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Secretion of PDGF isoforms during osteoclastogenesis and its modulation by anti-osteoclast drugs

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ABSTRACT

In an attempt to identify secretory products of osteoclasts that mediate the coupling of bone formation to resorption, we found that along with osteoclast differentiation, PDGF-A gene expression increase occurred first, by 12 h after stimulation of bone marrow macrophages with M-CSF and RANKL, and peaked at 36 h. This was next followed by a progressive increase in PDGF-B gene expression until a peak at 60 h, when mature osteoclasts formed. Isoform-specific ELISA of the conditioned medium collected every 24 h revealed that all three of the isoforms of PDGF-AA, AB and BB were secreted, in this temporal order as differentiation proceeded. Their secretion was enhanced when osteoclasts were activated by placing them on dentin slices. The secretion of all three isoforms was decreased in cathepsin K-deficient osteoclasts compared with wild-type osteoclasts. Pharmacological inhibition of cathepsin K with odanacatib also inhibited the secretion of all three isoforms, as was also the case with alendronate treatment. The secretion of sphingosine-1-phosphate, which increased during osteoclastogenesis, was reduced from cathepsin K-deficient osteoclasts, and was inhibited by treatment with odanacatib more profoundly than with alendronate. Thus, all three isoforms of PDGF, which are secreted at distinct differentiation stages of osteoclasts, appear to have distinct roles in the cell–cell communication that takes place in the micro-environment of bone remodeling, especially from the osteoclast lineage to mesenchymal cells and vascular cells, thereby stimulating osteogenesis and angiogenesis.

1. Introduction

Bone remodeling is a fundamental process by which the quality as well as the quantity of bone is maintained after skeletal maturity [1]. It is a complex process involving a multitude of cell types and biological activities, especially the resorption of calcified matrix by osteoclasts and the subsequent filling-in with essentially the same amount of new bone by osteoblasts. The most dynamic and critical step in the whole process is the reversal from the destructive to constructive phase, or the “coupling” of formation to the preceding resorption. A decline in this coupling function with aging underlies the development of osteoporosis and fragility fractures, and is therefore an especially attractive target for intervention. Thus, the factors mediating the coupling process and the mechanisms involved have attracted considerable attention since the 1970's [2].

The release and activation of matrix-embedded growth factors during bone resorption, such as TGF-β and IGF-I, have long been favorite candidates, and have recently gained some experimental support from mouse genetics studies [3,4]. In addition, emerging evidence points to the involvement of “clastokines”, or certain secretory products of osteoclasts, in the communication from osteoclasts to osteoblasts, and the number of reported clastokines has recently been increasing [5].

Following the reports on the roles of TGF-β and IGF-I in bone coupling [3,4], Xu Cao et al. reported in 2014 that the platelet-derived growth factor (PDGF)-BB secreted by pre-osteoclasts was a potential coupling factor [6]. They reported that PDGF-AA is not secreted, and that PDGF-BB is secreted mainly by pre-osteoclasts rather than mature osteoclasts [6]. In the course of searching by microarray profiling for potential coupling factors derived from osteoclasts, we had previously found a progressive increase in PDGF-B as well as PDGF-A gene expression during osteoclastogenesis, which prompted us to study in more detail the production and secretion of PDGF isoforms and their modulation by the anti-resorptive drugs alendronate and odanacatib, which are known to affect the coupling process in opposite directions [7].

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2. Materials and methods

2.1. Reagents

Alendronate sodium hydrate and odanacactib were purchased from Teijin Pharma Ltd. (Osaka, Japan) and Medchemexpress Co., Ltd. (Princeton, NJ), respectively. Odanacatib was dissolved in DMSO. Recombinant murine M-CSF and GST-RANKL were prepared as described previously [8].

