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Platelet-rich plasma suppresses osteoclastogenesis by promoting the secretion of osteoprotegerin

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Background and Objective: Platelet-rich plasma is characterized by containing fundamental protein growth factors. Although many *in vitro* studies have documented the capability of platelet-rich plasma to induce the growth of osteoblasts or osteoblast-like cells, the effect of platelet-rich plasma on osteoclastogenesis has not yet been studied. The aim of the present study was to evaluate the effects of platelet-rich plasma and platelet-poor plasma on osteoclastogenesis with rat bone marrow cell culture.

Material and Methods: Platelet-rich plasma and platelet-poor plasma were produced from the whole blood of rat. For cell culture, rat bone marrow cells were isolated from rat tibiae and then treated with 1,25α dihydroxy vitamin D_3 and with different concentrations of platelet-rich plasma or platelet-poor plasma. After 4 d of culture, rat bone marrow cells were stained with tartrate-resistant acid phosphatase (TRAP), and TRAP-positive cells that had more than three nuclei (TRAP-positive multinucleated cells) were counted as osteoclast-like cells. Osteoprotegerin, known as an osteoclastogenesis-related factor, cells was quantified using an enzyme-linked immunosorbent assay (ELISA).

Results: Although platelet-poor plasma had no effect on the formation of TRAP-positive multinucleated cells, platelet-rich plasma decreased the number of TRAP-positive multinucleated cells in a dose-dependent manner. The amount of osteoprotegerin produced from rat bone marrow cells and from MC3T3-E1 cells was enhanced in platelet-rich plasma-treated groups.

Conclusion: Under our experimental conditions, platelet-rich plasma decreased the formation of TRAP-positive multinucleated cells and increased the secretion of osteoprotegerin. This study suggests that platelet-rich plasma suppresses osteoclastogenesis, therefore inhibiting bone resorption. In addition we also demonstrated that platelet-rich plasma increased the secretion of osteoprotegerin, an inhibitor for osteoclast formation, thus suggesting that the enhancement of osteoprotegerin secretion induces this inhibitory effect.

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Platelet-rich plasma is defined as a volume of autologous plasma that has an abundant platelet concentration (1). Platelets are primarily involved in wound healing through clot formation and the release of growth factors that initiate and support wound healing. Many studies have investigated the effect of various growth factors on bone healing and have established the actual roles and mechanisms of these growth factors. Several growth factors contained in platelets have also been demonstrated to be effective on cell proliferation, chemotaxis, differentiation and extracellular matrix synthesis *in vitro* (2–5) and consequently to facilitate bone healing and regeneration *in vivo* (6–10) and in clinical trials (11,12). Platelet-rich plasma is used to deliver high concentrations of growth factors to sites requiring bone healing and regeneration and therefore considerable interest has recently emerged of the potential benefits, in addition to those of bone healing and regeneration, of the use of platelet-rich plasma.

Recently, a number of articles have been published that document basic research, both *in vitro* (13–22) and *in vivo* (23–30), and the clinical use (31–36) of platelet-rich plasma. To the authors' knowledge, the first clinical dental results with platelet-rich plasma were reported by Marx *et al*. (37). Their data suggest that platelet-rich plasma accelerates the rate and degree of bone formation. In addition, several authors have demonstrated that the application of platelet-rich plasma might improve bone healing and regeneration as well as the healing of soft tissue (21,38,39). Some authors have also published *in vitro* studies, the results of which indicate that the biological effects of platelet-rich plasma take place at the cellular level (13–21). However, most of these studies examined the biological effects of platelet-rich plasma on osteoblasts or osteoblast-like cells, not on osteoclasts, in spite of the fact that bone remodeling is accompanied by the interactions of osteoblasts and osteoclasts.

Osteoclasts that are primarily involved in bone resorption are multinucleated cells derived from hematopoietic precursors of the monocyte/macrophage lineage. Osteoclast formation requires a cell-to-cell interaction between osteoclast precursor cells and osteoblasts, and can be achieved by coculturing bone marrow precursor cells with osteoblasts/stromal cells (40). In other words, the effects of a number of cytokines that modulate the differentiation of osteoclasts are mediated by osteoblasts/stromal cells. The protein involved in this interaction has been identified as receptor activator of nuclear factor-κB ligand (RANKL), the central mediator of osteoclastogenesis in osteoblasts/stromal cells (41–43). The interaction of RANKL with receptor activator of nuclear factor (RANK) not only provides the signal for osteoclastogenesis from hematopoietic progenitor cells, but also contributes to the bone resorption activity and survival of osteoclasts. On the other hand, osteoblasts/stromal cells secrete a glycoprotein called osteoprotegerin, a soluble member of the tumor necrosis factor receptor superfamily that acts as a decoy receptor for RANKL and prevents its interaction with RANK (41). With regard to bone resorption, the number and function of osteoclasts are the main determinant of the rate of bone resorption, so it is of great interest to understand the effects of platelet-rich plasma on osteoclasts in the regeneration of bone tissue.

Therefore, the aim of the present study was to examine the molecular basis of the effects of platelet-rich plasma on osteoclastogenesis in bone marrow cultures and the involvement of osteoprotegerin production by osteoblast-like cells, and to determine the effect of platelet-rich plasma on bone resorption.

