Flexor Tendon Healing *In Vitro:* Effects of TGF-β on Tendon Cell Collagen Production

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Flexor tendon healing is complicated by adhesions to the surrounding sheath. Transforming growth factor beta (TGF-β) is a cytokine with numerous activities related to wound healing. We examined the effects of TGF-β–1, –2 and –3 on tendon cell proliferation and collagen production. Three separate cell lines—sheath fibroblasts, epitenon and endotenon tenocytes—were isolated from rabbit flexor tendons and cultured separately. Cell culture media was supplemented with 1 or 5 ng/mL of TGF-β–1, –2, or –3. Cell number and collagen I and III production were measured and compared with unsupplemented control cultures. The addition of TGF-β to cell culture media resulted in a decrease in cell number in all 3 lines that did not reach statistical significance. There was a significant increase ($p < .05$) in collagen I and III production with the addition of all 3 TGF-β isoforms. Modulation of TGF-β production may provide a mechanism to modulate adhesion formation clinically. (J Hand Surg 2002;27A: 615–620. Copyright © 2002 by the American Society for Surgery of the Hand.)

Key words: Tendon, TGF-β, collagen.

Flexor tendon healing is complicated by adhesions to the surrounding fibrosseous sheath. Adhesions between the tendon and sheath impair the gliding mechanism of tendons and result in poor finger range of motion. Attempts to modulate adhesion formation clinically have, for the most part, proven unsuccessful.[1–5] It is now clear that to modulate adhesion formation, a better understanding of the molecular basis of repair is needed.

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The traditional paradigm of flexor tendon healing as either simply intrinsic or extrinsic has been expanded to reflect differential collagen production at 3 distinct sites: the endotenon, epitenon, and tendon sheath.[6] Further investigation is required to better define the molecular pathway from tendon injury and repair to adhesion formation at each distinct location.

Transforming growth factor beta (TGF-β) is a cytokine with numerous biologic activities related to wound healing including fibroblast and macrophage recruitment, stimulation of collagen production, down-regulation of proteinase activity, and increase in metalloproteinase inhibitor.[7,8] There are 3 mammalian isoforms of TGF-β: β-1, β-2, and β-3. The isoforms are 60% to 80% homologous and are dimers of 12-kd polypeptides that are proteolytically cleaved from larger precursors.[9] All 3 isoforms are produced by most cells active in wound healing and tendon healing. Platelets are major contributors.

Transforming growth factor–β has been found to accelerate the wound healing process in several mod-

els.[10] It has now become widely appreciated that TGF-β is a key cytokine in the pathogenesis of fibrosis and scar formation, resulting from excessive disordered collagen deposition.[11,12] The neutralization of TGF-β with both antibodies and natural inhibitors has resulted in decreased fibrosis in models of dermal wound healing and glomerulonephritis. Shah et al[13] recently showed reduced dermal scarring after simultaneous neutralization of TGF-β–1 and TGF-β–2 or addition of TGF-β–3, suggesting differential effects with the 3 mammalian isoforms of TGF-β.

Recent studies have shown the importance of TGF-β in flexor tendon wound healing.[14–16] Our laboratory has shown up-regulation of TGF-β messenger RNA in both endotenon and epitenon tenocytes as well as tendon sheath fibroblasts.[14] Furthermore our laboratory has shown decreased adhesion formation and increased range of motion after the administration of TGF-β antibodies after tendon repair.[15] Most recently we have reported that TGF-β receptors are present in the tendon sheath, epitenon, and endotenon, and changes in receptor number correlate with tendon injury and subsequent repair.[16]

To better understand the clinical relevance of TGF-β on adhesion formation, one must examine the direct effects of TGF-β on collagen production by cells of the tendon sheath, epitenon, and endotenon. We have previously described an *in vitro* model of flexor tendon healing by using cells isolated from the tendon sheath, epitenon, and endotenon.[6] By using this model we found that lactate—an early mediator after tissue injury—significantly increased collagen production by sheath, epitenon, and endotenon cells. In this study we investigated the effects of varying concentrations of TGF-β–1, –2, –3 on collagen I and III production by each cell line—sheath, epitenon, and endotenon.