2.2. Osteoclastogenesis ex vivo

Bone marrow macrophages (BMM) were selected from bone marrow cells freshly isolated from 8 to 10-week-old C57BL/6 mice (Clea Japan Inc., Shizuoka, Japan) and cultured in αMEM (Gibco, Life Technologies Corp., Carlsbad, CA, USA) containing 10% FBS, 1/10 vol. of CMG culture supernatant as a source of M-CSF and penicillin/streptomycin (Gibco) as described previously [9]. BMM were then stimulated with RANKL (100 ng/ml) in the presence of M-CSF, and osteoclasts were identified by TRAP staining. In some experiments BMM isolated from cathepsin K-deficient mice were used to generate the osteoclasts, as described previously [10]. In collecting the conditioned medium for the determination of PDGF concentrations, BMM were plated at 20,000 cells/96-well plate. Experiments were performed in accordance with NCGG ethical guidelines for animal care, and the experimental protocols were approved by the animal care committee.

2.3. RNA extraction and RT-PCR

Total RNA was isolated from cells with the RNeasy mini kit (Qiagen, Hilden, Germany), and isolated RNAs were reverse-transcribed using a High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). For quantitative PCR, samples were analyzed using PowerSYBR Green PCR master mix and an ABI7300 real time PCR system (Applied Biosystems); the primers used are summarized in Supplemental Table 1. The relative abundance of each target mRNA was normalized by that of β-actin mRNA. Data of the previous microarray analysis, performed using the mouse genome 430 2.0 Array GeneChip (Affymetrix) and Affymetrix GeneChip Operating Software version 1.1 [11], were extracted from the deposition in GEO (accession no. GSE45656).

2.4. Assays for PDGF isoforms, CTX and S1P

The PDGF-AA, AB and BB concentrations in the conditioned medium were determined by Quantikine ELISA kits for Human/Mouse PDGF-AA Immunoassay, Mouse/Rat PDGF-AB Immunoassay and Mouse/Rat PDGF-BB Immunoassay (R&D Systems, Inc., Minneapolis, MN), respectively. The CTX and S1P concentrations in the culture medium were determined using a CrossLaps assay (Nordic Bioscience Diagnostics) and a Sphingosine 1 Phosphate Assay Kit (Echelon Biosciences Inc., Salt Lake City, UT), respectively.

2.5. Statistical analysis

Quantitative data are presented as means ± SD and were analyzed with Student's *t* test. A *P* value of <0.05 was considered statistically significant.

3. Results

Microarray analysis at distinct stages of osteoclast differentiation revealed the PDGF-A gene was expressed at low levels, while PDGF-B gene expression increased progressively as the process moved toward mature osteoclasts; the PDGF-C and D genes were not expressed (Supplemental Fig. 1). Detailed analysis was then performed with RNA harvested every 12 h throughout the typical 4-day course of osteoclastogenesis *ex vivo* [12]; following this schedule, bone marrow macrophages (BMM) became committed pre-osteoclasts by 48 h and mature osteoclasts formed at approximately 60 h. Quantitative PCR revealed that PDGF-A mRNA had increased significantly by 12 h, peaked at 36 h and declined rapidly thereafter, while PDGF-B mRNA progressively increased until 60 h, when mature osteoclasts formed (Fig. 1A). We also found that PDGF-R α and β gene expression was induced rapidly by 12 h and declined sharply after 36 h (Fig. 1B).

In order to assess the secretion of PDGF proteins, we employed isoform-specific ELISA. As shown in Supplemental Fig. 2, preliminary analysis with ELISA revealed PDGF-BB secretion was markedly increased during the second half of the period, 48–96 h. Significantly more PDGF-AB was detected in the second half as well, while more PDGF-AA was detected in the first half, i.e. 0–48 h. The concentration of all three isoforms in the culture medium with 10% FBS was zero (data not shown), indicating no cross-reactivity of the ELISA with the bovine PDGF isoforms derived from FBS. In order to see the time course of PDGF secretion in more detail, we collected the conditioned medium every 24 h, starting with the stimulation of BMM with RANKL and M-CSF at 0 h. As shown in Fig. 2A, PDGF-AA secretion peaked during the period of 24–48 h before the pre-osteoclast stage, followed by PDGF-AB, which peaked during 48–72 h; PDGF-BB secretion increased substantially after 48 h and remained high during 72–96 h after the formation of mature osteoclasts. The secretion of each isoform was further increased when osteoclasts were formed on dentin slices, i.e. when osteoclasts were actively engaged in resorption (Fig. 2B).

