

Inhibitory Effects of Platelet-rich Plasma on Osteoclast Differentiation in RAW264.7 Cells

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Abstract: Platelet-rich plasma (PRP) is an autologous source of platelet-derived growth factors, and has been used successfully in oral surgery repair and in the placement of osseointegrated implants. However, little is known about the underlying release mechanisms of these factors or the manner in which inhibition of osteoclastic cells is regulated. The present study investigated the efficacy of PRP as an inhibitor of receptor activator of NF- κ B ligand (RANKL)-induced TRAP-positive multinucleated cell formation in RAW264.7 cells. RANKL-induced formation of osteoclasts was significantly inhibited by treatment with PRP, while washed platelet treatment was more effective in inhibiting osteoclast formation. These results suggest that PRP and washed platelets are potent inhibitors of osteoclast differentiation.

Introduction

Platelet-rich plasma (PRP) is an autologous source of platelet-derived growth factors, and has been widely used and studied in the field of oral surgery repair. Recently, it has been used in the placement of osseointegrated implants for the purpose of bone regeneration^{1,2)}. Although, bone formation and resorption are coupled through the actions of osteoblastic stromal cells and osteoclasts^{3,4)}, there is no convincing evidence of direct responses to these growth factors in osteoclast lineage cells. RAW264.7 cells are known to have characteristics of murine macrophages, and the ability to differentiate into osteoclast-like cells by RANKL stimulation⁵⁾. The use of osteoclast precursor cell lines provides numerous advantages in studying the mechanisms of osteoclast differentiation via these growth factors. We focused on clarifying the direct action of PRP on cells of the osteoclast lineage, and the present study investigated the efficacy of PRP as an inhibitor of receptor activator of NF- κ B ligand (RANKL)-induced TRAP-positive multinucleated cell formation in RAW264.7 cells.

Materials and Methods

1) Reagents: Recombinant soluble RANKL (sRANKL) and recombinant human transforming growth factor- β (TGF- β) were obtained from Peprotech EC Ltd. (London, UK). Recombinant mouse TNF- α was from Roch Diagnostics (Penzberg, Germany). RPMI 1640 was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and α -MEM was from Gibco BRL. Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA).

2) Cells: The mouse macrophage cell line RAW264.7 and the osteoblastic cell line MC3T3-E1, derived from C57BL/6 mouse calvaria, were routinely maintained in α -MEM supplemented with 10% FBS in a 5% CO₂ incubator, and were subcultured at a split ratio of 1:10 approximately every 4 days.

3) Osteoclast formation assay: RAW264.7 cell D clones were kindly provided by Dr. T. Kukita of Kyusyu University (Fukuoka, Japan). Cells were cultured in α -MEM containing 10% FBS (5 \times 10³ cells in 150 ml/well of 96-well plates) in the presence of sRANKL (100 ng/ml) and various sample concentrations. After 3 days, TRAP-positive multinucleated cells were counted.

4) Cytotoxic activity assay: MC3T3-E1 cells were inoculated at 12 \times 10³ cells/well in 96-well plates, unless otherwise stated. After 24 hours, medium was removed with an aspirator, and was replaced with 0.1 ml of fresh medium containing different samples. Cells were incubated for a further 24 hours, and relative viable cell number was determined by MTT assay.

5) Platelet preparation: Venous blood was drawn from the antecubital fossa of healthy volunteers into citrated tubes (final concentration, 0.38%). Volunteers had not taken any medication known to affect platelet aggregation within the preceding month. Platelet-rich plasma (PRP) and platelet poor plasma (PPP) were prepared by general methods. Washed platelets (WPLT) were prepared by centrifugation of PRP (1200 \times g; 20 min), and the platelet pellet was resuspended in an equal volume of α -MEM.

Results and Discussion

We initially investigated whether RANKL induces formation of osteoclast-like multinucleated cells in RAW264.7. TNF- α synergistically stimulated osteoclast-like cell formation in the presence of low concentrations of RANKL, as previously described by Watanabe et al.⁵⁾ However, numerous large multinucleated cells were formed at higher concentrations (100 ng/ml) of RANKL alone (Fig. 1). We then tested whether WPLT inhibits the formation of osteoclast-like cells. RAW264.7 cells were pretreated with or without various concentrations of WPLT and were subsequently stimulated with RANKL. As shown in Fig. 2, RANKL-induced formation of osteoclasts was significantly inhibited by treatment with PRP. However, WPLT treatment was more effective in inhibiting osteoclast formation, and this inhibition was dose dependent. Figure 3 depicts the microscopic observation of cells inhibited by WPLT in the presence of 100 ng/ml RANKL.

Osteoblastic stromal cells play an important role in modulating the development of osteoclast progenitors^{3,4)}; therefore, we examined the effects of WPLT and PPP on cell proliferation of mouse osteoblastic MC3T3-E1 cells. As shown in Fig. 4, PPP markedly inhibited WPLT-induced cell proliferation of mouse osteoblasts. The morphological changes in osteoblasts are shown

in Fig. 5.

