REVIEW ARTICLE

Sports Med 2003; 33 (5): 381-394
0112-1642/03/0005-0381/$30.00/0

The Roles of Growth Factors in Tendon and Ligament Healing

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Abstract

Tendon healing is a complex and highly-regulated process that is initiated, sustained and eventually terminated by a large number and variety of molecules. Growth factors represent one of the most important of the molecular families involved in healing, and a considerable number of studies have been undertaken in an effort to elucidate their many functions. This review covers some of the recent investigations into the roles of five growth factors whose activities have been best characterised during tendon healing: insulin-like growth factor-I (IGF-I), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). All five are markedly up-regulated following tendon injury and are active at multiple stages of the healing process. IGF-I has been shown to be highly expressed during the early inflammatory phase in a number of animal tendon healing models, and appears to aid in the proliferation and migration of fibroblasts and to subsequently increase collagen production. TGFβ is also active during inflammation, and has a variety of effects including the regulation of cellular migration and proliferation, and fibronectin binding interactions. VEGF is produced at its highest levels only after the inflammatory phase, at which time it is a powerful stimulator of angiogenesis. PDGF is produced shortly after tendon

damage and helps to stimulate the production of other growth factors, including IGF-I, and has roles in tissue remodelling.

In vitro and *in vivo* studies have shown that bFGF is both a powerful stimulator of angiogenesis and a regulator of cellular migration and proliferation. This review also covers some of the most recent studies into the use of these molecules as therapeutic agents to increase the efficacy and efficiency of tendon and ligament healing. Studies into the effects of the exogenous application of TGFβ, IGF-I, PDGF and bFGF into the wound site singly and in combination have shown promise, significantly decreasing a number of parameters used to define the functional deficit of a healing tendon. Application of IGF-I has been shown to increase in the Achilles Functional Index and the breaking energy of injured rat tendon. TGFβ and PDGF have been shown separately to increase the breaking energy of healing tendon. Finally, application of bFGF has been shown to promote cellular proliferation and collagen synthesis *in vivo*.

Tendons are the connective tissue that attach muscle to bone, and allow the transduction of force of a contracting muscle to be exerted via the attached skeletal structure.[1] They consist primarily of water and type I collagen, with smaller amounts of other collagens and matrix materials, and various types of cells, most notably fibroblasts.

The process of tendon healing represents an interesting paradigm for medical science. Although most tendons have the ability to heal spontaneously after injury, the scar tissue that is formed is almost always mechanically inferior and therefore much less able to perform the functions of a normal tendon, and is also more susceptible to further damage.[2] Because the formulation of effective treatments for tendon injuries based on traditional tissue-level reparative procedures, surgical or otherwise, has presented such a problem to clinicians in the field, much research has been directed toward the understanding of the mechanisms of tendon healing at the molecular level. This has ultimately been in an effort to develop therapies to facilitate tendon healing through the use of individual molecules or groups of molecules known to have beneficial roles in the process.

The process of tendon healing follows a pattern similar to that of other healing tissues (table I).[2] Upon tissue damage, blood vessels will rupture and signalling molecules released by intrinsic cells will trigger a coagulation cascade that will coordinate the formation of a clot around the injured area. The clot will contain cells and platelets that will immediately begin to release a variety of molecules, most notably growth factors (such as platelet-derived growth factor [PDGF], transforming growth factor β [TGFβ], and insulin-like growth factor [IGF]-I and -II), causing acute local inflammation. During this inflammatory phase, there is an invasion by extrinsic cells such as neutrophils and macrophages which clean up necrotic debris by phagocytosis, and together with intrinsic cells (such as endotenon and epitenon cells) produce a second battery of cytokines to initiate the reparative phase. This stage sees collagen deposition and granulation tissue formation, as well as neovascularisation, extrinsic fibroblast migration and intrinsic fibroblast proliferation. These fibroblasts are responsible for synthesising the new extracellular matrix, consisting largely of collagens and glycosaminoglycan. Finally, a remodelling phase begins, which sees decreases in the cellular and vascular content of the callus tissue, and increases in collagen type I content and density. Eventually, the collagen will become more organised and is orientated and cross-linked with the healthy matrix outside the injury area. After the healing process is complete, cellularity, vascularity, and collagen makeup will return to something approximating that of the normal tendon, although the diameters and cross-linking of the collagen fibrils often remain inferior after healing.[3] This mechanically inferior

Table I. Summary of the healing process in tendons and ligaments

Time (days)	Phase	Process
0	Immediately post-injury	Clot formation around the wound
0–1	Inflammatory	First battery of growth factors and inflammatory molecules produced by cells within the blood clot
1–2	Inflammatory	Invasion by extrinsic cells, phagocytosis
2–4	Proliferation	Further invasion by extrinsic cells, followed by a second battery of growth factors that stimulate fibroblast proliferation
4–7	Reparative	Collagen deposition; granulation tissue formation; revascularisation
7–14	Reparative	Injury site becomes more organised; extracellular matrix is produced in large amounts
14–21	Remodelling	Decreases in cellular and vascular content; increases in collagen type I
21+	Remodelling	Collagen continues to become more organised and cross-linked with healthy matrix outside the injury area. Collagen ratios, water content and cellularity begin to approach normal levels

repair tissue is weaker and more susceptible to tendon creep than uninjured tendon, and is therefore at higher risk of further damage.

