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Platelet-Derived Growth Factor (PDGF)-BB Stimulates Osteoclastic Bone Resorption Directly: The Role of Receptor β[1]

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The growth-promoting activity of PDGF-BB was studied on the adult osteoclasts in the present study. The PDGF receptor β was detected on the osteoclast membrane through immunohistochemistry (LSAB method) and immunomicroscopy. The PDGF-BB was exerted on the osteoclasts that adhered to the bone slice at concentrations of 0, 10, 20, 30, and 40 ng/ml. The volume of Howship's lacuna augmented significantly and the number of resorption pits also increased with its dose ($p < 0.01$). The activity of both total acid phosphatase (ACP) and tartrate-resistant acid phosphatase (TRAP) increased significantly. These results suggest that PDGF-BB promotes adult osteoclastic bone resorption directly through PDGF receptor β and is believed to play important roles in the bone healing process and reconstruction.

Key Words: **PDGF-BB/PDGF β receptor; osteoclasts; ACP; TRAP.**

Platelet-derived growth factor is a dimmer composed of two related polypeptide chains, A and B, which are encoded by two distinct genes located in separate chromosomes (1, 2). All three possible isoforms of PDGF[3] has been identified, (AA, AB, BB), linked by disulfide bonds. Two types of PDGF receptor (PDGFR) subunits, α and β exist accordingly. It has been found that PDGFR-α binds all three isoforms of PDGF, but the PDGFR-β binds only to PDGF-BB (3–5). Generally, the PDGF-BB is thought the most potent mitogen among the three isoforms. Other researchers and we have found that the PDGF stimulates DNA synthesis and cell replication in primary cultures of osteoblast-enriched (ob) cells. Unfortunately, there is little focus on the PDGF and osteoclasts. In this study, we detected the PDGFR-anon the membrane of human osteoclasts and find that the PDGF-BB can promote the osteoclastic bone resorption directly.

MATERIALS AND METHODS

Materials. RPMI medium 1640 without phenol red and fatal bovine serum (FBS) was purchased from GibcoBRL, monoclonal antibody (mouse anti human) to PDGFR-Rβ and PDGF-BB were obtained from DAKO Co., LSAB kit was from Boster Co. (Wuhan, China). Gold particles (10 nm in diameter) conjugated with goat IgG anti mouse IgG were bought from Yat-Sen Biological Co. (Beijing). Collagenase II and the toludine blue were from Sigma Chemical Co. Acid phosphatase Kit was bought from bioMérieux (France).

Cell isolation procedures. The culture method used was described in detail previously (6). Briefly, the human iliac spongy bone was obtained from adult patients who needed to be operated fusion by spongy bone grafting (permitted by patients). After rinsing in 0.01 M phosphate-buffered saline (PBS, pH 7.2), the spongy bone was minced and then digested with collagenase II (1 mg/ml) for 90 min at 37°C. The cells were rinsed in PBS twice and suspended in the medium 1640. The cells were plated in culture plates in medium 1640 at the density of 10^4 cells/ml. At 4 h of culture, culture plates were rinsed with medium 1640 to wash away the unattached cells (7). The attached cells were collected again with a rubber policeman and suspended in medium 1640 without phenol red + 10% FBS. The phenol red was not used due to its faint estrogen effects (8).

Immunohistochemistry. The osteoclasts were plated on the coverglasses in the culture plates and then incubated at 37°C, 5% CO_2, 95% air. The coverglasses were taken out at 72 h. Rinsed in PBS, the samples were fixed in 10% formalin in PBS, and then immunohistochemically stained using LSAB kit. The monoclonal primary antibodies were diluted 1:200 in 1% BSA/PBS. DAB was used as the chrosmogen to color the reaction and the samples were finally counter-stained in hematoxylin.

Immunoelectronmicroscopy. The osteoclasts were taken out of culture flask at the third culture day and rinsed in PBS (0.01 M, pH 7.2). Then, monoclonal antibodies to PDGF R-β (100 ng/ml) were added to the cell suspension (10^5 cells/ml) before incubation at 37°C, 5% CO_2, 95% air for 5 min. After washed twice in PBS, cells were resuspended in medium at the same concentration, and the gold-labeled IgG (1:100) was added and incubated an additional 5, 10, and

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[3] Abbreviations used: PDGF, platelet-derived growth factor; ACP, acid phosphatase; TRAP, tartrate-resistant acid phosphatase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; LSAB, labeled-streptavidin-biotin.

