Effect of platelet-rich plasma on migration and proliferation of SaOS-2 osteoblasts: role of platelet-derived growth factor and transforming growth factor-β

Fabio Celotti, MD[1]; A. Colciago, PhD[1]; P. Negri-Cesi, PhD[1]; A. Pravettoni, PhD[1]; R. Zaninetti, PhD[1]; M.C. Sacchi, PhD[2]

1. Institute of Endocrinology, University of Milano, Milano, Italy, and
2. Laboratory of Hematology, Department of Transfusional Medicine and Hematology, ''SS. Antonio e Biagio e C. Arrigo Hospital,'' Alessandria, Italy

Reprint requests:
Prof. Fabio Celotti, Institute of Endocrinology, University of Milano, Via Balzaretti 9, 20133 Milano, Italy.
Fax: + 39 0250318204;
Email: fabio.celotti@unimi.it

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ABSTRACT

Platelet-enriched plasma (PRP) is used in therapy as a source of growth factors in bone fracture and wound healing; however, few data exist on its role in the different aspects of the healing process. The effect of PRP and of the two main growth factors present in this preparation (platelet-derived growth factor [PDGF] and transforming growth factor-β [TGF-β]) was evaluated in vitro using the human osteoblastic cell line SaOS-2, which was shown by reverse transcription-polymerase chain reaction to express both PDGF-α and -β receptors. Batroxobine-activated PRP was added in different concentrations to SaOS-2 cells to assess cell migration (by a microchemotaxis assay) and cell proliferation (by [^{3}H]-thymidine incorporation into the DNA). Immunoneutralization with anti-PDGF-β or anti-TGF-β antibodies allowed the assessment of the specific role of these growth factors. The overall results obtained indicate that PRP dose-dependently stimulates both chemotaxis and cell proliferation. PDGF and TGF-β appear to exert distinct effects on the two parameters, the former involved in stimulating cell migration and the latter in inhibiting cell proliferation. It is concluded that the different growth factors present in activated PRP can specifically contribute to the main processes of tissue regeneration.

Many growth factors, cytokines, and other bioactive compounds are released by platelets upon activation when wound healing is initiated in either soft or in bone tissue. The most abundant growth factors present in platelets are platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), followed by insulin-like growth factor (IGF)-1.[1–3] The PDGF family consists of four members, the well-characterized PDGF-A and PDGF-B, and two new members, PDGF-C and PDGF-D.[4,5] Biosynthesis and processing of the PDGFs produce full-length disulfide-linked homodimers PDGF-AA, -BB, -CC, and -DD and the heterodimer PDGF-AB. Additional processing is not required for the biological activity of PDGF-AA, -BB, and -AB. Two distinct PDGF receptors, α and β, mediate the effects of the PDGFs on target cells. The PDGF-A and -C chains selectively bind the α receptor, whereas PDGF-D preferentially binds the β receptor and PDGF-B displays a similar affinity for both receptors. Real-time polymerase chain reaction (PCR) performed on platelet RNA revealed a high level of expression for PDGF-A, PDGF-B, and PDGF-C but no expression was observed for PDGF-D. Like PDGF-A and -B, PDGF-C protein was shown by immuno-gold electron microscopy to be stored in platelet granules.[4]

Platelet-enriched plasma (PRP) has been shown by radioimmunoassay or enzyme immunoassay to contain PDGF-AB as the main PDGF family member, followed by PDGF-BB, present in much lower levels.[6,7] The other members of the family have not yet been assayed in PRP. PDGF stimulates DNA and protein synthesis in bone and acts as a potent mitogen for mesenchymal cells. It is also a powerful chemotactic factor for smooth muscle cells, fibroblasts, macrophages, leukocytes, and mesenchymal progenitor cells (MPCs).[8–11]

TGF-β is a two-chain polypeptide with a molecular mass of about 25 kDa present as three different gene products in humans: TGF-β1, TGF-β2, and TGF-β3. These peptides are 70–80% homologous and encoded by distinct genes located on different chromosomes.[12] TGF-β1 is reported to be highly concentrated in platelets[1] and to increase bone formation modulating osteoblast differentiation, increasing secretion of bone matrix protein, and inhibiting osteoclast formation.[1,13] TGF-β also represents

DMEM	Dulbecco's modified Eagle's medium
FCS	Fetal calf serum
IGF	Insulin-like growth factor
MPCs	Mesenchymal progenitor cells
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenylterazolium bromide
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RT	Reverse transcription
TGF-β	Transforming growth factor-β

a potent chemotactic stimulus to several cell populations.[14–16]