Materials and Methods

Animal Model

All rabbit experiments were performed with adherence to our institutional animal protocols.

Six adult male New Zealand white rabbits (4.0–4.5 kg) were anesthetized with an intramuscular injection of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (0.01 mg/kg). The middle digit flexor digitorum profundus equivalent was identified and isolated in each forepaw of each rabbit. The tendon and tendon sheath were transected and dissected separately under the operating microscope.

Cell Culture and Morphology

Cells from the digital flexor sheath, epitenon, and tendon were isolated and cultured by using a published protocol with minor modifications.[17] Briefly the intact flexor tendons and tendon sheath were separated by sharp dissection. The tendon sheath was then digested with 0.5% collagenase (Sigma, St Louis, MO) in 20 mmol/L N-2-hydroxethylpiperazine-N′-2′ethanesulfonic acid (HEPES) buffer for 10 minutes at room temperature. The tendon sheath fibroblasts were then plated and cultured in Hamm's F12 medium supplemented with 10% fetal bovine serum.

The intact tendons were then treated with 0.25% trypsin at 37°C for 20 minutes to release the epitenon tenocytes. The epitenon tenocytes were plated and cultured in Hamm's F12 medium. Finally the remaining tendon was then treated with 0.5% collagenase to release endotenon tenocytes. These were similarly plated and cultured in Hamm's F12 medium.

Effects of TGF-β on Cell Growth

Each cell line was grown to confluence at 37°C in a humidified, 5% CO_2 incubator. At confluence cells were passaged by washing with phosphate-buffered saline (PBS) and detached with trypsin/ethylenediamine tetra-acetic acid. The cells were then plated at a density of 2.0×10^4/well. The cells were grown in media supplemented with serum as described previously. After 1 day the cells were washed with PBS and grown in serum-free media supplemented with TGF-β–1, TGF-β–2, or TGF-β–3 (R&D Systems, Minneapolis, MN) each at concentrations of 1 and 5 ng/mL. Cell counts were done in triplicate 3 days later by using a hemacytometer and calculated as average +/− SEM. A 2-tailed Student's *t*-test was used to determine statistical difference between control and the 1 ng/mL group and the 1 ng/mL and 5 ng/mL groups.

Immunocytochemical Staining of Collagen I and III Production

The same 3 cell lines—sheath, epitenon, and endotenon—were grown on glass coverslips in Hamm's F12 medium. On day 4 the cells were fixed in 2% formalin. The cells were then rinsed in PBS and the endogenous peroxidase was quenched with 0.3% hydrogen peroxide and permeabilized with 0.1% saponin. Cells were stained separately with antibodies to collagen I (Sigma), II (Neomarkers,

Union City, CA), and III (Chemicon, Temecula, CA) and incubated for 1 hour at room temperature. Cells were washed with PBS and biotinylated secondary antibodies. Next Vectastain (Vecta, Burlingame, CA) reagent was added and the cells were stained with diamionbenzidine and mounted with Permount (Fisher, Pittsburgh, PA) and photographed.

Effects of TGF-β on Collagen I and III Production

The production of collagen types I and III by each cell line (sheath, epitenon, and endotenon) was measured by using an enzyme-linked immunosorbent assay (ELISA). Cells were grown in a 48-well plate in media with 10% rabbit serum until an optimal density of 2×10^4 cells/mL was achieved as determined by a dose curve. Then the culture media of each cell line was supplemented with 1 or 5 ng/mL of TGF-β–1, TGF-β–2, or TGF-β–3. Unsupplemented cultures were maintained as controls. Three days later the cells were fixed with 1% formalin and the endogenous peroxidase was quenched with 0.3% H_2O_2 in PBS. The primary antibodies, anticollagen type I and type III (Sigma), were added at 100 μL/well. The cells were washed and peroxidase-conjugated secondary antibody (Sigma) was added at 100 μL/well. Both primary and secondary antibodies were allowed to incubate for 1 hour; the optimal concentrations were determined via titration. The cells were again washed and 3,3′,5,5′-tetramethylbenzidine liquid substrate system (Sigma) was added for 30 minutes. The reaction was stopped by the addition of 100 μL 0.5 N H_2SO_4, and 100 μL of the reaction well was added to a 96-well microtiter plate. The plate was read on a microtiter plate reader at OD_{450} nm. All measurements were performed in triplicate.

