Plasma Rich in Growth Factors Stimulates Proliferation and Mineralization in Mesenchymal Stem Cells from Human Bone Marrow

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Abstract
Plasma rich in growth factors (PRGF) can be rapidly obtained from patient blood. PRGF has been used in regenerative therapy for soft tissue and bone formation, and represents a new and potentially useful adjunct in oral and maxillofacial bone reconstructive surgery. However, few studies have investigated the biological functions of PRGF in bone regeneration. Human mesenchymal stem cells (hMSC) isolated from human bone marrow have the capacity to commit to multiple cell types such as the osteoblastic lineage, and have been widely applied in tissue engineering studies in recent years. The aim of this study is to evaluate the effects of PRGF on osteogenic differentiation in hMSC. PRGF was prepared from whole blood centrifuged at 460 × g for 8 min. PRGF F2 was incubated with 10% calcium chloride solution at 37°C for 1 h to trigger platelet activation and growth factor release. Activated PRGF Fraction 2 was centrifuged at 3,000 × g for 15 min, and the supernatant was then isolated. We examined the effects of soluble factors in PRGF on proliferation and mineralization in hMSC culture supplemented with PRGF. The proliferation of hMSC was increased in osteogenic induction medium (OIM) supplemented with PRGF. Alkaline phosphatase activity increased in hMSC by PRGF. Staining for alizarin red S and von Kossa was strong in hMSC supplemented with PRGF. These results suggest that PRGF is able to promote bone generation.

Keywords:
plasma rich in growth factors, human mesenchymal stem cells, bone generation

Introduction
Platelet concentration products, which have high concentrations of platelets containing various growth factors, are autologous constituents of inductive factors obtained from blood (1). Platelet-rich plasma (PRP) has been used as a bone formation reagent (2, 3), although PRP formulations have different biological activities. Because PRP is isolated from whole blood depending on the various protocols, its biological activities can vary (4, 5). The preparation of plasma rich in growth factors (PRGF) relies on a method for concentrating platelets (6, 7) and is advantageous as it requires only one centrifugation step and is leukocyte-free, thus avoiding high levels of pro-inflammatory cytokines (6).

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PRGF allows delivery to the site of injury of a cocktail of proteins and growth factors that promotes wound healing and regeneration of tissue and bone (8, 9). Numerous studies have described the benefits of PRGF in vivo. However, most studies on the use of PRGF have only described the final outcome at the tissue level and have not investigated the mechanisms for tissue and bone formation in PRGF. Therefore, investigation of the role of PRGF in bone and/or tissue regeneration should involve a study of the biological properties and molecular functions using a cell culture system.

Human mesenchymal stem cells (hMSC) from bone marrow have the following characteristics: 1. adherent to plastic in culture; 2. presence of CD105, CD73 and CD90, but absence of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR molecules; and 3. capacity to differentiate into...
osteoblasts, chondrocytes and adipocytes (10–13). hMSC in
the presence of dexamethason, ascorbic acid, and β-
glycerophosphate enter a differentiation pathway that leads
to mineralized deposition of the necessary components to
form bone matrix (10, 14). Thus, hMSC have been used as
ideal seed cells in tissue engineering in recent years and
have been widely applied in tissue engineering studies.

In this study, we examined the effects of soluble factors in
PRGF on proliferation and alkaline phosphatase activity in
hMSC treated with PRGF supernatant. We evaluated the
efficacy of PRGF as a substitute for engineered mineraliza-
tion tissue.

Materials and Methods

Preparation of different blood products

For blood product preparation, whole blood from four
young healthy donors was collected from the external
jugular vein after informed consent was obtained. Whole
blood was immediately placed into 5-ml sterile extraction
tubes containing 0.5 ml of 3.8% sodium citrate as an
anticoagulant. PRGF prepared from whole blood was
centrifuged in accordance with Anitua’s protocol (6–8).
Briefly, human whole blood (10 ml) was centrifuged at 460 ×
g for 8 min. The plasma fraction (1 ml over theuffy coat)
was collected as Fraction 2 (F2), whereas Fraction 1 (F1)
was the layer above F2. PRGF F2 was incubated with 0.2%
calcium chloride solution at 37°C for 1 h to trigger platelet
activation and growth factor release. Activated PRGF F2
was centrifuged at 3,000 × g for 15 min, and then the
supernatant was isolated (Fig. 1). PRGF F2 supernatant was
stored at −80°C until use.

