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Activated Platelets Positively Regulate RANKL-Mediated Osteoclast Differentiation

Barbara Weicht,[1] Philipp Maitz,[1] Barbara Kandler,[1] Michael B. Fischer,[2] Georg Watzek,[1] and Reinhard Gruber[1]*

[1]Department of Oral Surgery, Medical University Vienna, Vienna, Austria
[2]Department of Transfusion Medicine, Medical University Vienna, Vienna, Austria

Abstract Platelets induce osteoclastogenesis in total bone marrow cultures where hematopoietic cells can interact with stromal cells. Whether or not activated platelets directly act on hematopoietic cells to promote their differentiation into osteoclasts remains unknown. Here we report that platelet releasates (PRS) increase osteoclastogenesis in stroma-depleted, macrophage colony-stimulating factor (M-CSF)-dependent bone marrow cells when cultured in the presence of receptor activator of NF-kappaB ligand (RANKL). The increased number of tartrate-resistant acid phosphatase-positive multinucleated cells (MNC) was paralleled by an enhanced transcription of osteoclast specific genes. Osteoclastogenesis was observed with hematopoietic cells previously depleted of B-cells or T-cells. Immunoprecipitation of transforming growth factor-beta (TGF-β) decreased the osteoclastogenic capacity of the PRS. PRS enhanced phosphorylation of Smad-2, a downstream signaling mediator of TGF-β. PRS increased phosphorylation of p38 and c-Jun NH(2)-terminal kinase (JNK), whereas only blocking of p38 but not JNK signaling suppressed osteoclastogenesis. These results suggest that activated platelets can enhance osteoclastogenesis by providing a source of TGF-β and by activating osteoclastogenic signaling pathways. J. Cell. Biochem. 102: 1300–1307, 2007.

Key words: osteoclast; platelets; transforming growth factor-beta; mitogen-activated protein kinase; inflammation; bone resorption

Osteoclasts, the exclusive bone-resorbing cells, are derived from hematopoietic progenitors of the monocytic lineage [Chambers, 2000; Boyle et al., 2003; Teitelbaum and Ross, 2003]. Osteoclasts and the bone-forming osteoblasts of the mesenchymal lineage are colocalized in multicellular units, where they control bone remodeling [Riggs and Parfitt, 2005]. The coupling of bone formation and resorption involves the expression of receptor activator of NF-kappaB ligand (RANKL) by osteogenic cells, which in turn activates RANK signaling in osteoclast progenitors [Suda et al., 1999; Chambers, 2000; Teitelbaum and Ross, 2003]. Osteoprotegerin (OPG), also being expressed by osteogenic cells, competes with RANK, thereby acting as an inhibitor of osteoclastogenesis [Suda et al., 1999]. The expression of RANKL and OPG is regulated by local factors and systemic hormones [Suda et al., 1999]. This indirect process of modulating osteoclastogenesis requires the presence of osteogenic cells or other cell types such as lymphocytes [Suda et al., 1999; Kawai et al., 2006]. Local factors such as transforming growth factor-beta (TGF-β) and inflammatory cytokines can increase osteoclastogenesis by directly targeting hematopoietic progenitors in the presence of permissive levels of RANKL [Chambers, 2000; Fuller et al., 2000; Lam et al., 2000; Quinn et al., 2001; Wei et al., 2005].

Platelets provide a major source of TGF-β and accumulate under pathologic conditions of increased bone resorption [Klinger and Jelkmann, 2002; Wagner and Burger, 2003; Weyrich et al., 2003; Gawaz et al., 2005], for example in rheumatoid arthritis [Endresen, 1981; Myers

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*Correspondence to: Reinhard Gruber, PhD, Department of Oral Surgery; Medical University Vienna, Währinger-strasse 25, A-1090 Vienna, Austria.
E-mail: reinhard.gruber@meduniwien.ac.at

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and Christine, 1982; Farr et al., 1984; Endresen, 1989] and in periodontal disease [Steinberg et al., 1995]. The functional link between platelets and osteoclastogenesis remains to be proven. In vitro, platelet releasates (PRS) can induce osteoclastogenesis in total bone marrow cultures [Gruber et al., 2002]. It is currently not clear whether platelet-mediated osteoclastogenesis is the sole consequence of an increased ratio of RANKL over OPG produced by osteogenic cells [Gruber et al., 2002], or whether platelet-derived molecules such as TGF-β can also directly stimulate hematopoietic cells to become osteoclasts. Although it seems plausible, possible direct effects of the PRS on hematopoietic cells cannot be predicted because activated platelets release a myriad of molecules which may either stimulate or suppress osteoclastogenesis [Klinger and Jelkmann, 2002; Dittrich et al., 2005; Macaulay et al., 2005].