Material and methods

All animal experiments were carried out in compliance with the laws and guidelines for experimental use and care of animals.

Preparation of platelet-rich plasma

To prepare platelet-rich plasma, whole blood was obtained from the heart of Sprague–Dawley rats (male, 4 wk of age). The rats were anesthetized with an intraperioneal injection of pentobarbital sodium (Somnopentyl®; Schering–Plough Animal Health, Kenilworth, NJ, USA) and the blood was drawn with a 4.5 mL tube containing citrate solution as the anticoagulant (Vacutainer®; Becton Dickinson, Franklin Lakes, NJ, USA). The tube was centrifuged at 140 *g* for 10 min using a standard electronically controlled bench-top centrifuge. After the first centrifugation step, whole blood was separated into three components: platelet-poor plasma, buffy coat (which is thought to be platelet-rich plasma) and red blood cells. The entire platelet-poor plasma and buffy coat components, including some red blood cells at the very top of the red blood cell layer, were pipetted out and transferred into another sterile tube. This pipetted material was centrifuged again at 140 *g* for 10 min, and the top yellow platelet-poor plasma was removed except for about 0.5 mL of plasma at the bottom of the tube. The remaining plasma was defined as platelet-rich plasma and the number of platelets was counted and adjusted to 1500×10^3 platelets/μL. Platelet-rich plasma or platelet-poor plasma was added to each well after five repeated cycles of freeze–thawing to release the growth factors contained in platelets.

Effect of platelet-rich plasma and platelet-poor plasma on the formation of osteoclast-like cells

To examine the effect of platelet-rich plasma and platelet-poor plasma (10×10^3 platelets/μL) on the formation of osteoclast-like cells, rat bone marrow cells were used. For the formation of osteoclast-like cells, bone marrow cells were flushed out from the tibiae of the Sprague–Dawley rats used in this study. These bone marrow cells were seeded in 24-multiwell plates at a density of 1.0×10^6 cells/well and were cultured in α-minimum essential medium (Gibco, Grand Island, NY, USA) containing 15% fetal bovine serum in the presence of 10^{-8} M 1α,25-dihydroxy vitamin D_3. Platelet-rich plasma or platelet-poor plasma was added at 0 (control group), 0.2, 1.0, 2.0, 4.0 or

10 μL/mL. After 4 d of culture without any changes being made to the medium, the cells were fixed and stained with tartrate-resistant acid phosphatase (TRAP) using a commercial kit (Sigma, St Louis, MO, USA). Tartrate-resistant acid phosphatase-positive cells that had more than three nuclei were defined as TRAP-positive multinucleated cells and were counted as osteoclast-like cells ($n = 32$ in each group).

Enzyme-linked immunosorbent assay for osteoprotegerin secreted into the culture medium by rat bone marrow cells

The amount of osteoprotegerin secreted into the cell culture medium was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®; R&D systems, Mineapolis, MN, USA). Rat bone marrow cells, derived as described above, were seeded in 24-multiwell plates at a density of 1.0×10^6 cells/well and were incubated for 4 d in the absence or presence of 10^{-8} M vitamin D_3. After 4 d of culture under the same culture conditions used for the formation of osteoclast-like cells, the culture media were collected and the amounts of osteoprotegerin were measured according to the manufacturer's instructions ($n = 16$ in each group).

Culture of MC3T3-E1 cells and osteoprotegerin ELISA, with or without platelet-rich plasma in the absence or presence of vitamin D_3

MC3T3-E1, a mouse osteoblastic cell line, was inoculated at a density of 0.5×10^5 cells/mL into the wells of 96-multiwell plates. These cells were cultured in α-minimum essential medium containing 10% fetal bovine serum. After the adhesion period of 5 h, the medium was changed to α-minimum essential medium containing 2% fetal bovine serum in the absence or presence of platelet-rich plasma (0.2, 1.0, 2.0, 4.0 or 10 μL/mL) and culture was continued for 4 d. After 4 d of culture without any culture medium change, the MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay was used to estimate the cell number. The cells were incubated with α-minimum essential medium containing 2% fetal bovine serum and MTT in the last 3 h of the culture period after collection of culture medium for analysis by osteoprotegerin ELISA. At the end of culture, the media were changed into 100 μL of dimethylsulphoxide and after 6 hours culture, the absorbance of formazan salts were determined at 570 nm in an ELISA reader. In addition, the amount of osteoprotegerin secreted into the culture medium over 4 d of incubation was measured using an ELISA kit according to the manufacturer's instructions ($n = 16$ in each group). Similarly to the rat bone marrow cell culture, both types of ELISA were also performed after culture of MC3T3-E1 cells in the absence or presence of 10^{-8} M vitamin D_3.

Statistical analysis

All of the results were expressed as the mean ± standard deviation. A comparative study of means was performed using the analysis of variance statistical test. The results were considered significantly different when the p-value was < 0.05. The Scheffe *post hoc* test for multiple comparisons was used when the analysis of variance was significant.

