The Effect of Growth Factors on the Corneal Stroma Extracellular Matrix Production by Keratocytes

LaTia Shaquan Etheredge

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The Effect of Growth Factors on the Corneal Stroma Extracellular Matrix

Production by Keratocytes

by

LaTia Shaquan Etheredge

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
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Date of Approval:
October 30, 2009

Keywords: cornea, proteoglycans, extracellular matrix, wound healing, collagen

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Dedication

I dedicate this dissertation to my family and friends.

*Being confident of this very thing, that he which hath begun a good work in you
will perform it until the day of Jesus Christ:* - Philippians 1:6
Acknowledgments

Thank you God for your agape love and faithfulness, for with You all things are possible. My parents, J.C. and Mary Etheredge, thank you for your prayers and encouragement when I wanted to give up. To my sisters, Lakisha, Dontrelle, TaTanya, and Johnnise; thank you all for being just a phone call away. To my adopted sisters, Toni, Shelly, Natasha, Nicole, and Kathryn; thank you all for your endless love, wit, and patience. To my host of nieces and nephews, thank you all for being my inspiration.

I will always be grateful to my mentor Dr. John R. Hassell, who has molded me into an individual that embraces scientific inquiry with diligence, curiosity, and integrity. It has been both a pleasure and an honor to be your student. Countless people have contributed to my work at South Florida. In particular, Dr. Patricia Kruk, Dr. David Birk, Dr. Hormuz Wadia, Dr. Santos Nicosia and Dr. Marian Young, thank you all for your time, support, and expertise. In addition, Mr. Bernard Batson, Bradley Kane, and Sheila Adams, thank you all for your patience and help.

Many new friends and church family added value and enjoyment to my time at USF, as they quickly became a significant part of my life. Thank you all for providing me with a home away from home.
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ABSTRACT

The corneal wound healing process is a complex process, which often leads to the development of scar tissue with loss of transparency. When the cornea is wounded, some of the viable keratocytes are activated by growth factors to proliferate, and repair the wound by the production of a new extracellular matrix (ECM) that is either normal or is disorganized (fibrotic). The first part of this dissertation aims to show that the growth factors IGF-I, TGF-β, FGF-2, and PDGF stimulate keratocytes to synthesize different levels of collagens and proteoglycans and are therefore responsible for initiating the wound healing repair process. FGF-2 stimulated keratocytes to proliferate but did not stimulate collagen production; IGF-I and PDGF stimulated keratocytes to proliferate and produce a collagenous ECM that could restore transparency; while, TGF-β stimulated keratocytes produce a fibrocollagenous ECM that is opaque.

The second part of this dissertation aims to evaluate collagen fibril content, distribution, and orientation in the ECM deposited by keratocytes cultured in IGF-I, TGF-β, FGF-2, and PDGF under a layer of agarose: a culture
modification that enhances the formation of ECM in vitro. FGF-2 agarose cultures had little ECM and the keratocytes were in close cell contact while IGF-I, TGF-β, and PDGF agarose cultures had the least cell contact with an extensive fibrillar ECM. This newly developed agarose overlay cell culture system increases ECM formation with the cell layer only when the synthesis of collagen is stimulated and that the ECM morphology is growth factor specific.

Cell culture has proven to be a reliable technique to study the keratocytes response to trauma and disease; however, limitations exist. Primary keratocytes that possess quiescent phenotype are unable to be rapidly expanded or subcultured without the addition of a mitogen(s). Most commonly, keratocytes are cultured and passaged in the presence of fetal bovine serum (FBS), which activate the cells to proliferate and differentiate into fibroblasts or myofibroblasts as well as lose expression of their unique transparency enabling gene products. The third part of this dissertation aims to develop a defined culture media that can be used to expand and subculture keratocytes that express keratocyte specific markers. Culture medium supplemented with FGF-2 combined with ITS was used to: expand and subculture primary bovine keratocytes while maintaining their expression of keratocan and to restore keratocan expression to bovine keratocytes expanded and subcultured with media containing 2.5% FBS.

This dissertation shows the significance of signaling molecules in vitro to produce keratocyte cultures useful for understanding normal stromal biology and its repair process.
Chapter One

Introduction
**The Cornea**

The cornea is a uniquely transparent structure that has a powerful refractive surface, which produces 2/3 of the eye's total focusing power (Fig. 1). The cornea is void of blood supply; therefore it receives its nutrients by diffusion from the tear fluid and aqueous humour. In humans, the cornea has a thickness of 0.5-0.8mm and a diameter of about 11.5mm. The cornea is made up of three distinct cellular layers that are separated by basement membranes (Fig. 2) [see reviews [1, 2]]. The epithelial layer is the outermost region that functions as a barrier and to absorb oxygen and other cell nutrients located in the tear fluid. The stroma is the thickest layer and functions to provide the cornea with strength and shape. It is separated from the epithelium anteriorly by bowman's membrane and from the endothelium posteriorly by descemet's membrane, both of which are acellular. The endothelial layer is a single layer of cells that pumps excess fluid out of the stroma. All of these layers work together to transmit and refract light on the surface of the retina.

Development of the Cornea

Corneal morphogenesis results from a sequence of controlled developmental events. In some lower vertebrates, the corneal epithelium secretes and assembles a primary stroma that is acellular and contains collagen types II, IX, and I. The primary stroma is composed of one or two layers of orthogonal layers of fibrils and has been shown to serve as a template for the subsequent invasion of neural crest cells that differentiate into keratocytes. The presumptive keratocytes proliferate and synthesize the secondary or mature stroma. The development of the mature stroma is initiated by the swelling of the primary stroma that is triggered by the removal of collagen type IX from the primary stroma and the production of hyaluronic acid. The stroma then undergoes dehydration and compaction; thus the extracellular (ECM) components of the stroma become more concentrated, resulting in the highly organized and uniform array of collagen fibrils that is transparent. The mature stroma primarily consists of collagen types I and V, and proteoglycans.

ECM Components of the Mature Cornea

The transparency of a mature cornea is mostly attributed to the cornea stroma, which constitutes 90% of the mature corneal thickness. The cornea stroma is comprised of a highly organized, uniquely transparent ECM, sparsely populated with keratocytes. The ECM is composed primarily of collagen types I and V and two major types of small leucine-rich proteoglycans; chondroitin sulfate (decorin) and keratan sulfate (lumican and keratocan) [2]. The collagen
fibrils present in the corneal stroma are heterotrophic and are arranged in alternate layers and are separated by proteoglycans [3, 4]. The small size of the proteoglycans determines the spacing of fibrils and the core proteins of the proteoglycans and collagen type V regulate the fibril diameter [5, 6]. Embedded in the corneal collagenous matrix are keratocytes. They account for 2.4% of the stromal volume and are responsible for the ECM production. They are characterized by a dendritic morphology and form a three-dimensional network by contact with their extensive processes [7-9].

**Collagen**

Collagen is the most abundant protein in vertebrate corneas and makes up approximately 80% of the total corneal protein. There are three major families of collagens: fibril-forming collagens, fibril-associated collagens with interrupted triple helices, and nonfibrillar collagens. The collagen stroma contains collagen types I and V, which are fibril-forming and the basement membrane of the epithelium and endothelium contains collagen type IV, which is a non-fibril forming collagen. Although there are differences among collagens, they all contain regions where a glycine is every third residue and are comprised of 3 polypeptide chains [10].

These chains undergo extensive post-translational modifications such as hydroxylation, glycosylation, and disulfide formation [11]. The hydroxylation of selected proline and lysine residues is necessary to stabilize the triple helical regions and ascorbate is co-factor needed for hydroxylation [12].
The disulfide bonds aid in aligning the modified polypeptide chains and initiate the formation of the triple helix starting at the C-terminal end. The resultant procollagen molecule is comprised of a triple helical domain, which is 33% glycine and non helical N- and C-terminal globular regions [11]. The procollagen N- and C-propeptides are cleaved by enzymes: a procollagen N-proteinase and procollagen a C-proteinase, respectively, producing collagen molecules that associate into collagen fibrils [13]. These collagen fibrils are the major components of most ECMs.

**Proteoglycans**

Proteoglycans are also part of most ECMs and are associated with collagen fibrils. Proteoglycans are composed of a core protein with covalently linked glycosaminoglycan (GAG) chains via serine residues. Glycosaminoglycans are linear, repeated disaccharides that are usually sulfated and have a molecular weight of 10-100 kDA. Hyaluronan is an unsulfated GAG. Sulfated GAGs are chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin sulfate, and heparan sulfate. Proteoglycans are classified by their core proteins and are grouped into three major families: modular, cell-surface, and small leucine-rich proteoglycans (SLRPs).
Modular proteoglycans contain large core proteins comprised of multiple and often repeating subunits and can be highly glycosylated [14]. These can be further divided into two subfamilies: hyalectans (versican, aggregan, neurocan, and brevican), which bind hyaluronan, and lectin-binding proteoglycans (perlican, agrin, and testican). The cell-surface proteoglycans are also divided into two subfamilies: core proteins with a membrane-spanning domain (syndecan) and with a glycosyl-phosphatidylinositol (GPI) linkage (glypican). The SLRPs are characterized by tandem arrays of leucine-rich repeats and the presence of at least one GAG side chain (Fig. 3) [15]. There are two subfamilies of SLRPs: dermatan/chondroitin sulfate (decorin and biglycan) and keratan sulfate (lumican, keratocan, and fibromodulin) proteoglycans. Lumican and keratocan are important for the transparency of the cornea by regulating the spacing and diameter of the collagen fibrils [5, 6, 16].

Figure 3. Small leucine-rich proteoglycan (SLRP) structure
Growth Factors and Receptors

Growth factors are cell-secreted soluble proteins that bind receptors on the cell surface of the same cell or in other cells [17]. There are two families of cell surface receptors: receptor tyrosine kinase and G-protein-coupled receptor [18]. Both the receptor and growth factor may have multiple isoforms that have different binding affinities and functions [17]. The binding of growth factors to their respective receptors can promote the proliferation, migration, differentiation, or survival of cells during development, tissue repair, and pathologies. The effect of growth factors can vary depending on when it is activated, in which cell type it is produced, and how it interacts with other factors such as extracellular matrix components.

There are many different growth factors and the research conducted in this thesis has focused on insulin-like growth factor (IGF-I), transforming growth factor-beta (TGF-β), Fibroblast growth factor-2 (FGF-2), platelet derived growth factor (PDGF). IGF-I and FGF-2 are single chain polypeptides. IGF-I has a 50% amino acid homology to insulin and IGF-2, and it binds to the IGF-I receptor [18]. FGF-2 belongs to the heparin-binding growth factor family. FGF-2 is composed of 146 amino acids and binds with a low affinity to heparin and heperan- sulfate proteoglycans. This association prevents the growth factor from being degraded and increases the affinity for 1 of 4 cell surface receptors (FGFR1-4) for FGF-2.

Unlike IGF-I and FGF-2, PDGF is a dimer composed of two polypeptide chains, A and B, which can form three different isoforms (PDGF-AA, PDGF-AB, and PDGF-BB). The A and B polypeptide chains are approximately 100 amino
acid residues long and have a 60% amino acid homology [19]. These proteins are cleaved intracellularly and are secreted in their active forms [19]. Each of these isoforms can bind to the alpha-receptor but PDGF-BB is the only isoform that can bind to the beta-receptor.

