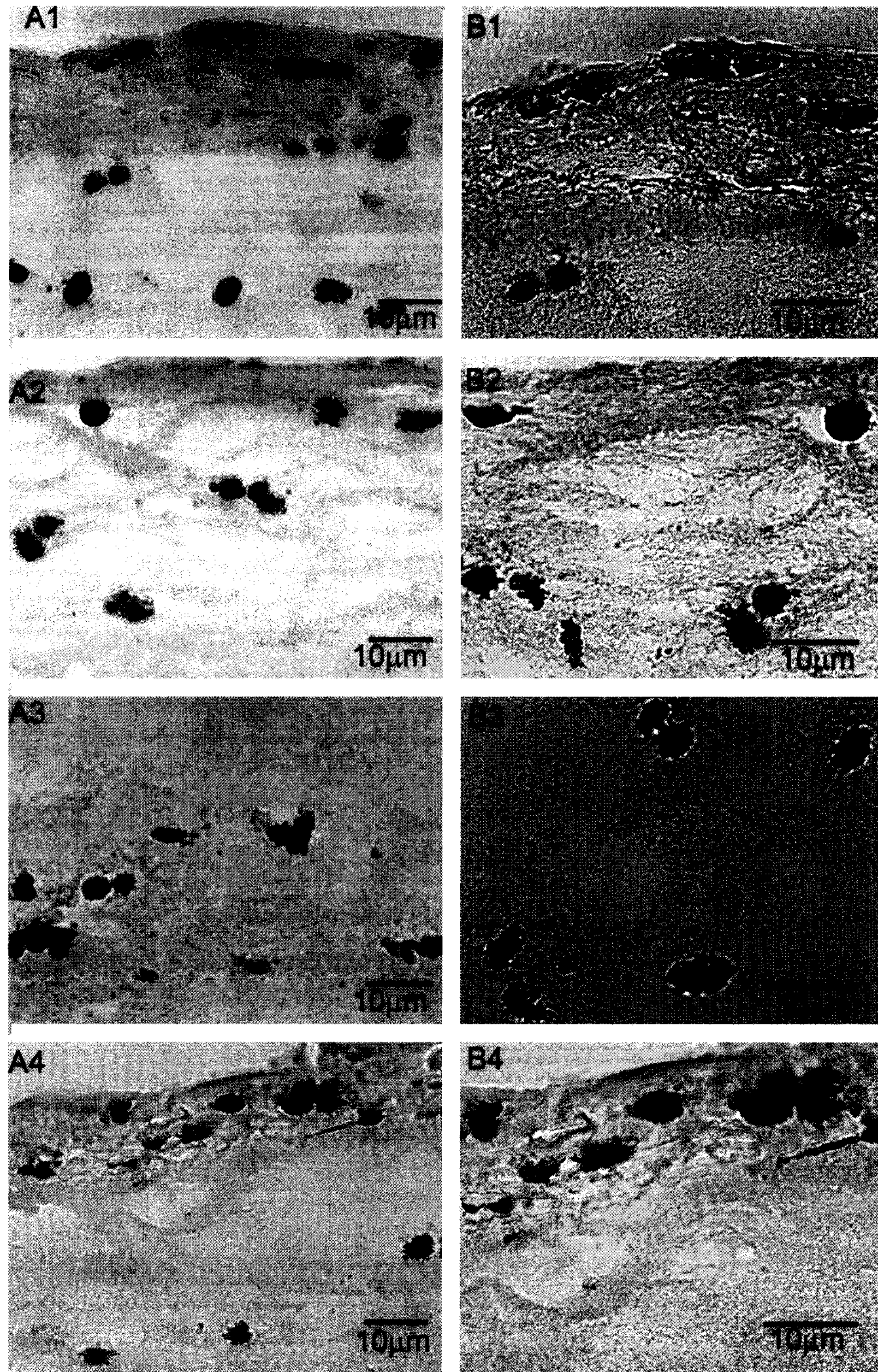


Fig. 17: H&E stained sections showing PMN migration within fibrin + P (NIPAAm)-co-AAc matrices in response to FMLP exposure for 4h (A1), 6h (A2) or 8h (A3). Untreated controls are shown in A4. B1-4 are corresponding to higher magnifications.

