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Concise Report

Platelet-released growth factors enhance the secretion of hyaluronic acid and induce hepatocyte growth factor production by synovial fibroblasts from arthritic patients

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Objectives. Autologous platelet-secreted growth factors (GFs) may have therapeutic effects in osteoarthritis (OA) capsular joints via multiple mechanisms. Our aim was to examine the effect of a platelet-derived preparation rich in growth factors (PRGFs) in OA synovial cell biology.
Methods. Synovial cells were isolated from 10 osteoarthritic patients and cultured in serum-free media (basal conditions) and exposed to either a platelet-poor preparation or PRGF for 72 h. Cells activated with interleukin-1β (IL-1β) for 48 h were also exposed to PRGF. Changes in several events relevant to joint homeostasis including (i) hyaluronic acid (HA) secretion, (ii) the balance between metalloproteinase-1, -3 and -13 (MMP-1, MMP-3 and MMP-13) and tissue inhibitor-1 (TIMP-1) and (iii) the secretion of transforming growth factor-β1(TGF-β1), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), were all assessed.
Results. PRGF significantly enhanced HA secretion compared with platelet-poor preparations, $P < 0.05$; at the same time release of TIMP-1, MMP-1, MMP-3 and MMP-13 were not affected. An increased HGF production was observed ($P < 0.05$) but VEGF and TGF-β1 levels remained unchanged. PRGF significantly enhanced the secretion of HA induced by IL-1β activation, $P < 0.05$, but it did not modify the IL-1β-induced rise in MMP-1, MMP-3 and VEGF. In contrast, PRGF-induced HGF production was abolished by the presence of IL-1β during PRGF treatment, $P < 0.05$.
Conclusions. Intra-articular administration of PRGF might be beneficial in restoring HA concentration and switching angiogenesis to a more balanced status but does not halt the effects of IL-1β on synovial cells.

KEY WORDS: Platelet-rich plasma, Osteoarthritis, Synovial cells, IL-β, Growth factors.

This article describes indications for use that are not cleared by the FDA.

Introduction

Osteoarthritis (OA) is a clinically heterogeneous and poorly understood disease. Although most research has looked at destruction of cartilage, arthritis is now often considered in terms of organ failure. OA pathogenesis can involve all of the major articular tissues including cartilage, synovial membrane, subchondral bone and other connective tissues such as ligaments and tendons [1].

Current research efforts seek to identify key biochemical pathways that can be targeted therapeutically through biological intervention. Therapies for OA include the use of inhibitors of interleukin-1β (IL-1β) that reverse cartilage destruction [2, 3]. Also being examined is the potential use of specific growth factors (GFs) as therapeutic proteins for cartilage repair [4]. According to the limited success of current efforts, substantial challenges remain. One critical point is that a pool of molecules is likely to be required according to the complexity of the OA process. One approach involves the intra-articular delivery of autologous platelet-rich preparations that deliver a large pool of GFs and proteins implicated in tissue repairing mechanisms.

Our group has standardized and characterized a platelet derivative, known as 'preparation rich in growth factors' (PRGFs) [5–8]. Released proteins in PRGF such as insulin-like growth factor (IGF-I), platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) could favour cartilage stabilization by regulating metabolic activities of resident chondrocytes and subchondral bone [4]. In addition, potential inhibitory factors such as TGF-β1 may regulate tissue inhibitor-1 (TIMP-1) and metalloproteinase (MMP) gene expression by chondrocytes while increases in TIMP-1 in the extracellular space may accelerate complex formation with active MMPs [9]. Furthermore, angiogenic regulatory proteins contained in PRGF [10–12], may modulate the as yet ill-defined disease-specific changes in angiogenesis found in capsular joints.

In this study, we have set up *in vitro* assays for exploring the activity of synovial cells treated with PRGF in order to study the effect of applied factors on cellular events relevant in arthritis such as hyaluronic acid (HA) secretion, the MMP/TIMP balance and angiogenesis. As disease progression is mediated by overproduction of proinflammatory cytokines such as IL-1β, we also examined the capability of PRGF to moderate the effects of IL-1β in the synovium.