Recent reports indicate that the preparation of an autologous PRP by centrifugal concentration is potentially useful as an adjunct to allograft and xenograft materials in implant reconstructive surgery, and in fracture and wound healing. PRP represents a cost-effective way to obtain high concentrations of growth factors in a limited volume of plasma potentially useful in tissue healing and bone regeneration. Although several studies have shown the efficacy of PRP both in preclinical[17–21] and clinical[22–27] settings, few studies exist on the role of the different PRP components on the complex series of events finally leading to tissue regeneration.

The aim of our studies was to investigate the effect of the supernatant obtained after PRP activation and centrifugation, on the migration of an osteoblast-like cell line, and on cell proliferation, focusing on the role played by the two major growth factors present in PRP: PDGF and TGF-β.

MATERIALS AND METHODS

SaOS-2 is a mature osteoblastic cell line derived from a human osteosarcoma. The cell line was originally obtained from the American Type Culture Collection (Manassas, VA) and routinely grown at 37 °C in a humidified atmosphere (5% CO_2 and 95% air) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μg/mL), and sodium pyruvate (1 mM).

PRP preparation and activation

Fifty-four milliliters of venous blood was withdrawn from the arm of a normal healthy volunteer into a 60 mL citrate syringe. Citrate phosphate dextrose was added to the syringe at a ratio of 6 : 54 mL of whole blood, achieving anticoagulation through calcium binding. The whole blood, collected in sterile test tubes of 15 mL each ($n = 4$), was centrifuged at $180 \times g$ per 20 minutes. At the end of the first centrifugation, the blood was separated into its two basic components as a function of density. The PRP represented the top layer; the red blood cells with white blood cells were contained in the lower layer. The top layer was then transferred into clean tubes and added with the first 100–300 μL of the RBC layer, which contained the larger and more recently synthesized platelets. The tubes were centrifuged at $580 \times g$ for 15 minutes to create a precise separation of the platelet pellets from the platelet poor plasma (PPP). A small volume of PPP (about 6 mL) was used to resuspend the platelet pellets, giving the final PRP fraction. Repeated analysis of these preparations indicated a platelet enrichment of about four to five times ($1.0–1.2 \times 10^6$ platelets/μL on average). Four milliliters of this fraction was transferred into a sterile syringe with 1 mL of calcium gluconate/batroxobine (Pentapharm, Basel, Switzerland) mixed and gently shaken. Within 1–3 minutes, this mixture solidified because batroxobine produced polymerization of the fibrin into an insoluble gel; at the same time, platelets degranulated releasing growth factors and cytokines, which remained trapped in the gel. The platelet gel was centrifuged at $1400 \times g$ for 10 minutes at room temperature, to separate the liquid phase, enriched in growth factors, from the solid phase. After collection, the supernatant was divided into sterile microtubes and frozen at –80 °C. Under these storage conditions, the preparation was equally effective for many months. Aliquots from the same preparation were used for all of the experiments described here.

PRP antibody treatments and fractionation

The stock preparation of PRP was used at different dilutions (1 : 10, 1 : 500, 1 : 1,000, 1 : 2,000, and 1 : 10,000) in serum-free DMEM (+ glutamine, antibiotics, and sodium pyruvate). PDGF and TGF-β contained in PRP were neutralized by adding specific polyclonal antibodies raised against human PDGF (cat. No. P6101: Sigma-Aldrich, St. Louis, MO) and human TGF-β (cat. No. Sc-7892; Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies were added to PRP 1 : 1,000 at a final concentration of 14, 28, and 56 μg/μL for TGF-β and of 14, 28, 56, 114, 228, and 457 μg/mL for PDGF. Each preparation was heated at 37 °C for 1 hour. Serum-free DMEM and DMEM + 1% FCS were added as negative and positive controls, respectively.

Anti human-PDGF is a polyclonal antibody developed in goats and purified by affinity chromatography. It recognizes all human homodimeric and heterodimeric forms and shows no cross-reactivity with other cytokines tested. According to the manufacturer, the concentration able to neutralize half maximum biological activity of PDGF is 3–5 μg/mL. Anti human-TGF-β is a rabbit polyclonal antibody raised against amino acids 301–412 of TGF beta 1/2/3. 1–2 μg immunoprecipitates 100–500 μg of total protein.