Results

TRAP staining

Rat bone marrow cells grown in the presence of vitamin D_3 for 4 d showed the formation of TRAP-positive multinucleated cells. The effects of platelet-rich plasma or platelet-poor plasma on osteoclastogenesis were evaluated at various densities. Although many TRAP-positive multinucleated cells were formed in the absence of platelet-rich plasma (control group), in conjunction with significant growth of bone marrow stromal cells (Fig. 1A), platelet-rich plasma markedly inhibited the formation of TRAP-positive multinucleated cells without suppressing the growth of stromal cells (Fig. 1B). By contrast, none of the platelet-poor plasma groups had any effect on the formation of TRAP-positive multinucleated cells (data not shown).

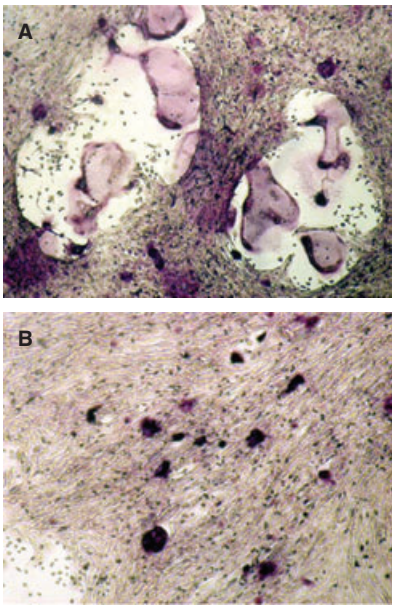

Fig. 1. Formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells in rat bone marrow cells without or with platelet-rich plasma. (A) TRAP-positive multinucleated cells were formed in the absence of platelet-rich plasma. (B) Platelet-rich plasma suppressed the formation of TRAP-positive multinucleated cells without suppressing the growth of stromal cells.

To provide quantitative data of the effect of platelet-rich plasma or platelet-poor plasma on osteoclastogenesis, the number of TRAP-positive multinucleated cells per well was counted. Platelet-rich plasma decreased the number of TRAP-positive multinucleated cells per well in a dose-dependent manner (Fig. 2A) and statistical analysis revealed significant differences between the control group and all platelet-rich plasma groups. However, TRAP-positive multinucleated cells were induced normally in all platelet-poor plasma groups and no inhibition was observed (Fig. 2B).

Osteoprotegerin ELISA in rat bone marrow cells

To determine the mechanisms of platelet-rich plasma-mediated inhibition of osteoclastogenesis, the levels of osteoprotegerin in the culture medium were measured by ELISA. In the

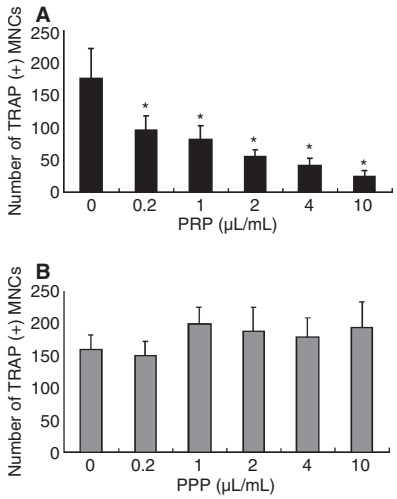

Fig. 2. Number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells with platelet-rich plasma or platelet-poor plasma. The values represent the mean ± standard deviation of 32 indivdual wells. (A) Platelet-rich plasma significantly suppressed the formation of TRAP-positive multinucleated cells in a dose-dependent manner compared with the control group (0 μL/mL) (*$p < 0.01$, analysis of variance, Scheffe). (B) By contrast, platelet-poor plasma had no significant effect on the formation of TRAP-positive multinucleated cells. PPP, platelet-poor plasma; PRP, platelet-rich plasma; TRAP(+)MNCs, tartrate-resistant acid phosphatase-positive multinucleated cells.

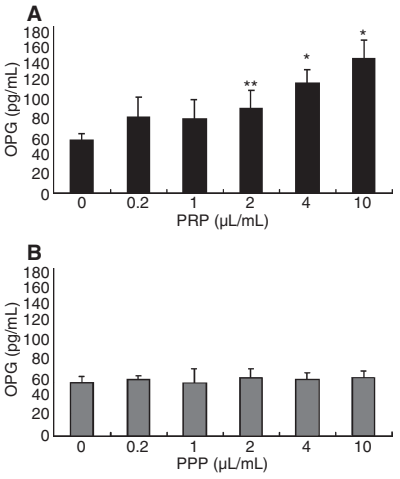

Fig. 3. The amount of osteoprotegerin in the culture medium of rat bone marrow cells treated with platelet-rich plasma or platelet-poor plasma was examined by enzyme-linked immunosorbent assay (ELISA). Rat bone marrow cells were cultured with platelet-rich plasma or platelet-poor plasma and the culture medium was collected. (A) Platelet-rich plasma caused a marked increase in the concentration of osteoprotegerin compared with the control group (0 μL/mL) (*$p < 0.01$, **$p < 0.05$, analysis of variance, Scheffe). (B) By contrast, platelet-poor plasma did not show any significant effect. OPG, osteoprotegerin; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

