Autologous fibrin matrices: A potential source of biological mediators that modulate tendon cell activities

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Abstract: The use of autologous fibrin matrices has been proposed as a therapeutic strategy for the local and physiological delivery of growth factors in the treatment of several clinical conditions requiring tendon healing or tendon graft remodelling. In the present work, we investigated the proliferation, synthesis of type-I collagen and angiogenic factors by tendon cells seeded on platelet-rich (PR) and platelet-poor (PP) matrices. Furthermore, *in vivo* cellular and vascular effects of each treatment were examined after infiltration in Achilles tendon in sheep. Results showed that the presence of platelets within the fibrin matrices increased significantly the proliferation of tendon cells. Additionally, cultured tendon cells synthesised type I collagen and angiogenic factors such as VEGF and HGF. The synthesis of VEGF, but not of HGF, was significantly higher when platelets were present within the matrix. In the sheep model, the injection of pre-clotted plasma within tendons increased cellular density and promoted neovascularization. These results indicate that administration of fibrin matrices is a safe and easy strategy that may open new avenues for enhancing tissue healing and remodelling and influences the process of regeneration in clinical situations characterised by a poor healing outcome. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 285–293, 2006

Key words: fibrin matrices; tendon; growth factors; platelets; angiogenesis

INTRODUCTION

Tendon tissue plays a central role in force transmission from skeletal muscle by anchoring them to bones, and injuries may result from overuse or stress associated with physical activities. Among sportsmen and women, incapacitating conditions, such as tendinopathy, marked by a degenerative process and a poor healing response, are frequent.[1] Current therapies to treat these conditions involve long-term conservative and palliative cares that do not change the intrinsically poor-healing properties of the tendon.[2] Given this situation, biologically based strategies involving the stimulation of cell activities through the delivery of growth factors have attracted considerable interest.[3,4]

The use of tendon grafts in both ACL reconstruction and tendon–tendon grafting is frequent in orthopedic surgery. In the former approach, the injured ligament is removed by arthroscopic surgery, and the joint is reconstructed with a tendon graft. However, to succeed, it is necessary to ensure the long-term survival of the graft by the controlled administration of chemotactic, mitogenic, and angiogenic factors that will promote cell metabolism, the formation of new blood vessels, and tissue remodeling.[5] In both operative and nonoperative treatments, a complex source of growth factors essential to natural wound repair and tissue promotion must be released at the required time and level, mimicking the physiological wound-healing process.[6]

To address these issues, the use of autologous activated platelets retained in fibrin matrices as a source of growth factor release in a three-dimensional scaffold has been proposed.[7] The human clinical applications of this fibrin matrix are widespread, including chronic leg ulcers,[8] implant consolidation in dentistry,[7] and tissue engineering.[9] Platelets embedded in the fibrin scaffold secrete a myriad of factors including transforming growth factor β-1 (TGF-β1), platelet-derived growth factor (PDGF), vascular endothelial

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growth factor (VEGF), epithelial growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factor type I (IGF-I) among others.[7] The progressive release of these proteins from the fibrin matrix and the intrinsic flexibility and biocompatibility of the scaffold provide unique properties for tissue remodeling, wound–healing, and angiogenic promotion.[10]

In view of these properties, we have proposed the use of autologous platelet-rich fibrin matrix to enhance tendon healing and remodeling. In this context, we have recently demonstrated that releasates from platelet-rich (PR) and platelet-poor (PP) matrices stimulated tendon-cell proliferation and the synthesis of VEGF and HGH from cultured tendon cells.[11] We have now further evaluated the potential role of growth factors released from a fibrin matrix on tendon cell proliferation and metabolism in culture. We have also assessed the *in vivo* activity and biosafety of the gel after infiltration in an animal model. By comparing the *in vitro* and *in vivo* effects of PR- and PP- matrices on tendon cells, we will shed light on the role of the platelets and the most suitable therapeutic approach. The present study was undertaken with the aim of addressing these issues and confirming the potential impact of PR-fibrin matrices as a source of growth factors for tendon-healing and tendon graft surgery.

MATERIALS AND METHODS

Isolation and culture of tendon cells

Human tendon samples were obtained from an otherwise healthy young donor during surgical reconstruction of the anterior cruciate ligament with semitendinosus tendon autograft. Informed consent and project approval from the local ethics committee were obtained. The tendon was cleaned of surrounding adipose tissue and cut into fragments that were placed in phosphate-buffered saline (PBS) supplemented with antibiotics (penicillin–streptomycin solution and amphotericin B; Sigma Chemical Company, St Louis, IL). The fragments were then minced and treated with 0.3% collagenase II (Gibco Life Technologies, Gaithersburg, MD) at 37°C for 2 h. The resulting cell suspension was filtered and centrifuged at 460*g* for 10 min. Cells were seeded into culture flasks and maintained with D-MEM/F12 (1 vol:1 vol) (Gibco) culture medium supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 2 m*M* L-Glutamine (Sigma), antibiotics, and amphotericin B in a humidified atmosphere at 37°C with 5% CO_2. All experiments were performed on cells obtained between the third and fifth passage. The cells were characterized immunocytochemically by staining for collagen type I (Biodesign, Saco ME), a determinant of the tendon-cell phenotype.[12]