PRP was also fractionated by Microcon® YM-30 (30,000 MW cutoff) centrifugal filter devices (Amicon Bioseparation, Millipore, Bedford, MA). Five hundred microliters of undiluted PRP was centrifuged at $14{,}000 \times g$ for 12 minutes at room temperature. The fluid collected at the bottom of the vial was diluted 1 : 10 and utilized in the microchemotaxis assay.

Microchemotaxis assay

The microchemotaxis assay was performed using a 48-well Boyden chamber according to the manufacturer's instructions (Neuroprobe, Cabin John, MD). Cells were plated 48 hours before use at a density of 20,000 cells/cm^2 in order to avoid confluence. For chemotaxis experiments, 28 μL of control media (DMEM) or chemoattractants were placed in the lower compartment of the chamber. Cells, collected by trypsinization, were resuspended in DMEM + 0.1% bovine serum albumin and placed (10^5 cells/well) in open-bottom wells of the upper compartment. Each pair of wells were separated by a polyvinylpyrrolidone-free polycarbonate porous membrane (8 μm pores) precoated with gelatin (0.2 mg/mL in phosphate buffered saline solution) for 5 days at 4 °C. The chamber was kept overnight at 37 °C, in humidified air with 5% CO_2. At the end of the incubation period, the migrated cells were adherent to the underside of the membrane; they were fixed by methanol and stained according to a Diff-Quik kit (Biomap, Milano, Italy). For quantitative analysis, cells were observed and counted

using a 40× objective on an optical microscope. Three random objective fields of stained cells were counted for each well, and the mean number of migrating cells was calculated.

Mitogenic assay

Proliferation of human SaOS-2 cells was measured by incorporation of [^{3}H]-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, England) into replicating DNA produced during mitosis. In brief, SaOS-2 cells were seeded at a density of 5,000 cells/cm^2 in 35 mm cell culture dishes and grown for 24 hours in DMEM plus 10% FCS. This medium was changed to a serum-free medium supplemented with PRP with and without different concentrations of the above-specified antibodies (14, 28, 56, 114 μg/mL) for 24 hours. Cells were pulse labeled with [^{3}H]-thymidine (1 μCi/dish) for the last 3 hours of culture (37 °C, 5% CO_2). After this incubation period, cells were washed extensively with phosphate buffered saline without Ca^{2+} and Mg^{2+}, incubated with 5% trichloroacetic acid for 30 minutes at 4 °C, washed with ethanol 70%, and then collected with NaOH (0.2 M). The amount of [^{3}H]-thymidine incorporated into the SaOS-2 cells was measured with a liquid scintillation counter (Perkin Elmer, Boston, MA). Cells treated with DMEM + 10% FCS were added as positive controls.

Cell viability assay

To examine the effects of PRP on SaOS-2 viability, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenylterazolium bromide (MTT) assay was performed. Cells were plated in 35 mm cell culture dishes (2,500 cells/cm^2) in DMEM + 10% FCS; 24 hours later, the medium was replaced with DMEM + 5% FCS and cells were treated with PRP 1 : 40 and 1 : 400. After 6 days, cells were collected and the MTT assay was performed. The absorbances were read on a microplate reader at 550 nm.

Expression of PDGF receptor-α and -β gene transcripts

Total RNA was isolated from SaOS-2 cell lysate in 4 M guanidinium isothiocyanate. After a phenol-chloroform extraction, total RNA was purified and quantified. The expression of PDGF receptor-α and -β was evaluated on total RNA by reverse transcription (RT)-PCR using a GeneAmp RNA PCR kit (Applera Italia, Italy) according to the protocol described by the manufacturer. RT was performed on 2 μg of total RNA, at 42 °C for 45 minutes, followed by 5 minutes at 95 °C. PCR condition and primers for PDGF receptors have been published previously. [16] β-actin was used as control and was amplified with the following primers: 5′-GAGAAGATGACCCAGATCATGTTTG-3′ 3′-GATGTCCACGTCACACTTC-5′ amplification product: 523 bp.

The amplified products were separated by electrophoresis on 2% agarose gels and identified by ethidium bromide staining. Specificity was confirmed by the size of the amplified products. RNA from human placenta, known to express both PDGF receptors,[28] was used as positive control.

Statistical analysis

Statistical analysis of the data was performed by one-way analysis of variance (ANOVA), followed by the Tukey's post hoc test for multiple comparisons using the Systat statistical software (version 5.2 for Macintosh, Evanston, IL).