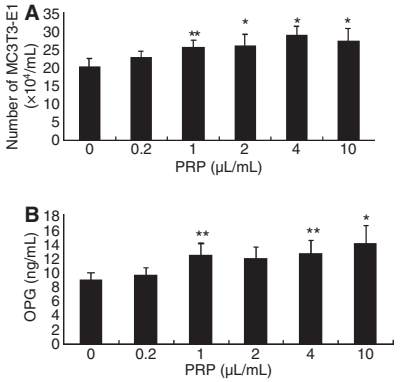

Fig. 4. The influence of different concentrations of platelet-rich plasma on the proliferation of MC3T3-E1 cells. Platelet-rich plasma stimulated the proliferation of MC3T3-E1 cells in a dose-dependent manner compared with the control group (0 μL/mL), both in the absence (A) and presence (B) of vitamin D_3 (*$p < 0.01$, **$p < 0.05$, analysis of variance, Scheffe). PRP, platelet-rich plasma.

absence of vitamin D_3, higher levels of osteoprotegerin were detected in the platelet-rich plasma-treated culture medium (Fig. 3A). Platelet-rich plasma (2.0, 4.0 and 10.0 μL/mL) enhanced the production of osteoprotegerin significantly compared with the control group. However, we found no significant effect of platelet-poor plasma on osteoprotegerin secretion from rat bone marrow cells (Fig. 3B). In the presence of vitamin D_3, osteoprotegerin was not detected in the culture medium, regardless of the presence of platelet-rich plasma or platelet-poor plasma (data not shown).

The effect of platelet-rich plasma on the proliferation of MC3T3-E1 cells in the absence or presence of vitamin D_3

Figure 4 shows the proliferation data of MC3T3-E1 cells, a mouse osteoblastic cell line, with or without platelet-rich plasma, on day 4 by use of the MTT assay. In the absence of vitamin D_3, treatment of the cultures with 1.0, 2.0, 4.0 or 10 μL/mL of platelet-rich plasma significantly increased the number of MC3T3-E1 cells compared with the control group (platelet-rich plasma 1.0 μL/mL, $p < 0.05$; and platelet-rich plasma 2.0, 4.0 and 10 μL/mL, $p < 0.01$) (Fig. 4A). The cells treated with 0.2 μL/mL of platelet-rich plasma showed only a slight increase in proliferation, which was not statistically significant. In the presence of vitamin D_3, platelet-rich plasma also stimulated the proliferation of MC3T3-E1 cells in all platelet-rich plasma groups, but this stimulation was weaker when compared with the culture in the absence of vitamin D_3 (Fig. 4B).

Osteoprotegerin ELISA of MC3T3-EI cells in the absence or presence of vitamin D_3

To investigate in greater detail the effect of platelet-rich plasma on osteoprotegerin production, we estimated the level of osteoprotegerin secreted by MC3T3-E1 cells into the culture medium by using a commercial osteoprotegerin ELISA kit in both culture systems (i.e. with or without vitamin D_3). Higher levels of osteoprotegerin were detected in the culture medium of MC3T3-E1 cells treated with platelet-rich plasma in the absence of vitamin D_3. In particular, platelet-rich plasma at 1.0, 4.0 and 10 μL/mL significantly enhanced the secretion of osteoprotegerin in comparison with the control group (1.0 and 4.0 μL/mL, $p < 0.05$; 10 μL/mL, $p < 0.01$) (Fig. 5A). In the presence of vitamin D_3, platelet-rich plasma also significantly enhanced the secretion of osteoprotegerin, but the amount of osteoprotegerin secreted was less than in the culture without vitamin D_3, as found for the proliferation assay (Fig. 5B).

Figure 6 showed osteoprotegerin secretion from a unit number of cells (1.0×10^4 cells). The amount of osteoprotegerin secreted was determined using the following formula:

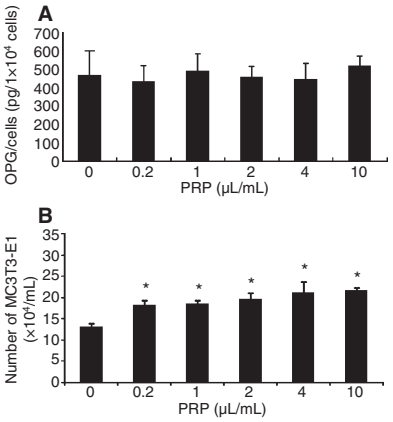

Fig. 5. The concentration of osteoprotegerin in the culture medium of MC3T3-E1 cells treated with platelet-rich plasma for 4 d was determined by enzyme-linked immunosorbent assay. In both groups [with (A) or without (B) vitamin D_3], treatment with platelet-rich plasma significantly increased the secretion of osteoprotegerin from MC3T3-E1 cells compared with the control group (0 μL/mL) (*$p < 0.01$, **$p < 0.05$, analysis of variance, Scheffe). OPG, osteoprotegerin; PRP, platelet-rich plasma.