PRS may exert their effects on osteoclastogenesis by the modulation of signaling pathways required for the differentiation and fusion of the respective hematopoietic progenitors. Signaling via p38 and c-Jun NH_2-terminal kinase (JNK) is involved in osteoclast formation and activation [Asagiri and Takayanagi, 2007]. Extracellular-regulated kinases1/2 (ERK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways are associated with regulation of cell survival, motility, and cytoskeletal rearrangement. Smad-2, in conjunction with Smad-4, can suppress the anti-osteoclastogenic activity of cytokines such as interferon-beta [Teitelbaum and Ross, 2003; Fox and Lovibond, 2005]. In vivo, blocking of p38 but not JNK signaling prevents osteolysis in models of chronic inflammation [Koller et al., 2005; Medicherla et al., 2006; Zwerina et al., 2006]. Pharmacologic molecules that block p38 and JNK signaling also suppress osteoclastogenesis in vitro [Lee et al., 2002]. Recombinant TGF-β can activate p38, JNK, and Smad-2 signaling in osteoclast progenitors [Lam et al., 2000; Karsdal et al., 2003; Fox and Lovibond, 2005; Wei et al., 2005]. However, the question whether or not PRS can directly modulate osteoclastogenesis by controlling the signaling pathways mentioned above in hematopoietic progenitors remains to be answered. The rationale for this in vitro study was therefore to determine if and by which mechanisms activated platelets can directly affect the process of osteoclastogenesis.

MATERIALS AND METHODS

Preparation of Platelet Releasates (PRS)

Leukocyte-depleted platelet concentrates from adult volunteer donors were obtained from the Department of Transfusion Medicine (Medical University Vienna, Austria). Aliquots of platelet concentrates containing 3×10^9 cells were washed in Tyrode's buffer, pH 6.4, and centrifuged at 1400*g* for 7 min. Pellets were resuspended in 3 ml αMEM (Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Linz, Austria), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco). Platelet suspension was incubated with 3U of human thrombin (Sigma, St. Louis, MO) for 30 min at room temperature. After centrifugation, PRS was sterile filtered and stored frozen in aliquots. PRS stock was diluted at a 1:6 ratio, corresponding to platelet numbers of 1.7×10^8/ml (PRS I); 2.3×10^7/ml (PRS II); 4.6×10^6/ml (PRS III); 7.7×10^5/ml (PRS IV); and 1.3×10^5/ml (PRS V).

Osteoclastogenesis From Stroma-depleted Bone Marrow Cells

Bone marrow cells were prepared by flushing the femora and tibiae of 4 to 6 weeks old female mice (strain HIM:OF-1 Swiss, SPF, Institute for Experimental Animal Research of the Medical University Vienna, Himberg, Austria) and incubated in T-75 flasks containing α-MEM supplemented with 10% FCS at 37°C in 5% CO_2. After 24 h in the presence of 10 ng/ml recombinant murine macrophage colony-stimulating factor (M-CSF) (416-ML/CF, R&D systems), the stroma-depleted nonadherent cell population containing osteoclast progenitors was harvested. For indicated experiments, the harvested cells were further depleted of T- and B-lymphocytes with magnetic beads linked to the antibodies Thy1.2 and B220, respectively, following the protocol of the manufacturer (Mouse Pan B, #114-41D; Mouse pan T, #114-43D, both Invitrogen Corporation). One million stroma-depleted bone marrow cells per cm^2 were seeded in 96-well culture plates. Culture medium was supplemented with M-CSF at 30 ng/ml, RANKL at 30 ng/ml (462-TR/CF; mouse RANKL fused to the signal peptide of CD33 and six histidine residues, R&D systems), with and without the addition of one sixth (v/v) of PRS. Culture medium was replenished on day 3 and day 5. On day 7 of culture, cells were fixed

and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma). TRAP-positive ($TRAP^+$) cells appeared dark red. $TRAP^+$ multinucleated cells (MNC) containing at least three nuclei were counted as osteoclasts.