The description of tendon healing above is somewhat generalised, and it is important to note that there are slight differences in the way different tendons heal, for example intrasynovial versus extrasynovial tendons. Whereas extrasynovial tendons can be easily influenced by growth factors and cytokines produced by extrinsic cells, for example from the paratenon, intrasynovial tendons are more reliant on intrinsic cells such as those derived from the epitenon and endotenon. These differences are most probably due to differences in the local environment and the ease with which needed growth factors can be provided to the injured area.[4] Although cells that originate from different regions of the tendon can have somewhat different influences during tissue repair, for most types of tendon both extrinsic and intrinsic cells will contribute to healing.[5]

Growth factors have a number of crucial roles in tendon healing. Growth factors such as TGFβ, IGF-I, PDGF, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are markedly up-regulated throughout tendon repair. They can potentially be produced by both intrinsic (for example epitenon) and extrinsic (for example macrophage) cells, often have dose-dependent effects, require specific receptors to be active, and usually work in synergy with other signalling molecules. Almost all are up-regulated when transcriptional factors (such as early growth response-1, which stimulates the production of acidic fibroblast growth factor, bFGF, TGFβ, PDGF, hepatocyte growth factor, VEGF and IGF-II, among others[6]), bind to their (often common) regulatory sites.[7] Whilst a large amount of data on these molecules have been produced in recent years, much work still needs to be undertaken to fully understand their varied functions and multiple synergies. This review will cover some of the more recent studies on the functions and clinical applications of five of the best studied growth factors during tendon healing: IGF-I, TGFβ, VEGF, PDGF and bFGF.

1. Characterisational Studies

Growth factors represent one of the largest of the molecular families involved in the healing process, and a considerable number of studies have been undertaken in an effort to elucidate their many functions and behaviours during healing progression (table II). Some of this work, with a focus on IGF-I, TGFβ, VEGF, PDGF, and bFGF, is outlined below.

1.1 Insulin-Like Growth Factor-I (IGF-I)

IGF-I is a single chain polypeptide that shows structural homology to proinsulin, and is involved in both normal body growth and healing.[26] It binds to two types of receptors, type I IGF receptor and type II mannose-6-phosphate receptor,[27] and is regulated by a group of specific IGF binding proteins.[28] It is an important mediator in all phases of wound

Table II. Summary of the roles of five growth factors during tendon and ligament healing

Growth factor	Phase in which growth factor is most active	Roles	Reference
IGF-I	Inflammation, proliferation	Promotes the proliferation and migration of cells, stimulates matrix production	8-13
TGFβ	Inflammation	Regulates cell migration, proteinase expression, fibronectin binding interactions, termination of cell proliferation, and stimulation of collagen production	14-19
VEGF	Proliferation, remodelling	Promotes angiogenesis	20,21
PDGF	Proliferation, remodelling	Regulates protein and DNA synthesis at the injury site, regulates the expression of other growth factors	10,22
bFGF	Proliferation, remodelling	Promotes cellular migration, angiogenesis	23-25

bFGF = basic fibroblast growth factor; **IGF-I** = insulin-like growth factor-I; **PDGF** = platelet-derived growth factor; **TGF**β = transforming growth factor; **VEGF** = vascular endothelial growth factor.

healing, particularly during the inflammatory and proliferative stages.[8] Injured tissues lacking the growth factor are significantly disadvantaged in healing.[29] Several studies[8,30-34] have shown that IGF-I is locally increased during and after inflammation following soft tissue injury, both at the mRNA and protein levels, and is associated with a corresponding up-regulation of its receptors.[35] Sciore et al.[8] demonstrated that IGF-I mRNA levels were more than 5-fold higher compared with controls 3 weeks after injury to the rabbit medial collateral ligament (MCL), then decreased (yet still remained at levels twice of that of the control) by weeks 6 and 14 (figure 1). Hansson et al.[9] showed that this up-regulation could also be seen at the protein level.

Because IGF-I is such a versatile and widespread signal molecule, it has numerous and varied activities during tendon healing, particularly when working in concert with other growth factors.[10] Its primary roles seem to be to stimulate the proliferation and migration of fibroblasts and other cells at the site of injury, and to subsequently increase the production of collagens and other extracellular matrix structures in these cells during the remodelling stages.[11,12] This proliferative activity was demonstrated by Jones and Clemmons[11] in various cell types, including fibroblasts. The ability of IGF-I to stimulate cells to produce collagen and fibronectin *in vitro* has been shown in rat calvarial cultures.[13]

As with many other cytokines, synergism with other molecules is important for its stimulatory activity. It is thought that IGF-I works to promote cell proliferation when in the presence of other growth factors, such as the PDGF isomer PDGF-BB, discussed in section 1.4. This was shown in *in vitro* work by Tsuzaki et al.[30] in which mitogenesis and subsequent cell division of tendon fibroblasts and tendon surface cells was highest when both growth factors were applied together, compared with their individual application.

