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ORIGINAL ARTICLE

Platelet-rich fibrin modulates cell proliferation of human periodontally related cells *in vitro*

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Background/purpose: Platelet-rich fibrin (PRF) is a second-generation platelet concentrate which allows one to obtain fibrin membranes enriched with platelets and growth factors, after an anticoagulant-free blood harvest. However, limited information is currently available concerning the biologic effects of PRF on periodontally related cells. To provide clear evidence for the clinical use of PRF, we investigated the biologic effects of PRF on human gingival fibroblasts (GFs), periodontal ligament (PDL) cells, oral epithelial cells, and osteoblasts.
Materials and methods: Blood collection was carried out on 10 healthy volunteers. PRF was obtained by centrifugation at 3000 rpm for 12 minutes with a PC-02 table centrifuge. Primary cultured human GFs and PDL cells, the GNM oral epithelial cell line, and the U2OS osteoblast cell line were used to evaluate cell viability and proliferation resulting from PRF according to trypan blue and tetrazolium bromide reduction assays.
Results: PRF did not interfere with cell viability of periodontally related cells ($P>0.05$). PRF stimulated cell proliferation of osteoblasts (135% of the control), PDL cells (130% of the control), and GFs (120% of the control) during a 3-day culture period (all $P<0.05$). However, PRF suppressed oral epithelial cell growth to as low as 80% of the control ($P<0.05$). In addition, GFs, PDL cells, and osteoblasts were observed to attach at the margins of PRF by phase-contrast microscopy.
Conclusion: Our results suggest that PRF modulates cell proliferation in a cell type-specific manner. These cell type-specific actions may be beneficial for periodontal regeneration.

Introduction

Periodontal wound healing after surgery requires a series of cell–cell interactions between epithelial cells, gingival fibroblasts (GFs), periodontal ligament (PDL) cells, and osteoblasts, whereas disruption of the vasculature leads to fibrin formation, platelet aggregation, and release of several growth factors into tissues from platelets.[1] These processes involve molecular signals which are primarily mediated by cytokines and growth factors. Platelets contain various growth factors and cytokines that play key

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roles in inflammation and wound repair.[2] Platelets also secrete fibrin, fibronectin, and vitronectin, which act as a matrix for connective tissue and as adhesion molecules for more efficient cell migration.[3] This has led to the idea of using platelets as therapeutic tools to improve tissue repair particularly in periodontal wound healing.

Platelet-rich fibrin (PRF) described by Choukroun et al.[4] is a second-generation platelet concentrate which allows one to obtain fibrin membranes enriched with platelets and growth factors, after starting from an anticoagulant-free blood harvest.[5,6] PRF looks like a fibrin network and leads to more efficient cell migration and proliferation and thus cicatrization. PRF was initially used in implant surgery to improve bone healing.[4] Despite a lack of scientifically proven clinical benefits, the homogeneous fibrin network that is obtained is considered by the promoters of this technique to be a healing biomaterial and is commonly used in implant and plastic periodontal surgery procedures to enhance bone regeneration and soft-tissue wound healing.[7,8] Compared with other autologous platelet concentrates, there are few references in the literature about the biologic properties of PRF.

PRF contains platelets, growth factors and cytokines that may enhance the healing potential of both bone and soft tissues.[1,2] However, the literature mostly contains studies of the experimental use of PRF in animals and humans, and only a few *in vitro* studies on the effects of PRF on cell proliferation and functions have been carried out. The aim of this study was to assess the effects of the PRF on periodontally related cells. We investigated the biologic effects of PRF on the proliferation of the GMN human oral epithelial cell line, the U2OS human osteoblast cell line, primary human GFs, and PDL cells.

Materials and methods

PRF preparation

After receiving approval of the institutional review board at Chung Shan Medical University Hospital, blood collection was carried out on 10 healthy nonsmoking volunteers. Blood samples were treated according to the PRF protocol with a PC-02 table centrifuge and collection kits provided by Process (Nice, France).[3–5] Briefly, samples were taken without an anticoagulant in 10-mL glass-coated plastic tubes (Becton Dickinson Vacutainer, Becton, Dickinson & Co., Franklin Lakes, NJ, USA) and immediately centrifuged at 3000 rpm for 12 minutes. A fibrin clot formed in the middle part of the tube (Fig. 1A), while the upper part contained acellular plasma, and the bottom part contained red corpuscles (Fig. 1A). The fibrin clot was easily separated from the lower part of the centrifuged blood. The PRF clot was gently pressed into a membrane with sterile dry gauze (Fig. 1B). PRF membranes were minced at 0.5×0.5 cm for the following experiments.