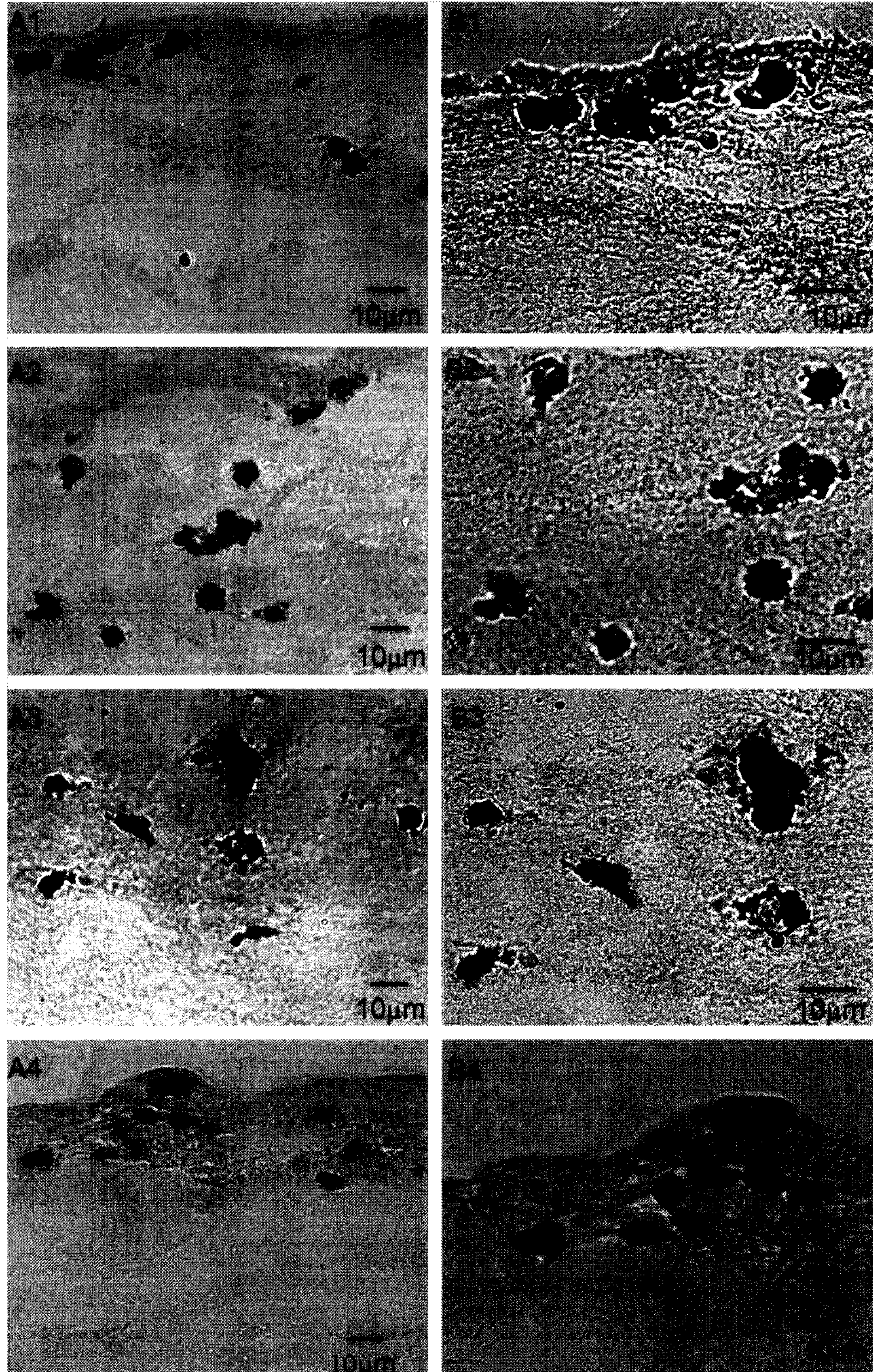


Fig. 18: H&E stained sections showing migration of differentiated HL60 cells within fibrin + P (NIPAAm)-co-AAc matrices in response to FMLP exposure for 4h (A1), 6h (A2) or 8h (A3). Untreated controls are shown in A4. B1-4 are corresponding higher magnifications to A1-4.

