Effect of Platelet-Rich Plasma on Cell Adhesion, Cell Migration, and Myofibroblastic Differentiation in Human Gingival Fibroblasts

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Background: Platelet-rich plasma is a blood-derived fraction that contains a high concentration of platelets and growth factors. It was proposed that the use of this platelet concentrate stimulates tissue repair. However, little is known about the biologic response of gingival fibroblasts to platelet's derived growth factors. In the present study, we evaluated whether platelet-rich plasma modulated cell adhesion, cell migration, and myofibroblastic differentiation in primary cultures of human gingival fibroblasts.

Methods: We studied the response of primary cultures of gingival fibroblasts to thrombin-activated platelet-rich plasma fractions. Cell adhesion was evaluated through a colorimetric assay. Cell spreading, actin cytoskeleton remodeling, and focal adhesion distribution were assessed through light and immunofluorescence microscopy. Cell migration was analyzed using a bicameral cell culture system. Smooth muscle actin production was studied through Western blotting.

Results: Exposure of gingival fibroblasts to platelet-rich plasma stimulated adhesion and spreading of cells on fibronectin matrices, the development of actin-enriched cellular extensions, and formation of focal adhesions. Platelet-rich plasma also promoted cell migration and invasion through a reconstituted basement membrane matrix. Differentiation into the myofibroblastic phenotype, assessed through the production of smooth muscle actin, was also stimulated by platelet-rich plasma preparations.

Conclusion: Platelet-rich plasma may modulate several cell responses potentially involved in wound healing such as cell adhesion, cell migration, and myofibroblastic differentiation. *J Periodontol 2008;79:714-720.*

KEY WORDS

Adhesion; migration; myofibroblast; platelet-rich plasma.

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Wound healing requires the coordination of a variety of physiological processes that follow a specific time sequence. After tissue injury, several cell responses, such as cell adhesion and migration, extracellular matrix synthesis and remodeling, cell proliferation, and differentiation are observed in a highly coordinated fashion.[1] It is generally accepted that growth factors play a significant role in wound healing.[2] In fact, almost all stages of tissue repair are controlled by a wide variety of cytokines and growth factors acting locally as regulators of basic cell functions.[1,2]

Cell adhesion and migration are striking events observed during wound healing, and these responses are modulated significantly by several polypeptidic growth factors released into the wounded tissue after injury.[1,2] A key event observed in this phenomenon is the differentiation of specialized cells termed myofibroblasts.[1] It has been postulated that this cell phenotype is derived from resident tissue fibroblasts, which are activated by growth factors released by platelets and activated macrophages present in the wound.[2] Transforming growth factor β1 (TGF-β1) has been identified as an inducer of the myofibroblast marker smooth muscle actin (α-sma), an actin isoform that takes part in

cell-mediated granulation tissue contraction during tissue repair.[3-5]

Platelets contribute to hemostasis by preventing blood loss at sites of vascular injury, and they contain a large number of growth factors and cytokines that play a key role in inflammation and tissue repair.[1,6] This has led to the idea of using platelets as therapeutic tools to improve wound healing, particularly in patients or conditions in which tissue repair is impaired or delayed significantly.[6] Platelet-rich plasma (PRP) is a fraction of concentrated human platelets in a small volume of plasma.[6] It contains concentrated amounts of at least eight growth factors, including three isoforms of platelet-derived growth factor (PDGF-AA, -BB, and -AB), two forms of transforming growth factor (TGF-β1 and -β2), insulin-like growth factor-I, vascular endothelial growth factor, and epidermal growth factor (EGF).[7,8] Although initial results were promising regarding the use of PRP in oral wound repair, a subsequent study[9] demonstrated contradictory results. In fact, although some investigators reported significant improvements in wound healing, others failed to detect those effects.[10-14] Moreover, in vitro studies[15-21] analyzing the response of cells to PRP demonstrated variable results. These discrepancies may be explained by the still poorly characterized cellular responses to PRP fractions.

Several studies[7,19-23] have assessed the biologic response of oral cells to PRP. Most of them evaluated the capacity of these preparations to induce cell proliferation and bone differentiation. However, studies designed to analyze other cellular responses, such as modulation of cell–matrix interactions and migration, are limited. The main aim of the present study was to assess, in primary cultures of human gingival fibroblasts (GFs), the potential of PRP to modulate relevant mechanisms potentially involved in wound repair including cell adhesion and spreading, cell migration, and myofibroblastic differentiation.

