Platelet-Rich Plasma Increases Matrix Metalloproteinases in Cultures of Human Synovial Fibroblasts

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Background: The effect of platelet-rich plasma on chondrocytes has been studied in cell and tissue culture. Less attention has been given to the effect of platelet-rich plasma on nonchondrocytic cell lineages within synovial joints, such as fibroblast-like synoviocytes, which produce cytokines and matrix metalloproteinases (MMPs) that mediate cartilage catabolism. The purpose of the present study was to determine the effect of platelet-rich plasma on cytokines and proteases produced by fibroblast-like synoviocytes.

Methods: Platelet-rich plasma and platelet-poor plasma from harvested autologous blood were prepared with a commercially available system. Fibroblast-like synoviocytes were treated with platelet-rich plasma, platelet-poor plasma, recombinant PDGFββ (platelet-derived growth factor ββ), or phosphate-buffered saline solution and incubated at 37°C for forty-eight hours. The concentrations of IL-1β (interleukin-1β), IL-1RA (IL-1 receptor antagonist), IL-6, IFN-γ (interferon-γ), IP-10 (interferon gamma-induced protein 10), MCP-1 (monocyte chemotactic protein-1), MIP-1β (macrophage inflammatory protein-1β), PDGFββ, RANTES, TNF-α (tumor necrosis factor-α), VEGF (vascular endothelial growth factor), MMP-1, MMP-3, and MMP-9 in the culture medium were determined by multiplex immunoassay.

Results: Platelet-rich plasma cultured in medium contained multiple catabolic mediators in substantial concentrations, including MMP-9 (15.8 ± 2.3 ng/mL) and MMP-1 (2.5 ± 0.8 ng/mL), as well as proinflammatory mediators IL-1β, IL-6, IFN-γ, IP-10, MCP-1, MIP-1β, RANTES, and TNF-α in concentrations between 20 pg/mL and 20 ng/mL. Platelet-poor plasma contained significantly lower concentrations of these compounds. Platelet-rich plasma was used to treat human fibroblast-like synoviocytes, and the resulting concentrations of mediators were corrected for the concentrations in the platelet-rich plasma alone. Compared with untreated fibroblast-like synoviocytes, synoviocytes treated with platelet-rich plasma exhibited significantly greater levels of MMP-1 (363 ± 94.0 ng/mL, $p = 0.018$) and MMP-3 (278 ± 90.0 ng/mL, $p = 0.018$). In contrast, platelet-poor plasma had little effect on mediators secreted by the synoviocytes. PDGFββ-treated fibroblast-like synoviocytes exhibited a broad proinflammatory cytokine response at four and forty-eight hours.

Conclusions: Platelet-rich plasma was shown to contain a mixture of anabolic and catabolic mediators. Synoviocytes treated with platelet-rich plasma responded with substantial MMP secretion, which may increase cartilage catabolism. Synoviocytes responded to PDGF with a substantial proinflammatory response.

Clinical Relevance: The multiple catabolic mediators in platelet-rich plasma and the secretion of MMPs by fibroblast-like synoviocytes treated with platelet-rich plasma could potentially accelerate cartilage catabolism, and this warrants further investigation.

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Platelet-rich plasma (PRP) is an autologous blood product that is used in multiple musculoskeletal applications including the treatment of bone defects, tendinopathies, and intra-articular pathology of the synovial joints[1]. The scientific rationale for this treatment is the fact that PRP is a biologically active autograft that delivers growth factors, cytokines, and other molecular mediators that increase healing. Use of PRP for intra-articular pathology of synovial joints is a potentially important application that has received attention both as a primary treatment and as an adjunct to surgical treatment[2,3].

The desired therapeutic effect of PRP is often attributed to the high concentration of growth factors, particularly platelet-derived growth factor (PDGF)[4]; however, PRP preparations contain multiple molecular mediators including growth factors, cytokines, and plasma proteins[5,6]. The molecular complexity of PRP preparations necessitates further study to determine the net overall effect for each clinical application[4,7]. For intra-articular applications, the effect of PRP on chondrocytes has been studied in cell and tissue culture[8,9]. Mesenchymal stem cell cultures differentiated into chondrocytes when exposed to buffered PRP treatments[10], and treatment of chondrocytes with PRP increased cell division and collagen synthesis[11], suggesting that PRP might aid cartilage regeneration. These studies have characterized the direct effect of PRP on chondrocytes, but not the indirect effect on chondrocytes or articular cartilage that is mediated by its effect on nonchondrocytic cell lineages. This is important since clinical studies of PRP for intra-articular use have shown mixed results. For example, Kon et al. demonstrated improved functional outcome at twelve months[3], but Filardo et al. showed that the effect in the same cohort diminished at twenty-four months of follow-up[12].

