

Molecular Studies in Flexor Tendon Wound Healing: The Role of Basic Fibroblast Growth Factor Gene Expression

James Chang, MD, Daniel Most, MD, Richard Thunder, BS,
Palo Alto, CA, Babak Mehrara, MD, Michael T. Longaker, MD,
New York, NY, William C. Lineaweaver, MD, Palo Alto, CA

Basic fibroblast growth factor (bFGF) is a cytokine that plays a fundamental role in angiogenesis. This study examines bFGF messenger RNA (mRNA) expression in a rabbit flexor tendon wound healing model. Thirty-four New Zealand white rabbit forepaws underwent transection and repair of the middle digit flexor digitorum profundus tendon in zone II. Tendons were harvested at increasing time intervals and analyzed by *in situ* hybridization and immunohistochemistry. Few tenocytes and tendon sheath cells expressed bFGF mRNA in unwounded tendons. In contrast, tendons subjected to transection and repair exhibited an increased signal for bFGF mRNA in both resident tenocytes concentrated along the epitenon and infiltrating fibroblasts and inflammatory cells from the tendon sheath. These data demonstrate that (1) normal tenocytes and tendon sheath cells are capable of bFGF production, (2) bFGF mRNA is upregulated in the tendon wound environment, and (3) the upregulation of this angiogenic cytokine occurs in tenocytes as well as in tendon sheath fibroblasts and inflammatory cells. (J Hand Surg 1998;23A:1052–1058. Copyright © 1998 by the American Society for Surgery of the Hand.)

The biochemical mechanisms of flexor tendon wound healing in the hand remain controversial: both intrinsic and extrinsic mechanisms of nutrition and tissue repair have been implicated.¹ One of the early

events of wound healing is angiogenesis, in which neovascularization promotes delivery of inflammatory cells and fibroblasts to the wound site. In tendon wound healing specifically, the recruitment of wound repair cells may involve tendon sheath synovial diffusion, migration via intact vincula, as well as neovascularization of the tendon sheath.

In recent years, there has been increasing interest in the role growth factors or cytokines play in the flexor tendon repair process. Growth factors are proteins released by 1 cell that affect other cells via protein-receptor-specific interactions. Recombinant forms of insulin-like growth factors I and II, 2 growth factors structurally related to insulin, were able to stimulate proteoglycan synthesis and cell proliferation in rabbit flexor tendons *in vitro*.² Additional work by Abrahamsson and Lohmander³ documented higher levels of proteoglycan synthesis and lower levels of collagen synthesis in response to

From the Divisions of Hand and Plastic Surgery, Stanford University Medical Center, Stanford, CA; the Division of Plastic Surgery, Veteran's Administration Health Care System, Palo Alto, CA; and the Institute of Reconstructive Plastic Surgery, New York University Medical Center, New York, NY.

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Reprint requests: James Chang, MD, Division of Plastic Surgery, NC-104, Stanford University Medical Center, Stanford, CA 94305.

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recombinant insulin-like growth factor-I in intrasynovial rabbit flexor tendons compared with extra-synovial specimens. Furthermore, cyclic strain in addition to insulin-like growth factor-I and platelet-derived growth factor stimulated overall DNA synthesis.⁴ Therefore, several growth factors have already been implicated in flexor tendon wound healing. Recent successful gene transfer into tendon and tendon sheath cells by Lou et al⁵ provides the possibility of manipulation of growth factor genes.

In addition to the growth factors mentioned above, basic fibroblast growth factor (bFGF) also may play a role in flexor tendon wound healing. Basic fibroblast growth factor is a 146 amino acid polypeptide within the heparin-binding growth factor family.⁶ It has been shown to be a potent stimulator of angiogenesis in multiple *in vivo* and *in vitro* models. Purified bFGF is able to induce migration and proliferation of endothelial cells in tissue culture.⁷ Ingrowth of new blood vessels results from application of exogenous bFGF into embryonic chick chorioallantoic membranes and rabbit corneas.⁸ Duffy et al⁹ implicated bFGF protein in a canine model of flexor tendon wound healing based on heparin-Sepharose chromatography studies. We hypothesize that bFGF is upregulated during flexor tendon wound healing. The purpose of this study was to provide direct localization and semiquantitation of bFGF gene expression as a marker of angiogenesis and tissue repair during the flexor tendon healing process.