There are three homodimeric mammalian isoforms of TGF-beta [20] that are 60-80% homologous. The most characterized member of the family of TGF-beta proteins is TGF-beta 1. TGF-beta is synthesized as a precursor protein that requires activation into a mature form for receptor binding and subsequent activation of signal transduction pathways. The TGF-beta family can bind to three membrane receptors: RI, RII, and RIII.

IGF-I, FGF-2, and PDGF act through receptor tyrosine kinases by binding simultaneously to two receptor chains. After binding, the receptors and sometimes the ligands themselves are phosphorylated on tyrosines, thus may initiate multiple intracellular signaling pathways. The major pathway associated with these growth factors is mitogen-activated protein kinase (MAP-Kinase). TGF-beta is slightly different because it acts through receptor serine/threonine kinases by activating a type-II receptor that recruits, phosphorylates, and activate type-I receptor; thus initiating the Smad-dependent signaling pathway.

**Wound Healing Overview**

There are three major phases of the wound healing process: inflammatory phase, the proliferative phase, and the remodeling phase [21, 22]. The inflammatory phase begins immediately after wounding by the invasion of
lymphocytes and macrophages and is characterized by the clotting cascade. The cells that make up the clot and the invading cells release growth factors and cytokines such as PDGF and TGF-β [21-23]. Subsequently, the proliferative phase begins as invading fibroblasts proliferate and synthesize a new ECM [21]. During the final remodeling phase, there is an increase in ECM production and breakdown. The recently formed ECM becomes more organized and cross-linked, eventually providing approximately 80% of the strength of the uninjured tissue [22].

*Corneal Wound Healing*

The cornea's ability to remain transparent can be compromised when the cornea encounters trauma, infection, ulceration, and other pathologies. The wound repair process of the cornea is similar to skin, tendon, and other tissue types; however, the distinct avascular nature and the uniquely organized ECM of the cornea cause differences in the mechanism [24, 25].

In a healthy cornea, there are very few resident inflammatory cells present [26, 27] but they have been shown to infiltrate the corneal stroma immediately after wounding [28, 29]. These cells along with the lacrimal gland, the epithelium, and the native stromal cells (keratocytes) produce the growth factors that are supplied to the site of injury [30]. Upon wounding, TGF-beta and IL-1 are released from the disrupted epithelium into the stroma. IL-1 has been shown to promote apoptosis of damaged keratocytes and keratocytes flanking the wound [31]. In addition, IL-1 increases the production of PDGF and other growth
factors, and activates neutrophils, which are cells that clear the wound site of invading bacteria and cellular debris [32]. Metalloproteinases are also released to further clear the wound site by degrading collagen, proteoglycans, and other ECM proteins [33, 34].

The initial stromal apoptosis and ECM degradation in the cornea is followed by the activation, proliferation, and migration of the remaining viable keratocytes into the depleted stroma, to begin to replenish the stroma. Some of the activated keratocytes are initiated by growth factors to transition into divergent phenotypes. FGF-2 has been found to cause keratocytes to become more spindle-shaped [35], increase their proliferation and migration [20], and promote keratan sulfate proteoglycan synthesis [35]. TGF-beta causes keratocytes to become myofibroblasts, which are associated with a highly fibrotic wound phenotype. These cells are characterized by decreased transparency, which is associated with the reduction of corneal crystallins and the expression of the cytoplasmic protein alpha smooth muscle actin [36]. Studies have shown that TGF-beta also stimulates the production of additional growth factors, collagen and other proteins associated with the remodeling of the ECM [37]. IGF-II has been shown to be a normal component of the stromal ECM and to be made by keratocytes during corneal development [38, 39].

The remodeling of the corneal stroma can end in corneal fibrosis or restoration of corneal transparency. It has been proposed that an altered ratio of collagen types I and V, an altered expression of proteoglycans in the wounded corneal stroma, and a decrease in corneal crystallin production by keratocytes
may be responsible for the opacity. The reason that injured keratocytes fail to regenerate a well-organized ECM remains unknown. It is probable that growth factors play an important role in regulating the expression of the genes needed for both wound healing and restoration of transparency.

**Causes of Blindness**

In 2004, approximately 3.6 million Americans suffered from blindness and vision impairment [40]. Diseases of the cornea represent a significant cause of blindness and vision impairment and have been shown to be the second leading cause of blindness in children [41, 42]. In the United States, corneal disease accounts for 0.7% of total number of new incidence of blindness, however, ophthalmic treatment of diseases affecting the cornea accounts for 25% [41].

Keratoconus is a non-inflammatory eye disease where the cornea develops a conical shape. Keratoconus is characterized by the thinning of the corneal stroma and is typically manifested during puberty. In the general population, reported incidence are approximately 1 per 2000 [43]. In keratoconus corneas, the thickness of lamellae is unaltered, but the number of lamellae is less in normal tissue [44]. Additionally, proteoglycans and keratocyte density are reduced in keratoconus corneas when compared with normal corneas. The biochemical analysis of the stromal matrix of the keratoconus cornea remains inconclusive.

In developed countries, less than 2% of blindness is caused by corneal disease in children, while in the poor areas of Asia and Africa 25-50% attributed
to corneal scarring [45]. Eye injuries are another cause of blindness leaving 1.6 million people blind, 2.3 million people with bilateral vision impairment, and 19 million with unilateral blindness or vision impairment [46]. Approximately, 750,000 of these eye injury cases will require hospitalization each year [46]. Overall, blindness and vision impairment is a growing economical concern, with the projected increase of about 70% by 2020 [1].

Outline

Corneal wound healing is a complex process of events that occurs as a result of the synthesis of new ECM proteins. The ECM components produced during corneal wound healing can aid in regeneration or induce scar formation which is characterized by a disorganized corneal stroma ECM [47]. The reason that injured corneas fail to regenerate a normal stroma remains unknown. It has been proposed that keratocytes produce an altered ratio of collagen types [48, 49] and have an altered expression of proteoglycan types [49, 50]. However, these changes alone cannot fully explain corneal wound healing and the subsequent scar formation. It is probable that the absence or presence of other ECM components, may contribute to the disorganized and opaque corneal stroma.

There are four phases of corneal wound healing: inflammation phase, proliferation phase, hypercellular phase, and matrix remodeling phase. Growth factors are produced primarily during the inflammation phase by lymphocytes and macrophages that invade the wound. We hypothesize that the subsequent
phases of corneal wound healing are stimulated by specific growth factors that stimulate keratocytes to proliferate and produce a specific ECM. We also propose that these different phases of corneal wound healing can be replicated in vitro by culture in serum free media containing growth factors.

Studies have shown that TGF-β, FGF-2, and PDGF can be found in tear fluid where they could act on keratocytes exposed by wounds and can be made by the invading lymphocytes and macrophages [51, 52]. TGF-β and FGF-2 have also been found in corneal wounds [53]. IGF-II and IGF-I have been shown to be present in the normal corneal stroma [39].

Therefore we will use serum free media containing IGF-I, TGF-β, FGF-2, and PDGF to activate keratocytes in vitro. Then, we will characterize the level of proliferation and ECM synthesis produced by the action of these growth factors on keratocytes and compare this to the levels of proliferation and ECM synthesis seen in vivo during the hypercellular and ECM matrix restoration phases of corneal stroma wound healing. As a result of this approach, we expect to be able to identify the growth factors that stimulate the different phases of corneal wound healing and replace the question marks shown in [Fig. 4] with specific growth factors.
Specific Aims

The remainder of this dissertation will present the findings of my research project of understanding the effect of IGF-I, TGF-β, FGF-2, and PDGF on corneal stroma ECM production by keratocytes. My first specific aim was to determine if insulin and IGF-I have the same effect on keratocyte proliferation and on ECM synthesis. This data is discussed in the Appendix. My second specific aim was evaluate the effect of IGF-I, TGF-β, FGF-2, and PDGF to stimulate keratocytes to proliferate and produce different ECM components. These findings are summarized in Chapter Two. My third specific aim was to evaluate collagen fibrils produced by keratocyte cultures in growth factors by using a novel agarose overlay cell culture system. These findings are summarized in Chapter Three.

Figure 4. Phases of corneal stromal wound healing.
My fourth specific aim was to develop a defined culture medium that can be used to expand and subculture keratocytes that express keratocyte specific markers. These findings are summarized in Chapter Four. A brief discussion of the major findings of my dissertation research is found in Chapter Five.
Chapter Two

The effect of growth factor signaling on keratocytes in vitro and its relationship to the phases of stromal wound repair

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Published in Investigative Ophthalmology & Visual Science 2009 July; 50(7): 3128-3136
Abstract

**Purpose:** To determine the relationship between signaling by different growth factors and the phases of corneal stromal wound repair. We hypothesize that the process involves sequential signaling, resulting first in proliferation, followed by extracellular matrix (ECM) synthesis.

**Methods:** The effect of IGF-I, TGF-β1, FGF-2, and PDGF on proliferation and ECM production by primary cultured bovine keratocytes was evaluated. DNA synthesis was determined by ³H-thymidine incorporation and maximal cell density was determined by measuring the DNA content. Relative levels of ECM components synthesized by keratocytes and secreted into the media was evaluated by ³H-glycine incorporation into total ECM protein and collagen, by ³H-glucosamine incorporation into chondroitin sulfate, keratan sulfate, and hyaluronan, and by western blots with antibodies specific to procollagen types I and III.

**Results:** FGF-2 stimulated the highest level of proliferation, the lowest level of glycosaminoglycan synthesis, and inhibited the synthesis of collagen collagen types I and III. IGF-I, in contrast, stimulated the lowest level of proliferation and the highest levels of collagen synthesis. PDGF and TGF-β1 had intermediate effects on proliferation and collagen synthesis. Although FGF-2 inhibited collagen production, it could be restored by subsequent treatment with IGF-I, TGF-β1, and PDGF.

**Conclusions:** The results of this study showed that the levels of proliferation induced by the growth factors were inversely related to the level of collagen
production. We suggest that FGF-2 initiates the hypercellular phase of corneal wound healing, while IGF-I and PDGF are involved in the restoration of a normal ECM.

**Introduction**

The mature corneal stroma consists of a distinctively organized and transparent extracellular matrix (ECM) which is comprised of mainly fibrillar collagen types I and V, and three of small leucine-rich proteoglycans; one with chondroitin sulfate chains (decorin) and two with keratan sulfate chains (lumican and keratocan). Collagen is present in the corneal stroma as fibrils that are separated by small spaces containing proteoglycans and are arranged in plywoodlike layers. Collagens play a fundamental role in the structure and function of the cornea. The organization of fibrils and lamellae, as well as the crosslinking of the collagen provide the cornea with its tensile strength while the uniform diameter and spacing of the collagen fibrils provide the cornea with its ability to transmit light onto the retina [for reviews see [2, 54, 55]. Collagen type V initiates fibril formation and acts in conjunction with lumican and keratocan and decorin to regulate fibril diameter growth [3, 6, 56-59]. Type III collagen also has been reported to be normally present in bovine and rabbit corneas [60, 61]. More recent studies in mice suggest collagen type III may be developmentally regulated and expressed primarily in neonatal corneas [62].