Cell cultures

Human PDL cells[9,10] and GFs[11,12] were cultured using an explant technique as described previously. Human PDL cells were cultured from the roots of extracted

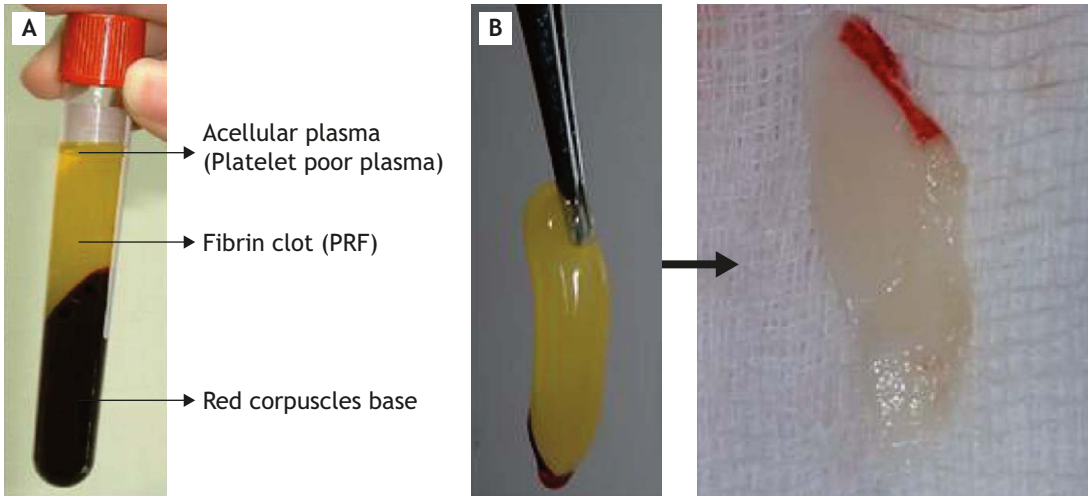

Fig. 1 (A) Platelet-rich fibrin (PRF) formed in the middle part of the tube. The upper part contained acellular plasma, and the bottom part contained red corpuscles. (B) The fibrin clot was easily separated from the lower part of the centrifuged blood. The platelet-rich fibrin clot was gently pressed between two layers of sterile dry gauze to form a membrane.

third molars. After extraction, teeth were rinsed with Hanks' buffered saline solution and then placed in 60-mm Petri dishes containing Dulbecco's modified Eagle's medium (DMEM) and 100 units of penicillin and 100 μg of streptomycin per millimeter. To avoid contamination from the gingiva, the PDL was carefully removed from the middle third of the root with a scalpel. Clinically healthy gingival connective tissues from a third molar extraction were used to culture GFs. The fragments from the PDL and gingiva were grown in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell cultures between the third and eighth passages were used.

U2OS cells (American Tissue Type Collection HTB 96), derived from a human osteogenic sarcoma, were cultured in DMEM supplemented with 10% FCS, streptomycin at 100 μg/mL, and penicillin at 100 mg/mL.[13] The GNM oral epithelial cell line, derived from a patient with T2N2aM0 gingival carcinoma and metastasis to the cervical lymph node,[14] was grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FCS and antibiotics. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 minutes, and aliquots of separated cells were subcultured. Cells were subcultured at a 1:4 split ratio every third day.

Cell viability

Each PRF membrane was covered with a 5-mL suspension of cells at a concentration of 5×10^4 cells/mL in 35-mm culture dishes. After a 3-day culture period, the medium was removed, and 0.5 mL of 0.25% trypsin in phosphate-buffered saline (PBS) was added to each culture dish to detach the cells. One milliliter of medium was added to 0.5 mL of this cell suspension. Then 0.5 mL of calcium- and magnesium-free PBS containing 0.25% trypan blue (wt/vol) was added to 0.5 mL of the cell suspension to stain nonviable cells. Fifteen microliters of the cell suspension was dropped into a hemocytometer chamber (Cambridge Instruments, Buffalo, NY, USA), and cell numbers were counted under a phase-contrast microscope.[15] Cell viability is represented as number of viable cells as a percentage of total cells.