In one set of experiments, PRS was replaced by human thrombin at 2 U/ml (Sigma). In another set of experiments, a pan-specific TGF-β neutralizing antibody (AB-100-NA; R&D Systems; 0.5 μg/ml of the antibody neutralizes 50 % of the bioactivity of 0.25 ng/ml TGF-β) at a maximum concentration of 5 μg/ml was added to the culture medium. To test the specificity of the antibody, PRS was spiked with recombinant human TGF-β (240-B, R&D Systems). For immunoprecipitation of TGF-β, PRS was incubated with the neutralizing antibody or a rabbit IgG (AB-105-C, R&D systems), both at 100 μg/ml, for 1 hour before being adsorbed and precipitated with Protein G immobilized on agarose (P7700, Sigma).

One-step Quantitative Reverse Transcription-PCR Analysis

Cellular RNA was extracted from spin columns (RNeasy, Qiagen). Reverse transcription (RT) and PCR were performed in a one-step methodology (SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit, Invitrogen) in a 7000 Real-Time PCR System (Applied Biosystems). The protocol was performed according to the one-step qRT-PCR Kit. The following primers were used [Yang et al., 2002; Schurigt et al., 2005]: matrix metalloproteinase-9 5′-cattcgcgtggataagaagt-3′ (sense primer); 5′-attttggaaactcacacgcc-3′ (antisense primer), cathepsin K 5′-gggccaggatgaaagttgta-3′ (sense primer); 5′-cactgctctcttcagggctt-3′ (antisense primer), TRAP 5′- gggagtggcccgcactcagctgtcc-3′ (sense primer); 5′- ggcagcgcgtgggtccgtgct-3′ (antisense primer), calcitonin receptor 5′- acaactgctggctgagtg-3′ (sense primer); 5′-gaagcagtagatagtcgccac-3′ (antisense primer), RANK 5′-cgaggaagattcccacagag-3′ (sense primer); 5′-agtgaagtcacagccctca-3′ (antisense primer), and β-actin 5′-ccacagctgagagggaaatc-3′ (sense primer); 5′-tctccagggaggaggaggat-3′ (antisense primer). RT-PCR conditions were 1 cycle of 50°C for 3 min followed by 1 cycle of 95°C for 5 min; 40 cycles of 95°C for 15 s, and 60°C for 30 s, followed by a final cycle of 40°C for 1 min. Ct value was used to calculate the fold-up regulation by substracting the Ct value for β-actin from the Ct value for the target gene, and comparing the experimental (E) result with the control (C) using the following equation: fold-upregulation $= 2^{-}\Delta\Delta Ct$, where $\Delta\Delta Ct = \Delta E^{-}\Delta C$, $\Delta E =$ CtE target$-$CtE β-actin and $\Delta C =$ CtC target$-$CtC β-actin. PCR amplification was performed in triplicates.

Cell Signaling Analysis by Western Blot and Pharmacologic Inhibitors

For Western blot analysis, stroma-depleted bone marrow cells were grown in 6-well plates in the presence of M-CSF at 30 ng/ml for 5 days followed by incubation with RANKL at 30 ng/ml or PRS I, or their combination for 15 min. Cells were lysed in SDS-buffer containing protease inhibitors and debris was eliminated by centrifugation. Cell extracts were separated by SDS–PAGE and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked in Tris-buffered saline, 0.1% Tween-20 with 5% w/v nonfat dry milk and incubated with a 1:1,000 dilution of antibodies against pERK1/2 (clone E-4, Santa Cruz Biotechnology, Santa Cruz, CA), ERK1 (K-23, Santa Cruz), pp38 MAPK (#9211, Cell Signaling Technology Inc., Beverly, MA), p38 MAPK (C-20, Santa Cruz), pJNK (#9251, Cell Signaling), JNK (C-17, Santa Cruz), pAkt (Ser473) (#9271, Cell Signaling), pAkt (Thr308) (#9275; Cell Signaling), pSmad-2 (#3101, Cell Signaling), and Smad-2/3 (#3102; Cell Signaling). The primary antibody was detected with the appropriate secondary antibody (Dako, Glostrup, Denmark) using the enhanced chemiluminescence method (Amersham). Blocking of p38 and JNK signaling was performed with SB203580 (Alexis Corporation, San Diego, CA) and SP600125 (Calbiochem, San Diego, CA), respectively. To evaluate cytotoxicity of the inhibitors, stroma-depleted bone marrow cells were incubated with SB203580 and SP600125 at 5–20 μM for 24 h and subjected to MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/ml; Sigma) assay. Formazan crystals were solubilized in dimethylsulfoxide and optical density was determined with a microplate reader.