Embedded in this unique ECM are keratocytes, neural crest-derived cells that produce the proteoglycans and collagens [63]. Keratocytes are
characterized by a dendritic morphology and form a three-dimensional network in contact with one another by their extensive processes [63, 64]. Keratocytes proliferate and are biosynthetically active during corneal development. In adult corneas, they are considered quiescent because they are locked in G-0 of the cell cycle [65] and they have a low level of proliferation and matrix synthesis [54, 63, 66]. Keratocytes also are characterized by high levels of crystallins (aldehyde dehydrogenase and transketolase) in their cytoplasm, which are proteins that are thought to provide cellular transparency [67].

The cornea’s ability to remain transparent can be compromised when the cornea encounters trauma, infection, ulceration, or chronic corneal inflammation. Upon injury, damaged keratocytes and keratocytes flanking the wound undergo apoptosis [66, 68]. Some of the remaining keratocytes are stimulated to lose their quiescence and transition into divergent or “activated” phenotypes, which proliferate, resulting in regions of hypercellularity that then go on to produce the new matrix needed to repair the wound [4, 66, 69, 70]. These divergent phenotypes can either aid in regeneration of a normal stromal matrix or become a scar by remaining hypercellular or producing a disorganized matrix [47, 71]. It has been proposed that an altered ratio of collagen types and proteoglycans in the ECM and/or the loss of the “crystallins” from the keratocytes cause the opacity [48, 49, 67, 72].

Peptide growth factors, extracellular signal proteins (ligands), which promote the growth, proliferation, and differentiation of cells in animal tissues by interacting with cell-surface receptors are partially credited for the regulation of
collagen and proteoglycans expression during corneal wound healing [73].

Growth factors are able to reach the wound site through tear film, aqueous fluid, and the epithelial layer of the cornea [73] [74, 75]. Insulin-like growth factor-II (IGF-II) has been shown to be present in the aqueous humor and in the cornea [38, 76]. Both insulin-like growth factor-I (IGF-I) and IGF-II, which act on the same receptors, have been shown to cause keratocytes to proliferate and maintain their dendritic morphology in vitro [38, 75]. Transforming growth factor-beta (TGF-β), fibroblast growth factor-2 (FGF-2), and platelet-derived growth factor (PDGF) have all been found in tear fluid [51, 52]. These growth factors can activate keratocytes to proliferate, differentiate into fibroblasts or myofibroblasts, and migrate, similar to that of in vivo keratocyte response to injury [69, 75, 77, 78].

Although a number of studies have reported the effect of growth factors on keratocyte morphology, crystallin content, alpha smooth muscle actin expression, and proteoglycan synthesis [35, 36, 38, 75, 77, 79-81]; comparatively, little attention has been given to their effect on collagen synthesis [37, 82], which is the single most important structural element of the cornea. Consequently, in this study, we analyzed the effects of different growth factors (IGF-I, TGF-β1, FGF-2, and PDGF) on keratocyte proliferation and matrix synthesis to test our hypothesis that stromal wound healing involves sequential signaling leading to keratocyte proliferation followed by extracellular matrix synthesis and deposition.
**Materials and Methods**

Chemicals, growth factors, papain, and hyaluronate lyase were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Isotopes were purchased from Perkin Elmer (Boston, MA). CyQuant kit, gels, reagents, and equipment that were used for protein separation and transfer were purchased from Invitrogen (Carlsbad, CA). Endo-beta-galactosidase and chondroitinase ABC were purchased from Associates of Cape Cod (East Falmouth, MA). Collagenase Form III for measuring $^3$H-glycine incorporation into collagen was purchased from Advanced Biofactures Corp. (Lynbrook, NY)

**Keratocyte isolation and culture**

Bovine keratocytes were collagenase-isolated as previously described [35, 50]. In brief, freshly harvested corneas were obtained from 12-month old calves and sequentially digested. Initially, the tissue was subjected to a 45-minute collagenase digestion to remove the endothelium and epithelium and then keratocytes were released from the stroma by a second 150-minute digestion. Keratocytes were plated in serum-free DMEM/F12 containing antibiotics and 1mM 2-phospho-ascorbic acid at 20,000 cells/cm$^2$ to allow attachment (day 0) and incubated at 37°C with 5% CO$_2$. Medium was changed the next day (day 1) and every third day thereafter with DMEM/F12 or DMEM/F12 supplemented with: 10 ng/ml IGF-I, 2 ng/ml TGF-β1, 10 ng/ml FGF-2, or 10 ng/ml PDGF-BB. Cultures were harvested on days 1, 7, 10, and 13.
DNA content

DNA was extracted from the cell layers of each well of harvested cultures using cyquant buffer. DNA in the extract was measured using a Cyquant Assay Kit consistent with the manufacturer's instructions and expressed as ng DNA/well. Calf thymus DNA was used as a standard.

Radiolabeling of keratocytes

Keratocytes were incubated with 10 µCi/ml $^3$H-thymidine for DNA synthesis or 25 µCi/ml $^3$H-glycine for protein/collagen synthesis, beginning on days 4, 7, and 10 then harvested 3 days later. Keratocytes were also incubated with 25 µCi/ml $^3$H-glucosamine for glycosaminoglycan synthesis beginning on day 7 and harvested on day 10.

DNA synthesis. Incorporation of $^3$H-thymidine into DNA was determined as previously described [35]. In summary, aliquots of cyquant buffer extract of the cell layer was adjusted to contain 10% trichloricacetic acid (TCA), carrier protein bovine serum albumin (BSA) was added and the mixture held at 4°C overnight to precipitate DNA. The precipitate was collected onto glass filters and the unincorporated isotope was removed by washing with ice-cold 5% TCA. Incorporation was measured by liquid scintillation counting and expressed as counts per minute/ng DNA.

Protein/collagen synthesis. Incorporation of $^3$H-glycine into collagen and total protein secreted into the media was measured as previously described [12]. Briefly, unincorporated radioactivity was removed by chromatography on PD-10
columns in 4 M guanidine-HCl. The guanidine was removed by dialysis against water. Aliquots of 400 µl were incubated with 1mg of papain in papain digestion solution (PBS containing 10mM cysteine and 10mM EDTA) then, digested for 3 hours at 55°C, precipitated with 10% TCA and the supernatant counted to determine incorporation of $^3$H-glycine into total protein. Additional 400 µl aliquots were digested with collagenase at 37°C for 3 hours in collagenase digestion solution (3 mM N-ethyl maleimide, 50 mM Tris, 150 mM NaCl, and 5 mM CaCl$_2$) and processed as described for papain digests, to determine incorporation into collagen. The incorporated radiolabeled radioactivity in collagen and in total protein were expressed counts per minute/µg DNA.

_Glycosaminoglycan synthesis._ Incorporated $^3$H-glucosamine secreted in the medium was isolated by chromatography on PD-10 columns in 4 M guanidine and dialyzed against distilled water overnight. Aliquots were digested with: H$_2$O (blank), chondroitinase ABC (.05 U/µl), endo-beta-galactosidase (.5 mU/ µl), and hyaluronate lyase (ampule dissolved in 250 µl of H$_2$O, used 5 µl/sample), for 3 hours at 37°C. Undigested GAGs were precipitated out by the addition of 2% dextran sulfate and 1% KAc in 95% ETOH at 4°C. The precipitate was removed by centrifugation and the radioactivity of the supernatant was measured by liquid scintillation spectrophotometry. Chondroitinase ABC digests both CS and HA, therefore, the amount of HA was subtracted from the amount of CS to determine CS content. The incorporated radioactivity in CS, KS, and HA was expressed per µg DNA.
**SDS/PAGE/Simply-blue stain**

Medium was collected from each condition and concentrated 10-fold using 10 000 MWCO Amicon Ultra spin-concentrators (Millipore Corp., Milford, MA). Samples were loaded based on DNA content and electrophoresed under reducing conditions on 3-8% tris-acetate acrylamide gels. Gels were stained with Simply Blue Safe Stain according to the manufacturer’s instructions.

**Pepsin digestion**

Eight milliliters of medium pooled from 4 wells was adjusted to 0.5 M acetic acid by the addition of 230 µl of glacial acetic acid. Two milliliters of 0.5 M acetic acid was added to each of the 4 wells. A pepsin stock solution (4mg/ml of 0.5 N acetic acid) was prepared and, after the samples had chilled to 4°C, 100 µl of the stock was added to the medium and 25 µl of the stock was added to each well. The samples were digested overnight at 4°C. The cell layer samples were combined and 50 µl of pepsin stock was added to the cell layer samples and digested for an extra 5 hours at 4°C. The samples were adjusted to neutrality with NaOH to inactivate pepsin. Samples were dialyzed against water, lyophilized, and reconstituted in 1X LDS sample buffer. Samples were loaded based on DNA content, determined on parallel cultures, to 3-8% tris-acetate gels and electrophoresed as described above.
**Western blotting**

Proteoglycan levels, fibronectin levels and procollagen levels in the media were determined by western blot as described previously [35]. Medium was digested with endo-beta-galactosidase to detect keratocan and lumican core proteins, or with chondroitinase ABC to detect decorin and biglycan core proteins. All samples were loaded based on DNA content and electrophoresed as described above. Proteins were transferred to a nitrocellulose membrane, and then blocked with I-Block solution. The primary antibodies were added at 4°C overnight: keratocan [50], lumican [50], decorin (DS1, Hybridoma Bank, U. of Iowa), biglycan (gift from Dr. Larry W. Fisher), extra domain A (EDA) fibronectin (Accurate Chemical & Scientific Corp., Westbury, NY), fibronectin (Chemicon International, Temecula, CA), procollagen type I (SP1.D8, Hybridoma Bank, U. of Iowa) or procollagen type III (US Biological, Swampscott, MA). The corresponding secondary was added, which is horseradish peroxidase tagged, and then detected using the ECL detection kit consistent with manufacture's instructions.

**Statistics**

All DNA values were the mean of 3 or 4 determinations. $^{3}$H-thymidine incorporation values were the mean of 4 determinations. $^{3}$H-glucosamine incorporation values were the mean of 3 determinations. All other values shown were based on a single determination but all experiments were conducted 2 or more times using different batches of primary cells and the results of a
representative experiment is shown. Statview Version 5 (SAS Institute, Cary, NC) was used for statistical comparisons. Samples were analyzed using a paired t test. Standard error used n>3 and standard deviation was used when n=3.

Results

Each growth factor stimulated a different rate of keratocyte proliferation. Cells were incubated with media containing $^3$H-thymidine for three days, beginning on days 4, 7, and 10, and incorporation per ng DNA was determined on days 7, 10, and 13 (Fig. 5A). All of the growth factors significantly stimulated higher levels of incorporation relative to the control for all three-time points except for IGF-I on day 10. At day 7, FGF-2 stimulated incorporation the most and IGF-I the least with TGF-β1 and PDGF stimulating intermediate levels of incorporation. Incorporation in the FGF-2 treated cultures decreased from day 7 to 10, to be levels lower than that of TGF-β1 and PDGF but still higher than that of IGF-I. Each of the growth factors stimulated the highest levels of incorporation on day 7, except TGF-β1, which stimulates the greatest incorporation on day 10.