Although less attention has been given to the effect of PRP on nonchondrocytic cell lineages within synovial joints, cells such as fibroblast-like synoviocytes, monocytes, macrophages, and T cells are relatively abundant in the synovial space. PRP treatment of monocyte cultures isolated from blood resulted in suppression of MCP-1 (monocyte chemotactic protein-1) expression and upregulation of RANTES (regulated upon activation, normal T-cell expressed and secreted) expression[13]. Synovial tissues have an active biological role in health and disease. In particular, it is well established that fibroblast-like synoviocytes respond to proinflammatory cytokines and produce metalloproteinases and cytokines that mediate cartilage destruction even in nonrheumatoid disease[14,15]. In principle, the mixture of molecular mediators present in PRP could result in either upregulation or downregulation of protein synthesis by fibroblast-like synoviocytes, resulting in complex changes in the net concentrations of proteases and cytokines in the synovial fluid that extend beyond the mediators in the PRP itself. To our knowledge, no studies have addressed this potentially important phenomenon in cell culture models of synovial tissues.

The purpose of the present study was fourfold. First, the cytokine and protease concentrations in PRP prepared from human blood using a commercially available system was characterized using multiplex protein assays. Second, the effect of PRP on the levels of cytokines and proteases produced by fibroblast-like synoviocytes was examined (controlling for the levels of these mediators in the added PRP itself). Third, the effect of exogenous PDGF on fibroblast-like synoviocytes in culture was studied to isolate the effect of PDGF in the response of the cells to PRP. Finally, these experiments were repeated with platelet-poor plasma (PPP) to assess its biologic activity in relation to PRP in the same model.

Materials and Methods

Subjects

Independent approval for the study was obtained (Sterling Institutional Review Board, Atlanta, Georgia), and all patients provided informed consent for study participation. Patients who were eighteen years of age or older and were undergoing elective arthroscopic or open surgery for degenerative and traumatic conditions of the foot and ankle were considered candidates for autologous blood harvesting. Autologous blood harvesting was performed by venipuncture with the patient anesthetized, and PRP was prepared according to the manufacturer's instructions (Harvest Technologies, Plymouth, Massachusetts). A portion of the PRP was utilized intraoperatively for the procedure, and a second portion of PRP as well as PPP were sent to the laboratory on ice and used to treat cells cultures within six hours of processing.

Patients who were at least eighteen years of age (mean and standard deviation, 45.6 ± 19.1 years) and were undergoing elective arthroscopic knee surgery for a degenerative condition were considered candidates for synovial tissue harvesting. During arthroscopic capsular debridement, a portion of the shavings was collected and sent to the laboratory on ice.

Cell Culture

Synovial fibroblasts were enriched according to previously published protocols[16-18]. Briefly, synovial tissue from a soft-tissue biopsy of a patient undergoing routine arthroscopy was rinsed with sterile phosphate-buffered saline solution (PBS) and digested with a 100-fold greater volume (w/v) of PBS containing 2 mg/mL collagenase D (Sigma-Aldrich, St. Louis, Missouri) for three hours at 37°C, rocking at 100 rpm. Cells and debris were centrifuged at 500 ×g for five minutes at ambient temperature, then resuspended in 4 mL of Dulbecco modified Eagle medium (DMEM) containing 2 mM L-glutamine (Life Technologies, Grand Island, New York) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1000 IU/mL of penicillin and 1000 μg/mL of streptomycin (cellgro; Mediatech, Manassas, Virginia). Adherent cells were allowed to attach to tissue culture plates overnight, and nonadherent cells were washed away with fresh medium once per day for the next three days, followed by repeated washings and addition of fresh media every three days for a total of two weeks. At the end of this time, the cultures consisted of spindle-shaped cells, which were identified as fibroblast-like synoviocytes. Fibronectin secretion and cell growth of these synoviocyte cultures were monitored over a period of four days. The rate of fibronectin secretion was determined by enzyme-linked immunosorbent assay (ELISA) to be 1.5 ± 0.3 pg/cell/day. The cultures were expanded, digested with trypsin, and split 1:4 in fresh medium. All experiments were performed when cells had been cultured for less than four weeks (approximately eleven cell doublings) and were growing exponentially.