RESULTS

The effect of the PRP supernatant on SaOS-2 cell migration is shown in Figure 1. There was a progressive increase in the chemotactic potency of PRP with decreasing dilutions of the preparation. The effect of PRP, starting from 1 : 2,000 dilution, was significantly higher than that of the negative control (DMEM alone). An effect significantly higher than that observed with 1% FCS, the positive control, was present at a dilution of 1 : 10. The 1 : 1,000 dilution, utilized in most of the experiments described here, behaved similarly to FCS 1%.

Heating PRP (at 70 °C for 20 minutes) strongly decreased the chemotactic effect of PRP, which was completely abolished by filtering the preparation with a 30 kDa molecular cutoff membrane (Figure 2).

Figure 3 shows a representative experiment in which the expression of the α- and β-PDGF receptor genes was studied by RT-PCR amplification and separation by an agarose gel electrophoresis. It appears from the bands obtained that two single gene products of 423 and 417 bp were amplified utilizing SaOS-2 RNA in the presence of the specific primers. The size of these cDNA fragments matched the expected size based on the primer selection. The same fragments have been obtained on analyzing the RNA extracted from the human placenta evaluated as positive control. The expression of β-actin was utilized as a control of the quality and concentration of RNA in each sample. Even if the method adopted is only indicative of the amount of receptor expressed, it appears from the data

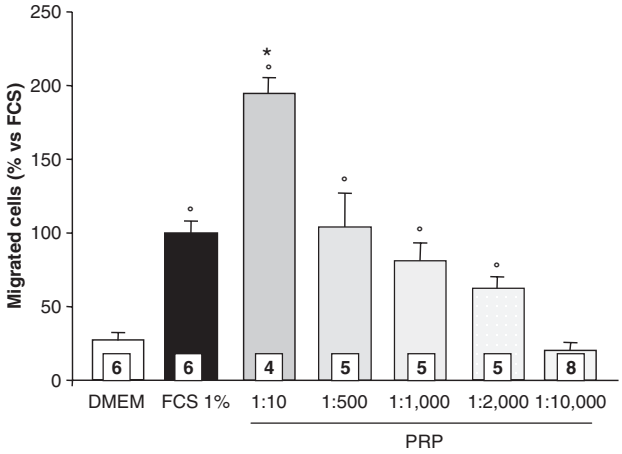

Figure 1. Effect of different PRP dilutions on SaOS-2 cell migration. Values are expressed as means ± SD of the percentage of migrated cells compared with 1% FCS. The number of samples analyzed is shown in the squares at the bottom of the columns. °$p < 0.05$ vs. DMEM; *$p < 0.05$ vs. FCS 1%.

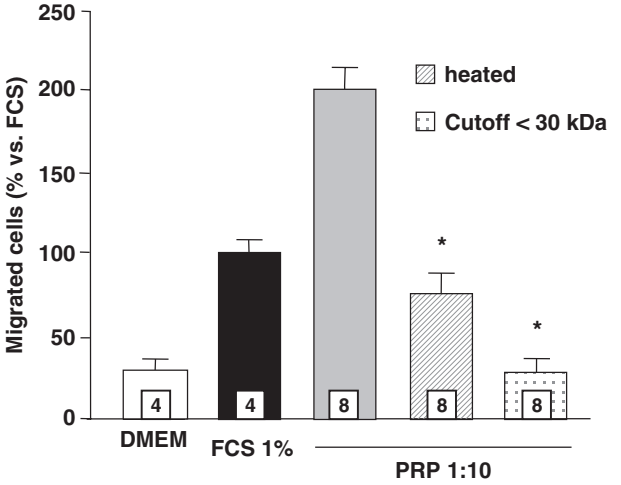

Figure 2. Effect of heating and of a molecular cutoff membrane on PRP-stimulated migration of SaOS-2 cells. Values are expressed as means ± SD of the percentage of migrated cells compared with 1% FCS. The number of samples analyzed is shown in the squares at the bottom of the columns. *$p < 0.05$ vs. PRP 1 : 10.