Preparation of PP- and PR-fibrin matrices

Blood was collected into 3.8% (wt/vol) sodium citrate from six healthy donors. Samples were centrifuged either at 4500*g* for 12 min at 4°C to obtain PP-plasma or at 460*g* for 8 min to obtain PR-plasma. Platelet counts were performed with a Beckman-Coulter A^{c}T Differential Analyzer (Galway, Ireland) in both the PP-plasma and PR-plasma before clotting. Calcium chloride was added to PP- and PR-plasma of each donor at a final concentration of 22.8 m*M*. Immediately after calcification, 150 μL of either PP- and PR-plasma were dispensed onto 48 well plates; all fibrin matrices were formed "*in situ*" within 15 min, covering the whole surface of the wells. Control cultures were set using purified human fibrinogen (3.5 mg/mL, Calbiochem, Darmstadt, Germany) that was converted into fibrin by addition of 1.5 U of human thrombin (Stago, France) and 22.8 m*M* calcium chloride.

Cells cultured on fibrin matrices

Subconfluent cultures of tendon cells maintained with serum-free medium for 24 h[13] were trypsinized, and cell viability was assessed by trypan blue dye exclusion. Cells were plated at a density of 1000/cm^2 on the wells coated with either PP- or PR-matrices and covered with 350 μL of culture media (D-MEM/F12 (1 vol:1 vol), 2 m*M* glutamine, and antibiotics) without any serum supplement. Additional wells coated with fibrin matrices and incubated with media, but without tendon cells, were used for background correction.

Cell proliferation was evaluated using the WST-1 (tetrazolium salt, (4-[3,4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) colorimetric assay (Roche, Basel, Switzerland). All experiments designed to study proliferation were performed three times and gave essentially the same results.

HGF, VEGF, and TGF-β1 were measured using commercially available Quantikine ELISA kits (R&D, Minneapolis, MN). Conditioned media were collected on day 6 of treatment, centrifuged for 5 min at 460*g*, and stored at −80°C until assayed. VEGF, HGF, and TGF-β1 were quantified in the culture media conditioned by tendon cells. Culture media obtained from control fibrin matrices without cells, after 6 days of incubation, were used for background correction.

Collagens are synthesized as precursor molecules, procollagens; these contain additional peptide sequences that are cleaved during secretion but that reflect stoichiometrically the amount of collagen molecules synthesized. Human procollagen type I C-peptide was measured in the media conditioned by tendon cells after 6 days of culture using an *in vitro* solid phase enzyme immunoassay (EIA) kit, according to the manufacturer instructions. (Takara, Japan) The results were normalized for cell number and expressed as ng or μg/10^6 cells.

Animal model

Six skeletally mature sheep weighing 35–40 kg were used in the study; each animal was examined for general health

by a licensed veterinarian. The study was carried out in accordance with guidelines for experimentation on animals in Spain (Decree 2223/88). Infiltrations and surgery were always performed under sterile conditions in an experimental operating room; both hind limbs were shaved and prepared in the standard sterile fashion.

Intratendinous injection of preclotted preparations rich in growth factors

Blood was drawn into 3.8% (wt/vol) sodium citrate from the inferior cava vein prior to anesthesia. PR-plasma was separated by centrifugation at 630*g* for 8 min. PP-plasma was prepared by further centrifugation of these tubes at 4500*g* for 12 min. Calcium chloride was added at a final concentration of 22.8 m*M* just prior to the injection into the tendon. Animals were anaesthetized by intramuscular injection with ketamine. Each animal was injected in the Achilles tendon of hind limbs with different preparations. Four tendons were injected with calcified PR-plasma into the Achilles tendon; four tendons were injected with PP-plasma; and the other four with saline. All injections (2 mL) were performed using a 26-gauge needle at 2.5 cm proximal to the bone insertion; the needle was displaced in parallel alongside the fascicles while the injection was made. Subsequently, the fibrin matrix developed *in situ* within the tendon. Injections were administered once a week for four weeks, and tissue was sectioned for histological examination in the fifth week, seven days after the last injection. Additionally, Achilles tendon tissue was harvested from normal sheep that had not been injected.