In situ hybridization is a molecular technique used to identify and localize intracellular messenger RNA (mRNA). This technique allows relative quantitation of cells producing specific mRNA transcripts. Gelberman et al¹⁰ reported the use of this technique to investigate expression of procollagen type I mRNA in canine flexor digitorum profundus repairs.¹⁰ They described upregulation of procollagen type I mRNA in cells along the epitendon surface in healing tendons. The work presented here represents initial studies using *in situ* hybridization to identify, localize, and quantitate bFGF gene expression in a flexor tendon wound healing model. Immunohistochemistry also was performed to confirm the presence of the resulting bFGF protein.

Materials and Methods

Tendon Repair

All procedures followed the guidelines of the animal research committees of the Stanford University

Medical Center and the Palo Alto Veteran's Administration Hospital. Anesthesia was administered to adult New Zealand white rabbits (4.0–4.5 kg) using an intramuscular injection of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (0.01 mg/kg). Rabbits also received 100 mg cefazolin immediately before surgery for antibacterial prophylaxis. Thirty-four adult New Zealand white rabbits underwent isolation of the middle digit flexor digitorum profundus equivalent. The tendons underwent sharp transection in zone II and immediate repair using the standard Kessler technique with a 6-0 nonabsorbable monofilament suture. Thereafter, the skin was closed using a 4-0 nonabsorbable monofilament suture without repairing the tendon sheath. The forepaws were cast in plaster in flexion to protect the tendon repair and to minimize variability in postoperative movement from rabbit to rabbit. The animals were killed at 1, 3, 7, 14, 28, and 56 days after surgery by intracardiac buthanasia overdose (0.2 mg/kg). In 6 of these rabbits, contralateral forepaw middle digit flexor digitorum profundus tendons were also harvested at the time of death by excising a portion of the uninjured tendon from zone II (total forepaws examined = 40).

Tendon Harvest

During harvesting of the tendon specimens, the original cutaneous incisions in the rabbit forepaws were reopened sharply. Meticulous dissection using $\times 3.5$ loupe magnification was performed to excise the preserved tendon sheath *en bloc* with the tendon repair site. The specimens were immediately embedded in Optimal Cutting Temperature compound (Miles Inc, Elkhart, IN), snap frozen in liquid nitrogen, and stored at -70°C until use.

In Situ Hybridization

In situ hybridization was performed using previously described methods.¹¹ Briefly, tendon specimen tissues were cut into 6- μm -thick sections at -20°C and mounted on ribonuclease-free, uncoated glass slides. A representative section of each specimen underwent hematoxylin-eosin staining. The remaining sections were immediately fixed for 10 minutes in 4% paraformaldehyde in phosphate-buffered saline solution with 5 mmol/L magnesium chloride, pH 7.3, and dehydrated with a graded series of ethanol-water solutions. Tissue sections were then prehybridized with a ribonuclease-free 40% formamide-transfer RNA-Denhardt's hybridization solution (FTD;

Denhardt's reagent; Boehringer Mannheim, Indianapolis, IN) for 1 hour at 42°C in a chamber with 100% humidity.

Slides were hybridized with a 1.5 ng/mL solution (in FTD) of 3' end-labeled digoxigenylated oligomer (20–30mer) DNA, sealed with ribonuclease-free coverslips, and incubated overnight at 42°C in a chamber with 100% humidity.