Preparation of Blood Fractions, PRP, and PPP

PRP and PPP were prepared from peripheral whole blood using the Harvest SmartPReP 2 APC+ platelet concentrate system (Harvest Technologies) according to the manufacturer's protocols. Fresh human blood was obtained by venipuncture from patients undergoing routine clinical treatment that included PRP treatment and arthroscopy for intra-articular pathology.

Treatment of Cells with PRP or PPP

Testing of the cultures utilized fibroblast-like synoviocytes isolated from six patients (A through F) and PRP or PPP from seven patients (1 through 7). On the day prior to the start of the treatment, 1.25×10^5 fibroblast-like synoviocyte

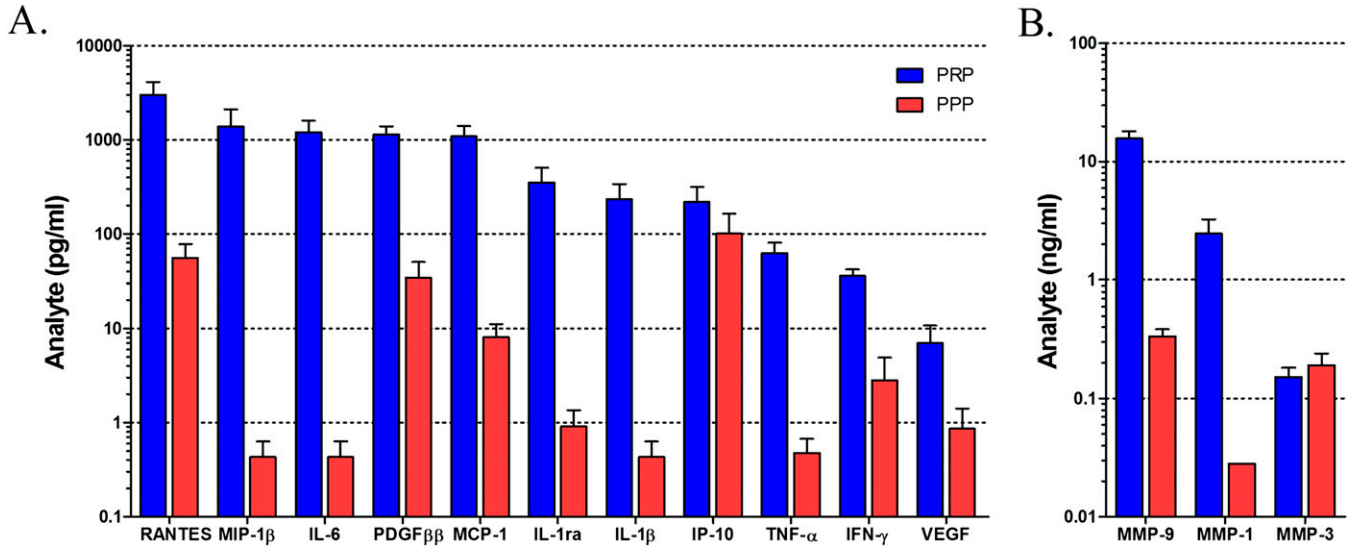

Fig. 1

Cytokine (**Fig. 1-A**) and matrix metalloproteinase (MMP) (**Fig. 1-B**) profiles of platelet-rich plasma (PRP) and platelet-poor plasma (PPP) diluted thirtyfold and incubated for two days in culture. The y axis is on a logarithmic scale in pg/mL or ng/mL. Values are shown as the mean for all seven patient PRP or PPP samples and the standard error of the mean. Analyte names are defined in the text.

cells were seeded in 1.5 mL of medium in a 24-well plate. Each culture was treated with 50 μL (equivalent to a thirtyfold dilution) of PRP, PPP, or PBS and incubated at 37°C in 5% CO_2 and 95% air. Specifically, the PRP and PPP from Patients 1 and 2 were separately used to treat cultures from Patients A and B; PRP and PPP from Patients 3 and 4 were tested on the culture from Patient C; and PRP and PPP from Patients 5, 6, and 7 were separately tested on cultures from Patients D, E, and F. PRP or PPP fractions were also added to 1.5 mL of medium without cells, representing inocula-only controls. After two days of incubation, the conditioned medium was harvested and centrifuged at 4000 ×g for ten minutes to remove cells and debris. All analyses were performed in triplicate. The effect of PRP or PPP on the cultures did not differ notably according to donor. Thus, the results of the PRP or PPP treatment are presented as the mean for the treatment of all cultures with the corresponding PRP or PPP from the seven donors.