Statistical Analysis

Collagen production for each cell line was calculated as average OD_{450}/cell number +/− standard error of the mean (SEM). Statistical differences were determined by using analysis of variance with a level of significance of $p < .05$.

Results

Effects of TGF-β on Tendon Cell Proliferation

Three different cell lines were isolated from each tendon specimen: sheath fibroblasts, epitenon tenocytes, and endotenon tenocytes. The addition of all 3 TGF-β isoforms—1, 2, and 3—decreased the cell number in culture when compared with unsupple-

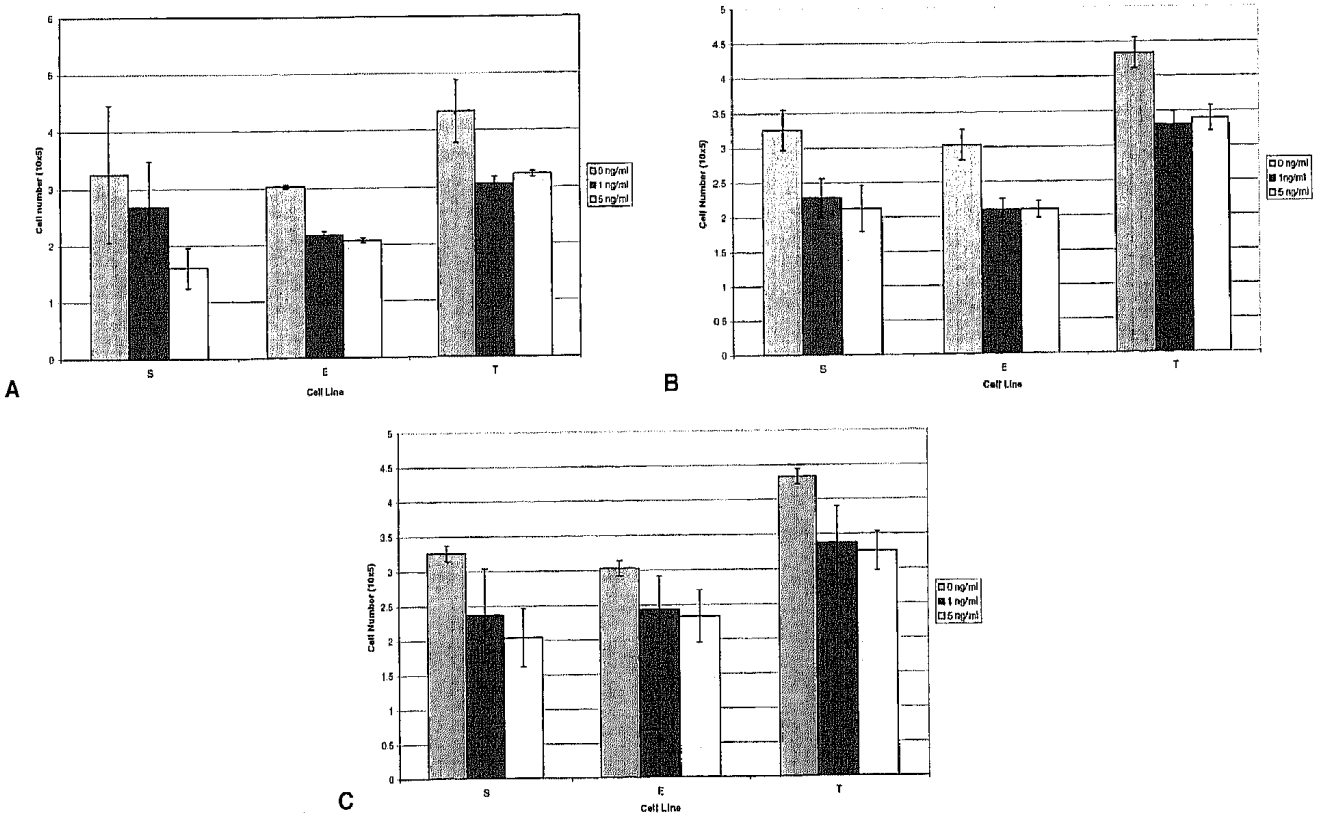

Figure 1. Cell growth of sheath (S), epitenon (E), and endotenon (T) cells at varying concentrations of (A) TGF-β–1, (B) β-2, and (C) β-3. Average cell number +/− SEM.

