Neuropeptide, Mast Cell, and Myofibroblast Expression After Rabbit Deep Flexor Tendon Repair

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Purpose Increased numbers of myofibroblasts, mast cells, and neuropeptide-containing nerve fibers have been found in a number of fibrotic processes in connective tissues. The purpose of the present study was to investigate the occurrence of factors implicated in a hypothesized profibrotic neuropeptide–mast cell–myofibroblast pathway in deep flexor tendon healing.

Methods In a rabbit model of flexor tendon injury, with repair of the sharply transected deep flexor tendon using a modified Kessler and a running circumferential peripheral suture, segments of flexor tendons and sheaths were analyzed. The time points chosen—3, 6, 21, and 42 days after tendon repair—represent different stages in tendon healing. The messenger RNA levels of transforming growth factor-β1 and α-smooth muscle actin were measured with conventional reverse transcription–polymerase chain reaction, and the numbers of myofibroblasts, mast cells, and neuropeptide-containing nerve fibers were determined with immunohistochemistry.

Results The messenger RNA levels for transforming growth factor-β1 and the myofibroblast marker α-smooth muscle actin were significantly increased in deep flexor tendons after injury and repair, at all studied time points, but remained unchanged or even down-regulated in the sheaths. Myofibroblasts, mast cells, and neuropeptide-containing nerve fibers all increased significantly in the healing tendons, exhibiting similar patterns of change in percentages of total cell number over time, reaching levels resembling that of the tendon sheaths with 33% to 50% of the total cell population.

Conclusions After injury to the deep flexor tendon in a rabbit model, the proportion of myofibroblasts, mast cells, and neuropeptide-containing nerve fibers increases significantly. These findings support the hypothesis that the profibrotic neuropeptide–mast cell–myofibroblast pathway is activated in deep flexor tendon healing. *(J Hand Surg 2010;35A:1842–1849. Copyright © 2010 by the American Society for Surgery of the Hand. All rights reserved.)*

Key words Flexor tendon healing, mast cell, myofibroblast, neuropeptide, TGF-β1.

FLEXOR TENDON INJURIES in the hand can result in permanent functional impairment caused by adhesion formation between the tendon, the tendon sheath, and the underlying bone. During the last decades, improvements have been made in both suture techniques, limiting ruptures through increased repair strength, and rehabilitation protocols that attempt to counteract the formation of adhesions.[1–4] However,

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flexor tendon injuries can still heal with a limited range of motion, especially when associated with damage to surrounding tissues. Other approaches, targeting the biomolecular aspects of the healing process, might be useful in achieving further progress in decreasing adhesion formation and optimizing the recovery and strength of flexor tendon repair.[1–4] Growth factors and inflammatory agents have been the major focus of research, but no single factor has yet proven to be the ultimate solution to either limit adhesion formation or augment tendon strength. The relative importance of different molecular and cellular participants during different phases, as well as potential negative effects of manipulation with down-regulation or up-regulation of a certain factor still remain, to a large extent, unknown.[1,3,4]

Fibrosis and contractures can negatively influence the outcome of the healing process. Several participants—among others, transforming growth factor-β1 (TGF-β1)—have been implicated in promoting fibrotic processes.[5] Recently, a neuropeptide–mast cell–myofibroblast pathway has been suggested in the development of posttraumatic capsular fibrosis with joint contracture.[6] Neuropeptides, such as substance P (SP) and calcitonin gene-related peptide, have been reported to stimulate the degranulation of mast cells. Mast cells, present in many connective tissues, increase after injury and can release a number of factors that have a potential profibrotic effect, including TGF-β1. Mast cells are also believed to contribute to the involvement of myofibroblasts in fibrosis.[7,8] Hypertrophic scars in skin, contractures in joint capsules, and Dupuytren's contracture in the hand exhibit increased numbers of mast cells, neuropeptides, and myofibroblasts.[6,9,10] Higher levels of neuropeptides have also been described in chronic tendinopathy of the patellar and the Achilles tendons.[11–13] However, little is known of the occurrence of the factors implicated in this neuropeptide–mast cell–myofibroblast pathway during flexor tendon healing *in vivo*.

