THE EFFECTS OF BASIC FIBROBLAST GROWTH FACTOR OR EPIDERMAL GROWTH FACTOR ON EQUINE TENDON FIBROBLASTS AND DERMAL FIBROBLASTS IN VITRO

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NOMENCLATURE

DMEM	Dulbecco's Minimum Essential Medium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extra-cellular signal Regulated Kinase
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FGF	Basic Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
HBSS	Hank's Balanced Salt Solution
HSPG	Heparan Sulfate Proteoglycan
IGF-I	Insulin-like Growth Factor – I
IgG	Immunoglobulin G
IL-2	Interleukin – 2
M-ELISA	Monolayer Enzyme Linked Immunosorbent Assay
mRNA	Messenger Ribonucleic Acid
OD	Optical Density
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween 20
PDGF	Platelet Derived Growth Factor
PG	Proteoglycan
rER	Rough Endoplasmic Reticulum
RPM	Revolutions Per Minute
SDF	Superficial Digital Flexor muscle
SDFT	Superficial Digital Flexor Tendon
TGF-β	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor

CHAPTER I

INTRODUCTION

Introduction and Review of Relevant Literature

Equestrian events such as horse racing, rodeos, and horse shows, as well as pleasure riding, have become a significant part of entertainment throughout the United States. Each year many horses are injured while training for, or performing in these events. A large proportion of these injuries are musculoskeletal related (Peloso et al., 1994). One of the most common injuries, involving approximately 30% of horses in training (Birch et al., 1998; Goodship et al., 1994), consists of lacerations involving the flexor tendons (Taylor et al., 1995; Foland et al., 1991; Webbon, 1977). These lacerations can be costly both in treatment modalities and in loss of revenue while the animal recuperates. In addition, there are reports of exercise-related, gradual degeneration of the central portion of flexor tendons resulting in rupture (Fackelman, 1973; Webbon, 1977). This fact being based on the sensitivity of intrinsic fibroblasts (fibroblasts responsible for the original makeup of the tendon) to hypoxia which is the suspect cause of these gradual degenerative changes (Birch, 1997). Causes of traumatic injuries include interference from another horse during racing, trailer accidents, and kicking or stepping on sharp objects (farm implements, tin, glass, barbed wire, buckets or water barrels) (Taylor et al., 1997).

The flexor tendons of the rear limbs are more commonly associated with traumatic injuries. The greater lateral movement of the back of the horse exposes the rear limbs more so than the front limbs, thus making the flexor tendons and overlying skin particularly prone to lacerations, puncture wounds or severe avulsion injuries (Baxter, 1987) compared to the front limbs. Wounds to the rear limbs most commonly occur in the

mid-metatarsal region or mid-pastern region (Baxter, 1987) where a large portion of the superficial digital flexor tendon (SDFT) and deep digital flexor tendon reside just beneath the skin.

After tendon wounding, a complex series of reparative events occur that constitute tendon healing. Tendon wound healing is a multi-step process not unlike skin wound healing. These events include cellular processes amounting to migration, proliferation and secretion of cytokines and chemokines. Two well known cytokines involved in wound healing are epidermal growth factor and fibroblast growth factor. These growth factors participate in many aspects of both tendon and dermal wound healing. To better understand the mechanisms involved in tendon wound healing, including the importance of these two growth factors, a discussion on the basic anatomy of the tendon, its cellular and extracellular matrix components, the biology of epidermal growth factor and fibroblast growth factor, and their effects on wound healing, are in order.

Tendon: An Introduction to the Anatomy

Tendons are dense fibrous elongated collagenous structures, which connect their sarcomeric contractile elements (i.e. skeletal muscle fibers) to bone. Two major groups of tendons are recognized: sheathed and unsheathed. Sheathed tendons are those surrounded by a synovial sheath and bathed in synovial fluid, which is a large contributor to their nutrition. The one to two layers of cells on the external surface of the tendon proper are called epitenon cells. The cells on the inner surface of the sheath are called parietal synovial cells. The sheath is not continuous, it surrounds approximately 85% of the tendon. The remaining 15% is filled with a connective tissue band called the mesotenon.

The mesotenon maintains communication between the tendon proper and the adjacent soft tissue structures via extensions called vincula (single = vinculum). These are areas in which blood vessels and nerves traverse. Non-sheathed tendons are connected directly to the surrounding soft tissue structures by a loose areolar connective tissue coat known as the paratenon. The paratenon is composed primarily of type III collagen and fibroblasts, with nerves and blood vessels traversing through it. Tendons surrounded by paratenon have been referred to as vascular tendons because vessels enter many points on the periphery and anastomose with a longitudinal system of capillaries. In both sheathed and non-sheathed tendons, blood supply is also received from proximal and distal tissue associations (muscle, bone)(Gelberman, 1985). A well-established blood supply is necessary in order for tendon cells to survive and participate in repair (Ark et al., 1994).

Tendons are composed primarily of type I collagen. Tendons can have anywhere from 1 to numerous fascicles, which are composed of multiple bundles of collagen fibrils (fibers) and fibroblasts. These fibers and fibroblasts are collectively known as primary bundles. The fibroblasts of primary bundles (tenocytes/tenoblasts) are the main source of type I collagen that makes up the tendon fibers (Riederer-Hendrickson et al., 1983). Fascicles and primary bundles within the tendon are bound together by loose connective tissue, the endotenon*, which permits longitudinal movement of collagen fascicles and

* the suffix -tenon is equivalent to -tendon or -tendineum

supports blood vessels, lymphatics and nerves. Type III collagen and elastin predominate in this area. The endotenon is continuous with the epitenon of sheathed tendons and the paratenon of non-sheathed tendons.

Tendon cells can be divided into two groups based on their location in reference to wound healing. The intrinsic group includes the tenocytes and the fibroblasts of the endotenon and epitenon. The extrinsic group includes paratenon fibroblasts, parietal synovial cells, and fibroblasts of the adjacent subcutis, dermis, and bone.

Collagen: Microanatomy and Synthesis

Collagen is a linear, unbranching, poorly soluble glycoprotein that is present in all tissues and organs of the body. Collagen is mostly secreted by fibroblasts (chondrocytes and osteoblasts secrete collagen also). It is the most abundant protein found in the body, and is one of a number of extracellular matrix (interstitium) proteins that participate in the framework/scaffolding of the body. (Eyre, 1990). Type I collagen alone makes up about 90% of the collagen present in the body, comprising most of the collagen of skin, bones and tendons. Two other prominent forms of collagen are type II, found primarily in cartilage, and type III, found mostly in skin and tendon (Woodhead-Galloway, 1980).

Collagen is composed primarily of amino acids with a small carbohydrate component. The amino acids that form the body of collagen are glycine, hydroxylysine and hydroxyproline. The structure of collagen is intricate. A single collagen fibril (fiber) is made of many macrofibrils (subfibril). Each macrofibril in turn is composed of many microfibrils. Each microfibril is composed of numerous molecules of tropocollagen (collagen). Tropocollagen molecules are held together by hydrogen and covalent bonds.

Covalent bonds occur between lysine residues present in the amino and carboxyl terminal ends to hydroxylysine residues within the body of an adjacent tropocollagen molecule. Hydroxylysine is not a naturally occurring amino acid. It is formed by the hydroxylation of lysine by lysyl hydroxylase, which requires ascorbic acid [vitamin C] as a cofactor (Kuhn, 1987). The crosslinking of a hydroxylysine residue to a lysine residue is by the enzyme lysyl oxidase, which requires copper as a cofactor (Kuhn, 1987). Tropocollagen is composed of three polypeptide chains twisted together into a right-handed triple helix. These three chains are in parallel and are staggered by one residue with respect to each other. The three chains are held together by hydrogen bonds, the main stabilizing force, and covalent bonds. Each individual polypeptide chain (approximately 1000 residues long) is called an α chain and is arranged in a left handed helix, with 3 residues per turn of the helix. Two primary α chains (α_1 and α_2) exist, each forming multiple subspecies (i.e. α_1(I), α_2(III)). The amino acid sequence of each polypeptide is generally a repeating tripeptide unit of Gly-X-Y, where X can be any amino acid and Y is predominantly one form of proline (primarily hydroxyproline formed by the hydroxylation of proline by prolyl hydroxylase). The size and spacing of the glycines makes the triple helix of the tropocollagen possible. The three α chains are cross linked by hydrogen bonds between the amino group of the glycine from one tripeptide of one chain and the carboxyl group of a residue in position X of a tripeptide in a different chain. The individuality of each α chain (amino acid composition) is what determines the type of collagen produced (Beck and Brodsky, 1998).

Synthesis of collagen starts in the rough endoplasmic reticulum (rER). In the rER, collagen polypeptide chains are synthesized as pro-α-chains that contains extension

peptides at the carboxyl and amino terminal ends. Within the rER cisternae, these pro-α-chains assemble into triple helices to form procollagen molecules. These procollagen molecules are transferred to the Golgi complex, packaged into secretory vesicles, and released by exocytosis. Therefore, the ability of identifying pro-collagen in the cell is very similar to identifying the collagen that is secreted. The extracellular enzymatic cleavage of the extension peptides by procollagen peptidases yields tropocollagen molecules. These in turn assemble in the extracellular matrix to form collagen fibrils (fibers) with subsequent intramolecular and intermolecular covalent cross-linking occurring (Miller and Gay, 1982; Gay and Miller, 1983). These extensive linkages contribute to the formation of tendons.

Growth Factors: An Introduction and Their Effects on Wound Healing

Basic Fibroblast Growth Factor

Fibroblast growth factors are a family of growth factor peptides with eighteen presently identified members. The members of the fibroblast growth factor family are known to evoke proliferative responses, as well as modulating cell motility (Slavin, 1995), integrin expression (Klein et al., 1993), cell differentiation, cell adhesion (Rusnati et al., 1997), cell to cell attachment (Richard et al., 1995), and cell survival in vitro (Fuks et al., 1994; Morrison et al., 1986; Gospodarowicz et al., 1978). Increasing evidence implicates these growth factors in normal physiological processes in vivo: these include embryonic and fetal development, neovascularization, and responses to wounding (Mason, 1994). Fibroblast growth factor members stimulate the proliferation of tissue derived from all three germ layers (mesoderm, ectoderm, and endoderm) as well as

neuroectodermal cells (Gospodarowicz, 1991) by autocrine (Yayon and Klagsburn, 1990; Sato and Rifkin, 1988), and paracrine regulation. Some fibroblast growth factor members have the ability to modulate the synthesis as well as the vectorial deposition of various extracellular matrix (ECM) components, including collagen type I and III, fibronectin, and laminin (Tseng et al., 1982; Gospodarowicz, 1981). Some of the growth factor members can also repress synthesis of collagen type I, II, V, XI and fibronectin (Tseng et al., 1982; Horton et al., 1989; Kypreos and Sonenshein, 1998). Fibroblast growth factor members also have the ability to stimulate the activity of various proteases and collagenases (Rifkin and Moscatelli, 1989). These growth factors tend to be conserved strongly between species, including mammals, birds, and amphibians. The sequence identity between human and mouse fibroblast growth factor homologs is typically greater than 90% at the amino acid level (Miller and Rizzino, 1994). This homology is thought to occur across multiple mammalian species, including the horse, although this has not been demonstrated through amino acid sequencing. The sequence similarities tend to be greatest within the so-called "core" region of homology, which comprises nearly the full length of basic fibroblast growth factor (FGF) (Miller and Rizzino, 1994). Therefore, basic fibroblast growth factor (FGF) is considered to be a prototype fibroblast growth factor and has been used extensively to study the structural and functional relationships of fibroblast growth factors (Miller and Rizzino, 1994). Basic fibroblast growth factor is widely distributed and present in many tissues including brain, kidney, adrenal gland, ovary, uterus, myocardium, dermis and tendon, as well as in numerous circulating cells including platelets (Brunner et al., 1993), macrophages (Sprugel et al., 1987) and T-lymphocytes (Blotnick et al., 1994). FGF has also been identified in plasma (Baird et al.,

1986), and in many cell culture lines (Slavin, 1995). FGF is both a mitogen and a morphogen. FGF, through activation of mitogen activated protein kinase, mediates protective effects against apoptosis triggered by tumor necrosis factor alpha, and highlights the important role that integration of multiple intracellular signaling pathways plays in the regulation of cell growth and death (Gardner and Johnson, 1996).

FGF is a peptide with four isoforms (Florkiewicz and Sommer, 1989), composed of approximately 146 amino acids. The classical form is 18KDa and is primarily located in the cytoplasm near the cell surface, and occasionally on the cell surface (Florkiewicz et al., 1991). The three other isoforms (22 KDa, 22.5 KDa, 24KDa), classified as high molecular weight forms, are primarily present in the nucleus (Florkiewicz et al., 1991). All four forms have been shown to induce cell growth, however, the 18KDa FGF has also been shown to be expressed more in migratory cells (i.e. fibroblasts, macrophages, etc.) than the high molecular weight forms (Bikfalvi et al., 1995). The cytoplasmic form also induces down regulation of fibroblast growth factor receptors (Mason, 1994).

The ability to bind heparin and heparan sulfate proteoglycans (HSPG) is apparently shared by all fibroblast growth factors, and cell surface heparin-like molecules appear to be necessary for some, if not all, of the biological activities of fibroblast growth factors (Yayon et al., 1991; Roghani et al., 1994; Flaumenhaft et al., 1990). Fibroblast growth factors are produced at low levels, and are strongly concentrated in the extracellular matrix (ECM) of most tissues, in an insoluble form, because of the high levels of insoluble HSPGs in the ECM (Taipale and Keski-Oja, 1997).

Fibroblast Growth Factor Receptors (FGFR)

Fibroblast growth factors have two receptor types that are necessary for signal transduction: 1) low affinity receptor, 2) high affinity receptor (Bikfalvi et al., 1989). The low affinity receptors are accessory molecules, present in much higher numbers than high affinity receptors (Moscatelli, 1987), and are for the most part required for binding of fibroblast growth factors to the high affinity receptors (Rapraeger et al., 1991). Low affinity receptors have high association as well as high dissociation rates compared to high affinity receptors which have low dissociation rates (Schlessinger et al., 1995; Moscatelli, 1992). The precise mechanism behind why a low affinity receptor is necessary is unknown, but thoughts include the low affinity receptor stabilizes the fibroblast growth factor- fibroblast growth factor receptor (FGF-FGFR) complex. FGF-FGFR complexes exhibit rapid lateral mobility on the cell surface. This lateral mobility will allow frequent encounters between occupied high-density low affinity receptors and the unoccupied, less abundant, high affinity receptors. Keeping in mind that low affinity receptors have a high dissociation rate, it is probable that dissociation from low affinity receptors in the vicinity of high affinity receptors will lead to formation of the more stable complex with the signaling receptor (Schlessinger et al., 1995). Another hypothesis is that it facilitates ligand receptor binding and receptor activation through conformational changes induced in either the ligand, receptor or both (Thomas, 1987; Yayon et al., 1991). Low affinity receptors are identified as HSPG found anchored in cell membranes and within the ECM (Hardingham and Fosang, 1992). Two major families of HSPG are associated with cell surfaces. These are the syndecans consisting of 4 transmembrane proteins, and the glypicans which are implanted in the membrane by

a glycosylphosphatidyl inositol anchor (David, 1993). Perlecan, a basal lamina proteoglycan, is a component of all basement membranes. Perlecan has also been shown to strongly bind FGF, thus making it a major candidate for a FGF low affinity receptor (Aviezer et al., 1994). HSPGs influence FGF utilization by increasing the stability of FGFs by limiting proteolytic degradation (Gospodarowicz and Cheng, 1986; Saksela et al., 1988; Sommer and Rifkin, 1989), serve as a reservoir for FGF by providing extracellular sites for long term storage (Flaumenhaft et al., 1989), and FGF complexed to heparan sulfate diffuses more rapidly in an in vitro model, which suggests that HSPGs cleaved from the cell surface, could increase the distribution of FGFs in vivo (Flaumenhaft et al., 1990). The release of FGF from heparan sulfate comes about by the solubilization of the ECM by proteoglycosidases. These enzymes are released by platelets when they attach to subendothelial connective tissue and released by activated macrophages; two common occurrences seen in wounds. The bound FGF is non-soluble and may act as a local growth regulator and induce the regeneration of tissues damaged in wounds.

High affinity receptors are highly glycosylated (comprising approximately 25-35% of the receptor mass) proteins with intrinsic tyrosine kinase activity, which is characteristic of growth factor receptors. There are low numbers of these receptors per cell. The fibroblast growth factor receptors constitute a gene family of five members (FGFR-1, FGFR-2, FGFR-3, FGFR-4, FGFR-5) (Coutts and Gallagher, 1995). The expression of fibroblast growth factor receptors vary throughout the body. FGFR-1 expression is most prominent in mesenchyme (Peters et al., 1992). FGFR-2 expression is strongest in surface ectoderm and in the epithelia of several developing organs (Orr-

Urtreger et al., 1991). In several instances where organ formation is likely to involve reciprocal epithelial-mesenchymal interactions, FGFR-1 and FGFR-2 exhibit complementary patterns of expression. FGFR-3 expression is most commonly seen in the central nervous system, intestine, lung, and bone (Miller and Rizzino, 1994). FGFR-4 is most conspicuous in muscle precursors and in differentiated skeletal muscle, as well as in definitive endoderm (Stark et al., 1991). FGFR-5 is structurally distinct from the other FGFRs. It is an integral membrane protein, and can bind FGF-1, FGF-2, FGF-3, and FGF4 (Burrus et al., 1992). The four primary FGFRs (FGFR-1 – 4) share a common structure consisting of an extracellular domain (approximately 360 amino acids), a short transmembrane domain (21 amino acids), and a cytoplasmic domain (approximately 420 amino acids) (Bikfalvi et al., 1997).

Epidermal Growth Factor

Epidermal growth factor (EGF) is a member of the epidermal growth factor family (Cohen, 1983). There are at least seven other members of this family. Epidermal growth factor is among the first growth factors discovered. In 1962, Cohen described a protein isolated from submaxillary glands of male mice that caused premature eyelid opening and tooth eruption in neonatal mice (Cohen, 1962). Based upon histological maturation and growth of the epidermis, the factor was called epidermal growth factor.

EGF is a cytokine that promotes cell proliferation, regulates tissue differentiation, and modulates organogenesis (Tebbs et al., 1997). EGF is found numerous organs and in most body fluids including milk, urine, tears and saliva. It is stored within alpha granules of platelets (Kurobe et al., 1986; Oka and Orth, 1983), and is released when platelets are

activated. In the eye it is formed by the lacrimal gland, regulates normal corneal cell turnover, and is involved in corneal healing. It is also found in epithelial cells and endothelial cells of the cornea (Swank and Hosggod, 1996), in chondrocytes of growth plates (Ren et al., 1997), and in Leydig cells of the testis where it appears to be involved in the development of the testis and in spermatogenesis (Yan et al., 1998). EGF is mitogenic and chemotactic for rat enterocytes (Blay and Brown, 1985), and mitogenic for vascular smooth muscle cells (Bhargava et al., 1979). EGF is also mitogenic for hepatocytes (Sand and Christoffersen, 1987), and regulates mammary gland proliferation (Dickson and Lippman, 1995). EGF regulates cell-to-cell adhesion through modulation of the interaction of E-cadherin with the actin cytoskeleton (Hazan and Norton, 1998), and like most other growth factors, EGF is involved in the induction of many neoplastic processes (Salomon et al., 1995).

The initial transcript of EGF is approximately 110 kb (Bell et al., 1986). Following splicing and export from the nucleus, a 4.8 kb mRNA codes for a 1207 amino acid precursor, named preproEGF, which contains a hydrophobic domain which is assumed to be required for anchoring to the cell membrane (Rall et al., 1985). The precursor is glycosylated, and has been shown to possess biological activity (Prigent and Lemoine, 1992) suggesting that EGF might be able to activate its receptor via an auto- or paracrine mechanism, while they still remain bound to the membrane (Voldborg et al., 1997).

EGF is a single chain acidic polypeptide of 53 amino acid residues containing three intramolecular disulfide bonds which are required for a proper tertiary structure. The three intramolecular disulfide bonds are characteristic for all members of the EGF family,

and are required for biological activity (Taylor 1972). The EGF peptide appears to be non-glycosylated and is very stable. Homology of EGF between species is thought to occur, however no amino acid sequencing data has been reported in the horse.

Epidermal Growth Factor Receptors (EGFR)

The EGF receptor (EGFR) is highly homologous amongst animal species including man, mice and chickens (Lax et al., 1988), and presumably horses, but this has not been proven. EGF receptors are present as high and low affinity classes (King and Cuatrecasas, 1982), the high affinity class being responsible for the EGF induced effects (Bellot et al., 1990; Defize et al., 1989). The specific role of the low affinity class remains unknown at this time. The high affinity EGF receptors are also known as ErbB receptors. ErbB is based on the oncogene Erb-B present in the erythroblastosis virus, a retrovirus of avian origin that produces tumors of the erythroblastic lineage (Downward et al., 1984). Erb-B oncogene and EGF receptor have very similar amino acid sequences, enough so that the EGF receptor family is also named the ErbB receptor family (Downward et al., 1984). There are four members to the ErbB family: ErbB1(epidermal growth factor receptor, also called HER-1[human epidermal growth factor receptor1] (Ullrich et al., 1984), ErbB2 [also Neu, HER-2] (Semba et al., 1985), ErbB3 [HER-3] (Kraus et al., 1989), and ErbB4 [HER-4] (Plowman et al., 1993).

The EGF receptor is a transmembrane glycoprotein of approximately 170KD. The external domain of the EGF receptor contains the amino terminus of the molecule. The hydrophobic transmembrane domain is 23 residues long. The internal domain of the receptor is composed of 542 amino acid residues. It contains a region of approximately

300 amino acid residues that is homologous to the catalytic domain of the protein kinase encoded by the src gene family of oncogenes. Thus the EGF receptor belongs to the tyrosine kinase class of receptors. The C- terminal domain is believed to have a number of regulatory functions. The first element is the Caln domain, necessary for receptor internalization and increased cytosolic calcium (Chen et al., 1989). The autophosphorylation of the C-terminal end removes an alternate substrate/competitive inhibitor conformation, permitting access of cellular substrates to the tyrosine kinase domain (Voldborg et al., 1997). This regulatory domain contains a number of tyrosine residues that can be phosphorylated by the receptor itself (Downward et al., 1984). The autophosphorylation tyrosines bind receptor substrates by their SH2 domains (Margoli, 1992).

The differential activation and coupling of EGF family receptors is complex. This complexity involves the ability of single EGF family hormones to bind multiple receptors, and single receptors with the ability to bind multiple hormones (except for ErbB2). Receptors that do not bind a particular hormone when expressed alone can be cross-activated (transmodulated), if a binding competent receptor is also present. For example, although EGF does not bind or activate ErbB2 expressed by itself, EGF induces the tyrosine phosphorylation of both ErbB1 and ErbB2 in cells expressing both receptors (King et al., 1988; Pinkas-Kranarski et al., 1996; Sliwkowski et al., 1994; Riese et al., 1995). Heterotypic interactions are now known to occur extensively among other combinations of ErbB family receptors, and there is a graded hierarchy of heteromeric interactions that may reflect differences in affinities of the various hormone-receptor-receptor complexes The mechanism(s) by which a non-binding receptor is recruited

into a receptor complex and can modulate ligand binding affinity is/are unknown. One theory is that each growth factor molecule binds to a high affinity site on one EGFR molecule and a low affinity site on the heterotopic EGFR molecule (Riese and Stern, 1998). EGF binding to the high affinity binding site is required to stabilize receptor dimers and for receptor activation. The activation of the high affinity receptor may cause it to undergo a conformational change and bind to a heterotopic receptor. This binding allows the growth factor to bind to the low affinity site on the heterotopic receptor. In return, both receptors are activated (Riese and Stern, 1998). The activation of a second receptor through binding of the primary receptor leads to a diverse array of signal pathway activations (Olayioye et al., 1998; Pinkas-Kranarski et al., 1998).

The mechanism of activation of the EGF receptor involves binding of EGF causing a conformational shift of the receptor molecule leading to increased affinity of the receptor for neighboring receptors. This increase in affinity leads to dimerization and hence tyrosine autophosphorylation (Schlessinger and Ullrich, 1992; Ullrich and Schlessinger, 1990; Yardern and Schlessinger, 1987).

Basic Fibroblast Growth Factor in Wound Healing

Wound healing is an orderly and continuous sequence of events leading ultimately, to near normal structural and functional ability of the tissue wounded. Healing consists of inflammation, tissue formation, and remodeling. Cell signals, many involving growth factors, direct an organized cellular infiltration, proliferation, activation, and eventual inhibition of wound healing (Brew et al., 1995). FGF essentially influences all of the diverse cells involved in wound healing (Figure 1). The immediate response to any

injury is initiation of coagulation with fibrin clot formation and platelet activation. Platelets degranulate and release many cytokines, including FGF (Brunner et al., 1992). At the same time, proteases and endoglycosidases, responding to basement membrane or endothelial cell damage, release FGF bound in the ECM, as does thrombin (Benezra et at., 1993). Syndecans within the extracellular matrix can control these proteases by reducing their proteolytic activities (Kainulainen et al., 1998). Syndecans can also bind FGF (Coutts and Gallagher, 1995) thus being a regulator of protease release and activity. FGF has a strong affinity for fibrin that is laid down during coagulation phase of wounding (Sahni et al., 1998). Fibrin and FGF have a synergistic effect for stimulating neovascularization. FGF directs cell migration and proliferation resulting in a local inflammatory response. Neutrophils, lymphocytes and macrophages are cells specifically involved in this inflammation. Neutrophils and lymphocytes act primarily to debride the wound and provide resistance to infection by eliciting the secretion of antimicrobial agents from other inflammatory cells. Neutrophils also contribute to the release of FGF by endothelial cells (Totani et al., 1994), and T lymphocytes synthesize and secrete FGF (Blotnick et al., 1994). Macrophages elaborate FGF (Sprugel et al., 1987) that stimulates fibroblast proliferation and new collagen formation. Fibroblasts also release FGF to affect active collagen turnover, wound contraction (Rudolph, 1979), and proteoglycan synthesis for as long as six months after injury. The tissue repair stage (granulation tissue) and remodeling stage of wound healing also depends on FGF-activated endothelial and epithelial cells for their contribution to angiogenesis (Slavin, 1995), re-epithelialization in skin (Hebda et al., 1990; O'Keefe et al., 1988; Shipley et al., 1989), and cell proliferation (Bikfalvi et al., 1997). Fibroblasts and endothelial cells release

FGF (Totani et al., 1994; Lorenzet et al., 1992; McNeil et al., 1989) that helps direct the final stage of wound healing.