Cytokine probe sequences (bFGF and tumor necrosis factor- α) were obtained from GenBank (Los Alamos Natural Laboratory, Los Alamos, NM) and were chosen in an antisense orientation to the desired mRNA, with a GC:AT ratio between 0.7 to 0.8 if possible to standardize hybridization kinetics (Table 1). Tumor necrosis factor- α , an unrelated cytokine, was tested as a control. A sense probe for interleukin-6 mRNA served as a negative control and a polythymidylate nucleotide probe was used as a positive control. Our panel also included hybridizations with probe-free FTD to determine background signal from nonspecific binding. Control sections (uninjured tendon specimens) were hybridized concurrently with experimental tissues. All probes (Operon Technologies Inc, Alameda, CA) were end-labeled before use with the digoxigenin-labeling kit (Boehringer Mannheim) and stored at 4°C.

Sections were washed for 30 minutes each in ribonuclease-free 5× saline sodium citrate (SSC), 2× SSC, 1× SSC, 0.5× SSC, and 0.25× SSC. Slides were then blocked with 2% normal sheep serum in 0.1 mol/L Tris-buffered saline solution, pH 7.5, for 1 hour at room temperature and incubated with sheep serum-blocked 0.5% antidigoxigenin sheep Fab fragments conjugated with alkaline phosphatase. Sections were developed with McGadey's reagent (nitroblue tetrazolium + 5-bromo-4-chloro-3-indolyl-2-phosphate in 0.1 mol/L Tris buffer, pH 9.5, +10 mmol/L levamisole) for 10 minutes at room temperature. No counterstain was used. Developing reactions were stopped with tap water, and the sections were mounted with Glycergel (Dako Inc, Carpinteria, CA) and secured with a coverslip.

Table 1. Probe DNA Sequences

Tumor necrosis factor- α ¹⁶

5'-ACA CCA GGG AGC CCC TGG GCC CCA-3'

bFGF¹⁷

5'-AGC CAG GTA CCG GTT CGC ACA CAC TCC-3'

Rat interleukin-6 sense strand¹⁸

5'-CGG AGA GGA GAC TTC ACA GAG GAT ACC-3'

Scoring Tissue Sections

Tendon specimens, in a blinded fashion, were visualized at $\times 100$ magnification with a Leica microgrid (Leica, Inc, Deerfield, IL). All positively stained cells within a 1-mm microgrid square of tissue at the repair site (in the tendon and in the surrounding tendon sheath as marked by the suture remnant) were scored. Positive staining was defined as a cell with a dark purple-blue cytoplasm.

Statistics

Positive staining cells per square millimeter were recorded as mean values \pm SEM. Differences between the experimental time points and the control time point were compared using the unpaired *t*-test and were considered significant at $p < .05$.

Immunohistochemistry

Eight specimens (2 control specimens and 1 specimen from each time point tested) within the total number of experimental tendons (34) also underwent immunohistochemical analysis to confirm the presence of bFGF protein. Immunohistochemistry was performed using previously described methods.¹² Frozen samples of rabbit tendon and sheath were sectioned into 5- μ m-thick slices and placed onto glass slides. Each section was fixed in ice-cold acetone, allowed to air dry, and rehydrated in phosphate-buffered saline. Endogenous peroxidase activity was inhibited by treatment with 0.1% hydrogen peroxide (Sigma Chemical Co, St Louis, MO) in phosphate-buffered saline followed by a rinse with phosphate-buffered saline. Normal horse serum (1.5%; Vector Laboratories, Burlingame, CA) was applied for 20 minutes and was followed by monoclonal antisera for bFGF (1:100 dilution; Upstate Biotechnology, Lake Placid, NY) for 2 hours at room temperature. Biotinylated horse anti-mouse secondary antibody (Vector Laboratories) and avidin-biotin peroxidase complex were successively applied and sections were immersed in 3,3' diaminobenzidine (Sigma Chemical Co). Immunohistochemical staining was analyzed using a Zeiss photomicroscope II (Carl Zeiss, Inc, Thornwood, NY) at $\times 100$ magnification.