MATERIALS AND METHODS

Cell Culture

Primary cultures of human GFs were established by the explant method.[24] Tissue explants were obtained from the retromolar tissue of three individuals (two females and one male; age range: 20 to 28 years) undergoing extraction of third molars at a private dental practice in Santiago, Chile between June and December 2006. Tissue samples were harvested with the informed consent of the patients, and the protocol for tissue obtainment was approved by the Ethical Committee of the Faculty of Dentistry, University of Chile. No history of inflammation of the retromolar tissue was reported. Patients reported no relevant preexisting medical or drug histories during the last 6 months. Cells were cultured in Dulbecco's modified Eagle's medium‡ (DMEM) containing 10% fetal bovine serum,§ 100 µg/ml penicillin,‖ 100 µg/ml streptomycin,¶ and 50 µg/ml gentamicin# at 37°C in a 5% CO_2 atmosphere. All experiments were performed using cells from these three donors and with cells expanded between passages four and 10.

Collection and Preparation of PRP

PRP was obtained from two healthy male volunteers using a commercially available system** following the method recommended by the manufacturer as described previously.[25] PRP was prepared from blood derived from these volunteers on two separate occasions, and all experiments were performed with these PRP samples. The average platelet count of donors was 230,000 (± 35,000) platelets/mm³. The average platelet count in the PRP preparation was 1,560,000 platelets/mm³ corresponding to 678.3% of the normal venous blood count. To induce platelet activation, PRP was added to serum-free DMEM at a 1:10 ratio, and bovine thrombin†† (10 units/ml) was added to the diluted PRP solution and incubated at 37°C for 30 minutes. Incubation with thrombin resulted in the formation of a gel that was centrifuged at 1,800 revolutions per minute for 7 minutes at 4°C. The supernatant was used to stimulate cells in all experiments. A fraction of platelet-poor plasma (PPP) was obtained according to the manufacturer's instructions and mixed with serum-free medium and thrombin as described for the PRP fractions. Activated PRP was diluted with DMEM to reach concentrations corresponding to 10%, 25%, and 50% of the PRP/DMEM mixture.

Cell Migration Assay

The ability of GFs to migrate was assayed using transwell chambers‡‡ with 8.0 µm–pore polycarbonate filters coated with 10 µg/ml reconstituted basement membrane§§ on the upper side of the filter.‖‖ Cells were resuspended in serum-free medium and seeded on the upper compartment of the chamber. Different PRP concentrations were added to the lower chamber. Migration was allowed to occur for 16 hours. Following the removal of the non-invading cells from the upper surface of the reconstituted basement membrane with a cotton swab, the invading cells were fixed and stained with 0.2% crystal violet. Cell migration was evaluated by counting five (×20) fields per chamber as described previously.[26] All assays were performed in quadruplicate in three separate sets of experiments.

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‡ Gibco BRL, Grand Island, NY.
§ Gibco BRL.
‖ Sigma, St. Louis, MO.
¶ Sigma.
# Sigma.
** Harvest Technologies, Munich, Germany.
†† Calbiochem, San Diego, CA.
‡‡ Matrigel, Costar, Cambridge, MA.
§§ BDBioscience USA, Bedford, MA.
‖‖ Collaborative Research, Bedford, MA.

Cell Adhesion

For cell adhesion, tissue culture 96-well plates were coated overnight at 4°C with 10 μg/ml fibronectin¶¶ in phosphate buffered saline (PBS), and non-specific binding sites were blocked by denatured bovine serum albumin## (BSA). GFs were stimulated previously with different concentrations of PRP for 16 hours. Cells were detached from the cell culture plates by a brief exposure to trypsin/EDTA and counted; 1×10^3 cells were seeded onto fibronectin-coated plates for 7 minutes. Cell adhesion was stopped by pouring off the medium, fixing cells with methanol for 2 minutes, and incubating with 0.2% crystal violet for 5 minutes. After removing excess dye, cells were solubilized in 0.1 M NaH_2PO_4 in 50% methanol for 10 minutes at room temperature. The absorbance at 570 nm was analyzed on a microplate reader*** as described previously.[26] All assays were performed in quadruplicate in three separate sets of experiments.