Our hypothesis was that, as in other types of connective tissues, there would be an increase in the elements involved in the neuropeptide–mast cell–myofibroblast pathway after injury and subsequent repair in the deep flexor tendon. The purpose of this study was to define the temporal relationships for elements of the pathway in a rabbit model.

MATERIALS AND METHODS

Animal model of flexor tendon injury

An animal model of flexor tendon injury described previously[14] was used, with the approval of the local ethics review board. Thirty-five female, young adult New Zealand white rabbits, weighing 3 kg (±0.3 kg) were divided into 5 groups, including one control group. The rabbits, allowed unrestricted cage activity, were provided with a standard laboratory diet and water *ad libitum*. They arrived 2 weeks before surgery to allow acclimatization. Four groups had surgery under general anesthesia with division and subsequent repair of the deep flexor tendons in the third digits bilaterally. The repaired tendons and sheaths were harvested at different time points (3, 6, 21, and 42 days, respectively).

Surgical procedure

The animals were anesthetized with intramuscular doses of fentanyl-fluanisone (0.3 mL/kg body weight; Hypnorm, Janssen, Beerse, Belgium) and midazolam (2 mg/kg body weight; Dormicum, Roche, Basel, Switzerland) and, immediately before surgery, they received a single intravenous prophylactic dose of the antibiotic cefuroxim (100 mg; Zinacef, GlaxoSmithKline, London, UK). All surgeries were performed by one of the authors (M. B.) in an animal operating facility under sterile conditions, using magnification and microsurgical instruments. After the hind paws were shaved, the deep flexor tendons were partially unloaded to prevent ruptures and allow unrestricted cage activity without immobilization. The same partial division at the tendon–muscle interface was performed in all animals having tendon repair, resulting in a diminished tensile load of the phalangeal section of the tendons.[14–17] A central longitudinal volar incision was used for tendon and sheath exposure. The tendon sheaths were carefully opened between the first and second annular pulleys, and the superficial flexor tendons were divided and resected. The deep flexor tendons were sharply divided transversely between the pulleys, the area corresponding to injuries in zone II. Repair was performed combining a modified Kessler core suture (5-0 Prolene; Ethicon, Johnson & Johnson, Sollentuna, Sweden) with a running circumferential peripheral suture (6-0 PDS; Ethicon, Johnson & Johnson). The core and the peripheral suture were performed the same way in all animals, with an average 10 throws of the peripheral suture, 1 mm apart. The tendon sheath was closed with a running 6-0 PDS suture, and the skin with a running 5-0 suture (Ethilon; Ethicon, Johnson & Johnson). A single dose of local analgesic (Marcain; AstraZeneca, Södertälje, Sweden) was injected into the hind paws for postoperative pain relief.

Sample collection

At 3, 6, 21, and 42 days after surgery (or after 2 wk acclimatization time, for the control group) rabbits were given a lethal dose of pentobarbital natrium (Apoteket,

Uppsala, Sweden) after first having received a sedative dose of midazolam (Dormicum, Roche). Tendons and tendon sheaths of the third digit were bilaterally harvested in 8-mm segments that included the repair site and were rinsed in physiological saline. For controls and at all time points after surgery, tissue samples intended for measurement of messenger RNA (mRNA) levels (n = 7) were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. Tendons and tendon sheaths for immunohistochemistry (n = 3) were fixed in formalin after removal and rinsing.

Reverse transcriptase–polymerase chain reaction

The RNA extraction was performed as described previously.[18,19] Reverse transcription with random primers (Omniscript RT Kit; Qiagen, Mississauga, Ontario, Canada) was followed by polymerase chain reaction (PCR), with all samples processed at the same time for each molecule with the number of cycles within the linear range of PCR amplification, using a PTC-100 PCR machine (MJ Research, Bio-Rad, Mississauga, Ontario, Canada), primers for α-smooth muscle actin (α-SMA) (F: gtg tga gga aga gga cag ca; R: tac gtc cag agg cat aga gg, 32 cycles), TGF-β1 (F: cgg cag ctg tac att gac tt; R: agc gca cga tca tgt tgg ac, 30 cycles), and the housekeeping gene 18S (F: gtc ccc caa ctt a; R: cac cta cgg aaa cct tgt tac, 30 cycles) in a 25-μL PCR mix (7.5 μL cDNA, 10.875 μL H_2O, 2.5 μL PCR buffer, 1 μL 50 mM $MgSO_4$, 0.5 μL nucleoside triphosphate mix, 1.25 μL forward and reverse primer, and 0.125 μL Taq polymerase). Non-reverse-transcribed total RNA constituted the negative control. The PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, and photographed; the negatives were analyzed with densitometry (MasterScan Interpretive Densitometer and RFLP Scanalytics software; CSPI Inc., Billerica, MA) as described previously.[20] Obtained values were normalized to those of the housekeeping gene 18S.

