EXPERIMENTAL STUDY

Effect of BMP-12, TGF-β1 and autologous conditioned serum on growth factor expression in Achilles tendon healing

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Abstract

Purpose Achilles tendon ruptures are devastating and recover slowly and incompletely. There is a great demand for biomolecular therapies to improve recovery, yet little is understood about growth factors in a healing tendon. Here, the role of growth factors during tendon healing in a rat model and their reaction to single and multiple growth factor treatment are explored.

Methods Rat tendons were transected surgically and resutured. The expression of bFGF, BMP-12, VEGF and TGF-β1 was assessed by immunohistochemical analysis one to 8 weeks after surgery. Paracrine effects of TGF-β1 or BMP-12 added by adenoviral transfer, as well as the effect of autologous conditioned serum (ACS) on growth factor expression, were evaluated.

Results bFGF, BMP-12 and VEGF expression was highest 1 week after transection. bFGF and BMP-12 declined during the remaining period whereas VEGF expression persisted. TGF-β1 expression dramatically increased after 8 weeks. ACS treatment increased bFGF ($P = 0.007$) and BMP-12 ($P = 0.004$) expression significantly after 8 weeks. Also overall expression of bFGF, BMP-12 and TGF-β1 regardless of time point was significantly greater than controls with ACS treatment ($P < 0.05$). Both BMP-12 and TGF-β1 treatments had no significant effect. No effect was observed in VEGF with any treatment.

Conclusion bFGF, BMP-12, VEGF and TGF-β1 are differentially expressed during tendon healing. Additional BMP-12 or TGF-β1 has no significant influence, whereas ACS generally increases expression of all factors except VEGF. Staged application of multiple growth factors may be the most promising biomolecular treatment.

Keywords Growth factors · Tendon healing · Biological repair · Animal model

Abbreviations

ACS	Autologous conditioned serum
bFGF	Basic fibroblast growth factor, a.k.a. FGF-2
BMP	Bone morphogenic protein
CDMP	Cartilage-derived morphogenic protein
GDF	Growth and differentiation factor
IGF-1	Insulin-like growth factor 1
PDGF	Platelet-derived growth factor
TGF-β1	Transforming growth factor beta 1
VEGF	Vascular endothelial growth factor

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Introduction

Tendon ruptures and tears are common injuries that heal slowly and are often treated surgically. However, clinical outcomes are not always satisfactory [21]. Growth factors play an important role in tendon healing [15], and there is increasing confidence that accelerated and improved healing may be achievable by biological intervention.

The physiological course of tendon healing with an emphasis on growth factor expression has been studied for various models of tendon pathology, including rotator cuff tears

[10, 23, 32, 40] and Achilles tendon ruptures in a rat model [3, 8, 9, 19, 29]; in vitro studies have used tenocyte cultures [6].

There have been a number of experiments in which recombinant growth factors have been applied in attempts to accelerate tendon healing in an animal model, but most have used only a single factor and results have been variable. Experiments designed to study the endogenous expression of growth factors have measured expression over only a few days or even just at a single time point. Thus, the natural expression of growth factors over time and the influence of growth factors added to promote tendon healing remains unclear [15, 22].

To address this issue, a rat model of acute traumatic Achilles tendon rupture without tendinopathy was chosen and four growth factors—basic Fibroblast growth Factor (bFGF), bone morphogenic factor 12 (BMP-12), vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β1)—that have an established and prominent role during tendon healing [22, 24] were measured.

Previous experiments have shown that basic fibroblast growth factor (bFGF, FGF-2) stimulates proliferation and thereby increases the cellularity of a healing tendon [1, 10, 36]. Bone morphogenic factor 12 (BMP-12, a.k.a. CDMP-3 or GDF-7) is important for tenoblast and fibroblast differentiation [5, 19, 23, 32], and vascular endothelial growth factor (VEGF) plays an essential role in angiogenesis [26, 40]. Transforming growth factor beta one (TGF-β1) is known to influence the healing of fibrous tissues including tendons. It is secreted by infiltrating macrophages and platelets and is known to stimulate proliferation and matrix organisation [2, 9, 13, 16, 27].

The hypothesis was that growth factors would be elevated at the beginning of tendon healing and would then gradually decrease as healing progressed. Stimulating this process with exogenous growth factors would lead to a prolonged endogenous expression of the growth factors over time.