It is also interesting to note that Tsuzaki et al.[30] observed that normal avian flexor tendon cells con-

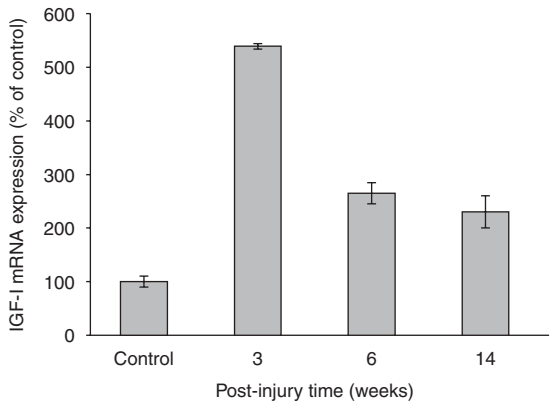

Fig. 1. Semi-quantitative reverse transcription polymerase chain reaction analysis of insulin-like growth factor-I (IGF-I) mRNA expression (expressed as percentage of control) from the injured medial collateral ligament of the New Zealand white rabbit at 3 weeks (n = 4; eight ligaments), 6 weeks (n = 4; eight ligaments), and 14 weeks (n = 4; eight ligaments) post-injury, and uninjured controls (n = 3; six ligaments). All were found to be significantly different from controls by analysis of variance ($p < 0.05$) [reproduced from Sciore et al.,[8] with permission from Elsevier Science].

tained a relatively high abundance of IGF-I protein as quantified by radioimmunoassay, but expression of IGF-I mRNA measured by reverse transcription polymerase chain reaction was very low. This can be explained by the observation that low-level expression of the gene within the normal tendon produces IGF-I protein which is immediately bound by specific binding proteins (such as binding protein-3). These binding proteins keep IGF-I in an inactive form and protect it from degradation. It was suggested that this reservoir of inactivated IGF-I protein is kept extracellularly until tissue injury occurs, at which time enzymes are released that free the bound IGF-I, activating it. This strategy of synthesising a comparatively small number of enzymes to activate a large reservoir of inactive regulatory molecules ensures a rapid response to tissue injury.

1.2 Transforming Growth Factor β (TGFβ)

TGFβ has shown to be active in almost all stages of tendon healing[14] and has such varied effects as stimulating extrinsic cell migration, regulation of proteinases,[15] fibronectin binding interactions,[16] termination of cell proliferation via cyclin-dependent kinase inhibitors[17] and stimulation of collagen production.[18] Three 25-kDa homodimeric mammalian isoforms exist (β1, β2 and β3), and studies in knockout mice have shown that each of these gives rise to a distinct phenotype.[36] They can be produced by most cells involved in the healing process[14] and bind to three distinct classes of membrane receptors, RI, RII and RIII.[37]

TGFβ-1 mRNA expression has been shown to dramatically increase a short time after tendon injury (figure 2) and is thought, in particular, to play an important role in the initial inflammatory response to tissue damage. Studies using lactate, one of the earliest mediators in wound healing due to its rapid build-up during tissue hypoxia, showed that it had the ability to directly stimulate TGFβ-1 production in flexor tendon cells.[5] Natsu-ume et al.[19] demonstrated a significant and early elevation in TGFβ-1 levels in the healing rat patellar ligament which remained high for at least 8 weeks. Immunohistochemical methods showed that initially the

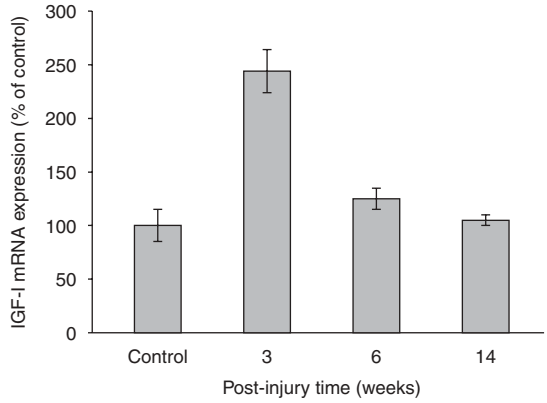

Fig. 2. Semi-quantitative reverse transcription polymerase chain reaction analysis of transforming growth factor β (TGFβ-1) mRNA levels (expressed as percentage of control) from the injured medial collateral ligament of the New Zealand white rabbit at 3 weeks (n = 4; eight ligaments), 6 weeks (n = 4; eight ligaments), and 14 weeks (n = 4; eight ligaments) post-injury, and uninjured controls (n = 3; six ligaments). TGFβ-1 mRNA levels at week 3 only were significantly different from controls by analysis of variance ($p < 0.05$) [reproduced from Sciore et al.,[8] with permission from Elsevier Science].

TGFβ-1 was extracellular, probably due to degranulation by platelets, but later was cell-associated, reflecting *de novo* synthesis.