mented controls (Fig. 1). There was a decrease in cell number between the control (no TGF-β) and the cell lines grown in 1 ng/mL of TGF-β, as well as a decrease in cell number between the control and higher concentrations of TGF-β (5 ng/mL). Although neither difference reached statistical significance for all cell lines, the change was greatest between 0 and 1 ng/mL. The largest overall (from 0–5 ng/mL) decreases occurred in the sheath cell line for each TGF-β isoforms—1, 2, and 3 (50%, 32%, and 37%, respectively).

Effects of TGF-β on Sheath Cell Collagen Production

Collagen I and III production by sheath cells was confirmed initially by using immunocytochemistry. With the addition of all 3 isoforms of TGF-β to culture media there was a significant increase in collagen production ($p < .05$) (Fig. 2). The increase in collagen I production for each TGF-β isoform was concentration dependent. In the TGF-β–1 and TGF-β–3 groups the most significant increase occurred between the 1 and the 5 ng/mL group, the largest increase in the TGF-β–2 group occurred between 0 and 1 ng/mL. Overall the greatest increase was observed in the TGF-β–1 group with an increase >80% in collagen I production between the control and 5 ng/mL cell lines. Similar trends were observed in collagen III production. The most significant increase in collagen III production occurred with the addition of TGF-β–1 to cell culture media—with a >100% increase in collagen III production between the control and the 5 ng/mL group.

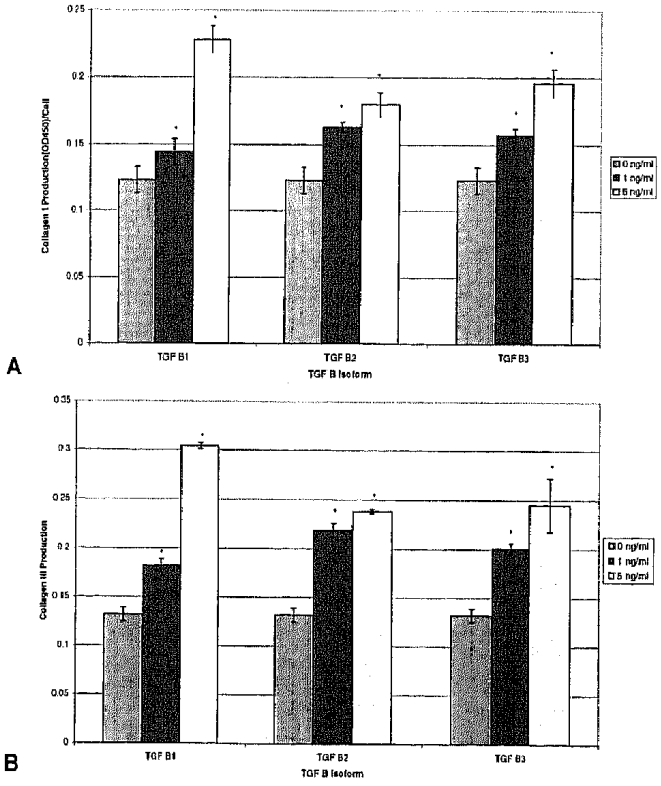

Figure 2. Collagen I (A) and III (B) production by sheath cells as measured spectrophotometrically at an optical density of 450. Average OD_{450}/cell number +/− SEM ($*p < .05$).