Evaluation of Cell Spreading

After stimulation with PPP or PRP, cells were detached from the substratum by a brief exposure to trypsin/EDTA and plated onto fibronectin-coated plates (10 μg/ml) for 20 minutes. Cell spreading was stopped by pouring off the medium, washing adhered cells once with PBS, fixing them with methanol for 2 minutes, and incubating with 0.2% crystal violet for 5 minutes. After several washes, spread cells were scored from at least four fields of different regions of the dish. Images were captured with a camera††† through an inverted microscope.‡‡‡ These assays were performed in three separate sets of experiments.

Actin Remodeling and Focal Adhesion Distribution

As described for the cell adhesion assay, cells were stimulated previously with PPP and PRP. After this, cells were plated on fibronectin-coated coverslips, washed with PBS and fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.25% Triton X-100 for 4 minutes, and incubated with PBS containing 5% BSA for 30 minutes at 37°C. Primary antibodies diluted in PBS containing 1% BSA§§§ were used in a dilution of 1:100 for anti-paxillin.‖‖‖ The antigen-antibody complex was washed and incubated with fluorescein 5-isothiocynate conjugated anti-rabbit immunoglobulin G.¶¶¶ F-actin was stained with phalloidin-rhodamine.### Fluorescence images were examined with a microscope**** and photographed using a 63× immersion objective and a camera.†††† These experiments were done on three separate occasions.

Western Blotting to Detect α-sma and β-Actin

Cells were lysed with a buffer containing 50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM ethylene glycol tetra acetic acid, 1% Triton X-100, 10% glyc-erol, 2 mM phenylmethylsulphonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 1 mM orthovanadate at 4°C. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride transfer membrane.‡‡‡‡ Membranes were exposed to primary antibodies (α-sma§§§§ or β-actin‖‖‖‖) and secondary antibodies coupled to horseradish peroxidase and developed.¶¶¶¶ Smooth muscle actin production, stimulated by PRP, was assessed in three independent experiments.

Statistical Analysis

The statistical significance for each data set was tested using the Student t and Tukey tests with the significance level set at $P < 0.05$.

RESULTS

Effect of PRP on Cell Adhesion

The ability of cells to adhere to a fibronectin matrix was evaluated in GFs stimulated with PPP and different concentrations of PRP (10%, 25%, 50%, and 100%). As shown in Figure 1, after 7 minutes of cell attachment, cell adhesion was stimulated in cells pretreated with PPP and all of the PRP preparations in a dose-dependent manner. Statistically significant increases in cell adhesion were only observed in cells stimulated with 25%, 50%, and 100% PRP compared to control (non-stimulated) cells ($P < 0.01$).

PRP Stimulates Cell Spreading and Formation of Focal Adhesions

To further characterize the morphological and molecular basis of the enhancement in cell adhesion stimulated by PPP and PRP fractions, we evaluated the ability of fibroblasts to spread over a fibronectin matrix, to develop focal adhesions, and to polymerize F-actin fibers.

After 20 minutes of cell adhesion, cells stimulated with 25% PRP spread well over a fibronectin matrix compared to control or PPP-stimulated cells (Fig. 2A). Quantification of the number of spread cells demonstrated that PRP significantly stimulated this response compared to PPP or no stimulation ($P < 0.01$) (Fig. 2B).
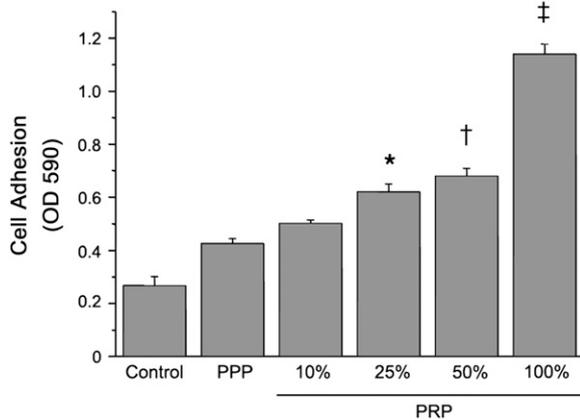
Figure 1.
Effect of PRP fractions on fibroblast adhesion. Human GFs were stimulated with 100% PPP and different PRP concentrations (10%, 25%, 50%, and 100%) for 16 hours. Cells were detached from the cell culture plates, counted (1×10^3), and plated onto 10 μg/ml-fibronectin–coated dishes. After 7 minutes, unattached cells were removed, and adhered cells were fixed with methanol and stained with 0.2% crystal violet. Cells were solubilized, and the released crystal violet was read in a microplate reader. The y axis indicates mean absorbance (optical density [OD]) at 590 nm. Bars indicate standard error. All assays were performed in quadruplicate. *Statistically significant difference between control and 25% PRP; †statistically significant difference between control, PPP, and 50% PRP; ‡statistically significant difference between 100% PRP and all of the other conditions tested.