Statistical Analysis

Statistical analysis was based on triplicates of at least three experiments with platelets from different donors. Data appearing in Figure 1A were analyzed by unpaired Student's *t*-test. A multiple comparison procedure to discriminate

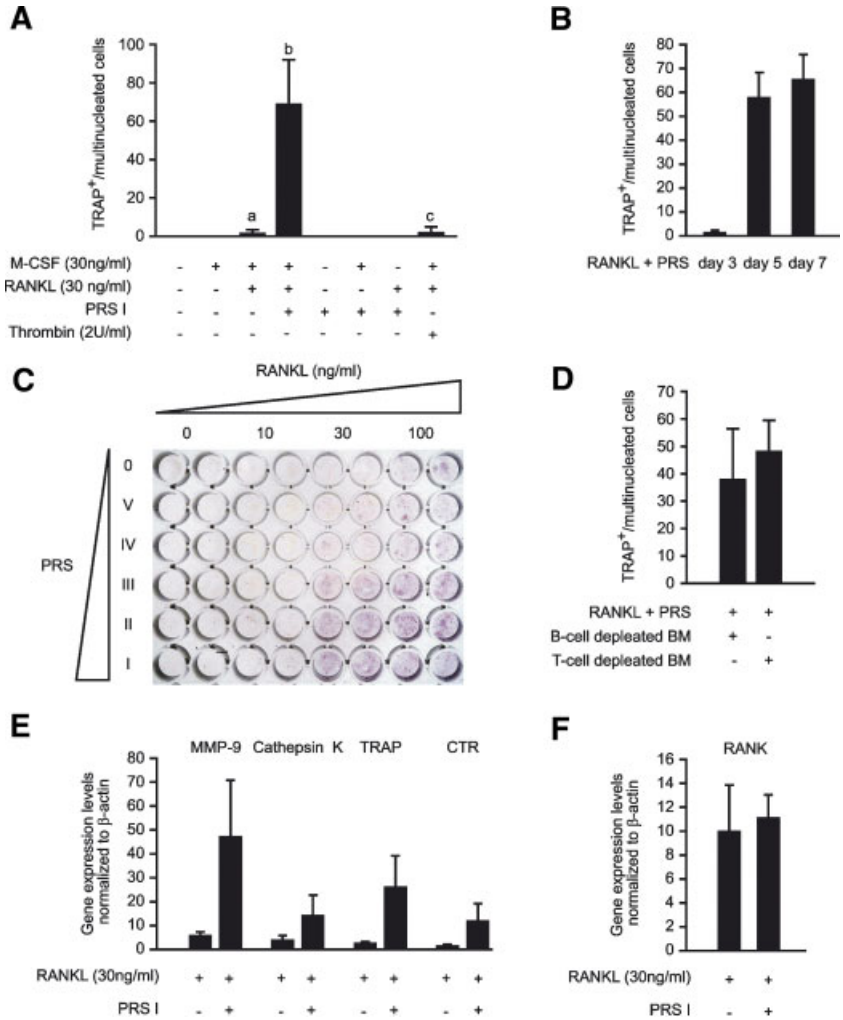

Fig. 1. Osteoclastogenesis in the presence of platelet releasates and permissive levels of RANKL. **A:** Nonadherent M-CSF-dependent bone marrow cells were cultured with different combinations of M-CSF, RANKL, platelet releasates supernatant (PRS), or thrombin with medium change on day 3 and day 5. After 7 days of culture, multinucleated cells staining positive for tartrate resistant acid phosphatase (TRAP) were counted. **B:** TRAP staining was also performed on day 3 and day 5. **C:** Dose-response experiments showed a maximum staining intensity for TRAP with RANKL at 30 ng/ml and the two highest concentrations of PRS. **D:** Osteoclastogenesis was observed in marrow cells depleted of B-cells and T-cells by magnetic beads linked to the antibodies Thy1.2 and B220. **E:** One-step quantitative reverse transcription-PCR analysis of the expression levels of osteoclast genes; matrix metalloproteinase-9 (MMP-9), cathepsin K, TRAP, calcitonin receptor (CTR), and RANK (**F**) in bone marrow cultures following incubation with M-CSF and RANKL, in the presence and absence of PRS. Bars represent the mean ± SD of three replicates from two representative experiments. $^{a\ versus\ b}P < 0.05$, $^{a\ versus\ c}P > 0.05$, $^{b\ versus\ c}P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

among the means by the Fisher's least significant difference (LSD) procedure was performed for all other data. The results are expressed as mean + standard deviation. Significance was assigned at the $P < 0.05$ level.