Klein[38] further showed in work on cultured rabbit sheath, epitenon and endotenon cells that each of the three TGFβ isoforms (TGFβ-1, -2 and -3) has effects on collagen production and cell viability. All three isoforms at two different concentrations (1 and 5 μg/L) decreased the number of cultured cells compared with controls; however, the differences did not reach statistical significance. Production of collagen types I and III (the most abundant types of collagen found in tendon) was significantly increased ($p < 0.05$) in all cell types, although higher growth factor concentration was generally not correlated with further increases in production.

Growth factors lack biological activity unless they bind to their specific receptors, so it follows that TGFβ receptors are also seen to be up-regulated during tendon healing. During healing of transected middle digit flexor digitorum profundus tendons, Ngo et al.[37] used immunohistochemical staining to show up-regulation of all three classes of TGFβ receptor proteins. Levels peaked at postoperative

day 14, and had decreased by day 56. They were abundant along both the tendon sheath and epitenon, suggesting that both intrinsic and extrinsic mechanisms of tendon healing were active in this model.

Similar to other growth factors, TGFβ-1 works in a dose-dependent manner and in synergy with other growth factors.[39] For example, *in vitro* studies on canine anterior cruciate ligament fibroblasts showed that low doses of TGFβ-1 act positively with the PDGF isomer PDGF-AB to promote fibroblast proliferation, whereas at increased concentrations, this was reversed.[20]

1.3 Vascular Endothelial Growth Factor (VEGF)

The growth factors discussed in sections 1.1 and 1.2 become active almost immediately following tissue injury and continue to regulate the function of various processes at almost all phases of healing. However, this early and almost continuous activation is not common to all growth factors. VEGF for example, while having some role in early cellular migration and proliferation, is most active after inflammation, most notably during the proliferative and remodelling phases where it has been shown to be a powerful stimulator of angiogenesis.[40] A number of different isoforms of VEGF exist which appear to have unique biological functions, although all bind to three structurally related receptor tyrosine kinases called VEGF receptor (VEGFR)-1, -2, and -3.[41] The proliferative and mitogenic activities of VEGF chiefly depend on its interactions with VEGFR-2.[42]

Expression of the VEGF gene can be up-regulated in response to both biological and biomechanical stimuli, including hypoxia,[43] other growth factors,[21] interleukins,[43] and, during osteogenesis, bone distraction. Increased levels of angiogenic growth factors such as VEGF within an injury site are correlated with a well-defined pattern of vascular ingrowth from the epi- and intra-tendinous blood supply toward the site of repair. This neovascularisation proceeds along the surface of the epitenon, through a normally avascular area, and provides extrinsic cells, nutrients, and growth factors to the injured area.

Boyer et al.[20] quantified VEGF mRNA levels in the canine intrasynovial flexor tendon at various time points following tendon transection using Northern blot analyses (figure 3). It was found that at days 0 and 4 following injury, levels remained approximately at baseline, which was followed by a peak at day 7 (with levels at approximately 210% that of normal), and then a steady decline back to baseline by day 21. This kind of temporal expression profile is consistent with observed neovascularisation in and around the tendon repair site following inflammation. For example, studies by Gelberman et al.[21] on the canine flexor tendon showed an increase in vessel length and density starting from post-operative day 3, which peaked at day 17, and was followed by a decrease in vessel density at day 28.

Using *in situ* hydridisation on the injured canine flexor tendon, the spatial pattern of VEGF expression has also been determined.[40,44] VEGF mRNA accumulation was detected in around 67% of cells at the injury site, whereas only 10% of the epitenon cells directly adjacent showed accumulation, and had levels comparable to those epitenon cells distant from the site of repair. This extremely apparent stratification of gene expression between adjacent

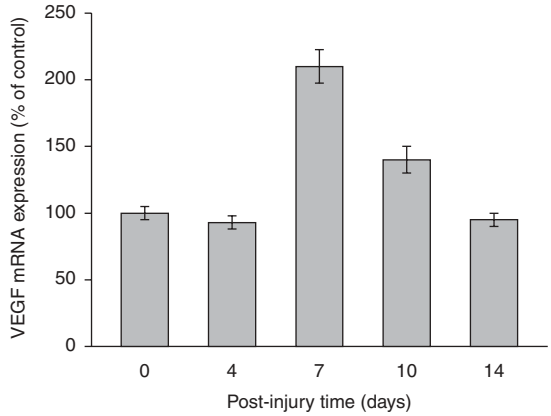

Fig. 3. Northern Blot analyses of vascular endothelial growth factor (VEGF) mRNA expression in the canine flexor tendon post injury, compared with controls. A statistically significant elevation in VEGF mRNA was shown at days 7 and 10 post-injury ($p < 0.05$) [reproduced from Boyer et al.,[20] with permission from Elsevier Science].

cells effectively demonstrates how highly coordinated the mechanisms of healing are. This coordination is brought about largely through the specificity of action and tight regulation of growth factors and other molecules during each of the phases of healing.