Cell proliferation assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was developed to monitor cell proliferation *in vitro*.[16] To elucidate PRF's role in cell proliferation, cells were seeded in 96-well plates at an initial density of 2×10^4 cells per well in DMEM containing PRF for 3 days. In the final 4 hours, 50 μL of the MTT solution was added to each well. Only the mitochondria of viable cells can reduce MTT to formazan. The produced insoluble formazan was dissolved with 150 μL of DMSO to each well. Reduced MTT was then measured spectrophotometrically in a dual-beam microtiter plate reader at 570 nm with 650 nm as a reference. Optical density values of the experimental groups were divided by the control value and are expressed as a percentage of the control.

Statistical analysis

Triplicate experiments were performed throughout this study. All assays were repeated three times to ensure reproducibility. The significance of the results obtained from the control and treated groups were statistically analyzed by Student's *t* test.

Results

The results of cell viability by trypan blue dye are shown in Fig. 2. PRF exhibited no cytotoxic effects to the four types of periodontally related cells ($P>0.05$). Each type of cell maintained its original morphology. Cells from gingiva (Fig. 3A) and the PDL (Fig. 3B) on the flat surface of the culture dishes exhibited a spindle-shaped morphology. Cells of the GMN oral epithelial cell line (Fig. 3C) and U2OS osteoblast cell line (Fig. 3D) have a cuboid/flat appearance. Moreover, GFs, PDL cells, and osteoblasts were also found to attach at the margin of PRF under observation by phase-contrast microscopy.

Figure 4 shows the effects of PRF on periodontally related cells. The cell density and number

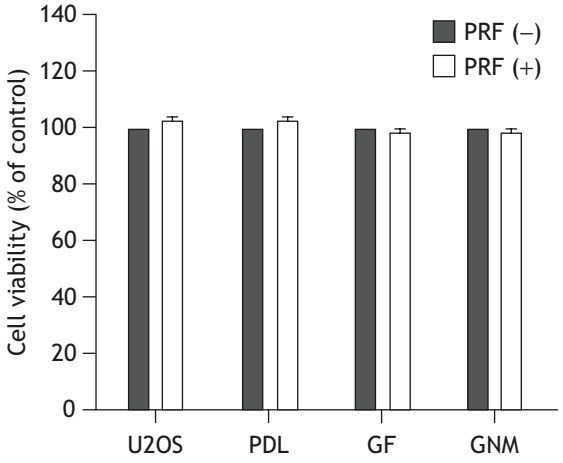

Fig. 2 No differences in cell viability between controls and platelet-rich fibrin (PRF) groups with periodontally related cells ($P>0.05$). PDL=periodontal ligament cells; GF=gingival fibroblasts.