RESULTS

PRS Enhances Osteoclastogenesis in the Presence of Permissive Levels of RANKL

PRS at concentrations corresponding to physiologic platelet counts in the blood synergized with RANKL to stimulate osteoclast differentiation. PRS or thrombin alone, even in the presence of M-CSF did not induce osteoclastogenesis (Fig. 1A). Osteoclastogenesis reached a maximum on day 5 and the number of TRAP$^+$/MNCs remained constant until day 7 (Fig. 1B). Dose-response experiments showed a maximum staining intensity for TRAP with RANKL at 30 ng/ml and the two highest concentrations of PRS (Fig. 1C). Osteoclastogenesis was also observed in marrow cells depleted of B-cells and T-cells (Fig. 1D). The supplementation of the

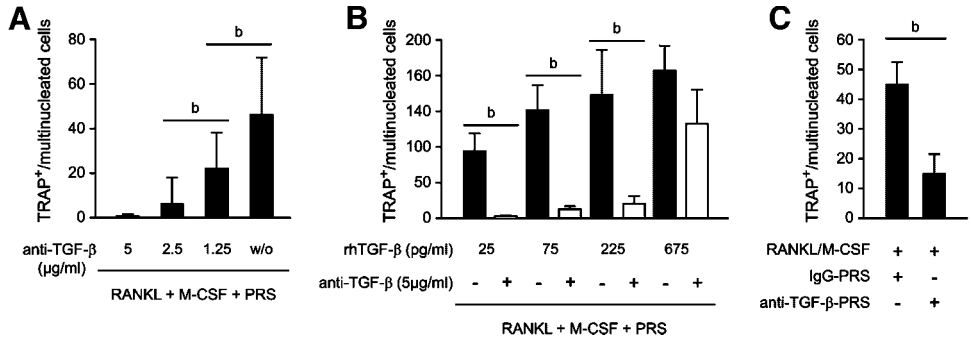

Fig. 2. Role of TGF-β in osteoclastogenesis. To determine the role of TGF-β in platelet-released supernatant (PRS)-induced osteoclastogenesis, experiments were performed in the presence of a neutralizing antibody. The TGF-β neutralizing antibody profoundly attenuated osteoclastogenesis (**A**). To test the specificity of the antibody, PRS was spiked with recombinant human TGF-β. Osteoclastogenesis was abrogated by the neutralizing antibody raised against TGF-β (**B**). For depletion of TGF-β from PRS, immunoprecipitation with the neutralizing antibody or a rabbit IgG, both at 100 μg/ml, was performed. Bars represent the mean ± SD of three replicates from four (A) and two (B) representative experiments. $^{b}P < 0.05$.

culture medium with PRS caused an increased expression of MMP-9, cathepsin K, TRAP, and calcitonin receptor (Fig. 1E). The expression level of RANK remained unchanged upon incubation of the cells with PRS (Fig. 1F).