1.4 Platelet-Derived Growth Factor (PDGF)

PDGF describes a group of dimeric polypeptide isoforms made up from three types of structurally similar subunits. Its activity is mediated through its interaction with two related tyrosine kinase receptors, one of which binds all three PDGF chains, and the other binds only one.[45] Work by Duffy et al.[44] has shown that PDGF is elevated in the healing canine digital flexor tendon, suggesting a role in the healing process. It is thought to play a significant role in the early stages of healing, at which time it induces the synthesis of other growth factors, such as IGF-I.[10] *In vitro* studies by Yoshikawa and Abrahamsson[46] on PDGF have further demonstrated that this growth factor also plays an important role during tissue remodelling. PDGF was observed to stimulate both collagen and non-collagen protein production, as well as DNA synthesis, in a dose-dependent manner.

One theory that has been put forward as to how PDGF increases protein production involves its induction of TGFβ-1 expression.[22] However, *in vivo* studies by Hildebrand et al.[47] in which the PDGF isomer PDGF-BB was applied to the healing MCL of the rabbit with and without TGFβ-1 showed no such complementary effect. In fact, addition of PDGF-BB and TGFβ-1 together resulted in poorer healing (as determined by ultimate load, energy absorbed to failure, and ultimate elongation values) than addition of PDGF-BB alone. Stimulation of DNA synthesis by PDGF has also been postulated to occur through a growth factor second messenger. In this case, increases in PDGF have been shown to result in up-regulation of IGF-I and IGF receptors, that once activated stimulate DNA synthesis.[10] A significant amount of the PDGF produced for this end is thought to come from an exogenous source, probably from platelets.[30]

Interestingly, the level of stimulation has been shown to be specific to the site and type of tendon examined. In studies by Yoshikawa and Abrahamsson[46] DNA synthesis was stimulated to higher levels in intermediate compared with intrasynovial tendons, and protein synthesis was higher in proximal intrasynovial tendon segments than in extrasynovial peroneal tendon segments.

1.5 Basic Fibroblast Growth Factor (bFGF)

The final growth factor that will be discussed here is bFGF. It is a single chain polypeptide composed of 146 amino acids, and is a member of the heparin-binding growth factor family.[35] Through its interaction with a number of isoforms of four cell surface receptors,[48] it has been shown to be a potent stimulator of angiogenesis and cellular migration and proliferation in both *in vivo* and *in vitro* studies.[23]

Stimulation of cellular migration and proliferation by bFGF has been demonstrated by Chan et al.[49] using cultured rat patellar tendon fibroblasts. In this study, an '*in vitro* wound' was created by mechanically generating a uniform cell-free zone in a culture dish. The progression of closure of the *in vitro* wound was measured at various time points after the addition of four different concentrations of bFGF, ranging from 0–50 μg/L. It was observed that the addition of as little as 2 μg/L of bFGF accelerated the rate at which wound closure progressed, and a concentration of 10 μg/L was most effective. Cellular proliferation was confirmed as the mechanism of wound closure and distinguished from cell chemotaxis by the measurement of 5-bromo-2′-deoxyuridine incorporation.

A later study by Chang et al.[25] used a rabbit flexor tendon model to localise and quantify bFGF mRNA during tendon healing. *In situ* hybridisation showed that bFGF expression was increased in both the tendon parenchyma and the tendon sheath from the first postoperative day, and remained elevated up to the last time point, day 56. The highest levels of expression within the tendon came from intrinsic tenocytes and fibroblasts migrating from the epitenon, along the edge of the wound. Inflammatory

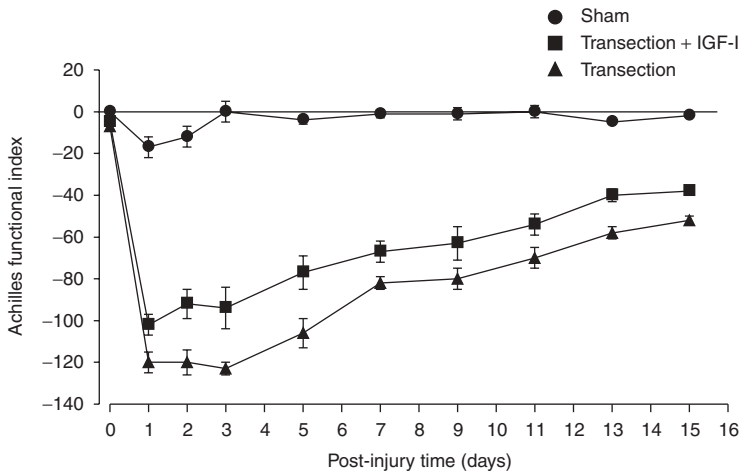

Fig. 4. 15-day time course showing the effects of insulin-like growth factor-I (IGF-I) [± SEM] on the Achilles Functional Index of the rat following tendon transection. Within a given time point all groups are significantly different ($p < 0.05$) [reproduced from Kurtz et al.,[50] with permission].

leucocytes and fibroblasts in the surrounding synovial tendon sheath also displayed high bFGF messenger levels. These observations again suggest that both intrinsic and extrinsic cells are important in tendon healing as sources of growth factors.