Histological examination

Tissue specimens were obtained by dissecting sections (1.5 cm long, 0.5 cm wide) from the middle axis to the peripheral area in contact with the sheath. Specimens were removed and immediately fixed in 4% formaldehyde for 48 h and embedded in paraffin wax. Longitudinal 5-μm sections were stained with hematoxylin/eosin. Total cell counts on the tendon tissue were done under conventional light microscopy (Leica DMLB, Leica GmbH, Bensheim, Germany), by two independent observers unaware of the treatment. Cells were counted within the field of view at regular intervals along the length of the section. Three longitudinal sections were examined per specimen. Four randomly chosen intrafascicle regions (0.216 mm^2 each) were evaluated per section, and the mean cell density per square millimeter was calculated (Table I). Cell density was also determined in normal Achilles tendon tissue. The interobserver variability was 14.1%. Additionally, some other tissue aspects including rounding of the nuclei, cell arrangement, and vascularity were also examined.

Statistical analysis

Results from cell culture studies and histological cell densities are expressed as mean ± SEM of a representative experiment that has been performed in triplicate. Distribution of data was analyzed using the Kolmogorov-Smirnov test. The means were compared using the Student's *t*-test. For nonparametric distributions, comparisons between groups were performed using the Mann–Whitney-U Wilcoxon test. A difference at a level of $p < 0.05$ was considered to be statistically significant (Statgraphics Plus, Manugistic, MS).

TABLE I
Cell Density Within Fascicles after 4 Weekly Injections of Saline, PP, or PR Plasma

	Cell Density (cells/mm^2) mean ± SD (*N*)
Untreated tendon	366 ± 116 (2)
Injected Achilles tendon	
Saline	463 ± 181 (4)
PR matrix	1546 ± 421[a,b] (4)
PP matrix	1801 ± 454[a,b] (4)

Number of Achilles tendons (*N*).
[a]Significant differences comparing with untreated tendon.
[b]Significant differences comparing with saline injected control.

RESULTS

Proliferation of tendon cells on different fibrin matrices

Tendon cells were cultured on the surface of homologous fibrin and PR- and PP-derived matrices. As shown in Figure 1, an increased tendon-cell proliferation was observed on PR- and PP- matrices [Fig. 1(A,C)] compared to homologous fibrin [Fig. 1(E)]. The increased cell density was better appreciated after hematoxylin staining [Fig. 1(B,D,F)].

A quantitative analysis confirmed that tendon-cell proliferation was not induced when cells were seeded on matrices prepared from purified fibrin (number of cells = 1927 cells/cm^2), which provided structural support for cell attachment but lacked growth factors (Fig. 2). The count of platelets in whole blood was 231×10^3 platelets/μL (SD: 41) ranging from 207×10^3 to 311×10^3 platelets/μL and were all within the normal human range; the PR plasma increased the platelet number to 542×10^3 platelets/μL (SD: 69) while the PP plasma decreased to 5×10^3 platelets/μL (SD: 3). As shown in Figure 2, an increased presence of platelets within the matrices increased significantly the tendon-cell proliferation (PP-matrices, 47,362 ± 5624 cells/cm^2: PR-matrices, 65,268 ± 8221 cells/cm^2; $t = -4.40$, $p = 0.0013$)

Synthesis of growth factors: TGF-β1, VEGF and HGF

A major finding in our work, which could be especially relevant to the vascular status of tendon, is that

