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Autologous preparations rich in growth factors promote proliferation and induce VEGF and HGF production by human tendon cells in culture

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Abstract

Blood platelets become activated and aggregate at the site of vessel injury. Upon activation by thrombin, platelets release storage pools of proteins and growth factors (GFs), including those involved in tissue repair. Our goal was to evaluate the potential beneficial effect of proteins released from platelet-rich clots on tendon healing. PDGF, TGF-β-1, IGF-I, HGF, VEGF and EGF were measured in human platelet-poor plasma (PPP) and in the releasates collected from either platelet-poor or platelet-rich clots prepared in vitro. We then studied the effects of the releasates on human tendon cells in culture. Releasates from both platelet-rich and platelet-poor clots stimulated tendon cell proliferation, in contrast to un-clotted PPP. The mitogenic activity of the supernatants was not decreased by the thrombin inhibitor, hirudin. Cultured tendon cells synthesise VEGF and HGF in the presence of PPP-clots and PRP-clot releasates, thus the synthesised amount was significantly higher with supernatants from platelet-rich clots than supernatants from a platelet-poor clot ($p < 0.05$). These results suggest that administering autologous platelet-rich clots may be beneficial to the treatment of tendon injuries by inducing cell proliferation and promoting the synthesis of angiogenic factors during the healing process.

Keywords: Platelets; Growth factors; Tendon; Healing; Cell culture

Introduction

Acute tendon injuries and tendon pathologies resulting in joint dysfunction are a growing problem in traumatology. The commonest form of tendon healing is by scarring, which affects function and is accompanied by an increased risk of further damage. In general, the tendon heals at a relatively slow rate compared with other connective tissues; a reason for its limited healing capability could be its poor vascularization [8,12]. Growth factors are liberated at sites of injury and inflammation. Platelets are known to actively participate in healing processes by delivering growth factors and other active molecules to the injured site by exocytosis following adhesion or their stimulation by thrombin or other strong stimuli [1]. Growth factors secreted by platelets include platelet-derived growth factor (PDGF), endothelial growth factor (EGF), insulin-like growth factor (IGF-I) and transforming growth factor-β-1 (TGF-β-1), vascular endothelial growth factor (VEGF),

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hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF). Some such as TGF-β-1, PDGF and IGF-I variously promote proliferation, cell migration and synthesis of extracellular matrix proteins including collagen [1,2,24]. Other released substances such as VEGF, HGF and bFGF are chemotactic and mitogenic for endothelial cells promoting angiogenesis and vascularisation, a key step in healing [9,21]. Other proteins from platelets, such as PF4, negatively regulate angiogenesis so the net effect will depend on the balance and this will depend on the tissue localization. Thrombin, a multifunctional serine protease, formed from plasma prothrombin upon calcium addition, is a key enzyme in blood coagulation. Apart from converting fibrinogen into fibrin, thrombin can directly stimulate cells inducing, for example, mitogenesis and causing them elaborate growth factors and cytokines [6]. Thrombin is also considered to be a regulator of inflammatory phases of reparative processes of tissues [25].

Autologous platelet releasates have been used in a number of clinical situations. One example is for the treatment of chronic leg ulcers, thereby improving the microcirculation in patients with peripheral neuropathy and/or vascular complications [14,17]. Platelet-released supernatants promote cell proliferation in periosteal implants [11]. Autologous platelets are being increasingly used as a source of growth factors in bone reconstruction and implant consolidation in dentistry [1]. A convenient and safe way to apply them is in an autologous platelet-rich fibrin clot. In another context, growth factors have a key role in tendon repair mechanisms [18]. Platelet preparations provide an autologous natural combination of rapidly secreted growth factors; consequently their clinical use to improve the healing capacity of tendons merits investigation. Furthermore, the addition of platelet-releasates to tendon cells in culture provides an interesting model to study the cooperative effects of a mixture of growth factors. The purpose of our present study is to compare the effects of releasates from platelet-rich and platelet-poor clots on tendon cell proliferation, to identify the role of platelets and to ascertain their regulation of the endogenous synthesis of growth factors by tendon cells in culture.