We first examined the effect of cathepsin K inhibition on PDGF secretion by a pharmacological approach. Since PDGF is a potential mediator of bone coupling [6] and since it is known that treatment with odanacatib (ODN), unlike alendronate (ALN), does not impair coupling, but rather maintains bone formation by osteoblasts [7], it would be expected that the secretion of PDGF as a coupling factor would be impaired by ALN, but not by ODN. Treatment of osteoclastogenic cultures with increasing doses of ODN had no effect on the differentiation of osteoclasts, as determined by TRAP staining (Supplemental Fig. 3A); at 300 nM ODN, osteoclast formation appeared only slightly decreased. In contrast, ODN inhibited bone resorption dose-dependently, as determined by CTX assay with the conditioned medium (Supplemental Fig. 3B). Based on these results, we used the submaximal dose of 200 nM in the subsequent experiments in comparing the effect of ODN with that of ALN on the secretion of PDGF isoforms. ALN markedly inhibited bone resorption at 1 μM, while it did not exhibit any toxic effect toward osteoclast differentiation until the dose of 5 μM (Supplemental Fig. 4). Therefore, we used the dose of 1 μM in analyzing the effect of ALN on PDGF secretion. As shown in Supplemental Fig. 5, ALN at 1 μM inhibited the secretion of PDGF-AA and BB significantly, while ODN at 200 nM was comparable to ALN in inhibiting the secretion of PDGF-AA and BB. The secretin of PDGF-AB was least affected by ALN or ODN treatment (Supplemental Fig. 5).

We also adopted a genetic approach, and made cathepsin K-deficient osteoclasts by harvesting the BMM from cathepsin K-deficient as well as wild type mice. As shown in Fig. 3A, the absence of cathepsin K had no effect on osteoclast differentiation per se, while bone resorption by cathepsin K-deficient osteoclasts was reduced by 62–80% compared with wild-type osteoclasts, as determined by CTX in the conditioned medium (Fig. 3B). Consistent with the results of odanacatib treatment, the secretion of PDGF-AA and BB isoforms by cathepsin K-deficient osteoclasts was 35–38% lower than that by wild-type osteoclasts (Fig. 3C); the reduction in