The mitogenic and chemoattractant effects of FGF are directed primarily towards cells derived from embryonic mesoderm or neuroectoderm, especially fibroblasts and endothelial cells, two cell populations vital to wound healing. For endothelial cells, FGF is an angiogenic factor guiding endothelial cell proliferation, protease production, cell migration and invasion necessary for new blood vessel formation (Montesano et al., 1986; Terranova et al., 1985; Joseph-Silverstein and Rifkin, 1987). Angiogenesis is stimulated directly and indirectly by FGF. Capillary growth is directly stimulated as endothelial cells are induced to migrate and proliferate (Terranova et al., 1985). Indirect stimulation occurs as FGF initiates macrophage production of additional FGF and other angiogenic factors. Plasminogen activators, stromelysin and collagenase production are induced by FGF (Mignatti et al., 1989; Montesano et al., 1986; Flaumenhaft et al., 1992; Davidson and Broadley, 1991). This expression initiates the breakdown of the extracellular matrix and facilitates the migration of endothelial cells through the matrix to the wound site.

At least two regulatory mechanisms for preventing excessive neovascularization are known. The most recent being platelet factor 4 that is released by platelets and inhibits fibroblast growth factor receptor dimerization, a critical step in signal transduction (Perollet et al., 1998). The second chemical mediator is interleukin 8, a proinflammatory chemokine which causes the down regulation of fibroblast growth factor receptors by a protein kinase C dependent, non-competitive mechanism of action (Presta et al., 1998).

The interesting aspect of this chemokine is that it is a known in vivo angiogenic compound.

Animal Models of Skin Wound Healing Using Basic Fibroblast Growth Factor

Fibroblast growth factor is detectable within the wound fluid and wounded tissue in the first 14 days of dermal wound healing in both young and aged animals (Ashcroft et al., 1997). Because of its presence in dermal wounds, it may play a role in the healing process. FGF clearly influences angiogenesis, mitogenesis and chemotaxis in cell culture (Paris and Pouyssegur, 1991;Hill et al., 1991; Belford, 1997; Ihn et al., 1995), all processes involved in wound healing. Extension of these findings to wound healing in vivo has been attempted in a number of different animal models.

Incisional implants. A FGF impregnated sponge implantation model simulating an artifical wound space has been examined in rats and guinea pigs. Marks et al. (1991) implanted type I collagen sponges into the subcutaneous tissue of the dorsum of guinea pigs. They reported a stronger breaking strength of sponges impregnated with fibroblasts and FGF (10 μg/ml) compared to sponges alone. They attributed the stronger strength of the FGF impregnated sponge to the abundant neovascularization within the sponge. It also appeared to them that the collagen network formed in the FGF impregnated and fibroblast seeded sponges, was more advanced (mature appearing) than in the control collagen sponge, also contributing to the increased tensile strength. Broadley et al. (1990) injected FGF (50μg/ml) into polyvinyl alcohol sponges and implanted them into the dorsum of diabetic rats and normal rats to evaluate the effects of FGF on wound healing. The concentrations of FGF and other growth factors have been shown to be lower at the

wound site (Grotendorst et al., 1985) of diabetic animals compared to control animals. Broadley et al. measured multiple biological parameters within the sponges. The parameters for their project included DNA levels, protein accumulation, collagen formation, and connective tissue tensile strength. Each of these parameters is delayed by diabetes. Seven days after implantation a dramatic increase in accumulation of DNA and protein was observed in both treated normal rats and treated diabetic rats compared to control diabetic rats. However, FGF caused a decrease in both total and relative amounts of collagen at 7 and 9 days postimplantation in both normal and diabetic rats, respectively. Broadley et al. (1989) also implanted normal rats with sponges containing anti-FGF. They noticed that a large percentage of rats exhibited impaired wound healing when treated with the anti-FGF compared to controls. Granulation tissue formation, total collagen, and angiogenesis were either inhibited or greatly reduced. Collagen reduction in the anti-FGF sponges was presumably due to the reduced numbers of collagen producing cells (i.e. fibroblasts), since collagen was also reduced in the previous experiment in which FGF was added and cell numbers were increased.

Incisional models. Numerous incisional (partial and full thickness) wounding experiments investigating the effects of FGF on wound healing have been reported. A full thickness incisional model, such as the rabbit ear dermal ulcer model, has been used to evaluate FGF. Mustoe et al. (1991) evaluated the ability of FGF to promote granulation tissue formation and re-epithelialization of the epidermis. They concluded that FGF (5 μg), when topically applied in a collagen solution, appeared to have increased new vessel formation, but did not produce any more collagenous matrix than those levels found in control, non-treated wounds. However, FGF did promote a significant increase in re-

epithelialization. They suggested that FGF may be effective in accelerating repair of partial thickness clinical wounds, such as donor graft sites, where enhanced epithelialization, and not granulation tissue formation, is required. Pierce et al. (1992), also using the rabbit ear dermal ulcer model, determined the composition, quantity, and rate of extracellular matrix deposition afforded by the topical application of FGF in a phosphate buffered saline solution. Their results demonstrated that FGF (2 μg) induced a predominantly angiogenic response in wounds, with a marked increase in endothelial cells, new blood vessels, and collagenolytic activity. The FGF treated wounds did not evolve into collagen containing scars and continued to accumulate only provisional matrix well past wound closure causing macroscopic nodules to form. Tsuboi and Rifkin (1990) made incisional wounds in genetically afflicted diabetic mice. They noticed that there were significant increases in breaking strength, degree of re-epithelialization, granulation tissue thickness, matrix density, numbers of infiltrated cells, and numbers of capillaries within normal and diabetic mice treated with topically applied FGF (5 μg), compared to diabetic mice not treated with FGF. Ishiguro et al. (1994) observed an increase in viability of skin flaps on rats when FGF (20 μg) was injected subcutaneously, compared to controls. They observed a larger increase in vascularity, which would provide an increased blood supply to the area.

The effects of FGF on partial thickness incisional wound models abound. Lynch et al. (1989) investigated the effect of topically applied FGF (2 ng/ml), in a methyl cellulose gel, on epidermal thickness, dermal thickness, dermal cellularity, hydroxyproline content, and epidermal/dermal interface area when injected into partial thickness incisional wounds in pigs. They concluded that FGF increased epidermal thickness and

hydroxyproline content, but had no effect in the other parameters. Hebda et al. (1990), who also topically applied FGF (10 μg), in PBS, onto partial thickness incisional wounds of pigs, showed an increase in re-epithelialization of the epidermis. Breuing et al. (1997) on the other hand observed that when FGF (10 ng/ml), in PBS, was placed into a surface chamber overlying a partial thickness skin incision on pigs, FGF had no effect on re-epithelialization. However, when Breuing et al. evaluated the wound fluid being produced, they did notice that FGF was present in the first day of wounding, but then declined over the preceding days. They hypothesized that FGF may have a role in early wound healing because of its increased presence, but not specifically on re-epithelialization. Cooper et al. (1991) observed the effects of topically applied FGF (600 ng) mixed into a slow release gel on partial thickness meshed skin grafts in mice. FGF caused an absence of regenerated epithelium, and produced an abundant bed of granulation tissue. Mazue et al. (1991) topically applied FGF (500 ng) mixed within a cream onto suction induced blisters (epidermal erosion) on human being's skin. They observed a significant increase in wound healing (decreased healing time) compared to control.

The Effects of Basic Fibroblast Growth Factor on Tendon

Duffy et al. (1995) observed FGF protein expression in uninjured canine tendon. Khan et al. (1996) observed in an in vivo tendon injury model, that FGF protein was expressed in rabbit endotenon cells and may be the only growth factor to produce a preferential effect on endotenon cells (fibroblasts). Endotenon cells being intrinsic fibroblasts may ultimately lead to less scar formation by enhancing type I collagen

presence more abundantly early in wound healing. However, Duffy et al. (1995) did not observe FGF protein expression in a canine healing tendon model. To observe if FGF had any effect on tendon fibroblasts, Banes et al. (1993) exposed avian flexor tendon tenocytes, from 8 week old chickens, to FGF (20 ng) and observed only modest DNA increase (2.2 fold) compared to controls. However, Chan et al. (1997) observed the effects of FGF (50 ng/ml) on patellar tendon fibroblasts of 8 week old rats and noted that FGF caused significant proliferation of these extensor tendon tenocytes in an in vitro wound closure model, but was not chemotactic for them, leading them to hypothesize that FGF may enhance tendon healing by a cell proliferative response, and not by chemotaxis.

Epidermal Growth Factor in Skin Wound Healing

Many studies have confirmed that EGF stimulates cell proliferation, and increases DNA content and collagen content in fibroblasts (Godspdarowicz and Moran, 1975; Gauger et al., 1985; Huey et al., 1980; Walthall and Ham, 1981; Steinmann et al., 1982; Ji et al., 1997), factors important in wound healing (Figure 1). Epidermal growth factor (EGF) is present in wound fluid during healing at concentrations higher than what is measurable in serum. Peak levels of EGF are observed at the beginning of wound healing and taper off to pre-wound control level as healing progresses (Vogt et al., 1998). The presence of epidermal growth factor decreases with age. In mouse skin, EGF is normally localized to fibroblasts, epithelial structures and hair follicles. In the epidermis, EGF is highly concentrated in the basal layer, and sparse in the stratum corneum layers. The concentration of EGF within skin of older animals is diminished compared to younger animals. In wound repair, EGF protein expression is still significantly less in older

animals than young animals, and this decrease contributes to a delay in re-epithelialization and maturity of granulation tissue, as well as a marked decrease in cellular infiltrate (Ashcroft et al., 1997).

Fibroblasts originally secreted the type I collagen which is present in normal dermis. After wounding, type III collagen is initially laid down by the surrounding fibroblasts, with slow but eventual replacement with type I collagen, again through the efforts of fibroblasts. Epidermal growth factor (5 μg/ml) has been shown to decrease the ratio of type III/type I collagen in high-density human dermal fibroblasts in vitro (Steinmann et al., 1982). Collagen type I expression was decreased while type IV collagen expression was increased 3 fold over controls in NIH-3T3 cells treated with EGF (10 ng/ml) (Grande et al., 1997). Epidermal growth factor (10 ng/ml) decreased collagen synthesis of human skin fibroblasts in vitro, but in the presence of ascorbic acid, collagen synthesis increased by 7-10%. Human skin fibroblasts secrete primarily type I collagen (> 90%) with lesser of Type III and IV collagens being produced (< 10%). Type I and III collagens were increased proportionally in adult human skin fibroblast cell culture with 0.1 mM ascorbic acid and 50 ng/ml EGF together (Hata et al., 1988). EGF (1 ng/ml) does not affect the concentration of collagenase (increase synthesis or decrease synthesis) in rat and human skin fibroblast cell culture (Buckley-Sturrock et al., 1989), however EGF (100 ng/ml) has been shown to increase the levels of procollagenases in human foreskin fibroblast cell culture (Chua et al., 1985). The ability to release collagenases would aid in the reorganization of the extracellular matrix after wounding.

Animal Models of Skin Wound Healing Using Epidermal Growth Factor

Numerous studies are available reporting the effects of EGF on dermal wound healing in a variety of animal models.

Subcutaneous implants: Laato et al. (1986) used subcutaneous cellulose sponge implants injected daily with EGF to observe granulation tissue formation in rats. They reported that epidermal growth factor's effects were dose dependent (0.2, 1, and 5 μg/ml), and caused a significant increase in cellularity, and accumulation of collagen and glycosaminoglycans compared to non-EGF controls. Davidson et al. (1988) observed the same granulation tissue response with daily-injected EGF (1 μg) into subcutaneous sponges in rats. Buckley et al. (1985) also observed in rats an increase in granulation tissue formation when subcutaneous polyvinyl alcohol sponge implants were prepared with slow release pellets containing EGF (10 μg) compared to non-EGF controls. They made the observation that EGF accelerates the process of wound repair, specifically neovascularization, organization by fibroblasts, and accumulation of collagen. They postulated that although collagen content increased, this was attributed to the relative increase in fibroblasts. Laato et al. (1987) confirmed this observation using EGF (5 μg) impregnated subcutaneous cellulose sponges in rats.

Incisional models: Niall et al. (1982) observed that sialectomized mice had slower healing times of full thickness skin wounds than control animals. They also noticed that addition of EGF (1 μg/ml/day) to control animals and sialectomized mice significantly decreased wound closure time. They speculated that grooming by mice is important in aiding wound closure, because of the presence of EGF in saliva. Brown et al. (1988) using a skin incision model in rats, observed a 200% increase in tensile strength of the

incision compared to the control. They related this to increased collagen formation and fibroblast proliferation observed microscopically. They also observed that continual application of EGF (10 μg/ml), through slow-release liposomes, is necessary for a proliferative response. Jijon et al. (1989) observed no significant difference in full thickness wound closure between EGF treated and control pigs. Although they dosed twice daily, they used a dose (0.3 μg in saline vehicle) that may not have been high enough to be effective in swine. Franklin and Lynch (1979), using a rabbit ear ulcer model, observed faster orientation of fibroblasts, vascularization, and maturation of dermis compared to controls. In their study, EGF (100μg) was placed in an ointment on the ear daily for upto 42 days.

Partial thickness incisional models: Breuing et al. (1997) showed no significant increase in dermal or epidermal healing in swine when EGF (10 ng/ml) was topically applied. They even showed an increase in healing time, based on microscopic evaluation of epidermal cell proliferation, with high doses of EGF (1000 ng/ml). Lynch et al. (1989) had previously shown no significant changes in wound healing in swine dosed topically with EGF (3.5 ng/ml).

Dermal healing is an important aspect of skin repair, however, epidermal repair is just as important. Reports have shown that EGF administered to partial and full thickness skin wounds significantly enhanced epidermal growth, proliferation and re-epithelialization of wounds (Mustoe et al., 1991[5 μg]; Brown et al., 1989 [10 μg/ml]; Brown et al., 1986 [10 μg/ml]). Andree et al. (1994) also proved this by inserting a gene that expressed EGF into porcine epidermal cells and observed quicker healing times in partial thickness skin wounds of swine.

The Effects of Epidermal Growth Factor on Tendon

Very little research has concentrated on the presence and effects of EGF on the cells of tendons, including parietal synovial fibroblasts, epitenon and endotenon cells. Duffy et al. (1995) observed a rise in levels of EGF immediately after intrasynovial superficial digital flexor tendon wounding in dogs, suggesting that EGF is involved in the initial healing of tendons. Khan et al. (1996) observed that epidermal growth factor receptors (EGFR) were expressed in high numbers immediately after tendon injury on the synovial sheath fibroblasts of rabbits, but not observed in the epitenon or endotenon cells. Khan et al. (1998) confirmed this initial observation by demonstrating a significant mitogenic response of rabbit synovial sheath fibroblasts to EGF (10^{-8} mol/L) in vitro. Gauger et al. (1985) originally observed the effects of EGF on primary avian tendon cell growth. They observed that EGF caused a significant increase in cell number that was concentration dependent (10-300 ng/ml), but had very little effect on collagen synthesis. Franklin et al. (1986) injected EGF (20 μg/kg) twice daily for 15 days into transected achille's tendons of rats and observed a significant increase in cell number, DNA and collagen content. Over time (15 days), the stimulatory effect of EGF subsided, but the increase in collagen content continued.

Summary and Statement of Research Problem

Traumatic tendon injuries, especially of the flexor tendon of the metacarpal and metatarsal regions, are common in horses (Foland et al., 1991; Taylor et al., 1995). Many of these injuries lead to biomechanical impairment, which for the most part eliminates a large percentage of these horses from the show arena and race track (Taylor et al., 1997).

This impairment occurs in part because of the complications of scarring, based on fibroblastic responses that occur within the tendon (Gelberman et al., 1987). The ability to enhance mechanical strength of equine tendons after wounding is critical for return of the horse to the show circuit and race track.

Wound healing of the dermis and tendon consists of the coordinated effort between fibroblasts, endothelial cells, and inflammatory cells. During wound healing, fibroblasts proliferate, migrate to the wound edges and into the wound gap, and synthesize and secrete collagen in an attempt to re-create the collagen makeup and orientation in the normal dermis and tendon. The biomechanical strength of the dermis and tendon are dependent on the deposition and organization of collagen type I into the wound. Collagen type III deposition, which normally occurs during wound repair, composes the primary scar formed. Collagen type III is inferior in strength to collagen type I and thus can lead to improper wound healing (increased chance for re-injury). The ability to manipulate fibroblasts within wounds so as to enhance the deposition of collagen type I into the wound gap, would facilitate greater biomechanical strength.

One potential way to enhance these processes is through the use of exogenous growth factors, in particular, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). It has been shown in the tendon and dermis of other species, that EGF and bFGF can enhance fibroblastic activity, including proliferation and migration of fibroblasts and stimulate synthesis of collagen type I in numerous tissues (Gospodarowicz and Moran, 1975; Paris and Pouyssegur, 1991; Chan et al., 1997; Blay and Brown, 1985). All these processes are necessary for proper wound healing (Veomett et al., 1989; Holley and Kiernan, 1968). Little is known about the effects of growth

factors on equine tendons and skin. To my knowledge, only two articles have been published on the effects of growth factors on equine tendon healing (Murphy and Nixon, 1997; Jann et al., 1999). Murphy and Nixon (1997) observed significantly increased levels of hydroxyproline content and enhanced proliferation of equine superficial digital flexor tendon explant fibroblasts when exposed to IGF-I. Jann et al. (1999) concluded that EGF has a stimulatory effect on tenoblast migration along suture material in vitro. They also observed that IGF was stimulatory at low concentrations, and inhibitory at high concentrations, to epitenon fibroblast migration on suture material in vitro. Studies investigating the effects of growth factors on equine skin are limited to one report which demonstrated mitogenic activity of EGF, but not FGF, on equine dermal fibroblasts (Cochrane, 1996). Understanding the effects of EGF and bFGF on equine tendon fibroblast and dermal fibroblast populations, especially in the same topographical region, may provide a new strategy to improve healing of these tissues, based on results seen in other species.

Therefore, my hypothesis is that EGF or FGF can enhance proliferation, migration and type I collagen synthesis, while suppressing type III collagen synthesis, in a dose-dependent fashion, as seen in other animal models, and contribute to wound healing in these tissues. My objectives for this study are to observe the in vitro effects of epidermal growth factor or basic fibroblast growth factor on proliferation, migration, and collagen type I and type III synthesis of equine tendon and dermal fibroblasts. The fibroblasts used in this study are from the superficial digital flexor tendon and overlying skin of the mid-metatarsal region of the hind limbs.

Figure 1.

The Elaboration and Participation of FGF and EGF in Wound Healing

Inflammation

- Platelets degranulate and release FGF and EGF (Brunner et al., 1992; Kurobe et al., 1986)
- Proteases release FGF from ECM (Benezra et al., 1993)
- FGF binds to fibrin being laid down (Sahni et al., 1998)
- Neutrophils trigger release of FGF by endothelial cells (Totani et al., 1994)
- T-lymphocytes and macrophages secrete FGF (Blotnick et al., 1994; Sprugel et al., 1987)

Repair

- Fibroblasts release FGF (Rudolph, 1979)
- FGF and EGF enhance fibroblast proliferation (Bikfalvi et al., 1997; Gospodarowicz and Moran, 1975)
- FGF enhances angiogenesis (Slavin, 1995)
- FGF and EGF enhance re-epithelialization of skin (Hebda et al., 1990; Mustoe et al., 1991)
- FGF induces activation of ECM proteases to facilitate migration of new blood vessels (Mignatti et al, 1989; Montesano et al., 1986; Flaumenhaft et al., 1992)

Remodeling

- FGF enhances fibroblast migration, proliferation and collagen synthesis (Brew et al., 1995)
- FGF and EGF induce activation of ECM proteases to aid in remodeling of ECM (Davidson and Broadley, 1991; Flaumenhaft et al., 1992; Chua et al., 1985)
- EGF decreases the ratio of collagen type III/I secretion by dermal fibroblasts (Steinmann et al., 1982)

CHAPTER II

EXTRACTION AND ISOLATION OF FIBROBLASTS FROM EQUINE SUPERFICIAL DIGITAL FLEXOR TENDON AND OVERLYING SKIN

Introduction

Trauma to equine tendons and skin of the extremities is common among performance horses (Taylor et al., 1995; Taylor et al., 1997; Bertone, 1989; Bertone, 1995). Delayed or improper healing of these structures can lead to loss of revenue for the owner, and potential euthanasia of the animal. One of the primary cells involved in wound healing is the fibroblast. The ability to understand the mechanisms in which fibroblasts participate in wound healing greatly enhances the chances of manipulating them to positively influence wound healing. Growth factors have been shown to enhance fibroblast activities in tendon and skin wound healing models in other species (Evans, 1999; Brew et al., 1995; Bikfalvi et al., 1997; Bennett and Schultz, 1993), but very little has been done in the horse (Jann et al., 1999; Murphy and Nixon, 1997;Cochrane et al., 1996). Although extensive data may have been obtained for a specific growth factor (ex. epidermal growth factor) in certain animals (ex. pigs, mice), that does not guarantee that the same effects will occur in all species. Preliminary studies are in order to define the effects of epidermal growth factor and basic fibroblast growth factor, on equine tendon and dermal fibroblasts. In vitro experiments can provide an environment in which to perform these preliminary studies.

In some species, studies utilizing growth factors in vivo are feasible based on the dose levels required, availability of recombinant growth factor, and cost of the animal models (i.e. mice, rats). However, there are numerous species (humans, primates, horses, large ruminants, exotics, zoo animals) in which preliminary effects of growth factors in vivo are not feasible. In order to identify some of the potential positive or negative biological effects, an in vitro system is often utilized to evaluate diverse cell populations.

This greatly reduces cost and animal numbers needed to identify potential clinically applicable data. Today, hundreds of cell lines from numerous species are available commercially for some preliminary studies (i.e. American Tissue Culture Collection), however, not every cell line is available for all species, especially the horse.

My goal was to establish superficial digital flexor tendon and dermal fibroblasts cell lines from the mid-metatarsal region of horses.

Materials and Methods

Tissue Preparation

Tissues from 11 horses between the ages of 3 years and 20 years were utilized (Table 1). Each of these animals were euthanatized for reasons unrelated to this project, and free of diseases or disorders that may have affected musculoskeletal or dermal tissues used. The plantar skin of the right hind limb was shaved from the tarsal-metatarsal joint to the fetlock joint and scrubbed using sterile technique. The skin was elevated, subcutaneous tissue was bluntly dissected away, a piece of skin removed, and placed in Hank's balanced salt solution (HBSS). Next, the paratenon was sharply dissected away, and a tendon segment was excised (approximately 6-8 cm) and placed in HBSS. Under a biological safety hood, both tissue samples were rinsed separately twice in HBSS supplemented with antibiotics (6 mg gentamicin, 500 μg amphotericin B, 10,000 units penicillin, and 10 mg streptomycin per 100 ml HBSS). The skin was cut into 2-4 mm segments with a scalpel, and placed into a sterile 250 ml Erlenmeyer flask. The tendon segment was cut into small strips (approximately 5 mm x 3 mm x 2 mm) with the use of a scalpel and placed into a separate sterile 250 ml Erlenmeyer flask.

Digestion

A collagenase solution was prepared using 100 mg collagenase type IA (Sigma, St. Louis, MO) dissolved in 100 ml HBSS supplemented with antibiotics as described above. To this solution was added 5 ml fetal bovine serum (FBS). Into each Erhlenmeyer flask containing tissue was placed 200 ml of collagenase solution. A sterile magnet stir rod was placed into each flask. The flasks were each placed separately into a 37°C water bath with an underlying magnetic stir plate and tissues digested for a total of 10-16 hours.

At every 3-4 hour interval of digestion, the collagenase solution from each flask was transferred into separate sterile centrifuge tubes. After the flasks were empty, 10 ml of HBSS supplemented with antibiotics was added to each flask, swirled, and also transferred into the respective centrifuge tubes. This rinse step was performed to remove cells partially adhered to the walls of the flask. The tubes were centrifuged for 5 minutes at 210 x g and 4 °C. The supernatant from the centrifuge tubes was poured back into the appropriate flasks and returned to the water baths for continued digestion. Approximately 25% of the total tissue was digested at each 3-4 hour digestion period. The pellet (cell and collagen fragments) was re-suspended in growth media. Growth media was composed of 100 ml Dulbecco's modified eagles medium nutrient mixture F-12 Ham, 3 mg gentamicin, 250 μg amphotericin B, 10,000 units penicillin, 10 mg streptomycin (Antimycotic solution[Sigma, St. Louis, MO]), 15.5 ml of FBS (Sigma) and 1 ml of ITS+3 (insulin, transferrin, selenium [Sigma]). The tube was recentrifuged as above, supernatant removed and pellets re-suspended in growth media. This rinse step was used to remove any residual collagenase. The suspension was then transferred to 25 cm^2 culture flasks which were placed into a humidified incubator at 5% CO_2 and 37°C.

The flasks were incubated for 3 days, after which the media was replaced with fresh media, then observed for cell attachment and/or growth.

Cell Culture

Once cells were confluent in a flask, they were rinsed with trypsin + EDTA, suspended into growth media and split into two separate flasks for continued growth. Cells obtained from sequential digestions of each tissue were pooled to establish primary cell lines.

Epithelial Cell Contamination

Occasionally epithelial cells were harvested during the digestion process of the skin. These epithelial cells grew into individual islands surrounded by fibroblasts. To remove these epithelial "contaminants", dispase (protease [Sigma]) was added to tissue culture flasks (Paraskeva et al., 1984). The dispase was reconstituted in DMEM + Ham's F-12 media and placed onto the monolayer for 10 minutes at 37°C at a concentration of 2 units/ml. If epithelial cells remained after this time, the dispase was removed and fresh dispase was placed into the flask and incubated for an additional 5 minutes at 37°C and 5% CO_2. After the incubation, the dispase was removed and growth media was added.