PRGF extraction was performed according to the
guidelines established by the Ethics Committee of Nihon
University School of Dentistry at Matsudo (Recognition
number: EC14-13–029-1).

Cell culture

hMSC (Pioetics™, human mesenchymal stem cells, 2nd
doubling passage) harvested and cultured from normal
human bone marrow were purchased from Lonza (Basel,
Switzerland). hMSC were cultured in mesenchymal stem
cell growth medium (GM) consisting of mesenchymal stem
cell basal medium and SingleQuots growth supplements that
comprised fetal bovine serum (FBS), L-glutamine and
penicillin/streptomycin (Lonza) at 37°C in the presence of
95% air and 5% CO2. These cells were positive for CD44,
CD73, CD90, CD105, and CD166, and negative for CD19,
CD14, CD34, CD45, and HLA-DR. hMSC from the 4th to
the 5th passage were used for the following experiments.

For induction of osteogenic differentiation, hMSC were
seeded at 3.1 × 10³ cells/cm² in GM. After 24 h, media were
changed to MSC osteogenic induction medium (OIM) con-
sisting of osteogenic basal medium, osteogenic SingleQuots
that contained L-glutamine, penicillin/streptomycin, dexam-
ethasone, ascorbate, and β-glycerophosphate (Lonza), and
supplemented with or without 10% PRGF F2 supernatant.
The medium was replaced every 3 days.

Experiments using hDFCs were performed in accordance
with the guidelines established by the Ethics Committee of
Nihon University School of Dentistry at Matsudo
(Recognition number: 10-036).

**Cell numbers**

hMSCs were seeded in a 24-well plate at a density of $1 \times 10^4$ cells/well in GM. After 24 h, medium was replaced with GM, OIM-FBS, or OIM-PRGF. Cell numbers were counted with a Z1 Counter Particle Counter (Beckman Coulter, Miami, FL).

**Alkaline phosphatase assay**

Alkaline phosphatase (ALP) activity was assayed using a StemTAGTM Alkaline Phosphatase Activity Assay kit (Cell Biolabs, San Diego, CA). Briefly, cells were lysed in 0.5 ml of cell lysis buffer per 35-mm dish. Cell lysates were incubated with the substrate solution at 37°C for 30 min, and the reaction was stopped by addition of stop solution. The amount of p-nitrophenol released during the enzymatic reaction was determined by measurement of the absorbance at 405 nm. ALP activity was determined from a p-nitrophenol standard curve; 1 U indicates the release of 1 mmol of p-nitrophenol per min at 37°C. ALP activity is expressed in terms of milliunits per well (munits/well).

**Alizarin red S and von Kossa staining**

Cell monolayers were washed twice with phosphate-buffered saline (PBS), fixed with 10% formalin solution for 30 min, and then washed twice with pure water. For alizarin red S staining, cells were placed in 1% alizarin red S (Kanto Chemical, Tokyo, Japan) solution for 10 min, washed three times with pure water, and then air-dried.

We performed von Kossa staining according to the method of Jaiswal et al. (15). Briefly, cell monolayers were placed in a 5% silver nitrate (Kanto Chemical) solution and kept in the dark for 10 min. After washed with pure water, cell monolayers were exposed to bright light for 15 min.

**Statistical analysis**

Data are expressed as means±SD of three samples. Significance of differences between culture samples was determined using Student’s t-test and a value of $p<0.05$ was considered to be statistically significant.

**Results**

**Cell proliferation**

We measured the cell number of hMSC cultured with GM, OIM, and OIM supplemented with 10% PRGF F2 supernatant (OIM-PRGF). Cell numbers in each hMSC culture increased in a time-dependent manner. Although the cell proliferation of hMSC decreased in OIM culture compared to GM culture, proliferation increased in OIM-PRGF (Fig.2). On culture day 3, cell number was significantly higher in hMSC cultured with OIM-PRGF when compared with OIM (Fig.2).
Alkaline phosphatase activity

We next measured ALP activity in MSC cultured with GM, OIM, and OIM-PRGF. ALP activity in hMSC cultured with OIM-PRGF was significantly higher in cells cultured with OIM on days 7 and 10 (Fig.3). ALP activity was significantly higher in hMSC culture using OIM and OIM-PRGF when compared with GM on days 7 and 10 (Fig.3). Cell number was not different significantly among cultures of GM, OIM and OIM-PRGF on days 7 and 10 (data not shown).