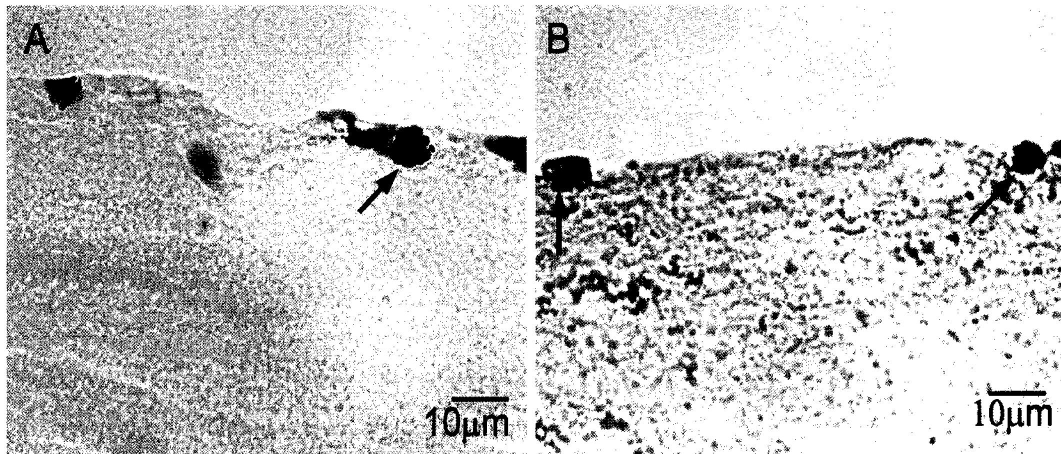


Fig. 19: H&E stained sections showing only minimal PMN migration within a collagen matrix in response to FMLP exposure for 8h (B). Untreated control is shown in A.

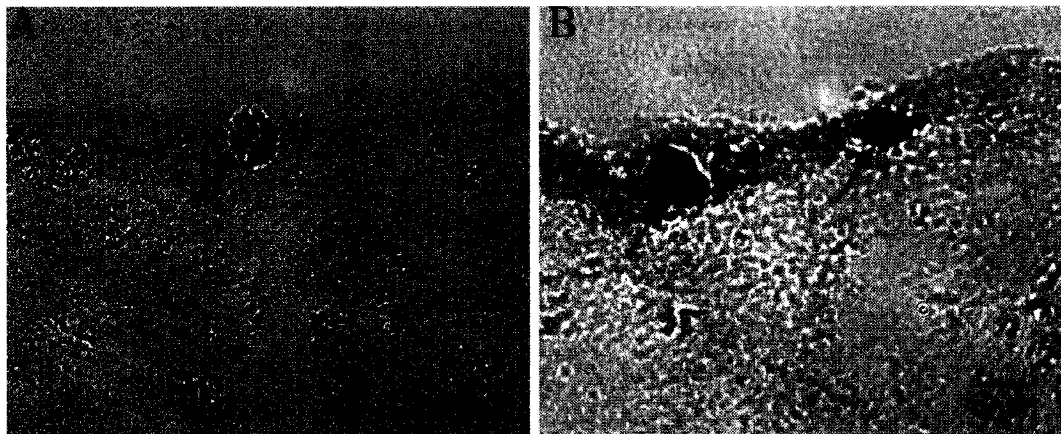


Fig. 20: H&E stained sections showing very minimal migration of differentiated HL60 migration within collagen matrix in response to exposure to FMLP, even after 8h (B). Untreated control is shown in A.