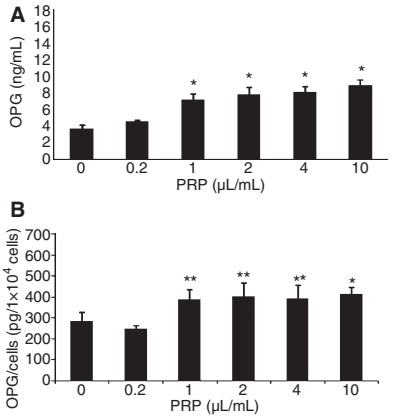

Fig. 6. Osteoprotegerin secretion from a unit number of cells (1.0×10^4 cells) was determined theoretically. In contrast to the total amount in the culture medium, osteoprotegerin secretion from a unit number of MC3T3-E1 cells was not enhanced in any experimental group in the absence of vitamin D_3 (A). However, when vitamin D_3 was added, osteoprotegerin secretion from a unit number of cells was stimulated significantly in some experimental groups (B). (*$p < 0.01$, **$p < 0.05$, analysis of variance, Scheffe). OPG, osteoprotegerin; PRP, platelet-rich plasma.

$$\text{OPG secretion}(\text{pg}/1.0 \times 10^4\text{cells}) = (\text{the amount of OPG}(\text{pg}) / \text{the number of cells}) \times 1.0 \times 10^4.$$

Although an increase in the total amount of osteoprotegerin was observed, no significant difference was detected in any of the groups in the absence of vitamin D_3 (Fig. 6A). However, in the presence of vitamin D_3, platelet-rich plasma stimulated the secretion of a significant amount of osteoprotegerin, relative to the control, from a unit number of cells, indicating that platelet-rich plasma augmented the specific activity of each cells to produce osteoprotegerin (Fig. 6B).

Discussion

Platelet-rich plasma has been considered to be effective for bone repair and regeneration and has been increasingly applied in the field of oral surgery, periodontal surgery and oral implant surgery. One of the most dominant factors in the regeneration of bone tissues is osteoblasts and therefore many *in vitro* systems have been used to study various aspects of function (such as proliferation, matrix maturation and mineralization) of osteoblasts. Although some studies have been carried out to determine the effects of platelet-rich plasma on osteoblasts (13–21), few studies have examined the effects of platelet-rich plasma on osteoclasts. Bone remodeling requires bone resorption mediated by osteoclasts, which is tightly coupled with bone formation by osteoblasts. In the present study, we explored the effects of platelet-rich plasma on osteoclastogenesis to provide insight into a new biological function of platelet-rich plasma.

In this study, we demonstrated that platelet-rich plasma markedly inhibited the formation of TRAP-positive multinucleated cells, which were defined as osteoclast-like cells, in rat bone marrow cultures treated with vitamin D_3, whereas platelet-poor plasma did not influence the formation of TRAP-positive multinucleated cells. These results suggest that platelet-rich plasma suppresses osteoclastogenesis, therefore inhibiting bone resorption. As noted above, the previous reports mainly described the regulatory function of platelet-rich plasma on the proliferation or differentiation of osteoblasts, and only a few reports have described the effect of platelet-rich plasma on osteoclasts. Gruber *et al*. (43) reported on an *in vitro* study evaluating the effect of platelet-released supernatants on osteoclastogenesis in murine bone marrow cells. They concluded that the supernatants of activated platelets can induce the formation of osteoclast-like cells. Their findings are completely different from ours; however, we identifed some factors that may explain the contrasting results. For example, the number of platelets added to the culture was different from the number used in our study and the number of TRAP-positive multinucleated cells formed in the control group was much lower than found in the control group of the present study. Taking into consideration these contrasting results, the effect of platelet-rich plasma on osteoclastogenesis is controversial. However, under our experimental conditions, we successfully showed that platelet-rich plasma markedly inhibited osteoclastogenesis from rat bone marrow cells.

RANKL and osteoprotegerin, which are expressed by osteoblasts, are the main regulators in osteoclastogenesis (42,43). In the present study, to investigate this regulatory process in greater detail, we examined the effect of platelet-rich plasma and of platelet-poor plasma on the secretion of osteoprotegerin, a potent inhibitor of RANKL-induced osteoclastogenesis, using rat bone marrow cells. In the absence of vitamin D_3, treatment of these cultures with platelet-rich plasma stimulated the production of osteoprotegerin and these stimulations were significant among three of the platelet-rich plasma groups. By contrast, platelet-poor plasma did not influence the secretion of osteoprotegerin. However, in the presence of vitamin D_3, osteoprotegerin was not detected in the culture medium with the ELISA kit used in this study. In addition, we also showed significant stimulation of

platelet-rich plasma on the secretion of osteoprotegerin using MC3T3-E1 cells in the absence or presence of vitamin D_3, although osteoprotegerin secretion was decreased by the addition of vitamin D_3. Before we discuss the effect of platelet-rich plasma on the secretion of osteoprotegerin, the effect of vitamin D_3 should be noted. The results of previous studies showed that vitamin D_3 up-regulated the expression of RANKL and down-regulated osteoprotegerin in a reciprocal manner (44). Under our experimental conditions, rat bone marrow cells secreted 60 pg/mL of osteoprotegerin at baseline (control group) in the absence of vitamin D_3. According to the previous study and the present study (osteoprotegerin secretion from MC3T3-E1 cells), vitamin D_3 decreased osteoprotegerin secretion by 30% or 50%, respectively (44). In addition, in the presence of vitamin D_3, the expression of RANKL was induced, and RANKL may bind to osteoprotegerin. RANKL expresses on the cell membrane, so RANKL-osteoprotegerin complex could not be detected by ELISA kit. We thought that osteoprotegerin in the culture medium was lower than the minimum detectable dose of the ELISA kit for these reasons. Vitamin D_3 also suppressed the proliferation of MC3T3-E1 cells, as indicated in Fig. 4. Some previous studies have reported similar results and are helpful in analyzing the results of the present study (45). The correlation between osteoprotegerin secretion and osteoprotegerin proliferation will be discussed in the following paragraph. Platelet-rich plasma stimulated the secretion of osteoprotegerin significantly, in spite of the addition of vitamin D_3. From these results, it seems reasonable to believe that the increase of osteoprotegerin secretion is the major cause of the inhibition of osteoclastogenesis by platelet-rich plasma.