Matrix mineralization

Mineralization of hMSC was examined by staining with alizarin red S and von Kossa. The hMSC cultured with OIM-PRGF stained strongly with alizarin red S on day 10, whereas the OIM culture stained weakly (Fig.4). hMSC were subjected to von Kossa staining on day 24 of culture. OIM-PRGF culture stained strongly with von Kossa compared to OIM culture (Fig.4). In contrast, no staining of hMSC that had been cultured in GM was observed.

Discussion

Platelet concentration products such as PRGF are frequently applied to bony defects. This product is readily and rapidly obtained from patient blood, and because it is a 100% autologous product, there are no immunological concerns regarding their use. PRGF was recently used following tooth extraction and around dental implants to improve healing of the peri-implant bone and was reported to accelerate bone regeneration (6, 7, 9, 16, 17). It has been reported that PRGFs exert positive effects on periodontal ligament fibroblasts and alveolar bone osteoblasts, which could be positive for periodontal regeneration (6, 7). On the other hand, adult tissue stem cells contribute to the functional maintenance of organs and to cell renewal, tissue remodeling and repair (18, 19). hMSC from bone marrow are well-established stem cells that are currently used for tissue regeneration. In this study, we investigated the biological effects of soluble factors in PRGF F2 for osteoblast differentiation/mineralization using hMSC.

Proliferation of hMSC increased in OIM supplemented with PRGF supernatant (Fig.2). PRGF contains several growth factors for guided tissue regeneration (20). We previously reported that PRGF contained platelet-derived growth factor (PDGF) -AB and -BB, insulin-like growth factor (IGF)-1, and transforming growth factor (TGF)-β. It is known that these growth factors stimulate mitogenic responses in MSC (21). PRGF including several growth factors may have a positive influence in clinical situations requiring rapid healing and tissue regeneration.

ALP is an early marker of osteogenic differentiation. The activity of ALP was also increased in hMSC by PRGF (Fig. 3). In addition, staining of Alizarin red S and von Kossa was stronger in hMSC cultured with OIM-PRGF as compared to
This suggests that PRGF promotes mineralization in hMSC. TGF-β and PDGF are present in PRGF, and stimulate osteogenic differentiation in MSC (22, 23). These growth factors are stimulators of both chondrogenic and osteogenic MSC differentiation, thus suggesting that this factor plays a critical role in early and mid-phase processes in the endochondral bone healing pathway (24). IGF, which is also present in PRGF, stimulates ALP activity and mineralization capacity of stem cells (25) and plays an important role in bone development (26). Thus, PRGF may induce matrix mineralization in the osteoblast lineage and accelerate bone healing.

In this study, the PRGF F2 supernatant was isolated by centrifugation of PRGF F2 clots in order to investigate the biological effects of soluble factors in PRGF F2. Our previous studies have shown that the concentration of IGF-1 is slightly elevated in the supernatant when compared with PRGF F2, whereas the concentration of TGF-β is markedly decreased in the supernatant (27). In addition, the concentrations of PDGF-AB, PDGF-BB and VEGF were decreased by about 50% in the supernatant (27). Furthermore, the biological effects of PRGF for regeneration involved soluble factors released from platelets such as growth factors and scaffolding by fibrin. PRGF clots are three-dimensional fibrin matrices that can provide the site for cell proliferation and migration. At the same time, the clots act as a biomolecule delivery system and are transplanted into lost or damaged bones, such as the cavity following tooth extraction, in clinical and in vitro studies. New in vitro experimental models are necessary to elucidate all the biological functions of PRGF, including scaffolds and release of growth factors.

This study showed that the soluble factors in PRGF increased the proliferation and mineralization associated with osteogenic induction using hMSC cultures. PRGF is a source of autogenous growth factors that help with bone regeneration and may be convenient for clinical application.

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Conflict of interest

There are no conflicts of interest associated with this study.

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