Figure 2.
*Effect of PRP on cell spreading. **A)** Human GFs were stimulated with 25% PPP and 25% PRP for 12 hours. Cells were detached, counted (1×10^3), and plated onto 10 μg/ml fibronectin–coated dishes. After 20 minutes, attached cells were stained with crystal violet, and images were recorded for each experimental condition. Bar = 15 μm. **B)** Spreading of cells was calculated by counting at least four fields from different regions of the culture dish. Data are the mean value of the number of spread cells detected in three separate experiments. Bars indicate standard error. *Statistically significant differences between control and PRP-stimulated cells (P <0.01).*

We also evaluated the distribution of paxillin-enriched focal adhesions and the formation of F-actin fibers through immunofluorescence. After 1 hour of cell adhesion, unstimulated cells showed a rounded morphology with no signs of spreading (Fig. 3). However, at equivalent time-points, PPP- and PRP-stimulated cells showed an elongated morphology, more actin stress fibers, and a higher number of paxillin-enriched focal adhesions (Fig. 3). Under the stimulus of PRP fractions, these results suggest that, GFs developed a more active cytoskeleton arrangement that predisposed cells to adhere.

PRP Promotes Cell Migration
To analyze whether PRP also constitutes a chemotactic stimulus to GFs, we evaluated the ability of fibroblasts to migrate toward a PRP gradient by a cell migration assay using a bicameral cell culture system. As shown in Figure 4, in every condition tested (PPP and 25% and 100% PRP), cell migration was stimulated at statistically significant levels compared to untreated cells (*P* <0.01).

PRP Stimulates α-sma Production
Finally, we assessed whether PRP has a role in myofibroblastic differentiation. To this end, we measured the production of the actin isoform α-sma, a long-term marker of this phenomenon, by exposing GFs to 10%
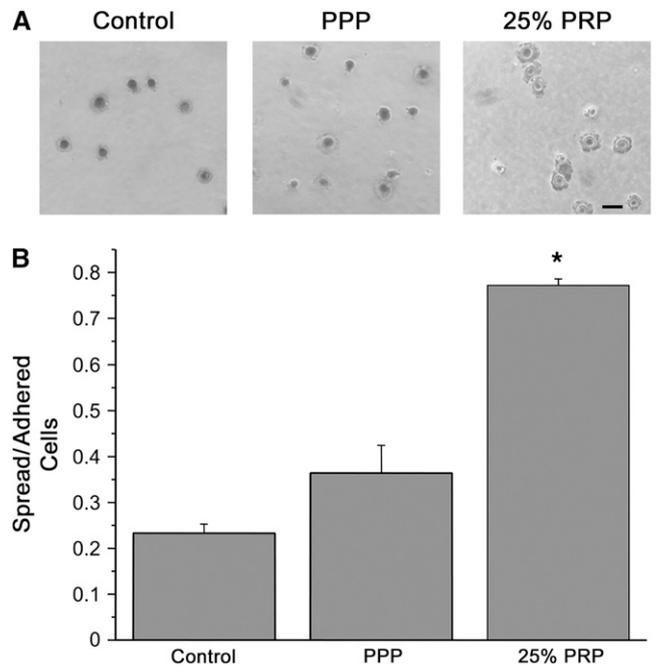
and 25% PRP for 72 hours. Figure 5 shows that α-sma production, assessed through Western blotting, was stimulated strongly by PRP in a dose-dependent manner.

DISCUSSION
Although PRP and platelet concentrates have been proposed as a therapeutic tool to improve tissue repair, the underlying cellular-level mechanisms remain poorly characterized. In the present study, we used primary cultures of human GFs to evaluate whether PRP fractions may modulate several cell responses that have been involved in wound healing. Our results showed that PRP may stimulate adhesion to fibronectin, cell spreading, focal adhesion formation, cell migration, and differentiation into the myofibroblastic phenotype.