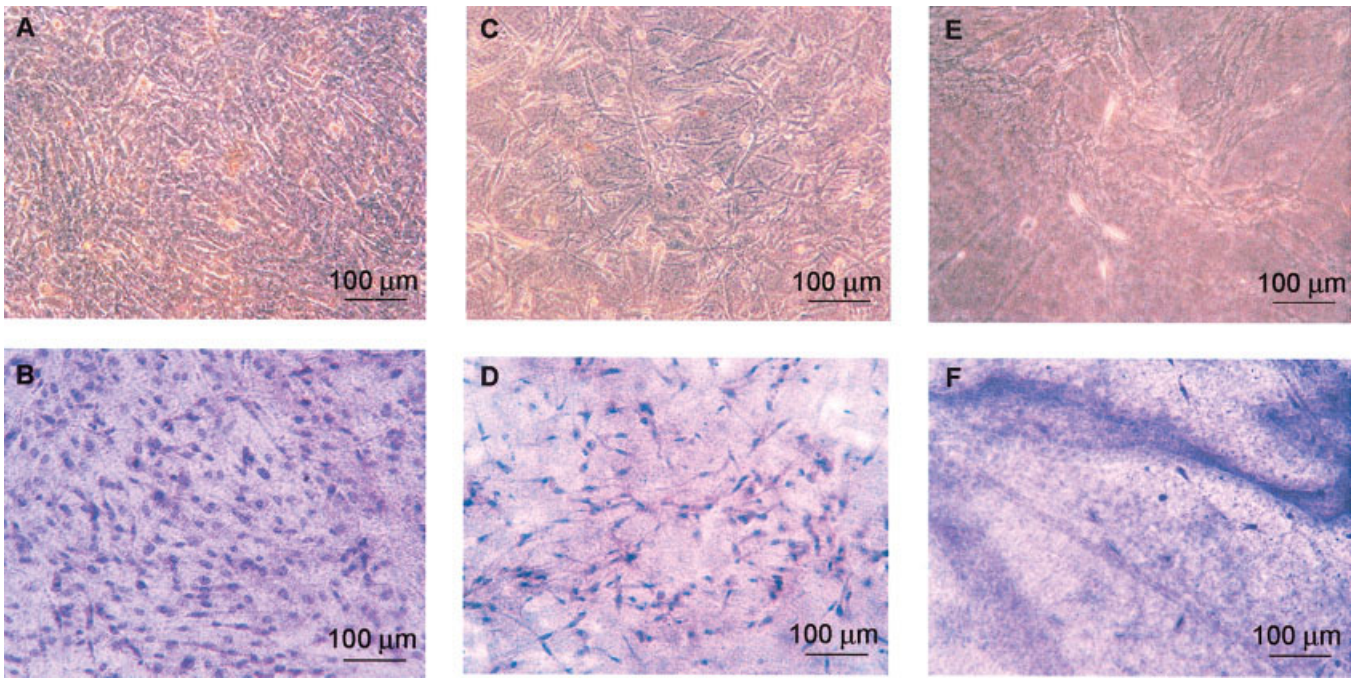

Figure 1. Representative photomicrographs of human tendon cells demonstrating proliferation induced after 6 days of culture on different fibrin matrices. (A, B) Autologous platelet-rich fibrin, (C, D) autologous platelet-poor fibrin, and (E, F) purified human fibrin; (B), (D), and (F) illustrate cellular density in the different matrices after staining cell nucleus with hematoxylin. Photographs were taken with an inverted optical microscope equipped with a camera. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tendon cells synthesized considerable amounts of VEGF and HGF when cultured on the fibrin matrices. As shown in Figure 3(A), the synthesis of VEGF was again dependent on the number of platelets within the scaffolds ($t = -5.77474$, $p = 0.000179$). Furthermore, it was observed that tendon cells synthesized HGF when grown on fibrin matrices, although the production of HGF was similar by cells grown on either PP- or PR-fibrin matrices ($t = 1.37469$, $p = 0.1992$) [Fig. 3(B)].

TGF-β1 released from PR-matrices without cells was significantly higher than that from PP-matrices, 1,4500 ± 2004 ng/mL versus 731 ± 58 ng/mL, respectively (mean ± SD) ($t = -16.82$, $p = 1.15 \times 10^{-8}$). Moreover, cells cultured on either PP- or PR-matrices synthesized TGF-β1. However, the TGF-β1 production by tendon cells growing on PR-matrices was significantly higher ($W = 32$, $p = 0.0306$) [Fig. 3(C)].

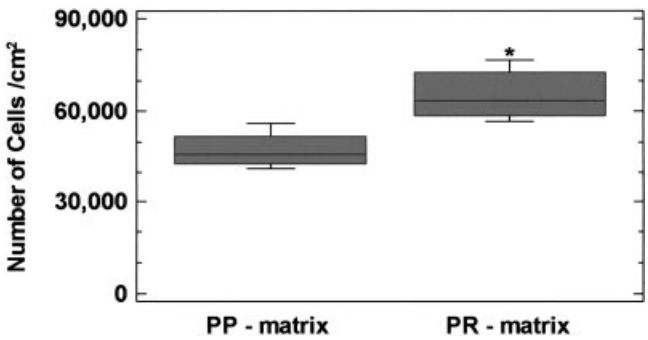

Figure 2. Proliferation of tendon cells on PP- and PR-matrices. Box plots summarize the combined results obtained for six different donors; * $p < 0.05$ as compared to PP-fibrin.

Production of type I collagen

The extracellular matrix of tendons is composed predominantly of type I collagen fibers; therefore, its production is a key event in the healing and remodeling of tendons. Cells seeded on PR- and PP-matrices produced type I collagen after 6 days of culture in contrast to cells seeded on homologous fibrin. Unexpectedly, we observed that collagen production was similar on PP- and PR-matrices despite the lower concentration of TGF-β1 released from the PP-matrix (Fig. 4).

Animal studies

In vivo injection of recalcified PP- and PR-matrices provoked a large increase in cell density within the fascicles [Fig. 5(A–D)]. The cellular density was similar in tendons injected with PR-matrices and with