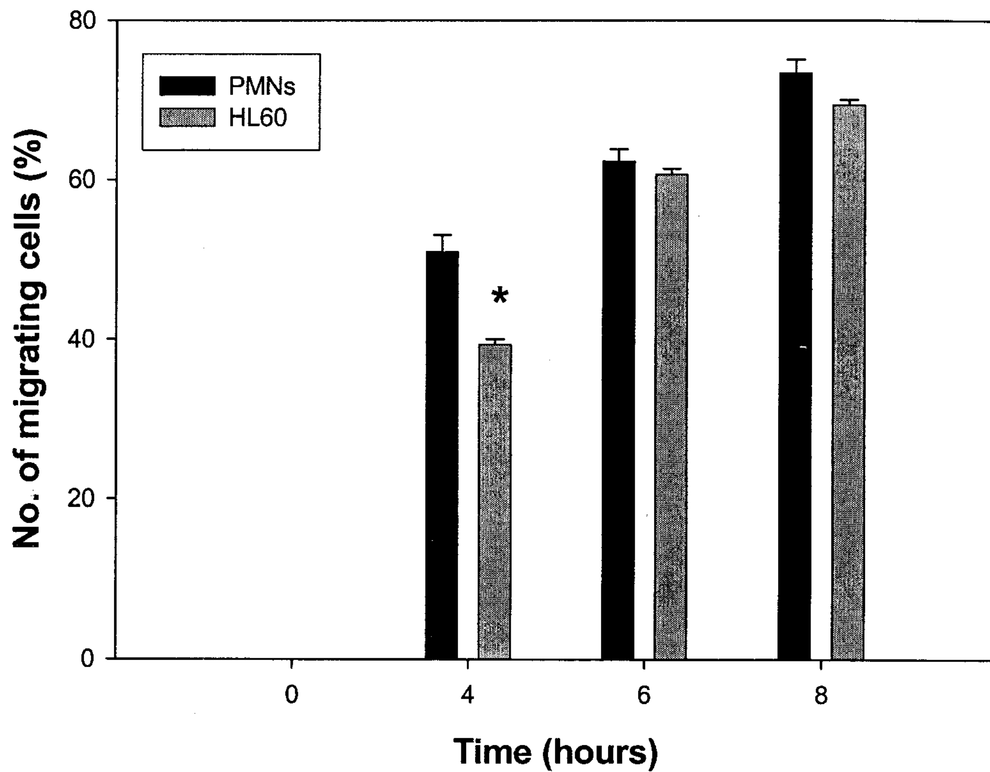


Fig. 21: FMLP effects on migration of PMNs and HL60 cells through fibrin + P (NIPAAm)-co-AAc matrix. A time dependent manner in cell migration was observed. There was no statistical difference between PMNs and HL60 migration except at 4h. n=3 samples each.
*: Difference in number of migrating cells by t-test, with statistical significance set at $P < 0.05$.