During wound healing, cells must attach to a newly formed matrix structure to migrate.[27] Soon after wounding, a provisional matrix of fibronectin, fibrinogen, fibrin, and vitronectin is formed in the wound area.[28] Activated fibroblasts must move from surrounding collagenous connective tissues into a fibrin/fibronectin-filled wound and subsequently synthesize
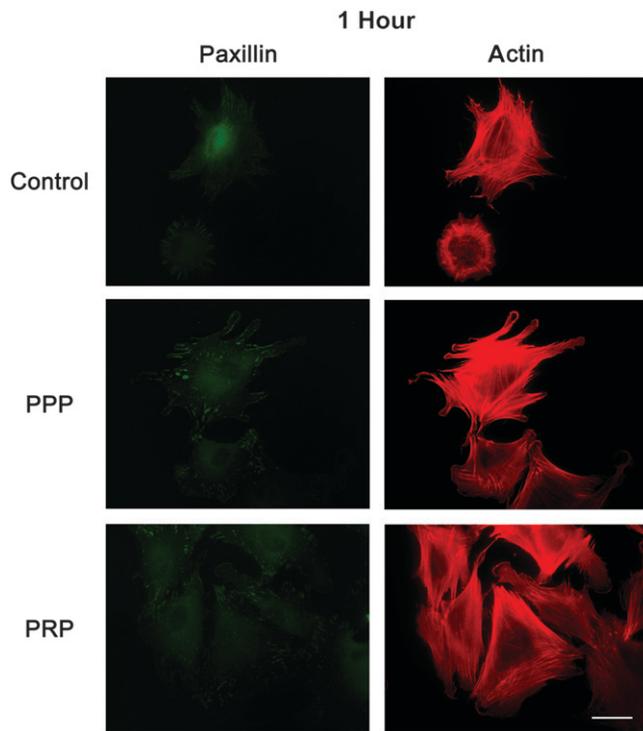
1 Hour

Paxillin | Actin

Control

PPP

PRP

Figure 3.
Effect of PRP on focal adhesion formation and actin cytoskeleton arrangement. Human GFs were stimulated with 25% PPP and 25% PRP for 12 hours. Cells were detached from the cell culture plates and plated onto 10 μg/ml fibronectin–coated dishes. After 1 hour, cells were fixed, and paxillin and F-actin were detected through immunofluorescence. Bar = 15 μm.

Figure 4.
*PRP stimulates fibroblast cell migration. **A)** Quantification of migration assay is expressed as the average number of migrating cells detected on the lower side of the filter. Bars indicate standard error. All assays were performed in quadruplicate. *Statistically significant difference between control and PPP; †statistically significant difference between control, PPP and 25% PRP; ‡statistically significant difference between 100% PRP and all of the other conditions tested. **B)** Human GFs were placed in the upper compartment of a transwell chamber with 8.0 μm-pore polycarbonate filters coated with 10 μg/ml reconstituted basement membrane. In the lower chamber, different PRP concentrations were added. Migration was allowed to occur for 16 hours. Following the removal of the non-invading cells from the upper surface of the reconstituted basement membrane, the invading cells were fixed, stained with 0.2% crystal violet, and cell migration was evaluated by counting five (×20) fields per chamber.*

a new collagenous matrix. Therefore, cell adhesion and migration, modulated by growth factors, are essential for the healing process to progress.[29] Integrins are the molecules primarily responsible for cell adherence to extracellular matrix, and expression of these molecular components is modulated actively by different growth factors.[30] TGF-β1 upregulates β1- and β3-integrin receptors.[31,32] Adhesion of GFs to fibronectin is also stimulated by physiological concentrations of TGF-β1.[33] EGF stimulates β1-integrin expression in Swiss 3T3 cells, and PDGF-BB induces α5-integrin mRNA levels in skin fibroblasts.[29,34] In the present study, fibroblast adhesion to fibronectin matrices was stimulated significantly by PRP in a dose-dependent manner. Our observations also revealed that PRP stimulated cell spreading over fibronectin and the development of actin stress fibers and paxillin-enriched focal adhesions. These results suggest that molecular components present in PRP preparations may stimulate the ability of cells to adhere and spread over extracellular molecules, such as fibronectin.