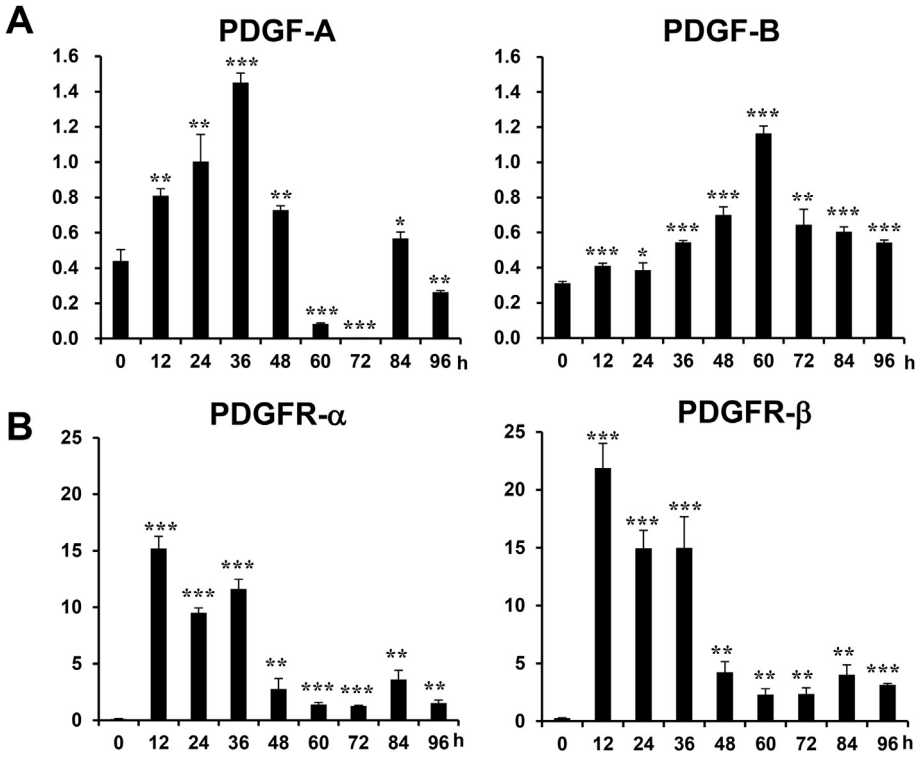

Fig. 1. PDGF (A) and PDGFR (B) gene expression during osteoclastogenesis. RNA was isolated every 12 h at the indicated times and mRNA expression was quantified by RT-PCR as described in the Methods. The abundance of target mRNA was normalized by that of β-actin mRNA. Data are shown as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 0 h.

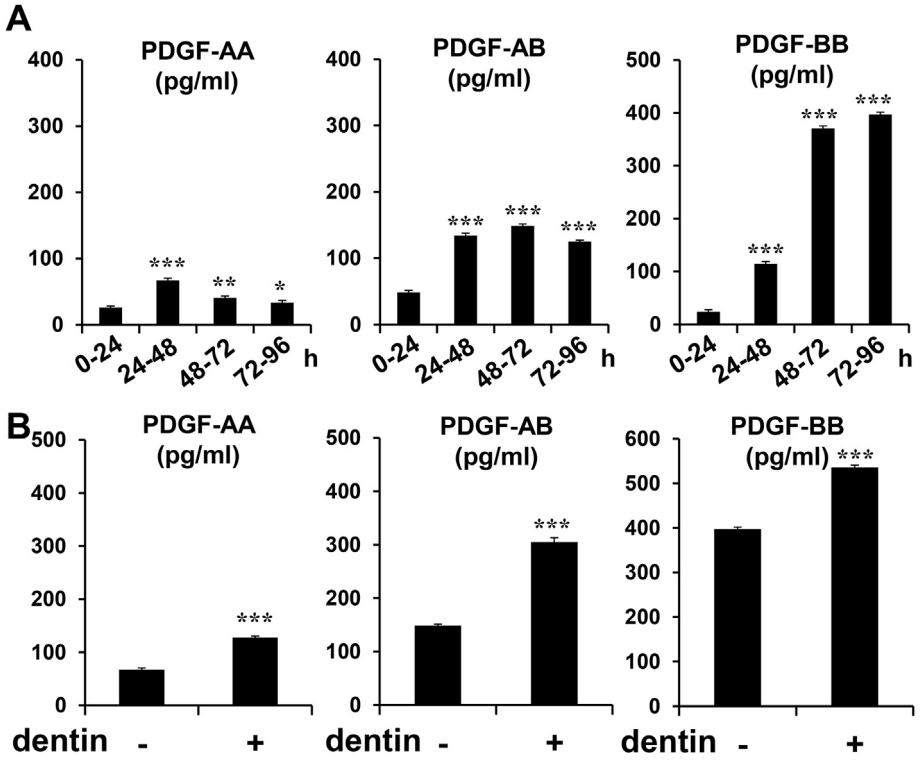

Fig. 2. All three isoforms of PDGF-AA, AB and BB are secreted along with osteoclast differentiation. After the stimulation of BMM with M-CSF and RANKL, the conditioned medium was collected every 24 h as indicated and the concentration of each PDGF isoform was determined by isoform-specific ELISA, as described in the Methods (A). Data are shown as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 0–24 h. In B, osteoclasts were formed on dentin slices (+dentin), while the concentration of PDGF-AA, AB and BB (72–96 h) was compared with that in osteoclasts without dentin (-dentin). Data are shown as the mean ± SD (n = 3). $^{***}p < 0.001$ vs. − dentin.