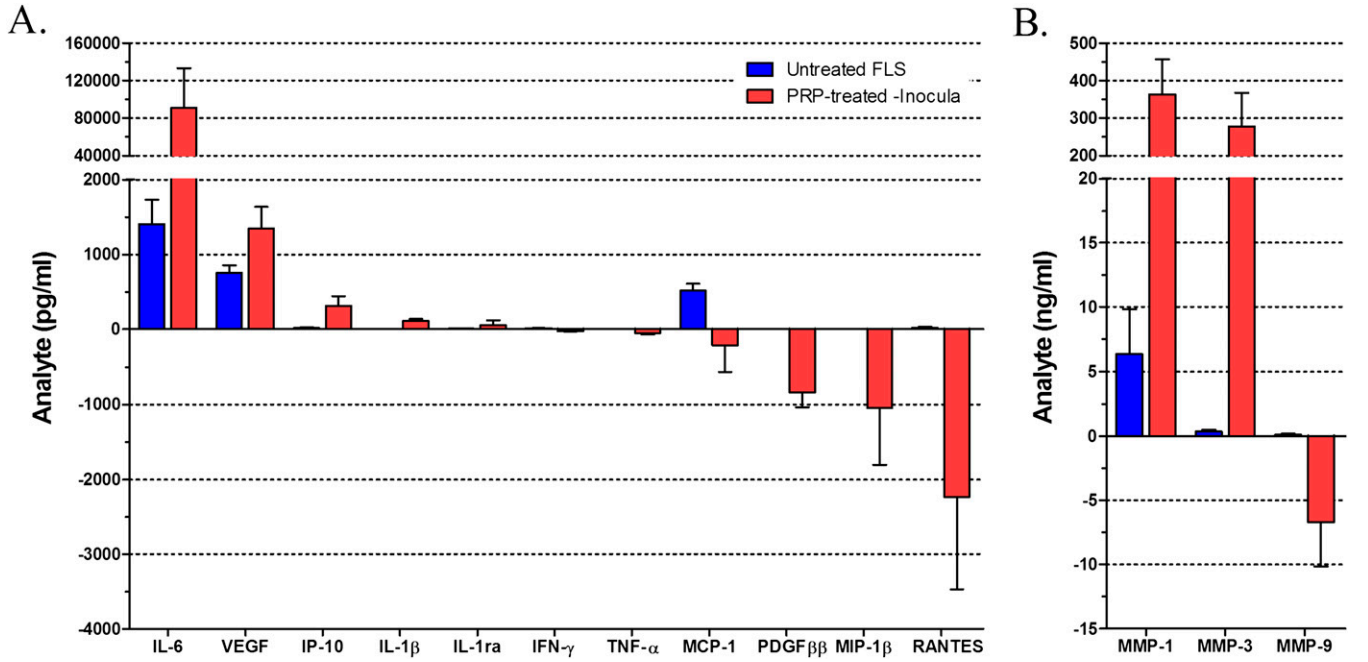

Fig. 2

Cytokine (**Fig. 2-A**) and matrix metalloproteinase (MMP) (**Fig. 2-B**) profiles of fibroblast-like synovioctye (FLS) cells treated with or without a thirtyfold dilution of PRP and incubated for two days in culture. Analyte values in the PRP-only controls were subtracted from those in the PRP-treated cells to better illustrate the effect of PRP treatment. Values are shown as the mean and the standard error of the mean. Analyte names are defined in the text.

Treatment of Cells with PDGFββ

On the day prior to treatment with PDGFββ, 1.25×10^5 fibroblast-like synoviocyte cells from patients D, E, and F were independently treated with or without 150 ng/mL of PDGFββ in 1.5 mL of medium in a 24-well plate. Synoviocyte cultures were incubated for either four or forty-eight hours. All analyses were performed in triplicate, and the results for treatment and no treatment are each presented as the mean for all cultures.