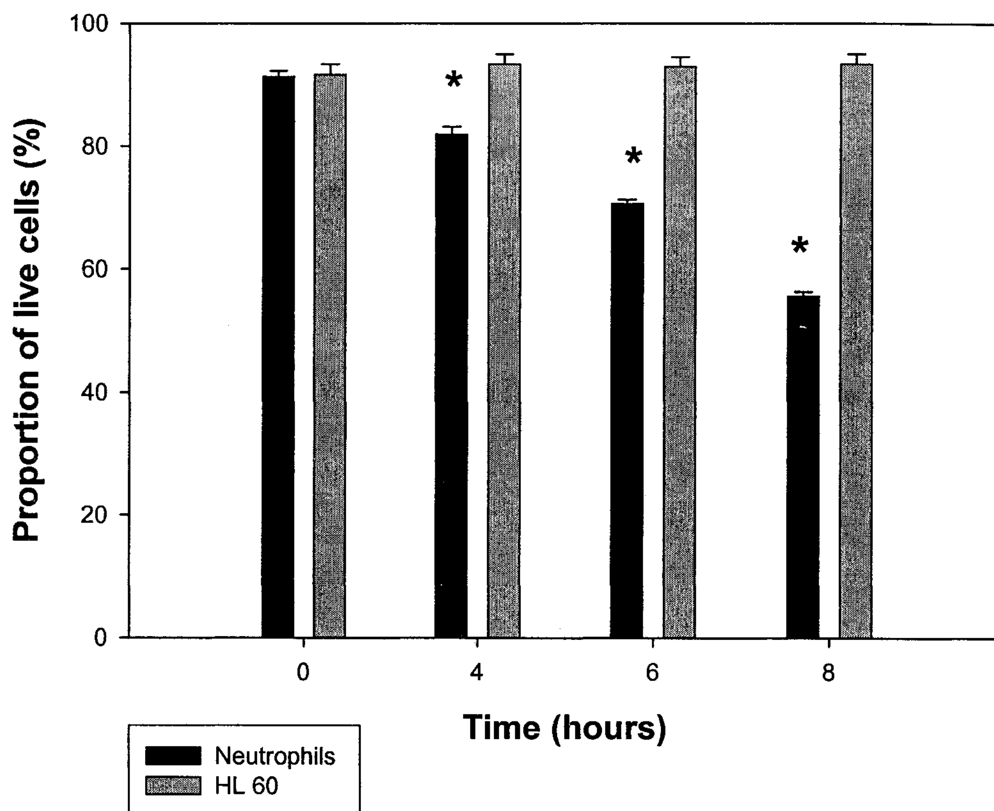


Fig. 22: Viability of PMNs and HL60 cells within three-dimensional fibrin + P (NIPAAm)-co-AAc matrix. The columns represent percentage of cell survival. The number of HL60 alive (90-95%) did not change during the experiment, but human PMNs were dying. N=3 samples each.
*: Statistical significance by t-test, $P< 0.05$

## III.9. MMPs release by granulocytes

Granulocytes extracted from fresh human peripheral blood stained positive for CD 15 and therefore a marker for differentiated granulocytes. Zymography revealed that granulocyte supernatants on top of the collagen-based matrix contained MMP-2 and MMP-9. The maximum secretion occurred when the cells were exposed to $10^{-7}$ Mol FMLP for 4 hours (Fig. 24).

When granulocytes were put on top of a 0.3% fibrin + 0.5% p(NIPAAm)-co-AAc(1:1) matrix, however, there was no MMP-2 and MMP-9 production detected (Fig.25), and the matrix was completely degraded after 24 hours.

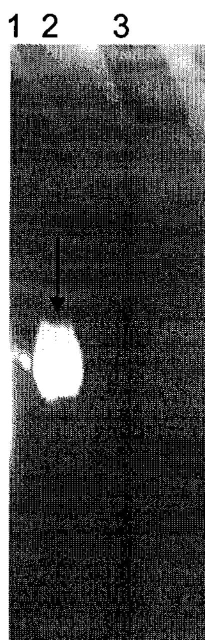


Fig. 23: Gelatin zymography for MMP detection, showing a control gel loaded with MMP-9

Well Samples
1. MMP-9 (2ng) 92 kDa
2. Neutrophils without FMLP; T: 4h
3. Neutrophils without FMLP; T: 6h

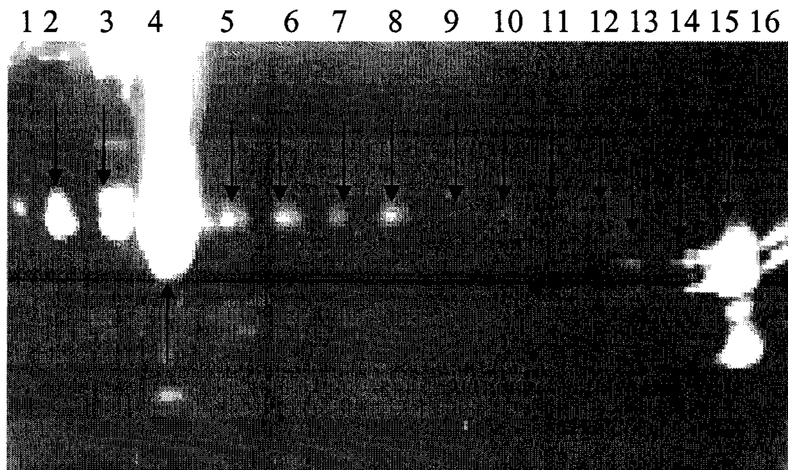


Fig. 24: Gelatin zymography for detection of MMP production by cells within a collagen matrix.

| Well | Samples |
|---|---|
| 1. | Blank |
| 2. | MMP-9 (2ng) |
| 3. | MMP-9 (10ng) |
| 4. | Activated MMP-9 (2ng) 92 kDa |
| **5.** | **Neutrophil: $10^{-7}$ M FMLP; T: 4h** |
| 6. | Neutrophil: $10^{-7}$ M FMLP; T: 6h |
| 7. | Neutrophil: $10^{-7}$ M FMLP; T: 8h |
| 8. | Neutrophil: $10^{-6}$ M FMLP; T: 4h |
| 9. | Neutrophil: $10^{-6}$ M FMLP; T: 6h |
| 10. | Neutrophil: $10^{-6}$ M FMLP; T: 8h |
| 11. | Neutrophil: $10^{-5}$ M FMLP; T: 4h |
| 12. | Neutrophil: $10^{-5}$ M FMLP; T: 6h |
| 13. | Neutrophil: $10^{-5}$ M FMLP; T: 8h |
| 14. | MMP-2 (10ng) |
| 15. | MMP-9 (2ng) |
| 16. | Blank |