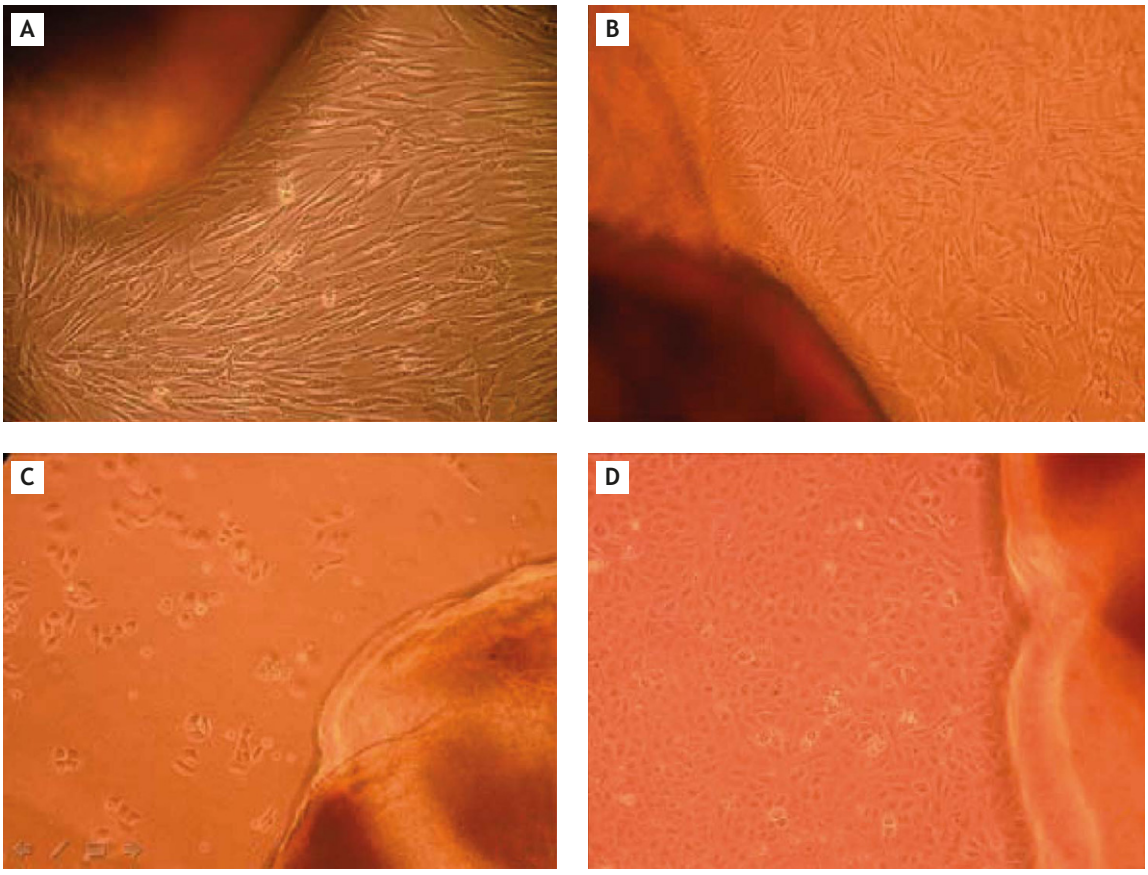

Fig. 3 Periodontally related cells maintained their original morphology when cultured with platelet-rich fibrin membranes. (A) Gingival fibroblasts; (B) periodontal ligament cells; (C) epithelial cells; and (D) osteoblasts.

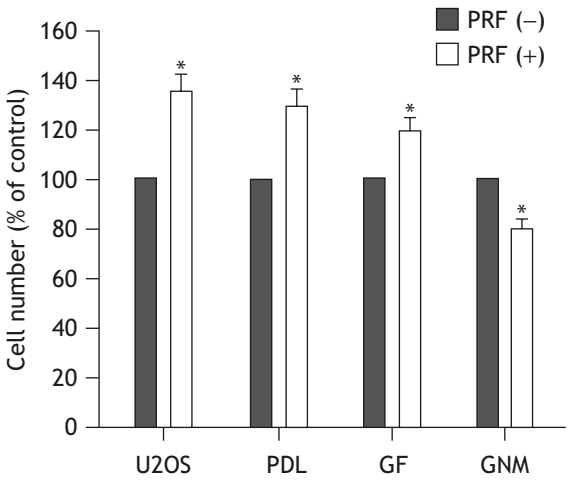

Fig. 4 Effects of platelet-rich fibrin (PRF) on periodontally related cells. Cells were exposed to PRF for 3 days. Viable cell numbers were measured with an MTT assay. The percentage of absorbance of each cell with PRF compared with that of the control was calculated. Each point and bar represent the mean±standard deviation. *Significant difference from the control value at $P<0.05$. PDL=periodontal ligament cells; GF=gingival fibroblasts.

gradually increased during the 3-day incubation period. PRF was found to increase PDL, GF and osteoblast proliferation, and cell numbers increased about 1.2-, 1.3- and 1.35-fold, respectively ($P<0.05$). However, PRF was found to reduce the epithelial cell number by about 20% compared with the untreated control ($P<0.05$).

Discussion

The simple and open-access technique of PRF was first developed in France by Choukroun et al.,[4] and PRF is produced in a totally natural manner, without using an anticoagulant during blood harvesting or bovine thrombin or calcium chloride for platelet activation and fibrin polymerization. The protocol is very simple and cheap. Venous blood is collected in dry 10-mL glass tubes, and centrifuged at about 400*g* for 12 minutes.[3–6] After centrifugation, three layers are formed in the tube: a base of red blood cells at the bottom, acellular plasma on the top (supernatant), and a clot of PRF between them.