Growth factors differentially increased keratocyte culture density. The DNA content of the keratocyte cultures were measured on days 1, 7, 10, and 13 to determine if cultures treated with each of the growth factors increased the cell number (Fig. 5B). The DNA content of the control on day 1 was not significantly different from the control on day 7 (data for day 1 control not shown on figure). The DNA content of the control and growth factor treated cultures were
significantly different on day 7: FGF-2 was 3.2-fold higher than control, PDGF was 2.0-fold higher, TGF-β1 was 1.8-fold higher, and IGF-I was 1.6-fold higher. The DNA content for the control and growth factor treated cultures on days 10 and 13 followed the same pattern as day 7.

Dermal fibroblasts and keratocytes in standard monolayer culture do not readily remove the N-and C-terminal globular domains on the procollagen they synthesize. Procollagen cannot form collagen fibrils that would associate with the cells and as a result, the procollagen is secreted into the media [83, 84]. In the absence of collagen fibrils, the proteoglycans and other components of the ECM that associate with collagen fibrils are also secreted into the media. Therefore, the media was analyzed for the components of the ECM. Each growth factor stimulated a different level of total ECM protein and collagen synthesis. Keratocytes were cultured in media containing ³H-glycine for 3 days beginning on days 4, 7, and 10. The sensitivity of the incorporated radiolabel secreted into the media to papain and to collagenase was used to determine the amount of total ECM protein synthesis and collagen synthesis, respectively.

The results show that compared to controls, IGF-I, TGF-β1, and PDGF stimulated incorporation into total ECM protein from as little as 4.0-fold for TGF-β1 on day 7, to as much as 15-fold for IGF-I on day 13. Keratocytes cultured in IGF-I synthesized the highest levels of total ECM protein; FGF-2 the lowest; while PDGF and TGF-β1 had intermediate levels of total ECM protein synthesis, for all three-time points (Fig. 6A). The levels of total ECM protein synthesized by the keratocytes cultured in IGF-I, TGF-β1, and PDGF increased from day 7 to 10
and from day 10 to 13 and increased the most in the TGF-β1 treated cultures. The levels of collagen synthesis followed the same pattern as the total ECM protein synthesis, but was ~20-40% lower (Fig. 6B).

The proteins that were secreted into the medium by keratocytes also were evaluated by SDS/PAGE/simply-blue staining. Analysis of DNA-normalized aliquots from day 10 cultures showed that each of the tested growth factors caused keratocytes to secrete proteins in the medium that produced a similar banding pattern, but the band intensity was growth factor dependent (Fig. 7). IGF-I and TGF-β1 treated cultures secreted the most proteins, FGF-2 the least and PDGF secreted intermediate levels of proteins. Compared to day 10 cultures, the band intensity of proteins in the medium was less in samples from day 7 cultures and greater in samples from day 13 cultures, but the banding pattern for each of the growth factors was comparable at all three time points (data not shown). These results suggest that IGF-I, TGF-β1, PDGF, and FGF-2 stimulate the cells to secrete essentially the same matrix proteins into the media; however, the expression levels of the proteins are growth factor specific.

The major proteins secreted into the media were identified using western blotting. We analyzed DNA-normalized aliquots of media harvested at day 10, for EDA fibronectin, an isoform of fibronectin found after wounding [85], and fibronectin. Antiserum to EDA fibronectin reacted with a single band of 210 kD present in the media (Fig. 8A). This protein was most abundant in the media of TGF-β1 treated cultures with lesser amounts in the media of cultures treated with the other growth factors and only trace amounts in media from controls. A
western blot using an antibody that recognizes all forms of fibronectin, including EDA fibronectin, indicated that the levels of fibronectin produced by the control were similar to that of the FGF-2 treated cultures (Fig. 8B). The blue staining band at 210 kD in Figure 3 is likely to be fibronectin and is identified as FN.

Western blot analysis using an antibody specific for type I procollagen on DNA-normalized aliquots of medium from day 10 of culture, demonstrated that type I procollagen was readily detected in the medium of keratocytes cultured in IGF-I, TGF-β1, and PDGF (Fig. 9A). When compared to control, IGF-I, TGF-β1, and PDGF stimulated the production of type I procollagen while FGF-2 inhibited the production of type I procollagen. The production of type III procollagen by growth factor treated cultures also was evaluated by western blot using an antibody specific for type III procollagen. Compared to control, type III procollagen was stimulated to the greatest extent in cultures treated with IGF-I and TGF-β1 and was inhibited in cultures treated with FGF-2 (Fig. 9B). The major blue staining bands that react with these antibodies are identified in Figure 3 as proα1(1)(III) and pNα1(1)(III).

Fibril-forming collagen (types I and III) content in the media and cell layer from day 10 cultures was determined by pepsin digestion and SDS/PAGE/simply blue staining (Fig. 10). Pepsin readily degrades globular proteins under acidic conditions, but those regions of collagen molecules that form a stable triple helix are resistant to this protease. Keratocytes cultured in IGF-I had the highest levels of fibrillar collagen in the media. TGF-β1 and PDGF induced production of intermediate amounts of collagen deposited into the medium. Medium from FGF-
2 treated cultures had levels of fibrillar collagen similar to that of the control. Fibrillar collagen associated with the cell layer was seen only in the TGF-β1 treated cultures.

Glycosaminoglycan synthesis by cultured keratocytes in response to the different growth factors also was evaluated. Cells were radiolabeled with $^3$H-glucosamine from days 7 to 10 and the glycosaminoglycans secreted into the media were digested with chondroitinase ABC, endo-beta-galactosidase, or hyaluronate lyase to determine the relative levels of chondroitin sulfate, keratan sulfate, and hyaluronan synthesis, respectively. The results show that when compared to control, cultures treated with TGF-β1 and PDGF synthesized the highest levels of chondroitin sulfate and FGF-2 the least (Fig. 11A). Furthermore, cultures treated with PDGF and IGF-I produced the highest levels of keratan sulfate synthesis while FGF-2 did not stimulate keratan sulfate synthesis (Fig. 11B). Hyaluronan synthesis was 20-fold higher than the control in keratocytes cultured in media containing TGF-β1 (Fig. 11C). These data indicate that the levels of glycosaminoglycan synthesis stimulated by each of the growth factors generally follows the pattern of total ECM protein synthesis.

Western blot analyses with antibodies to the core proteins of decorin and, biglycan, or keratocan, and lumican, respectively were performed. Aliquots of media harvested on day 10 from keratocytes cultured in each of the growth factors were normalized for DNA content, digested with either chondroitinase ABC or endo-beta-galactosidase and analyzed (Fig. 12). Decorin and keratocan production was stimulated by all of the growth factors. Lumican production was
only stimulated by IGF-I. Biglycan was only detected in media from keratocytes cultured in TGF-β1.

FGF-2 induced proliferation the most, a hallmark of the initial phase of wound healing, but inhibited the synthesis of procollagen I, which is essential for a corneal reconstruction. Therefore, we determined if keratocytes that have been cultured in FGF-2 could still respond to other growth factors that stimulate collagen synthesis and up-regulate procollagen type I synthesis. Keratocytes were treated with FGF-2 for 7 days, followed by 6 days of treatment with DMEM F/12, IGF-I, TGF-β1, FGF-2, or PDGF. DNA content of the cell layer and type I procollagen in the medium were determined (Fig. 13). The DNA content of cultures continually treated with FGF-2 significantly increased from day 1 (C1) to day 7 (F7), and then declined a small amount from day 7 (F7) to day 13 (F-F). Compared to continuous culture in FGF-2 (F-F), cultures switched to PDGF (F-P) and the IGF-I (F-I) had significantly higher DNA content (Fig. 13A). Aliquots of medium normalized for DNA content were analyzed using western blots with the antibody to type I procollagen (Fig. 13B). Type I procollagen levels in the media of keratocytes treated with FGF-2 for 7 days (F7) and 13 days (F-F) were less than the controls (C7 and C13) but cultures switched to IGF-I (F-I), TGF-β1 (F-T), and PDGF (F-P) showed increased type I procollagen levels.

Discussion

When the cornea stroma is wounded, injured keratocytes and keratocytes surrounding the wound undergo apoptosis [66, 86]. Proliferation by some or all
of the remaining cells would be essential to replace the cells lost due to apoptosis. The results of this study show, IGF-I, TGF-β1, FGF-2, and PDGF stimulate different rates of DNA synthesis and different levels of maximal cell density. FGF-2 treated keratocytes synthesized DNA at the highest rate at the day seven time point and reached the highest level of maximal cell density. PDGF treated keratocytes synthesized DNA at a lower rate at the day 7 time point but then exceeded FGF-2 at the days 10 and 13 time points and eventually achieved nearly the same cell density as cells treated with FGF-2. Keratocytes treated with TGF-β1 synthesized DNA at a rate similar to PDGF and achieved the third highest level of maximal cell density. IGF-I treated keratocytes had the lowest rate of DNA synthesis and lowest level of maximal cell density. These results indicate that, for these growth factors the level of maximal cell density achieved is related to the rate of DNA synthesis induced by that growth factor. All of these growth factors, however, would be potential candidates for regulatory roles in controlling proliferation involved in the hypercellular phase of corneal stromal wound healing.

The synthesis of an ECM containing collagen is essential subsequent to proliferation in restoring the integrity of the corneal stroma after wounding. The results of this study show that IGF-I, TGF-β1, and PDGF stimulate significantly different levels of collagen synthesis; IGF-I stimulated higher levels of collagen synthesis than did TGF-β1 and PDGF. FGF-2 did not stimulate collagen synthesis. This same pattern was observed in: $^3$H-glycine labeling for total ECM proteins and collagenous proteins, simply blue staining of matrix proteins,
western blots with antibodies to types I and III procollagen, and levels of
collagenous proteins revealed by pepsin digestion of the medium. The level of
collagen synthesis induced by each of the growth factors is, then, inversely
related to the level of confluence achieved by keratocytes cultured in that growth
factor.

In addition to the collagens, the proteoglycans and glycoproteins play a
major role during wound healing. Keratan sulfate proteoglycans are normally
present in the corneal stroma at high levels and function to direct collagen
assembly. In contrast, hyaluronan is only present in mature corneas during
wound healing and may reduce transparency by disrupting the spacing of
collagen fibrils [4, 87]. We found that while all of the growth factors stimulated
chondroitin sulfate synthesis, FGF-2 stimulated its synthesis the least. FGF-2
was also the only growth factor that did not stimulate keratan sulfate synthesis.
TGF-β1 treatment, however, also stimulated the synthesis of biglycan and
hyaluronan, known markers of ECM scarring [4, 88, 89].

In addition, TGF-β1 stimulated the highest levels of EDA fibronectin and
the greatest amount of collagen associated with the cells. Fibronectin is known
to assist in the formation of ECM by enhancing the conversion of procollagen to
collagen [11]. These results suggest that in addition to stimulating the synthesis
of collagens, IGF-I and PDGF also stimulated the synthesis of the collagens,
proteoglycans, and glycoproteins that more closely resemble normal corneal
stromal composition. Therefore, regeneration of normal stroma after injury is
likely to involve these growth factors while production of a scar-type ECM would involve TGF-β1.