Results

All animals included in the study survived the operative procedure and casting period without infections. Hematoxylin-eosin staining identified the tendon-tendon sheath interface for each specimen

(Fig. 1). *In situ* hybridization was first performed on control unwounded rabbit flexor tendons. In the unwounded state, there was low-level expression of bFGF mRNA within the tendon parenchyma (17 ± 5 cells/mm²) from tenocytes exclusively. The surrounding tendon sheath in these unwounded specimens also revealed low numbers of fibroblasts and inflammatory cells expressing bFGF mRNA (77 ± 14 cells/mm²).

In contrast, flexor tendons subjected to transection and repair exhibited a significantly elevated number of cells expressing bFGF mRNA from resident tenocytes concentrated at the repair site and most densely along the epitendon surrounding the repair site. Inflammatory cells were also found to infiltrate the tendon parenchyma. These cells also expressed bFGF mRNA (Fig. 2A). The tendon sheath of post-repair specimens exhibited increased numbers of infiltrating fibroblasts and inflammatory cells with positive staining for bFGF mRNA. At each time point postrepair (from 1 day to 56 days), the tendon sheath was several-fold more cellular than the underlying tendon (Table 2). There was a high expression of bFGF mRNA from these cells. The area of increased

mRNA signal included the portion of the tendon sheath previously incised and the uninjured sheath overlying the tendon repair. Levels of bFGF mRNA expression remained elevated up to 2 months (56 days) following injury and repair.

Tumor necrosis factor- α , a protein that does not appear to have a prominent role in tissue repair,¹³ was not expressed in the tendon and tendon sheath in both control specimens and in specimens after injury. As expected, interleukin-6 sense controls also exhibited no signal (Fig. 2B).

Immunohistochemical staining revealed diffuse bFGF protein signal patterns in both control and postrepair tendon specimens. Basic fibroblast growth factor protein was found within the tendon parenchyma and within the tendon sheath (data not shown). However, because of the diffuse intracellular and extracellular distribution of the bFGF protein signal, it was not possible to quantify protein levels. Nevertheless, this confirmed the results of the *in situ* hybridization studies by demonstrating that bFGF mRNA is actively translated into bFGF protein.



Figure 1. Hematoxylin-eosin stain of a rabbit flexor tendon 1 week after transection and repair. Note the tendon cells (large arrow) and tendon sheath cells (small arrow). (Magnification $\times 200$.)