PRF presents a complex tridimensional architecture which truly makes it a platelet- and leukocyte-rich fibrin biomaterial.[5,6] When delicately pressed between two layers of gauze, the PRF clot becomes a strong membrane with high potential, both for clinical applications[7,8,17,18] and tissue engineering.[19]

In this study, PRF exhibited no cytotoxicity to periodontally related cells. Our results are in agreement with Dohan et al.,[20] who reported that PRF exhibited no cytotoxicity toward preadipocytes, keratinocytes, osteoblasts, or GFs. Thus, the biocompatibility of PRF is not cell type-specific. Taken together, PRF acts as a biomaterial to periodontally related cells.

To the best of our knowledge, we first found that PRF stimulated osteoblast, GF, and PDL cell proliferation as a mitogen. The mechanism responsible for the cell proliferation by PRF might be explained as follows. Many growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β, are released from PRF.[5,6,21] Recently, Dohan et al.[21] demonstrated that the PRF membrane has a very significant slow sustained release of key growth factors for at least 1 week, which means that the membrane stimulates its environment for a significant time during remodeling. The properties of this natural fibrin biomaterial thus offer great potential during wound healing.

Interestingly, PRF was found to inhibit epithelial cell proliferation. These biologic actions seem very similar to those of previous reports. PDGF and TGF-β stimulate mitogenic activity in osteoblasts,[22,23] GFs,[24] and PDL cells,[23,24] but TGF-β acts as a growth inhibitor for epithelial cells.[25,26] Taken together, with our data on growth factor levels, these findings suggest that PRF possibly modulates cell proliferation by PDGF- and TGF-β-related mechanisms.

In this study, PRF exhibited no cytotoxicity to epithelial cells according to a trypan blue assay. The MTT assay is also a kind of cytotoxicity assay. However, epithelial cells seemed to be negatively influenced by PRF according to the MTT assay. The reasons are not conflicting and may be explained as follows. The trypan blue assay exhibits the percentage of cell viability. The MTT assay is a colorimetric method for quantifying viable cell numbers. The methyl-tetrazolium ring is cleared by mitochondrial dehydrogenases in viable cells to formazan, which has a blue color and can be measured with a spectrophotometer.[27] Dead cells are unable to produce the colored formazan product; this assay can distinguish live from dead cells. In addition, epithelial cells presented a typical cuboid/flat appearance under observations by phase-contrast microscopy in the present study. Taken together, PRF did not interfere with epithelial cell viability, but it retarded the proliferation of epithelial cells.

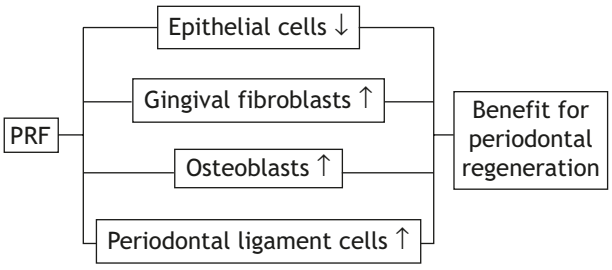

Fig. 5 Platelet-rich fibrin (PRF) modulates cell proliferation in a cell type-specific manner and may be beneficial to periodontal regeneration.

As far as we know, this is the first attempt to evaluate the role of PRF in human periodontally related cells *in vitro*. We demonstrated that PRF exhibits non-cytotoxic effects toward periodontally related cells. PRF can stimulate osteoblast, GF and PDL cell growth and retard epithelial cell proliferation. PRF may modulate cell proliferation in a cell type-specific manner (Fig. 5). The ability of PRF to suppress epithelial cell proliferation seems beneficial for periodontal regeneration. The retardation of the down-growth of junctional epithelium to the root surfaces in the regeneration procedure might avoid interference by the epithelium with the formation of new attachment on root surfaces.

It is our opinion that *in vitro* experiments are very helpful for assaying the biologic effects of PRF on periodontally related cells, but they may be limited in their ability to simulate clinical conditions. It may be unrealistic to translate *in vitro* findings to *in vitro* situations. These *in vitro* observations are very likely to be extrapolated to animal studies such as periodontitis models or critical-sized bony defect models to clarify the potential benefits of using PRF in periodontal regeneration.

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