Immunohistochemistry

Tissue samples were embedded in paraffin blocks, sagittally cut in 6-μm sections, and mounted on glass slides, normal tendon sheaths excluded. Technical difficulties due to the small size of the normal tendon sheath samples did not allow successful embedding. A triple labeling immunohistochemical protocol developed previously[6] was used to measure mast cells (tryptase), myofibroblasts (α-SMA), and neuropeptide-containing (SP) nerve fibers. The slides were treated with the primary monoclonal α-SMA antibody (clone 1A4, Sigma-Aldrich, St. Louis, MO) at 37°C for 60 minutes, followed by the secondary antibody (sheep antimouse IgG horseradish peroxidase conjugate in 1:1000 dilution; Röche Molecular Biochemicals, Laval, Quebec, Canada) for another 60 minutes at room temperature. After application of DAB(3,3-diaminobenzidine solution)/peroxide substrate (1:10, Röche) they were treated with polyclonal tryptase goat antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C and the secondary antigoat antibody conjugated with Cy3 (1:1000; Peninsula Laboratories, Houghton, MI) for 60 minutes at room temperature, followed by polyclonal guinea pig antibodies to SP (1:200; Peninsula Laboratories) for 2 hours and anti-guinea pig secondary antibody (1:1000) conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 60 minutes. Slides were then treated with DAPI (4′-6-diamidine-2-phenyl indole; Vector Laboratories, Burlington, Ontario, Canada) and viewed under a Zeiss light microscope (Axioskop 2 plus; Zeiss, Toronto, Ontario, Canada). A digital camera (Axiocam; Zeiss) was used to photograph 5 areas from each section. The photographs were analyzed with the program Image-Pro Plus (Media Cybernetics, Silver Spring, MD). For each image, blinded counts of the total cell number (nuclear marker DAPI, Vector Laboratories), myofibroblasts, mast cells, and SP were repeated 3 times and averaged. This was followed by determining the average for each section and, consequently, each sample.

Data analysis

The statistical analysis was performed using Statistica software (StatSoft, Tulsa, OK). The levels of mRNA for α-SMA and TGF-β1 in the repaired tissues are expressed as values normalized to the housekeeping gene 18S. Significant differences between the control values and mRNA levels after repair were determined using analysis of variance combined with Tukey's post hoc test. For comparison of differences in the normalized mRNA levels between tendons and tendon sheaths at the same time points, analysis of variance and Tukey's test were used. Data from samples subjected to immunohistochemistry were analyzed with the same methods. For all analyses, p values $<.05$ were considered significant.

RESULTS

Levels of TGF-β1 mRNA

As found previously,[21] the mRNA levels for TGF-β1 in tendons increased after injury and repair, with levels twice those detected in normal, uninjured tissue as early as 3 days after surgery. They remained elevated for the entire duration of the study period, out to 42 days after