To verify this either bone morphogenic factor 12 (BMP-12) as a specific inductor of tendon and ligament tissue, transforming growth factor beta (TGF-β1) as a treatment with diverse effects or autologous conditioned serum (ACS) as a representative of biological treatment with multiple growth factors was added.

Materials and methods

Rat Achilles tendon healing model

Approval for all animal procedures was obtained beforehand from the local Institutional Animal Care and Use Committee of Harvard Medical School (Boston, MA, USA). Sixty male adult Sprague-Dawley rats (weight 400–425 g, >10 weeks old) were used.

Fifteen animals per group were used for natural expression of growth factors, controls, BMP-12, TGF-β1 and ACS groups. For each group, 3 animals each were euthanised after 1, 2, 4 and 8 weeks. The unoperated contralateral tendons of the animals not treated were used to establish a baseline for the stainings.

Animals were placed under general anaesthesia with isoflurane. The right hind leg was shaved and disinfected with Betadine. After placement on a warm sterile field, a 3-cm incision was made above the right Achilles tendon. The peritendon was opened and the Achilles tendon cut at a right angle to the collagen fibres, creating a 5-mm gap proximal to the calcaneal insertion. The plantaris tendon was transected, preventing a splinting phenomenon.

In animals used for examination of endogenous expression of growth factors and animals in the control and ACS group, Achilles tendon, peritendon and skin were sutured back together.

For animals in groups with BMP-12 or TGF-β1 treatment, the transfected muscle grafts were placed with two single stitches around the side of tendon injury after end-to-end suture repair.

After regaining consciousness, the rats received buprenorphine injections twice daily for 2 days.

Growth factors

BMP-12 or TGF-β1 cDNA was transferred to 4-mm round muscle flaps harvested from a donor rat by gene transfer using recombinant adenovirus vectors as described previously [19]. In short, serotype 5 recombinant adenoviral vector was used, recombinant adenoviruses (Ad.BMP-12 or Ad.TGF-β1) were propagated in 293/Cre8 cells and muscle grafts incubated with particles in DMEM medium with 5% foetal bovine serum for 24 h before implantation. Expression of BMP-12 and TGF-β1 has been found to be increased 4–7 days after transfection [19].

ACS was used from a previous study as described [20]. In short, pooled rat blood was incubated for 9 h at 37°C in Orthokine syringes (Orthogen AG, Duesseldorf, Germany) containing glass beads. Samples were then centrifuged and frozen at −20°C. For injection, aliquots were thawed on ice and allowed to reach room temperature. A volume of 170 µl of ACS were injected once every 24 h for 3 days directly into the area of the sutured repair using a 1-ml insulin syringe. According to previous studies, ACS contains increased levels of TGF-β1, bFGF and VEGF compared to basal serum concentrations, as well as inflammatory and anti-inflammatory cytokines. However, Platelet-derived growth factor-BB (PDGF-BB) in rat derived ACS and insulin-like growth factor 1 (IGF-1) in human derived ACS were not substantially increased [20, 37].

Immunohistochemical analysis

Animals were placed under general anaesthesia with isoflurane and asphyxiated with CO_2. To harvest the whole muscle–tendon–bone units a 5-cm incision was made from below the knee down to the plantar fascia of the foot. Muscle–tendon–bone units were explanted by transection of the middle part of the gastrocnemius muscle and of the calcaneus.

The tendons were immediately placed in 4% buffered formalin (pH 7.4) for 24 h, dehydrated and embedded in paraffin wax. Endogenous peroxidase was blocked by 30-min incubation in 1% hydrogen peroxide in phosphate-buffered saline (PBS). After washing in PBS, sections were incubated for 1 h with 2% powdered milk in PBS to block nonspecific protein binding. Sections were incubated with the primary antibody over night at 4°C, followed by three 5-min washes. For amplified detection with biotin–avidin–peroxidase, sections were incubated with biotinylated secondary antibody (rabbit anti-mouse biotin label or goat anti rabbit biotin label, Dako, Denmark) for 30 min at room temperature, washed three times with PBS and incubated with avidin-biotinylated-peroxidase complex (Vectastain ABC Kit Elite Standard, Vector, CA, USA) for 30 min at room temperature. This was followed by three 5-min washes with PBS. Substrate (solution with Tris, peroxide and 3,3 Diaminobenzidine tablets, Sigma, Munich) was added for 5 min. Sections were washed in tap water three times and stained with haematoxylin and eosin. Primary antibodies used were against bFGF (rabbit polyclonal; Abcam, Cambridge UK), BMP-12 (rabbit polyclonal; AbD Serotec, Oxford, UK), VEGF (mouse monoclonal; Abcam, Cambridge, UK) and TGF-β1 (rabbit polyclonal; Santa Cruz, CA, USA).