Fibroblast Determination

Immunocytochemical stains utilizing antibodies against vimentin (mesenchymal cells), cytokeratin (epithelial cells), and factor VIII related antigen [von Willebrand factor] (endothelial cells) were used to identify the isolated cells as fibroblasts. For each

of the cell lines, $1x10^4$ cells were suspended in growth media were placed into one chamber of a 4-chamber slide (Lab-Tek, Naperville, Ill.). The slides were incubated at 37°C and 5% CO_2 for 5-10 days until a semi-confluent monolayer was formed. The media was removed and the cells were fixed for 10 minutes by filling the chambers with 95% ethanol. The ethanol was then poured out, the chamber walls and rubber seal removed, and the slides air dried at room temperature. The slides were then placed into a DAKO® auto-immunostainer (Dako, Carpinteria, CA). The programmed procedure was as follows: 3% hydrogen peroxide was applied to each slide for 10 minutes and then washed with phosphate buffered saline (PBS). 10% FBS serum was then applied for 20 minutes and then removed by airflow. Antibodies to either factor VIII-related antigen (von Willebrand's factor), cytokeratin, or vimentin were then applied for 30 minutes, and slides rinsed with PBS. A biotinylated secondary antibody (anti-IgG) was applied for 20 minutes and slides rinsed with PBS. Horseradish peroxidase-streptavidin conjugate was applied for 20 minutes followed by rinsing with PBS. 3,3'-diaminobenzidine tetrahydrochloride (DAB) was then applied for 5 minutes and slides rinsed with PBS. A counterstain of Mayer's hematoxylin was then applied for 2 minutes, followed by rinsing with distilled water, and the slide removed from the auto-stainer. A dehydration process encompassing two - 1 minute soaks in 95 % ethanol, and two - 1 minute soaks in 100% ethanol were performed. The slides were then cleared with toluene which was performed 3 times at 2 minutes each time. Resin was then placed on the slide and a cover slip was placed. Microscope slides containing multiple paraffin embedded equine tissues cut at 5 μm thickness were stained as described above and used as positive (with antibody) and negative (without antibody) controls.

Cell Count

Cell counts were performed for all experiments. After cells were trypsinized and re-suspended in growth media, a 100μl sample of cell suspension was added to 900μl of 0.25 % trypan blue in PBS. A small volume of this was added to a hemocytometer and unstained cells (no blue coloration) were counted in five bold chambers (Figure 2). This procedure was performed four times, each from a different sample from the cell suspension solution, and the average count was used. The count was then multiplied by 20,000 to give the number of cells/ml. The rationale for the cell count is as follows: The volume under all five squares is 0.5 μl. The dilution factor with trypan blue is 1:10. Therefore, the true volume of cells is 0.05 μl. To determine the numbers of cells/ ml (cells/volume), the average cell count is multiplied by [(1/.05) x 1000)] = 20,000. The 1000 is obtained by converting μl to ml.

Results

Of the eleven horses in which tendon and dermal fibroblast harvesting was attempted, five horses representing three cell lines each of equine superficial digital flexor tendon fibroblasts and distal extremity dermal fibroblasts were obtained for use in this study (Table 1). The remaining cell lines were either not established (no cell growth after digestion), became contaminated with bacteria or fungi, or had altered phenotypic expression after a few cell splits.

The cultured cells were spindle shaped with two or more cytoplasmic projections. However, isolated islands of epithelial cells characterized by polyhedral shape and

intercellular bridges between cells were observed amongst the dermal fibroblasts. Exposure of these cells to dispase caused loosening and dissociation of the cells, as one unit, from the flask without affecting fibroblast adherence. A simple rinse of the cells with media removed any and all observable epithelial cells from the fibroblast culture.

Fibroblasts grew primarily in isolated aggregates with eventual convergence of aggregates. To determine that these were indeed fibroblasts, immunocytochemical stains were utilized. All cell lines (tendon and dermal fibroblasts) stained positive for vimentin (mesenchymal cells) and negative for both cytokeratin (epithelial cells) and factor VIII related antigen (endothelial cells). (Figure 3**).** Positive control tissues stained appropriately for each of the antibodies used. Background staining was not observed with any of the negative control tissues. Based on spindle-shaped morphology, staining characteristics, and tissue harvested, dermal and intrinsic tendon fibroblasts were determined to be the primary cell harvested. Other cell types such as adipocytes, myocytes (erector pili muscle), neurons (ganglia), neural crest origin cells, epidermal cells (skin), endothelial cells and pericytes, were excluded based on their morphology, growth characteristics, immunostaining patterns, and low numbers normally present in the skin and tendon.

Discussion

The ability to isolate cells of interest from fresh tissue has become a routine practice performed in many laboratories around the world. However, only within the past two decades have the techniques to specifically identify many cell types come about through advances in immunocytochemistry and *in situ* hybridization. Light and electron

microscopy can be used to determine morphological characteristics of many cell lines, but immunocytochemistry can add a new dimension. The cells isolated in this project were determined to be fibroblasts by exclusion of other cells through cell morphology, probability (normal numbers of cells present within the tendon or skin), and immunocytochemical staining. Banes et al (1988) isolated two populations of cells, synovial cells and intrinsic tendon fibroblasts, from the sheathed portion of the superficial digital flexor tendon of chickens based on morphologic features. However, definitive determination of either fibroblast population, through use of exclusion techniques, was not performed. In this study, immunocytochemical stains specific for epithelial cell intermediate filaments (cytokeratin), mesenchymal cell intermediate filaments (vimentin) such as fibroblasts, and factor VIII-related antigen, specific granules found in endothelial cells, along with morphological characteristics were utilized to determine fibroblasts were the primary cells harvested.

Although single cell cloning by limiting dilution technique is an option after cell isolation, it was deemed not to be necessary for this project. The dermis is separated into two parts, the papillary dermis and reticular dermis, in all mammalian species, including horses. In each of these areas a variation in morphology and growth characteristics has been observed in humans (Novotny and Gnoth, 1991; Harper and Grove, 1979). The tendon fibroblasts in this study also are a combination of cells that include tenocytes (primary internal fibroblasts) and endotenon fibroblasts. Cloning would eliminate this diversity normally seen in the tendon and dermis. The goal of this study was to look at the effects of epidermal growth factor or basic fibroblast growth factor on four biologic parameters of isolated tendon and dermal fibroblasts relevant to in vivo wound healing.

(The possibility of using the results obtained in these experiments, for in vivo experiments, would be unduly confusing if the use of clonal cultures was implemented because of the numbers of different cells involved).

Two fibroblast extraction procedures, explant and collagenase digestion, are commonly used to establish tendon and skin cell lines. The explant procedure appears to be more commonly used (Becker et al., 1991; Abrahamsson et al., 1989; Evans and Trail, 1998; Birch et al., 1997; Agren et al., 1999; He et al, 1999; Belford, 1997) than the digestion procedure (Banes et al., 1988; Gauger et al., 1985; Shipley et al., 1989). The digestion procedure was performed for all procedures in this study because of previously successful use in the laboratory.

There are benefits and potential problems with both of the procedures. In explants, the cells of the tissue touching the flask are those that migrate out and eventually proliferate, thus small numbers of fibroblasts are obtained and used to represent the whole tissue. Deeper cells cannot get out of the matrix. On the other hand, digestion releases many more fibroblasts from all areas of the tissue. But in the process of digestion, death of many cells occurs as well as the potential for a change in the cell's phenotype or metabolic processes due to prolonged exposure to a harsh proteolytic environment. Unfortunately, literature was not found that addresses these differences, and potential problems associated with each of them. Future studies may evaluate morphologic and growth characteristics of fibroblasts obtained by collagenase digestion compared to tissue block explants.

Cell Line	Horse Breed	Age (years)	Gender
Skin # 1	Quarter Horse	6	Male Intact
Skin # 2*	Quarter Horse	8	Female Intact
Skin # 3	Thoroughbred	5	Female Intact
Tendon # 1	Thoroughbred	6-8	Male Castrated
Tendon # 2	Mixed	11	Female Intact
Tendon # 3*	Quarter Horse	8	Female Intact
NA	Shetland pony mix	7-10	Male Intact
NA	Shetland pony mix	20+	Female Intact
NA	Thoroughbred	6	Male Castrated
NA	Quarter Horse	3.5	Female Intact
NA	Thoroughbred	3	Male Castrated
NA	Thoroughbred	3	Male Castrated

Table 1. Breed, age and gender information on all horses from which tendon and dermal fibroblast harvest was attempted. All horses with a cell line name were utilized in the projects. All horses with NA under cell line column were not used in the projects. A horse in which two cell lines were harvested is denoted with a * . NA = not available.

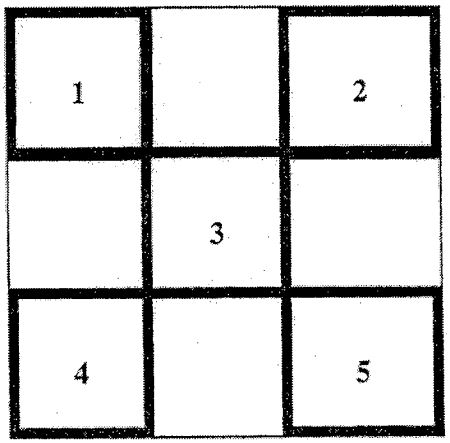

Figure 2. A schematic of chambers seen on a hemocytometer. The bold faced squares (chambers) are those in which cells were counted at 100 x magnification. Cell count was the total for all bold faced squares.

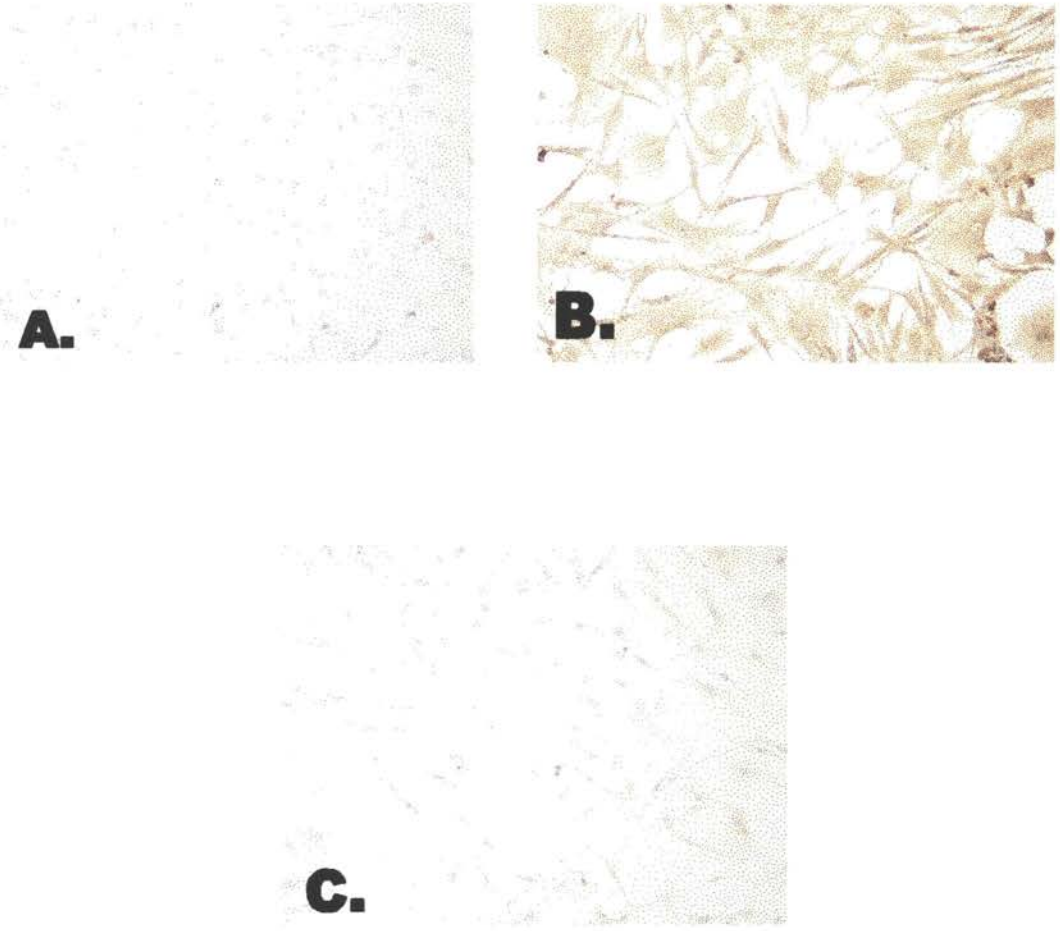

Figure 3. Immunocytochemical staining of cultured fibroblasts for determination of cell types. A. Cytokeratin stain for identification of epithelial cells. B. Vimentin stain to identify mesenchymal cells. C. Factor VIII-related antigen stain to identify endothelial cells. Note positive brown staining of vimentin stain only. Peroxidase-labeled streptavidin-biotin method, Mayer's hematoxylin counterstain. 100 x.

CHAPTER III

THE MITOGENIC EFFECTS OF BASIC FIBROBLAST GROWTH FACTOR OR EPIDERMAL GROWTH FACTOR ON EQUINE TENDON AND DERMAL FIBROBLASTS

Introduction

The processes involved in tendon and dermal wound healing (i.e. inflammation, tissue formation, remodeling) are quite similar. After the initial extravasation of blood cells (red blood cells and white blood cells) and formation of a clot, one of the primary events to occur next involves the proliferation of adjacent cells including fibroblasts, endothelial cells and epithelial cells. The proliferative phase is necessary to replace those cells irreversibly damaged or lost during wounding. Fibroblasts are essential for wound healing because of their ability to bridge the wound gap through proliferative and migratory efforts.

Although the basic mechanisms of wound healing are well documented, more information is needed to understand variations within each individual. In horses, dermal wounds on extremities have slower healing rates than on other parts of the body (i.e. trunk, abdomen) (Jacobs et al., 1984; Britton 1970). The reasons for this are not clear but may involve complications with exuberant granulation tissue or sarcoid formation (Cochrane et al., 1996), wound infection (Jacobs et al., 1984), poor blood supply, lesser retraction of the wound edges on extremities (Jacobs et al, 1984; Walton and Neal, 1972) due to tension of the skin, a delay of myofibroblasts infiltrating into the wound (Swaim 1980), or to the effects of the cutaneous musculature subjacent to the body wounds causing early reduction in wound size (Kennedy and Cliff 1979). In vitro experiments utilizing skin from 22 horses demonstrated a slower growth rate of equine extremity dermal fibroblasts than trunk dermal fibroblasts (Miller et al., 2000) which may partially explain the delay in wound healing.

During tendon wound healing, complications arise when paratenon fibroblasts proliferate and migrate to the wound gap, in effect trying to repair a tissue that was originally deposited by intrinsic fibroblasts (tenocytes) (Garner et al., 1989). The intrinsic fibroblast population (tenocytes and endotenon cells) do not participate in wound healing until many weeks after initiation of healing (Siddiqi et al., 1992).

Numerous mitogenic growth factors, including basic fibroblast growth factor, platelet derived growth factor, transforming growth factor-β, and epidermal growth factor, are present during the initial phases of wound healing. The concentration of these individual growth factors within wounds may help enhance, to some degree, the healing process. Fibroblast growth factor and epidermal growth factor have been shown to be mitogenic for dermal fibroblasts from several rodent species and human beings (Baird et al., 1986; Bikfalvi et al., 1997; Brew et al., 1995; Carpenter and Cohen, 1976; Davidson et al., 1988; Godspodarowicz, 1978). However, their mitogenic effects on equine dermal fibroblasts is limited to one study in which EGF was mitogenic after 20 days in culture, and FGF was not mitogenic (Cochrane et al., 1996). Both of these growth factors have been shown to be less effective inducing proliferation of extensor tendon fibroblasts (Chan et al., 1997), with no known studies on equine tendon fibroblasts.

The objective of this study is to determine the mitogenic potential of epidermal growth factor or basic fibroblast growth factor on equine superficial digital flexor tendon fibroblasts and adjacent dermal fibroblasts. The ability to enhance/modify mitogenesis of these specific fibroblast populations, through the use of growth factors, may provide a method to decrease healing time in the equine athlete.

Materials and Methods

Mitogenesis assay

The following assay was performed individually on each of three dermal and three tendon fibroblast lines (Table 1 – Chapter II). Using 96-well flat bottom sterile plates (Corning-Costar, Corning, NY), human recombinant EGF (Sigma, St. Louis, MO) and 18 KDa human recombinant basic FGF (Sigma), each at concentrations of 0 ng/ml (control), 2 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml in modified growth media containing 3% FBS, were separately applied to 1 x 10^4 cells/ well in triplicate. Each of these triplicate assays was performed at least twice for a total of at least six results per growth factor concentration. After incubation for 3 days at 37°C at 5% CO_2, cell numbers were determined with the use of CyQuant®, a semi-quantitative fluorescence nucleic acid assay (Molecular Probes, Eugene, OR). Plates were read on Cytofluor® 2300 Fluorescence Measurement System (Millipore, Bedford, MA) at an excitation wavelength of 480 nm and emission wavelength of 520 nm.

A second plate (standard curve) for each of the fibroblast lines was made using cell densities of 1 x 10^4 to 3 x 10^4 cells/well (in 10,000 cell increments), in quadruplicate, in modified growth media containing 3% FBS. These plates were incubated at 37°C and 5% CO_2 for 8 hours and evaluated using CyQuant® as described above. Linear regression was utilized on the optical density readings obtained from this plate to derive a cell number standard curve for each cell line. The optical density readings from each well in the growth factor concentration groups were converted to cell numbers using the standard curve. Cell numbers from each growth factor concentration group were pooled and compared to the 0 ng/ml treatment (control) group using two-tailed, paired T-test

with a $p < 0.05$ being considered significant. Dose-dependent responses were determined using linear regression on the pooled samples of all three horses with a $p<0.05$ being considered significant.

Results

The number of cells available during each experiment varied. This occurred because cell lines were not confluent monolayers, or contained clumped cells after trypsinization decreasing the number of individual cells available for use. This variation in cell number affected the number of replicates performed. At least 2 triplicate runs were performed per cell line per growth factor concentration. Replicated runs were quite reproducible with similar results occurring on different runs performed on different days.

Mitogenic Effects of EGF on Equine Tendon Fibroblasts

Only one concentration of EGF for 1 cell line (tendon cell line # 2) was significantly mitogenic compared to control values (Figure 4). Tendon cell line #1 (ten #1) ($n = 6$) control had a mean of 13972 ± 2663 cells compared to the highest of 14294 ± 2648 cells for 5 ng/ml ($p = 0.28$). Tendon cell line #2 (ten #2) ($n = 9$) had a control mean of 11822 ± 855 cells and a high of 12992 ± 807 cells for 20 ng/ml ($p = 0.002$). Tendon cell line #3 ($n = 10$) had a control mean of 16613 ± 991 which was the highest and a low of 16107 ± 1053 cells for 2 ng/ml ($p = 0.23$). No significant dose-dependent response was seen in any cell line.

Mitogenic Effects of FGF on Equine Tendon Fibroblasts

No concentration of FGF was significantly mitogenic for any of the tendon cell lines compared to control values (Figure 5). Ten # 1($n = 6$) control mean was 11885 ±1391

cells with a high of 12597 ± 2209 cells at 10 ng/ml (p = 0.56). Ten # 2 (n = 8) control mean was 15729 ± 1170 cells and a high of 15932 ± 989 cells at 2 ng/ml (p = 0.24). Ten # 3 (n = 10) control mean was 15947 ± 1107 cells with a high of 16386 ± 754 cells at 10 ng/ml (p = 0.30). (Figure 5). No significant dose-dependent response was seen in any cell line.

Mitogenic Effects of EGF on Equine Dermal Fibroblasts

No concentration of EGF was significantly mitogenic for any of the skin cell lines compared to control values (Figure 6). Skin cell line # 1 (skin #1) (n = 10) control mean was 13314 ± 1951 cells which was the highest and 12819 ± 2131 cells for 2 ng/ml (p = 0.15) being the lowest. Skin cell line # 2 (skin #2) (n = 11) had a control mean of 10351 ± 653 cells and a high of 10927 ±1098 cells for 10 ng/ml (p = 0.24). Skin cell line # 3 (skin #3) (n = 6) had a control mean of 11562 ± 928 cells and a high of 11802 ± 1596 cells for 2 ng/ml (p = 0.52). No significant dose-dependent response was seen in any cell line.

Mitogenic Effects of FGF on Equine Dermal Fibroblasts

No concentration of FGF was significantly mitogenic for any of the skin cell lines compared to control values (Figure 7). Skin # 1 (n = 10) mean control was 12679 ± 1838 cells with a high of 12708 ± 1211 cells for 20 ng/ml (p = 0.96). Skin #2 (n = 11) control mean was 11299 ± 846 cells as a high with a low of 10705 ± 761 cells at 2 ng/ml (p = 0.11). Skin # 3 (n = 6) control mean was 11659 ± 1644 cells with a high of 11910 ± 1150

cells at 2 ng/ml ($p = 0.72$). No significant dose-dependent response was seen in any cell line.

Discussion

Once a tissue is wounded, numerous cells within the wound plane are destroyed. In order for them to be replaced, proliferation of cells within surrounding tissues is necessary. If these cells do not proliferate, wound healing may be prolonged. Epidermal and fibroblast growth factors have been shown to enhance the mitogenic response of numerous cell types in vivo and in vitro therefore enhancing wound healing (Martin et al., 1992; Bennett and Schultz, 1993a; Bennet and Schultz, 1993b; Rudkin and Miller, 1996). However in this in vitro study, EGF and FGF did not enhance proliferation of dermal fibroblasts at the dose levels utilized. There was a significant increase in cell number for one tendon cell line (ten #2) at the highest concentration of EGF (20 ng/ml) (Figure 4). This effect was not seen in the two other tendon cell lines.

The concentration of growth factors utilized in this project were extracted from published *in vitro* experiments on rats (Chan et al. 1997), and have been shown to be mitogenic for a variety of cell lines from several rodent species and human beings (Baird et al., 1986; Bikfalvi et al., 1997; Brew et al., 1995; Carpenter and Cohen, 1976; Davidson et al., 1988; Godspodarowicz, 1978). The highest concentration (20 ng/ml) of both growth factors (EGF, FGF) utilized in this project may not have been high enough to stimulate mitogenesis in these equine cell lines, except for ten #2 where 20 ng/ml was the only concentration at which significance was seen (Figure 4). Chan et al. (1997) showed a 4 fold increase in proliferation of juvenile rat patellar tendon fibroblasts after exposing them to 2 ng/ml of FGF for 24 hours. Their technique involved an in vitro wound closure

model. Hata et al. (1988) observed a greater than 50% increase in mitogenesis, after a 5 day incubation period, when they added 2 ng/ml of EGF to adult human dermal fibroblasts. Kang and Kang (1999) recently demonstrated a 3 fold increase in proliferation of rabbit flexor tendon cells, using 100 ng/ml of EGF and an incubation of 6 days.

Duration of exposure of cells to growth factors is also an important aspect in enhancing wound healing. The cell lines used were exposed to a single dose of growth factor and incubated for three days. This may have not been enough exposure to enhance mitogenesis. Hata et al. (1988) observed a 20-25% increase in proliferation of human dermal fibroblasts exposed to EGF (2 ng/ml) after 3 days of incubation, but observed twice that increase after 5 days. Shipley et al. (1989) exposed human embryonic lung fibroblasts in vitro to both EGF (20 μg/ml) and FGF (50 μg/ml) for seven days with additions of new media and fresh growth factor every other day and observed significant proliferative responses compared to media controls. Perhaps if the media in my experiment was replaced on the second day of experimentation with fresh growth factor, or higher concentrations of growth factors were used, more of the equine cell lines would have been responsive.

The addition of 3% FBS may mask the effects of growth factors. Growth factors as well as other mitogenic compounds are known to be present in serum. However, the concentration of these factors in serum is not known. Therefore these serum factors may be maximally enhancing proliferation of the equine tendon and dermal fibroblasts so addition of EGF or FGF alone has no effect. However, Carpenter and Cohen (1976) showed significant proliferative activity of EGF (2 ng/ml) + 10% FCS (fetal calf serum)

on human foreskin fibroblasts compared to 10 % fetal calf serum alone after six days of incubation. Nishiyama et al. (1991) showed similar significant mitogenic results using EGF (50 ng/ml) or FGF (100 ng/ml) in 10% fetal bovine serum on human dermal fibroblasts incubated for 5 days. Therefore, the use of 3 % FBS in these experiments should not have masked the effects of mitogenesis.

The majority of proliferation/mitogenic experiments utilize rodent species. Although both EGF and FGF, as well as their receptors, are for the most part homologous among species (~ 90%), differences in their proliferative activity across species may be anticipated. Rabbit tendon and dermal fibroblasts have been shown to proliferate more readily than their human counterparts in the same in vitro conditions (Evans and Trail, 1998). Thus, the horse cell lines used in this study may not be as sensitive or responsive to growth factors as other species used in previous mitogenic studies. An important hypothesis to test before considering clinical application

Gender differences may have played a role. Estrogen treatment in post-menopausal women have been shown to increase rate of skin wound healing and increase levels of TGF-β, a growth factor known to be beneficial in wound healing (Bello and Phillips, 1999). In intact male rats, testosterone has been shown to enhance the effects of EGF in enhancing skin wound healing (Niall et al., 1982). Although both sexes were used in this study, preliminary observation of the mitogenic results does not demonstrate a gender difference. However, this factor was not reviewed by statistical analysis because of the small number of animals used. And although gender differences were not the scope of this project, it is interesting to note that the intact male dermal fibroblasts appeared to have a higher basal metabolic rate compared to the females. After careful evaluation of

Figure 6 and 7, one can see that the male (skin #1) proliferative rates were higher for all dosage groups, including control, compared to the two females (skin #2 and #3). A factor that may need to taken into consideration in future projects.

Age plays an important role as well in matters of wound healing. In older individuals, fewer numbers of cells are seen in normal tissue (Novotny and Gnoth, 1991), healing is delayed, even in the presence of known mitogenic growth factors (EGF, FGF, PDGF-AA) (Agren et al., 1999), and the normal expression of growth factors (FGF, VEGF [vascular endothelial growth factor]) seen in early wound healing is decreased as well (Swift et al., 1999). Interestingly enough, the tendon cell line that demonstrated a stronger mitogenic response to EGF compared to control was the oldest horse used (11 years [youngest = 5 years]). However, once again the biological significance of this is questionable due to animal number.