Material and methods

Specimens

Synovial tissue was obtained from a total of 10 OA patients undergoing joint replacement surgery, after informed consent. The study was conducted following the International Conference Harmonised Good Clinical Practice guidance. Mean age was 69 ± 5 yrs, three were men and seven women. The study was approved by the United Surgical Partners hospital Institutional Review Board.

Cell isolation and characterization

Cells were isolated from the superficial layer of the synovium following our standard protocol with minor modifications [11]. All experiments were performed between passages 3 and 5 inclusive. Cell number was determined using the WST-1 colorimetric assay (Roche Penzberg, Germany). Calibration curves relating the absorbance to cell number were established in parallel for non-stimulated and IL-1β-stimulated cells and for each culture.

Synovial fibroblasts were identified using a monoclonal antibody directed against prolyl 4-hydroxylase (Dako Cytomation,

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TABLE 1. Effects of IL-1β activation on secretion from OA synovial fibroblasts

	HA	TIMP-1	MMP-1	MMP-3	VEGF	TGF-β1
	(μg/10^6 cells)		(ng/10^6 cells)			
Non-stimulated	11.05 ± 4.57	4.47 ± 2.39	16.37 ± 11.28	5.05 ± 2.13	7.68 ± 4.00	1.80 ± 0.99
IL-1β-stimulated	119 ± 26***	4.22 ± 3.84	206 ± 125**	2054 ± 867**	21.49 ± 16.09**	1.69 ± 0.69

Cells were maintained simply in serum-free media to examine constitutive secretion (non-stimulated cells, NS) or stimulated with IL-1β (1 ng/ml) for 48 h to examine the effects of IL-1β activation. Levels were measured by EIA and ELISA from the supernatants of OA synovial fibroblasts. IL-1β stimulation increased the levels of MMP-1 and MMP-3 above the constitutive secretion by 12.5 ± 11.1-fold and 539 ± 326-fold, respectively. HGF was poorly detected, being barely present for only five out of the ten patients (detection limit 0.70 ng/10^6 cells) and was not detectable after IL-1β stimulation while VEGF levels were increased. Concentrations are expressed as mean ± S.D. ($n = 10$ individual patients); (**$P < 0.01$, ***$P < 0.001$).

Glostrup, Denmark). Fluorescence was analysed by flow cytometry for each culture; 86 ± 6% of the cells were positive for prolyl 4-hydroxylase (synoviocytes B) and negative for CD14 or CD68 (synoviocytes A) (data not shown).

Plasma preparations

Blood was collected into 3.8% (wt/vol) sodium citrate from a healthy adult donor and centrifuged either at 4500*g* for 12 min at 4°C to separate platelet-poor (PP) plasma or at 460*g* for 8 min to obtain PRGF. PP- and PRGF-fibrin matrices were formed by adding calcium chloride at a final concentration of 22.8 mM to samples in glass tubes and incubating at 37°C. The matrices were allowed to retract for 1 h and after centrifugation at 4500*g* for 10 min at 4°C the released material was collected by aspiration.

Cell culture experiments

Cells were seeded in 48-well plates at a density of 20 000 cells per well and maintained for 48 h simply in serum-free DMEM/F12 to examine constitutive secretion (non-stimulated cells, NS) or stimulated with 1 ng/ml of IL-1β (R&D Systems, Abingdon, UK) to analyse the effects of IL-1β activation. Then, NS cells were incubated over 3 days with 20% of PP-preparation or 20% PRGF and IL-1β-activated cells with 20% PRGF plus 1 ng/ml of IL-1β. In the latter group of experiments, additional cultures were performed omitting IL-1β activation during PRGF treatment. Conditioned media were stored at −80°C until assay.