Two hypotheses could account for the mechanisms of the stimulation of osteoprotegerin secretion. The first is that osteoprotegerin secretion is stimulated by increasing the number of osteoprotegerin-secreted cells, namely stromal/osteoblastic cells. The second is that platelet-rich plasma can enhance the amount of osteoprotegerin secreted from each cell. From our experimental results, the amount of osteoprotegerin secreted from a unit cell number (1.0×10^4 cells) was calculated by using the formula described in the Material and methods. Our results showed that platelet-rich plasma could contribute to the stimulation of proliferation and secretion of osteoprotegerin in MC3T3-E1 cells in the presence of vitamin D_3. Regarding other factors involved in osteoclastogenesis, such as RANKL, further investigations are necessary to examine their expression, but our results imply that the effect of platelet-rich plasma on the proliferation of osteoblasts/stromal cells and the enhancement of osteoprotegerin secretion may be attributed to the inhibition of osteoclastogenesis.

Elevated levels of osteoprotegerin are found in the culture medium, raising the question of which factors, especially growth factors in platelet-rich plasma, result in the outcome observed in the present study. As already noted, if the contribution of platelet-rich plasma to the proliferation of osteoblasts/stromal cells is the most important factor in the enhancement of osteoprotegerin secretion, there are some studies that focused on the effect of platelet-rich plasma on the proliferation of osteoblastic cells. According to these studies, platelet-rich plasma stimulates the proliferation of rat bone marrow cells and human osteoblastic cells (13). In our previous study, we also reported that platelet-derived growth factor and transforming growth factor-β play an important role in the proliferation of osteoblastic cells (46). From the standpoint of the interaction between growth factors and osteoclastogenesis, it is widely recognized that transforming growth factor-β plays a pivotal role in the regulation of osteoclastogenesis (47). In fact, our data, and the effect of transforming growth factor-β on osteoclastogenesis reported in previous studies, share certain similarities in that both platelet-rich plasma and transforming growth factor-β suppress the formation of TRAP-positive multinucleated cells and stimulate the production of osteoprotegerin from osteoblasts/stromal cells (48–50), although transforming growth factor-β has been shown to have biphasic effects or dose-dependent effects on osteoclast differentiation, and the effects of transforming growth factor-β on osteoclastogenesis *in vitro* depend on the model system (51–53). Takai *et al*. (48) and Thirunavukkarasu *et al*. (50) reported that transforming growth factor-β increased osteoprotegerin mRNA expression. Additionally, many other studies have evaluated the effect of other growth factors on osteoclastogenesis (54–56) and the action of each growth factor and their interaction with and effect on osteoclastogenesis might be complicated. Future studies will attempt to define the role of each growth factor in osteoclastogenesis.

Although some points require further elucidation regarding the effect of platelet-rich plasma on osteoclastogenesis, especially the expression of RANKL and RANK, the results of the present study clearly show that platelet-rich plasma can suppress the formation of osteoclast-like cells and suggest that the enhancement of osteoprotegerin secretion is involved in these results. Future studies are required to elucidate, in more detail, the molecular mechanisms by which platelet-rich plasma regulates the osteoclastogenesis and the precise mechanism of action of platelet-rich plasma in the complicated network of various cell types in the bone microenvironment.

References

1. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? *Implant Dent* 2001;**10:**225–228.
2. Jiang D, Dziak R, Lynch SE, Stephan EB. Modification of an osteoconductive anorganic bovine bone mineral matrix with growth factors. *J Periodontol* 1999; **70:**834–839.
3. Strayhorn CL, Garrett JS, Dunn RL, Benedict JJ, Somerman MJ. Growth factors regulate expression of osteoblast-associated genes. *J Periodontol* 1999;**70:** 1345–1354.
4. Spinella-Jaegle S, Roman-Roman S, Faucheu C *et al*. Opposite effects of bone morphogenetic protein-2 and transforming growth factor-beta1 on osteoblast differentiation. *Bone* 2001;**29:**323–330.