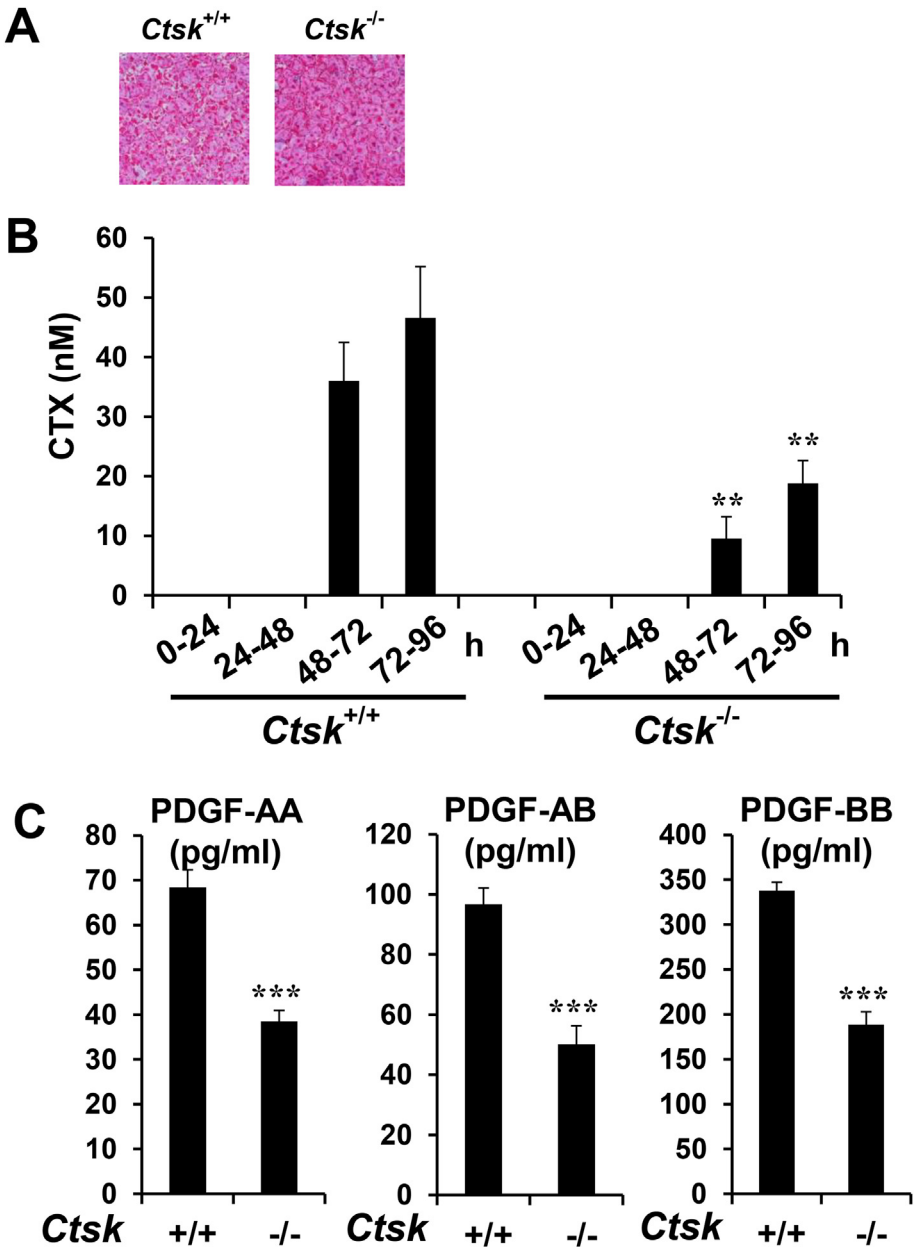

Fig. 3. Reduced PDGF secretion from cathepsin K-deficient osteoclasts. Osteoclasts differentiate normally from BMM isolated from cathepsin K-deficient mice (A); however, they are defective in bone resorption, as determine by CTX assay (B). In C, the concentration of each PDGF isoform in the conditioned medium from wild-type (+/+) and cathepsin K-deficient osteoclasts (−/−) was determined by isoform-specific ELISA, as described in the Methods. Data are shown as the mean ± SD (n = 3). **p < 0.01, ***p < 0.001 vs. *Ctsk*$^{+/+}$.

PDGF-AB secretion by cathepsin K-deficient osteoclasts was more substantial than that by pharmacological inhibition with ODN.