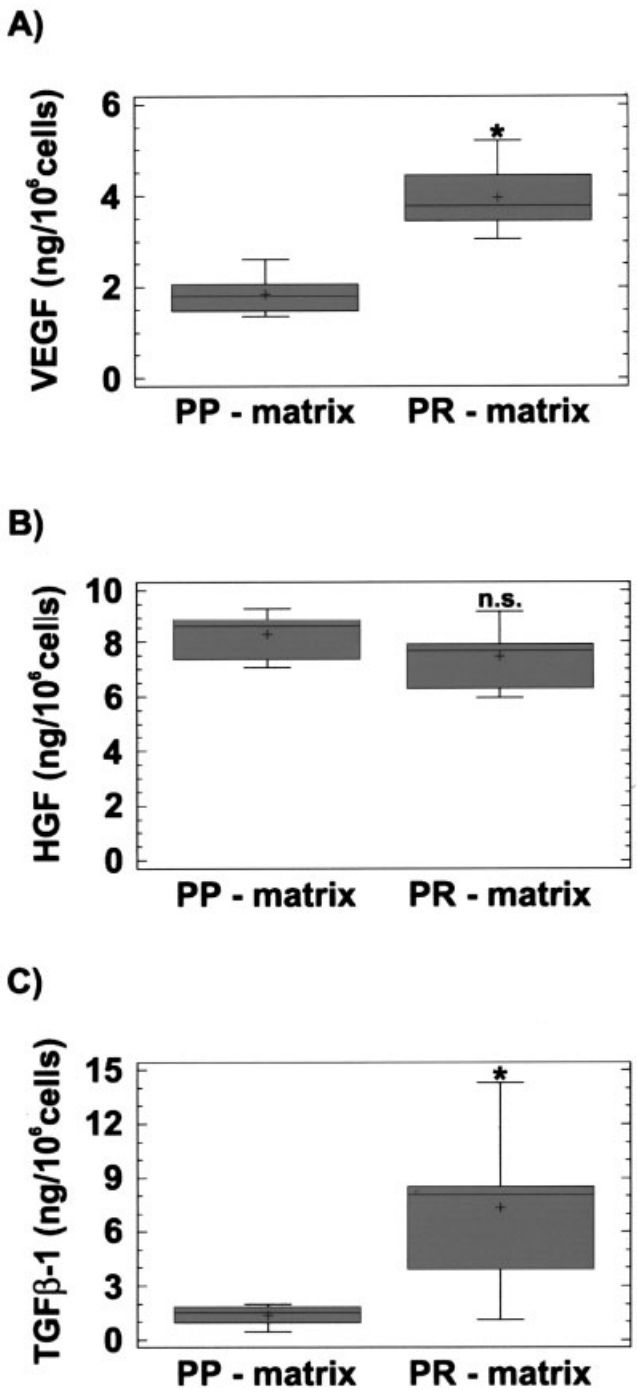

Figure 3. Synthesis of growth factors (A) VEGF, (B) HGF, and (C) TGF-β1. Results are normalized for cell number and the box plots summarize the combined values obtained for six different donors, in three independent experiments. * $p < 0.05$ as compared to PP-fibrin.

PP-matrices; nevertheless, both were significantly higher than saline-injected tendons or normal untreated controls (Table I).

In tendons injected with PP- and PR-matrices, concurring with an increase in cell density, there was a change in cell morphology. In fact, when PP- and PR-matrices were injected, tendon cells had an ovoid shape, in contrast to spindle shape-morphology in intact tendon. However, these ovoid cells appeared aligned along the collagen fibers, showing organization along lines of tension. The latter contrasted with tendons injected with saline where disorganized and disordered cells accumulated in limited areas at the injured site [Fig. 5(E)]. Most areas in the same saline-injected specimen showed a more regular appearance with scarce spindle-shaped fibroblasts laying down the collagen matrix and oriented in the direction of stress mechanical forces as in natural tendon tissue [Fig. 5(F)]. In the connective tissue layer of the endotenon, a high concentration of cells with large hyperchromatic ovoid nuclei was detected. In either PP- or PR-plasma-injected tissue, endotenons were more prominent through an accumulation of round cellular elements.

Concerning the vasculature, high vasodilatation, collapse, and activation of endothelial cells were the most striking features in tendons treated with PR- and PP-matrices [Fig. 6(A,B)]. An intense neovascularization and an absence of inflammatory cells were observed in all specimens treated with fibrin matrices.

DISCUSSION

There is growing evidence suggesting that the growth factors released from a fibrin matrix may be suitable for accelerating tissue-healing and remodeling.[3,6,14] Fibrin matrix preparations made by regulated polymerization of fibrinogen in plasma through the induced formation of native thrombin represent a clinical strategy for the delivery of biological mediators that has the advantages of being technically simple and autologous, thus obviating the risk of immunological reaction or transmitted diseases. The entrapped platelets release a natural pool of growth factors including PDGF, TGF-β1, IGF-I, VEGF, HGF,

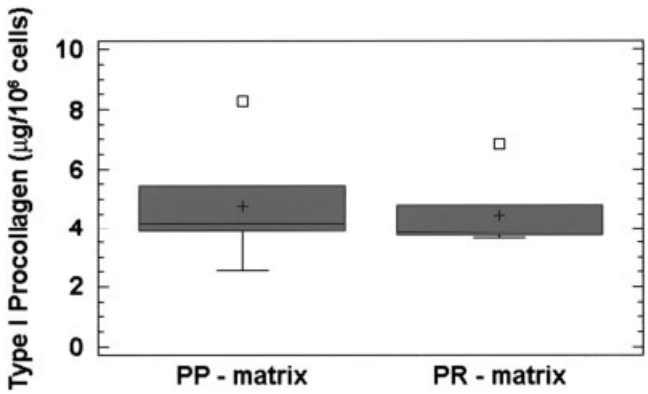

Figure 4. Production of type 1 collagen. Box plots summarize the combined values obtained for six different donors. n.s., no significant difference as compared to PP-fibrin.