Histological scoring was supported by digital image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA). Samples were taken from 5 distinct locations, including the central part of the rupture, the left and right borders, borders towards the muscle insertion and the calcaneal insertion. Scores of the 5 sections were then averaged to a whole score for the tendon.

The immunoreactive score used is widely accepted for quantification of immunohistological stainings [4, 14, 28, 30, 35, 39]. The characterisation of relative amount and location of the protein of interest and the high sensitivity are of great advantage. The score was calculated by assigning points for overall staining (1 for no staining to 4 for strong staining) and for overall stained cells (1 for no cells to 5 for over 80% of cells) and multiplying both to a general score. Sections were scored by two independent blinded observers with high inter-observer reliability (Spearman's correlation coefficient 0.86). Scores also correlated strongly with digital image analysis of overall stained area (coefficient 0.85).

Statistical analysis

Analysis of variance (ANOVA) was performed on time courses and differences between groups. Where ANOVA was significant, multiple comparisons were performed with the Tukey-HSD post hoc test. Results were considered significant with a P-value of <0.05. Statistics, plots of means and standard deviations were produced with R v. 2.10.1.

Results

Natural course of growth factor expression during tendon healing

bFGF, BMP-12, TGF-β1 and VEGF were detected in all samples and displayed clearly distinct time courses of expression (Fig. 1), thereby validating the immunohistological scoring performed.

Expression levels of bFGF ($P = 0.012$) and BMP-12 ($P = 0.008$) were significantly higher 1 week after transection compared to unoperated rat Achilles tendons whereas no significant difference was observed for TGF-β1 and VEGF.

After 2, 4 and 8 weeks bFGF expression was close to the expression found in unoperated tendon. Similarly, BMP-12 expression decreased rapidly over time. From 2 weeks onwards, expression of BMP-12 was similar to the expression observed in untreated tendons.
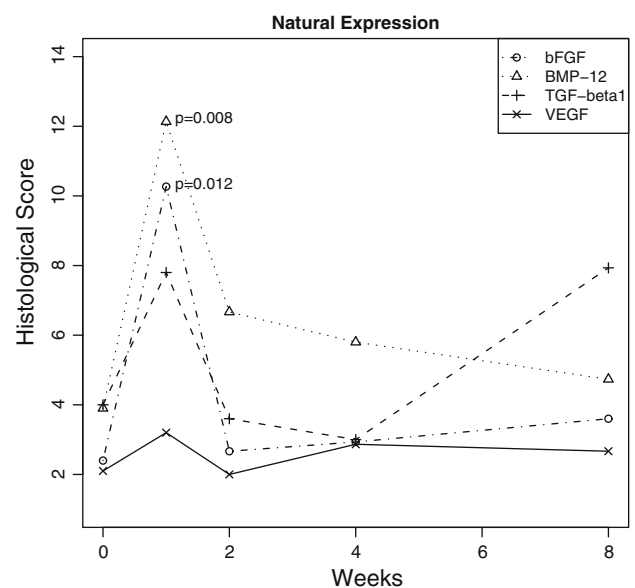

Fig. 1 Immunoreactive scores for primary antibodies against bFGF, BMP-12, VEGF and TGF-β1 at different time points after tendon transection. The scores are plotted as means. Every point in the plot represents the mean value of 3 samples for the respective time point. Points at '0 weeks' represent the mean score of normal tendon. P-values refer to the difference to normal tendon. See text for further description

VEGF expression was elevated after 1 week, decreasing to normal at week 2 and slightly increasing to 3 points at weeks 4 and 8.

