Accelerated Healing of the Rat Achilles Tendon in Response to Autologous Conditioned Serum

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Background: Despite advances in the treatment of ruptured Achilles tendon, imperfections of endogenous repair often leave patients symptomatic. Local administration of autologous conditioned serum (ACS) in patients with inflammatory, degenerative conditions has shown beneficial effects.

Purpose: Because ACS also contains growth factors that should accelerate tendon healing, we studied the effect of ACS on the healing of transected rat Achilles tendon.

Study Design: Controlled laboratory study.

Methods: In preliminary in vitro experiments, rat tendons were incubated with ACS and the effect on the expression of Coll A1 and Col3A1 was assessed by real-time quantitative polymerase chain reaction. To test its effect in vivo, the Achilles tendons of 80 Sprague Dawley rats were transected and sutured back together. Ten rats from each group (ACS group, n = 40; control group, n = 40) were euthanized at 1, 2, 4, and 8 weeks postoperatively for biomechanical (n = 7) and histologic (n = 3) testing. Lysyl oxidase activity was assayed by a fluorometric assay. The organization of repair tissue was assessed histologically with hematoxylin and eosin- and with Sirius red-stained sections, and with immunohistochemistry.

Results: Tendons exposed to ACS in vitro showed a greatly enhanced expression of the Coll A1 gene. The ACS-treated tendons were thicker, had more type I collagen, and an accelerated recovery of tendon stiffness and histologic maturity of the repair tissue. However, there were no differences in the maximum load to failure between groups up to week 8, perhaps because lysyl oxidase activities were unchanged.

Conclusion and Clinical Relevance: Overall, our study demonstrates that treatment with ACS has the potential to improve Achilles tendon healing and should be considered as a treatment modality in man. However, as strength was not shown to be increased within the parameters of this study, the clinical importance of the observed changes in humans still needs to be defined.

Keywords: growth factors; autologous conditioned serum; animal model; biological repair

Once a rare occurrence, rupture of the Achilles tendon has become the most common type of sports-related tendon rupture, with an estimated annual incidence of 10 ruptures per 100 000 people.30 Despite advances in the treatment of Achilles tendon rupture such as percutaneous suturing21,81 or functional treatment,22 recovery times are still long and rerupture rates are substantial.39

Further major improvements in Achilles tendon rupture healing are most likely to result from the implementation of novel, biological approaches. In particular, numerous studies have documented the beneficial effects of individual growth factors on tendon healing in animal models. Such growth factors include vascular endothelial growth factor (VEGF),40 transforming growth factor-beta (TGF-β1),27 platelet-derived growth factor (PDGF),35 growth and differentiation factors,28 insulin-like growth factor-1,6 basic fibroblast growth factor (FGF-2, also bFGF),5 and bone morphogenetic proteins (BMP-12 and BMP-13).8 Exposure in vitro of patellar tendon fibroblasts to bFGF was found to affect cell proliferation and collagen type III expression, both early events in tendon healing.8 Additionally, an angiogenic effect was observed with bFGF application.10
However, of all growth factors studied, VEGF appears to have the strongest angiogenic activity. During embryogenesis of tendon tissue, BMPs—in particular, BMP-12 and BMP-13—affect the expression of elastin and collagen type I. Additionally it was found in animal experiments that BMP-12 stimulated the healing of the patellar tendon. Among the other growth factors that play critical roles in tendon healing is TGF-β1, which has been reported to induce the expression of collagen type I and fibronectin mRNA in experiments in tissue culture. Upregulation of mRNAs expressing the α-chains of type I and III collagens has been reported to occur in tenocytes isolated from sheep cultured with and without serum. It appears that optimal tendon repair requires high expression of collagen type I. As healing progresses, there is a switch from collagen type III, which is expressed early in the healing process, to collagen type I, which provides greater mechanical strength. As newly synthesized collagen is laid down, the enzyme lysyl oxidase converts certain lysine and hydroxylysine groups into aldehydes, which spontaneously form cross-links that stabilize collagen fibers.