Materials and methods

Isolation and culture of tendon cells

Human tendon samples were obtained during the surgical reconstruction of the anterior cruciate ligament with semitendinous tendon autograft, from an otherwise healthy young donor after informed consent and project approval from the local ethics committee. The tendon samples were cleaned of surrounding adipose tissue. Tendon fragments collected in phosphate-buffered saline (PBS) solution supplemented with antibiotics (Penicillin–Streptomycin solution and amphotericin B, Sigma Chemical Company, St. Louis, IL, USA) were 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 (1vol:1vol) (Gibco) culture medium supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 2 mM 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 are characterized immunocytochemically by staining for collagen type I (Biodesign, Saco ME), a determinant of the tendon cell phenotype [15].

Plasma preparations rich in growth factors

Blood was collected into 3.8% (wt/vol) sodium citrate from four healthy donors, three middle aged men and one 31 year old woman from our group. Samples were centrifuged either at 4500*g* for 12 min at 4 °C to separate platelet-poor plasma (PPP) or at 460 g for 8 min to obtain platelet-rich plasma (PRP). PPP- and PRP-clots were formed by adding calcium chloride at a final concentration of 22.8 mM to samples in glass tubes and incubating at 37 °C. Clots were allowed to retract for 1 h and the released supernatants were collected by aspiration. For each donor, three different preparations were used to supplement the culture medium: PPP, supernatant released from a PPP-clot (PPCR) and supernatant released from a PRP-clot (PRCR).

Cell culture assays

Subconfluent cultures of tendon cells were trypsinized and cell viability was assessed by trypan blue dye exclusion; cells were plated at a density of 800 cells per well in 48 tissue-culture well plates and maintained with serum-free medium for 24 h [7]. Then, culture medium was replaced by serum-free medium supplemented with either: (i) PPP 20% (vol/vol), (ii) 20% (vol/vol) PPCR, or (c) 20% (vol/vol) PRCR. Additional cultures analysed the effect of thrombin on the proliferation of tendon cells. These were identical to the above except PPP or releasates were incubated at 37 °C with excess hirudin (10 U/ml) (Stago, Asnières, France) for 5 min prior to their being added to the culture medium. Control cultures were also performed in the absence of PPP or releasate. The study period was six days, and during this time the culture medium was not changed. All experiments were run in parallel. Cell proliferation was evaluated using the WST-1 (tetrazoliumsalt,(4-[3,4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]- 1,3-benzenedisulfonate) colorimetric assay (Roche, Basel, Switzerland). Absorbance at 450/620 nm was directly proportional to the number of living cells in the culture. All experiments designed to study proliferation were repeated in triplicate on three different occasions and gave essentially the same results. The mean data of repeated analyses are shown for each experiment. As an index of cell number, calibration curves ranging from 800 cells to 50,000 cells per well were established using the WST-1 cell counting kit. Under our experimental conditions, cell number is related to absorbance as follows: $\text{Absorbance} = 0.112 + 6.199 \times 10^{-5} \times \text{cell number}$; ($n = 11$; $r = 0.9986$).

Determination of growth factor concentrations

Platelet counts were performed with a Beckman-Coulter A^{c}T Differential Analyzer (Galway, Ireland) not only on peripheral blood but also in the PPP and PRP before clotting. Growth factors were measured in PPP, PPCR and PRCR using commercially available Quantikine colorimetric sandwich ELISA kits (R&D, Minneapolis, MN, USA). Samples were assayed for IGF-I, TGF-β-1, PDGF-AB, VEGF, HGF, and EGF. The concentration of the above-mentioned growth factors was also measured in the culture medium conditioned by tendon cells, in three independent experiments. Culture medium was collected on day 6 of treatment, centrifuged for 5 min at 460*g* and stored at −80 °C until assayed. Control cultures without cells were used to assess the stability of the studied GFs, during a six day incubation at 37 °C and as background correction. Growth fac-