2. *In Vivo* Studies

Shortly after the initial investigations which discovered and characterised some of the more important growth factors employed in tendon and ligament healing, clinical studies commenced in a variety of animal models. Because tendon healing is a complex process involving the interaction of a large number of different molecules, cells and tissues, results have often been unpredictable and disappointing. However, some success has been achieved, which suggests that the speed and quality of tendon healing may eventually be improved by the application and/or regulation of growth factors and other molecules. The major challenges seem to be in predicting the synergies and antagonisms among growth factors and between growth factors and other molecules, and how to temporally and spatially apply different growth factors for best effect. Another major technical challenge common to all *in vivo* studies is the delivery of the therapeutic molecules to the target cells in a specific and sustained manner.

In vivo use of TGFβ, IGF-I, PDGF, bFGF, singly and in combination has shown some promise in recent years. The following is a brief summary of the most recent *in vivo* work for each of these growth factors.

2.1 IGF-1

IGF-I has been successfully used by Kurtz et al.[50] to increase the rate of healing in the transacted rat Achilles tendon (figure 4). Following transection, each tendon was treated with 25μg of a recombinant variant form of IGF-I (a form which has much less binding affinity to circulating proteins) in a methylcellulose gel vehicle. An obvious positive effect on the healing tissue was observed as early as 24 hours after the transection and addition of IGF-I (as shown by measurements of the Achilles Functional Index), and this effect continued up until the tenth and last measurement, on day 15.

The same study also showed the ability of IGF-I to reduce inflammation and its resulting functional deficit in damaged tendons. In experiments similar to those described above, 20 rats underwent Achilles tendon transection followed by an injection of the inflammatory agent carrageenan. The injury was

subsequently treated with the recombinant IGF-I, and functional and biomechanical data collected. It was observed that the rats that received IGF-I had a much less functional deficit induced by the carrageenan than rats that did not receive IGF-I. Although the exact mechanism by which IGF-I regulates inflammation is unknown, it was postulated that instead of simply preventing inflammatory cells from migrating into the injury area (as there was no significant decrease of these types of cells in the wound), it may act through a negative feedback loop. As one of the main products of the inflammatory cascade, high concentrations of IGF-I may act to switch off early inflammatory cascade genes in the cells involved in this process.

The ability of IGF-I to augment ligament healing when in combination with bFGF has also been studied. In one study,[51] a small incision in the MCL of the rat was treated with a collagen emulsion/IGF-I/bFGF preparation and left to heal (with no ligament repair) for 12 days, after which the ligament was extracted and its biomechanical properties tested. A statistically significant increase in the breaking energy of 58% (±83%, $p < 0.05$, $n = 10$) was observed; however, measurements of rupture force and stiffness were found not to be significantly different from the controls.

2.2 TGFβ

In vivo studies do not always involve the exogenous application or up-regulation of a particular growth factor; for many growth factors, too high a dose can in fact be detrimental. High levels of TGFβ-1, for example, have been implicated in tendon adhesion formation, which can significantly decrease the range of motion of a tendon.[49] In an effort to counter this, Chang et al.[14] have conducted studies on TGFβ-1 and -2 within the healing rabbit zone II flexor tendon. Their work used neutralising TGFβ-1 and -2 antibodies in an attempt to decrease TGFβ-1 and -2 activity and the associated loss of range of motion. Twenty-two animals underwent a transection of the zone II middle digit flexor digitorum profundus followed by a treatment of either phosphate-buffered saline, TGFβ-1 antibody, or a combination of TGFβ-1 and -2 antibodies. They observed that the animals that received antibodies to TGFβ-1 had around twice the range of motion (defined as the combined angular measurement of flexion at the proximal and distal interphalangeal joints) than those that did not. Interestingly, animals that received antibodies to both TGFβ-1 and -2 did not show a significantly higher range of motion than those that received only TGFβ-1 antibodies.

Other members of the TGFβ superfamily have been used *in vivo* with some success. Forslund and Aspenburg[52,53] used a single direct injection of cartilage-derived morphogenetic protein-2 (CDMP-2; also known as GDF-6 or bone morphogenetic protein-13), into the transected Achilles tendons of rats, and observed an increase in the force at failure of 39% in rats treated with CDMP-2 versus the control. Also, the tendons treated with CDMP-2 were thicker and appeared more dense than the non-treated controls. CDMP-1 was used in an earlier study from the same laboratory, but it seemed less potent as shown by a two-way ANOVA. In a third study, Aspenburg and Forslund[53] used the TGFβ family member osteogenic protein-1, but this was shown to induce bone formation in the tendon and had a detrimental effect on mechanical strength.