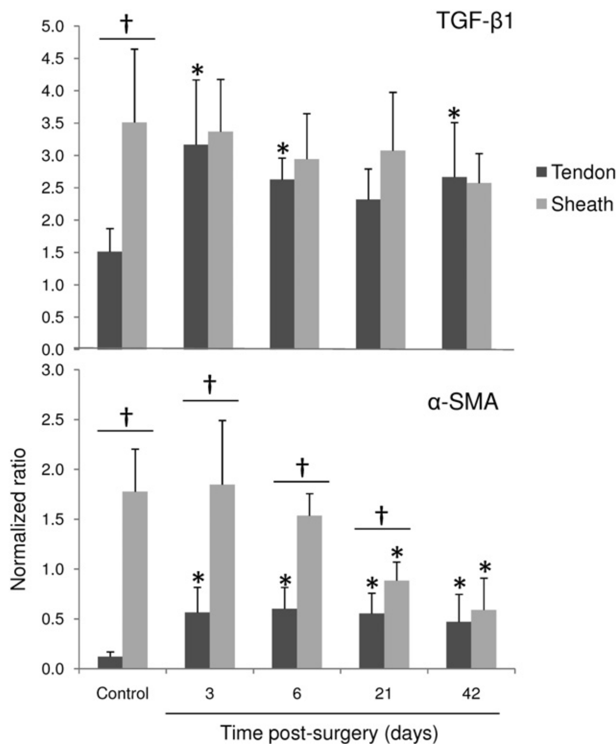

FIGURE 1: TGF-β1 and α-SMA mRNA expression, presented as normalized ratio to housekeeping gene 18S. Significant differences ($p < .05$) compared to uninjured control values are indicated by *, and significant differences between tendon and sheath at the same time point are indicated by †.

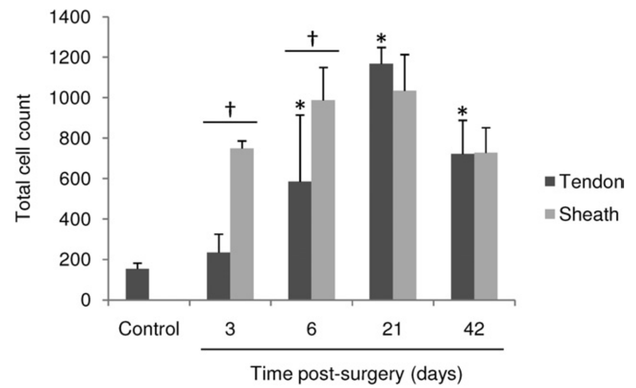

FIGURE 2: Total cell count in tendons and tendon sheaths after repair, and control tendons. (No data for normal, uninjured control sheath tissue.) Significant differences ($p < .05$) compared to uninjured control values are indicated by *, and significant differences between tendon and sheath at the same time point are indicated by †.

repair. Also in accordance with previous results, the mRNA levels for TGF-β1 in tendon sheaths did not significantly increase after tissue repair (Fig. 1). However, when comparing the levels in the tendon sheaths before and after repair with the levels in tendon tissue, the levels in the sheaths were initially more than twice as high as the levels in the tendons. After repair, the levels of TGF-β1 mRNA in the tendon tissue increased and matched the unaltered levels in the sheaths, remaining high until the last time point at 42 days.

Levels of α-SMA mRNA

There was a significant increase of α-SMA mRNA during the first week in the repaired tendons, with levels 4 to 5 times as high as in uninjured controls (Fig. 1). The levels in the tendon sheath tissue, however, did not change significantly during the first week (3 and 6 d) after injury and repair. Levels remained increased in the tendons, while significantly decreasing after 21 days in the tendon sheaths, ending with no significant differences between tendons and tendon sheaths 6 weeks after injury. In the uninjured control group of tendons and tendon sheaths, there was a significant difference in α-SMA mRNA, with levels more than 14 times higher in the tendon sheath tissue.

Myofibroblasts, mast cells, and neuropeptide-containing nerve fibers

From the immunohistochemical staining analysis, the total cell count in the repaired tendons steadily increased over time, reaching levels more than 7 times higher than in uninjured controls after 21 days (Fig. 2). At the last time point, the total cell count was decreasing but still elevated (4.7 times higher than control values). The tendon sheaths, assessed at the different time points after injury and repair, had a significantly higher cellular content at the earlier time points but were at comparable levels to the tendons after 21 and 42 days.

Myofibroblasts and mast cells constituted <10% of the total cell population in the tendon controls. After repair, levels increased to 30% to 40% after 3 days and remained at high levels (>4.3 to 5.5 times higher than the controls, respectively, 37% to 50% of total cell count) for the whole time period of the study, until 42 days after repair (Figs. 3, 4). The same pattern was seen for the neuropeptide assessed. The repaired tendon sheaths tended to exhibit somewhat lower neuropeptide-containing nerve fiber, mast cell, and myofibroblast percentages of total cell count, with levels significantly lower than the tendons at early and late time points.