Multiplex Immunoassays

The cytokines investigated were IL-1β (interleukin-1β), IL-1RA (IL-1 receptor activator), IL-6, IFN-γ (interferon-γ), IP-10 (interferon gamma-induced protein 10), MCP-1 (monocyte chemotactic protein-1), MIP-1β (macrophage inflammatory protein-1β), PDGFββ, RANTES, TNF-α (tumor necrosis factor-α), and VEGF (vascular endothelial growth factor). The concentrations of these cytokines in the supernatant were determined by multiplex immunoassay (Bio-Plex; Bio-Rad, Hercules, California) according to the manufacturer's protocol. The concentrations of matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9 in the supernatant were also determined by multiplex immunoassay (Meso Scale Diagnostics, Gaithersburg, Maryland) according to the manufacturer's protocol.

Statistical Analysis

Data were analyzed with use of the t test and the Wilcoxon signed-rank test. An a priori power analysis assuming a very large effect size (Cohen d = 1.4) indicated that the power of the Wilcoxon signed-rank test for seven subjects and an alpha value of 0.05 was 84%.

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No extramural or intramural funding was employed or necessary for design and conception, data collection, data interpretation and analysis, or preparation of the manuscript. Biochemical assays were provided by Cytonics (Jupiter, Florida), which has no current commercial interest in PRP, including production or distribution of PRP-related products.

Results

Seven patients (three male and four female) with a mean age (and standard deviation) of 51.6 ± 8.9 years underwent venipuncture and collection of whole blood between June 2011 and August 2011. All patients underwent local autografting with PRP. Four patients underwent arthroscopic debridement for degenerative pathology of the tibiotalar joint, two underwent open reduction and internal fixation of an ankle fracture, and one underwent forefoot reconstruction for a degenerative deformity.

The molecular mediators in PRP and PPP include both soluble factors and factors released from cells (e.g., platelets and leukocytes). The cytokine and MMP profiles of PRP and PPP from each of seven patients were analyzed after two days of incubation in culture medium to obtain a more complete picture of the molecular mediators released from both PRP and PPP (Fig. 1). PRP was shown to contain a number of anabolic and catabolic cytokines (IL-1β, IL-1RA, IL-6, IFN-γ, IP-10, MCP-1, MIP-1β, RANTES, TNF-α) at concentrations between 20 pg/mL and 20 ng/mL, PDGFββ (1.14 ± 0.26 ng/mL), and proteases MMP-1 (2.5 ± 0.8 ng/mL) and MMP-9 (15.8 ± 2.3 ng/mL). T tests indicated that the concentrations of all mediators except IP-10 and MMP-3 differed significantly ($p < 0.05$) between PRP and PPP.

Fibroblast-like synoviocytes were cultured for forty-eight hours after treatment with PRP. The concentration of each mediator in the PRP-only control was subtracted from its final concentration (to account for the effect of the inoculum) before comparison with the concentration in cells cultured in the absence of PRP (Fig. 2). PRP treatment of fibroblast-like