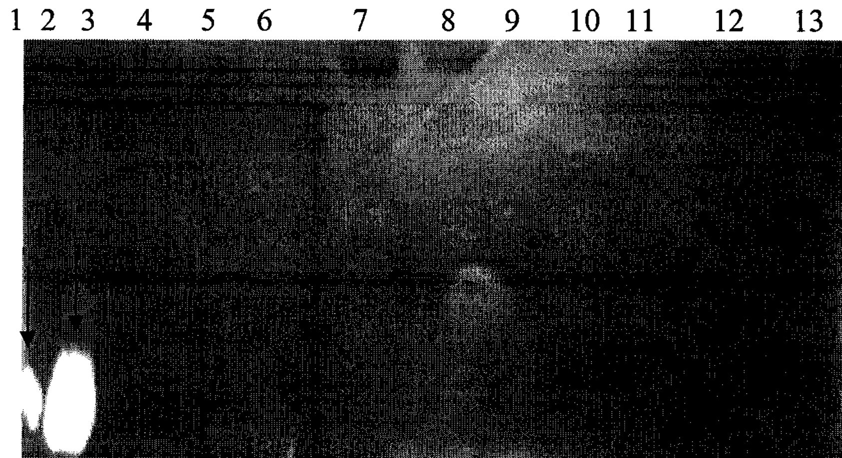


Fig. 25: Gelatin zymography for detection of MMP production by cells within a fibrin + P (NIPAAm)-co-AAc matrix.

| Well | Samples |
|---|---|
| 1. | MMP-2 (2ng) 72 kDa |
| 2. | Activated MMP-9 (2ng) 92 kDa |
| 3. | Neutrophil without FMLP; T: 4h |
| 4. | Neutrophil: $10^{-7}$ M FMLP; T: 4h |
| 5. | Neutrophil: $10^{-7}$ M FMLP; T: 6h |
| 6. | Blank |
| 7. | Neutrophil: $10^{-7}$ M FMLP; T: 8h |
| 8. | Neutrophil: $10^{-6}$ M FMLP; T: 4h |
| 9. | Neutrophil: $10^{-6}$ M FMLP; T: 6h |
| 10. | Neutrophil: $10^{-6}$ M FMLP; T: 8h |
| 11. | Neutrophil: $10^{-5}$ M FMLP; T: 4h |
| 12. | Neutrophil: $10^{-5}$ M FMLP; T: 6h |
| 13. | Neutrophil: $10^{-5}$ M FMLP; T: 8h |

# IV. Discussion

The goal of this study was to develop a very basic prototype tissue engineered human conjunctiva-sclera that could be further developed for use in *in vitro* toxicology testing of various chemicals, compounds and drugs. In particular, the focus was on the development of an optimal scaffold for *in vitro* cellular differentiation and maintenance of the differentiated phenotype of the multiple cell types within the engineered tissue, using a combinatorial approach.

## IV.1. Immortalized Vascular Endothelial Cell Line

Different experiments require different properties of endothelial cells. For some experiments primary cell cultures are a prerequisite, but for my study, immortalized cells were preferred as the aim was to have a large, homogeneous population of cells that can be used to fabricate a large number of constructs for use in *in vitro* testing. Human endothelial cells, like other normal diploid human cells, have a limited lifespan, and will become senescent. The establishment of immortalized cell lines by integration of DNA encoding the HPV16 E6-E7 into the genome of cultured primary human cells of different origin has been shown to be highly efficient (Reznikoff et al., 19941; Willey et al., 1991; Pecokaro et al., 1989; Duist et al., 1987; Tsao et al., 1995). For this project, I developed HUVEC lines with extended lifespans using retroviral transfection of HPV 16 E6 E7. As documented in previous reports (Rhim et al., 1998; Gagnon et al., 2002), HPV 16 E6 E7 transfected HUVECs retained the characteristics of a primary cell. Rhim et al (1998) have previously shown that HPV16 E6-E7 immortalized HUVECs had

3 times the lifespan of the non immortalized HUVECs and have been in continuous culture for over 1 year. The line I developed has been in culture, with cryogenic storage in liquid nitrogen and thawing when needed, for 3 years.