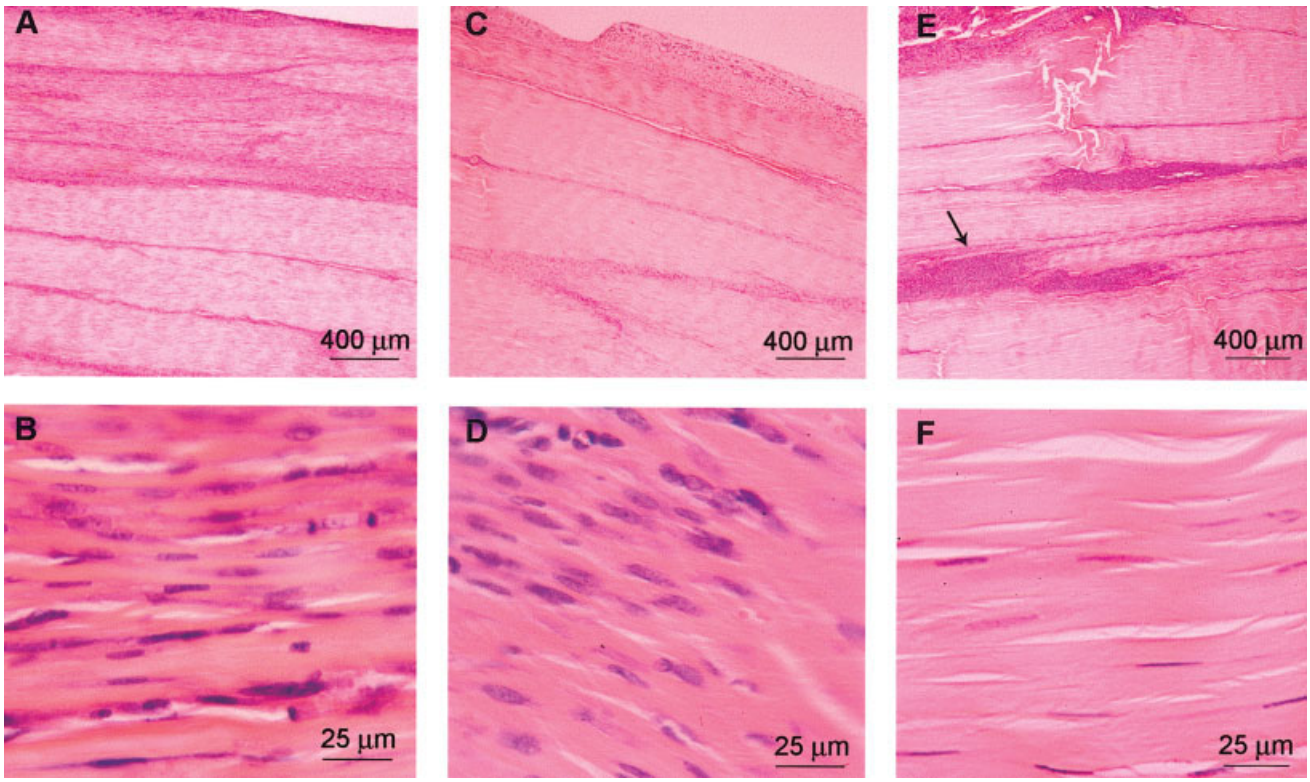

Figure 5. Longitudinal sections of Achilles tendons seen by light microscopy and hematoxylin/eosin staining. (A) Tendon treated with PR-fibrin; and (B) high cellular density was observed at higher magnification in a mid area. (C) Tendon treated with PP-fibrin and (D) central area at higher magnification showing cell density. (E) Tendon treated with saline showing randomly oriented cells concentrated in limited areas (black arrow) and (F) mid area at higher magnification shows low cell density. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and EGF, some of which diffuse rapidly into the milieu while others bind to the fibrin and are released slowly.[7] This pool of growth factors mimics native physiology stimulating cell surface receptors and intracellular signaling proteins that control both repair and remodeling mechanisms.[15] The fibrin matrix also contains adhesive glycoproteins such as vitronectin and thrombospondin-1, both able to bind growth factors, thereby influencing their storage or presentation toward cellular receptors.[16,17] These proteins may also react with cell receptors and influence motility and the cellular response.

Assuming this knowledge and the potential role of fibrin matrices in enhancing tendon-healing, we have examined whether the local and physiological delivery of biological mediators from autologous fibrin matrices can influence tendon cell function. To address this issue, tendon cells were cultured for 6 days on homologous fibrin and PR- and PP-matrices and cell proliferation, production of type I collagen and synthesis of growth factors by the tendon cells were determined. The information gathered from this study may be very useful to understand the *in vivo* role of the fibrin matrix after it is injected within the tendons.