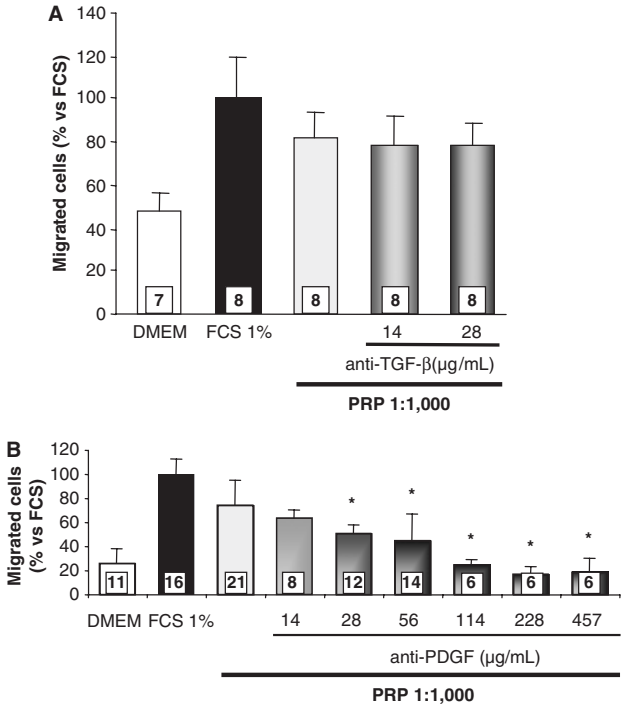

Figure 4. Effect of anti-TGF-β and anti-PDGF antibody treatment of PRP on SaOS-2 cell migration. (A) Anti-TGF-β; (B) anti-PDGF. Values are expressed as means ± SD of the percentage of migrated cells compared with 1% FCS. The number of samples analyzed is shown in the squares at the bottom of the columns. *$p < 0.05$ vs. PRP 1 : 1,000.

that both receptors are expressed by SaOS-2 cells in an apparently similar abundance.

Preincubation with antiserum directed against TGF-β appeared ineffective on cell migration (Figure 4, upper panel). On the other hand, the incubation of the PRP supernatant with an antiserum directed against PDGF produced a dose-dependent decrease in the chemotactic effect of PRP up to the concentration of 114 μg/mL; at this dose level, the chemoattractive effect was not significantly different from that of the negative control—DMEM. Further increases in the antibody concentration had no effect on the migratory effect of SaOS-2 cells (Figure 4, lower panel).

Figure 5 shows the effect of PRP on cell proliferation as measured by [^{3}H]-thymidine uptake. It appears that PRP produced a dose-dependent stimulation of SaOS-2 cell proliferation when compared with DMEM. All the three dilutions utilized (1 : 1,000, 1 : 500, and 1 : 100) produced a statistically significant effect versus controls (Figure 5, upper panel, left side). The effect of PRP was strengthened by the addition of the antibody directed against TGF-β (Figure 5, upper panel, right side), indicating that TGF-β has an inhibitory effect on the mitotic activity of this osteoblastic cell line. In contrast, immunoneutralization of PDGF, even with concentrations of antibodies able to suppress the chemotactic stimulus completely, does not produce any significant effect on the thymidine incorporation by the cells. In these studies, cell proliferation has been studied in cells maintained in short-term cultures (24 hours) without FCS.

The stimulatory effect of PRP on the cell proliferation has also been assessed in a more chronic setting in which cells were maintained in culture with low amounts of FCS (5%) for 15 days in the absence (C) or presence of PRP 1 : 100. The results indicate that a significant stimulatory effect of PRP might also be observed in these culture conditions (Figure 6).

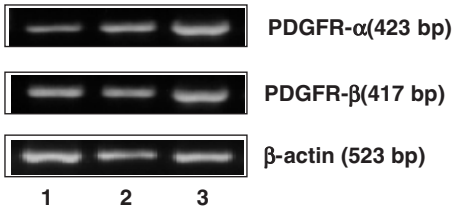

Figure 3. Representative experiment showing PDGF receptor-α and -β mRNA expression in SaOS-2 cells amplified by reverse transcription-polymerase chain reaction. Lanes 1 and 2: SaOS-2 cells; lane 3: human placenta.