DISCUSSION

In this study with a rabbit model of deep flexor tendon injury, components of the potential profibrotic neuropeptide–mast cell–myofibroblast axis were significantly in-

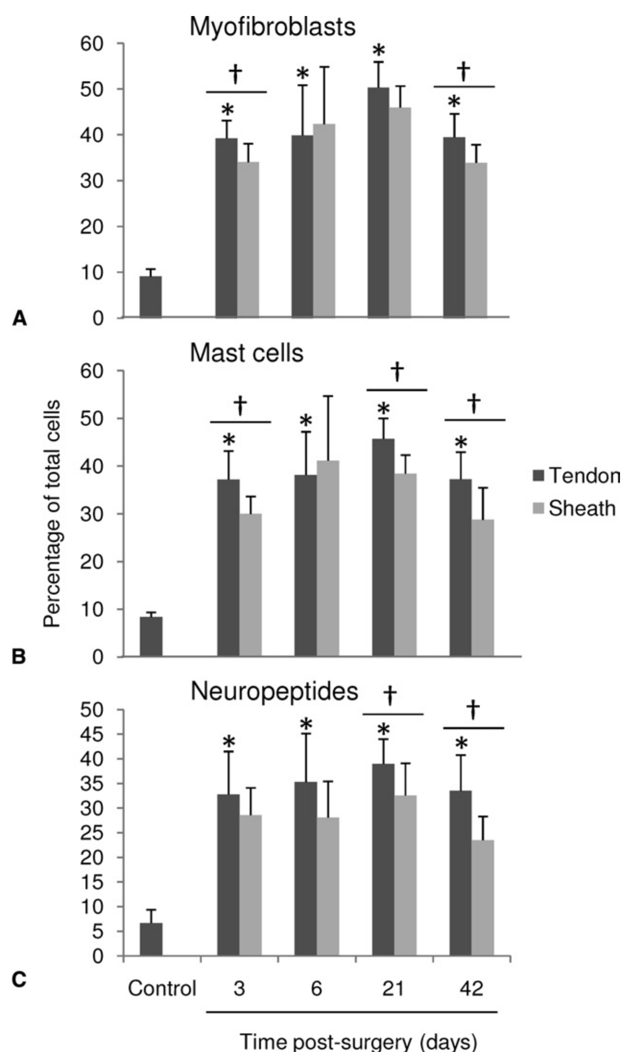

FIGURE 3: A Myofibroblasts, **B** mast cells, and **C** neuropeptide-containing nerve fibers—percentage of total cell count in controls and at different time points after repair. (No data for normal, uninjured control sheath tissue.) Significant differences ($p < .05$) compared to uninjured control values are indicated by *, and significant differences between tendon and sheath at the same time point are indicated by †.

creased and showed changes over time during the first 6 weeks after tendon injury. Myofibroblasts and mast cells, as well as neuropeptide-containing nerve fibers, that under normal conditions in uninjured tissues constitute <10% of the total cell population, reached levels ranging from 33% to 50% after tendon injury and subsequent repair. These levels are comparable with those described for changes in joint capsules after injury and immobilization in a rabbit knee model[6,22] as well as in specimens from patients with elbow contractures.[23] Their increase also corresponds well with the proliferative phase of healing, when tendon apoptosis is inhibited and cellularity increases.[24]

Mast cells play a role in connective tissue remodeling, including repair after direct mechanical injury,[8] degranulating as a reaction to the physical trauma and releasing a number of different factors contributing to the first inflammatory stage of the healing process.[7,25] Increased numbers of mast cells have also been demonstrated in fibroproliferative skin wound healing in red Duroc pigs, and administration of a mast cell stabilizer abrogates many features of the abnormal phenotype, apparently via the neuropeptide–mast cell–myofibroblast axis.[26] Myofibroblasts, easily distinguishable from normal fibroblasts by their expression of α-SMA, can generate greater amounts of contractile force. They are present in granulation tissue, where a contraction of the wound is a desired and necessary effect for healing,[27,28] but they are also frequent in conditions in which the tissue is exhibiting excessive contraction, such as hypertrophic scarring and Dupuytren's disease.[29,30] TGF-β1 induces the myofibroblast phenotype and has been suggested to contribute to progression of Dupuytren's contracture.[31–34] TGF-β1 has also been studied in deep flexor tendon healing, increasing after repair.[35,36] In the analysis of mRNA levels in this study, TGF-β1 increased in the repaired tendons, resembling the pattern of α-SMA mRNA expression. The increase in the flexor tendons remained for the whole study period, until 42 days after surgery, which is in accordance with earlier findings in a rabbit model[37] but differs from the results in a chicken model, in which levels returned to normal after the first week.[36] Attempts to improve tendon healing, limiting adhesion formation by blocking TGF-β1, have shown promising results.[37–39] This study suggests the existence of a fibrotic pathway in deep flexor tendon healing, involving TGF-β1 activity through a potential connection to mast cells and myofibroblasts. However, the effect of TGF-β1 blocking agents on the neuropeptide–mast cell–myofibroblast pathway remains to be determined.