Differences among animals of the same species also exist. Dermal fibroblasts from ponies demonstrated proliferative activity (tritiated thymidine incorporation) quicker than horse dermal fibroblasts in the same in vitro basal media conditions (Miller et al., 2000), and ponies also have a faster second intention healing response than horses in vivo (Wilmink et al., 1999a; Wilmink et al., 1999b). Five of six horses evaluated were Quarter horses or Thoroughbreds. No differences were observed between these horse breeds in this project. However, ten #2 which demonstrated the only significant response to EGF came from a hybrid horse. Unfortunately, the exact breed cross that the horse represents is not known. The significance of this is unknown, but may relate to the genetic makeup of this horse, which may affect the way in which its fibroblasts respond to growth factors.

Regional variations within the same animal exist. Normal in vitro growth patterns between dermal fibroblasts of the extremities of horses and those of the trunk and abdomen differ (Miller et al., 2000; Jacobs et al., 1984). Differences in in vitro growth of rabbit ligament fibroblasts from different ligaments (i.e. medial collateral and cranial cruciate) are also observed (Schmidt et al., 1995). In vivo, flexor tendons also heal poorly and much more slowly than extensor tendons in (Evans and Trail, 1998). Chan et al. (1997) showed significant proliferative responses when rat patellar tendon fibroblasts were exposed to FGF (2 ng/ml) in vitro. However, in this study, equine flexor tendon fibroblasts were used, and showed no significant response. No comparisons were done in this project for regional variations between differing dermal fibroblast locales and different tendons to evaluate this aspect closer.

Regional variations within the same tissue also exist. Abrahamsson et al.(1989), showed that there were variations in proliferative rate and matrix synthesis among fibroblasts isolated from different regions of the same sheathed tendon (superficial digital flexor) of rabbits. Novotny and Gnoth (1991) and Harper and Grove (1979) showed a difference in morphology as well as differences in growth potential, in vitro, between papillary dermis and reticular dermis from the same area of human skin. Regional variations within the same tissues were not compared in this study. The dermal fibroblasts were a mixture of both papillary and reticular fibroblasts. Separation procedures were not undertaken to differentiate the two. The flexor tendon was removed from the same leg , and within the same region, on each of the animals evaluated.

Assay validation and interpretation are very important aspects in making decisions on the value of results obtained from experimentation. Unfortunately, positive controls

were not used to validate the sensitivity of the proliferation assay performed. By utilizing IGF-I, which has been shown to enhance the proliferative activity of equine flexor tendon intrinsic fibroblasts (Murphy and Nixon, 1997), I might have been able to determine if the CyQuant assay was measuring proliferation effectively. Another problem encountered was the semi-quantitative nature of the CyQuant assay. The results were based on the amount of nucleic acid bound by the fluorescent in the kit. Nucleic acids are represented in DNA (proliferation) as well as RNA (protein synthesis). Since, growth factors may also alter mRNA expression of other proteins such as collagen type I and III, this may affect the results of the proliferation assay. Therefore the results obtained in the proliferation assay may be overshadowed by increased mRNA expression, or under-represented by down-regulation of mRNA expression. Other procedures that might have been used and that are common for proliferation assays include tritiated thymidine incorporation or bromo-deoxyuridine incorporation. Both of these assays are specific for DNA.

Determination of significance can be evaluated on statistical grounds or biological relevance grounds. Although the proliferative effects of 20 ng/ml EGF on ten #2 was determined to be statistically significant, the biological relevance appears to be minimal, since less than a 1.2 fold increase in proliferation was observed. Most other reports demonstrated a minimum of 2 fold or greater proliferative response as being statistically significant, and hence having a greater chance of being biologically significant (Miller et al., 2000; Cochrane et al., 1996; Chan et al., 1997).

All the factors mentioned above are important considerations when comparing and interpreting results between equine tendon and dermal fibroblasts, with those of other projects utilizing similar cell lines and growth factors.

In conclusion, no mitogenic results were observed for the cell lines used when exposed to five concentrations of EGF or FGF. Placed into the context of equine distal limb, neither EGF or FGF would be expected to significantly enhance the proliferation of fibroblasts involved in wound healing. Re-evaluation of the proliferative effects of these growth factors on other equine cells lines, using higher dose levels or longer exposure times may clarify this apparent species difference.

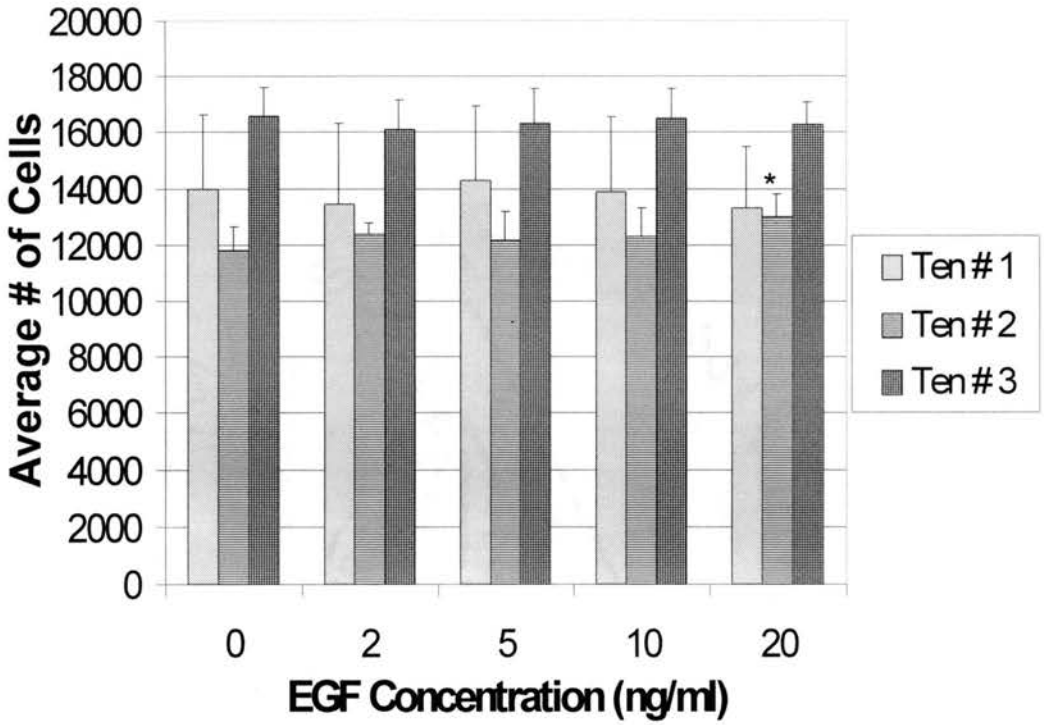

Figure 4. The mitogenic response of equine tendon fibroblasts to EGF at varying concentrations (0, 2, 5, 10, and 20 ng/ml). Results are expressed as the means with standard deviations (n = 6, 9, 10 respectively for ten #1, # 2, and # 3). Statistically significant difference between control (0 ng/ml) and EGF groups indicated by * ($p<0.05$).

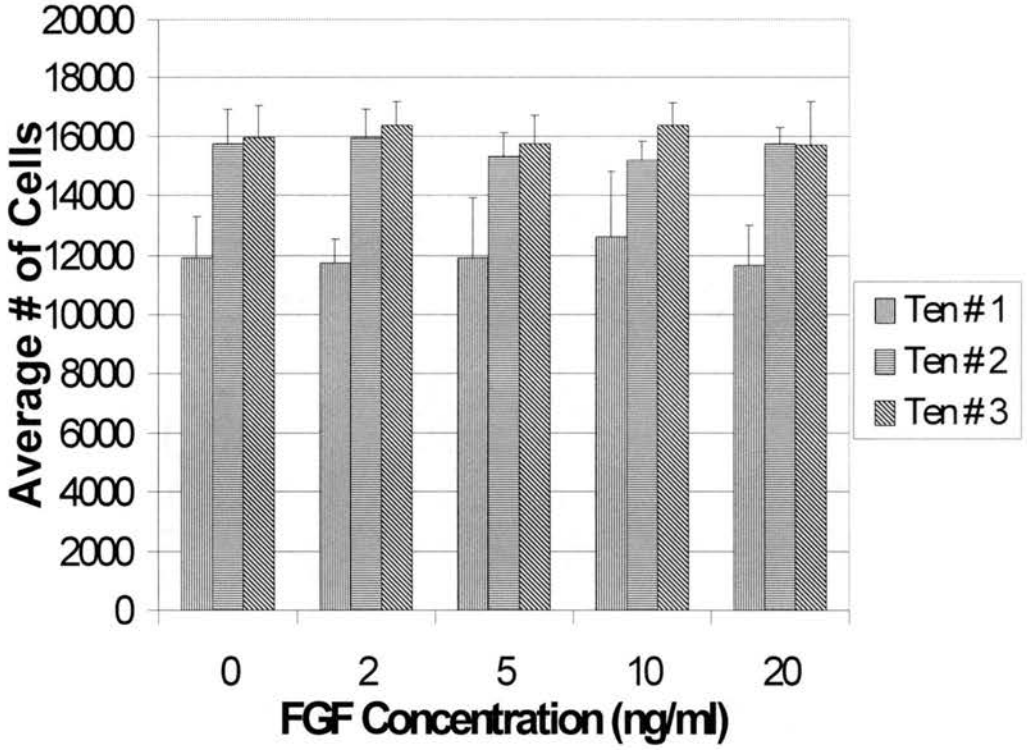

Figure 5. The mitogenic response of equine tendon fibroblasts to FGF at varying concentrations (0, 2, 5, 10, and 20 ng/ml). Results are expressed as the means with standard deviations (n = 6, 8, 10 respectively for ten #1, # 2, and # 3).

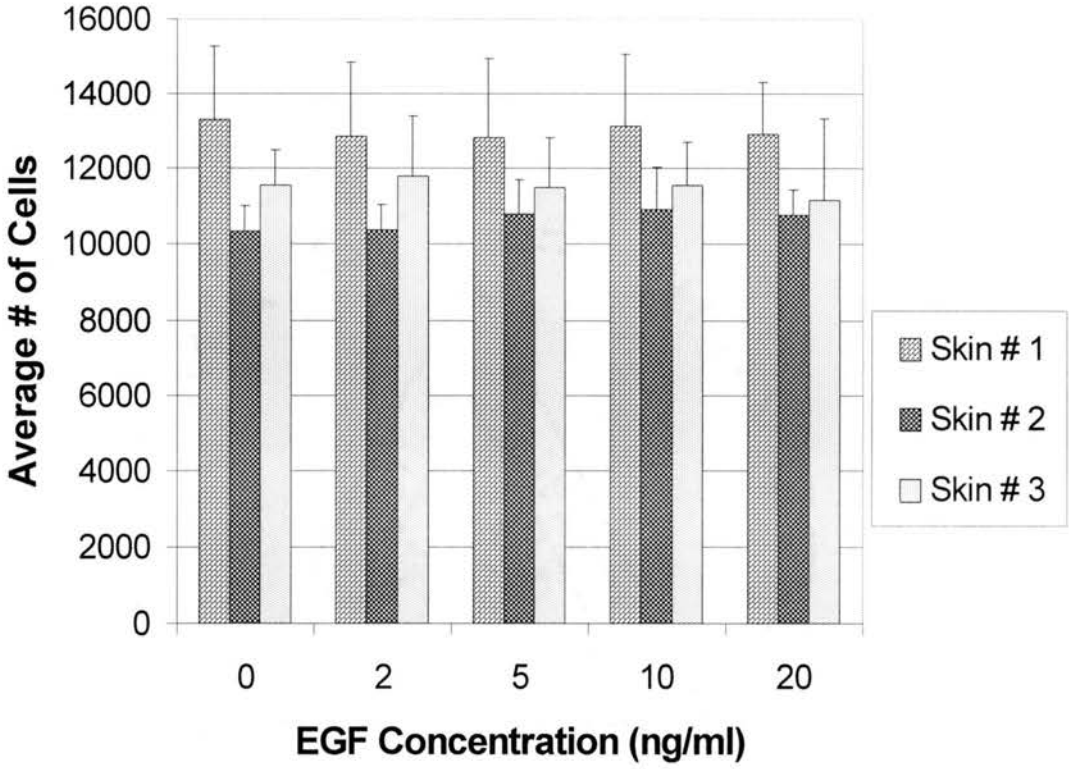

Figure 6. The mitogenic response of equine dermal fibroblasts to EGF at varying concentrations (0, 2, 5, 10, and 20 ng/ml). Results are expressed as the means with standard deviations (n = 10, 11, 6 respectively for skin # 1, # 2, and # 3).

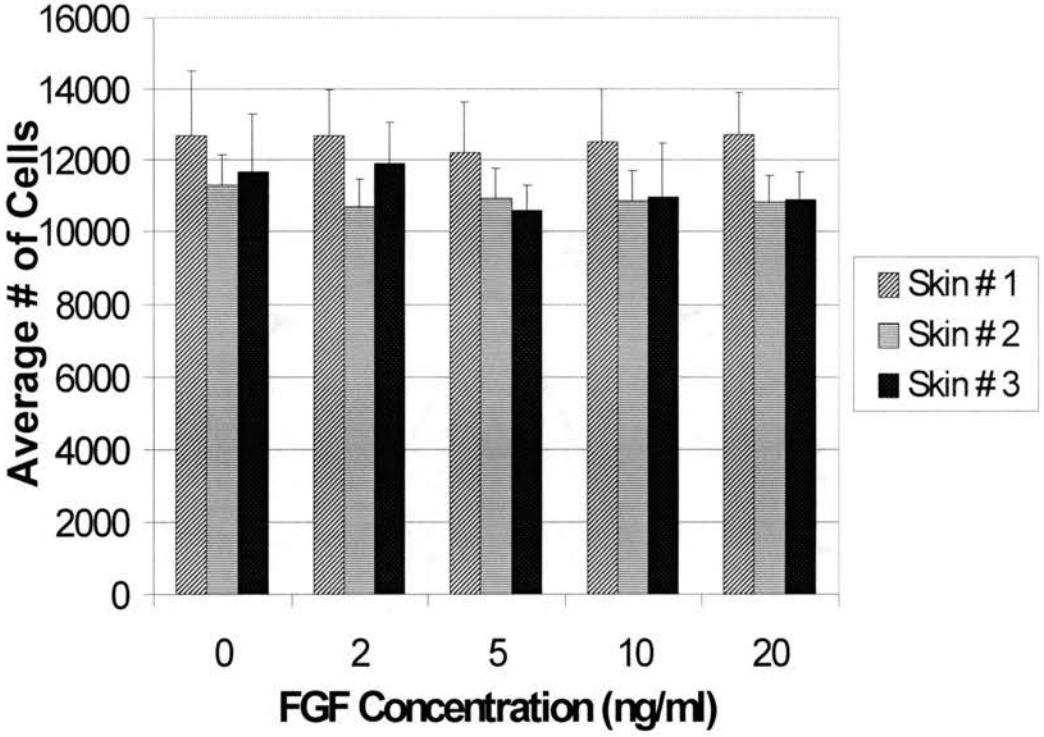

Figure 7. The mitogenic response of equine dermal fibroblasts to FGF at varying concentrations (0, 2, 5, 10, and 20 ng/ml). Results are expressed as the means with standard deviations (n = 10, 11, 6 respectively for skin # 1, # 2, and # 3).

CHAPTER IV

THE CHEMOTACTIC ACTIVITY OF BASIC FIBROBLAST GROWTH FACTOR OR EPIDERMAL GROWTH FACTOR ON EQUINE TENDON AND DERMAL FIBORBLASTS

Introduction

Wound healing in both tendon and skin is a coordinated effort between multiple cell types including fibroblasts, endothelial cells and epidermal cells (skin only) (Witte and Barbul, 1997; Dyson, 1997). The ability of two apposed wound edges to fuse and heal relies significantly on the ability of adjacent cells to migrate into the wound gap during the acute stage of wound healing. Once present in the wound gap these cells (i.e. fibroblasts) can proliferate and secrete extracellular matrix components in order to return the damaged area back to "normal" functional status (Lawrence, 1998). Fibroblast migration into a wound is a prerequisite for the formation of granulation tissue, and in turn, successful wound closure (Schreier et al., 1993). If fibroblasts are not able to migrate into the wound gap, a marked delay in wound healing occurs because of the inability of the wound gap to close. In order for the body to solicit repair cells (i.e. fibroblasts) to the wound area during the acute phases of wound healing, numerous cells including platelets (from damaged blood vessels), neutrophils and macrophages, which have migrated in by chemotactic stimuli, secrete growth factors into the wound gap. These growth factors along with other chemotactic stimuli, some of which may be bound to the extracellular matrix, attract fibroblasts from the surrounding undamaged tissue into the wound gap. Once present, fibroblasts begin to proliferate, secrete matrix proteins (Gailit and Clark, 1994) and chemotactic cytokines that further stimulate their own migration and the migration of other fibroblasts (Sporn and Roberts, 1986).

Numerous growth factors, including fibroblast growth factor and epidermal growth factor, are known to be chemotactic for multiple cell types (Steed, 1997; Rothe and Falanga, 1989). Both FGF and EGF individually have been shown to be chemotactic for

bovine nuchal ligament, human skin, and rat dermal granulation tissue fibroblasts (Buckley-Sturrock et al., 1989). Fibroblast growth factor has also been shown to be chemotactic for rat patellar tendon fibroblasts (Chan et al., 1997).

The ability of cells to migrate depends largely on the expression of integrins. Integrins are proteins that allow communication between the cell and surrounding extracellular matrix (Albelda and Buck, 1990; Ruoslahti, 1991). Harwood et al (1998) recently observed an increase in expression of specific integrins involved in cell migration during *in vivo* healing of canine flexor tendons. Harwood et al (1999) later demonstrated that FGF and PDGF-BB up-regulated expression of these integrins indicating that the chemotactic effects of certain growth factors are related to up-regulating integrins.

Successful equine tendon and skin wound healing requires migration of fibroblasts into wound gaps (Goodship et al., 1994; Bertone, 1989). To date, the chemotactic effects of growth factors, especially fibroblast growth factor and epidermal growth factor, on equine fibroblast cell lines has not been described. Therefore, the objective of this study was to determine if fibroblast growth factor or epidermal growth factor were chemotactic for equine tendon and/or dermal fibroblasts.

Materials and Methods

Chemotaxis Assay

The following procedure was performed in triplicate on each of three tendon and three dermal fibroblast cell lines using EGF and bFGF individually. Blind-well chemotactic chambers (Whatman-Nucleopore®, Clifton, NJ) with polyvinylpyrrolidone (PVP) free, 13 mm in diameter, polycarbonate chemotaxis membranes with 8 μm pores

coated with 0.1% gelatin (Bio-Rad®, Hercules, CA), were used for this experiment (Figure 8). Into the bottom well of each chamber was placed 200 µl of one of the following concentrations of growth factor: 0 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml and 20 ng/ml. The growth factors were dissolved in serum-free, ITS+3-free growth media. The membrane was then placed over the bottom well, the top chamber put into place and a 200 µl cell suspension containing $1x10^5$ cells in growth media containing 3% FBS was placed into the top chamber. The chambers were incubated for 4 hours at 37°C and 5% CO_2. The top chamber was removed and any remaining media was removed by micropipetting. A sterile swab was used to remove cells from the top of the membrane. The membrane was lifted with forceps and placed on a clean glass microscope slide bottom-side down. Diff-Quick® solutions were placed on the membrane at 15-30 second intervals, carefully tapped off on paper towels between solutions, a cover slip was placed, resin used to seal the edges, and the slides left at room temperature for 15-30 minutes to allow the resin to dry. Three fields of cells (nuclei) were counted at 20 X power objective (total magnification 200x) (Figure 9). The nine values (three counts x triplicate) were pooled and compared to the pooled negative control (0 ng/ml) values for statistical significance using paired, two tailed T-test with $p < 0.05$ being significant. To determine if a dose-dependent response occurred, linear regression was utilized on the pooled samples of all three horses, and a $p < 0.05$ was considered significant.

Results

Chemotactic Effects of EGF on Equine Tendon Fibroblasts

The chemotactic activity of epidermal growth factor was significantly greater for all concentrations tested compared to 0 ng/ml control for all tendon cell lines. The 2 ng/ml

concentration significantly enhanced chemotaxis (ten #1 (n = 9), p = .00004; ten # 2 (n = 9), p = .00001; ten # 3 (n = 9), p = .00002). A dose-dependent increase in chemotactic activity was observed (p <0.05). (Figure 10).

Chemotactic Effects of FGF on Equine Tendon Fibroblasts

Significant chemotactic activity for ten #1 (n = 9) was observed at 2 ng/ml (p = .001) but not for ten # 2 (n = 9) (p = .065) and ten #3 (n = 9) (p = .057). However ten #2 and ten # 3 did show significant chemotactic activity at the next higher dose (5 ng/ml; Ten #2, p = .0004; ten # 3, p = .00008) and continued for the remainder of the doses as did ten #1. A dose-dependent increase in chemotactic activity was observed (p <0.05) (Figure 11).

Chemotactic Effects of EGF on Equine Dermal Fibroblasts

All concentrations of epidermal growth factor (2, 5, 10, and 20 ng/ml) caused similar statistically significant increases in cell migration (ex. 2 ng/ml, skin #1 (n = 9), p = 0.00037; skin #2 (n = 9), p = .00035; skin #3 (n = 9), p = .0005) compared to 0 ng/ml control for all cell lines. A dose-dependent increase in chemotactic activity was observed (p <0.05) (Figure 12).

Chemotactic Effects of FGF on Equine Dermal Fibroblasts

The chemotactic effects of FGF at 2, 5, 10, and 20 ng/ml, on equine dermal fibroblasts, was significantly greater than the 0 ng/ml control group for skin #1 (n = 9), skin # 2 (n = 9), and skin #3 (n = 9). A dose-dependent increase in chemotactic activity was observed (p <0.05) (Figure 13).

Discussion

For the healing cascade to proceed after wounding in tendon and skin, fibroblasts must first migrate into the injury site during the inflammation and tissue formation stages. EGF and FGF have individually been shown to increase the chemotactic activity of multiple human and rodent cell lines *in vitro* at varying concentrations (10 ng/ml – 200 ng/ml)(Blay and Brown, 1985; Chen et al., 1993; Chen et al., 1994a; Chen et al., 1994b; Klemke et al., 1994; Fujii et al., 1995; Matthay et al., 1993; Basson et al., 1992; Hoying and Williams, 1996; Watanabe et al., 1995; Kondo et al., 1993; Jackson and Reidy, 1993; Grant et al., 1992; Mignatti et al., 1991). This study confirmed that both EGF and FGF increased the migratory behavior of both equine tendon and dermal fibroblasts *in vitro* in chemotactic chambers. In contrast, one report by Chan et al. (1997), using rat patellar tendon fibroblasts, did not show any significant chemotactic response with FGF (2, 10, and 50 ng/ml) in serum free DMEM, even though they utilized the same procedure as the one used in this experiment. The fact that they used a rat extensor tendon compared to an equine flexor tendon, may explain the differences seen, since extensor tendons heal slower, possibly due to decreased chemotactic activity of fibroblasts, than flexor tendons (Evans and Trail, 1998).

It is interesting that dermal fibroblasts responded at a lower dose of each growth factor (2 ng/ml) compared to tendon fibroblasts (5 ng/ml). The skin is continuously exposed to harsh and damaging environments. The probability of the dermal fibroblasts becoming metabolically reactive to mechanical injury to the skin (lacerations, crushing injury, burns), and participating in wound healing is most likely much higher than for tendon fibroblasts. Intrinsic tendon fibroblasts for the most part are quiescent, even

during the early stages of wound healing (Garner et al., 1989; Khan et al., 1996). Active participation of these cells in tendon wound healing does not occur until many days to weeks post-wounding (Manske and Lesker, 1984: Garner et al., 1989; Khan et al., 1996). Hence, dermal fibroblasts may be closer to the threshold of responding to a hormonal chemotactic response, or may have increased numbers of receptors, as compared to tendon fibroblasts.

Chemotaxis is regulated in large part by cell-matrix interactions (Gailit and Clark, 1994). Only in the presence of varying extracellular components (i.e. laminin, fibronectin, etc.) will chemotactic activity occur (Li et al., 1999). EGF (10 ng/ml), as well as FGF (2-200 ng/ml), have been shown to increase the expression of cell adhesion molecules, thereby promoting cell-matrix interactions (Zhang et al., 1997; Harwood et al., 1999; Collo and Pepper, 1999). A temporary matrix that is involved with cell migration, in respect to wound healing, is fibrin. Fibrin molecules released from damaged vessels deposit into a wound gap within minutes and provide a scaffold in which fibroblasts can migrate into the wound and deposit new extracellular matrix. In many instances, these fibroblasts will deposit collagen on these fibrin strands creating a temporary new extracellular matrix. Fibrin also has the capacity to bind FGF (Sahni et al., 1998), therefore providing a directed stimulus for fibroblast migration into the wound gap, thus enhancing wound healing (Quirinia and Viidik, 1998). The addition of 0.1% gelatin to membranes in this project provided the extracellular matrix, similar to that present in vivo, which consequently aided in migration of cells by providing a path on which to travel. Preliminary projects using cell membranes without gelatin coating afforded markedly fewer cells attaching, let alone migrating (personal observations).

Gelatin, a degraded product of collagen, maintains many of the peptide sites necessary for cell-matrix interactions, as well as provides a site for growth factor binding (Muniruzzaman et al., 1998). There is a possibility that an increase in concentration of gelatin or the use of collagen type I gel matrices might have afforded a greater chemotactic response by providing more cell-matrix interactions, which are necessary for cell migration.

The time frame at which events in wound healing occur are important. The enhanced chemotactic activity of in vitro fibroblasts over a four hour incubation period corresponds well to the time frame in which fibrin is first laid down during wounding. Combining the two demonstrates that FGF or EGF may enhance the chemotactic activity of fibroblasts adjacent to the wound the same time fibrin strands have just recently been deposited.