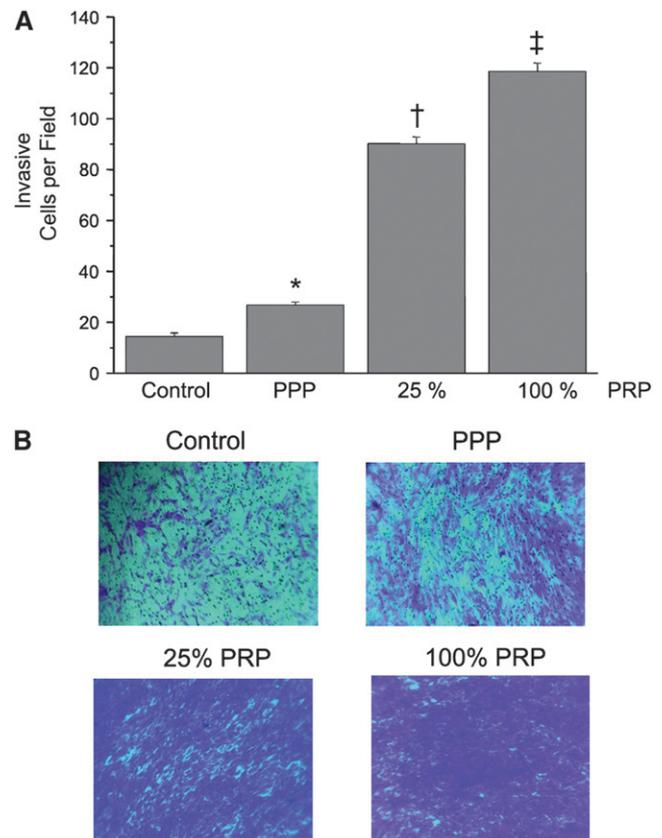After tissue injury, fibroblasts must migrate from the connective tissue near the wounded area and traverse through a provisional matrix largely composed of cross-linked fibrin and fibronectin.[1] This matrix also serves as a structural support for fibroblasts that migrate into the lesion.[1] Growth factors released after platelet activation and, in particular, PDGF isoforms are able to stimulate chemotaxis of fibroblasts into the wounded tissue.[35] In the present study, all PRP concentrations stimulated a dose-dependent increase in fibroblast chemotactic migration. A similar observation was described in the human osteoblastic cell line SaOS-2 and in bone marrow–derived mesenchymal progenitor cells in which PRP was able to stimulate cell migration.[17,36] However, to the best of our knowledge, this is the first study to show that GFs
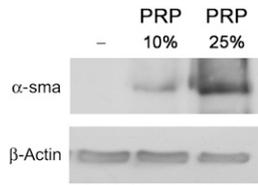
Figure 5.
PRP stimulates α-sma production in GFs. Human GFs were cultured in 35-mm culture dishes and stimulated with different PRP concentrations for 72 hours. After this, α-sma production was evaluated through Western blotting. β-actin was used as the loading control. This assay is representative of three independent experiments. − = non-stimulated cells.

are able to migrate and invade a reconstituted basement membrane in response to a chemotactic stimulus derived from PRP.

Myofibroblastic differentiation constitutes an essential step in wound healing, and this cell phenotype is responsible for cell-mediated matrix contraction.[4,5] Through contraction of their actin cytoskeleton, wound myofibroblasts are able to reduce the initial size of the wound and, thereby, contribute to tissue repair.[4,5] These cells are also characterized by the expression of the actin isoform α-sma.[5] In vivo, wound myofibroblasts are believed to arise from α-sma–negative fibroblasts, and α-sma expression has been associated with an increased ability of cells to contract their cytoskeleton.[3,4] TGF-β1, a growth factor detected at high concentrations in PRP fractions, has been identified as an important inducer of the myofibroblastic phenotype.[5,7,8] Although α-sma plays a significant role during granulation tissue contraction,[3,4] to the best of our knowledge, no previous study evaluated whether PRP modulates the expression of this phenotypic cell marker. Our findings showed that PRP fractions potently stimulate α-sma production in human GFs in a dose-dependent manner. This finding suggests that concentrated levels of growth factors present in PRP fractions may stimulate myofibroblastic differentiation in GFs.

CONCLUSIONS

PRP fractions promote important cell responses involved in tissue repair including fibroblast migration, cell adhesion, and α-sma production. These findings are interesting in view of the potential beneficial effects of PRP in gingival repair.

ACKNOWLEDGMENTS

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