Table 1
Platelet count and concentrations of a range of growth factors in three different preparations, PPP, PPCR, PRCR, from four donors

Plasma preparation	Donor	Plts × 10^6 (ml)	Concentration (ng/ml)					
			PDGF-AB	TGF-β-1	EGF	VEGF	HGF	IGF-I
Platelet rich-clot releasate (PRCR)	1	366	10.43	33.75	0.42	0.14	0.31	85.17
	2	642	20.70	59.75	0.52	0.31	0.58	83.79
	3	431	8.46	26.70	0.48	0.63	0.38	183.09
	4	402	13.73	31.15	0.35	0.11	0.47	110.82
Platelet poor-clot releasate (PPCR)	1	3	0.41	2.21	<0.02	<0.03	0.27	100.00
	2	11	1.03	5.28	<0.02	<0.03	0.36	85.11
	3	3	0.62	1.58	<0.02	<0.03	0.30	161.74
	4	4	1.04	3.04	<0.02	<0.03	0.41	123.07
Platelet poor plasma (PPP)	1	3	0.37	1.41	<0.02	<0.03	0.29	92.56
	2	11	0.16	1.10	<0.02	<0.03	0.42	68.79
	3	3	0.41	1.72	<0.02	<0.03	0.31	151.11
	4	4	0.89	1.25	<0.02	<0.03	0.43	98.75

tors concentrations assessed in the culture medium conditioned by tendon cells were adjusted for cell number and expressed as ng/10^6 cells.

Statistical analysis

Results are expressed as mean ± SEM. Significant differences among defined groups were tested using the non-parametric Kruskal–Wallis procedure followed by Bonferroni to discriminate among the means. Additionally, the Kolmogorov–Smirnov test was used to compare distributions of assessed growth factors in the response to the two treatments (PPCR versus PRCR). A difference at a level of $p < 0.05$ was considered to be statistically significant (Statgraphics Plus, Manugistic, MS, USA).

Results

Growth factor concentrations in supernatants from PRP- and PPP-clots and in PPP

PRP and PPP were prepared from citrated blood of four donors and the platelet counts established by electronic counting (Table 1). PRP- and PPP-clots were formed as described and the supernatants (or releasates) obtained 1 h after clot retraction. The concentrations of a range of growth factors were assessed in PPP and in