Role of TGF-β in Osteoclastogenesis

To determine the role of TGF-β in PRS-induced osteoclastogenesis, experiments were performed in the presence of a neutralizing antibody. The TGF-β neutralizing antibody at a concentration of 5 μg/ml profoundly attenuated osteoclastogenesis (Fig. 2A). Recombinant TGF-β dose-dependently increased osteoclastogenesis and the effect was abrogated by the neutralizing antibody raised against TGF-β (Fig. 2B). Depletion of TGF-β from PRS decreased its potential to stimulate osteoclastogenesis (Fig. 2C).

Cell Signaling in Osteoclastogenesis

Western blot analysis showed that incubation of bone marrow cells with PRS increased the phosphorylation of p38, JNK and Smad-2. Phosphorylation of AKT at Ser473 and Thr308 as well as ERK were not visibly changed (Fig. 3A). To determine whether the enhanced phosphorylation status of p38 and JNK mediates the effect of PRS on osteoclastogenesis the kinase-specific inhibitors SB203580 and SP600125 were added to the culture medium. SB203580 abolished osteoclast formation, whereas blocking of JNK signaling with SP600125 had no effect under these culture conditions (Fig. 3B). Viability of M-CSF-dependent marrow cells was not influenced by SB203580 and SP600125 at concentrations used in the osteoclast culture (Fig. 3C).

DISCUSSION

Activated platelets can induce osteoclastogenesis in murine bone marrow cultures containing hematopoietic progenitors and osteogenic cells of the mesenchymal lineage [Takahashi et al., 1988; Gruber et al., 2002]. Findings from this model suggest that PRS can increase RANKL expression in osteogenic cells which in turn causes the differentiation of hematopoietic cells into osteoclasts [Gruber et al., 2002]. Possible direct effects of PRS on osteoclastogenesis cannot be determined with this model. Based on a model with stroma-depleted bone marrow cells [Fuller et al., 2000; Lam et al., 2000], we observed direct effects of PRS on osteoclastogenesis when permissive levels of RANKL were present. The target cells are likely to be osteoclast progenitors, as the PRS also increased osteoclastogenesis in B-cell and T-cell depleted cell preparations. Whether or not PRS can change the expression of RANKL on B-cells and T-cells, thereby indirectly modulating osteoclastogenesis, remains to be determined [Kawai et al., 2006].

TGF-β was considered a candidate molecule to account responsible for the observed effect as TGF-β is released during platelet aggregation and is activated by a furin-like proprotein convertase which is simultaneously released [Blakytny et al., 2004]. Recombinant TGF-β can directly stimulate osteoclastogenesis [Fuller et al., 2000; Massey et al., 2001; Quinn et al., 2001]. We found that neutralization of TGF-β in

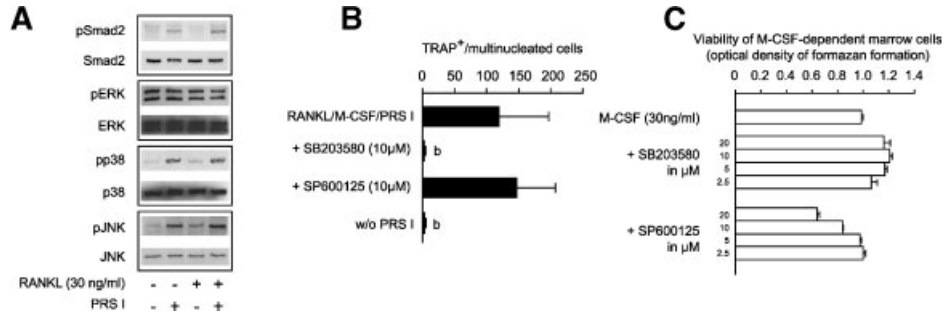

Fig. 3. Characterization of signaling pathways responsible for osteoclastogenesis. **A:** Bone marrow cells were cultured in the presence of M-CSF for 5 days before they were exposed to RANKL, platelet-released supernatant (PRS), or their combination for 15 min. Phosphorylated and unphosphorylated proteins were detected by Western blot analysis. **B:** Bone marrow cells were continuously exposed to RANKL, M-CSF, and platelet-released supernatant (PRS), with and without SB203580 (p38) and SP600125 (JNK). After 7 days of culture the number of multinucleated cells staining positive for TRAP was counted. **C:** Bone marrow cells were incubated with SB203580 and SP600125 for 24 h and subjected to MTT assay. Optical density of solubilized formazan crystals was determined. Bars represent the means ± SD of three replicates from two representative experiments. $^{b}P < 0.05$.