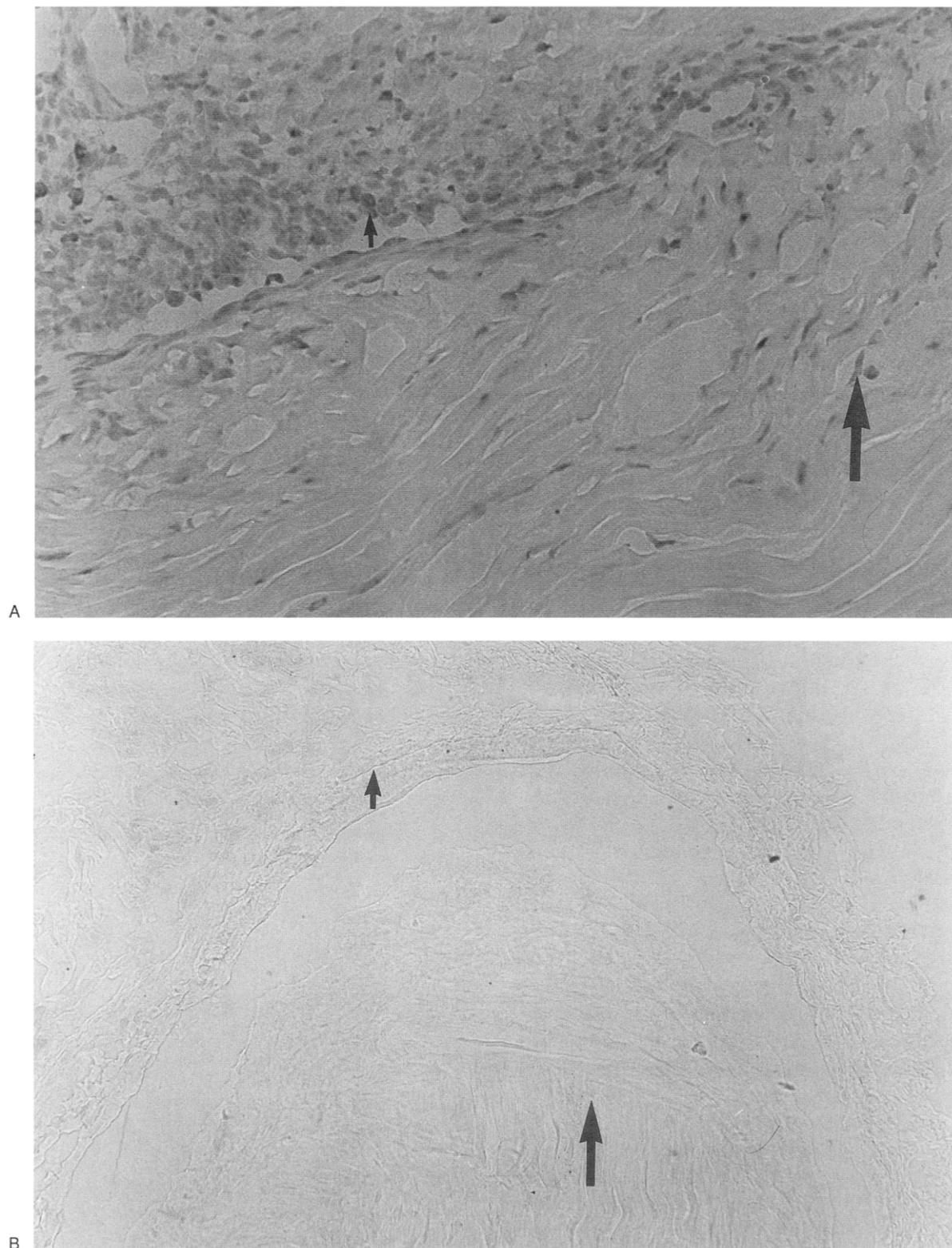


Figure 2. (A) *In situ* hybridization of a rabbit flexor tendon 1 week after transection and repair: the probe is specific for bFGF mRNA. Note the positive cells in the tendon (large arrow) and the tendon sheath (small arrow). (Magnification $\times 200$.) (B) *In situ* hybridization of a rabbit flexor tendon 1 week after transection and repair: the probe is specific for interleukin-6 sense strand. Note the absence of positive cells in the tendon (large arrow) and the tendon sheath (small arrow). (Magnification $\times 200$.)

Table 2. Positive-Staining Cells for Basic Fibroblast Growth Factor Messenger RNA by *In Situ* Hybridization (Cells/mm² of Tissue)

	Tendon		Tendon Sheath	
	Cells ± SEM	p Value*	Cells ± SEM	p Value*
Control (n = 6)	17 ± 5	—	77 ± 14	—
Day 1 (n = 4)	222 ± 61	.003	583 ± 140	.002
Day 3 (n = 4)	286 ± 114	.018	950 ± 230	.001
Day 7 (n = 4)	297 ± 30	<.0001	1,053 ± 114	<.0001
Day 14 (n = 8)	210 ± 41	.002	878 ± 131	.0002
Day 28 (n = 6)	237 ± 39	.0002	953 ± 88	<.0001
Day 56 (n = 8)	214 ± 42	.002	797 ± 140	.001

* Experimental time point compared with control time point.

Discussion

Growth factors or cytokines are multifunctional proteins that play important roles in the complex cell-cell interactions leading to wound healing. Growth factors including insulin-like growth factor, bFGF, platelet-derived growth factor, and epidermal growth factor have been implicated in flexor tendon wound healing and may act synergistically.^{2-4,9} Duffy et al⁹ used a variety of techniques to detect several of these growth factors. Heparin-sepharose chromatography of repaired canine flexor tendons demonstrated elution peaks consistent with platelet-derived growth factor and epidermal growth factor, but not bFGF. Instead, bFGF was found on elution of noninjured tendons. Furthermore, the mitogenic activity of tendon specimens was neutralized by bFGF antibodies. This work provided only indirect evidence of bFGF in flexor tendon wound healing.