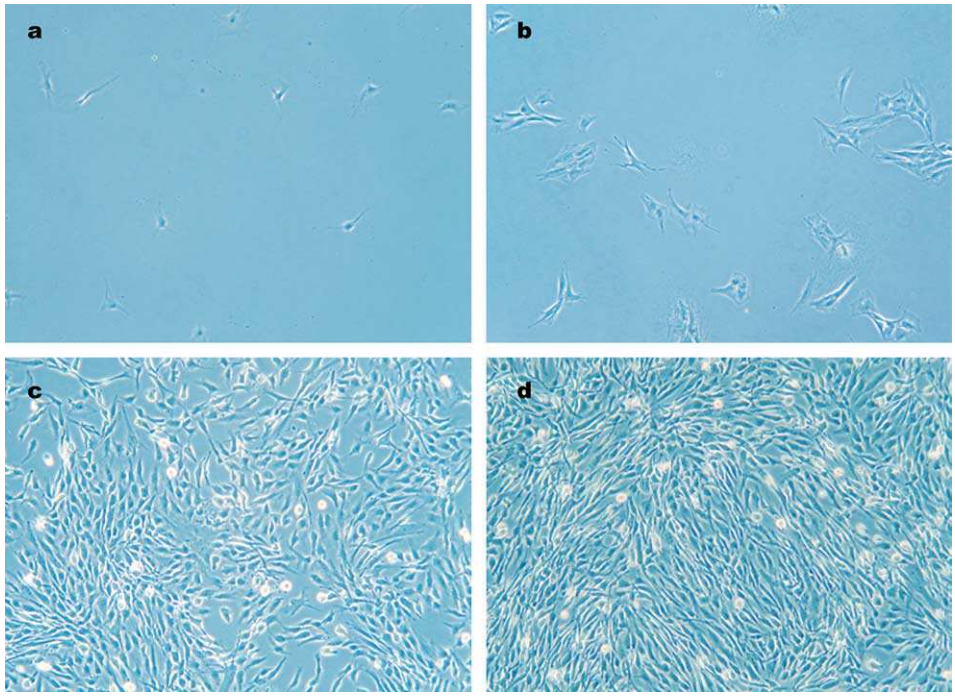

Fig. 1. Photomicrographs of representative human tendon cell cultures demonstrating proliferation induced by six days of treatment with platelet poor plasma (PPP), supernatant released from a platelet poor-clot (PPCR) and supernatant released from a platelet rich-clot (PRCR). Magnification: 80×. (a) Control cultures, (b) 20% PPP (vol/vol), (c) 20% PPCR (vol/vol), (d) 20% PRCR (vol/vol).

each supernatant (Table 1). The levels of each growth factor in the PPP and in the supernatant obtained after clotting of the PPP were basically similar. To be noted is the high level of IGF-I compared to the others. In contrast, the supernatants obtained from platelet-rich clots contained much greater amounts of PDGF and TGF-β-1 reflecting their release from the platelet α-granules. Moreover VEGF and EGF could only be quantified when platelets were present in the preparations. As well as showing inter-donor variability, the amounts of these GFs also showed a greater correlation with the platelet count.

Cell proliferation

Photomicrographs of tendon cells in 6 day cultures, either from control incubations or after the three described treatments, are shown in Fig. 1. As shown in Fig. 1c and d, either PRP- or PPP-clot (PRCR or PPCR) releasates induced a proliferation of tendon cells while only a weak effect was produced by the non-calcified PPP (Fig. 1b). Control cells in culture medium with 0.2% serum did not show any change in the number of cells after six days (Fig. 1d).

Although cells proliferated in the presence of PPCR, the proliferation was enhanced in PRCR and this difference was statistically significant ($p < 0.05$). Fig. 2a shows the inter-donor variability in the proliferation studies. Fig. 2b summarises the data for each category.

To analyse if thrombin could be a factor in the observed proliferation, or have synergistic effects with growth factors in PPCR and PRCR, samples were treated with hirudin, a highly selective exogenous thrombin inhibitor. Cells treated with PPP pre-incubated with hirudin did not show any change. Inhibition of the thrombin active site failed to significantly affect proliferation of tendon cells cultured with supernatants released from either PPP- or PRP-clots (Fig. 2c).

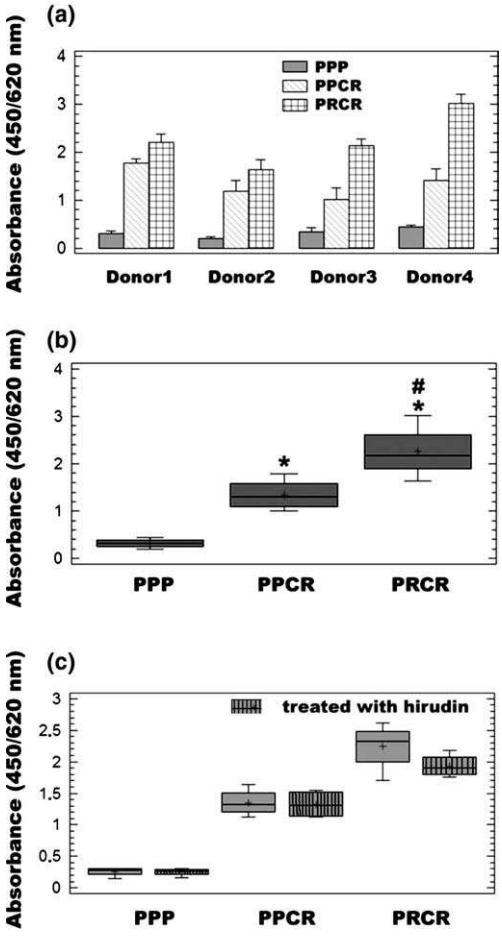

Fig. 2. Cell proliferation after six days of incubation with three treatments: platelet poor plasma (PPP), supernatant released from a platelet poor-clot (PPCR) and supernatant released from a platelet rich-clot (PRCR). (a) Inter-donor variability in proliferation studies, mean ± SEM of three independent experiments. (b) Summary of all individual data of Fig. 2a. ✱ $p < 0.05$ as compared to PPP. # $p < 0.05$ as compared to PPCR. (c) Pre-incubation with hirudin of PPP, PPCR and PRCR, not showing any significant difference in proliferation.