In this study, there were differences between the tendon sheath and the tendon in uninjured tissue when comparing the amount of myofibroblasts, as represented by the expression of α-SMA mRNA. The α-SMA mRNA levels were up to 14 times higher in the tendon sheaths, indicating that myofibroblasts were significantly more abundant in normal tendon sheath tissue than in tendons. These results are in accordance with earlier *in vitro* findings, in which it has been suggested to be a possible contributor to adhesion formation.[40] However, after injury, the amount of α-SMA mRNA remained unaltered or decreased in the sheaths, whereas it increased by up to 5 times in the tendon tissue. In the immunohistochemical analysis, the amount of myofibroblasts in tendons

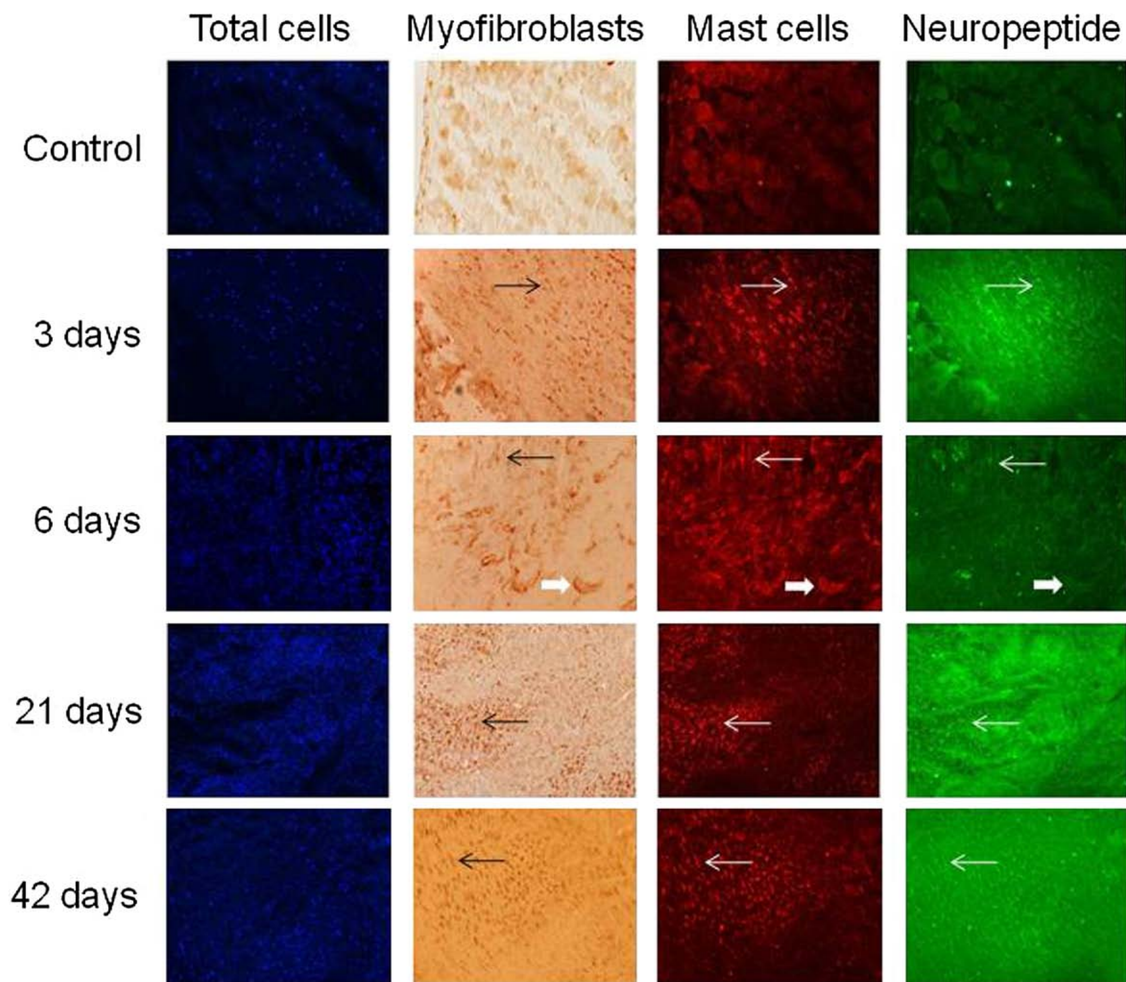

FIGURE 4: Representative flexor tendon tissue sections of controls and at different time points after tendon repair, with different light source and filters demonstrating staining for total cell count (nuclear marker DAPI, Vector Laboratories), myofibroblasts (α-SMA), mast cells (tryptase), and neuropeptides (SP) in tendons. Thick white arrows indicate one of the blood vessels. Black arrows point to groups of myofibroblasts, and thin white arrows indicate the corresponding areas with colocalization of mast cells and neuropeptides.

increased by 4.3 to 5.5 times from 3 until 21 days after tendon repair, as compared to controls. Apart from the first and last time points after repair, the levels were comparable to the proportion of myofibroblasts in the tendon sheaths, with no significant difference. Unfortunately, due to technical difficulties, there were no control samples of the uninjured sheath tissue. A possible explanation for the differences between the mRNA and immunohistochemical findings, regarding the myofibroblast content in the tendon sheaths, is the inability to discriminate between myofibroblasts in the tendon proper and myofibroblasts in the smooth muscle-containing walls of blood vessels through mRNA measurements with reverse transcriptase–polymerase chain reaction. Even though there was no separate staining for the blood vessels in the immunohistochemical analysis, it is still possible to manually identify blood vessels and exclude the α-SMA–positive staining cells localized in their walls from the count. The implications of the increased number of myofibroblasts after injury in the flexor tendon, regarding both possible adhesion formation and changed mechanical properties affecting ultimate tendon strength, invites further studies that include a longer time period after tendon repair before analysis.