Although the effect of growth factors on tendon healing is impressive, it has become increasingly clear that tendon repair is not triggered by a single growth factor but requires the interplay of various such factors. Collectively, they exert powerful and comprehensive effects on healing. Consistent with this hypothesis, injections of platelet concentrates that contain a rich mixture of growth factors into the hematoma of severed Achilles tendons increased tendon callus strength and stiffness in the rat, but no such improvement was apparent when individual growth factors TGF-β1 or FGF-2 were injected.

Autologous conditioned serum (ACS) preparations are a natural source of multiple growth factors. By exposing whole blood to glass beads, Meijer and coworkers obtained serum enriched in anti-inflammatory cytokines, including interleukins (IL-4, IL-10, IL-13) and interleukin-1 receptor antagonist but which also contained elevated concentrations of growth factors FGF-2, hepatocyte growth factor, and TGF-β1. In clinical trials, locally injected ACS was found to be beneficial in the treatment of equine osteoarthritis, lumbar radicular compression, and muscle injuries. We tested the hypothesis that the growth factors in ACS would improve the healing of ruptured and sutured Achilles tendons in rats.

MATERIALS AND METHODS

Autologous Conditioned Serum

Serum was conditioned by incubating pooled rat blood for 9 hours at 37°C in Orthokine syringes (Orthogen AG, Düsseldorf, Germany) containing glass beads (surface area 21 mm²). After incubation, samples were centrifuged and the conditioned serum collected and frozen in 0.2-mL aliquots at −20°C. For injection, aliquots were thawed on ice, allowed to reach room temperature, and 170 μL of ACS injected directly into the area of the sutured Achilles tendon using a 1-mL insulin syringe. The dose used was calculated from a study in mice, based on mean body weight. The exact location of the injection site was defined by the position of the stitches closing the skin. The gap was closed with 4 stitches and injections were made between stitches 2 and 3.

Rat Achilles Tendon Healing Model

Eighty adult male Sprague Dawley rats, weighing 400 to 425 g (Charles River Laboratories, Boston, Massachusetts) were used. The Institutional Animal Care and Use Committee approved this study. Animals were randomly assigned to an experimental and a control group of 40 rats each. The experimental group received ACS injections 24, 48, and 72 hours postoperatively directly into the sutured tendon area, while the control group did not receive any such injections. Injections of phosphate-buffered saline (PBS) alone were not considered as this has previously been shown to have no effect. Similarly, injection of unconditioned serum is of questionable value because platelet activation during its preparation cannot be avoided.

Rats were placed under general anesthesia with isoflurane and received buprenorphine (0.06 mg/kg) intramuscularly into the left thigh. The right hind leg was shaved and disinfected with Betadine-Scrub (Betadine, Stamford, Connecticut) and rinsed with 70% alcohol. Animals were placed in a sterile field on a heated surgery table and covered with a sterile surgical drape such that only the prepared limb remained exposed. A 3-cm incision was made above the right Achilles tendon, the superficial tendon was exposed, and the peritendon opened. The tendon was transected with a no. 15 scalpel blade perpendicular to the collagen fibers, creating a gap 5 mm proximal to the calcaneal insertion of the tendons. The plantar tendon was transected to prevent internal splitting. After transection, the Achilles tendon was sutured back together with PDS II 0-2 (Ethicon, Norderstedt, Germany) with a Kessler–Kirchmayer stitch. (In our experience from a pilot study, use of thinner thread increases rerupture rates and, according to the literature, decreases tendon strength.) The subcutaneous layer and skin were closed by a continuous suture with Prolene 0-4 (Ethicon). After regaining consciousness the rats received twice-daily buprenorphine (0.06 mg/kg) for 2 days. To simulate early functional therapy, operated legs were left without cast immobilization.