The establishment of immortalized cells using HPV E6 E7 fragments induces the reactivation of telomerase (Kim et al., 1994). Telomerase activity is restored in HPV-immortalized cells, so telomere length remains stable while cells undergo passaging in culture. Immunohistochemistry showed that telomerase enzyme was present in the HUVECs with extended lifespans, but was not detected in primary HUVECs. This demonstrated that HPV E6 E7 transfection activated telomerase in HUVECs to extend their lifespans, as previously reported by Rhim et al. (1998).

HUVECs with extended lifespans should retain the original characteristics of freshly isolated or low passage primary cells. I was able to select a HUVEC line that expressed Factor VIII-related antigen and took up Dil-Ac-LDL, which were documented markers for cells of endothelial origin in other previous studies (Fontijn et al., 1995, Gagnon et al., 2002). In a related study (Gagnon et al., 2002), we showed that these cells also had expressed receptors for vascular endothelial growth factor receptor-1 and receptor-2 (VEGFR-1 and R-2), and tissue-type plasminogen activator (tPA) and were able to undergo angiogenesis in the conjunctiva-sclera constructs.

The HUVECs with extended lifespans therefore resembled normal HUVECs in key characteristics except that they failed to senesce. The preservation of a normal phenotype in immortalized HUVEC makes it possible to

construct a tissue substitute that will look and behave like the real tissue. It can therefore be concluded that E6 and E7 immortalisation was effective in producing a human vascular endothelial cell line derived from the umbilical vein.

### IV.2. Conjunctiva-Sclera Model

In the sclera and conjunctiva of the eye, blood vessels develop by angiogenesis forming branches from the anterior ciliary arteries (Foster and Sainz de la Maza, 1994). This process was successfully emulated *in vitro* by HUVECs in a 3-dimensional matrix.

In a basic hydrated matrix composed of only fibrin, HUVECs can reorganize themselves into an extensive network of capillary-like structures and tubes upon reaching confluence, if provided with growth factors. I showed that bFGF and EGF were capable of inducing angiogenesis within the conjunctiva-scleral model. According to *in vivo* angiogenesis studies, one angiogenic factor may not stimulate complete angiogenesis and there may be a requirement for combination of two or more factors (Carmeliet, 2000). My results showed that indeed the combination of bFGF and EGF enhanced angiogenesis activity. There was also evidence for a dose-dependent effect of RAc on microvascular network complexity formation.

### IV.3. Effect of Extracellular Matrix Molecules and Synthesized Polymers

Extracellular matrices have diverse biological effects, including promoting the growth, migration, and differentiation of various cells (Hay, 1981; Madri and

William, 1983; Young and Herman, 1985). Cell migration through collagen (Kuntz and Saltzman, 1997) and fibrin (Herbert et al., 1996) gels is known to depend mainly upon the sensitivity of the material to proteases produced by the cells, the amount of enzyme produced by the cells, and the amount of material to be remodelled by the cells as they migrate through the material. In this case, while both fibrin-based matrices and collagen matrices supported angiogenesis, the fibrin matrices allowed migration of inflammatory leukocytes while collagen did not. However, the collagen matrices in general showed greater stability. This is discussed further in Section IV.5 below.

### IV.4. <u>Innervation of the Conjunctiva-Scleral Model</u>

The ophthalmic nerve or first division of the trigeminal supplies branches to the conjunctiva, sclera as well as the cornea (Duke-Elder and Wybar, 1961). Although DRGs isolated from chick embryos were used in place of the trigeminal ganglion to innervate the engineered conjunctiva–scleral models, DRGs had previously been shown to be a mechanistically realistic substitute for the trigeminal ganglia (Suuronen et al, 2004). In the present conjunctiva-scleral model, neurites extended from the DRG of embryonic chicks through the fibrin + P (NIPAAm)-co-AAC matrix. In a related project on the cornea, action potentials were found in these nerves, indicating that they are most likely functional (Suuronen et al., 2004).