Although FGF-2 stimulated keratocytes to proliferate at high levels and also stimulated the production of decorin and chondroitin sulfate, it did not stimulate the synthesis of collagenous proteins. Western blots, however, indicated that FGF-2 actually inhibited the synthesis of types I and III procollagen. The ECM produced by FGF-2 action on keratocytes would consist of essentially proteoglycans and fibronectin, and without collagen it would lack tensile strength. We also showed that the FGF-2 mediated inhibition of type I procollagen synthesis could be reversed by subsequent culture in media containing the growth factors that stimulate collagen synthesis, IGF-I and PDGF, growth factors that also stimulate the synthesis of the normal proteoglycans. This indicates that the inhibition of type I procollagen synthesis by FGF-2 was not a permanent phenotypic change.

In conclusion, we suggest that stromal wound healing occurs as a result of the sequential action of growth factors on keratocytes (Fig. 14). Quiescent keratocytes are activated to proliferate upon wounding to create regions of hypercellularity that consist of densely packed cells with a sparse or provisional matrix that contains keratocan [30, 70, 71]. We found that while FGF-2 stimulated proliferation and chondroitin sulfate synthesis, it did not stimulate keratan sulfate synthesis and it inhibited procollagen types I and III synthesis. This low level of total ECM synthesis by FGF-2 activated keratocytes would result in a sparse matrix.
Thus, we speculate that the regions of hypercellularity that are seen during the initial phases of wound healing are keratocytes that have been activated by FGF-2. In vivo, the provisional matrix in regions of hypercellularity can be replaced by a fibrillar matrix. We also observed that collagen synthesis can be restored to FGF-2 activated keratocytes by subsequent culture in media containing IGF-I, TGF-β1, and PDGF. Thus, we further speculate that IGF-I or PDGF could be acting on the hypercellular keratocytes to make them become collagenous keratocytes. These collagenous keratocytes replace the provisional matrix with a normal fibrillar matrix that restores stromal integrity and then the keratocytes later become quiescent again. TGF-β1 also restored collagen synthesis to FGF-2 activated keratocytes but this growth factor also stimulated the synthesis of biglycan and hyaluronan, markers of fibrosis.

We propose; therefore, that TGF-β1 would cause the hypercellular keratocytes to become fibrobrocollagenous keratocytes and produce a scar-type fibrillar matrix. TGF-β1 has also been shown to cause keratocytes to become myofibroblasts, based on the appearance of α smooth muscle actin [36]. Keratocytes made myofibroblastic by TGF-β1 would be synonymous with fibrocollagenous keratocytes. The absence of any subsequent growth factor would result in a hypercellular scar. The targeted modulation of these signaling molecules has considerable clinical relevance.
Figure 5. Keratocyte DNA Synthesis and cell density. Keratocytes were cultured in DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF. A: Keratocyte DNA Synthesis. Cultures were radiolabeled with $^3$H-thymidine on days 4-7, 7-10, and 10-13, the incorporation determined and expressed per ng DNA. Compared to control, IGF-I, TGF-β1, FGF-2, and PDGF significantly ($p < 0.011$) increased $^3$H-thymidine incorporation at all three-time points, except for IGF-I on day 10. B: Keratocyte Density. DNA content of keratocyte cultures measured on days 7, 10, and 13. Keratocytes cultured with IGF-I, TGF-β1, FGF-2, and PDGF contained significantly ($p < 0.044$) more DNA than the control, for all three-time points.
Figure 6. Protein and collagen synthesis by keratocytes in culture. Keratocytes were cultured with DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF and radiolabeled with $^3$H-glycine on days 4-7, 7-10, and 10-13. The sensitivity of the incorporated radiolabel secreted in the media to papain (A: Total Protein) and to collagenase (B: Collagenous protein) was determined and expressed per ng DNA.
Figure 7. Simply-blue stainable proteins secreted into the medium by keratocytes in culture. Keratocytes were cultured with DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF. The medium from the 7-10 day time period was harvested and analyzed by SDS/Page. The bands later identified by western blot in Figs 4 and 5 are labeled: fibronectin [FN], procollagen I [proα1(I) and pNα1(I)], and procollagen III [proα1(III) and pNα1(III)].
Figure 8. Western blots of media from day 10 keratocyte cultures using antibodies to EDA fibronectin for EDA fibronectin (A) and to fibronectin for total fibronectin (B). Keratocytes were cultured with DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF. The bands detected by these antibodies correspond to the bands marked FN on figure 3.
Figure 9. Western blots of media from day 10 keratocyte cultures using antibodies to procollagen type I (A) and procollagen type III (B). Keratocytes were cultured with DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF. The bands marked proα1(I), proα1(III), pNα1(I), and pNα1(III), corresponds to the migration position of the band marked proα1(I)(III) and pNα1(I)(III) on figure 3. The migration position of pepsin digested collagen types I and III are marked as α1(I), α2(I), and α1(III), respectively.
Figure 10. Fibrillar collagen present in the medium and cell layer of day 10 keratocyte cultures. Keratocytes were cultured with DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF. Cultures were harvested on day 10 and samples were digested with pepsin prior to electrophoresis. A pepsin alone control was added (Pepsin). The migration position of the α1(I), α2(I) and α1(III) chains of collagen are marked.
**Figure 11.** Glycosaminoglycan synthesis by keratocyte cultures. Keratocytes were cultured with DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF and radiolabeled with $^3$H-glucosamine on days 7-10. The incorporated radiolabel secreted in the media was then characterized by its sensitivity to chondroitinase ABC for chondroitin sulfate (A), to endo-beta-galactosidase for keratan sulfate (B), and hyaluronan lyase for hyaluronan (C). When compared to control, the synthesis of chondroitin sulfate (A) was significantly ($p<0.008$) higher in cultures treated with each of the growth factors; keratan sulfate (B) was significantly ($p<0.012$) higher in TGF-β1, FGF-2, and PDGF treated cultures; and hyaluronan (C) was significantly ($p<0.05$) higher in cultures treated with TGF-β1, FGF-2 and PDGF.
Figure 12. Western blots of medium from day 10 keratocyte cultures using antibodies to decorin, biglycan, keratocan, and lumican. Keratocytes were cultured with DMEM/F12 (Control) or DMEM F/12 supplemented with IGF-I, TGF-β1, FGF-2, or PDGF. Media was digested with chondroitinase ABC, for decorin and biglycan, or endo-beta-galactosidase, for keratocan, and lumican, prior to electrophoresis.
**Figure 13.** DNA content and procollagen type I production of keratocytes cultured first with FGF-2 and then changed to each of the growth factors. Keratocyte were cultured with FGF-2 for 7 days (F7), then medium was changed to DMEM F/12 (F-C) or DMEM F/12 supplemented with IGF-I (F-I), TGF-β1 (F-T), FGF-2 (F-F), or PDGF (F-P) and the keratocytes cultured for 6 additional days. Keratocytes cultured with DMEM/F12 on days 1 (C1), 7 (C7), and 13 (C13) were used as controls. A: Keratocyte density. DNA content of keratocytes cultured with FGF-2 for 7 days was significantly (p<0.0001) higher than the day 7 control. Keratocytes cultured in FGF-2 for 7 days and then switched to PDGF (F-P) or to IGF-I (F-I) had significantly (p<0.025) higher DNA content than keratocytes continuously cultured in FGF-2. B: Procollagen type I. Western blot using an antibody to procollagen type I of media harvested on days 7 and 13 of culture.
**Figure 14.** Schematic representation of possible growth factor mediated wound healing of the corneal stroma. Upon wounding, quiescent keratocytes are activated by FGF-2 to become hypercellular: keratocytes that proliferate and synthesize a provisional matrix, a matrix that lacks collagen but contains proteoglycans and fibronectin. The hypercellular keratocytes can be stimulated by IGF-I or PDGF to become collagenous keratocytes and synthesize a normal collagenous matrix abundant in collagen type I and keratocan. In this case, collagenous keratocytes eventually become quiescent. Hypercellular keratocytes can also be stimulated by TGF-β1 to become fibrocollagenous keratocytes and produce an ECM scar, rich in EDA fibronectin, biglycan, and hyaluronan. In addition, without subsequent activation by other growth factors, hypercellular keratocytes can remain hypercellular, which give rise to a hypercellular scar.
Chapter Three

Collagen processing by keratocytes cultured under agarose and in media containing IGF-I, TGF-β, FGF-2, and PDGF

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Abstract

The transparency of the corneal stroma extracellular matrix (ECM) is attributed to the unique arrangement and size of the collagen fibrils. Previous studies have shown that the processing of procollagen to collagen fibrils by keratocytes cultured in insulin is enhanced by an agarose overlay. We compared collagen processing by keratocytes treated with IGF-I, TGF-β, FGF-2, and PDGF in cultures with or without an agarose overlay.

Collagenase-isolated bovine keratocytes from bovine corneas were plated at 40,000 cells/cm² and then cultured with DMEM/F12 alone, or DMEM/F12 supplemented on day 4 with either 10ng IGF-I, 2ng TGF-β, 10ng FGF-2, or 10ng PDGF/ml. Cultures were maintained as standard cultures or overlayed with ~1mm of 3% agarose on day 6 and harvested for analysis on days 20. Pepsin digestion and was used to determine procollagen deposited into the media and collagen content of cell layer. Distribution of collagen type I and fibronectin in the ECM of the agarose cultures was determined by immunofluorescence. Collagen fibril size and orientation was evaluated by electron microscopy.

Only 6% of the collagen was processed in standard cultures for keratocytes cultured in media containing IGF-I and PDGF, and the agarose overlay increased processing 6-fold to 31%. Collagen processing for FGF-2 was at ~10% with or without agarose. TGF-β treated keratocytes processed 19% of the collagen in standard culture and agarose increased processing to 31% but it was not a significant increase. Immunofluorescence staining for collagen type I and fibronectin in the ECM were in different patterns for all growth factors, which
suggests they do not co-distributed. Electron microscopy showed that the FGF-2 agarose cultures had little collagen fibrils and the keratocytes were in close cell contact while IGF-I, TGF-β, and PDGF agarose cultures had an extensive ECM with abundant collagen fibrils. The results of this study indicate that the synthesis of procollagen and the processing of procollagen to collagen are independently regulated and growth factor specific.

**Introduction**

The avascular cornea functions as a major refractive element of the eye. The stroma is 90% of the thickness of the cornea and it consists of a uniquely transparent extracellular matrix (ECM) and keratocytes. The stromal ECM is comprised primarily of collagen types I and V that arranged in uniformly sized, and spaced fibrils with keratan sulfate proteoglycans (keratocan and lumican) or chondroitin sulfate proteoglycans (decorin) between the fibrils [54, 90-92]. The highly organized ECM is synthesized and secreted by keratocytes, which are dendritic in morphology and are sparsely scattered among the stroma [93, 94]. The transparency of the cornea is due to the precise arrangement of the collagen fibrils in the ECM [55]. This arrangement can be disrupted and transparency can be compromised upon wounding [4, 5, 72].