The results presented in this study demonstrate that fibrin matrices increase significantly the rate of tendon-cell proliferation when compared to homologous purified fibrin. In addition, the proliferation of tendon cells increased when PR-matrices containing a higher number of platelets were used. The latter agrees with our previous results using releasates from PR- and PP-matrices.[11] This implies that the physiological presence of biological mediators within the matrices is a necessary event to modify tendon cell metabolism. The increase in the rate of tendon-cell proliferation would be advantageous for *in vivo* tendon healing and remodeling.

Another interesting finding is that tendon cells synthesized considerable amounts of VEGF and HGF when cultured on the fibrin matrices. This could be especially relevant to the vascular status of tendon assuming that the low-healing capability of tendon is associated with a reduced blood supply when compared with other tissues. Therefore, tendon tissue-healing and remodeling requires angiogenesis, a process that is tightly controlled by growth factors.[18] In the case of VEGF, we found that its synthesis was dependent on the number of platelets within the scaffolds. Previous studies from our laboratory have showed that TGF-β1 secreted by platelets is involved in the production of VEGF by tendon cells.[19] This paracrine-acting factor, which is needed to maintain

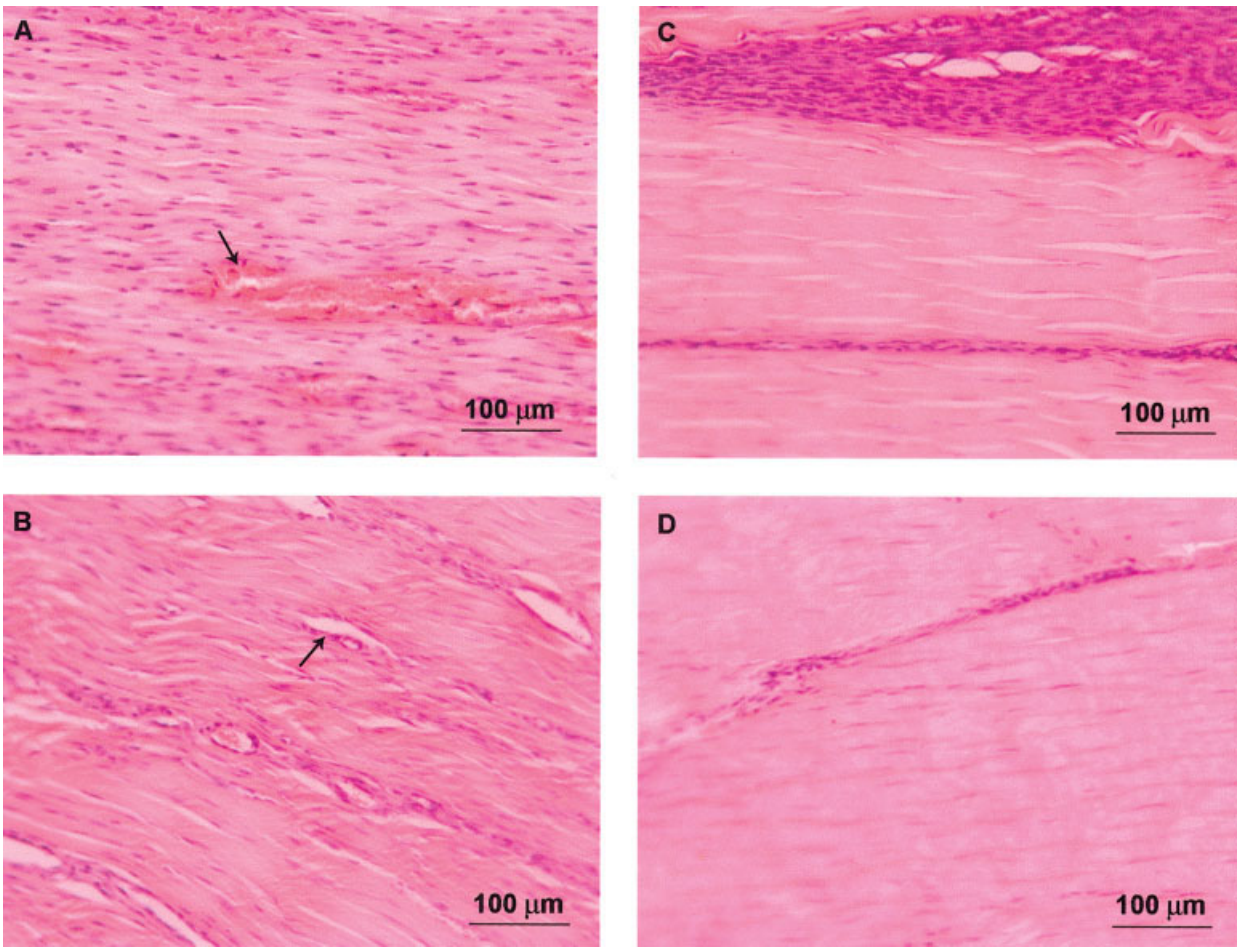

Figure 6. Histological sections of the Achilles tendons, hematoxylin/eosin staining, (A) treated with PR-fibrin, and (B) treated with PP-fibrin. Note the presence of blood vessels in PP- and PR-fibrin (black arrows). (C) tendon treated with saline, and (D) untreated tendon. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

vascular integrity, represents the most potent stimulator of vascular permeability by virtue of its effects on endothelial cell fenestration and proliferation.