Since PDGF production is linked to sphingosine-1-phosphate (S1P) in certain cell types, such as vascular smooth muscle cells [13] and since S1P is another coupling factor candidate [14,15], we also examined the secretion of S1P during osteoclastogenesis using a newly developed ELISA with a specific antibody against S1P [16]. As shown in Fig. 4A (left), S1P concentration in the conditioned medium increased progressively during osteoclastogenesis, and the secretion was increased substantially when osteoclasts were placed on dentin slices (Supplemental Fig. 6). The secretion of S1P by cathepsin K-deficient osteoclasts was decreased significantly compared with wild-type osteoclasts throughout the differentiation process (Fig. 4A, right). ALN at 1 μM inhibited the secretion of S1P by 35%, and ODN exerted a more profound effect than ALN, with 80% inhibition at 200 nM (Fig. 4B); under the same experimental conditions, ALN at 1 μM was comparable to ODN at 200 nM in inhibiting bone resorption, as determined by the CTX assay (Fig. 4C).

4. Discussion

PDGF was identified in the 1970's as a serum growth factor for fibroblasts, smooth muscle cells and glial cells [17]. The PDGF family includes four genes, PDGF-A, B, C and D, the former two of which produce three isoforms, PDGF-AA, AB and BB. These ligands act via two receptor tyrosine kinases, PDGFR-α and PDGFR-β, and the possible ligand–receptor interactions are both multiple and complex, although there is functional evidence for certain preferential

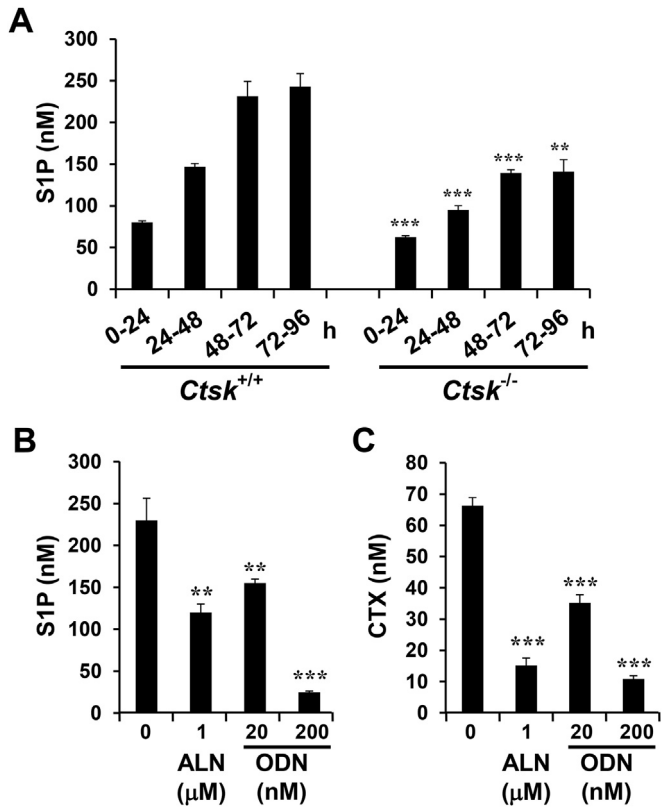

Fig. 4. Odanacatib as well as alendronate inhibits S1P secretion. After the stimulation of BMM from wild-type (*Ctsk*$^{+/+}$) and cathepsin K deficient (*Ctsk*$^{-/-}$) mice with M-CSF and RANKL at 0 h, the conditioned medium was collected every 24 h, and the concentration of S1P was determined by ELISA, as described in the Methods (A). In B and C, after the stimulation of BMM with M-CSF and RANKL, the cells on dentin slices were treated once with the indicated doses of odanacatib (ODN) or alendronate (ALN) at 48 h for collecting the conditioned medium during 72–96 h for S1P (B) and CTX (C) assays. Data are shown as the mean ± SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle control.

combinations *in vivo*, such as PDGF-AA acting via PDGFR-α, and PDGF-BB via PDGFR-β [17].