Measurements of growth factors [IGF-I, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and TGF-β1] and HA, TIMP-1, MMP-1, MMP-3 and MMP-13

HA was determined by an enzyme-linked binding protein assay (Corgenix Inc., CO, USA). TIMP-1, MMP-1, MMP-13 and MMP-3 (including free, pro-enzymes and complexed forms) were measured by the corresponding one-step sandwich enzyme immunoassay (EIA) from Amersham Biosciences (UK, Buckinghamshire, England) and the last from BioSource International, USA. Enzyme-linked immunosorbent assay (ELISA) kits were used to determine levels of VEGF, HGF, IGF-I and TGF-β1 (R&D). Culture media obtained from control cultures without cells, after 3 days incubation, were used for background correction. These results were normalized for cell number and expressed as ng/10^6 cells.

Statistical analyses

Data are expressed as mean ± S.D. Significant differences among defined groups were evaluated using the non-parametric Kruskal–Wallis procedure and differences were detected graphically using notched box plots. Additionally the magnitude of changes, obtained by omitting IL-1β during PRGF treatment, were expressed as percentages of variation (mean ± S.D.) relative to PRGF. Here, differences were assessed using the Wilcoxon test. A difference of $P < 0.05$ was considered to be statistically significant (Statgraphics Plus, Manugistic, MS, USA).

Results

Basal secretion from synovial fibroblasts and changes induced by IL-1β

The presence of HA, TIMP-1, MMP-1, MMP-3, MMP-13, IGF-I, TGF-β1, HGF and VEGF was confirmed in synovial fluid from 60 OA patients (data not shown). Secretion from NS synovial fibroblasts from 10 OA patients and changes induced by IL-1β activation are shown in Table 1.

Characterization of plasma preparations

The PRGF and PP-preparation used throughout the experiment differed in platelet number, 494×10^6 platelets/ml *vs* 15×10^6 platelets/ml consequently platelet-secreted factors diverged: 38.38 *vs* 2.62 ng/ml for TGF-β1, 16.87 *vs* 1.04 ng/ml for PDGF-AB. EGF and VEGF were 0.35 and 0.11 ng/ml in PRGF while not detected in PP-preparations. Both preparations contained broadly similar concentrations of HA (45.25 ± 2.83 ng/ml), TIMP-1 (241.5 ± 7.8 ng/ml), MMP-1 (21.65 ± 2.20 ng/ml), MMP-3 (3.63 ± 0.81 ng/ml), IGF-I (126.9 ± 5.5 ng/ml) and HGF (0.45 ± 0.03ng/ml).

Effects of PRGF on secretion from synovial fibroblasts

(i) 20% PRGF in DMEM significantly enhanced HA secretion compared with the PP-preparation $P < 0.05$ pointing to platelets as inducers of HA synthesis. IL-1β further enhanced the stimulatory effect on HA production by PRGF, $P < 0.05$ (Fig. 1A).

(ii) Synovial cells synthesized similar amounts of MMP-1 and MMP-3 in the presence of either PRGF (23.40 ± 14.80 and 15.70 ± 7.60 ng/10^6 cells) or PP-preparation (18.00 ± 10.80 and 12.50 ± 8.80 ng/10^6 cells). The secretion of TIMP-1 was also not altered by the platelet releasate. Changes induced by IL-1β on MMP-1 and -3 were not modified by PRGF treatment.

To be noted that in these experimental conditions, exogenous IL-1β was also present during the treatment with PRGF. When IL-1β was omitted during the PRGF treatment, MMP-1 and MMP-3 synthesis decreased by 90.7 ± 0.3% and 84.7 ± 8.9%, respectively, ($P < 0.001$), indicating that the secretion of MMP-1 and MMP-3 are dynamic and almost totally IL-1β-dependent.

(iii) Angiogenic factors:

VEGF. Synovial cells synthesized similar amounts of VEGF in the presence of PRGF or PP-preparation. The increase of VEGF induced by IL-1β was not modified by PRGF. However, when PRGF was applied unaccompanied by IL-1β, VEGF was reduced by 71.37 ± 11.34%, ($P = 0.002$), confirming that the increase was IL-1β-dependent (data not shown in Fig. 1B).