2.3 PDGF

Letson and Dahners[51] used treatments of PDGF alone, PDGF in combination with IGF-I, and PDGF in combination with bFGF, in an attempt to improve the healing of the rat MCL. 1.2μg of each growth factor in a collagen emulsion was injected into the transected ligament, and at day 12 post-injury the ligaments were harvested and their biomechanical properties tested. They observed that the PDGF-only treatment increased healed ligament strength by 73% (±55%, $p < 0.0025$), stiffness by 94% (±63%, $p < 0.0025$), and breaking energy by 101% (±104%, $p < 0.01$; not statistically significant). Likewise, the PDGF + IGF-I and PDGF + bFGF treatments also increased the quality of ligament healing to a similar level versus controls; however, in this case no synergistic interactions were observed. It was suggested that this was perhaps due to sub-

Table III. Structural properties of the healing rabbit femur-MCL-tibia complexes after treatment with a high (20μg) or low (0.04μg) dose of PDGF-BB or control. Data in the experimental/sham section are normalised and expressed as experimental divided by sham (reproduced from Hildebrand et al.,[47] with permission)

Property	Fibrin sealant	Low-dose PDGF-BB	High-dose PDGF-BB
Experimental			
Stiffness (N/mm)	22.4 ± 4.6	30.8 ± 2.6	24.4 ± 11.0
Ultimate load (N)	83.7 ± 28.4	119.4 ± 47.6	130.2 ± 86.4
Energy absorbed (J)	125 ± 25	350 ± 120	380 ± 340
Ultimate elongation (mm)	4.0 ± 0.5	4.7 ± 1.9	5.6 ± 2.1
Experimental/sham			
Stiffness (N/mm)	0.59 ± 0.15	0.68 ± 0.15	0.62 ± 0.23
Ultimate load (N)	0.33 ± 0.14	0.40 ± 0.21	0.51 ± 0.24
Energy absorbed (J)	0.19 ± 0.10	0.35 ± 0.18	0.44 ± 0.24
Ultimate elongation (mm)	0.56 ± 0.11	0.66 ± 0.28	0.88 ± 0.39

MCL = medial collateral ligament; **PDGF-BB** = platelet-derived growth factor isomer BB.

optimal dosing of the two molecules or that multiple doses over the healing period were required. The latter of these is perhaps most important as the three growth factors have somewhat different temporal profiles. PDGF exerts the greatest of its effects almost immediately after injury occurs, triggering the healing cascades during inflammation that mark the beginning of healing proper, whereas IGF-I and bFGF are important during the intermediate and later phases, particularly during cell proliferation and angiogenesis. An optimum therapy using these molecules would most likely involve the immediate addition of PDGF followed sometime later by the application of bFGF and/or IGF-I to up-regulate these later stages.

Subsequent to this study, Hildebrand et al.[47] demonstrated that the introduction of PDGF-BB into the injury site of the MCL of rabbits significantly increases its quality of healing, as shown by increases in the ultimate load, energy absorbed to failure, and ultimate elongation values of the femur-MCL-tibia complex (table III). However, these improved biomechanical properties were not apparent from histological examination as there was no significant difference in cellularity, vascularity or fibre alignment between treated ligaments and controls. It was thought that other structural components not examined, such as fibril diameters, must have been responsible for the increases.

This study was of interest as it also tested for a dose-dependent response using a fibrin sealant as a delivery vehicle to provide either 0, 0.4, or 20μg of PDGF-BB to the wound site. The high-dose treatment did indeed result in a femur-MCL-tibia complex with better biomechanical properties than the low-dose, successfully demonstrating a positive dose-response.

2.4 bFGF

Chan et al.[24] studied the effects of a single injection of bFGF on type III collagen expression, cell proliferation, ultimate stress and the pyridinoline content in the initial stages of healing in the rat patellar tendon. Three days after a defect was introduced into the mid-part of the petallar tendon, various doses of bFGF were injected directly into the wound site. It was observed that after 7 days increasing dosage of bFGF was correlated with increases in collagen type III expression and cellular proliferation, and although ultimate stress and pyridinoline content appeared to also increase, it was not found to be statistically significant.

In a study by Fukui et al.,[54] a defect in the MCL was treated with varying doses of recombinant human bFGF carried by a fibrin gel, and repair tissues examined at postoperative days 7, 14, 21 and 42. bFGF was found to promote the early formation of repair tissue compared with controls. Again, a dose-dependent response was shown, although in

this case higher doses had adverse effects. While a low dose resulted in the rate of tissue maturation being very similar to the controls, a high dose resulted in significant delays in maturation observed at the third and sixth week. A further observation was that type I procollagen expression was reduced in all bFGF-treated groups.

Somewhat similar results were found by Kobayashi et al.[55] in an investigation of the healing canine anterior cruciate ligament. In this work, cylindrical defects were introduced into the anteromedial bundle of the canine anterior cruciate ligament, a region known to have an extremely poor potential for healing, and treated with bFGF-impregnated pellets. The early stages of healing were shown to be positively influenced by the treatment, with defects quickly filling with new granulation tissue, as opposed to only partial filling in the control group. A dose-response was not investigated in this study, and although the amount of bFGF used was identical to the highest dose used in Fukui et al.'s[54] work and the progression of healing observed at the same time points, no significant disruption of maturation was reported as was in Fukui et al.'s study. These divergent results are likely due to the different animal models and/or delivery vehicle used in the two studies (for example Kobayashi et al.[55] postulated that it was unlikely that the bFGF pellet remained biologically active for more than 3 weeks, and Fukui et al.[54] only observed the interference of repair tissue maturation from week 3 onwards), as well as the different environments of the two ligaments studied (compared with the MCL, the anterior cruciate ligament has a poor supply of blood and early repair cells due to its intra-articular location[56]).