DISCUSSION

The data presented here indicate that SaOS-2 osteoblasts possess both types of PDGF receptors and that PRP in vitro has a strong, dose-dependent, chemoattractant activity. This preparation is also able to stimulate cell proliferation. PRP dilutions up to 1 : 2,000 have a significantly higher chemoattractant activity than the control medium in Boyden's chamber microchemotaxis assay. This

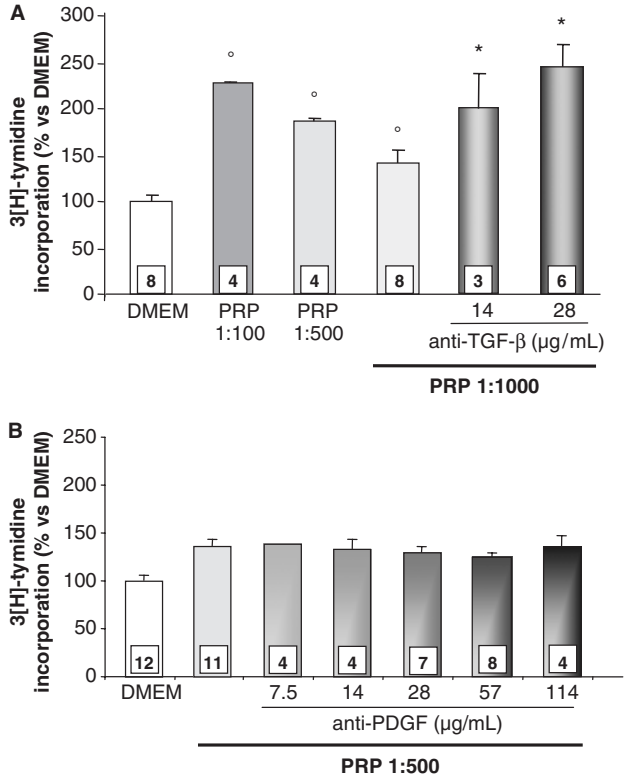

Figure 5. Effect of anti-TGF-β and anti-PDGF on SaOS-2 cell proliferation measured by [^{3}H]-thymidine incorporation. (A) Anti-TGF-β; (B) anti-PDGF. Values are expressed as means ± SD of the percentage of migrated cells compared with DMEM. The number of samples analyzed is shown in the squares at the bottom of the columns. °$p < 0.05$ vs. DMEM; *$p < 0.05$ vs. platelet-rich plasma 1 : 1,000.

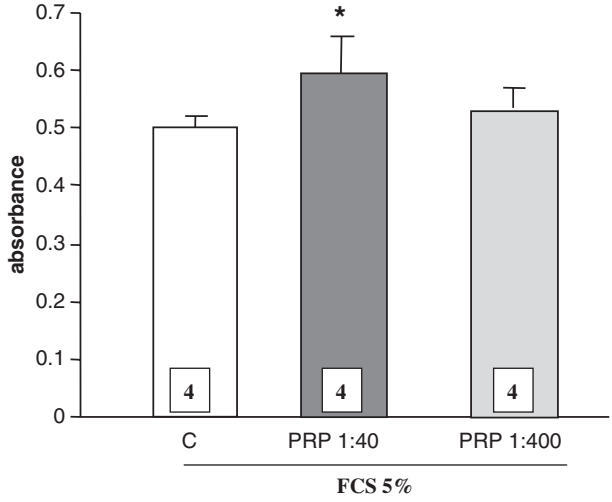

Figure 6. SaOS-2 cell viability after a 6-days treatment with PRP. Values are expressed as means ± SD of the absorbance values. The number of samples analyzed is shown in the squares at the bottom of the columns. *$p < 0.05$ vs. fetal calf serum 5% (C).

observation is in substantial agreement with similar results obtained with primary human[29,30] and rat bone marrow cells.[31,32] Preliminary observations obtained in our laboratory indicate that autologous and heterologous PRP produce a remarkable chemoattraction on primary human osteoblast. The effect appears to be slightly weaker than that observed with SaOS-2 cells and related to the degree of cell differentiation.

The effect of PRP on SaOS-2 cell migration was almost abolished by heating and by filtration on a 30 kDa cutoff membrane, indicating the involvement of proteins with a molecular weight around this value or above.

Among the factors released in PRP from platelet α-granules upon activation, PDGF, possessing a MW of approximately 30 kDa, was considered a good candidate to explain the chemoattractant activity of the preparation. TGF-β, which, together with PDGF, is the most abundant growth factor present in PRP, possesses a lower MW (around 13 kDa) in its active forms but it is secreted as a high-MW latent complex bound by a specific binding protein (125–160 kDa in platelets), which are possibly retained by the membrane.