At weeks 1, 2, 4, and 8, 10 animals from each group were euthanized with CO₂ after isoflurane anesthesia, and whole bone-tendon units were removed for testing (n = 7 for biomechanical testing, n = 3 for histology). To harvest the whole muscle-Achilles tendon-bone units, a 5-cm incision was made from below the knee down to the plantar fascia of the foot. The muscle-Achilles tendon-bone units were exposed, dissecting the subcutaneous layer from muscle, tendon, and calcaneus. Muscle-Achilles tendon-bone units were harvested by transection of the middle part of the gastrocnemius muscle and of the calcaneus.

Enzyme-Linked Immunoabsorbent Assay Tests

Cytokine levels were determined with Quantikine ELISA (enzyme-linked immunoabsorbent assay) kits (R&D Systems).
III was specifically detected using the primary rabbit antirat collagen type III polyclonal antibody (1:300, Batch 24042961, AbD Serotec, Düsseldorf, Germany) and visualized with conjugated secondary goat antirabbit immunoglobulin G antibodies (1:50, B8648, E88386, Sigma Aldrich, Hamburg, Germany). For staining, endogenous peroxidase was blocked by 30-minute incubation in 1% hydrogen peroxide in PBS. After washing in PBS, sections were incubated with 1:10 diluted rat serum to block nonspecific protein binding. Sections were incubated with the primary antibody for 1 hour at room temperature, followed by three 5-minute washes. For amplified detection with biotin-streptavidin, sections were incubated with biotinylated secondary antibody for 1 hour at room temperature, washed 3 times with PBS, and incubated with fluorescein 5-isothiocyanate–conjugated streptavidin for 20 minutes at room temperature. This was followed by three 5-minute washes with PBS.

To detect visualized areas of collagen III, 5 regions were evaluated for each sample (center of tendon, left border, right border, 2 mm above tendon center, and 2 mm below tendon center). Images (magnification ×400, area evaluated 100 μm × 100 μm) were captured with a digital camera (Olympus C-3030, Hamburg, Germany). The ratios were obtained by automated measurements of the area of visualized areas of collagen III versus nonvisualized areas of the images taken using digital imaging analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, Maryland). Results are expressed as the ratio of area of collagen. Slides were also evaluated in a blinded manner by visual inspection. There was excellent agreement between the scores obtained this way and the results of automated image analysis.

Sirius Red Staining/Cross-Polarization Microscopy

For cross-polarization microscopy, 5-μm sections were stained for 1 hour in picrosirius solution (Sirius red F3BA [0.1%] in saturated aqueous picric acid, pH 2) according to the method of Junqueira et al.16 Sections were washed for 2 minutes in 0.01 N HCl, dehydrated, cleared, and mounted in synthetic resin. Slides were viewed under polarized light microscopy. Thicker, mature collagen fibers, including type I collagen fibers, appeared red-orange, whereas thinner collagen fibers, including type III collagen, appeared pale green.16 To determine thicker, mature red-orange versus thinner pale green collagen ratios, 5 regions were evaluated for each sample (center of tendon, left border, right border, 2 mm above tendon center, and 2 mm below tendon center). Images (magnification ×400, area evaluated 100 μm × 100 μm) were captured with a digital camera (Olympus C-3030). Collagen ratios were obtained by automated measurements of the area of thicker mature red-orange versus thinner pale green using digital imaging analysis software (Image-Pro Plus, Media Cybernetics). Results are expressed as the ratio of area of collagen. Slides were also evaluated in a blinded manner by visual inspection. There was excellent agreement between the scores obtained this way and the results of automated image analysis.