5. Mott DA, Mailhot J, Cuenin MF, Sharawy M, Borke J. Enhancement of osteoblast proliferation in vitro by selective enrichment of demineralized freeze-dried bone allograft with specific growth factors. *J Oral Implantol* 2002; **28:**57–66.
6. Wang HL, Pappert TD, Castelli WA, Chiego DJ Jr, Shyr Y, Smith BA. The effect of platelet-derived growth factor on the cellular response of the periodontium: an autoradiographic study on dogs. *J Periodontol* 1994;**65:**429–436.
7. Giannobile WV, Finkelman RD, Lynch SE. Comparison of canine and non-human primate animal models for periodontal regenerative therapy: results following a single administration of PDGF/IGF-I. *J Periodontol* 1994;**65:** 1158–1168.
8. Lind M, Overgaard S, Glerup H, Soballe K, Bunger C. Transforming growth factor-beta1 adsorbed to tricalciumphosphate coated implants increases peri-implant bone remodeling. *Biomaterials* 2001;**22:**189–193.
9. Meraw SJ, Reeve CM, Lohse CM, Sioussat TM. Treatment of peri-implant defects with combination growth factor cement. *J Periodontol* 2000;**71:**8–13.
10. Vuola J, Bohling T, Goransson H, Puolakkainen P. Transforming growth factor beta released from natural coral implant enhances bone growth at calvarium of mature rat. *J Biomed Mater Res* 2002;**59:**152–159.
11. Nevins M, Camelo M, Nevins ML, Schenk RK, Lynch SE. Periodontal regeneration in humans using recombinant human platelet-derived growth factor-BB (rhPDGF-BB) and allogenic bone. *J Periodontol* 2003;**74:**1282–1292.
12. Sarment DP, Cooke JW, Miller SE *et al*. Effect of rhPDGF-BB on bone turnover during periodontal repair. *J Clin Periodontol* 2006;**33:**135–140.
13. Gruber R, Varga F, Fischer MB, Watzek G. Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes. *Clin Oral Implants Res* 2002; **13:**529–535.
14. Okuda K, Kawase T, Momose M *et al*. Platelet-rich plasma contains high levels of platelet-derived growth factor and transforming growth factor-beta and modulates the proliferation of periodontally related cells in vitro. *J Periodontol* 2003;**74:**849–857.
15. Arpornmaeklong P, Kochel M, Depprich R, Kubler NR, Wurzler KK. Influence of platelet-rich plasma (PRP) on osteogenic differentiation of rat bone marrow stromal cells. An in vitro study. *Int J Oral Maxillofac Surg* 2004;**33:**60–70.
16. Soffer E, Ouhayoun JP, Dosquet C, Meunier A, Anagnostou F. Effects of platelet lysates on select bone cell functions. *Clin Oral Implants Res* 2004;**15:**581–588.
17. Annunziata M, Oliva A, Buonaiuto C *et al*. In vitro cell-type specific biological response of human periodontally related cells to platelet-rich plasma. *J Periodont Res* 2005;**40:**489–495.
18. Ferreira CF, Carriel Gomes MC, Filho JS, Granjeiro JM, Oliveira Simoes CM, Magini Rde S. Platelet-rich plasma influence on human osteoblasts growth. *Clin Oral Implants Res* 2005;**16:**456–460.
19. Doucet C, Ernou I, Zhang Y *et al*. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol* 2005;**205:**228–236.
20. Ogino Y, Ayukawa Y, Tsukiyama Y, Koyano K. The effect of platelet-rich plasma on the cellular response of rat bone marrow cells in vitro. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005;**100:**302–307.
21. Graziani F, Ivanovski S, Cei S, Ducci F, Tonetti M, Gabriele M. The in vitro effect of different PRP concentrations on osteoblasts and fibroblasts. *Clin Oral Implants Res* 2006;**17:**212–219.
22. Tomoyasu A, Higashio K, Kanomata K *et al*. Platelet-rich plasma stimulates osteoblastic differentiation in the presence of BMPs. *Biochem Biophys Res Commun* 2007;**361:**62–67.
23. Jakse N, Tangl S, Gilli R *et al*. Influence of PRP on autogenous sinus grafts. An experimental study on sheep. *Clin Oral Implants Res* 2003;**14:**578–583.
24. Wiltfan J, Kloss FR, Kessler P *et al*. Effects of platelet-rich plasma on bone healing in combination with autogenous bone and bone substitutes in critical-size defects. An animal experiment. *Clin Oral Implants Res* 2004;**15:**187–193.
25. Aghaloo TL, Moy PK, Freymiller EG. Evaluation of platelet-rich plasma in combination with freeze-dried bone in the rabbit cranium. A pilot study. *Clin Oral Implants Res* 2005;**16:**250–257.
26. Thorwarth M, Rupprecht S, Falk S, Felszeghy E, Wiltfang J, Schlegel KA. Expression of bone matrix proteins during de novo bone formation using a bovine collagen and platelet-rich plasma (prp) – an immunohistochemical analysis. *Biomaterials* 2005;**26:**2575–2584.
27. Pryor ME, Polimeni G, Koo KT *et al*. Analysis of rat calvaria defects implanted with a platelet-rich plasma preparation: histologic and histometric observations. *J Clin Periodontol* 2005;**32:**966–972.
28. Thorwarth M, Wehrhan F, Schultze-Mosgau S, Wiltfang J, Schlegel KA. PRP modulates expression of bone matrix proteins in vivo without long-term effects on bone formation. *Bone* 2006;**38:**30–40.
29. Gerard D, Carlson ER, Gotcher JE, Jacobs M. Effects of platelet-rich plasma at the cellular level on healing of autologous bone-grafted mandibular defects in dogs. *J Oral Maxillofac Surg* 2007;**65:** 721–727.
30. de Vasconcelos Gurgel BC, Goncalves PF, Pimentel SP *et al*. Platelet-rich plasma may not provide any additional effect when associated with guided bone regeneration around dental implants in dogs. *Clin Oral Implants Res* 2007;**18:**649–654.
31. Camargo PM, Lekovic V, Weinlaender M, Vasilic N, Madzarevic M, Kenney EB. Platelet-rich plasma and bovine porous bone mineral combined with guided tissue regeneration in the treatment of intrabony defects in humans. *J Periodont Res* 2002;**37:**300–306.
32. Wiltfang J, Schlegel KA, Schultze-Mosgau S, Nkenke E, Zimmermann R, Kessler P. Sinus floor augmentation with beta-tricalciumphosphate (beta-TCP): does platelet-rich plasma promote its osseous integration and degradation? *Clin Oral Implants Res* 2003;**14:**213–218.
33. Raghoebar GM, Schortinghuis J, Liem RS, Ruben JL, van der Wal JE, Vissink A. Does platelet-rich plasma promote remodeling of autologous bone grafts used for augmentation of the maxillary sinus floor? *Clin Oral Implants Res* 2005;**16:**349–356.
34. Monov G, Fuerst G, Tepper G, Watzak G, Zechner W, Watzek G. The effect of platelet-rich plasma upon implant stability measured by resonance frequency analysis in the lower anterior mandibles. *Clin Oral Implants Res* 2005;**16:**461–465.
35. Consolo U, Zaffe D, Bertoldi C, Ceccherelli G. Platelet-rich plasma activity on maxillary sinus floor augmentation by autologous bone. *Clin Oral Implants Res* 2007;**18:**252–262.
36. Demir B, Sengun D, Berberoglu A. Clinical evaluation of platelet-rich plasma and bioactive glass in the treatment of intrabony defects. *J Clin Periodontol* 2007; **34:**709–715.
37. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998;**85:**638–646.
38. Carter CA, Jolly DG, Worden CE Sr, Hendren DG, Kane CJ. Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing. *Exp MolPathol* 2003;**74:**244–255.
39. Frechette JP, Martineau I, Gagnon G. Platelet-rich plasmas: growth factor content and roles in wound healing. *J Dent Res* 2005;**84:**434–439.