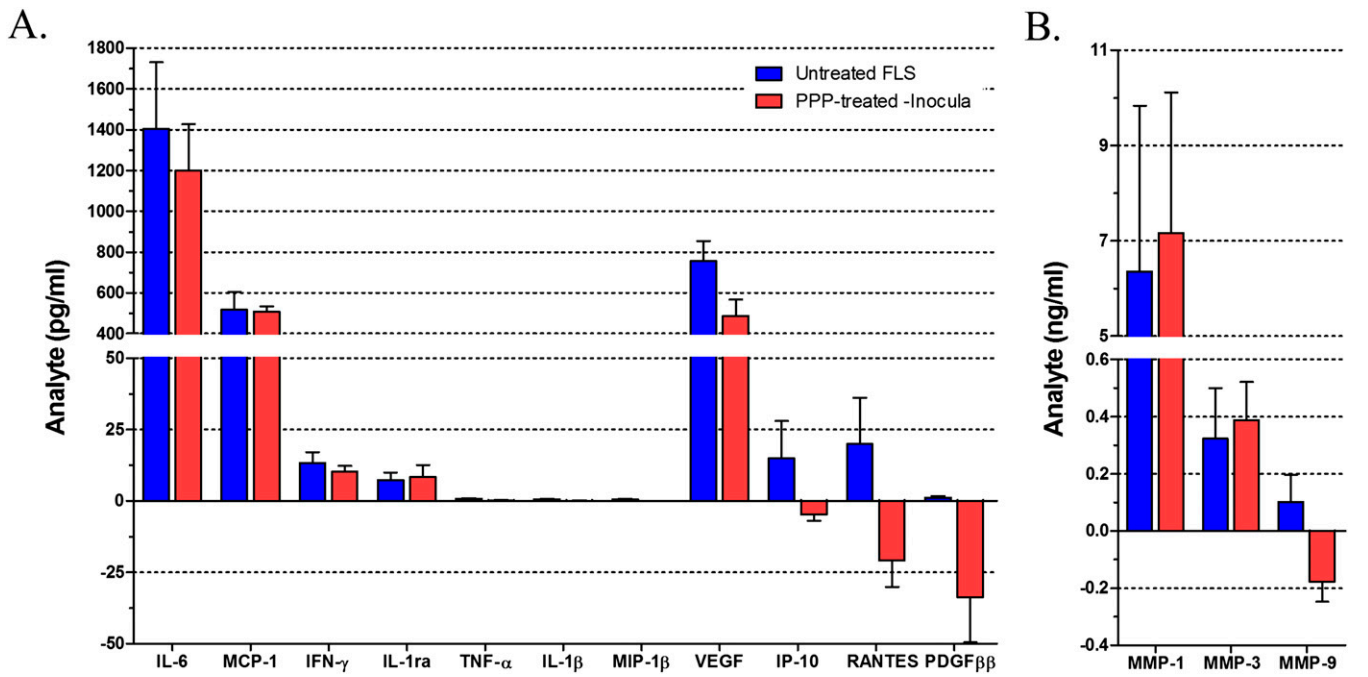

Fig. 3

Cytokine (**Fig. 3-A**) and matrix metalloproteinase (MMP) (**Fig. 3-B**) profiles of fibroblast-like synovioctye (FLS) cells treated with or without a thirtyfold dilution of PPP and incubated for two days in culture. Analyte values in the PPP-only controls were subtracted from those in the PPP-treated cells to better illustrate the effect of PPP treatment. Values are shown as the mean and the standard error of the mean. Analyte names are defined in the text.

synoviocytes led to a significant increase in IL-6 secretion in treated cells (90.7 ± 42.7 ng/mL) compared with untreated controls (1.4 ± 0.3 ng/mL, p = 0.043). PRP treatment also led to clearance of 838 ± 199 pg/mL of PDGFββ (p = 0.018) and clearance of chemotactic cytokines MIP-1β (p = 0.018) and RANTES (p = 0.028) as well as secretion of IL-1β (p = 0.028). In addition, treatment led to changes in the secretion of VEGF (p = 0.063) and IP-10 (p = 0.063) that did not reach significance (Fig. 2-A). Exposure of fibroblast-like synoviocytes to PRP also had a significant effect on the secretion of MMP-1 (363 ± 94 ng/mL in treated cells, p = 0.018) and MMP-3 (278 ± 90 ng/mL in treated cells, p = 0.018) relative to untreated cells (6.4 ± 3.5 ng/mL and 0.3 ± 0.2 ng/mL, respectively) (Fig. 2-B).

Fibroblast-like synoviocyte cells treated with PPP were cultured for forty-eight hours. Again, the concentration of each mediator in the PPP-only control was subtracted from its final concentration (to account for the effect of the inoculum) before comparison with the concentration in cells cultured in the absence of PPP (Fig. 3). Overall, a minimal response of fibroblast-like synoviocyte cells to PPP was noted.

Fibroblast-like synoviocytes treated with 150 ng/mL of PDGF were cultured for either four or forty-eight hours and