Growth factors in the culture medium: comparison of the three treatments based on their ability to induce the synthesis of growth factors

PDGF-AB, EGF, VEGF, IGF-I, TGF-β-1 and HGF were measured at the start of the treatment (day 0) and after six days of culture (day 6) for each of the three experimental conditions. TGF-β-1 was slightly increased in the PRCR cultures for three of the four donors tested. Concentrations of IGF-I did not vary from the start of the treatment to day 6 with any of the assayed conditions, whereas PDGF-AB and EGF levels decreased when cells were treated with the supernatants of PRP-clots (PRCR), ($p = 0.02$ and 0.03, respectively). Concurrently, VEGF and HGF showed a significant increase as shown in Fig. 3. Tendon cells treated with releasates of platelet poor clots (PPCR) also synthesized HGF and VEGF but the amounts were lower than with supernatants from PRP-clots (PRCR) ($p = 0.03$) (Fig. 3); though these differences could point out the effects of secreted platelet proteins. Increased amounts of growth factors on day 6 would reflect protein synthesis by tendon cells. A loss of growth factors would suggest their endocytosis after binding to the tendon cells or their degradation in the milieu through the action of released or cell surface proteases.

Discussion

Autologous platelets as a source of healing factors have been shown to promote tissue repair in several clinical situations [1]. When Ca^{2+} is added to PPP or PRP in glass tubes, coagulation is initiated. This is a slow proc-

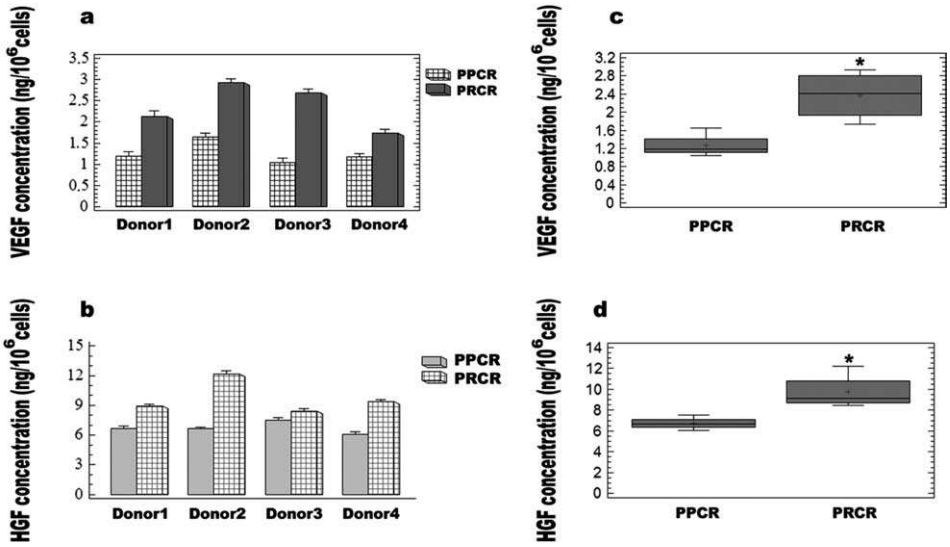

Fig. 3. Synthesis of VEGF and HGF, expressed as nanograms per 10^6 tendon cells, treated with either platelet poor-clot (PPCR) or platelet rich-clot (PRCR). (a) Inter-donor variability in VEGF synthesis, mean ± SEM of three independent experiments. (b) Summary of individual data of Fig. 3a. ✱ $p < 0.05$ as compared to PPCR. (c) Inter-donor variability in HGF synthesis, mean ± SEM of three independent experiments. (d) Summary of individual data of Fig. 3c. ✱ $p < 0.05$ as compared to PPCR.

ess that involves thrombin generation, platelet activation and secretion, and fibrin formation. The process ends when the clot retracts. The media around the retracted clots, contains proteins such as growth factors and other molecules that can be involved in healing. In the present study, we show that substances released from plasma clots and platelets act on human tendon cells, promoting proliferation and also inducing the synthesis of angiogenic growth factors.