## IV. 5. Introduction of Inflammatory Components into the Model

Conjunctivitis and scleritis are two frequent common inflammation disorders that may be associated with ocular complications. In both conditions, granulocytic neutrophils are the first cells to arrive at the site of inflammation and they participate in the subsequent inflammatory response. These were therefore the cells chosen to determine the efficacy of the model for use in subsequent work for studying immune and inflammatory reactions, i.e. neutrophils were used to test this very simplistic, prototype *in vitro* conjunctiva-sclera.

The results showed that FMLP induced migration of PMNs seeded onto fibrin + p(NIPAAm)-co-AAc matrices. No MMP-2 or MMP-9 release was detected. However, the matrix was readily degradable by the RPMI 1640 medium, suggesting that there is either a component of the medium that is not compatible with matrix stability, or that no permanent crosslinks were formed between the fibrin and p(NIPAAM)-co-AAc. This suggests that the better migration is most likely due to the matrix not being stable, rather than release of matrix digesting enzymes from the neutrophils. It is possible that in fibrin + p(NIPAAm)-co-AAc gels, MMP production could have occurred but the enzymes were released into the culture medium with the hydrogel degradation and were too diffuse to be detectable by zymography.

In collagen matrices, which showed greater stability in culture, however, neutrophils did not show strong adhesion to the matrices and were not able to migrate through them. On the other hand, collagen allowed MMP secretion but not attachment of leukocytes. Work by Labow et al. (unpublished results) on

adhesion of U937 macrophage cell line to different collagen-based matrices showed that there was poor cell adhesion to these matrices. Their results support our observations that leukocytes do not show an affinity for adhesion to collagen-based matrices. It is possible that the collagen matrices, which were more robustly covalently crosslinked with glutaraldehyde, did not allow surface remodelling by cells that could keep them firmly attached to the matrix.

Although PMNs isolated were able to migrate through fibrin matrices, they are short-lived as previously discussed in the Introduction. In this project, therefore, I searched for an established line of inflammatory cells that mimic the responses of neutrophils and selected the HL 60 granulocytic line for further examination. The HL60 line was of interest due to the ability of this promyelocytic line to differentiate when induced by different compounds (DMSO, RA) along either the granulocytic or the monocytic/macrophage pathway (Breitman et al., 1980; Collins, 1987, Collins et al., 1978, 1979; McCarthy et al., 1983; Sachs, 1981). Results obtained showed that RA induces terminal differentiation of HL60 cell lines in my cultures as expected, with mature cells strongly expressing the CD 15 granulocytic differentiation antigen. The expression of the CD 15 antigen recognized on RA-induced HL60 cells was compatible with the observations made on peripheral blood neutrophils. These early results therefore indicate that the HL60 cell line can provide a continuous source of human cells for use in an *in vitro* model for studying inflammation. Further studies using other physiological and biochemical criteria beyond the

scope of this thesis, however, will be required to establish their efficacy as immune and inflammatory components within an *in vitro* model.

## V. Conclusion and Future Work

The contribution of this thesis to new knowledge is through my demonstration that it is possible to tissue engineer a human conjunctiva-scleral tissue substitute using a combination of a scaffold composed of extracellular matrix proteins (with synthetic crosslinkers as required) and a combination of more than one cell type (vascular endothelial, epithelial, nerves, granulocytes). I optimized the matrices and showed that it was possible to support both processes of angiogenesis and nerve in-growth within a single conjunctiva-scleral model.

I also examined the ability of fibrin-based and collagen-based matrices to support basic immune and inflammatory components like granulocytes. The fibrin-based matrices were superior to collagen in allowing granulocytic neutrophil attachment and migration, while collagen allowed for MMP production. In the future, a combination of fibrin and collagen should probably be tried to allow for both migration and MMP secretion. The immune cells can also be combined with the angiogenic and innervation components in the future for a mechanistically accurate conjunctiva-sclera mimic. It will also test the potency of the vessel-like structures, if the immune cells are able to travel through them and migrate into the matrices. It is anticipated that with implementation of further desired characteristics and further fine tuning such model may in the future be useful as an alternative to animals in *in vitro* toxicology testing.

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