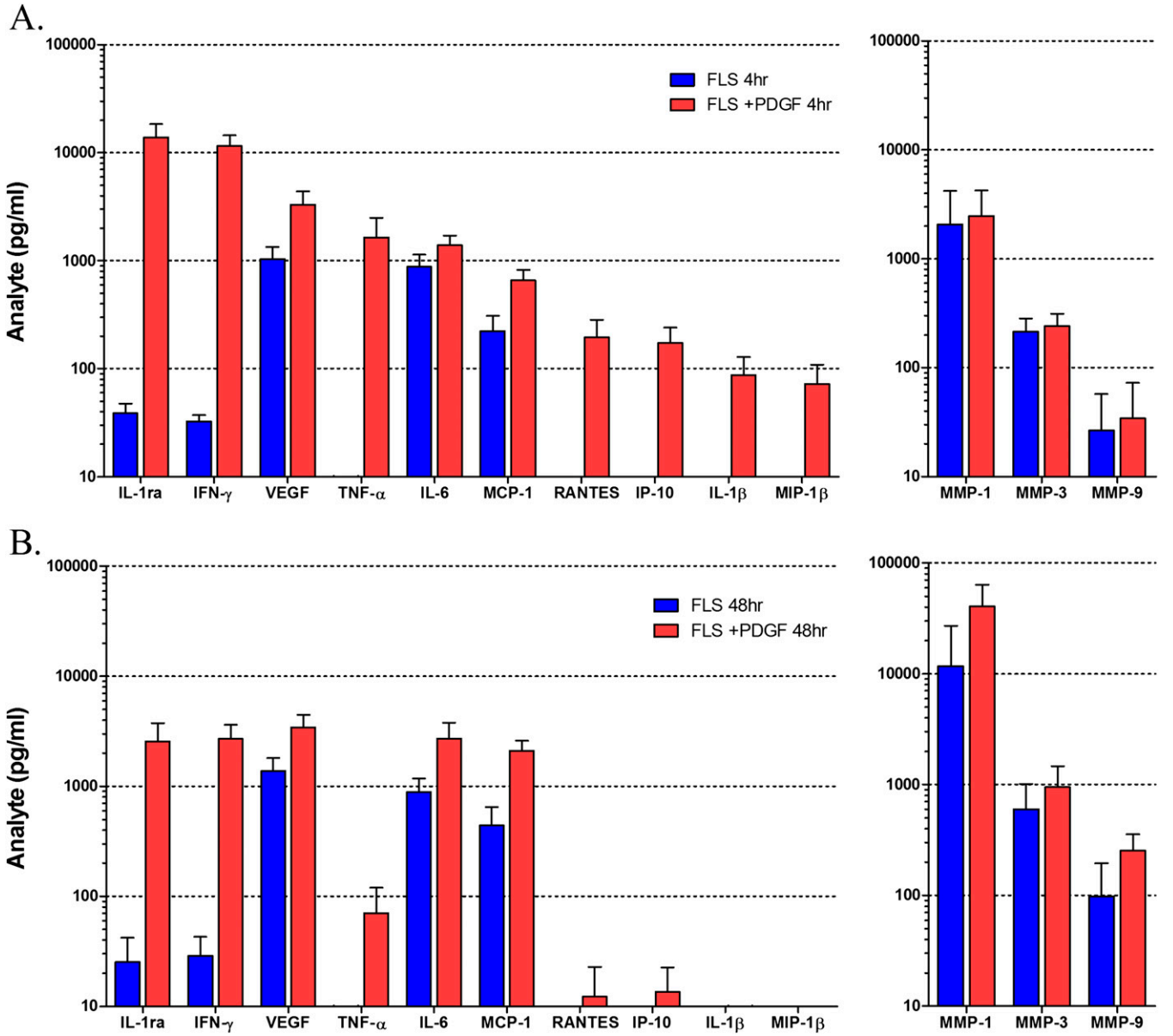

Fig. 4

Cytokine and matrix metalloproteinase (MMP) profiles of fibroblast-like synovioctye (FLS) cells after four hours (**Fig. 4-A**) and forty-eight hours (**Fig. 4-B**) of treatment with 150 ng/mL of platelet-derived growth factor ββ (PDGFββ). Values are shown as the mean and the standard error of the mean. Analyte names are defined in the text. The y axis is on a logarithmic scale.

compared with cells cultured in the absence of PDGF (Fig. 4). Treatment of the synoviocytes for four hours caused the acute secretion of several anabolic and catabolic cytokines (IL-1RA, IFN-γ, TNF-α, MCP-1, RANTES, IP-10, IL-1β, and MIP-1β). By forty-eight hours, the concentrations of many of these cytokines (IL-1RA, IFN-γ, TNF-α, RANTES, IP-10, IL-1β, and MIP-1β) had decreased, but most were still elevated relative to untreated cells. MMP secretion was not affected by PDGF treatment at four hours, but at forty-eight hours there was a trend toward increased MMP-1 secretion that did not reach significance ($p = 0.08$).