Previous work from our laboratory identified a role for transforming growth factor- β ,¹⁴ which is a cytokine important in the early inflammatory response associated with wound healing. Transforming growth factor- β mRNA was expressed in both intrinsic tenocytes and extrinsic inflammatory cells after injury and repair, suggesting dual mechanisms of flexor tendon repair. In this study, mRNA for the angiogenic cytokine bFGF was identified, localized, and quantitated in a similar model of rabbit flexor tendon wound healing.

In the rabbit flexor tendon specimens, the presence of bFGF protein was first documented using an antibody specific to bFGF. Because bFGF does not have a signal peptide, researchers have postulated that it is released in response to tissue injury.⁶ The immunohistochemical studies identified total extracellular and intracellular protein and was useful for

confirming the presence of bFGF in flexor tendon wound healing. However, immunohistochemistry was not useful for quantification between control and postrepair specimens because of the extracellular distribution of protein signal. As in our previous study examining transforming growth factor- β , we found that the *in situ* hybridization technique was excellent for mRNA analysis because it localized mRNA intracellularly to specific cell types. The cell types could in turn be identified based on morphology.

The technique of *in situ* hybridization was first used in tendon wound healing studies by Gelberman et al.¹⁰ Gene expression of procollagen type I mRNA was localized to epitelon cells using [³²P]deoxycytidine triphosphate isotope. The density of autoradiography signal peaked in epitelon cells at postoperative day 10. Adjacent endotenon tissue did not express procollagen type I mRNA, and tendon sheath results were variable. The nonisotopic method described here uses a color change, thereby allowing better histologic preservation of cell morphology and avoiding the need for radioactive substrates. Furthermore, it was possible to count the number of cells in a given region of tissue (positive cells/mm²) to semi-quantify the level of gene expression for a specific mRNA sequence.

Using this nonisotopic, colorimetric *in situ* hybridization technique, we observed upregulation of bFGF mRNA in wounded specimens from both the tendon parenchyma and the tendon sheath beginning on postoperative day 1. This gene expression of bFGF continued until the last time point (56 days). The rabbit forepaws remained in a cast for up to 56 days to protect the tendon repair. In future experiments, early removal of the casts and longer time periods of

tendon wound healing are planned. In the tendon itself, cells expressing bFGF mRNA were present in the parenchyma, but the majority were centered along the cut edge and consisted of intrinsic tenocytes and fibroblasts migrating from the epitenon. Inflammatory leukocytes were also found at the cut edge. In addition, the surrounding synovial tendon sheath was observed to have inflammatory leukocytes and fibroblasts expressing bFGF mRNA several-fold higher in concentration than an equivalent area of the underlying tendon. We conclude that both the epitenon and the tendon sheath are important sources of cells secreting this angiogenic growth factor.

These observations are supported by the earlier work of Gelberman et al.¹⁵ In a canine model of flexor tendon repair, a protocol of early controlled mobilization prevented ingrowth of capillary buds via extrinsic adhesion formation. Instead, neovascularization occurred primarily in the intrinsic epitenon. Vessels in the epitenon were observed to progress across normally avascular segments of tendon to the repair site. In our study, the marked upregulation of bFGF mRNA along the epitenon of the repair site may represent an early, sustained signal for angiogenesis.

Synovial diffusion and vascular perfusion both contribute to the nutrition of healing flexor tendons. In areas of injury where vinculae and their branches have been traumatically disrupted, angiogenesis creates capillary networks and granulation tissue to transport cells essential for inflammation and collagen synthesis. The initial signalling processes are now thought to be mediated by several growth factors, including bFGF. The upregulation of bFGF in both intrinsic tenocytes and tendon sheath cells demonstrated here suggests a role for bFGF in mechanisms of angiogenesis and flexor tendon repair.

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