HGF. Interestingly, both the PP-preparation and PRGF induced a significant production of HGF (14.42 ± 6.60 ng/10^6 cells and 18.66 ± 6.63 ng/10^6 cells, respectively) and VEGF and

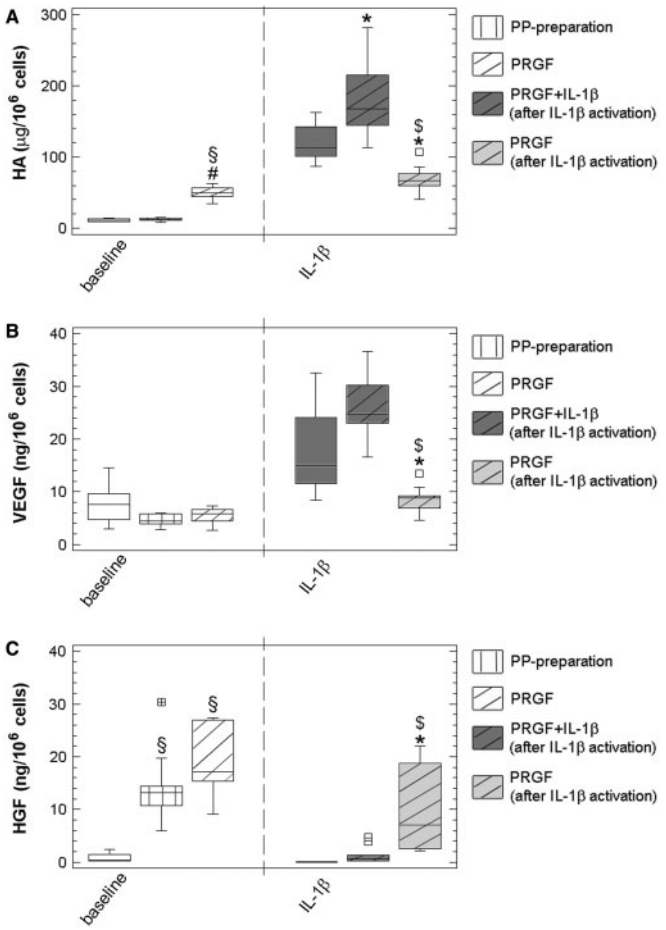

FIG.1. Secretion of HA (**A**), VEGF (**B**) and HGF (**C**) by synovial fibroblasts after 3 days of incubation in the presence of PRGF in two parallel group of experiments. Data summarize the values obtained for 10 patients with OA. Crosshatched bars indicate PRGF treatment in both the groups of experiments. Coloured boxes represent the group of experiments performed with IL-1β-stimulated cells; here IL-1β stimulation was discontinued during PRGF treatment in one group (light grey). Significant differences among defined groups were evaluated within the same group of experiments. §$P < 0.05$ for PRGF or PP groups *vs* baseline; # $P < 0.05$ for PRGF groups *vs* PP; *$P < 0.05$ for PRGF (after IL-1β activation) or PRGF+IL-1β (after IL-1β activation) *vs* IL-1β, $P < 0.05$ for PRGF (after IL-1β activation) *vs* PRGF+IL-1β (after IL-1β activation).

HGF showed a positive correlation after 3 days PR-treatment, $P < 0.05$. Importantly, HGF production is abolished when cells are activated with IL-1β, $P < 0.05$ (Fig. 1B) and remarkably, HGF levels were no longer correlated with VEGF. Significantly, PRGF maintained the stimulatory effect on HGF secretion if IL-1β was not added at the same time, $P < 0.05$.

TGF-β1. No differences in the secretion of TGF-β1 were detected in the presence of PP-preparation or PRGF with or without IL-1β.

Discussion

There are a large number of studies describing the use of different platelet-rich products with the purpose of accelerating tissue repair [6, 10, 13]. The use of specific GFs and proteins to promote cartilage repair and for the treatment of OA is also offering promising results [4, 14]. By joining both concepts and supported by the positive effects of PRGF in different clinical situations involving connective tissues [7, 8], we have hypothesized that the delivery of a natural mixture of biologically active molecules incorporated in a forming fibrin matrix within the joint compartment may target synovial fibroblasts thus inducing positive changes in the whole joint micro-environment.