Interestingly, TGF-β1 showed a different pattern of expression compared to the other three growth factors. It was elevated at week 1 and subsequently declined to the level of normal tendon at weeks 2 and 4. However, expression increased again after 8 weeks. Changes in TGF-β1 staining scores more strongly reflected the number of stained cells at each time point. Representative histological images are shown in Fig. 2.

Auto- and paracrine effects of BMP-12, TGF-β1 or ACS on growth factor expression

ACS had the greatest effect on growth factor expression. It increased bFGF significantly after 8 weeks compared to controls ($P = 0.007$) and BMP-12 ($P = 0.004$). A nearly significant elevation of bFGF was also observed with ACS in week 4 compared to BMP-12 ($P = 0.074$) and TGF-β1 ($P = 0.084$). Comparing the expression of BMP-12, TGF-β1 and VEGF between groups at single time points did not yield any significant differences (Figures 3, 4, 5, 6).

Overall bFGF expression regardless of time point was significantly greater in the ACS group compared to controls and the BMP-12 and TGF-β1 groups (all $P < 0.002$). Overall BMP-12 expression was also greater in the ACS group compared to controls and the BMP-12 and TGF-β1 groups (all $P < 0.05$). Overall TGF-β1 expression was greater in the ACS group compared to controls and the TGF-β1 group (all $P < 0.05$). There was no significant difference between groups for the overall expression of VEGF.

Discussion

The most important findings of the present study were the distinct time courses of the measured growth factors and their increase with ACS stimulation. Affirming the initial
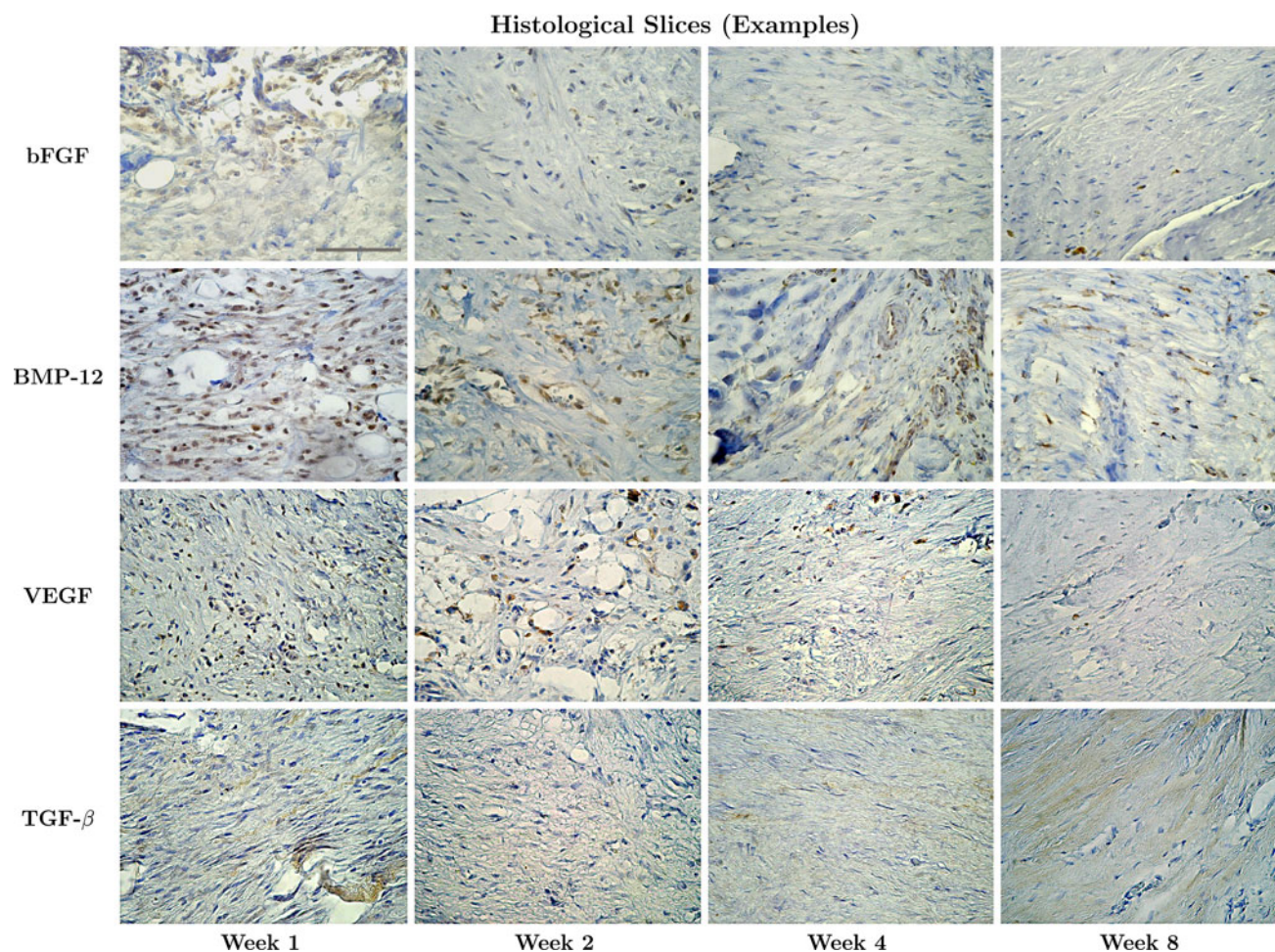