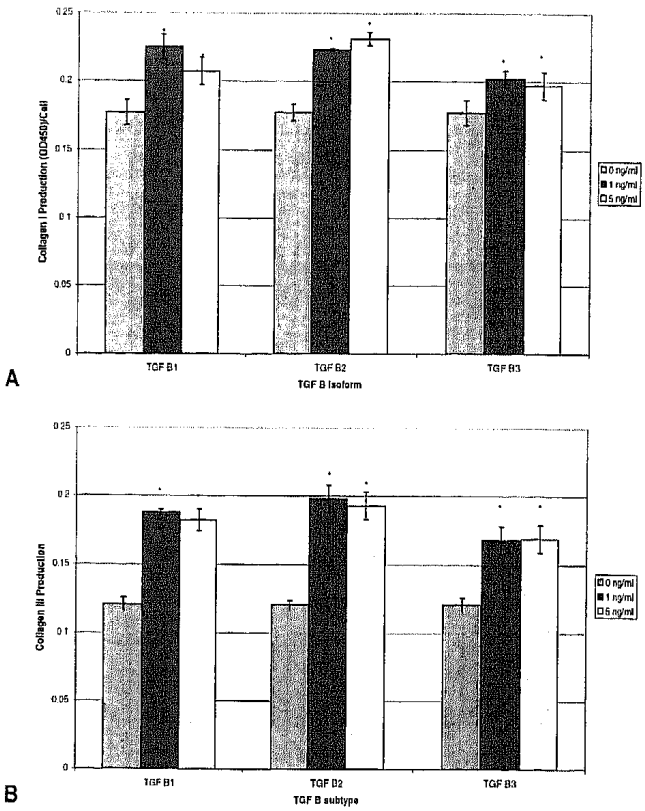

Figure 3. Collagen I (A) and III (B) production by epitenon cells as measured spectrophotometrically at an optical density of 450. Average OD_{450}/cell number +/− SEM ($*p < .05$).

Effects of TGF-β on Epitenon Collagen Production

Collagen I and III production by epitenon cells was confirmed initially by using immunocytochemistry. In the epitenon cell lines there was an increase in collagen I and III production with the addition of TGF-β to cell culture media (Fig. 3). These increases reached significance in all 3 isoform groups. Interestingly there was little difference in collagen I and III production with increasing TGF-β from 1 to 5 ng/mL.

Effects of TGF-β on Endotenon Collagen Production

Collagen I and III production by endotenon cells was confirmed initially by using immunocytochem-

istry. In the endotenon cell lines there was an increase in collagen I and III production with the addition of TGF-β isoforms to cell culture media (Fig. 4). The most notable increase in collagen I production occurred between the control and lower concentration of TGF-β–1, –2, and –3. There was little change in collagen production between the lower and higher concentration of TGF-β. Similar trends were observed in endotenon cell collagen III production.

Discussion

In this study we examined the effects of TGF-β–1, –2, and –3 on tendon sheath fibroblasts, epitenon tenocyte, and endotenon tenocyte proliferation and collagen production. All 3 TGF-β isoforms increased collagen production by all cell culture lines. Although collagen production increased, a decrease in cell number was observed for each cell line. This decrease in cell number did not seem to be concentration dependent because there was little difference in cell number between the control and higher concentration groups.