Increasing the chemotactic activity of fibroblasts can be a positive as well as a negative attribute of wound healing. In vitro, the goal was to increase the number of fibroblasts migrating to the other side of a membrane. However in vivo, it is necessary to direct the targeted fibroblasts to migrate into the wound gap without stimulating un-wanted fibroblasts to the same area. During the healing processes of unsheathed tendons, paratenon fibroblasts migrate into the wound gap and provide the initial scaffold on which all other steps involved in healing take place (Gelberman et al., 1987). Unfortunately, the effects of EGF of FGF on paratenon fibroblasts was not evaluated in this study. In an attempt to return the tendon to close to normal anatomical form and function, the ability to enhance migration of intrinsic fibroblasts into the wound gap would appear to be more beneficial, since these are the cells which originally created the tendon (Gelberman et al., 1987). In order to do that through the use of exogenous

chemotactic factors (i.e. growth factors), a proper vehicle for delivery of these factors is needed. In the past, direct injection of growth factors into a wound, or the placement of growth factor soaked sponges/graft material into a wound, were utilized (Takahashi, 1997; Laato et al., 1986; Sprugel et al., 1987; Fiddes et al., 1991). However, the chances for dilutional decreases in potency of the growth factors as well as the dissipation of growth factors into surrounding tissues, causing unwanted fibroblast migration, greatly increase (Takahashi, 1997; Davies et al., 1997). Recently, the ability to chemically, covalently bind biologically active growth factors to suture material has been shown to be promising (Rohrich et al., 1999). By strategically placing growth factors directly into the wound gap, via the suture material, chances of losing active concentrations of these growth factors, as well as preventing the potential for the migration of unwanted fibroblasts, will most likely decrease. Jann et al. (1999) have shown that intrinsic fibroblast migration does occur on suture material, and can be enhanced by EGF (80 ng/ml). It is therefore possible to have the cell of interest migrate on suture as well as enhance their motility.

The sex or breed of the animals used in this study do not appear to have a profound affect on the chemotactic activity. Although, age may have a slight influence on the effects of cell migration. Skin #3, which was from the youngest horse of the three, appeared to have higher numbers of cells migrating with all concentrations, including control, compared to the other two horses when exposed to FGF. The same age affect also occurred in Ten #1, the youngest horse, in FGF exposed assay. However, this was not seen with EGF. The relevance of this is unknown since age and gender comparisons were not the scope of this project, nor was their any statistical data obtained because of

the small number of animals used. However, one can speculate that the basal metabolic activity of younger horses is greater than that of old. Differences were not seen when comparing the effects of EGF to FGF on both dermal and tendon fibroblasts.

Growth factor concentrations utilized in this project were similar to those in the literature. Grant et al (1992) demonstrated a plateau of 200 ng/ml FGF and 50 ng/ml EGF for bovine corneal stromal fibroblasts. Although 20 ng/ml of FGF and EGF was the highest concentration used, there is a possibility that higher concentrations might have continued to increase the number of migratory cells observed, since a dose-dependent response was seen throughout all doses. In contrast, Chan et al (1997) did not see any significant increase in chemotactic activity when exposing rat patellar tendon fibroblasts to concentrations of FGF ranging from 2-50 ng/ml.

The use of low concentrations of FBS in the cell suspension should not have altered the affects of cell migration nor did it mask the effects of the growth factors since significant differences were observed between concentration groups. Trials were not performed to see if the same chemotactic effects occurred when serum free media was used. However, in vivo chemotaxis would also be subject to multiple potential chemotactic factors, and thus the addition of FBS seems warranted.

In summary, this is the first known report of the chemotactic effects of EGF and FGF on equine tendon and dermal fibroblasts. The experiment concluded that epidermal growth factor and fibroblast growth factor can enhance chemotaxis for both equine tendon and dermal fibroblasts, and thus may play a key role in tendon and dermal wound healing by regulating the migration of fibroblasts.

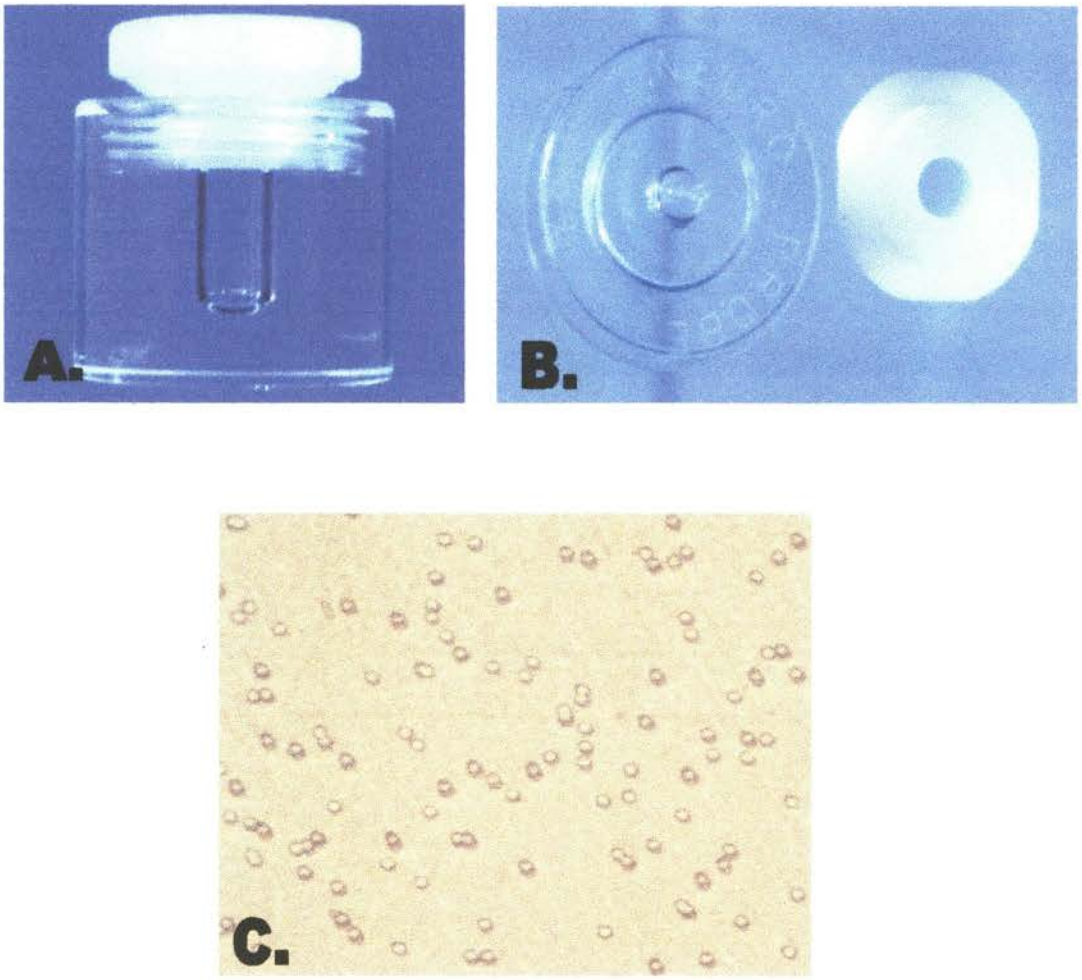

Figure 8. Chemotactic chamber. A. Front view of chamber (2 cm in diameter). Note bottom chamber in which growth factor is placed. B. Top view of chamber. Note plateau in left picture where membrane is placed. The top chamber, in which cells are placed, is present in the screw cap. C. Photomicrograph of the membranes used (200x). Note the multiple 8 μm pores through which cells can migrate.

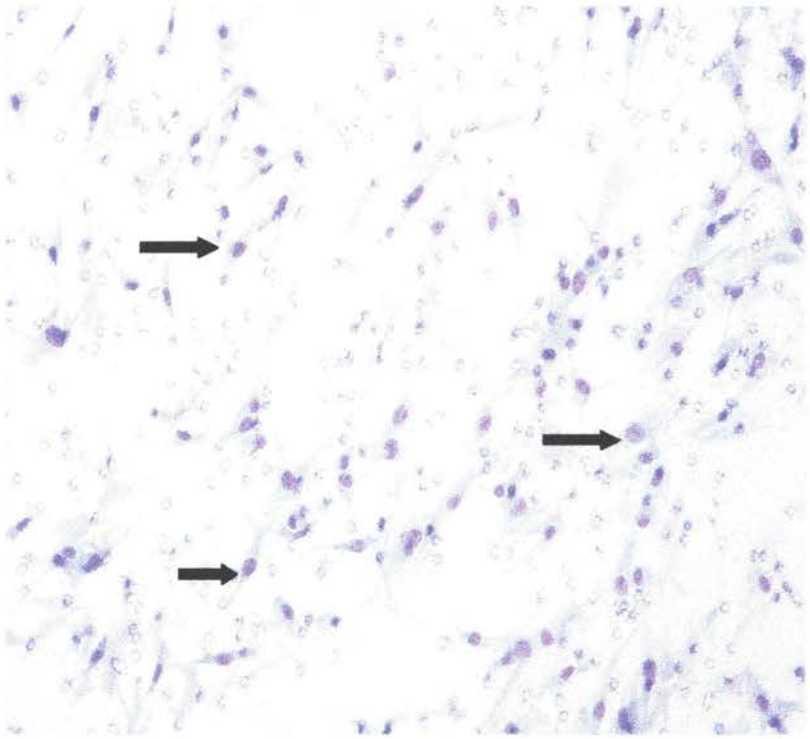

Figure 9. Photomicrograph of equine tendon fibroblasts adhered to a chemotactic membrane coated with 0.1% gelatin after 5 hours of incubation at 37°C and 5% CO_2. Diff-Quik stain ™. Note the prominent staining of nuclei (arrows). 200x.

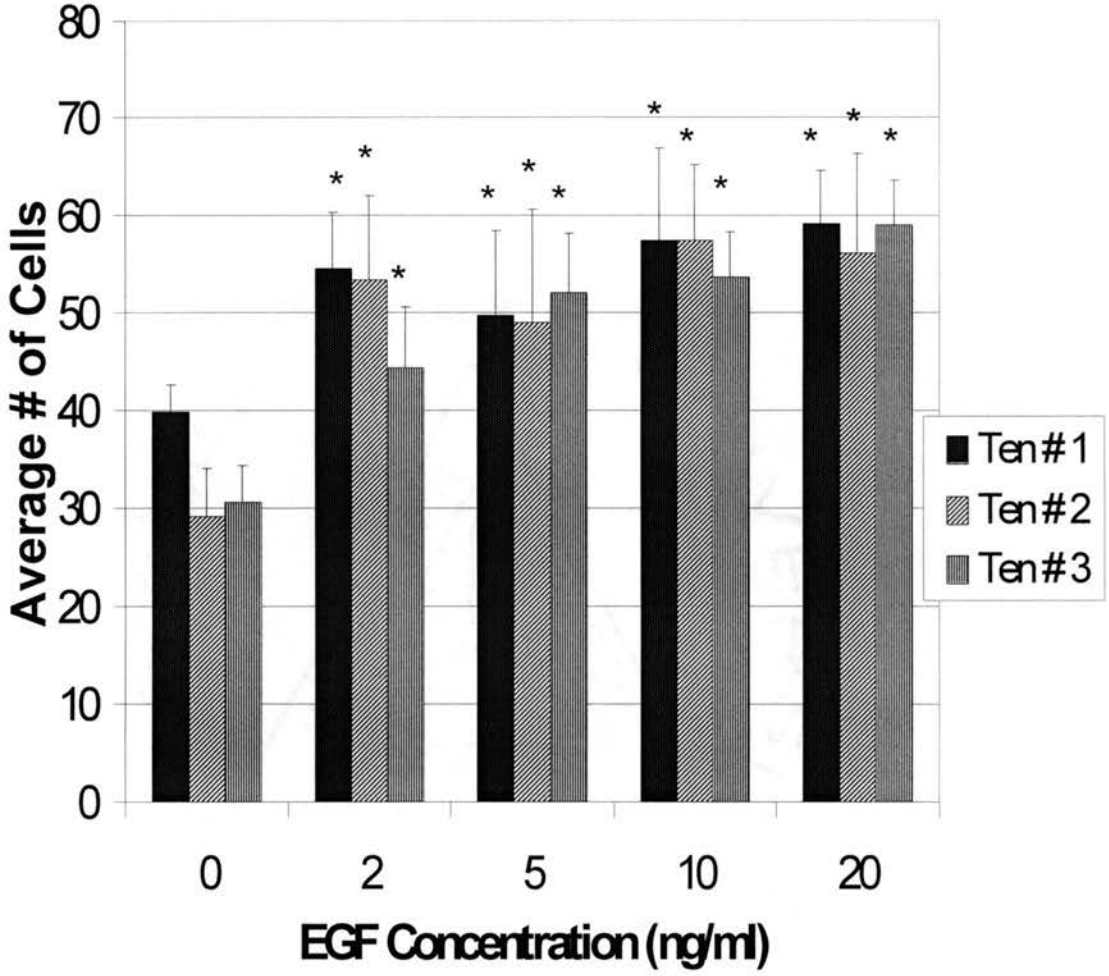

Figure 10. The chemotactic response of equine tendon fibroblasts to EGF at varying concentrations (0, 2, 5, 10, and 20 ng/ml). Results are expressed as the mean number of cells counted per 20 X objective field with standard deviations (n = 9, 9, 9, respectively for ten #1, # 2, and # 3). Statistically significant difference between the control (0 ng/ml) and experimental groups is indicated by * ($p < 0.05$).

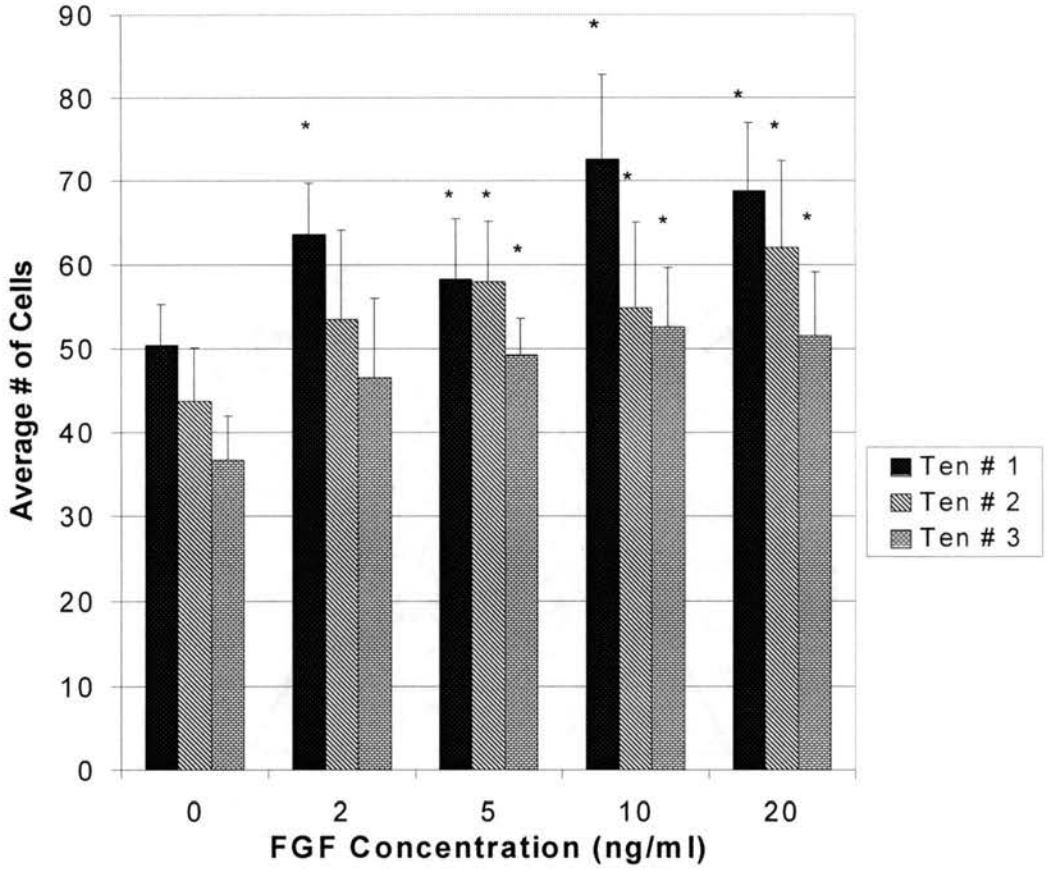

Figure 11. The chemotactic response of equine tendon fibroblasts to FGF at varying concentrations (0, 2, 5, 10, and 20 ng/ml). Results are expressed as the mean number of cells counted per 20 X objective field with standard deviations (n = 9, 9, 9, respectively for ten # 1, # 2, and # 3). Statistically significant difference between the control (0 ng/ml) and experimental groups is indicated by * ($p < 0.05$).

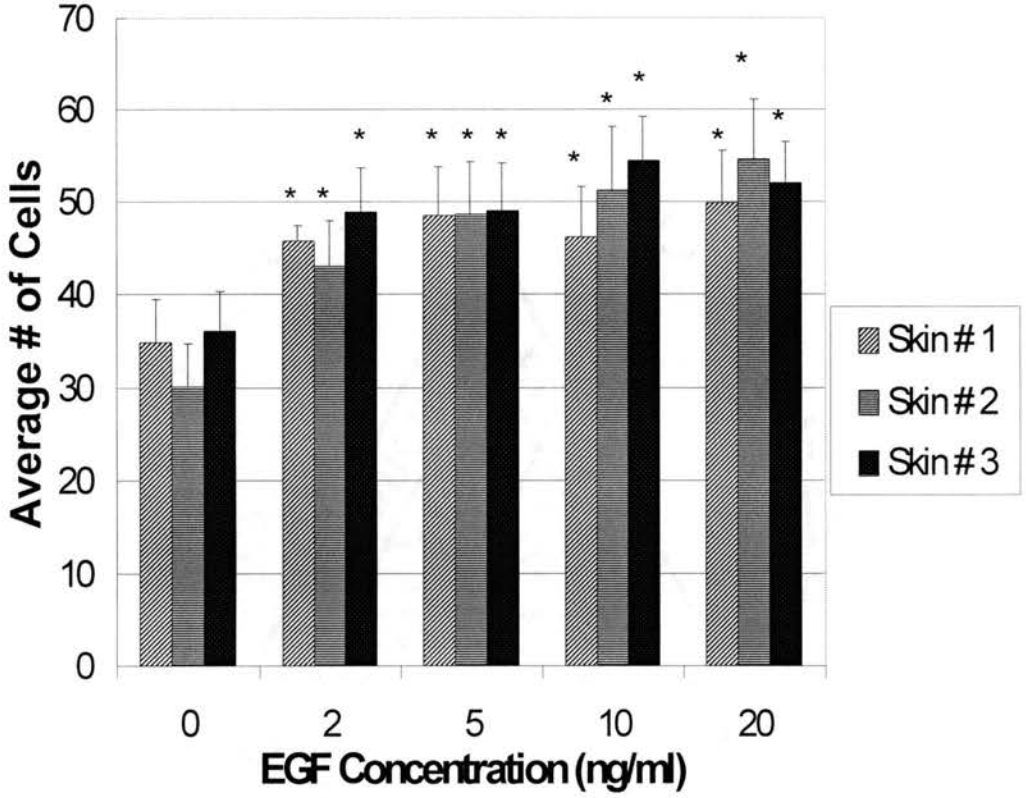

Figure 12. The chemotactic response of equine dermal fibroblasts to EGF at varying concentrations 0, 2, 5, 10, and 20 ng/ml). Results are expressed as the mean number of cells counted per 20 X objective field with standard deviations (n = 9, 9, 9, respectively for skin # 1, # 2, and # 3). Statistically significant difference between the control (0 ng/ml) and experimental groups is indicated by * ($p < 0.05$).

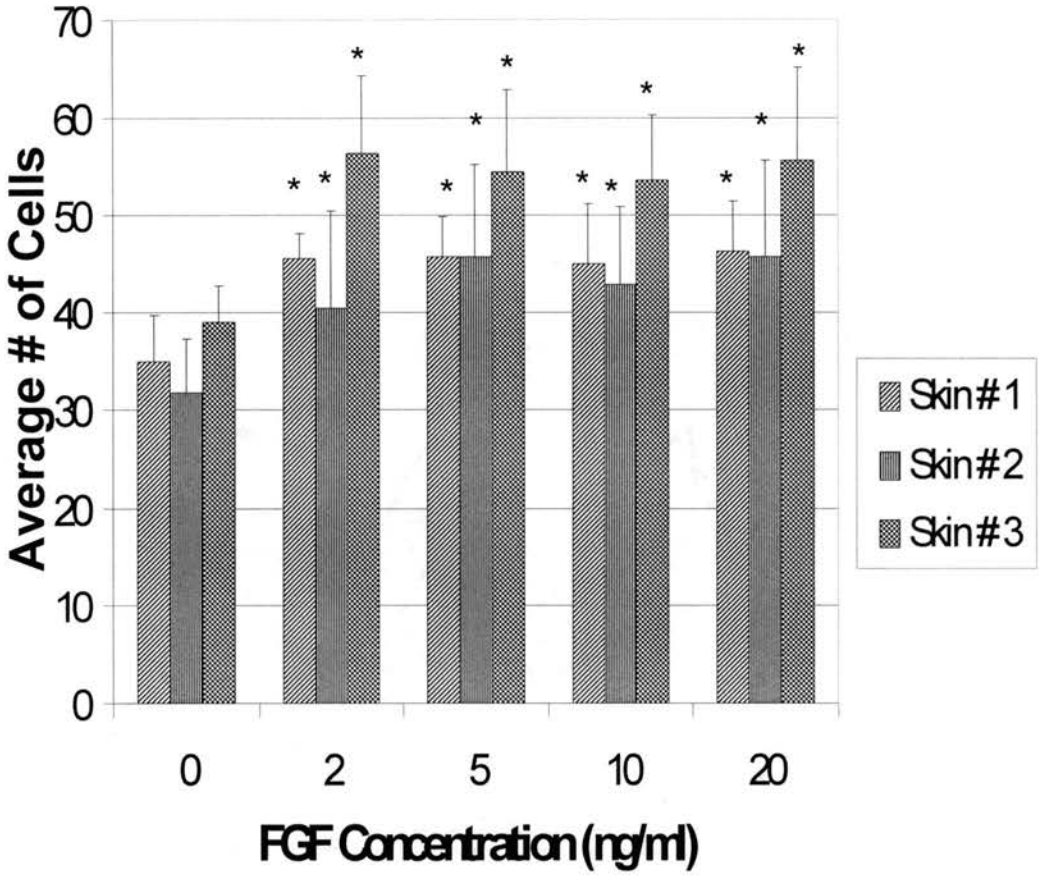

Figure 13. The chemotactic response of equine dermal fibroblasts to FGF at varying concentrations (0, 2, 5, 10, and 20 ng/ml). Results are expressed as the mean number of cells counted per 20 X objective field with standard deviations (n = 9, 9, 9, respectively for skin # 1, # 2, and # 3). Statistically significant difference between the control (0 ng/ml) and experimental groups is indicated by * ($p < 0.05$).

CHAPTER V

THE EFFECTS OF BASIC FIBROBLAST GROWTH FACTOR OR EPIDERMAL GROWTH FACTOR ON PRO-COLLAGEN TYPE I SYNTHESIS IN EQUINE TENDON AND DERMAL FIBROBLASTS

Introduction

Tendon and dermal wound healing constitute a complex, yet organized series of events in which fibroblastic migration, proliferation, and secretion of extra-cellular matrix components is necessary for proper healing to occur (Gelberman et al., 1987; Witte and Barbul, 1997; Goodship et al., 1994; Singer and Clark, 1999). Also intimately involved with healing are a cascade of growth factors that are secreted by resident as well as migrated cells within the wound site. These growth factors, individually and in coordination with each other, contribute to many of the processes involved in wound healing: cell migration, proliferation, matrix synthesis, and angiogenesis (Lawrence and Diegelmann, 1994; Brew et al., 1995; Martin et al;., 1992; Bennett and Schultz, 1993; Rothe and Falanga, 1989; Steed, 1997; Evans, 1999). However, even with the elaborate detailed instructions given internally to promote wound healing, the final outcome, especially in tendons, is sometimes not enough to return the wounded tissue back to normal anatomical and functional integrity (Woo, 1999). A large determining factor in returning skin and tendon back to their original function (granted neither fully returns to 100% normal function) is strongly based on the extra-cellular matrices that are secreted and deposited during the healing process (Gailit and Clark, 1994; Woo and Tkach, 1989).

The normal extracellular matrix of both dermis and skin is composed primarily of type I collagen. Type I collagen comprises approximately 30% of the total protein of the body (Woodhead-Galloway, 1980; Eyre, 1989; Woo et al., 1994). The largest percentage of type I collagen is in tissues of support and structural strength (i.e. bone, tendon, ligament, and skin). When an alteration in collagen type I occurs there is a decrease in functional strength and integrity. For example, one of the Ehrlo-Danlos syndromes as well as osteogenesis imperfecta are developmental defects in the skin and bone,

respectively, where spontaneous tearing of skin and fractures of bone occur because of the defect in normal collagen type I metabolism and secretion within these tissues (Cotran et al., 1999; Rosenberg, 1999).

In wound healing, the initial collagen secreted is type III, which has been shown to be inferior in strength and elasticity compared to type I collagen (Gelberman et al., 1987). Over time (weeks), collagen type III is slowly replaced by type I collagen which over many months to years is re-organized to resemble, as much as possible, the original extra-cellular makeup of the original tissue (i.e,. tendon or skin) (Gelberman et al., 1987). Therefore, an extended period of time is needed for successful wound healing to occur, given no external or internal variables interfere with the wound healing mechanisms. Variables that can affect wound healing include infection, immune status, age, wound location, metabolic disease (i.e. diabetes), as well as alterations in growth factor concentration and receptor regulation (Swift et al., 1999; Shukla et al., 1998; Broadley et al., 1989). Horses encounter a number of these variables. First and foremost, equine skin injury usually occurs on extremities. Skin in these areas has been shown to heal slower than other areas of the body based on decreased activity of fibroblasts (Miller et al., 2000; Jacobs et al., 1984; Wilmink et al., 1999). Complications also include exuberant granulation tissue and sarcoid formation that may result from growth factor/growth factor receptor dysregulation (Cochrane et al., 1996; Tuan and Nichter, 1998). In equine digital flexor tendons, rupture of the tendon can occur due to the strain placed on the wound. Rupture occurs because of the poor structural integrity of collagen type III that originally holds the two tendon ends together (Gelberman et al., 1987).

Enhancement of wound healing within the tendon and extremity skin lies in the ability of fibroblasts to secrete type I collagen, not type III collagen, at the onset of wound healing. Secretion of type I collagen would increase the structural strength of the wounded tissue sooner, allowing other aspects of wound healing to continue. Fibroblasts from the paratenon migrate into the wound gap, proliferate and secrete type III collagen (Williams et al., 1980; Williams et al., 1984; Woo et al., 1989). Participation of intrinsic fibroblasts, the cells of the tendon proper, does not occur until days or weeks after wounding (Garner et al., 1989). The ability to positively enhance collagen synthesis by intrinsic fibroblasts early in the course of wound healing may greatly enhance healing of flexor tendons. One way to enhance the secretion of type I collagen by these fibroblasts (tendon and dermal) is through exposure to growth factors which have been shown to increase collagen expression in numerous human and rodent cell lines (Marui et al., 1997; Murphy et al., 1994; Deie et al., 1997; Kang and Kang, 1999; Abrahamsson et al., 1991; Lepisto et al., 1995).