Fig. 2 Representative immunohistochemical sections of tendons 1, 2, 4 and 8 weeks after tendon rupture using antibodies against bFGF, BMP-12, VEGF and TGF-β1. The *grey bar* in the *top left* image represents 100 μm
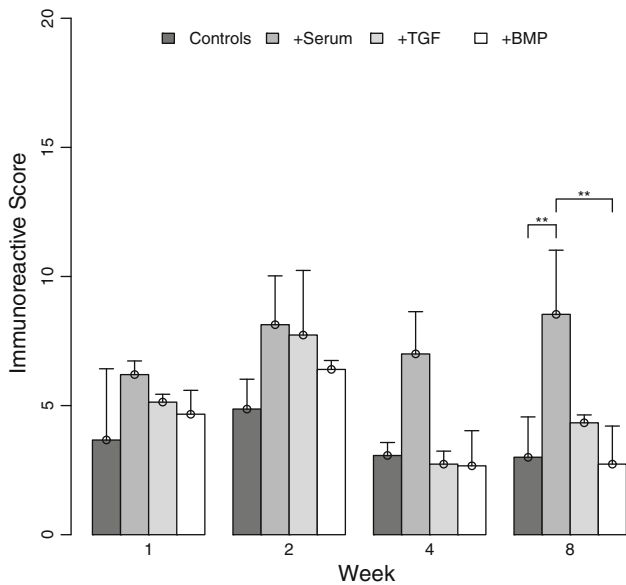
Fig. 3 Expression of bFGF over time as measured by immunoreactive score. For weeks 1, 2, 4 and 8, *grey boxes* indicate the mean values of immunoreactive scores for controls and groups stimulated with either ACS (+ACS), TGF-β1 (+TGF) or BMP-12 (+BMP) as indicated. *Whiskers* indicate the upper standard deviation; the lower symmetrical interval is omitted for reasons of clarity and comprehensibility. *Brackets* with stars on top indicate significant pairings, **$P < 0.01$
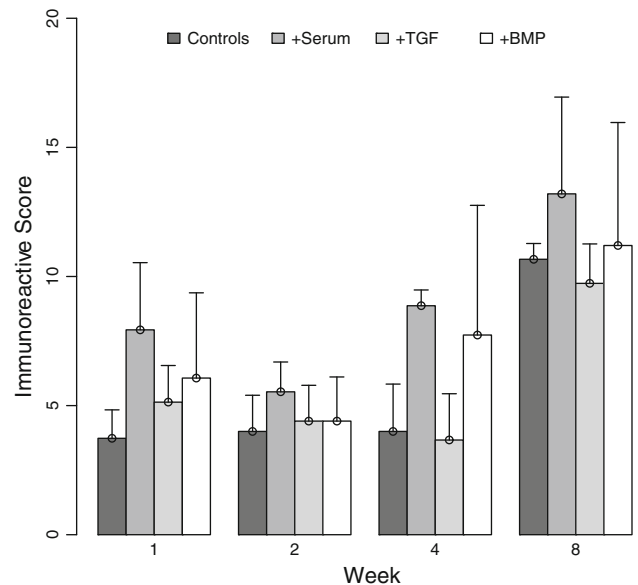