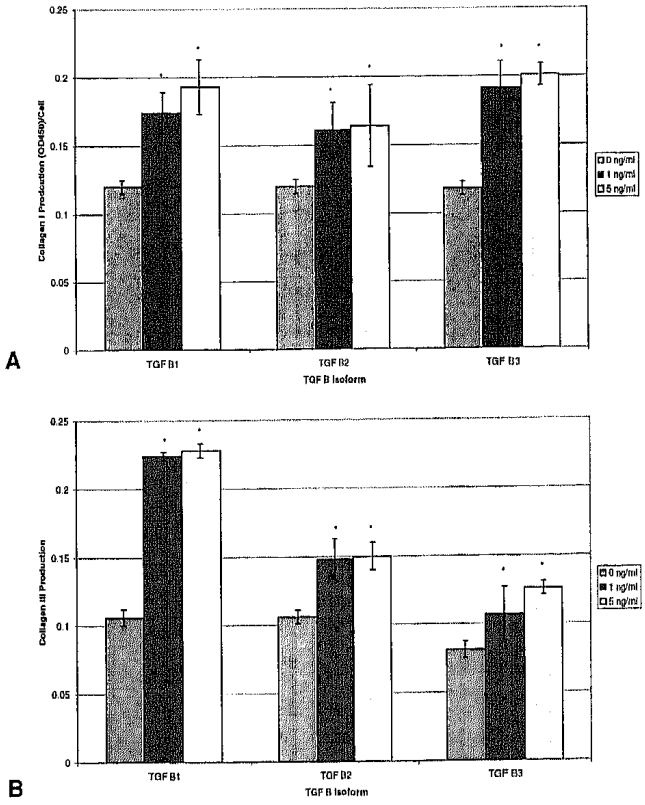

Figure 4. Collagen I (A) and III (B) production by endotenon cells as measured spectrophotometrically at an optical density of 450. Average OD_{450}/cell number +/− SEM (*$p < .05$).

Previous studies on flexor tendon healing have implicated basic fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor in the tendon healing process.[18] Our laboratory has recently shown a similarly important role for TGF-β in tendon healing.[14–16]

Transforming growth factor–β peptides bind to 3 membrane receptors named RI, RII, and RIII. All 3 membrane receptors are potentially expressed in almost every cell type examined to date.[19] Receptors RI and RII are transmembrane serine/threonine kinase receptors and are required for TGF-β signal transduction. RIII is a membrane-bound proteoglycan that lacks an intracellular signaling component. RIII acts mainly to present ligands to RII and also serves as a reservoir for ligands on the cell surface. Both TGF-β and its receptors are key modulators of wound repair.

Previous work has shown that application of exogenous TGF-β growth factors on normal and chronic wounds accelerates the healing process. Overexpression of TGF-β, however, could lead to scarring and fibrosis. Our laboratory has shown up-regulation of all 3 TGF-β receptors after tendon injury and repair, providing further evidence of the role of TGF-β in flexor tendon wound healing.[16]

Collagen I and III are typically the most abundant collagen subtypes found in tendons. Collagen I is usually found only in uninjured tendons, whereas collagen III is produced after tendon injury. Ultimately as scar matures, collagen III is replaced by collagen I.[20] The addition of TGF-β to cell culture media increased the production of both collagen I and collagen III in all 3 cell lines. For sheath and endotenon cell lines the most notable effects of TGF-β were observed with the addition of TGF-β–1. In the epitenon cell lines the trend of increased collagen production was similar in all 3 TGF-β subtypes. In all 3 cell lines the increase in collagen production did not increase proportionately with increasing TGF-β concentration. In general collagen production increased little from the lower and higher TGF-β concentration. Direct comparisons between collagen I and III cannot be made because different antibodies, with potentially different affinities, to collagen I and III were used.

This study increases our understanding of the events that occur during the healing process after flexor tendon injury and subsequent repair. It is becoming increasingly clear that TGF-β has a role in collagen production by tendon sheath cells and likely plays a role in tendon healing and possibly adhesion

formation. It has previously been shown that all 3 TGF-β receptor isoforms are up-regulated after tendon injury and repair.[16] In addition our group has shown neutralization of TGF-β–1 improves postoperative range of motion.[15] This study provides additional information about the effects of TGF-β on cell growth and collagen production—an important step in our attempts to better define the flexor tendon healing cascade. In addition this study confirms our previous observations that collagen production occurs at 3 distinct levels—the sheath, epitenon, and endotenon. Therefore as we consider strategies to modulate adhesion formation clinically, the relative contributions by all 3 cell types must be addressed. Furthermore in characterizing the steps involved in the tendon healing cascade, the temporal relationship (ie, early vs late mediators of adhesion formation) must also be addressed.

Transforming growth factor–β, which has been shown to have a pivotal role in the wound healing process in a variety of tissues, likely has a critical role in flexor tendon healing. Additional studies that better define the molecular events after tendon injury and repair are needed. Strategies that modulate TGF-β expression in other organ systems may be useful in the modulation of adhesion in flexor tendon healing.[21–23]

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