Although the keratocytes readily proliferate and are biosynthetically active during corneal development, they exhibit a relatively low level activity in the adult cornea and are considered “quiescent” [7, 8, 66]. However, the keratocytes become active again when a wound occurs to the cornea stroma. The
keratocytes damaged during wounding undergo apoptosis [66, 68]. Some of the viable remaining keratocytes are activated to proliferate and migrate to the wound site [66, 69, 95] to form regions of hypercellularity [70, 96, 97]; densely packed cells with only a sparse or “provisional” ECM. The keratocytes can remain hypercellular, resulting in a hypercellular scar or, can go on to stratify by producing an extensive collagenous ECM that is either a normal ECM which restores transparency or an abnormal fibrotic ECM, which is opaque [4, 72, 98, 99]. Thus, keratocytes first go through a proliferative, ECM deficient hypercellular phase that is then followed by either a collagenous phase or a fibrocollagenous phase that produces keratocyte stratification.

We found that the phases of ECM synthesis seen during wound healing in vivo could be replicated by the action of FGF-2, IGF-I, TGF-β, and PDGF on keratocytes in vitro [100]. FGF-2 stimulated the highest level of proliferation and maintained proteoglycan synthesis but did not stimulate collagen synthesis. IGF-I, TGF-β, and PDGF, in contrast, stimulated high levels of collagen and proteoglycan synthesis. TGF-β also stimulated hyaluronan, biglycan, and fibronectin synthesis, components of the fibrotic ECM. Therefore, we proposed [100] that in wound healing; 1) FGF-2 would induce the hypercellular phase, producing the sparse, collagen deficient, provisional matrix, 2) the action of IGF-I and PDGF would then induce the collagenous phase, producing the normal stromal ECM that restores transparency or 3) the action of TGF-beta would induce the fibrocollagenous phase, producing the opaque ECM that causes blindness.
Collagen is first made as procollagen: three polypeptides containing N- and C-terminal globular domains with glycine-rich central region that forms a triple helix [11]. In vivo, the globular domains are rapidly removed allowing the helical regions to associate and form collagen fibrils. Fibroblasts in standard cell culture do not readily process procollagen to collagen [83]. As a result, the procollagen accumulates in the culture medium and very little collagen is assembled into an ECM associated with the cells. We have found, however, that a 3% agarose overlay on keratocytes cultured in insulin increased collagen processing and the formation of an ECM associated with the keratocytes [84]. In the current study, we use this agarose overlay cell culture system to compare collagen processing and ECM assembly by keratocytes cultured in each of the growth factors that induce the different phases of cornea stromal wound repair.

**Materials and Methods**

All chemicals and growth factors were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. CyQuant kit, DMEM F/12, polyacrylamide gels, as well as pre-cast polyacrylamide gels and reagents that were used for protein separation and transfer were purchased from Invitrogen (Carlsbad, CA) and ECL Western blot analysis system was purchased from GE Healthcare (Piscataway, NJ). Costar cell culture plates, nunc 96 well plate and chamber slides were purchased from Fisher Scientific (Suwanee, GA). Rabbit polyclonal antibodies to collagen type I were obtained from Cosmo Bio Co. (Tokyo, Japan) and to fibronectin from Chemicon International (Temecula, CA). FITC
conjugated anti rabbit antibodies were obtained from Novus Biologicals (Littleton, CO). A mouse monoclonal antibody to fibronectin and an alkaline phosphotase conjugated goat anti mouse second antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) for use in enzyme-linked immunosorbent assay.

**Keratocytes isolation and culture**

Bovine eyes harvested from 12-month calves were purchased from Pel Freeze (Rogers, AR) and keratocytes were isolated as previously described [50]. Briefly, corneas were removed and keratocytes isolated by using two sequential collagenase digestions. The culture medium used throughout was serum-free DMEM/F12 containing antibiotics and 1mM 2-phospho-ascorbic acid (DMEM). Cells were plated at ~400,000 cells/well in six well plates; ~200,000 cells/well in gelatin coated, 2-well slides; and ~1,000,000 cells/60 mm dish on day 0. The medium was changed on day 4 with DMEM or to DMEM supplemented with 10ng IGF-I, 2ng TGF-β, 10ng FGF-2, or 10ng PDGF/ml.

Keratocyte cultures were overlayed with 3% agarose in DMEM on day 0 as previously described [84]. In brief, 3 grams of agarose in 50 mls of distilled water was autoclaved, cooled to 37 °C and mixed with an equal volume of 37°C 2x DMEM. The medium was removed from cultures and the cells were overlayed with a volume of 3% agarose sufficient to produce a layer ~1mm thick on top of the cells. The agarose was allowed to solidify at room temperature. DMEM supplemented with 10ng IGF-I, 2ng TGF-β, 10ng FGF-2, or 10ng
PDGF/ml was then added to the respective wells. Cultures were harvested on day 20 without intervening media changes.

**DNA measurement**

DNA was extracted from the cell layers of each well of harvested cultures using the buffer supplied in a Cyquant Assay Kit and the DNA measured using methods provided with the kit. Calf thymus was used as a standard.

**Collagen measurement**

Medium and cell layer of cultures were pepsin digested as previously described [84, 100] to isolate collagen. Briefly, eight milliliters of culture medium from a single well of a six well plate was adjusted to 0.5 M acetic acid. Eight milliliters of 0.5 M acetic acid was added to the corresponding well. After samples were chilled to 4°C, 100 µl of 4mg/ml pepsin stock was added to the medium and to each well. The samples were digested overnight at 4°C, neutralized to inactivate the pepsin, dialyzed against distilled water, lyophilized, and reconstituted in 100 µl of 1X LDS sample buffer (Invitrogen). Samples of 10 µl were reduced and electrophoresed on 3-8% tris-acetate gels. Gels were soaked in Simply blue safe stain according to the manufacturer's instructions (Invitrogen) to stain the collagen. The lanes were scanned and the optical density of the α and β chains of collagen was measured using Quantity One.

Collagen for use as standard in SDS/PAGE analysis, was also isolated from corneas by pepsin digestion, and specifically precipitated by dialysis against
cold distilled water. The precipitated collagen was solubilized in 0.01N HCl and quantified by measuring its absorbance at 228 nN.

**Fibronectin measurement**

Fibronectin levels in the media were determined by enzyme-linked immunosorbent assay using 96 well nunc plates. Media from cell cultures and fibronectin used as standard were coated on the wells in bicarbonate buffer. The wells were blocked with phosphate buffered saline (PBS) containing 1% bovine serum albumin. The primary and alkaline phosphotase conjugated second antibody was diluted in PBS with 0.5% tween-20. P-nitophenyl phosphate in diethanolamine was used as a substrate for color development and optical density was measured at 405 nm in a 96 well plate reader.

**Fixation and immunohistochemistry**

Cells on glass slides were fixed in 4% paraformaldehyde in PBS for 1 hr and rinsed with 1% glycine. The agarose layer was removed and nonspecific antigens were blocked with 2.5% BSA in PBS at room temperature. Slides were then incubated with a rabbit anti-bovine collagen type I antibody at 1:500 or rabbit anti-bovine fibronectin at 1:1000 in dilution buffer (1% BSA, 1% Tween 20 in 1x PBS) for 2 hrs. Unbound antibody was washed off with two 5-minute washes in PBS/Tween 20. The primary antibodies were detected by FITC conjugated anti-rabbit antibody at 1:500 in dilution buffer incubated for 1 hour in the dark. Afterward, slides were given two 5-minute washes with PBS in the dark. Slides
were allowed to dry. Cover slips were mounted with DAPI mounting medium, which stain the nuclei of all cells. Slides stained with the secondary antibody only were used to determine the levels of background fluorescence for recording digital images on slides stained with both primary and secondary antibodies. Positive controls were sections of fresh bovine corneal tissue.

Fixation and electron microscopy

Transmission electron microscopy was used to evaluate the organization of ECM deposited by keratocytes cultured under agarose. Keratocytes cultured in 60mm plates were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, 0.1M sodium cacodylate, and 8.0 mM calcium chloride. The agarose overlay was removed and cultures were post-fixed with 1% osmium tetroxide. The samples were dehydrated with a graded series of ethanol/water solutions. Cells were scraped off the dish with a cell scraper, transferred to a 1.5ml tube, further dehydrated with pure propylene oxide and then infiltrated and embedded in a mixture of Embed 812, nadic methyl anhydride, dodecenylsuccinic anhydride and DMP-30 (Electron Microscopy Sciences, PA). Sections of 90nm thickness were examined using a JEOL 1400 transmission electron microscope, and photographed with a Gatan Orius camera.

Statistics

All DNA and collagen values were the mean of 3 determinations. Statview Version 5 (SAS Institute, Cary, NC) was used for statistical comparisons.
Samples were analyzed using a paired t test and standard deviation was used when n=3.

**Results**

Keratocytes were cultured for days 4 to 20 in media containing IGF-I, TGF-β, FGF-2 or PDGF in standard culture and with an agarose overlay. The cultures were harvested on day 20 and the fibrillar collagen deposited with the cell layer was solubilized by pepsin digestion. The collagen in the pepsin digestion was measured by SDS/PAGE and the amount of collagen was expressed per DNA. IGF-I and PDGF agarose cultures accumulated significantly more collagen with the cell layer in agarose cultures than in standard culture by 2.8-fold (**Fig. 15**). In contrast, standard and agarose keratocyte cultures treated with TGF-β and FGF-2 had similar levels of collagen associated with the cell layer, although TGF-β cultures accumulated significantly more collagen than FGF-2 by 6-fold. These results indicate that the agarose overlay significantly enhanced collagen deposition with the cell layer in keratocytes cultured in media containing IGF-I and PDGF.

*In vivo*, procollagen, a precursor of collagen is readily processed and subsequently assembled into mature collagen fibrils [11]. However, in standard cell culture, procollagen is not efficiently processed and it accumulates in the media as procollagen [83, 84]. We used pepsin digestion to convert the procollagen in the media to collagen to make it compatible with measurement of collagen levels in the cell layer. Cultures treated with IGF-I, TGF-β, and PDGF
and overlayed with agarose had significantly lower (45-55% lower) levels of collagen in the media than the standard cultures (Fig. 16). This indicates that procollagen secretion into the media was significantly reduced by the agarose overlay for keratocytes cultured in all growth factors except FGF-2.

The amounts of collagen in the media and cell layers were then combined to determine total collagen produced in standard and agarose cultures treated with growth factors (Fig. 17). Keratocytes cultured in media containing IGF-I, TGF-β, and PDGF produced significantly less (37-39% less) total collagen when overlayered with agarose. This indicates that the agarose overlay significantly inhibits collagen synthesis in keratocytes cultured in all growth factors except FGF-2.

We next calculated the amount of collagen in the cell layer as a percent of the total collagen (cell layer and media) produced (Fig. 18). The results show that keratocytes cultured in IGF-I and PDGF in standard culture converted ~6% of the total collagen produced to fibrillar collagen and this was increased to 31% (5 -fold) by agarose. Keratocytes cultured in TGF-β in standard culture, however, converted significantly more (19%) than IGF-I and PDGF in standard culture and although the agarose increased conversion to 31% for TGF-β, it was not significantly more. Thus, for keratocytes cultured in TGF-β, the agarose overlay did not significantly enhance procollagen conversion to collagen over that achieved in standard cultures because of the already high levels of conversion in standard culture. Collagen conversion was at ~10% for FGF-2 treated cultures, with or without agarose. These results indicate the synthesis of procollagen and
the processing of procollagen are independently regulated and growth factor specific.