Fig. 5 Expression of TGF-β1 over time as measured by immunoreactive score. For weeks 1, 2, 4 and 8 *grey boxes* indicate the mean values of immunoreactive scores for controls and groups stimulated with either ACS (+ACS), TGF-β1 (+TGF) or BMP-12 (+BMP) as indicated. *Whiskers* indicate the upper standard deviation; the lower symmetrical interval is omitted for reasons of clarity and comprehensibility

Fig. 4 Expression of BMP-12 over time as measured by immunoreactive score. For weeks 1, 2, 4 and 8, *grey boxes* indicate the mean values of immunoreactive scores for controls and groups stimulated with either ACS (+ACS), TGF-β1 (+TGF) or BMP-12 (+BMP) as indicated. *Whiskers* indicate the upper standard deviation; the lower symmetrical interval is omitted for reasons of clarity and comprehensibility
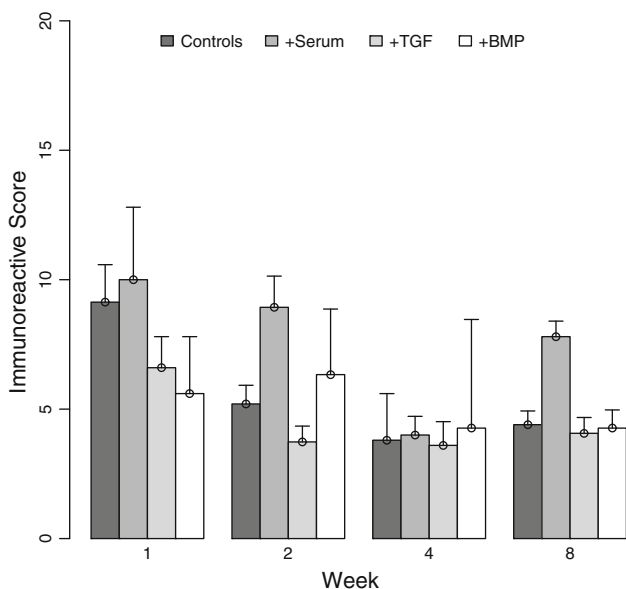

Fig. 6 Expression of VEGF over time as measured by immunoreactive score. For weeks 1, 2, 4 and 8, *grey boxes* indicate the mean values of immunoreactive scores for controls and groups stimulated with either ACS (+ACS), TGF-β1 (+TGF) or BMP-12 (+BMP) as indicated. *Whiskers* indicate the upper standard deviation; the lower symmetrical interval is omitted for reasons of clarity and comprehensibility
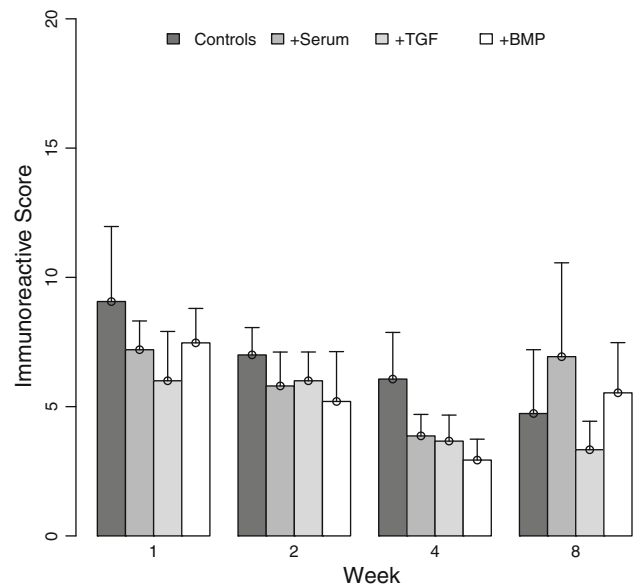
hypothesis, the expression of bFGF, BMP-12, VEGF and TGF-β1 was elevated in the 1 week of healing, possibly in response to the inflammation after rupture. As the healing progressed, each growth factor followed a slightly different path, most likely interacting with the effects of other growth factors. BMP-12 and bFGF expression both

declined rapidly after the 1 week of healing, possibly because inflammation was resolving at this stage. After 2–4 weeks, both growth factors approached their basal expression levels.