Biomechanical Testing

The muscle-tendon–bone units were kept wrapped in cotton gauze soaked in lactated Ringer's solution and stored at...
Lysyl Oxidase Activity

Twelve Achilles tendons of the left, unoperated leg were collected for the determination of lysyl oxidase activity. Tendons were explanted (6 per group) and cultured for 7 days in 1 mL of DMEM + 0.2% FBS with and without 10% ACS for 7 days. The activity of lysyl oxidase was determined according to the peroxidase-coupled fluorometric assay of Treckman et al.92 Tendon samples were homogenized in extraction buffer consisting of 0.15 M NaCl and 16 mM potassium phosphate, pH 7.8. The samples were centrifuged at 10 000 × g for 20 minutes. The pellets were extracted with 3 volumes of 4 M urea and 16 mM potassium phosphate, pH 7.8. After centrifugation as noted, lysyl oxidase was batch-extracted with diethylaminoethyl cellulose. Samples were allowed to adsorb to the resin for 30 minutes with gentle mixing. The resin was then washed with 4 M urea and 16 mM potassium phosphate buffer, pH 7.8. The bound lysyl oxidase was eluted with 0.5 mM of 0.5 M NaCl, 4 M urea, and 16 mM potassium phosphate buffer. The fluorometric assay was conducted on an Amino Bowman Series 2 Luminescence spectrophotometer (Perkin-Elmer, Waltham, Massachusetts) at 55°C with constant stirring. To the assay solution containing 0.25 mg of sodium homovanillate, 40 μg of horseradish peroxidase, 1.2 M urea, in 1.9 mL of 0.05 M sodium borate buffer, pH 8.2, a 100-μL test sample was added. Reactions were started by addition of 0.1 mL of a 50 mM solution of diaminopentane substrate solution. Fluorescence changes were monitored continuously for 10 minutes at excitation and emission wavelengths of 315 and 425 nm, respectively. The slope of the fluorescence change is proportional to the lysyl oxidase activity in the test solution. Fluorescence changes were completely inhibited by the lysyl oxidase inhibitor β-aminopropionitrile fumarate (2 × 10−4 M).

Statistical Analysis

One-tailed Student t tests with Kolmogorov-Smirnov test for assessing normal distribution of values was performed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, California). The level of significance was set to P ≤ 0.05.

RESULTS

In Vitro Data

Effect of Conditioning on Growth Factor Content in Rat Conditioned Serum. Conditioning of rat blood in Orthokine syringes for 9 hours significantly increased TGF-β1 levels 1.4-fold from 23 030 to 32 450 pg/mL (P = .0123). The PDGF-BB (7550 pg/mL, basal), 7980 pg/mL in ACS were essentially unaffected, and increases in VEGF (21 pg/mL basal to 28 pg/mL, P = .0476) were small.

Effect of Conditioned Serum on Collagen mRNA Expression. Incubation of Achilles tendons in vitro in ACS for 7 days caused substantial increases in Col1A1 and Col3A1 mRNA expression over levels in untreated tendons. The expression of Col1A1 mRNA was more than 5 times higher than that of Col 3A1 (Figure 1).

In Vivo Data—Effect of ACS on Achilles Tendon Healing

Histology and Immunohistochemistry. Sirius red staining did not reveal differences in collagen type deposition at weeks 1 and 4, but a distinct increase of thicker mature red-orange fibers was apparent at weeks 2 and 8 in tendons from animals with sutured tendons receiving ACS injections (Figures 2A and 3B).

This finding is supported by the semiquantitative immunohistochemical analysis with a collagen type III-specific antibody. In ACS-treated animals, the collagen III content at weeks 1, 2, and 8 was approximately one-third that of animals that did not receive ACS injections (Figure 2B).

Staining with hematoxylin and eosin revealed a much more rapid recovery of the normal histologic appearance of the tendon in response to ACS (Figure 3A). In the control group at week 1, cells were abundant and interlaced with adipose deposits. Occasional macrophages and fibroblastic elements were also apparent. Capillarization was distinct and some collagen fibrils were already bundled and properly aligned. At week 2, collagen deposition was markedly increased but tension fibers were not yet apparent. In addition to occasional fibrocytes, many fibroblasts were apparent. At week 4, thick fibers that often ran parallel to each other were present. Macrophages in close proximity to the sutures were also observed. At week 8, collagen crimp started to synchronize within big collagen bundles. In comparison with normal tendons, however, there were still more fibrocytes between the collagen fibers.
In tendons of ACS-treated rats, collagen fibers were apparent as early as week 1 postoperatively. These collagen fibers were visibly thicker and more bundled than in tendons of animals that did not receive ACS injections. This trend toward better organized collagen continued in week 2, at which time bundled collagen became apparent.