The third component in the suggested profibrotic axis is the neuropeptide-containing nerve endings, with the marker SP being significantly increased after tendon repair in our study. Neuropeptides have been connected to an increased number of myofibroblasts and contractile tissues in Dupuytren's disease[9] and can stimulate mast cell degranulation.[8] The neuropeptide SP has been postulated to have both potential positive and negative

effects on connective tissues,[10,11,13] and treatment with SP after acute Achilles tendon injury repair has resulted in improved biomechanical properties in the early phases of healing in an animal model.[41] Whether the determining factor between positive and negative effects is dose-dependent and related to the potential activation of the profibrotic neuropeptide–mast cell–myofibroblast pathway remains to be investigated.

A possible weakness of this study, as mentioned earlier, relates to the inability to separate α-SMA–containing myofibroblasts in the walls of blood vessels from those in the tendon proper, resulting in higher levels in the mRNA measurements. It most likely also results in somewhat higher numbers for the immunohistochemical analysis. However, even though an increase in blood vessels through angiogenesis after injury and repair to some extent also contributes to the increase in the immunohistochemical analysis, it likely does not represent the complete increase. The methodology of semiquantitative PCR is highly reproducible and the results are found to be comparable to those obtained by real-time quantitative PCR.[42]

We have shown that, in repaired tendon and tendon sheath samples, all 3 factors implicated in the suggested profibrotic neuropeptide–mast cell–myofibroblast axis exhibited similar patterns of increase, indirectly supporting the theory of interdependence in expression. The presence of mast cells and SP in uninjured control tendons also supports the hypothesis that they have a role in normal connective tissue homeostasis, with adaptations to load and stress.[8] The findings of this study support the concept that the suggested profibrotic neuropeptide–mast cell–myofibroblast axis is activated in deep flexor tendon healing.

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