TGF-β1 showed an initially high expression, declined rapidly to normal concentrations and increased again strongly in week 8, probably reflecting remodelling and scar formation in the late stages of tendon healing. VEGF expression was initially higher than normal and remained above normal for the entire 8-week period. This may reflect the importance of neoangiogenesis in tendon healing. However, it is surprising that VEGF expression did not decline at the end of this period because it coincides with an elevated expression of TGF-β1, which has been reported to suppress VEGF expression in tendon [8]. Also, from a tissue architecture point of view, a decrease in neoangiogenesis due to scar formation after 8 weeks might be expected [34].

In contrast to the second hypothesis, ACS displayed the greatest effect on the endogenous expression of growth factors, while the addition of TGF-β1 or BMP-12 did not affect or seemed to even decrease their expression. This may indicate a reactive suppression due to the addition of BMP-12 or TGF-β1 and may signify that addition of a single growth factor cannot emulate the complex interactions of healing.

According to previous studies, ACS contains increased levels of TGF-β1, bFGF and VEGF, as well as inflammatory and anti-inflammatory cytokines [20, 37].

Schippinger found no significant systematic influence and no significant increase of IGF-1 using ACP (autologous conditioned plasma) after intramuscular application [31]. Due to the fast absorption and degradation of the cytokines, it was not expected that the dosage of ACS used would have any generalised effect or an effect on the contralateral tendon.

Limitations of this study were the use of transected Achilles tendons without tendinopathy, the time of follow-up and the restricted number of growth factors to eminent key factors during tendon healing.

However, it was not intended to mimic the pathophysiology of human tendinopathy, the most frequent and devastating disease leading to Achilles tendon ruptures [17, 34]. Instead, the study concentrated on transected Achilles tendons that simulate a rupture in an otherwise healthy tendon in order to illuminate the normal process of tendon healing and to investigate the effect of additional growth factors. This knowledge is crucial because it allows one to approach the pathologically changed Achilles tendon with more understanding of the pathological differences.

The time points for examination of the growth factor expression were chosen based upon a similar study of rotator cuff healing in rats [38] and reflect the time course of the different phases of tendon healing, namely early inflammation, reorganisation and late tissue remodelling.

Tendon ruptures lead to a considerable loss of function of the injured extremity. During healing, the ruptured tendon forms a regenerate within the first few weeks, yet despite intensive remodelling over the following months a complete regeneration of the tendon is never achieved [15]. Tendon healing is a complex and highly regulated process that is initiated, sustained and terminated by a large variety of biomolecular and biomechanical stimuli [11, 22]. The scar tissue that is formed is mostly mechanically inferior and cannot perform as well as a normal tendon [3, 19, 25].

It is established that patients profit from an early functional therapy after Achilles tendon repair (e.g., [7, 33]). Current development of biomolecular therapies aims to further improve healing results [33]. Current trends may lead to a gross oversimplification of the biological processes and complex interactions between growth factors in a healing tendon.

Based on the results of the present study, staged application of different growth factors is likely to have a greater effect than the application of a single growth factor at one time point, a conclusion independently favoured by Molloy et al. [22] and Oliva et al. [24]. ACS provides a convenient means of applying multiple, autologous growth factors. Several studies have shown its beneficial effect on the healing tendon in rats, both in healing time and resulting mechanical properties of the healed tendon [12, 18, 20]. Further studies may determine the right dosage and intervals for an optimal therapy.

In view of a possible clinical application of biological augmentation of tendon healing in man, there is no doubt about the improvement by biological intervention. However, any treatment aiming to mimic the natural biological process should not focus on a single growth factor. Considering the highly complex process of healing and the influence of exogenous cytokines on endogenous growth factors, a truly practical treatment will require much development. Nevertheless, the future of growth factor based therapies is promising.

Conclusion

Tendon healing involves the coordinated, interactive mediation of many growth factors. The four endogenous growth factors studied here showed different expression patterns during tendon healing and were differentially affected by the exogenous application of BMP-12, TGF-β1 and ACS.

The present study suggest that the 1 week of tendon healing is a crucial period of growth factor stimulation and that ACS has the greatest influence on later expression especially of bFGF and BMP-12. Future research should focus on the complex interactions—synergism or antagonism—of growth factors in order to create a solid basis for biomolecular treatment.

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