Contrary to the recent report that PDGF-BB, not AA, is secreted mainly by pre-osteoclasts and not mature osteoclasts [6], we found in the present study that all three of the isoforms of PDGF-AA, AB and BB are secreted. Most importantly, they are secreted in this temporal order as osteoclast differentiation proceeds (Supplemental Fig. 7). In view of the findings that PDGF-A mRNA expression increased earlier and reached peak at 36 h, followed by progressive increase in PDGF-B mRNA expression until 60 h, it is assumed that the increased secretion reflects the increased intracellular production of each isoform. Since PDGFR-α and β are expressed transiently in the early part of the process during commitment to pre-osteoclasts, it is conceivable that osteoclast differentiation is modulated by PDGF in an autocrine or paracrine fashion. The more plausible target cells of osteoclast-derived PDGF are thought to be skeletal stem cells (SSC) and vascular mural cells (Supplemental Fig. 7). PDGFR-α has been used as a marker for mesenchymal [18] or SSC [19], which have the capacity to differentiate into osteoblasts, chondrocytes and adipocytes. Thus, PDGF-AA derived from early osteoclast progenitors can act on SSC by binding to the preferred PDGFR-α, and then contribute to osteogenesis when they are stimulated toward osteoblastogenesis by cues from the microenvironment (Supplemental Fig. 7).

PAGF-BB, which is mainly provided by mature osteoclasts as well as pre-osteoclasts, exerts an effect on perivascular mesenchymal cells, such as smooth muscle cells and pericytes, which evidently express PDGFR-β [17] (Supplemental Fig. 7). It has recently been reported that CD31^{hi}Endomucin^{hi} endothelial cells in the bone marrow are important for osteogenesis as well as angiogenesis [20], and that endothelial notch activity contributes to osteogenesis through the release of Noggin [21]. Thus, it is likely that osteoclast-derived PDGF-BB, by stimulating angiogenesis together with VEGF (which is also induced during osteoclastogenesis [12]), promotes osteogenesis indirectly, as proposed in a recent report [6]. The roles PDGF-PDGFR signaling directly plays in osteoblast lineage cells have been controversial, while several papers have reported mitogenic effects of PDGF-BB through PDGFR-β on mesenchymal stromal cells, anti-osteoblastogenic [22] and chemotactic effects toward osteoblastic cells *in vitro* [23], and also bone protective effects of systemically administered PDGF-BB in ovariectomized rats *in vivo* [24].

Given that PDGF is a candidate osteoclast-derived coupling factor, it is of interest to know how the secretion is modulated under drug treatment, especially ALN and ODN, which both target osteoclasts and yet affect coupling in opposite directions, negatively and positively, respectively. Although it was reported in a recent paper that the concentration of PDGF-BB in the serum or bone marrow of cathepsin K knockout mice, as well as wild-type mice treated with the cathepsin K inhibitor L-006235, was modestly higher than those in control mice [6], we observed in the current study that the secretion of all three isoforms by cathepsin K-deficient osteoclasts was reduced substantially compared with wild-type cells, and that the secretion of PDGF-BB as well as AA was inhibited by more than 40% by a single treatment with 200 nM odanacatib, a result similar to alendronate treatment. Thus, the cellular origin of the increased PDGF-BB in the bone marrow under cathepsin K inhibition remains to be determined.

S1P is a secretory product of osteoclasts and also a potential candidate mediator of bone coupling [14,15]. In the current study we confirmed by using a specific ELISA for S1P that its secretion increases progressively along with osteoclast differentiation. However, in contrast with the reports that cathepsin K-deficient osteoclasts secrete more S1P than control osteoclasts [25] and that cathepsin K-deficient pre-osteoclasts, as well as wild-type pre-osteoclasts treated with the cathepsin K inhibitor L-006235 secrete more S1P [6], we observed that the secretion of S1P by cathepsin K-deficient osteoclasts was reduced by more than 30% compared with wild-type cells, and that a single treatment with 200 nM odanacatib inhibited S1P secretion by 80%, even more profoundly than the effect of alendronate. Thus, in view of these data the maintenance of bone coupling under odanacatib treatment, in contrast to the suppressive effect of alendronate, cannot be fully accounted for by the amount of S1P secreted by osteoclasts.

In conclusion, we propose that all three of the PDGF isoforms derived from distinct osteoclastic differentiation stages have multiple roles in skeletal maintenance, with a direct effect on osteogenesis and an indirect effect via angiogenesis (Supplemental Fig. 7).

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.115.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.04.115.

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