40. Suda T, Udagawa N, Nakamura I, Miyaura C, Takahashi N. Modulation of osteoclast differentiation by local factors. *Bone* 1995;**17:**87S–91S.
41. Simonet WS, Lacey DL, Dunstan CR *et al*. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;**89:**309–319.
42. Lacey DL, Timms E, Tan HL *et al*. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;**93:**165–176.
43. Gruber R, Karreth F, Fischer MB, Watzek G. Platelet-released supernatants stimulate formation of osteoclast-like cells through a prostaglandin/RANKL-dependent mechanism. *Bone* 2002;**30:**726–732.
44. Kondo T, Kitazawa R, Maeda S, Kitazawa S. 1 alpha,25 dihydroxyvitamin D3 rapidly regulates the mouse osteoprotegerin gene through dual pathways. *J Bone Miner Res* 2004;**19:**1411–1419.
45. Atkins GJ, Kostakis P, Pan B *et al*. RANKL expression is related to the differentiation state of human osteoblasts. *J Bone Miner Res* 2003;**18:**1088–1098.
46. Ogino Y, Ayukawa Y, Kukita T, Koyano K. The contribution of platelet-derived growth factor, transforming growth factor-beta1 and insulin-like growth factor-I in platelet-rich plasma (PRP) to the proliferation of osteoblast-like cells. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;**101:**724–729.
47. Chambers TJ. Regulation of the differentiation and function of osteoclasts. *J Pathol* 2000;**192:**4–13.
48. Takai H, Kanematsu M, Yano K *et al*. Transforming growth factor-beta stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. *J Biol Chem* 1998;**273:**27091–27096.
49. Quinn JM, Itoh K, Udagawa N *et al*. Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. *J Bone Miner Res* 2001;**16:**1787–1794.
50. Thirunavukkarasu K, Miles RR, Halladay DL *et al*. Stimulation of osteoprotegerin (OPG) gene expression by transforming growth factor-beta (TGF-beta). Mapping of the OPG promoter region that mediates TGF-beta effects. *J Biol Chem* 2001;**276:**36241–36250.
51. Sells Galvin RJ, Gatlin CL, Horn JW, Fuson TR. TGF-beta enhances osteoclast differentiation in hematopoietic cell cultures stimulated with RANKL and M-CSF. *Biochem Biophys Res Commun* 1999;**265:**233–239.
52. Yan T, Riggs BL, Boyle WJ, Khosla S. Regulation of osteoclastogenesis and RANK expression by TGF-beta1. *J Cell Biochem* 2001;**83:**320–325.
53. Koseki T, Gao Y, Okahashi N *et al*. Role of TGF-beta family inosteoclastogenesis induced by RANKL. *Cell Signal* 2002;**14:**31–36.
54. Fiorelli G, Formigli L, Zecchi Orlandini S *et al*. Characterization and function of the receptor for IGF-I in human preosteoclastic cells. *Bone* 1996;**18:**269–276.
55. Hurley MM, Lee SK, Raisz LG, Bernecker P, Lorenzo J. Basic fibroblast growth factor induces osteoclast formation in murine bone marrow cultures. *Bone* 1998;**22:**309–316.
56. Zhang Z, Chen J, Jin D. Platelet-derived growth factor (PDGF)-BB stimulates osteoclastic bone resorption directly: the role of receptor beta. *Biochem Biophys Res Commun* 1998;**251:**190–194.