Kobayashi et al.[55] also noted that bFGF provided only a boost to the initial stages of healing, yet all subsequent steps proceeded with significantly more speed and efficacy than would take place naturally. It was hypothesised therefore that this initial 'kick-start' was all that was required to set in motion a cascade of other stimuli, most probably derived from invading cells and surrounding fluid, which resulted in greatly improved healing. This suggests that potential therapies may only have to control a small group or even a single key molecule to instigate or accelerate healing at a recalcitrant wound site – an important attribute for effective, practical, and economical therapies.

3. Future Directions

Each of the five growth factors discussed in sections 1 and 2 has important, varied roles within the healing tendon. IGF-I, PDGF and bFGF have vital functions during the early and intermediate stages of healing, during which they aid in the migration and proliferation of fibroblasts and stimulate extracellular matrix synthesis. TGFβ and VEGF also have some role in these processes, and in addition are instrumental in the remodelling phases, regulating angiogenesis within the wound site. Each of these molecules is involved in a myriad of interactions during these different stages of tendon healing, affecting both its own activity and expression as well as that of other molecules. If growth factors are to be successfully employed as therapeutics in the future, further research will be required. Work will need to focus on further defining the roles of each of the growth factors known as well as the strategies of regulation they employ, and most importantly will need to identify and clarify the synergistic and antagonistic influences they have on one another.

Some success has already been achieved utilising growth factors as therapeutics using a variety of delivery techniques, including direct injection, surgical implant, collagen or gel vehicles and gene therapy. In most of these studies, the application of a single molecule has shown some enhancement of healing; however, in general this temporary boost of a single 'healing signal' soon becomes diluted out, and has only a limited effect on the final outcome. Using a combination of patients' own growth factors to promote healing in injured tissue has become an important and potentially very fruitful area of research. Autologous growth factors are produced by platelets which are easily harvested from whole blood by a few centrifugation steps. Once a platelet-rich plasma specimen has been prepared, platelets can be activated to produce high titre growth factor

combinations which can then be delivered to the wound site. While little work has been performed on this type of growth factor treatment specifically for tendon and ligament healing, several studies on other tissues have shown promise. Tischler[57] for example, used autologous PDGF to treat decubitus ulcers, and observed much higher rates of healing in treated ulcers verses controls. Obviously the treatment in this study simply involved the topical application of the growth factor combination onto the wound site – delivering it to a healing ligament or tendon would present more of a problem.

In future, most success will likely come from the application of not one but multiple growth factors over the healing period in a similar way. A treatment programme could be envisioned in which a key molecule could be applied at the beginning of each phase of healing to significantly compress the healing process while simultaneously increasing the quality. Inherent in this approach however, is the problem of a subject requiring multiple treatments over a relatively short time. In this case, treatments involving surgery to deliver the molecule would most likely be infeasible; a direct injection of either the molecule or gene as part of a gene therapy solution would be more satisfactory. These options of course present their own problems, the most obvious of which is the need for sustained yet controlled release (or production) of the therapeutic molecule.

A viable alternative to the exogenous application of growth factors would of course be to use some other kind of stimuli to increase the production or activity of endogenous growth factors. Studies in this area have already been undertaken in a number of wound healing models using various stimuli, including hypoxia, ultrasound, mechanical, and electrical stimulation. Recently, Bouletreau et al.[58] used hypoxia, a stimuli present in the microenvironment of a fracture, to increase the production of bone morphogenetic protein-2 transcripts in cultured bovine endothelial cells. A 2- to 3-fold increase in bone morphogenetic protein-2 mRNA expression was observed after 24 and 48 hours in hypoxic cells compared with controls. Likewise, a study by Yeung et al.[59] showed TGFβ-1 production could be increased in response to mechanical stimulation in distracted fracture callus cells compared with normal fracture callus cells. These and other studies have shown that different types of physical and chemical stimuli are effectively translated into biological stimuli that result in the activation of the normal growth factor-mediated healing cascades. The use of these techniques to activate or amplify endogenous growth factors coupled with an effective exogenous application could prove to be extremely beneficial and obviate the need for invasive surgical procedures.

4. Conclusion

The processes of tendon and ligament healing are highly complex, but are slowly starting to become more well defined. Of the large number of molecules involved, growth factors play a central role. They are a diverse group of signal molecules whose effects are intricate and overlapping, and whose action is often dependent on dose, temporal expression, interaction with other growth factors, and even spatial distribution at the injury site.

There has been a steadily increasing number of *in vitro* and *in vivo* investigations into the action of growth factors over recent years which have provided vital information on the mechanics of the healing process. These data have been used to perform *in vivo* growth factor-based therapeutic studies which have shown some definite promise in increasing the efficiency and effectiveness of tendon healing. While a truly practical and effective treatment based on the application or regulation of growth factors *in vivo* may still be some time away, the future of cytokine-based therapies is promising.

Acknowledgements

The authors would like to acknowledge support provided by St George Hospital and South Eastern Area Health Service.

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