Fibronectin is a component of the ECM that has a molecular weight similar to procollagen type I. Therefore, we also measured the levels of fibronectin in the media of keratocytes treated with growth factors and cultured with and without agarose overlay (Fig. 19). The levels of fibronectin in the media of keratocytes in standard culture containing PDGF and TGF-β was 5-fold and 38-fold higher, respectively, than that for IGF-I or FGF-2 in standard culture. However, in contrast to that seen with the agarose-mediated reduction of procollagen in the media, agarose did not reduce the levels of fibronectin deposited into the media.

The distribution of collagen and fibronectin within the cell layer of the agarose cultures was determined by immunohistochemistry using rabbit antibodies to collagen type I and to fibronectin followed by a FITC labeled second goat anti rabbit antibody (Fig. 20). Nuclei were stained with DAPI. The location of the nuclei showed that keratocytes tended to cluster in aggregates when cultured in IGF-I but were more uniformly distributed when cultured in the other growth factors. The antibody to collagen type I showed that collagen in IGF-I treated cultures were deposited in linearly connected clumps that corresponded with the cell aggregates. The collagen deposited in the TGF-β treated cultures were distributed more uniformly in large homogenous patches. Staining for collagen in FGF-2 treated cultures was almost non-existent. The collagen in the PDGF treated cultures was deposited in both intensely staining clumps and in
faintly staining patches. The antibody to fibronectin revealed fibronectin was distributed in both thick and thin linear tracks: thick linear tracks in the IGF-I and PDGF treated cultures, much thinner and more homogeneously distributed linear tracks in the TGF-β treated cultures and both thick and thin linear tracks in the FGF-2 treated cultures. Staining for fibronectin was clearly more extensive than the staining for collagen in the FGF-2 treated cultures.

The size and organization of the collagen fibrils in the ECM of the cell layer of agarose cultures was determined by transmission electron microscopy. There was extensive ECM space between cells in the IGF-I, TGF-β and PDGF treated cultures that was filled with collagen fibrils (Fig. 21). The diameter of fibrils in the TGF-β treated culture were approximately one half the diameter and were more uniformly oriented than that seen in IGF-I treated cultures. The fibrils in the PDGF treated cultures showed the characteristics seen in both the TGF-β and IGF-I cultures. There was only limited ECM space in the FGF-2 treated cultures. The keratocytes were densely packed together and the collagen fibrils in the space between the cells were sparse. The cell surfaces in FGF-2 treated cultures showed extensive regions of cell-cell contact.

**Discussion**

The results of these studies shows that compared to standard cultures, the agarose overlay enhanced collagen accumulation associated with the cell layer of keratocytes cultured in media containing IGF-I and PDGF but not in keratocytes cultured in TGF-β. TGF-β, IGF-I and PDGF, all stimulated collagen
synthesis in standard cultures [100]. The results of the current study, however, show TGF-β treated keratocytes to be more efficient at processing procollagen to collagen in standard culture than IGF-I or PDGF and this may account for the lack of an enhancing effect of the agarose overlay on TGF-β treated keratocytes. The accumulation of collagen associated with the cell layer was also not enhanced by the agarose overlay of the FGF-2 treated cultures. FGF-2, however, does not stimulate collagen synthesis in standard culture [100]. This suggests that the agarose overlay only can enhance collagen processing when collagen synthesis is stimulated by some growth factors.

TGF-β has previously been shown to enhance the proliferation and cell stratification of human fibroblast in standard culture and in media containing fetal bovine serum [101] and these antibodies to fibronectin could inhibit both the basal levels and TGF-β stimulated levels of proliferation and stratification. We found TGF-β stimulated fibronectin synthesis 5 and 20-fold greater than that of PDGF and IGF-I, respectively and that the distribution of fibronectin in the ECM of the TGF-β treated cultures, as determined by immunoflorescent staining, was distinctively different than that of the IGF-I and PDGF treated cultures. Fibronectin has been shown to play an essential role in the assembly of collagen types I and III fibrils [102, 103]. The high levels of fibronectin production seen in TGF-β treated cultures and its unique distribution may be responsible for the high levels of procollagen to collagen conversion seen in the TGF-β treated keratocytes in standard culture.
The agarose overlay reduced the levels of procollagen, but not the levels of fibronectin in the media of keratocytes cultured in media containing IGF-I, TGF-β, and PDGF. We previously proposed [84] that the agarose overlay may be acting as a semi permeable barrier, or molecular sieve, to retard the diffusion of procollagen into the media and increase its concentration at the cell surface where the enzymes are that remove the N- and C-terminal globular domains from the procollagen. Fibronectin and procollagen have similar molecular weights and the differential effect that the agarose has on their diffusion into the media would suggest that the agarose is not acting as a molecular sieve.

The immunostaining for collagen and fibronectin showed they were deposited with the cell layer in distinctly different patterns for each of the growth factors. The keratocytes cultured in IGF-I formed aggregates and collagen was deposited in clumps while the fibronectin was in thick tracks associated with these cell aggregates. In contrast, the collagen was deposited in large patches and the fibronectin in thin tracks and both were more uniformly distributed in cell layer of keratocytes cultured in TGF-β. The pattern of collagen distribution in the PDGF treated cultures was intermediate between that seen in the IGF-I and TGF-β treated cultures. Similarly, the pattern of distribution of fibronectin deposited in the PDGF treated cultures also had characteristics of both the IGF-I and TGF-β cultures: it was distributed throughout the culture, but it was in thick bands, like the IGF-cultures. Fibronectin distribution is the FGF-2 treated cultures was considerably more extensive than that of collagen. These
observations suggest that the collagen and fibronectin deposited in the ECM of these cultures do no co-distribute.

Electron microscopy revealed differences in the collagen fibrils that were deposited in the ECM by keratocytes cultured in the different growth factors. The fibrils in the IGF-I treated cultures were organized in lamellae while the fibrils in the TGF-β treated cultures were thinner and were more uniformly oriented. Although there was abundant fibril deposition in the IGF-I, TGF-β, and PDGF treated cultures, there was only a few fibrils seen in the FGF-2 treated cultures. In the FGF-2 treated cultures, the keratocytes were densely packed together with extensive close cell contacts, leaving little space for an ECM. This is similar to that seen for keratocytes in the hypercellular phase of corneal stromal wound healing.
**Figure 15.** Collagen levels associated with the cell layer of keratocytes in standard and in agarose culture. Keratocytes were cultured in media containing IGF-I, TGF-β, FGF-2, or PDGF and harvested for analysis on day 20. Agarose overlay significantly enhanced collagen accumulation in keratocytes cultured in IGF-I and PDGF by 2.8 fold. TGF-β and FGF-2 agarose cultures had similar levels of collagen in the cell layer as the standard cultures.
Figure 16. Collagen levels in the media of keratocytes in standard and in agarose culture. Keratocytes were cultured in media containing IGF-I, TGF-β, FGF-2, or PDGF and harvested for analysis on day 20. Keratocytes cultured in media containing IGF-I, TGF-β, and PDGF and under agarose deposited significantly less collagen into the media than in parallel standard cultures.
Figure 17. Collagen levels associated with the cell layer and media combined of keratocytes in standard and in agarose cultures. Keratocytes were cultured in media containing IGF-I, TGF-β, FGF-2, or PDGF and harvested on day 20. IGF-I, TGF-β, and PDGF agarose cultures produced significantly less total collagen than standard cultures.
**Figure 18.** Procollagen to collagen conversion rates for keratocytes in standard and agarose cultures. Collagen amounts associated with the cell layer (Figure 1) were divided by the amounts in the cell layer and media combined and expressed as a percent. Compared to standard cultures, agarose significantly enhanced procollagen conversion to collagen in cultures treated with IGF-I and PDGF by 6-fold. TGF-β treated standard cultures were significantly more efficient at converting procollagen to collagen then IGF-I and PDGF standard cultures by ~3-fold.
Figure 19. Fibronectin levels in the media of keratocytes in standard and agarose cultures. Keratocytes were cultured in media containing IGF-I, TGF-β, FGF-2, or PDGF and harvested for analysis on day 20. In standard cultures, keratocytes cultured in TGF-β accumulated ~20-fold more fibronectin in the media than IGF-I and FGF-2 cultures and 5-fold more fibronectin in the media than PDGF cultures. However, compared to standard culture, agarose had no effect on the deposition of fibronectin into the media.
Figure 20. Immunofluorescent staining of the cell layers of keratocytes cultured under agarose. Keratocytes were cultured in media containing IGF-I (I), TGF-β (T), FGF-2 (F), or PDGF (P) and stained with antibodies to either collagen (c) or fibronectin (f) on day 20. Nuclei were stained with DAPI. Collagen was distributed in clumps and in large patches while fibronectin was distributed in thick and thin tracks.
Figure 21. Electron microscopy of the cell layers of keratocytes cultured under agarose. Keratocytes were cultured in media containing IGF-I (I), TGF-β (T), FGF-2 (F), or PDGF (P) and fixed for electronmicroscopy on day 20. Collagen fibrils were abundant in the ECM of the IGF-I TGF-β, and PDGF treated cultures while FGF-2 treated cultures exhibited only little ECM with few collagen fibrils.
Chapter Four

Expansion of Primary Cultures of Keratocyte with Retention of Native Phenotype
Introduction

The stroma is the thickest layer of the cornea and consists of a transparent ECM, comprised mainly of proteoglycans and fibrillar collagens [2]. The ECM is synthesized and secreted by keratocytes. In a mature cornea, keratocytes are quiescent and dispersed among the stroma, possessing a dendritic morphology and elevated levels of two keratan sulfate proteoglycans: lumican and keratocan. These cells also express crystallins, high levels of aldehyde dehydrogenase (ALDH) and transketolase, which contribute to the transparency of the cornea [4, 104, 105]. The expression of keratocan and high levels of ALDH are unique to keratocytes and they are considered markers of their native phenotype.

The standard culture medium for keratocytes in culture is DMEM/F12 containing 5-10% fetal bovine serum. Fetal bovine serum causes the keratocytes proliferate and to differentiate into fibroblasts or myofibroblasts [81, 106, 107]. These cells are characterized by their spindle-like morphology, increased rate of proliferation and expression of fibronectin, hyaluronan, and alpha-smooth muscle actin, and decreased expression of keratan sulfate proteoglycans and aldehyde dehydrogenase (ALDH) [80, 88, 106]. It has been suggested that the corneal fibroblasts and myofibroblasts are not terminally differentiated and can return to the keratocyte phenotype [108].