To my knowledge, no published literature is available detailing the effects of growth factors on collagen synthesis by equine tendon or dermal fibroblasts. Therefore, the objective of this study is to determine if fibroblast growth factor or epidermal growth factor can stimulate an increase in synthesis of type I pro-collagen by equine flexor tendon intrinsic fibroblasts and/or dermal fibroblasts of the extremity. The ability of identifying pro-collagen in the cell is very similar to identifying the collagen that is secreted because of their similar structure. Collagen is originally formed as pro-collagen in the endoplasmic reticulum of fibroblasts. As pro-collagen is released by the cell via

exocytosis, extension peptides present on pro-collagen are cleaved off, releasing collagen molecules.

Materials and Methods

Pro-Collagen Type I Detection Assay

The following procedures were performed on three equine tendon and three dermal fibroblast cell lines using EGF or FGF separately. All assays were performed in triplicate and at least two separate times. Epidermal growth factor or basic fibroblast growth factor diluted to concentrations of 0 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml in modified growth media containing 3% FBS and 50 μg/ml L-ascorbic acid (Sigma) were placed in triplicate into 96 well, flat bottom, cell culture plates (Corning-Costar, Corning, NY). Ascorbic acid was added to enhance hydroxylation of lysine residues in collagen alpha chains. To each of these wells was added $1x10^4$ cells suspended in modified growth media containing 3% FBS and 50 μg/ml L-ascorbic acid. (Dilutional effect of the growth factor concentrations by the cell suspension were taken into account. The final growth factor concentrations are those stated above). Plates were incubated for 24 hours at 37°C and 5% CO_2. The media was discarded and the cells fixed to the plate with 10% acetone in phosphate buffered saline (PBS) for 10 minutes. A modification of the monolayer enzyme-linked immunosorbent assay (M-ELISA) developed by Saliki et.al. (1997) was used to determine change in pro-collagen I synthesis when exposed to varying concentrations of EGF or FGF. Briefly, after fixation the plates were air-dried overnight. The wells were then washed with PBS +Tween 20 (PBST) for 1 minute, emptied and a mouse procollagen I IgG1 monoclonal antibody (Chemicon, Temecula, CA) diluted in PBST + 10% FBS was added. After 1 hour incubation at 37°C, the antibody was

removed, wells washed 4 times with PBST, and peroxidase-conjugated sheep anti-mouse IgG antibody (Sigma) diluted in PBST+ 10% FBS was added, and again incubated for 1 hour at 37°C. The wells were emptied, washed 4 times in PBST, and then 95 µl of substrate (0.01% H_2O_2 and 0.1 mg/ml of tetramethylbenzidine) in citrate buffer (pH 5.0) was added to each well and left for 25 minutes at room temperature on a plate shaker. The plate was observed for color change (blue). Twenty-five microliters of a 2M sulfuric acid solution was then added to each well to terminate the chemical reaction. The plates were read at 450 nm wavelength on a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). To compensate for variations in optical density (OD) readings between replicate plates, 0 ng/ml values were converted to 100% (control standard). Then all other OD data points for growth factor treatments were expressed as percentages of control value. The data for growth factor treatments were presented as percent of procollagen I synthesis compared to control. Comparisons of the pooled values of the various growth factor concentrations to control wells (0 ng/ml) were statistically analyzed using a one sample T-test with $p < 0.05$ being significant. To determine if a dose-dependent change occurred, linear regression on pooled samples from all three horses was utilized with a $p < 0.05$ being significant.

Results

The number of cells available during each experiment varied. This occurred because cell lines were not confluent monolayers, or contained clumped cells after trypsinization decreasing the number of individual cells available for use. This variation in cell number affected the number of replicates performed. At least 2 triplicate runs were performed

per cell line per growth factor concentration. Replicated runs were quite reproducible with similar results occurring on different runs performed on different days.

The effects of EGF on pro-collagen type I synthesis in equine tendon fibroblasts

For all three tendon cell lines a trend for a decrease in pro-collagen type I synthesis was observed when exposed to increased concentrations of epidermal growth factor (Figure 14). However, the dose at which a significant decrease was first seen differed among the 3 cell lines. For ten #1 ($n = 6$) a significant difference was not observed until 10 ng/ml ($p = 0.011$). Ten # 2 ($n = 12$) showed significance at 2 ng/ml ($p = 0.029$) and continued for all remaining concentrations. Ten # 3 ($n = 10$) showed a significant decrease in pro-collagen type I synthesis at 5 ng/ml ($p = 0.0002$) and maintained a continual decrease over the remaining concentrations. Another observation was that ten # 1 appeared to have reached a plateau at 10 ng/ml (91.11 ± 5.52 %) since similar results were observed for 20 ng/ml (91.50 ± 4.36). The remaining two tendon cell lines show a steady decline in pro-collagen type I synthesis through all concentrations. A dose-dependent decrease in pro-collagen type I synthesis was observed ($p<0.05$).

The effects of FGF on pro-collagen type I synthesis in equine tendon fibroblasts

A trend for an increase in pro-collagen type I synthesis was observed when all three equine tendon cell lines were exposed to fibroblast growth factor (Figure 15). The dose at which a significant response was observed differed between cell lines. Ten # 1($n = 6$) and ten #2 ($n = 12$) both showed a significant difference only at 20 ng/ml ($p = 0.020$, $p = 0.021$ respectively) compared to 0 ng/ml control. Ten # 3 ($n = 10$) however showed

significance starting at 5 ng/ml (p = 0.012). A dose-dependent increase in pro-collagen type I synthesis was observed ($p<0.05$).

The effects of EGF on pro-collagen type I synthesis in equine dermal fibroblasts

Epidermal growth factor showed a similar trend of decreasing pro-collagen type I synthesis in equine dermal fibroblasts as it did in equine tendon fibroblasts (Figure 16). However, all skin lines showed a significant difference starting with 2 ng/ml compared to 0 ng/ml, and maintained their significance through the remainder concentrations. The decreasing trend of synthesis with increasing concentration however, differed among cell lines. Skin # 1 (n = 8) trend decreased initially at 2 ng/ml (96.9t ± 3.01 %) , staying at that level for 5 ng/ml (96. 08 ± 1.69 %) , and then decreased to a plateau again for the last two concentrations (94.84 ± 2.19 % and 94.44 ± 3.45% respectively). Skin #2 (n= 10) declined steadily (96.86 ± 3.61%, 94.21 ± 1.96%, 92.23 ± 2.37% respectively) in 2% increments, but at 20 ng/ml it decreased 3 fold more (86.01 ± 3.43%) than between any of the other concentrations. Finally skin #3 (n = 12) declined steadily with a narrowing of the increments as the concentration increased (96.43 ± 3.93%, 93.83 ± 4.86%, 91.80 ± 8.27%, and 90.49 ± 6.35% respectively). A dose-dependent decrease in pro-collagen type I synthesis was observed ($p<0.05$).

The effects of FGF on pro-collagen type I synthesis in equine dermal fibroblasts

Fibroblast growth factor induced a significant increase in pro-collagen type I synthesis in all three equine dermal fibroblast cell lines as it did with equine tendon fibroblasts (Figure 17). However, the concentration at which significance was observed

differed among the three cell lines. Skin # 1 (n = 8) and skin # 2 (n = 10) had significant increases in synthesis at 2 ng/ml ($p = 0.0002$, $p = 0.014$, respectively) compared to 0ng/ml control. However, skin # 3 (n = 18) did not show a significant increase until 10 ng/ml ($p = 0.033$) when compared to 0 ng/ml control. All three cell-lines showed a trend of a gradual increase in pro-collagen type I synthesis for concentrations 2 –10 ng/ml. However the most significant changes occurred between 10 ng/ml and 20 ng/ml where each cell line increased dramatically (skin #1, 110.94 ± 5.49% (10 ng/ml) to 120.49 ± 6.68 % (20 ng/ml); skin #2, 112.62 ± 5.27 % (10 ng/ml) to 120.2 ± 5.94 % (20 ng/ml); skin #3, 106.28 ± 11.49% (10 ng/ml) to 112.54 ± 13.83 % (20 ng/ml)). A dose-dependent increase in pro-collagen type I synthesis was observed ($p<0.05$).

Discussion

The ability to return the skin or tendon to normal, or close to normal anatomical and functional integrity, would be a positive aspect in wound healing. One potential way of doing this is by enhancing type I collagen secretion by intrinsic fibroblasts of the tendon and dermal fibroblasts of the skin. In this study, fibroblast growth factor was shown to increase the steady-state level of pro-collagen type I synthesis by both tendon and dermal fibroblasts. Dermal fibroblasts had a higher increase in percent of type I collagen synthesized when exposed to FGF than tendon, and were shown to be significant at lower concentrations when compared to tendon fibroblasts. This may be explained by the fact that dermal fibroblasts may have higher receptor numbers than tendon fibroblasts, therefore initiating signal transduction pathways at lower growth factor concentrations to increase the production of collagen. FGF demonstrated only slightly higher increases in

pro-collagen type I synthesis by female horse tendon fibroblasts compared to the male horses. No differences were observed between genders with dermal fibroblasts. Age differences were not observed for either cell population exposed to FGF. It is important to note that evaluation of gender and age were not the scope of this study, and the trends observed are based on a small number of animals.

The increase in pro-collagen type I synthesis by FGF could be explained by the fact that FGF is normally present in the early stages of wound healing in both tendons and skin of different species (Yu et al., 1994; Chang et al., 1998;Duffy et al., 1995 Khan et al., 1996; Shukla et al., 1998). Therefore, it may, in some way, participate in the healing of skin and tendon. Interestingly, fibroblast growth factor has been described as decreasing the steady-state level of type I collagen. In human skin fibroblasts, exposure to FGF (2-50 ng/ml) for 24 hours to 4 days, was shown to decrease the steady-state level of type I collagen mRNA expression in both normal skin fibroblasts, keloid fibroblasts and scleroderma fibroblasts (Ichiki et al., 1997; Tan et al., 1993). Cochrane et al. (1996) also demonstrated decreased total collagen synthesis when exposing normal equine dermal fibroblasts, granulation fibroblasts, and sarcoid fibroblasts to FGF (10 ng/ml) for 5 days. The incubation time in Ichiki et al. study correlates with the 24 hour incubation time performed in this experiment. Interesting enough, the doses in that study, which decreased collagen type I production, varied from 2 ng/ml to 50 ng/ml. These dosages were similar to the ones used in my study. Therefore, the potential for dissimilarities between those studies and this one seem not to be related to dosage or incubation. However, the previous reports utilized northern blot analysis (mRNA for collagen type I). Northern blot is a more sensitive assay for proteins (pro-collagen type I included) than

determination by M-ELISA, which was used in this project. ELISAs are known as semi-quantitative assays, meaning they are highly specific but not as sensitive. The specificity of the M-ELISA used in this study was not determined. Pro-collagen type I was not available to establish a concentration curve nor to determine if the antibody used was specific for pro-collagen type I. However, the antibody was a monoclonal and the manufacturer guaranteed that the antibody did not cross react with other types of collagen. So the dose dependent changes observed would verify that the antibody was binding to type I collagen.

This inhibitory effect of FGF on collagen type I synthesis has been partially explained in a cell lines other than dermal or tendon fibroblasts. For example, in bovine vascular smooth muscle cells, FGF (1 ng/ml) increases the level of B-myb mRNA levels. B-myb is a transcription factor, that when up-regulated, has been shown to down regulate collagen expression (Kypreos et al., 1998). In a rat osteoblastic cell line, FGF (10 ng/ml) was shown to decrease the activity of lysyl oxidase. Lysyl oxidase is the enzyme required for cross-linking a hydroxylysine residue of one α chain to a lysine residue of a different α chain during pro-collagen formation (Feres-Filho et al., 1996). FGF (30 ng/ml), also in a murine osteoblast cell line, was shown to activate extracellular-signal regulated kinase (ERK) pathway of signal tranduction. It has been shown that inhibition of the ERK pathway upregulates type I collagen gene expression (Chaudhary and Avioli, 2000).

Not all studies demonstrated a decreased in collagen synthesis. In a few in vitro studies utilizing rabbit ligament fibroblasts (Marui et al., 1997[culture]; Murphy et al., 1994[explants]), and an established murine fibroblast cell line (Grande et al., 1997), FGF

(1 ng/ml, 10 ng/ml, and 10 ng/ml, respectively) did not alter the steady-state levels of collagen type I as determined by Northern Blot analysis in these studies. An explanation for why an increase in collagen type I synthesis was seen in my study and not in these three studies may involve the use of tendon and dermal fibroblasts. The cells used in the previous studies dealt with ligament and a transformed cell line. Also, the other studies used rabbits and a mouse cell line which are distinctly different than horses. And finally, the assays used by them involved northern blot analysis which is more sensitive and specific than M-ELISA which was used in my project.

Epidermal growth factor, in contrast to FGF, was shown to decrease the steady-state levels of pro-collagen type I equally in both tendon and dermal fibroblasts in a dose-dependent manner. Skin fibroblasts demonstrated a larger decrease in collagen expression at a lower concentration than tendon fibroblasts. Gender and age trends were observed in this study. EGF produced a larger decrease in pro-collagen type I synthesis among female horses, for both tendon and dermal fibroblasts, than in males. Also, the oldest horses demonstrated the strongest decreases at the highest concentration of EGF. The gender differences may have some association with sex hormones and their effects on cells. This parameter was not evaluated because it was not part of the scope of the study. The older horses may have demonstrated stronger responses due to a possible high number of cell surface receptors. However, this is pure speculation.

These results, in some respect, correlate with other studies. Human gingival fibroblasts in vitro were shown to have a proportionate decrease in collagen synthesis, over a 2 day incubation period, with increasing EGF concentration (0-10 ng/ml) (Huey et al., 1980), as demonstrated by northern blot analysis. Human skin fibroblasts also showed an EGF dose

dependent (2-50 ng/ml) inhibition of type I collagen synthesis over a four day incubation period (Hata et al., 1988) also demonstrated by northern blot analysis.

Ascorbic acid is a cofactor for the enzyme lysyl hydroxylase. This enzyme is necessary in the formation of hydroxylysine, a residue in collagen α chains necessary for crosslinking to occur among α chains. It is one of the limiting steps in collagen synthesis. That is why ascorbic acid (50-100 μg/ml) has been shown to stimulate collagen synthesis above steady state levels in human skin fibroblasts (Hata et al., 1988; Geesin et al., 1993). However, when ascorbic acid (50 μg/ml) was added to epidermal growth factor (2-20 ng/ml) in my project, a decrease was still observed in relation to type I collagen synthesis. Why a decrease in collagen synthesis was seen in my project, even in the presence of ascorbic acid, is unknown. But one can hypothesize that the signal transduction response to EGF receptor activation converges with the ascorbic acid signaling pathway. At this convergence, the signal from EGF may inhibit or override any further activator steps of collagen synthesis in equine tendon and dermal fibroblasts by ascorbic acid. Evaluation of the affects of EGF without ascorbic acid were not performed. However, one can speculate that there might have been similar results or even a larger decrease in pro-collagen synthesis without the addition of ascorbic acid.

Epidermal growth factor has been shown to produce a broad range of collagen secretory responses in different animal cell lines. When rabbit cruciate ligament fibroblasts were exposed to EGF (0.1 and 1.0 ng/ml), an increase in total collagen was observed (Marui et al., 1997). No change in collagen synthesis, compared to steady-state levels, were observed when medial collateral ligaments were used from the same animals (Marui et al., 1997). However, Deie et al (1997) exposed rabbit medial collateral

ligament fibroblasts to EGF (0.1 and 1.0 ng/ml) and observed an enhancement in collagen synthesis, with a stronger response seen in older animals than younger ones. These results demonstrate that location of cells (i.e. collateral and cruciate ligament) and age of animals can influence the effects of EGF on collagen synthesis. Seventeen-day old chicken embryo common calcanean tendon fibroblasts exposed to EGF (100 ng/ml) increased collagen synthesis, although not a statistically significant increase (Gauger et al., 1985). When rabbit deep digital flexor intrinsic tendon fibroblasts were exposed to EGF (100 ng/ml) plus ascorbic acid (50 μg/ml), they showed a dose dependent increase in collagen (Kang and Kang, 1999). These results differ from data reported here. The reason for this is unknown but may be associated with a difference in species, location of tissue, the dose used, as well as the type of assay performed to analyze collagen synthesis. Established epithelioid cells from normal rat kidney demonstrated slight, yet not significant, increases in type I collagen synthesis when exposed to EGF (25 ng/ml). However, when chronically exposed (7 days) to EGF (25 ng/ml), a marked decrease (50% of steady state levels) in collagen type I was observed (Creely et al., 1990). The fibroblasts in my study were exposed to a single dose of EGF for 24 hours before evaluating collagen synthesis. The differences in these results may be associated with exposure time to EGF. Possibly if the equine fibroblasts were exposed longer (2-4 days) with repeated changes in media and growth factor, a change towards increased collagen synthesis might have occurred. This being based on the premise that acclimation of the cells to decreased levels of FBS (15% to 3%) as well as to trypsinization, may be necessary in order for cells to respond properly to the effects of growth factors. However,

there is a possibility that the response to extended exposure might have just enhanced the decreased expression of pro-collagen type I synthesis to EGF.

This project involved the use of in vitro techniques. The establishment of primary fibroblast cell lines may have altered their expression of collagen types. Embryo chicken tendon fibroblasts freshly harvested were shown to normally express type I collagen but very few fibroblasts expressed type III collagen (Uitto et al., 1976). However, when these tendon fibroblasts were harvested and grown in culture monolayers, the expression of type III collagen increased with increasing passages to a point where 80% of the fibroblasts expressed type III collagen while still expressing type I collagen (Herrmann et al., 1980). Obviously, activation of a signal transduction pathway to increase type III collagen synthesis occurred. Therefore, established cell lines might also alter the effects of growth factors on collagen synthesis, since they too act through a signal transduction pathway. Since all cell lines in this project did not exceed 8 passages, this might partially explain the differences seen between this project, which demonstrated an increase in collagen synthesis by FGF, and the results obtained from immortalized cell lines such as human dermal fibroblasts and rat osteoblasts (Ichiki et al., 1997; Hurley et al., 1993), which demonstrated a decrease in collagen synthesis by FGF.

In summary, EGF decreased pro-collagen type I synthesis in a dose dependent manner in both equine dermal and tendon fibroblasts, demonstrating a significant response at a lower dose in dermal fibroblasts. On the other hand, FGF increased pro-collagen type I synthesis in a dose dependent manner in both cell lines, once again demonstrating a significant response at a lower dose, as well as demonstrating a higher synthetic rate at the final concentration (20 ng/ml), in dermal fibroblasts compared to tendon fibroblasts.

Based on these results FGF could potentially enhance the tissue formation stage of wound healing by stimulating the secretion of type I collagen which would greatly increase the tensile strength of the wound. However, EGF would not be a good candidate for enhanced wound healing based on the fact that it actually decreases the amount of type I collagen normally secreted by fibroblasts. Therefore, the wound site would theoretically maintain a higher level of type III collagen, which has been shown to be inferior to type I collagen in strength and elasticity, for a longer period of time. But, since there was a dose dependent response seen with both growth factors, there is a possibility of manipulating these doses so as to enhance wound healing with FGF while controlling excessive growth (exuberant granulation tissue) with EGF, a very real problem in horses.

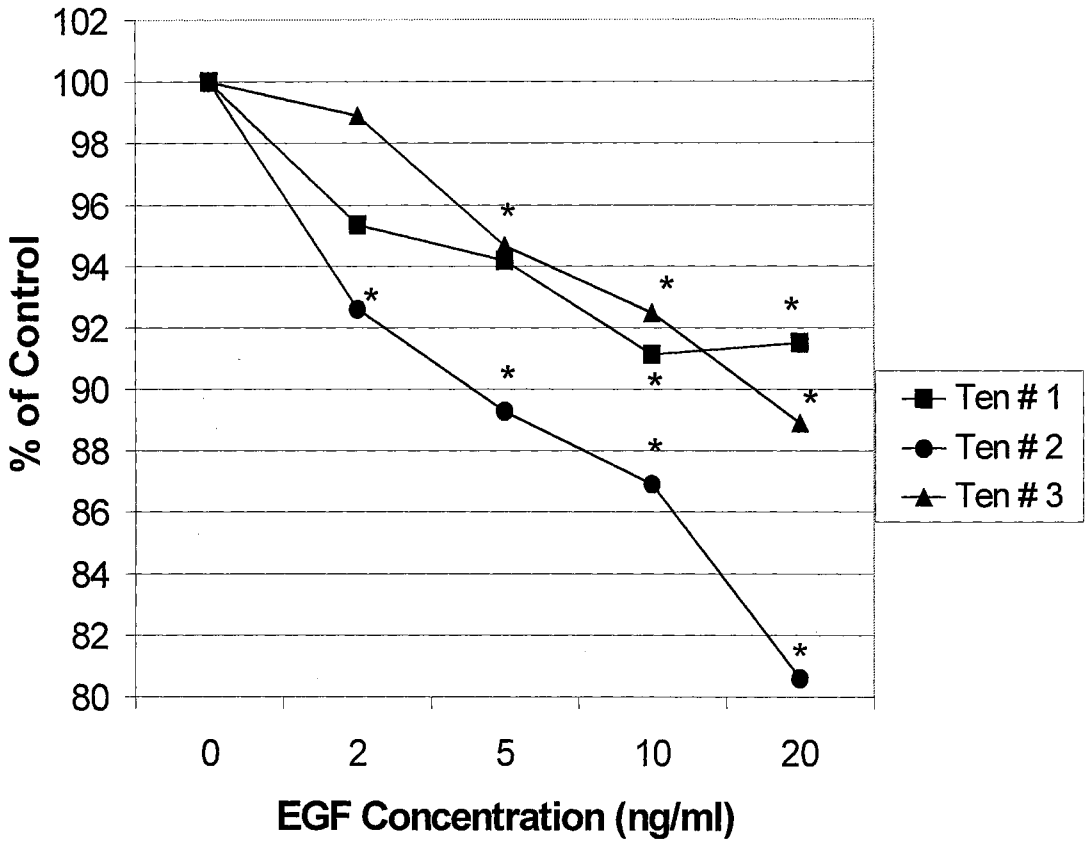

Figure 14. The effects of epidermal growth factor (EGF) on pro-collagen type I synthesis by equine tendon fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 2 (n = 6, 12, 10 respectively for ten # 1, # 2, and # 3). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

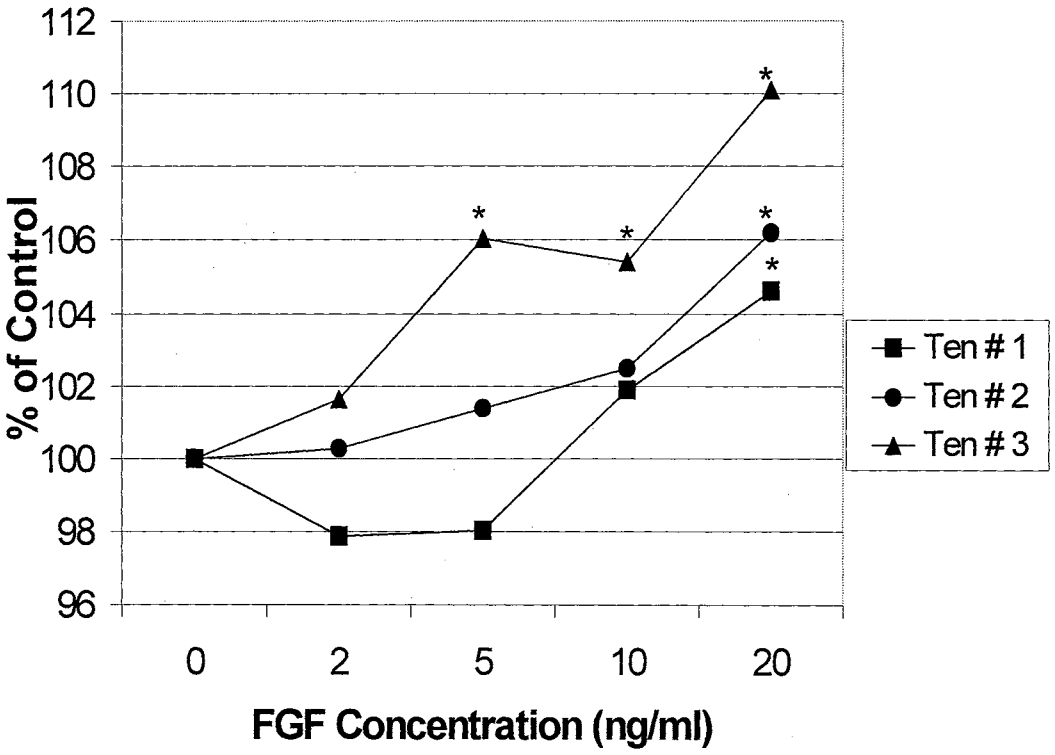

Figure 15. The effects of fibroblast growth factor (FGF) on pro-collagen type I synthesis by equine tendon fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 2. (n= 6, 12, 10 respectively for ten # 1, # 2, and # 3)). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