A major finding of our work is the ability of PRGF to stimulate HA synthesis driving the secretion of HA by the synovial fibroblasts. The stimulation induced by PRGF but not the PP-preparation supports the participation of platelet-secreted factors on HA synthesis. In fact, a stimulatory action of TGF-β1 and PDGF, which are major platelet secretory products had been previously reported. Particularly the former up-regulates the hyaluronan synthase isoform 1 [15] while PDGF primarily stimulates hyaluronan synthase isoform 2 [16]. By regulating the endogenous HA synthesis PRGF would restore HA levels thereby enhancing cartilage protection [17] and joint lubrication.

Another focus of interest is to examine the potential influence of PRGF on the degradative status of the joint. In line with recent reports [3], we have found that IL-1β induced a marked secretion of MMP-1 and MMP-3, not accompanied by increases in the amount of secreted TIMP-1, emphasizing the importance of synovial cells as a source of cartilage degrading enzymes under inflammatory conditions. Along with other studies [18], we failed to detect secretion of MMP-13 by OA synovial cells; a finding that is compatible with the low levels found in OA synovial fluids (data not shown). Although PRGF on its own is not involved in the regulatory mechanisms controlling TIMP or MMP synthesis by synovial fibroblasts, it will provide an exogenous source of TIMPs and α2-macroglobulin, natural inhibitors of active proteases. The latter, an endoproteinase inhibitor present in plasma and synovial fluid, can inhibit metalloproteinase activity besides preventing the breakdown of cartilage aggrecan by ADAMTS-4 and ADAMTS-5 [19]. Interestingly, PRGF therapy is also a relevant source of anabolic factors for cartilage including IGF-I and TGF-β1 and might influence cartilage metabolism positively [2, 14].

Although relatively little is known about vascular responses in OA, several lines of evidence suggest that a pro-angiogenic state exists in osteoarthritic joints. In fact, degeneration of cartilage has been associated with the presence of VEGF receptors [20] and with the invasion of cartilage by new vessels from underlying bone. Furthermore, neovascularization is accompanied by marginal soft tissue changes and osteophyte growth [21]. In these circumstances, VEGF may promote angiogenesis inducing inflammatory changes detrimental to the joint [1, 22]. Our study showed basal VEGF but not HGF secretion and confirmed the ability of IL-1β to further increase VEGF [23]. At the same time, HGF remained undetectable, implying that VEGF may act independently, promoting angiogenesis and perpetuating pain under inflammatory conditions [24]. In fact, experimental studies involving other tissues underline the synergy between HGF and VEGF, which likely can act cooperatively in normal physiology [25, 26] suggesting a role in non-inflammatory as opposed to inflammatory angiogenesis [27, 28]. Possibly, PRGF can modify the angiogenic balance by triggering the secretion of HGF [29].

In line with this hypothesis, we have shown that IL-1β potently inhibited HGF production stimulated by PRGF. This is intriguing essentially for three reasons. First previous reports [30, 31] have shown the involvement of mitogen-activated protein kinase (MAPK) signalling pathways for HGF induction, specifically through the activation of extracellular signal-regulated kinase (ERK) and p38, which mediate activation of many key transcription factors. The second observation contained in other reports [3, 32] is that MAPK signalling also controls IL-1β-induced MMP production. Lastly, signal pathway synergy studies [33] have identified p38MAPK as part of the signal transduction pathway effecting TGF-β1 and/or IGF-I actions. Thus, it is possible that inducers of HGF production target the same signalling pathways as IL-1β. All these data taken together emphasize the complex interplay between GFs and cytokines signalling cascades,

establishing a network that links inflammation, MMP production and GF effects.

The administration of a natural mixture of GFs within the joint could induce positive changes and may provide new opportunities for OA treatment.

Rheumatology key messages

- Autologous PRGF provides a complex combination of molecules that may induce positive cellular responses within the joint.
- Research on autologous therapies might yield a new therapeutic option for OA treatment.

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