Numerous fibrocytes but also some fibroblasts were found between the collagen fibrils. Four weeks postoperatively, there was a distinct overall tendonlike appearance with thick collagen bundles and cells oriented along the tension fibers, but occasional vascular conglomerates and fibrocytes were still present and around the sutures some macrophages could still be seen. At 8 weeks postoperatively, the tendons had a smooth appearance resembling very much normal tendons with small fibrocartilaginous areas in the center of the stress areas (Figure 3A).

Tendon Thickness and Biomechanical Properties. The ACS treatment caused consistent increases in tendon thickness at the site of healing (Figure 4).

Tendons of rats treated with ACS reached stiffness values 4 weeks earlier than the group that was not receiving ACS injections (Figure 5A). On the other hand, there were no differences in maximum load to failure between groups up to week 8 (Figure 5B). As judged from the continued increased thickness of ACS-treated tendons, remodeling of repair tissue is delayed with ACS treatment.

Activity of Lysyl Oxidase. The activity of lysyl oxidase in Achilles tendons obtained from control and ACS-treated tendon in vitro as measured by the slopes of fluorescence development was not significantly different (control, 0.04115 per minute; ACS, 0.0368 per minute; \( P = .7579 \)).

**DISCUSSION**

Patients suffering from complications after Achilles tendon rupture and suturing are common in clinical practice. The imperfections of endogenous repair often leave patients symptomatic and delay the time for return to work and sports activities. Because surgical treatment and post-treatment regimens are already refined, further major improvement of Achilles tendon healing is likely to come from the use of biological approaches, such as the administration of appropriate growth factors.
Disturbances of the tendon structure lead to considerable loss of function in the lower extremities. To fulfill its function, the Achilles tendon has special morphologic properties: the helical Achilles tendon is relatively thick and collagen-rich with a distinct parallel orientation of the collagen fibrils. This arrangement renders the tendon one of the most stress-resistant tissues in the human body. It is thus significant that, in our in vitro study, ACS supplementation of tendons increased markedly the expression of mRNA coding for collagen type I and collagen type III. The expression of type I collagen was increased to a greater degree than that of type III collagen, which is consistent with the in vivo findings. This explains the improved stiffness at 4 weeks produced by ACS, because type I collagen contributes to greater tensile strength. Measurements at a later time point showed that remodeling is delayed as postulated.

We speculate that this increased expression of mRNA encoding the α chains of collagen types I and III may result from conditioning of blood, which increased the concentration of TGF-β1 1.4-fold. Wright-Carpenter et al. and Meijer et al. reported a comparable 1.8-fold increase in TGF-β1 after conditioning of mouse serum. These authors documented an even larger 5.6-fold increase in FGF-2, a growth factor that we did not measure. The FGF injections have been shown to affect the initial events, cell proliferation and collagen type III expression, in a rat patellar tendon model without having a significant effect on ultimate stress. It is quite possible that the elevated concentration of TGF-β1 in ACS may have mediated part of the observed effects on tendon healing. This cytokine has pleiotropic effects on fibroblasts and has emerged as a pivotal factor in tendon and ligament healing. It has been shown to stimulate proliferation, migration, and collagen production by fibroblasts, and to inhibit the degradation of extracellular matrix proteins by catabolic enzymes. The stimulation of proliferation and migration may be of particular importance in the initial stages of repair, whereas the inhibition of matrix degradation may be crucial for the maintenance of tissue integrity during the remodelling phase.