On the other hand, cultured tendon cells also synthesized HGF when grown on fibrin matrices. However, the production of HGF was similar by cells cultured on either PP- or PR-fibrin matrices. Since HGF induces autophosphorylation of the c-Met receptor, it is a mitogen for endothelial cells, stimulating cell migration and the formation of vascular tubules,[20,21] thus being considered as a therapeutic tool for inducing angiogenesis.[22] Interestingly, coadministration of VEGF and HGF has been tested as an alternative strategy to promote angiogenesis without the typical inflammation associated with VEGF.[23] Min et al.[24] suggested that a combined treatment with VEGF and HGF could be superior to either factor alone for enhancing therapeutic angiogenesis while avoiding inflammation.

We also paid attention to the TGF-β1 released by the tendon cells cultured on PR- and PP-matrices. Platelets are the major source of TGF-β1 that is known to play an important role in the healing and remodeling process of connective tissue and in directing cellular response to injury. However, since TGF-β1 drives fibrogenesis, it potentially stimulates the development of scar tissue and is associated with the onset of fibrosis due to increased collagen formation and deposition. Our results showed the TGF-β1 released from PR-matrices was significantly higher than from PP-matrices (14,500 ng/mL versus 731 ng/mL, respectively). Additionally, TGF-β1 secretion from tendon cells cultured on PR-matrices was also higher than from cells seeded on PP-matrices. This data has created much controversy around the potential therapeutic role and biosafety of PR-matrices on tendon-healing. However, this increased TGF-β1 release does not necessary imply a higher collagen deposition and scar formation. We hypothesize that type I collagen production from tendon cells cultured in both matrices should be evaluated, since other biological mediators secreted by the fibrin matrices could counterbalance this effect.

To accomplish this, we measured collagen produc-

tion from cells seeded on both matrices and found that cells seeded on PR- and PP-matrices produced type I collagen after 6 days of culture in contrast to cells cultured on homologous fibrin. Unexpectedly, we also observed that collagen production was similar on PR- and PP-matrices despite the lower concentration of TGF-β1 released from the latter. Therefore, our data shows that cells cultured on both matrices produced type I collagen, which is a key event in the healing and remodeling of tendons and is essential for gaining tensile strength. However, we suggest that the lack of higher collagen synthesis in the presence of PR-matrices compared to PP-matrices is that the effect of TGF-β1 is neutralized by other platelet-released molecules, corroborating our previous findings.[19] One of these molecules could be HGF, which has been reported to antagonize the profibrotic action of TGF-β1 by inhibiting fibronectin and type I collagen expression.[25]

To aid knowledge of the potential *in vivo* effects of the fibrin matrices, we decided to inject a saline solution and PP- and PR-matrices into the Achilles tendon of sheep and compare the cellular and vascular effects exerted by each treatment in normal tendons. In fact, the administration of the fibrin matrices increased cell density within the fascicles; however, we could not see any difference between PP- and PR-matrices in spite of the higher amount of platelets in the latter. We also observed a change in cell morphology compared with saline injections and untreated control tendons. In the connective tissue layer of the endotenon, a high concentration of cells with large hyperchromatic ovoid nuclei was detected. This observation suggests the possibility of tenoblast division within the endotenon with subsequent migration into the tendon parenchyma. The angiogenic stimulation induced by the fibrin matrices was also clearly detected in the histological sections of tendons. No signs of fibrosis were observed, which implies that the use of fibrin matrices may be a safe strategy to initiate and promote healing in damaged tendons or remodeling of tendon grafts. Finally, neovascularization and an absence of inflammatory cells were observed in all specimens treated with fibrin matrices, which might be a consequence of the combined release of VEGF and HGF, as has previously been described.[23]

In summary, PP- and especially PR-matrices can be considered a safe and effective strategy to accelerate tendon-cell proliferation, stimulate the synthesis of type I collagen, and promote neovascularization both *in vitro* and *in vivo*. Another two major advantages of this treatment include the rapid and easy preparation of the matrices and the increased safety of the procedure, since both the fibrin matrix and the biological mediators are autologous. Overall, the use of autologous fibrin matrices releasing growth factors may open new avenues for enhancing tissue-healing and remodeling.

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