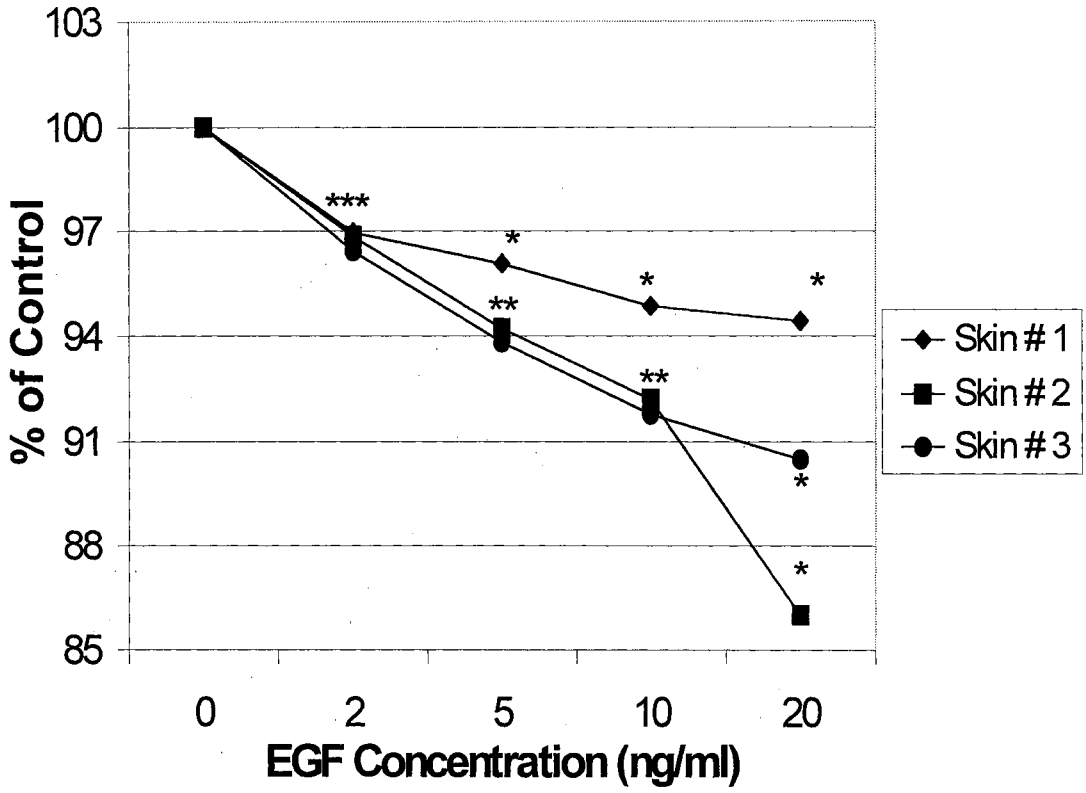

Figure 16. The effects of epidermal growth factor (EGF) on pro-collagen type I synthesis by equine dermal fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 3 (n = 9, 12, 10 respectively for skin # 1, # 2, and # 3). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

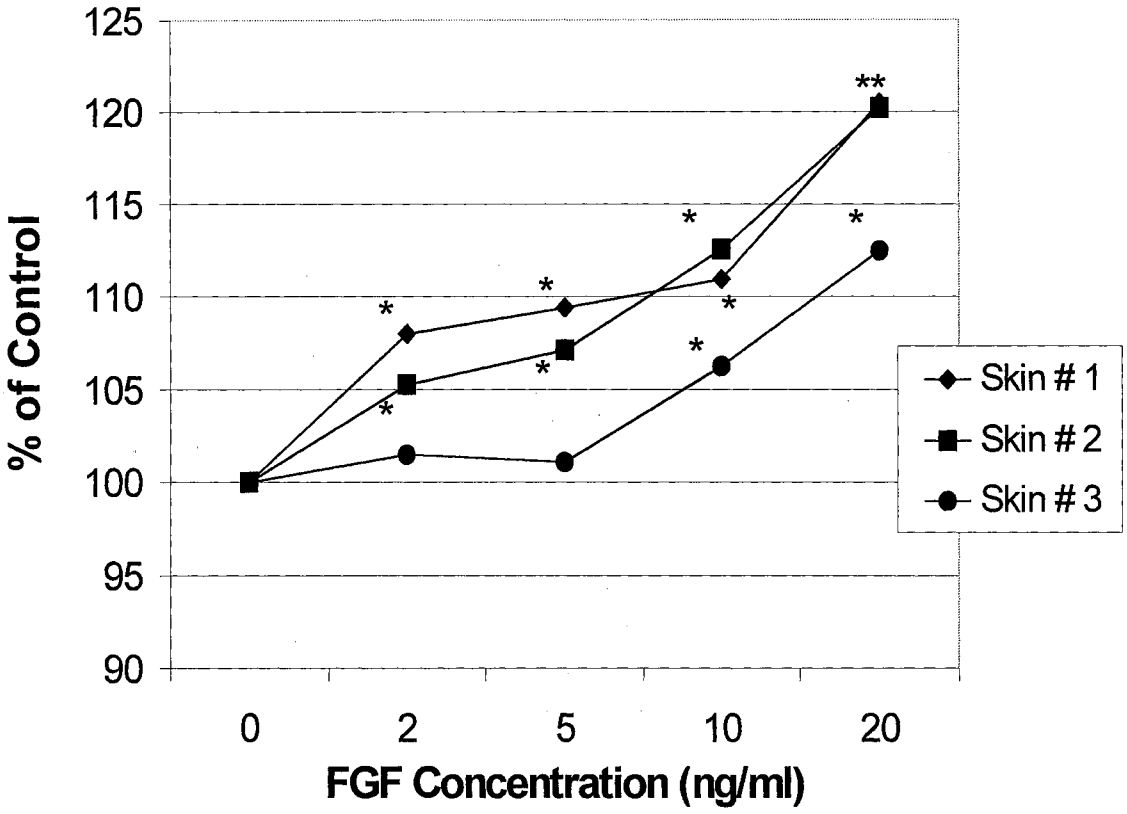

Figure 17. The effects of fibroblast growth factor (FGF) on pro-collagen type I synthesis by equine dermal fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 3. (n = 9, 12, 10 respectively for skin # 1, # 2, and # 3). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

EGF	0 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	N
Ten # 1	100 ± 0	95.329 ±5.626	94.1772 ±6.215	91.1132 ±5.52	91.5024 ±4.359	6
Ten # 2	100±0	92.5998 ±10.202	89.3036 ±11.66	86.9358 ±13.549	80.5957 ±13.565	12
Ten # 3	100±0	98.914 ±2.267	94.677 ±2.823	92.476 ±5.29	88.9 ±6.78	10
FGF	**0 ng/ml**	**2 ng/ml**	**5 ng/ml**	**10 ng/ml**	**20 ng/ml**	**N**
Ten # 1	100±0	97.8748 ±3.6767	98.0255 ±5.2795	101.908 ±4.4158	104.627 ±3.3623	6
Ten # 2	100±0	100.266 ±3.9964	101.370 ±4.4202	102.493 ±5.8501	106.216 ±8.0236	12
Ten # 3	100±0	101.654 ±4.0384	106.032 ±6.1291	105.424 ±5.6118	110.08 ±3.338	10

Table 2. Mean percentages with standard deviations for pro-collagen type I synthesis in equine tendon fibroblasts when exposed to epidermal growth factor (EGF) or fibroblast growth factor (FGF) for 24 hours. The control group (0 ng/ml = 100%) represent steady-state levels. N represents the number of data points per mean value in a given row.

EGF	0 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	N
Skin#1	100 ± 0	96.973 ±3.01	96.0751 ±1.6855	94.8434 ±2.1951	94.4403 ±3.4495	8
Skin#2	100±0	96.858 ±3.613	94.205 ±1.9619	92.228 ±2.3732	86.008 ±3.4305	10
Skin#3	100±0	96.4317 ±3.9259	93.8301 ±4.8576	91.8024 ±8.2653	90.4887 ±6.3483	12
FGF	**0 ng/ml**	**2 ng/ml**	**5 ng/ml**	**10 ng/ml**	**20 ng/ml**	**N**
Skin#1	100±0	107.978 ±3.1802	109.404 ±4.7691	110.942 ±5.4863	120.493 ±6.6816	8
Skin#2	100±0	105.239 ±5.4213	107.097 ±3.2841	112.622 ±5.2699	120.197 ±5.9445	10
Skin#3	100±0	101.444 ±10.404	101.055 ±7.3641	106.282 ±11.494	112.54 ±13.827	18

Table 3. Mean percentages with standard deviations for pro-collagen type I synthesis in equine dermal fibroblasts when exposed to epidermal growth factor (EGF) or fibroblast growth factor (FGF) for 24 hours. The control group (0 ng/ml = 100%) represent steady-state levels. N represents the number of data points per mean value in a given row.

CHAPTER VI

THE EFFECTS OF BASIC FIBROBLAST GROWTH FACTOR OR EPIDERMAL GROWTH FACTOR ON PRO-COLLAGEN TYPE III SYNTHESIS IN EQUINE TENDON AND DERMAL FIBROBLASTS

Introduction

Traumatic injuries to the extremities of horses are very common, especially among performance horses (i.e. racing, rodeo) (Peloso et al., 1994). Of all the structures of the limb, wounds to the skin and flexor tendons are the most common (Baxter, 1987; Bertone, 1995). Although the events in wound healing are similar between skin and tendon (Goodship et al., 1994: Witte and Barbul, 1997), variations in the time course of wound healing exist. For example, equine skin of the trunk heals faster than extremity skin (Wilmink et al., 1999a; Jacobs et al., 1984). It has been shown that equine fibroblasts from the extremity skin are less metabolically active than those of the trunk (Miller et al., 2000). Flexor tendons, especially of the hind limbs, are extremely prone to rupture because of the inferior strength of the initial granulation tissue formed, as well as the extreme tensile forces placed on the wound area (Woo and Tkach, 1989).

Wound healing of both the tendon and skin involves the coordinated effort of numerous cells, with fibroblasts playing the leading role. After wounding, the surrounding fibroblasts migrate to the wound gap, proliferate, and secrete collagen as well as other extracellular matrical proteins to provide a temporary scaffold/foundation to replace the tissue which was lost (Woo et al., 1994; Singer and Clark, 1999; Gelberman, 1985). Type III collagen is the most abundant collagen secreted at this time. Normally, the dermis and tendon are composed primarily of type I collagen. Because type I collagen is highly crosslinked, it is able to resist strong tensile forces while also being somewhat elastic. Type III collagen, however, has fewer crosslinks, and therefore has less tensile strength while being less elastic (Miller and Gay, 1982). Thus, when forces

are placed on an acute wound, there is a potential for breakdown of the wound at any time in which type III collagen is the predominant collagen type present.

In non-sheathed tendons, the paratenon is the primary participant in healing. The fibroblasts from the paratenon migrate into the wound gap, proliferate and secrete type III collagen (Williams et al., 1980; Williams et al., 1984 Woo and Tkach, 1989). Participation of intrinsic fibroblasts does not occur until days to weeks after wounding (Garner et al., 1989; Russell and Manske, 1990; Hamada et al., 1997). When these cells are activated, they primarily secrete type I collagen, the original makeup of the tendon. At the same time, these fibroblasts are thought to secrete metalloproteases similar to dermal fibroblasts during wound healing (Cochrane, 1997). Metalloproteases participate in removal of old, damaged, or improper collagen fibers, for replacement with type I collagen fibers secreted by resident fibroblasts (Docherty et al., 1992). The ideal situation for wound healing in the tendon would be for intrinsic fibroblasts to proliferate and secrete type I collagen in the initial stages of wound healing, thus providing a stronger foundation on which to enhance wound healing. One potential way of doing this is through the addition/enhancement of exogenous/endogenous growth factors in the wound gap. However, this might also enhance the chances for increasing type III collagen secretion. Murphy et al. (1994) exposed rabbit medial collateral ligaments to TGF-β and observed an increase in both type I and type III collagen synthesis. Lepisto et al. (1995) observed the same results when human dermal fibroblasts were exposed to PDGF-AB. Although growth factors have the ability to enhance type I collagen they might also increase type III collagen leading to inferior scar formation.

Basic fibroblast growth factor and epidermal growth factor have both been shown to alter (positively and negatively) the synthesis of type I and type III collagen in multiple rodent and human cell lines (Hurley et al., 1993; Pickering et al., 1997; Tseng et al., 1982; Creely et al., 1990; Gauger et al., 1985; Laato et al., 1987). The objective of this study was to determine if epidermal growth factor or basic fibroblast growth factor would decrease the synthesis of type III pro-collagen by both equine extremity dermal fibroblasts and tendon fibroblasts, with the recent knowledge (chapter V) that these growth factors can alter pro-collagen type I synthesis.

Materials and Methods

Pro-Collagen Type III Detection Assay

The following procedures were performed on three dermal and three tendon fibroblast cell lines using epidermal growth factor (EGF) or basic fibroblast growth factor (FGF) separately. All assays were performed in triplicate and at least two separate times. Epidermal growth factor or basic fibroblast growth factor diluted to concentrations of 0 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml in modified growth media containing 3% FBS and 50 μg/ml L-ascorbic acid (Sigma, St. Louis, MO) were placed in triplicate into 96 well, flat bottom, cell culture plates (Corning-Costar, Corning, NY). Ascorbic acid was added to enhance hydroxylation of lysine residues in collagen alpha chains. To each of these wells was added $1x10^4$ cells suspended in modified growth media containing 3% FBS and 50 μg/ml L-ascorbic acid (Dilutional effect of the growth factor concentrations by the cell suspension were taken into account. The final growth factor concentrations are those stated above). Plates were incubated for 24 hours at 37°C and 5% CO_2. The media was discarded and the cells fixed to the plate with 20% acetone in

PBS for 10 minutes. A modification of the monolayer enzyme-linked immunosorbent assay (M-ELISA) developed by Saliki et al. (1997) was used to determine change in pro-collagen III synthesis when exposed to varying concentrations of EGF or FGF. Briefly, after fixation the plates were air-dried overnight. The wells were then washed with PBST for 1 minute, emptied and a rabbit polyclonal procollagen III antibody (Chemicon) diluted in PBST + 10% FBS was added. After 1 hour incubation at 37°C, the antibody was removed, wells washed 4 times with PBST, and peroxidase-conjugated sheep anti-rabbit whole molecule antibody (Sigma) diluted in PBST+ 10% FBS was added, and again incubated for 1 hour at 37°C. The wells were emptied, washed 4 times in PBST and 95 µl of substrate (0.01% H_2O_2 and 0.1 mg/ml of tetramethylbenzidine) in citrate buffer (pH 5.0) was added and left for 25 minutes at room temperature on a plate shaker and the plate was observed for color change (blue). Twenty-five microliters of a 2M sulfuric acid solution was added to the wells containing the citrate buffer to terminate chemical reaction, and the plate was read at 450 nm wavelength on a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). To compensate for variations in optical density (OD) readings between replicate plates, 0 ng/ml values were converted to 100% (control standard). All other OD data points for growth factor treatments were expressed as percentages of control value. The data for growth factor treatments were presented as percent of procollagen III synthesis compared to control. Comparison of the pooled values of the various growth factor concentrations to control wells (0 ng/ml) were statistically analyzed using a one sample T-test with $p < 0.05$ being significant. To determine if a dose-dependent change occurred, linear regression on pooled samples from all three horses was utilized with a $p < 0.05$ being significant.

Results

The number of cells available during each experiment varied. This occurred because cell lines were not confluent monolayers, or contained clumped cells after trypsinization decreasing the number of individual cells available for use. This variation in cell number affected the number of replicates performed. At least 2 triplicate runs were performed per cell line per growth factor concentration. Replicated runs were quite reproducible with similar results occurring on different runs performed on different days.

The effects of EGF on pro-collagen type III synthesis in equine tendon fibroblasts

For all three tendon cell lines, a consistent, significant and gradual decrease in pro-collagen type III synthesis was observed starting at 5ng/ml (ten # 1($n = 9$), $p = 0.032$; ten # 2 ($n = 12$), $p = 0.00013$; ten #3 ($n = 10$), $p = 0.0056$) compared to 0 ng/ml control (Figure 18). The 20 ng/ml dose yielded the lowest percentage of pro-collagen type III synthesis observed for all three cell lines (ten # 1, 93.18 ± 2.14 %; ten # 2, 94.17 ± 3.02 %; ten # 3, 94.66 ± 3.44%). A dose-dependent decrease in procollagen type III synthesis was observed ($p < 0.05$).

The effects of FGF on pro-collagen type III synthesis in equine tendon fibroblasts

An inconsistent, yet significant increase in pro-collagen type III synthesis was observed for all three tendon cell lines (Figure 19). Ten #1 cell line behaved differently than the other two cells lines. A change in percentage was not seen until the 20 ng/ml dose when a significant increase in synthesis was observed (102.2 ± 2.66%, $p = 0.038$) compared to 0 ng/ml control (100%). The other two cell lines showed significant increases in synthesis at different concentrations. Ten # 2 showed a significant increase

compared to 0 ng/ml at 10 ng/ml (105.03 ± 3.14%, p = 0.00017). For Ten #3, a significant increase was observed starting with 2 ng/ml (102.54 ± 2.81%, p = 0.019). Both ten #2 and ten # 3 showed similar increments of increase for all concentrations of FGF compared to 0 ng/ml control. A dose-dependent increase in procollagen type III synthesis was observed ($p < 0.05$).

The effects of EGF on pro-collagen type III synthesis in equine dermal fibroblasts

A significant decrease was observed in skin # 2 and skin #3 beginning with at 2 ng/ml (skin # 2 (n = 10), p = 0.032; skin # 3 (n = 12), p = 0.0065) (Figure 20). The rate of decrease of pro-collagen type III synthesis was similar between skin # 2 and skin #3. Skin # 1 (n = 8) showed a decrease in percentage of pro-collagen type III compared to 0 ng/ml control, but was not statistically significant. A dose-dependent decrease in procollagen type III synthesis was observed ($p < 0.05$).

The effects of FGF on pro-collagen type III synthesis in equine dermal fibroblasts.

All three skin lines showed an inconsistent yet significant increase in pro-collagen type III synthesis compared to 0 ng/ml control. However, the concentration at which this was observed differed among the three cell lines (Figure 21). For skin #1 (n = 8) a significant increase in synthesis was observed immediately at 2 ng/ml (104.35 ± 4.59%, p = 0.032). Skin # 2 (n = 10) however did not show a significant increase in synthesis until 10 ng/ml (102.16 ± 2.42%, p = 0.014). Skin # 3 (n = 11) demonstrated a significant increase in synthesis at 2 ng/ml (101.44 ± 10.40 p = 0.045), lost its significance at 5 ng/ml (p = 0.15) because of a slight decline in percentage of collagen synthesis (101.06

± 7.36%) , but continued to significantly increase at 10ng/ml and 20 ng/ml ($p = 0.018$, $p = 0.006$, respectively). A dose-dependent increase in procollagen type III synthesis was observed ($p < 0.05$).

Discussion

The saying " a chain is only as strong as its weakest link" holds true for mechanical strength associated with wound healing. The "weakest link" in wound healing is the stage at which abundant type III collagen is secreted and laid down into the wound gap to form a temporary scaffold/foundation. This remains the "weak link" until such time that type I collagen can replace it through a series of complex yet coordinated events (Singer and Clark, 1999; Goodship et al., 1994). It needs to be re-emphasized that type I collagen is used as the primary collagen in the makeup of tendon and skin because of its superior strength and elasticity – two features attributed to crosslinking (Miller and Gay, 1982). However, type III collagen is somewhat the opposite of type I in that it has fewer cross-links and therefore is inferior in strength and elasticity. In tendons, there is a potential for rupture/re-rupture of the tendon, at the wound gap, because of the poor tensile strength of type III collagen that is present during healing. The present study was performed to determine if either epidermal growth factor or fibroblast growth factor could decrease the synthesis of type III pro-collagen by equine tendon and dermal fibroblasts.

Fibroblast growth factor increased type III pro-collagen synthesis in both equine tendon and dermal fibroblasts, in a dose-dependent manner. This could be detrimental to wound healing because of the increase of a collagen type that is known to have poor tensile strength.

FGF has not been extensively studied in relation to collagen type III synthesis. This could be explained by the fact that collagen type I comprises a large percentage of the collagen seen in normal tissue. Consequently, most studies have focused on looking at ways of returning tissues back to their original collagen makeup. In one study, adult rabbit medial collateral ligament explants exposed to FGF (10 ng/ml) in culture for 24 hours did not show any response in type III collagen synthesis. Radiolabeled proline incorporation was used to measure collagen synthesis (Murphy et al., 1994). And in two other reports in which vascular cells were used in vitro, exposure to FGF (100 ng/ml) for 48 hours was shown to enhance secretion of type III collagen in bovine aortic vascular endothelial cells (Tseng et al., 1982), while repressing type III collagen expression in human vascular smooth muscle cells which were exposed for 72 hours (Pickering et al., 1997). In both of these studies, radiolabeled proline and hydroxyproline incorporation was used to determine collagen synthesis. In relation to tendons, exposure to FGF (100 ng/ml) for 48 hours has been shown to enhance the synthesis of type III collagen in both normal and carpal tunnel syndrome human flexor retinacular (extrinsic) fibroblasts (Allampallam et al., 2000). The variation in response to FGF, once again, can be explained by numerous variables including, but not limited to species, tissue type, cell type, and age. In this study, the increase in collagen synthesis was higher overall in dermal fibroblasts than tendon fibroblasts. This could be explained by higher receptor numbers or closer proximity of receptors on the cell membrane, which would allow rapid dimerization and activation of receptors, on dermal fibroblasts. The skin is continually exposed to external hazards. This exposure may cause the skin to maintain a higher basal

rate than tendons, although not proven. Therefore, dermal fibroblasts may be more acutely responsive to changes in the environment than tendon fibroblasts.

Tendons exposed to FGF demonstrated stronger results for female horses (ten #2 and #3), which were also older, than the one castrated horse (ten#1). Although the population, n=3, is not large enough to consider this to be significant or not, a trend can be seen. The reasons for this may be associated with hormonal influence on the effects of FGF on tendon or an increased sensitivity or increased number of receptors on older animals. Interestingly enough, FGF demonstrated a slightly stronger response in dermal fibroblasts from the male horse (skin #1), but no difference in age was seen.

Equine tendon and dermal fibroblasts, when exposed to epidermal growth factor, responded opposite from the effects of FGF. A dose dependent decrease in pro-collagen type III synthesis was observed. When taken in the context of wound healing, this decrease could potentially be beneficial because it decreases the amount of type III collagen present, therefore allowing type I collagen synthesis to predominate. However, this is based on the assumption that type I collagen is being secreted at a sufficient amount to replace the originally secreted type III collagen into the wound. For example, Laato et al. (1987) observed the effects of EGF (1-100 μg/ml) on collagen type I and type III synthesis by granulation tissue fibroblasts cultured from subcutaneous sponge implants in rats. They observed a potentially positive effect of decreased collagen type III synthesis after 4 days incubation with FGF, but they also observed a decrease in type I collagen synthesis. Their results indicated that EGF actually significantly decreased the strength of the initial granulation tissue formation as compared to control animals. Unfortunately, the results on pro-collagen type I synthesis in this project concur with

Laato et al. results, in that type I pro-collagen was decreased from steady state levels when exposed to EGF. Therefore, EGF would not be a good candidate for in vivo wound healing in the context of extracellular matrix deposition because of its negative effects of pro-collagen type I and III synthesis.

The dose dependent decrease in pro-collagen type III is quite similar to what others have seen in human dermal and gingival fibroblasts (Huey et al., 1980; Steinmann et al., 1982; Hata et al., 1988). Although Hata et al. (1988) observed this decrease with EGF (50 ng/ml) alone after 4 days of incubation, when EGF and ascorbic acid (50 μg/ml) were combined and added to cells, they observed an increase in total collagen synthesis compared to controls. This synergistic effect is contradictory to what was observed in this study since ascorbic acid was also utilized. Ascorbic acid is a cofactor for the enzyme lysyl hydroxylase. This enzyme is necessary in the formation of hydroxylysine, a residue in collagen α chains necessary for crosslinking to occur amongst α chains. It is one of the limiting steps in collagen synthesis. This factor alone may explain why Hata et al. (1988) observed an increase in collagen type III production. Why a decrease in collagen synthesis was seen in this project, even in the presence of ascorbic acid, is unknown. But it may be related to convergence and integration of the signaling pathways for each of the compounds. EGF may inactivate ascorbic acid's collagen synthetic activity at a common point in their signal transduction pathways.

Different effects, compared to those seen in this study, have also been seen in tendon fibroblasts from other species. Twenty-eight day old chicken embryo tendon fibroblasts showed a significant increase in collagen type III synthesis when exposed to EGF (100 ng/ml) for 5 days (Gauger et al., 1985). The differences seen in this study may be

explained by the fact that these fibroblasts were from embryos that have been shown to heal differently as well as respond differently to growth factors. A rabbit deep digital flexor tendon fibroblast cell line showed a dose dependent increase in total collagen synthesis in the presence of EGF (10-100 ng/ml) for 6 days, with replenishment of growth factor and media every 2 days (Kang and Kang, 1999). The same has also been seen with rabbit ligament fibroblasts exposed to EGF for 24 hours (0.1-1.0 ng/ml)(Marui et al., 1997; Deie et al., 1997). The differences between these studies and mine include species of animal used, growth factor concentrations/doses, and methods of identifying collagen. Each of these studies utilized the incorporation of radiolabeled proline.

A trend for a stronger decrease in pro-collagen type III synthesis by fibroblasts from the skin of female horses (Skin #2 and #3) was observed compared to the male horse (skin #1). It is also interesting to note that the female horses were also older than the male horse. Therefore, sex and/or age may play a part in the response of EGF to dermal fibroblasts. A different response was seen with tendons. Only a slightly lower decrease in synthesis was observed for the castrated horse (ten #1) compared to the female horses (ten #2 and #3). These differences demonstrate that the effects of EGF as well as FGF, described above, can vary between age, sex, and location of cells.

Procedures and assay validation are crucial to any results observed during experimentation. Unfortunately, positive and negative controls were not used to validate if the antibody used in this study was specific. Positive controls would consist of obtaining pro-collagen type III and develop a standard curve through results obtained by the ELISA technique. Negative controls would consist of obtaining a cell line that is known not to secrete pro-collagen type III, or obtaining a cell line that is known not to

have altered pro-collagen type II synthesis when exposed to EGF or FGF. Comparisons between these data sets and those of published manuscripts also differ in the assay utilized. The majority of articles used northern or western blotting techniques which have become standard in the evaluation and quantitation of mRNA and proteins (Kang and Kang, 1999; Marui et al., 1997, Gauger et al., 1985; Hata et al., 1988). Therefore, the significant increases or decreases seen in their assays, compared to the results of this study, might be explained by this assay difference.

The results observed in this study are the first to demonstrate the effects of epidermal growth factor and fibroblast growth factor on pro-collagen type III synthesis by equine tendon and dermal fibroblasts. Although the present results differ from some previous reports, the differences could be due to the numerous variables involved including the methods utilized, and species, tissue type, cell type, and age of the animals used. In summary, pro-collagen type III synthesis was enhanced by fibroblast growth factor but depressed by epidermal growth factor in a dose-dependent manner for both equine tendon and dermal fibroblasts. Also, a greater increase in pro-collagen type III synthesis was observed in dermal fibroblasts than in tendon fibroblasts in relation to FGF exposure. This may be explained by the fact that dermal fibroblasts might express higher numbers of growth factor receptors, or may respond to a lower concentration of growth factor than tendon fibroblasts.