PDGF-AB is a known mitogenic factor for mesenchymal-derived cells [13]. An increase in proliferation of tendon cells promoted by IGF-I in the presence of PDGF has been reported [26]. Thrombin also stimulates cells involved in tissue repair [16,25]. When we looked into the possible effects of thrombin in cell proliferation, we found that hirudin blockade of thrombin had no significant effect on cell proliferation, nor in the synthesis of VEGF and HGF under our experimental conditions (data not shown).

A major finding in our work is that tendon cells synthesize considerable amounts of VEGF and HGF after exposure to the releasates. The increase in VEGF and HGF could be an intrinsic mechanism of local tissue for inducing angiogenesis as part of tissue repair. Angiogenesis is tightly controlled by GFs that favour either vessel growth or regression. In the injured tendon, local expression of angiogenic factors is related to an enhancement of the healing process. Up-regulation of VEGF at the injured site in the early stages of tendon repair has been already reported [3,5]. Several factors released from platelets during clotting and clot retraction can potentially induce VEGF production either alone or in combination. Interestingly, PDGF induced the synthesis of VEGF in osteoblast cultures [4]; IGF-I upregulated VEGF expression by osteoblast-like cells as reported by Goad [10]; furthermore TGF-β-1 induced VEGF expression in osteoblasts and osteoblast-like cells, and this event was dose-dependent [20]. All these growth factors could have an interactive effect on the synthesis of VEGF and HGF in our culture conditions.

Previous studies [27] showed that the combination of HGF and VEGF produced additive effects on endothelial cell proliferation and a synergistic effect on endothelial cell migration. In accordance with these results, we have found a strong angiogenic effect when this preparation rich in growth factors was infiltrated into the Achilles tendons of sheep (Sanchez et al., unpublished results). HGF is a heparin-binding protein with mitogenic, motogenic and activities for various cell types. It is a mitogen for endothelial cells, stimulating cell migration, and branching and/or tubular morphogenesis of epithelial and endothelial cells [28]. Nevertheless, HGF has not been studied with respect to tendon healing. The majority of the clinical evidence for its angiogenic effects comes from the treatment of chronic leg ulcers [19]. Interestingly, neutralization of HGF in cutaneous wounds decreased neovascularization and the formation of granular tissue [22]. HGF-induced expression of VEGF has been implicated in paracrine amplification of angiogenesis, contributing to angiogenic responses during inflammation and tissue healing [27]. Xin [28] showed synergistic effects of VEGF and HGF in promoting human endothelial cell survival and tubulogenesis, a response that did not occur with either growth factor alone.

In clinical situations, scarifications represent a frequent treatment for the damaged tendon; the goal being to induce blood clot formation. The application of supernatants rich in growth factors prepared ex vivo from autologous platelet-rich clots can provide an effective alternative approach when the desired strategy is to initiate healing in damaged tendons. The angiogenic effect of clot releasates may be of paramount importance in the context of tendon grafts when these are used to reconstruct anterior cruciate ligaments. The rapid promotion of neovascularization of tendon tissue would enhance the remodeling process of the graft which is essential for the optimal clinical outcome after anterior cruciate ligament reconstruction [23].

It is likely that VEGF and HGF are produced and act in concert with a multitude of other factors involved in angiogenesis during tendon repair or remodeling. It is important to define these factors and their interrelationship. Further understanding of these mechanisms will provide the foundation for therapeutic molecular developments in tendon repair.

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