Discussion

The present study investigated the molecular profile of PRP and its effect on protein expression by human fibroblast-like synoviocytes in cell culture. The effect of exogenous PDGF on synoviocytes in cell culture was also investigated. The strengths of this study include the use of a broad proteomic panel of cytokines, chemokines, growth factors, and proteases as well as the use of primary human cells in culture. The study demonstrated that the prepared PRP contained a mixture of anabolic and catabolic mediators, that synoviocytes responded to PRP with substantial MMP secretion, that synoviocytes responded to PDGF with a substantial proinflammatory response, and that PPP did not cause similar responses.

PRP is hypothesized to have a therapeutic effect on synovial joints because of its high concentration of growth factors, particularly PDGF[1,5]. PDGF treatment stimulates cell division[19], and multiple laboratory studies have demonstrated enrichment of PDGF in PRP[5,20]. The cellular components of PRP include platelets, at a concentration ranging from two to eight times that in whole blood, and other blood-derived cells, such as leukocytes, depending on the method of preparation[5]. Multiple cell-derived and plasma mediators combine in a complex mixture of plasma proteins, growth factors, and catabolic and anabolic cytokines in addition to PDGF. The growth factor content of PRP varies both among patients and according to the method of preparation[21,22], but it typically consists of variable concentrations of platelet factors released on degranulation, including (but not limited to) PDGFαα, PDGFαβ, PDGFββ, transforming growth factor (TGF) β-1 and β-2, insulin-like growth factor 1 (IGF-1), platelet-derived epidermal growth factor (PDEGF), platelet-derived angiogenesis factor (PDAF), basic fibroblast growth factor (bFGF), and platelet factor 4 (PF-4)[5,23,24]. Compared with the growth factor content, the cytokine and protease content of PRP has been less well characterized, and it varies depending on the leukocyte content of the preparation. Sundman et al. found variable concentrations of MMP-9 and IL-1β in PRP prepared using different systems, and these concentrations were correlated with the concentration of neutrophils[6].

Since PRP preparations contain multiple molecular and cellular mediators whose concentrations can vary depending on numerous factors, it is difficult to predict whether the net effect of PRP treatment will be catabolic or promote healing. To our knowledge, no studies have addressed this potentially important phenomenon in cell culture models of synovial tissues. Fibroblast-like synoviocytes can be cultured directly from human synovium[16-18] and represent a useful tool for studying the in vitro response of synovial fibroblasts to PRP treatment.

The MMP and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) families of endoproteinases catalyze the normal turnover of extracellular matrix macromolecules, which include collagen, proteoglycans (e.g., aggrecan, decorin, biglycan, and fibromodulin), and structural extracellular matrix proteins (e.g., fibronectin). Collectively, MMP and ADAMTS proteinases are responsible for the cleavage of nearly all extracellular matrix proteins[25]. The initial steps of cartilage catabolism involve extracellular processes triggered by MMPs and ADAMTS, and MMP production in chondrocytes[26] and fibroblasts[27,28] can be triggered by inflammatory stimuli. The presence of catabolic cytokines in PRP and the upregulation of MMPs in fibroblast-like synoviocytes exposed to PRP suggest that the application of PRP to synovial joints may be associated with increased catabolism of cartilage and extracellular matrix proteins. In the present study, the net effect of the mediators in PRP on fibroblast-like synoviocytes was substantially catabolic because of the greatly increased MMP concentrations.

The conclusions drawn from the study are limited by several factors. The chief limitation is the use of a cell culture model to investigate the complex physiology of the synovial joint. Human fibroblast-like synoviocytes were selected because of their known biological role in health and disease, which is mediated by expression of proteases in response to cytokine signaling[14,15]; however, additional cell types including monocytes, macrophages, and T cells are present in synovium and synovial fluid. Although experiments are currently underway to test these cells lines as well, the overall effect on cartilage metabolism in a living synovial joint is impossible to predict from cell culture models alone. In addition, PRP treatment of fibroblast-like synoviocyte cultures represents an allogeneic use of PRP, whereas PRP is used autologously in clinical practice. A further limitation is the fact that only a single commercial PRP preparation system was tested, although there are known differences among the output of systems from different vendors[5]. The sample size in this initial study was relatively small; therefore, confirmation of the results may require larger studies. Finally, medical comorbidities in human subjects may affect the content of PRP and the response of fibroblast-like synoviocytes in ways that are difficult to predict.

Despite these limitations, our data indicate that the multiple catabolic mediators in PRP and the secretion of MMPs by PRP-treated fibroblast-like synoviocytes justify caution when using PRP for clinical applications and warrant further studies to characterize this autologous product more carefully. ■

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