Figure 3. Effect of autologous conditioned serum (ACS) treatment on the histologic appearance of the repair site in the rat Achilles tendon. A, sections were made from tendons recovered 1, 2, 4, and 8 weeks after surgery (week 1 at top, week 8 at bottom) and stained with hematoxylin and eosin. Repair tissue formed with ACS injections (right) appears more structured, with a more developed crimp pattern, bigger collagen bundles than without (left). Sutured-only tissue shows more fibroblasts, fat cells, and blood vessels (left). Magnification ×200. B, sections were made from tendons recovered 1, 2, 4, and 8 weeks after surgery (week 1 at top, week 8 at bottom) and stained with Sirius red. Sirius red staining did not reveal differences in collagen type deposition at weeks 1 and 4, but a distinct increase of thicker mature red-orange fibers was apparent at weeks 2 and 8 in tendons from animals with sutured tendons receiving ACS injections (right) than those without ACS (left). Magnification ×400.
Figure 4. Autologous conditioned serum (ACS) application causes consistent increases in tendon thickness of the healing Achilles tendon. Significance was reached at week 1, $P = .0417$; week 4, $P = .0162$; and week 8, $P = .0125$. ○, mean ± standard error of values in sutured-only animals; •, values in sutured animals receiving ACS injections.

Stimulation of type I collagen mRNA expression by TGF-β1 was also documented by the studies in tissue culture of Ignotz et al.11 Our analysis of collagen types in tendons from ACS-treated animals showed a marked increase of collagen type I and a decrease of collagen type III. An increased content of type III collagen in the fibers would tend to reduce their tensile strength.13,14,20,25

Our investigation of collagen maturation and collagen type III deposition also showed differences between the control and treatment groups. The ACS-treated animals showed markedly more mature, thick reddish-orange collagen fibers at weeks 2 and 8 postoperatively as compared with controls. The quantitative evaluation of collagen type III showed a 3-fold reduced level of this collagen at weeks 1, 2, and 8 in ACS-treated animals compared with controls. This finding is in agreement with the reports of Aspenberg and Virchenko,2 who also documented a beneficial effect of platelet concentrate administration on the histologic appearance of the healing tendon, with a concomitant increased tendon callus strength and stiffness by about 30% after week 1, an improvement which persisted for as long as 3 weeks after the injection.39 Despite the improved collagen synthesis, which is apparent from the significantly greater tendon thickness at weeks 1, 4, and 8 (Figure 4), stiffness was significantly ($P = .038$) increased at week 4 only.

In summary, it is clearly evident that ACS injections accelerate the rate of organization of repair tissue (Figure 3); they increase collagen mRNA expression (Figure 1), collagen deposition as reflected by tendon thickness (Figure 4), and accelerate collagen fiber maturation (Figure 2). Tendon stiffness does not improve significantly over control until week 4 (Figure 5), likely because of the time required for collagen cross-linking and remodeling of the repair site to occur. The fact that repair tissue remodeling
2124 Majewski et al

is a slow process is evidenced by the finding that tendon thickness remained elevated even at week 8 (Figure 4). Tendon thickness should decrease with longer healing times, an aspect we did not address with this study. Clinically, this could mean that ACS treatment shortens functional recovery times. One would not expect that new tendon tissue, which is still undergoing remodeling, should be stronger than the original normal tendon. The fact that ACS-aided tendon repair fills in well-organized, strong collagen fibers suggests that the healed tendon should contain fewer imperfections than in repair tissue formed following spontaneous healing or tendon suture. Such tissue would be expected to be less prone to rerupture.

The biologically active component(s) in ACS that are responsible for the observed accelerated Achilles tendon healing remain to be defined. The ACS injections apparently do not critically limit inflammatory signals of the early healing phase but supplement critical growth factors that are present in serum in limiting concentrations. Overall, our study documents a beneficial effect of ACS administration on the collagen composition, histologic appearance, and mechanical strength of the tendon regenerates. In view of a possible clinical application of ACS in ruptured tendon healing in man, studies on the identity of growth factors and the dose-response relationship causing the observed accelerated healing as well as studies showing clinically important benefits in humans, such as early increase in strength, are indicated.

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