To understand the role of these two growth factors in PRP-induced chemotaxis, PRP was preincubated with specific neutralizing antibodies directed toward human PDGF and TGF-β. The concentration of the antibodies was selected based on the reported concentrations of these growth factors in PRP and on the neutralizing capacity of the antisera. PDGF and TGF-β have been reported to have a similar concentration of about 150–200 ng/mL in preparations containing $\sim 10^9$ platelets/μL.[2,7,26] Specifically, PDGF-AB (117 ± 63 ng/mL), and TGF-β1 (169 ± 84 ng/mL) were found in larger amounts, while PDGF-BB (10 ± 8 ng/mL) and TGF-β2 (0.4 ± 0.3 ng/mL) were found in much smaller amounts.[3]

The maximal amount of antisera added to neutralize PDGF activity (457 μg/mL) was considerably excess with respect to the reported PDGF concentration present in PRP. The antibody recognizes all of the classic PDGF isoforms, -AA, -AB, and -BB, but it has never been tested against the recently discovered proteins -C and -D.

Also, the maximal concentration of anti-TGF-β (28 μg/mL) was probably considerably in excess because this antibody concentration is reported to immunoprecipitate at least 2,800 μg/mL of protein (package inset).

The data presented show that the chemotactic activity exerted by PRP on SaOS-2 cells is completely neutralized by the anti-PDGF antibody at concentrations of 114 μg/mL or above, indicating that this growth factor is largely responsible for PRP-induced chemotaxis. These results are in substantial agreement with those of Gruber et al.,[29] who have shown that PRP of adult volunteers, obtained from thrombin-activated platelets, increases migration of bone marrow-derived MPCs. In these studies, the addition of an anti-PDGF antibody produced a 57% decrease in the chemotacic activity of the PRP preparation. It is very likely that the incomplete inhibition of the PDGF effect observed by these authors was due to the insufficient amount of neutralizing antibody used: 10 μg/mL utilizing a preparation corresponding to a platelet number of 4×10^7/mL.

Under our conditions, concentrations of 114 μg/mL or above, of an antibody with a reported neutralizing activity analogous to that utilized by these authors, produced a complete neutralization of PDGF contained in a preparation corresponding to 1×10^6/mL platelets. The key role of PDGF in the chemoattraction of osteoblast has been substantiated by the studies performed by Fiedler et al.[10,11] on human bone-derived cells at various stages of differentiation.

In studies performed utilizing the Boyden chamber assay with human MPCs,[11] rhPDGF-BB produced a powerful chemotactic stimulus, which declined with osteogenic differentiation. More recently,[10] the same authors showed that the chemotactic response of MPCs depended on the PDGF isoform utilized. Although rhPDGF-AA and rhPDGF-AB induced a rather weak chemotactic stimulus, the -BB isoform was very effective. Blocking PDGF-α receptors with specific antibodies, the chemotactic response decreased significantly in most cases, but not to a complete inhibition of migration, whereas the preincubation with specific antibodies against the PDGF-β receptor led to a complete inhibition of migration with all PDGF isoforms.

The neutralization study indicates that TGF-β does not produce a significant effect on SaOS-2 cell migration. In the TGF-β experiments, the amount of antiserum added was not increased beyond 28 μg/mL because of complete lack of effect at concentrations already in great excess compared with the expected TGF-β levels in PRP. In contrast, the same doses of antiserum were able to affect cell proliferation.

Although TGF-β was shown to be chemotactic in several cell types,[14–16] our observations are in substantial agreement with those of Fidler et al.,[11] who observed that TGF-β1 does not stimulate cell migration of human MPCs. In contrast, the same authors showed that primary osteoblasts and progenitor cells, once induced to differentiate, were able to respond to the chemotactic stimulus driven by TGF-β1. SaOS-2 cells although showing a relatively differentiated osteoblastic phenotype, represent an active proliferating tumor cell line[33] that might not show the same responsiveness to the growth factors as normal differentiated osteoblasts, even if these cells express TGF-β receptors.

PRP has a definite dose-dependent effect on SaOS-2 proliferation, even if the mitogenic stimulus appears to be less potent than that exerted on cell migration. The global efficacy of the preparation is probably the result of a positive stimulus exerted by growth factors present in PRP (possibly IGF-I) and of an opposite effect produced by TGF-β. The proliferative stimulus has been shown in our studies both in short-term (24 hours) and long-term (6 days) periods of stimulation. This result is in agreement with the recent paper of Kanno et al.,[34] in which 10% PRP preparation produced a 20–30% increase in SaOS-2 cell viability measured by the MTT assay.