These results suggest that WPLT is a potent inhibitor of RANKL-induced osteoclastic differentiation in RAW264.7 cells. Furthermore, plasma contains osteoblast-inhibiting factor(s). Therefore, improved preparation of platelets, which excludes the osteoblast-inhibiting factor(s) from PRP, may be needed for efficient reproduction of osseous tissue.

References

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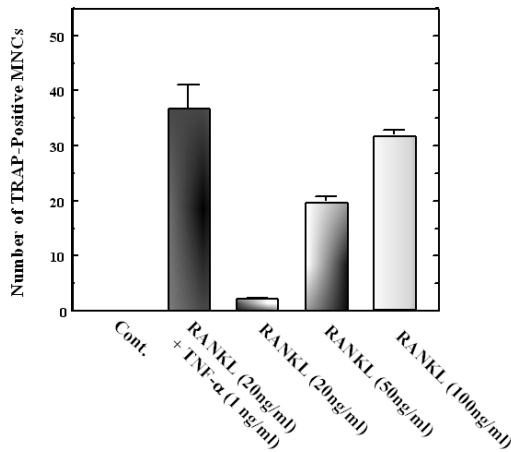


Fig. 1. RANKL-induced osteoclast-like cells in RAW264.7 cell cultures. RAW264.7 cells were treated with RANKL and TNF-α in 96-well plates, as described in Materials and Methods. Results are expressed as means ± S. D. of triplicate cultures.

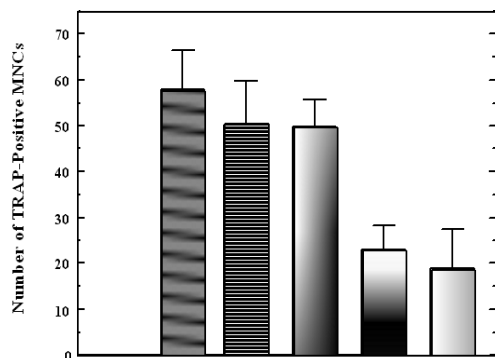


Fig. 2. Effects of PRP and W-PLT on RANKL-induced osteoclast-like cells in RAW264.7 cells. RAW264.7 cells were treated with RANKL (100 ng/ml) in the presence or absence of PRP and various concentrations of W-PLT. After 72 h, the number of TRAP-positive cells was determined and represented as the mean ± S.E.M. of quadruplicate cultures in 96-well plates.

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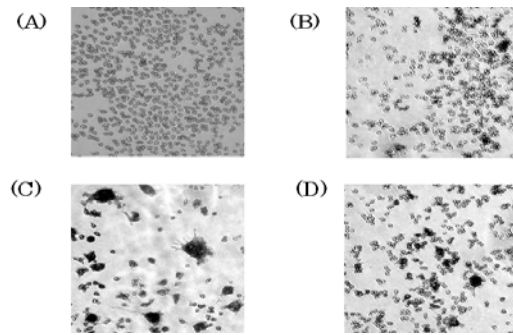


Fig. 3. Morphological demonstration of osteoclast-like cells formed from RAW264.7. Cells were cultured in 96-well plates, as described in Materials and Methods. (A) Control, (B) WPLT (10%), (C) RANKL (100 ng/ml), (D) RANKL (100 ng/ml) + WPLT (10%).

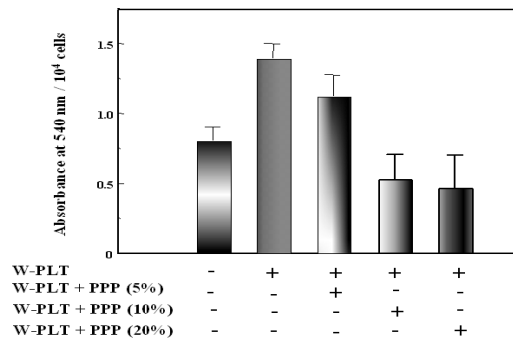


Fig. 4. Effects of PPP on cell proliferation of MC3T3-E1 cells. WPLT-stimulated MC3T3-E1 cells were incubated in the presence or absence of various concentrations of PPP in α-MEM. Cells were incubated for a further 24 h, and relative viable cell number was determined by MTT method assay.

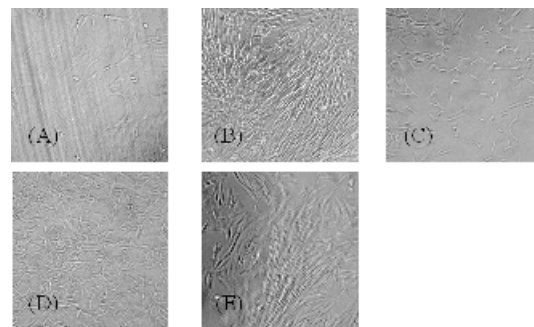


Fig. 5. Morphological changes in osteoblastic MC3T3-E1 cells. (A) Control MC3T3-E1 cells, (B) cells treated with 5% WPLT, (C) cells treated with 5% PPP, (D) cells treated with WPLT+PPP, (E) cells treated with human TGF-β at 2 ng/ml.