Considering the wide spread use of corneal fibroblasts in vision research, it would be useful if there was a simple way to restore the keratocyte phenotype to these differentiated cells. Studies have shown that human keratocytes can
retain their native morphology and keratocan expression in the presence of FBS, when cultured on an amniotic membrane [109]. Other studies have shown that KS synthesis and a dendritic morphology but not ALDH content could be restored to bovine corneal fibroblasts by switching to serum-free medium [110]. Additionally, keratocytes isolated from human corneas, cultured in a special stem cell medium and selected for a stem cell marker protein become fibroblastic, but keratan sulfate, keratocan, and ALDH expression could be restored in these cells by subsequent culture in medium containing FGF-2 and ITS (Insulin, Transferrin, and Selenium) [111].

In this study, we investigated the effect of media containing either insulin, ITS, or FGF-2 alone or in combination, on the proliferation and restoration of keratocan by bovine and human keratocytes. We expect FGF-2 to significantly stimulate proliferation and ITS and insulin to stimulate keratocan production.

**Materials and Methods**

The materials and methods used are the same as in Chapter 2.

**Results and Discussion**

We attempted to grow and pass primary bovine keratocytes by culture in medium containing either insulin, ITS, FGF-2, or FGF-2 with insulin or ITS. We found that keratocytes cultured in insulin and ITS could not be removed from the culture plate by standard trypsin digestion. Keratocytes cultured in FGF-2 proliferated and were efficiently removed and passed, but proliferation
discontinued after the first or second passage. FGF-2 treated keratocytes possessed spindled-shape morphology. Keratocytes cultured in FGF-2 combined with insulin or ITS, however, could be repeatedly passed using trypsin digestion and replated. These cells continued to proliferate and retained their dendritic morphology when cultured in insulin or ITS.

Therefore, we cultured bovine keratocytes in media containing FGF-2 combined with ITS and passed them 4 times at low density. Keratocytes were then evaluated for their ability to proliferate and produce keratocan by subsequent culture for 6 days in media insulin, ITS, or FGF-2 alone or in combination. The results showed that compared to control, the DNA content of the keratocytes cultured in FGF-2 alone increased 24% and the DNA content of the cultures in FGF-2 combined with either insulin or ITS increased 1.6 fold (Fig. 22A).

Media samples were digested with endo β galactosidase to remove the KS chains from KSPG’s. Then, digested media was DNA-normalized and levels of keratocan core protein were measured by Western blot using an antibody to keratocan (Fig. 22B). The results showed that both the control and the FGF-2 treated cultures expressed low levels of keratocan while cultures in FGF-2 plus insulin or plus ITS expressed higher levels but cultures in insulin or in ITS alone expressed the highest levels. The keratocytes cultured in insulin or in ITS also re-acquired a dendritic morphology (data not shown). This suggests that keratocytes maintain keratocan expression after growth and passage in media containing FGF-2 plus ITS. Additionally, keratocytes subsequently cultured in
insulin or ITS further enhances keratocan expression as well as their dendritic morphology.

We next determined if keratocan could be restored to cultured bovine keratocytes passed in fetal bovine serum by subsequent culture in insulin and ITS alone or in combination with FGF-2. Therefore, we grew keratocytes in media containing 2.5% FBS and they possessed myofibroblast-like morphology. The keratocytes were passed 6 times, and plated them at low density. We then tested their ability to proliferate (Fig. 23A) and produce keratocan (Fig. 23B) by subsequent culture in media with and without growth factors for 6 days. Compared to control, the DNA content of the cultures treated with FGF-2 alone increased 22%. The DNA content of the cultures treated with FGF-2 combined with insulin increased 1.4-fold, while the DNA content of cultures treated with FGF-2 combined with ITS increased 5-fold. Levels of keratocan were evaluated as previously described. We found that the control and the FGF-2 treated cultures contained undetectable levels of keratocan. Cultures treated with insulin alone, ITS alone, and FGF-2 combined with ITS contained readily detectable levels of keratocan. The keratocytes cultured in insulin alone and ITS alone reacquired a dendritic morphology (data not shown). This data showed that keratocan synthesis and a dendritic morphology can be restored to keratocytes passed in FBS by subsequent culture in insulin or ITS.

We next isolated human keratocytes from human corneas and cultured them in medium containing FGF-2 and ITS. When the human keratocytes are plated and allowed to attach, they possess a dendritic morphology. Unlike
bovine keratocytes, the human keratocytes become spindled-shaped after being cultured in media containing FGF-2 and ITS and passed only once. Thus, while we were able to grow and pass these human keratocytes in this defined culture medium, we were unable to detect keratocan by western blot, a hallmark of native keratocytes.

To assure that our antibodies to keratocan reacted with human keratocan if present, we extracted keratocan from human corneas and tested the antibodies by western blot. We found that both the antibody to bovine keratocan and the antibody we purchased to human keratocan reacted with human keratocan core protein. Therefore the failure to detect keratocan in media from cultured human keratocytes is not due to faulty antibodies. The human corneas were from 16, 17, and 42-year-old donors, while the bovine corneas were taken from 1-2 year(s) old cattle. Thus, it may be necessary to isolate keratocytes from corneas of younger human donors in order to successfully culture human keratocytes that maintain their in vivo phenotype.
Figure 22. Cell growth and keratocan expression by cultures derived from passed keratocytes. Primary cultures of bovine keratocytes were grown and passed 4 times in media containing FGF-2 plus ITS. The keratocytes were then plated at low density and cultured in media containing either FGF-2, insulin, ITS, insulin + FGF-2, or ITS + FGF-2 for 6 days. A: DNA content of cell layer. B: Western blot of endo-β-galactosidase digests of media using an antibody to keratocan core protein.
Figure 23. Cell growth and keratocan expression by cultures derived from passed corneal fibroblasts. Primary cultures of bovine keratocytes were grown and passed 6 times in media containing 2.5% FBS. The corneal fibroblasts were then plated at low density in media containing either FGF-2, insulin, insulin + FGF-2, ITS, or ITS + FGF-2 for 6 days. A: DNA content of cell layer. B: Western blot of endo-β- galactosidase digests of media using an antibody to keratocan core protein.
Chapter Five
Conclusions and Future Directions
When the corneal stroma is wounded the keratocytes become activated by specific growth factors to proliferate, form regions of hypercellularity (densely packed cells with little extracellular matrix), and then go on to stratify by producing an extensive collagenous matrix. These growth factors are made by macrophages, lymphocytes, and keratocytes themselves. Additionally, growth factors have also been found to be present in tear film, aqueous fluid, and epithelium layer of the cornea. Studies have shown that TGF-β is the key growth factor that aids in wound healing and causes the formation of a scar. We have found that other growth factors such as, IGF-I, FGF-2, and PDGF are also important during the corneal wound healing process in vitro.

We grew keratocytes in standard culture and in standard culture with an agarose overlay. The agarose overlay enhanced the conversion of procollagen to collagen (Fig. 24), thereby increasing the deposition of collagen fibrils with the cell layer. We postulated that the agarose overlay was acting as a molecular sieve. There is no agarose present in vivo, however, the basement membrane that is made by the corneal epithelium is similar in structure and is likely to have the same function as the agarose overlay in vitro.
Keratocytes were cultured in media containing IGF-I, TGF-β, FGF-2, and PDGF and compared their ability to stimulate cell proliferation and extracellular matrix (ECM) synthesis. Distribution and orientation of specific ECM proteins synthesized by keratocytes cultured in media containing IGF-I, TGF-β, FGF-2, and PDGF was evaluated by overlaying cells with agarose. We found that FGF-2 stimulated the highest level of proliferation but did not stimulate collagen synthesis. FGF-2 treated keratocytes were in close cell contact. Therefore we speculated that FGF-2 signaling is associated with the hypercellular phase of corneal wound healing.

IGF-I, TGF-β, and PDGF all stimulated proliferation and collagen synthesis. TGF-β also stimulated the highest levels of EDA fibronectin, collagen type III, hyaluronan, and biglycan, markers of the abnormal fibrotic ECM. IGF-I and PDGF cultures mostly stimulated collagen type I and keratan sulfate proteoglycans. TGF-β, IGF-I, and PDGF are likely to be involved in the

**Figure 24.** Agarose overlay enhances the conversion of procollagen to collagen.
remodeling and stratification phase of corneal wound healing. Presumably, IGF-I and PDGF are responsible for the restoration of a normal collagenous ECM, whereas TGF-β is involved in the formation of a collagenous scarred ECM. This sequential action of growth factors is depicted in (Fig. 25).

**Figure 25.** Proposed sequential action of growth factors to produce the phases of wound healing

Taken together, the data in this dissertation should help to explain the role of growth factors in the corneal wound healing process. We have discovered that IGF-I, TGF-β, FGF-2, and PDGF are likely responsible for stimulating the different phases of corneal wound healing *in vivo* based on keratocyte
morphology and extracellular matrix production. Therefore, we can possibly direct keratocytes to make a normal collagenous ECM upon wounding by manipulating the levels of growth factors during specific phases of corneal wound repair. As a result, we can reduce corneal opacity caused by scarring. Additionally, these growth factors can be used to stimulate keratocytes to produce collagen, in collagen-depleted corneas of keratoconus patients. Therefore, this dissertation is clinically relevant and a significant addition to the scientific literature. It serves to identify novel targets for treatment of corneal scarring and keratoconus.

To further this study, we plan to evaluate the levels of these growth factors in each phase of the corneal wound healing process *in vivo*. Then, inhibit select growth factors or use knockout animals to determine the effect on the morphology of keratocytes and the ECM components. Additionally, we plan to use the newly established agarose cell culture system to further visualize the corneal stroma ECM *in vitro*. More specifically, the agarose cell culture system will be used to further study the interactions between keratocytes and their surrounding extracellular matrix as well as to determine the expression of growth factor receptors during each phase of corneal wound healing.
References


Appendices
Appendix A

Pepsin readily degrades globular proteins, but the triple helical domains of collagen are resistant to this protease. Aliquots of pepsin digested media and cell layers, normalized for DNA, harvested from the day 7, 10, and 13 time points were fractionated on SDS/PAGE and the collagenous proteins visualized by simply blue staining (Fig. 26). Similar to that seen for the proteoglycans, the media (M) from the IGF-1 cultures contained substantially higher amounts of the α1(I), α2(I) and α1(V) chains of collagen than the control cultures and media from the days 10 and 13 insulin cultures contained slightly higher levels of these collagens than the IGF-1 cultures. This data demonstrate that insulin and IGF-I stimulate keratocytes to synthesize similar levels of collagen.

Figure 26. SDS/PAGE/Blue stain of pepsin digests from keratocyte cultures. Keratocytes were cultured in media alone (C) or in media containing either IGF-I (IG) or insulin (IN). Cultures were harvested on day 7, 10, and 17. The media (M) and cell layers (CL) were digested with pepsin prior to electrophoresis.
About the Author

LaTia Shaquan Etheredge was born on September 21, 1984 in Washington, DC. She was raised in Fort Washington, Maryland where she completed grade school. She graduated from Virginia University ‘Cum Laude’, with a Bachelor of Science in Chemistry and a minor in Biology. During her undergraduate studies, she completed summer internships at the University of Puerto Rico (Rio Piedras Campus), Texas A&M University, and University of Virginia. Her undergraduate experiences fostered her interests in Biomedical Science. To this end, she joined the lab of Dr. John R. Hassell to pursue her Ph.D in Medical Sciences with a concentration in Pathology and Cell Biology.