the culture medium substantially decreased osteoclastogenesis suggesting a key function of the pleiotropic molecule in this in vitro model. However, these blocking experiments do not rule out that TGF-β being produced by the bone marrow cells or supplied with serum might be essential for osteoclastogenesis. Other platelet-derived molecules could have mediated the osteoclastogenic effect [Kaneda et al., 2000]. We therefore immunoprecipitated TGF-β out of PRS and found a reduction in the osteoclastogenic potential when compared to preparations of PRS depleted with IgG control antibodies. The neutralizing capacity of the antibody could be confirmed by its ability to reduce osteoclastogenesis provoked by recombinant TGF-β. Moreover, the effects of PRS occurred independent of endogenous prostaglandin synthesis (data not shown), as observed with TGF-β in explant cultures and with hematopoietic progenitors and RANKL [Lerner, 1996; Quinn et al., 2001]. Moreover, Smad-2, which mediates signals from TGF-β receptors, was increasingly phosphorylated upon incubation of the bone marrow cells with PRS. Overall these findings suggest that at least part of the observed activity of the PRS on osteoclastogenesis is mediated by TGF-β.

Although not as characteristic as Smad-2 signaling for TGF-β, osteoclastogenesis enhanced by PRS may also involve mitogen-activating protein kinases and PI3K/AKT signaling [Lee et al., 2002; Boyle et al., 2003; Takayanagi, 2005]. Increased activation of p38 and JNK signaling in osteoclast progenitors suggests that the effects of PRS are similar to those of recombinant TGF-β [Koseki et al., 2002; Karsdal et al., 2003]. PRS increased p38 and JNK phosphorylation in hematopoietic progenitors but are not sufficient to induce osteoclastogenesis. Results from experiments with pharmacologic inhibitors indicate that only p38 was essential for osteoclastogenesis. Transgenic animal models and in vitro studies based on higher concentrations of the JNK-inhibitor, however, suggest that the JNK signaling pathway is also relevant in osteoclastogenesis [Ikeda et al., 2004; Lee et al., 2006]. Under conditions of chronic inflammation, blocking of p38 signaling in models of rheumatoid arthritis prevents inflammatory bone destruction [Medicherla et al., 2006; Zwerina et al., 2006], whereas destructive arthritis was not modulated in animal models with a JNK negative background [Koller et al., 2005]. We also observed that the phosphorylation status of ERK and AKT was not further increased by PRS. Both pathways were however constitutively phosphorylated, probably as a result of M-CSF in the culture medium, and may be involved in osteoclast survival and aspects required for resorption activity [Nakamura et al., 2003].

The question on whether or not these in vitro findings represent a pathophysiological mechanism of osteolysis associated with chronic inflammation and platelet activation remains open. This possible link is, however, reasonable to suggest because chronic inflammatory reactions as observed in rheumatoid arthritis [Endresen, 1981; Myers and Christine, 1982; Farr

et al., 1984; Endresen, 1989] and periodontal disease [Steinberg et al., 1995] are associated with accumulation and activation of platelets. Moreover, TGF-β levels are elevated in synovial fluid of patients with rheumatoid arthritis [Brennan et al., 1990; Schlaak et al., 1996; Lettesjo et al., 1998] and an increased concentration of TGF-β1 was observed in the gingival tissues as well as in fluid samples obtained from periodontal pockets [Skaleric et al., 1997]. In addition, megakaryocytes as the source of platelets are considered to inhibit osteoclastogenesis [Kacena et al., 2006]. Hence, our in vitro model provides only a limited insight into the possible interaction of platelets and osteoclasts in vivo. Our data indicate that platelets can directly target osteoclast progenitors and stimulate osteoclastogenesis in the presence of RANKL by a process involving TGF-β and activation of p38 signaling.

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