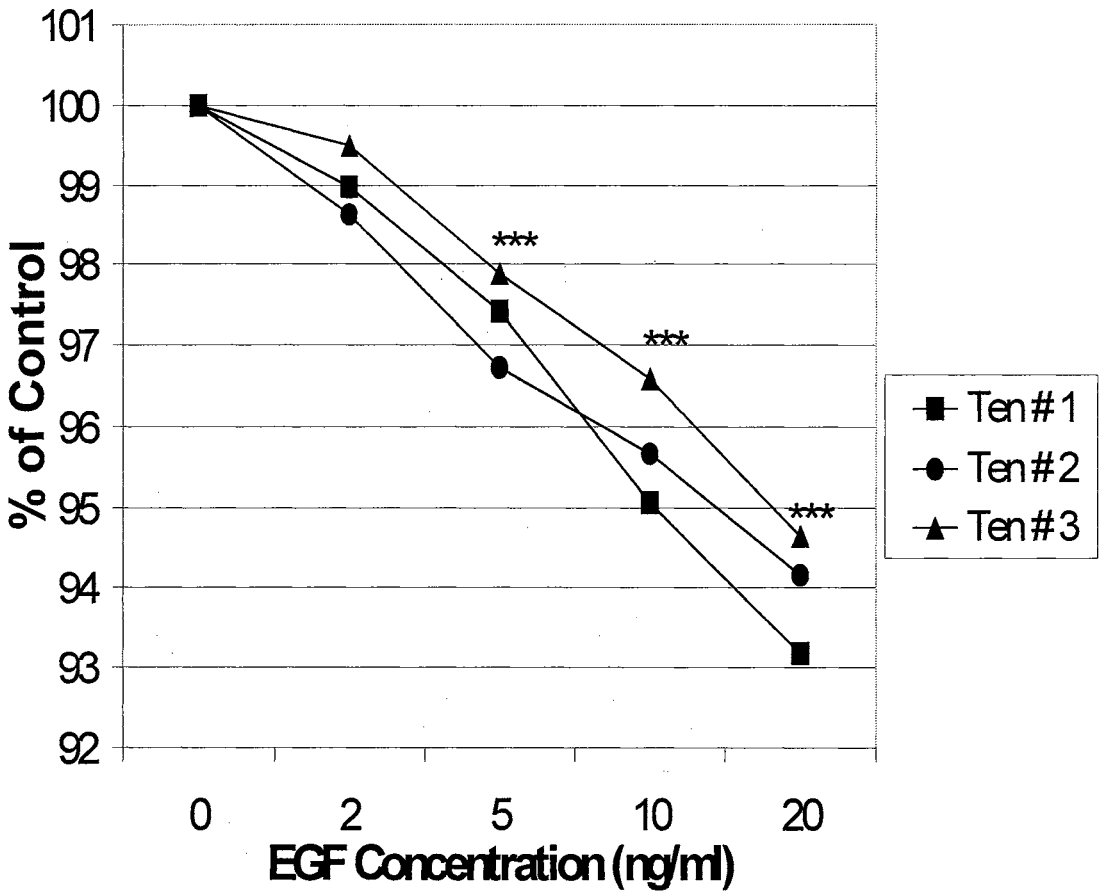

Figure 18. The effects of epidermal growth factor (EGF) on pro-collagen type III synthesis by equine tendon fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 4. (n=9, 12, 10 respectively for ten # 1, # 2, and # 3). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

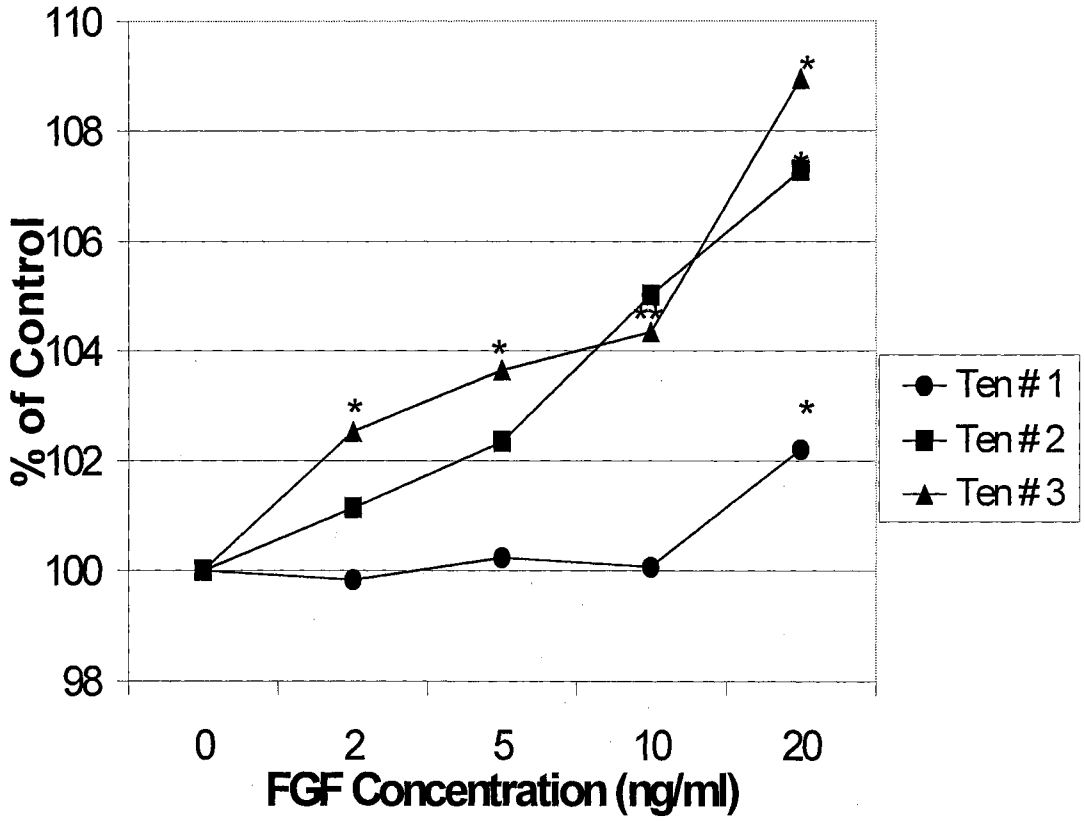

Figure 19. The effects of fibroblast growth factor (FGF) on pro-collagen type III synthesis by equine tendon fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 4. (n= 9, 12, 10 respectively for ten # 1, # 2, and # 3). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

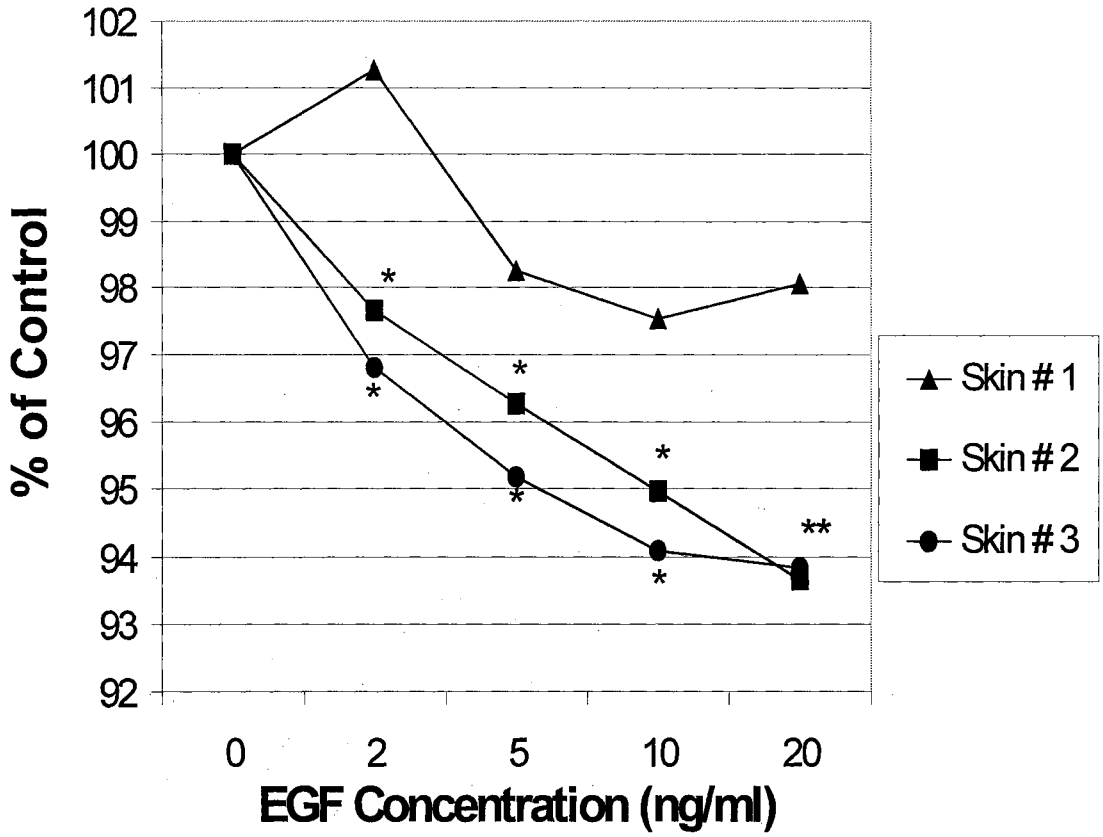

Figure 20. The effects of epidermal growth factor (EGF) on pro-collagen type III synthesis by equine dermal fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 5. (n= 8, 10, 12 respectively for skin # 1, # 2, and # 3). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

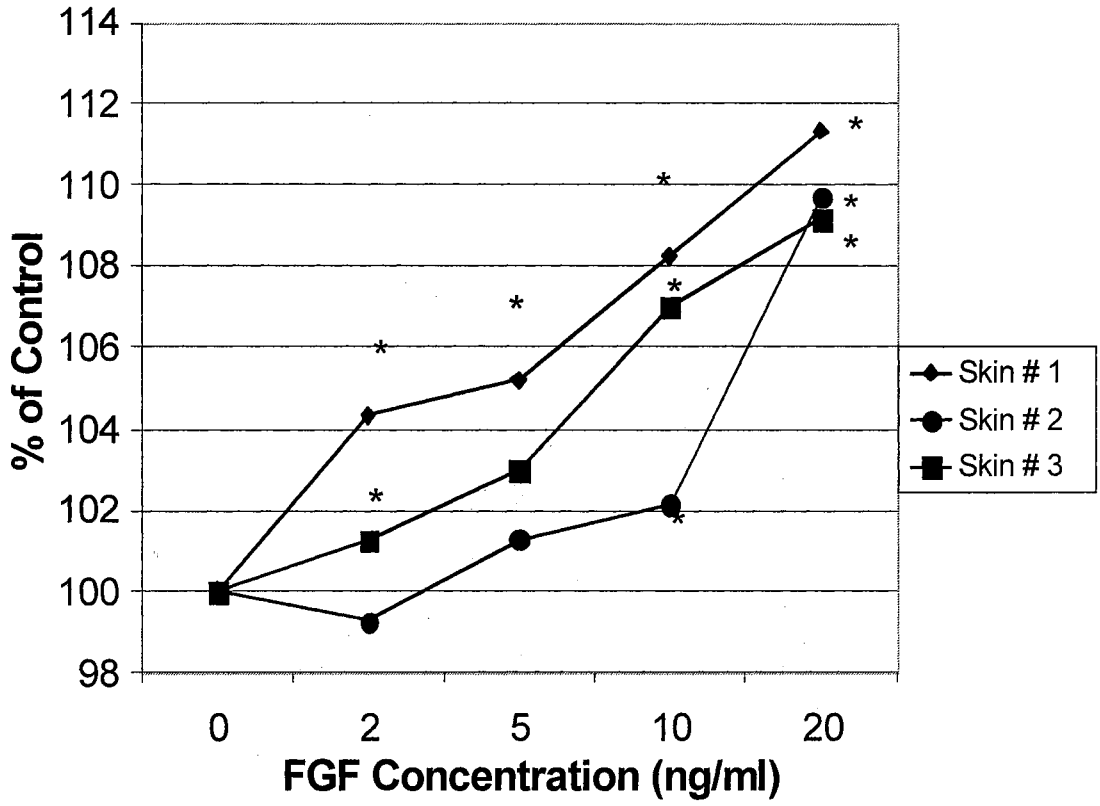

Figure 21. The effects of fibroblast growth factor (FGF) on pro-collagen type III synthesis by equine dermal fibroblasts. Results are expressed as percentage of control (0 ng/ml = 100%). Standard deviations are presented in Table 5. (n= 8, 10, 11 respectively for skin # 1, # 2, and # 3). Statistically significant difference between the control and experimental groups is indicated by * ($p < 0.05$).

EGF	0 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	N
Ten # 1	100 ± 0	98.9719 ±2.1481	97.4166 ±2.9893	95.0638 ±2.3951	93.176 ±2.135	9
Ten # 2	100±0	98.6259 ±3.0288	96.7233 ±1.9757	95.6752 ±2.4128	94.1675 ±3.0208	12
Ten # 3	100±0	99.4891 ±1.5937	97.8815 ±1.8529	96.578 ±1.7751	94.6559 ±3.4389	10
FGF	**0 ng/ml**	**2 ng/ml**	**5 ng/ml**	**10 ng/ml**	**20 ng/ml**	**N**
Ten # 1	100±0	99.828 ±2.0567	100.237 ±3.5118	100.072 ±2.337	102.198 ±2.6615	9
Ten # 2	100±0	101.136 ±3.7539	102.34 ±4.1017	105.035 ±3.1395	107.279 ±3.5729	12
Ten # 3	100±0	102.542 ±2.8106	103.651 ±3.794	104.356 ±2.6192	108.946 ±2.3316	10

Table 4. Mean percentages with standard deviations for pro-collagen type III synthesis in equine tendon fibroblasts exposed to epidermal growth factor (EGF) or fibroblast growth factor (FGF) for 24 hours. The control group (0 ng/ml = 100%) represents steady-state levels. N represents the number of data points per mean value in a given row.

EGF	0 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	N
Skin#1	100 ± 0	101.264 ±4.3229	98.2581 ±3.1698	97.5493 ±3.4908	98.0738 ±5.935	8
Skin#2	100±0	97.6584 ±2.8297	96.2716 ±3.7743	94.9617 ±3.5697	93.6701 ±3.572	10
Skin#3	100±0	96.8058 ±3.3306	95.1775 ±4.5619	94.0858 ±4.699	93.85 ±5.3746	12
FGF	**0 ng/ml**	**2 ng/ml**	**5 ng/ml**	**10 ng/ml**	**20 ng/ml**	**N**
Skin#1	100±0	104.349 ±4.5956	105.206 ±1.9092	108.242 ±3.42	111.335 ±5.2693	8
Skin#2	100±0	99.2724 ±2.3984	101.28 ±2.0952	102.159 ±2.242	109.687 ±3.256	10
Skin#3	100±0	101.268 ±1.8306	103.009 ±6.3548	106.996 ±8.228	109.158 ±8.7459	11

Table 5. Mean percentages with standard deviations for pro-collagen type III synthesis in equine dermal fibroblasts exposed to epidermal growth factor (EGF) or fibroblast growth factor (FGF) for 24 hours. The control group (0 ng/ml = 100%) represents steady-state levels. N represents the number of data points per mean value in a given row.

CHAPTER VII

SUMMARY

This study determined the effects of epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) on equine tendon and dermal fibroblasts in the context of fibroblast migration, proliferation and collagen synthesis (type I and type III).

The horse model was chosen because of the lack of data on growth factor involvement in wound healing either in vitro or in vivo. Three cell lines of intrinsic fibroblasts from the superficial digital flexor tendon were successfully established. This tendon was chosen because it is commonly damaged in traumatic events, which performance horses are more subjected to (Bertone, 1995). I was especially interested in determining if growth factors could enhance the activities of these intrinsic cells, since they are the original synthesizers of the tendon proper. Three cell lines of dermal fibroblasts from the skin overlying the flexor tendon were also successfully established. The skin was evaluated because it too is damaged by traumatic events to the lower extremities of performance horses. It was also studied because of the notion that equine skin on the extremities heals slower that other parts of the body (Jacobs et al., 1984). A summary of the results for each growth factor and their impact on proliferation, chemotaxis, collagen type I and collagen type III synthesis, on equine tendon and dermal fibroblasts are presented in tables 6 and 7.

Fibroblast growth factor significantly increased the chemotactic activity of both equine dermal and tendon fibroblasts in a dose dependent fashion, but was unable to enhance proliferative activity. In the context of wound healing, FGF would be able to stimulate migration of more fibroblasts to the wound site. Once fibroblasts reached the site, their proliferative activity would not be increased by FGF. FGF significantly increased the amount of type I and type III collagen synthesized by dermal and tendon

fibroblasts. This combined effect could be detrimental by potentially leading to exuberant granulation tissue formation. Excess granulation tissue would lead to increased healing times, and a higher chance of hemorrhaging and wound infection (Bertone, 1989). It appears that this potential problem would be worse in the skin. The dermal fibroblasts demonstrated a higher percent increase in pro-collagen type I and III synthesis by FGF than tendon fibroblasts.

The dermal fibroblasts responded to, or were more sensitive to FGF for chemotaxis and pro-collagen synthesis, based on the low concentrations at which significant changes were observed. In the skin, most significant responses were observed at 2 ng/ml, whereas in the tendon most responses occurred between 5 and 10 ng/ml, a 2 to 5 fold difference.

Both fibroblast populations responded similarly to epidermal growth factor in chemotactic and proliferative assays, with a significant dose dependent increase in chemotaxis, yet no significant response in proliferative activity. One tendon cell line (ten #2) showed a significant increase in proliferative activity at the highest EGF concentration used (20 ng/ml). This result may represent a spurious data point, or the lowest concentration at which EGF had a significant effect on proliferative activity of gelding equine cells relative to other species or age of the animal. The chemotactic response was significantly increased at 2 ng/ml of EGF for both cell lines. EGF significantly decreased collagen type I and type III synthesis compared to steady-state levels. In the context of wound healing, EGF would dramatically increase wound healing time because less collagen would be available to form a scaffold/foundation. Less collagen content would lead to a higher chance of dehiscence of wound edges because of

the lack of tensile strength, provided by collagen, necessary to compensate for normal movement of skin and tendon wound edges. Dermal fibroblasts responded significantly at a lower concentration (2 ng/ml) of EGF than tendon fibroblasts (5 ng/ml) for both collagen types. However, a similar percent decrease in both collagen types was observed at the highest concentration (20 ng/ml) of EGF for both fibroblast populations. This response is different than what was seen with FGF in regard to percent differences between cell populations. Dermal fibroblasts demonstrated a larger percentage change than tendon fibroblasts.

Trends, based on age and gender, were observed for each of the assays performed. However, the data was not thoroughly evaluated by statistical analysis because of the low population number. Male dermal fibroblasts and female tendon fibroblasts appeared to have a higher rate of proliferation, in the proliferative assay, for both EGF and FGF. Dermal fibroblasts from younger animals also appeared to have a higher rate of proliferation in the proliferative assay. However, this trend was not observed for tendon fibroblasts.

In the chemotaxis assay, the youngest horses for both skin and tendon demonstrated the highest number of cells which migrated when exposed to FGF. However, only a slight trend for increased migration was observed for EGF on the tendon cell population. No gender trends were observed for either cell line exposed to EGF. But the gelding tendon fibroblasts demonstrated a higher chemotactic response in comparison to the female tendon cell lines.

In the pro-collagen type I assay, EGF caused a larger overall decrease in synthesis for female tendon and dermal fibroblasts. Also, the oldest female in both tendon and

dermal cell lines demonstrated the largest decrease in synthesis. FGF produced a larger increase in procollagen type I in the gelding tendon fibroblasts, however no gender trend was observed for dermal fibroblasts. Age did not appear to affect FGF's ability to enhance collagen type I synthesis.

In the pro-collagen type III assay, a mixture of male and female trends were observed. Female tendon fibroblasts, which happened to be from the oldest animals, demonstrated a larger increase in collagen type III synthesis when exposed to FGF, however the young, male dermal fibroblasts demonstrated a slightly higher synthetic rate than females when exposed to FGF. Female dermal fibroblasts demonstrated a larger decrease in collagen production, however male tendon fibroblasts demonstrated a slightly larger decrease in collagen when exposed to EGF. No age trend was observed for either cell type when exposed to EGF.

Overall, it appears that female horses responded more strongly to growth factors in the majority of the assays performed. This difference may be associated with the affect sex hormones have on cell function in vitro. Age correlations were not observed across the different assays however.

In these studies, growth factors were not combined. But to speculate on possible results may provide insight into future studies. Although no significant differences were observed for the proliferation assays performed for either growth factor, there is a chance, albeit a slight one, that a synergistic effect might occur. This hypothesis is based on the concept that the concentration of growth factors used in this study, when combined together, may exceed the threshold needed to activate the mitogenic signal transduction pathway above which is normally activated in the presence of serum supplemented media

only. Chemotaxis was significantly enhanced in the presence of either growth factor. There is a possibility that a stronger response would be observed if a combination of growth factors were used, since each growth factor would utilize its own set of receptors, therefore compounding the chemotactic effects. Collagen synthesis, however, is an interesting story in itself. The results clearly showed that EGF decreased collagen type I and type III synthesis compared to steady-state levels, and FGF increased collagen type I and III synthesis compared to steady-state levels. The ability of FGF to enhance type I collagen synthesis in both cell lines in a dose-dependent manner was exciting, because the ability to enhance type I synthesis potentially means the ability to heal wounds sooner. Unfortunately, type III collagen was also enhanced. EGF was shown in this study to decrease collagen type I and III synthesis. If EGF was added at a concentration that may counter-act the affects of FGF on type III synthesis, but at the same time still allow a significant amount of collagen type I synthesized, then there is a possibility to enhance wound healing. Using the doses obtained in this project, it appears that there is a possibility of this occurring with dermal fibroblasts (10 ng/ml for each of EGF and FGF, or 20 ng/ml each of EGF and FGF). However, it appears that tendon fibroblasts would not respond similarly (tables 2, 3, 4, and 5 [Chapters V, VI]).

And finally, assay validation is necessary in determining if an assay functions properly or not. Unfortunately, positive and negative controls were not used for the pro-collagen assays in chapters V and VI. The antibodies used in these assays have not been used on horse tissues prior to these experiments. Fortunately, dose dependent changes were observed that helped to partially validate positive reactions were occurring. Also, pro-collagens I and III were not available to establish a standard curve on which to

extrapolate known concentrations of these pro-collagens. These values may have helped demonstrate a higher level of pro-collagen synthesis by the fibroblasts, than by converting results to percentages and comparing these numbers to each other.

This is the first study of the effects of fibroblast growth factor or epidermal growth factor on equine tendon and dermal fibroblasts in respect to proliferation, migration, collagen type I and collagen type III synthesis. Future aspects of this project may include the use of higher doses of growth factors to determine if plateau levels exist, or to determine if mitogenic responses can occur. Combining growth factors could be used to potentially enhance the results observed in these studies. Synthetic serum or serum free media could be used to eliminate unknown variables present in serum, that could potentially alter the results of the assays performed. For the chemotaxis assay, the use of higher concentrations of gelatin may be helpful in enhancing attachment and migration of cells through the porous membrane. And finally to utilize more highly sensitive, qualitative and quantitative assays, such as northern blot analysis for the collagen assays or thymidine incorporation for the mitogenic assays, to verify the results obtained in these studies. The results of these studies could then eventually be expanded into *in vivo* studies, where when placed into a complex, multifactorial environment, the effects of growth factors on wound healing can be better determined, and results compared to those obtained in this project.

Cell Line	Mitogenesis	Chemotaxis	Pro-collagen Type I	Pro-collagen Type III
Skin #1 Sex: M Age: 6 years	none	(+) 2, 5, 10, 20	(-) 2, 5, 10, 20	none
Skin #2 Sex. F Age: 8 years	none	(+) 2, 5, 10, 20	(-) 2, 5, 10, 20	(-) 2, 5, 10, 20
Skin #3 Sex: F Age: 5 years	none	(+) 2, 5, 10, 20	(-) 2, 5, 10, 20	(-) 2, 5, 10, 20
Ten #1 Sex: MC Age:6-8 year	none	(+) 2, 5, 10, 20	(-) 10, 20	(-) 5, 10, 20
Ten #2 Sex: F Age: 11 year	(+) 20	(+) 2, 5, 10, 20	(-) 2, 5, 10, 20	(-) 5, 10, 20
Ten #3 Sex: F Age: 8 years	none	(+) 2, 5, 10, 20	(-) 5, 10, 20	(-) 5, 10, 20

Table 6. Epidermal growth factor summary table. The concentration(s) (ng/ml) of epidermal growth factor (EGF) that showed statistically significant changes ($p < 0.05$) (+ = increase, - = decrease) on equine tendon (ten) and dermal (skin) fibroblasts as compared to control(s) (0 ng/ml).

Cell Line	Mitogenesis	Chemotaxis	Pro-collagen Type I	Pro-collagen Type III
Skin #1 Sex: M Age: 6 years	none	(+) 2, 5, 10, 20	(+) 2, 5, 10, 20	(+) 2, 5, 10, 20
Skin #2 Sex: F Age: 8 years	none	(+) 2, 5, 10, 20	(+) 2, 5, 10, 20	(+) 10, 20
Skin #3 Sex: F Age: 5 years	none	(+) 2, 5, 10, 20	(+) 10, 20	(+) 2,10, 20
Ten #1 Sex: MC Age:6-8 year	none	(+) 2, 5, 10, 20	(+) 20	(+) 20
Ten #2 Sex: F Age: 11 year	none	(+) 5, 10, 20	(+) 20	(+) 10, 20
Ten #3 Sex: F Age: 8 years	none	(+) 5, 10, 20	(+) 5, 10, 20	(+) 2, 5, 10, 20

Table 7. Fibroblast growth factor summary table. The concentration(s) (ng/ml) of basic fibroblast growth factor (FGF) that showed statistically significant changes ($p < 0.05$) (+ = increase, - = decrease) on equine tendon (ten) and dermal (skin) fibroblasts as compared to control(s) (0 ng/ml).

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