PRP was also shown to increase [^{3}H]-thymidine incorporation into primary human bone cell cultures.[17,35] Proliferative effects of platelet derivates have also been shown in bone-derived rat primary cells,[31,32] in peridontally related cells,[2] and in stromal stem cells.[36]

The proliferative stimulus observed in our study by PRP was not antagonized by the specific antibody directed against PDGF when added to the cultures at concentrations able to neutralize the chemotactic effect completely. We can conclude from these experiments that PRP exerts a chemotactic and proliferative stimulus on SaOS-2 osteoblasts but that only the increased cell migratory activity is mediated by PDGF. Our observations are in partial disagreement with the results obtained in in vitro experiments with human MPCs.[29,35] In those studies, the mitogenic potential of PRP was partially suppressed by neutralizing antibodies raised against PDGF. Moreover, there are studies indicating that PDGF-BB is mitogenic for human osteoblasts and in various osteogenic cell lines.[37,38] The proliferative effects of this growth factor are apparently mediated mainly by the activation of the β-receptor because its activation alone is sufficient for PDGF-BB-mediated anchorage-independent cell growth [39] at least in 3T3 fibroblasts.

To the authors' knowledge no report on the effect of PDGF in SaOS-2 cells proliferation has been previously published in the literature; therefore, it is possible that, even if both types of PDGF receptors are expressed in these cells (as shown by our result), the proliferative stimulus is mediated by other components present in PRP. It is now well recognized that PDGF-mediated cellular responses vary among cell types,[39] and that these variations are most likely due to innate differences in available signaling molecules even in cell types that express PDGF receptors.

Moreover, the expression of the PDGF-β receptor is tightly regulated during the cell cycle in normal cells, while an *Rb* null 3T3 cell line expresses a stable level of PDGF-β receptor after serum stimulation and exhibits a shorter G1 phase and a faster cell cycle than control cells. It has also been shown that p53 binding to the PDGF-β receptor promoter appears necessary to control the expression of the receptor at least in some situations.[40] SaOS-2 cells are defective of this gene, as well as of the Rb gene. Consequently, one could argue that SaOS-2 cells might not represent a suitable model to investigate the proliferative effects of PDGF.

The inhibitory effect produced by TGF-β is not surprising in view of the fact that TGF-β reversibly inhibits cell proliferation in epithelial, endothelial, hematopoietic, and neural cells[12] and SaOS-2 cells possess type I (but no type II) TGF-β receptors on the cell surface.[41] However, because TGF-β growth inhibition appears to be mediated by the inhibition of the phosphorylation of the retinoblastoma gene product pRb,[42] this result might be surprising because SaOS-2 cells, as mentioned previously, lack a functional *Rb* gene. On the other hand, it has been shown that inactivation of the *Rb* gene in other cell lines does not lead to loss of TGF-β receptors or response to TGF-β.[43] Few previous studies have evaluated the effect of TGF-β on SaOS-2 cell proliferation,[44,45] showing few effects on cell mitotic activity when added to co-cultures. It is likely that the proliferative effect observed in our studies neutralizing TGF-β occurs uncovering the effect of other molecules present in PRP.

It is possible to propose that IGF-1, which is the third growth factor contained in PRP in decreasing concentration,[3] might account for the mitogenic effect observed in SaOS-2 cells, because these cells express IGF-2-binding sites and IGF-1-treated cell lines display enhanced proliferation.[46,47]

In conclusion, PRP is an easily available blood derivative containing high concentrations of growth factors and cytokines, which, when applied locally, increases tissue and bone repair acting on osteoprogenitor cell recruitment, proliferation, and differentiation. The role of its individual constituents in promoting tissue repair remains to be fully elucidated. On the basis of the observations made utilizing an osteoblastic human cell line, the amounts of PDGF contained in the preparations appeared to be completely responsible for the chemotactic effect produced by PRP, whereas the role of this growth factor in cell proliferation appeared negligible. In contrast, TGF-β did not promote chemotaxis of SaOS-2 cells, but it showed an anti-proliferative effect. It is possible that this growth factor is important in increasing the differentiation of the cells after their migration in the lesions. The PRP component responsible for the mitogenic stimulus exerted by PRP on osteoblasts is yet to be identified. It is clear that the net effects of PRP represent the result of agonist and antagonist actions of the different components, which, when more clearly defined, might allow better use of this platelet derivative in the future.

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