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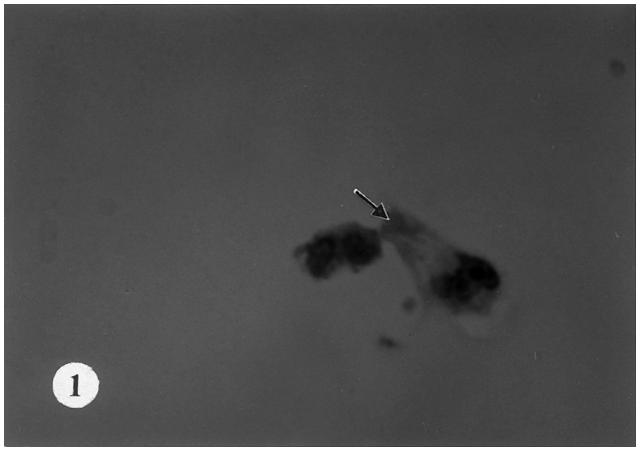

FIG. 1. Stained with LSAB, counter-stained in hematoxylin, the PDGFR-β was detected on the osteoclast because it was stained brown. DAB was used as the chrosmogen. ×650.

15 min at 37°C. Washed in PBS twice, then, the cells were centrifuged at 3000 rpm for 5 min in the Eppendorf tube (1.5 ml) and fixed in 2.5% glutaraldehyde 2 h. The followed procedures were according to Zhang and Piao (9), briefly, the sample was rinsed in PBS at 4°C for 6 h. Postfixed in 1% osmium tetroxide, dehydrated in serial acetone, embedded in Epon, ultrathin sections were cut, and observed under Hitachi H 7000 electron microscope.

Measurement of ACP and TRAP activity. Total acid phosphatase activity was measured by incubating samples of medium exposed to osteoclasts after centrifuged at 2000 rpm for 5 min at room temperature, with α-napthyl phosphate in citrate buffer (pH 5.4). Fast Red TR salt was then added to react with the products of ACP on the substrates and the azo dye produced to be used to calculate the enzyme activity. To measure the activity of TRAP, the sodium tartrate was added to the incubation to achieve a final concentration of 75 mM. The test was operated on the Olympus Au800 Random Access Chemistry Analyzer (Japan).

Resorption pits analysis. The method was followed as May and Gay (10). Briefly, the bone slices (20 μm) were prepared from human dense cortical bone, which was cut into pieces using a low speed saw cooled with cold water after the soft tissues were detached. Slices were cleaned by ultrasonication and rinsed in distilled water. Epoxy ethane was used to sterilize the slices 2 weeks before use. Cells were put onto the bone slices in RPMI medium 1640 + 10% FBS + PDGF-BB. Subsequent rinsing at 4 h and the same medium in 12-well culture dishes (5 slices/2 ml medium/per well) was added. At the 72-culture hour, the slices were taken out to rinse in PBS and stained for 2 min in 0.1% toludine blue in 0.1% sodium borate. Ultrasonic washed in 0.05M ammonium hydroxide for 5 min, the slices were stained for another 2 min in 0.1% toludine blue in 0.1% sodium borate. After the bone slices were dehydrated in 100% acetone for 5 min, the number and the area of Howship's lacuna was measured under the Leica Quantimet 500 system (Cambridge, British).

Statistics. All results are expressed as mean ± SD. Statistical significance is evaluated by one-way ANOVA test with the SPSS software.

RESULTS

Immunohistochemistry. The osteoclasts were positive for the immunohistochemical stain because that the cellular membrane was stained brown (Fig. 1). The cells had multiple nuclei, and the pseudopodia were seen clearly. Because of its high sensitivity and low level of background, the labeled-streptavidin-biotin (LSAB) technique has become one of the most widely used for histologic staining (11).

Immunoelectromicroscopy. The colloidal gold precipitated on the cellular membrane and the volume of the gold particles were homogeneous. Fewer gold particles were detected on the cells that incubated with gold-labeled IgG only for 5 min (Fig. 2). Much more gold particles were located on the cellular membrane after the secondary antibodies were added for 10 min (Fig. 3). At the 15 min of incubation, the cellular capsulated the gold particles to form coated pits (Fig. 4) and then endocytosed (Fig. 5).

Acid phosphatase release from isolated human osteoclasts. Culture medium (control) exposed to osteoclasts for 48 h on culture plate contained 1.65 U l^{-1} total acid phosphatase activity and among them 1.43 U l^{-1} was resistant to tartrate. Following the dose of PDGF-BB increased, the ACP activity rose from 1.65 to 2.88 U l^{-1} ($p = 0.014$) and the TRAP activity was from

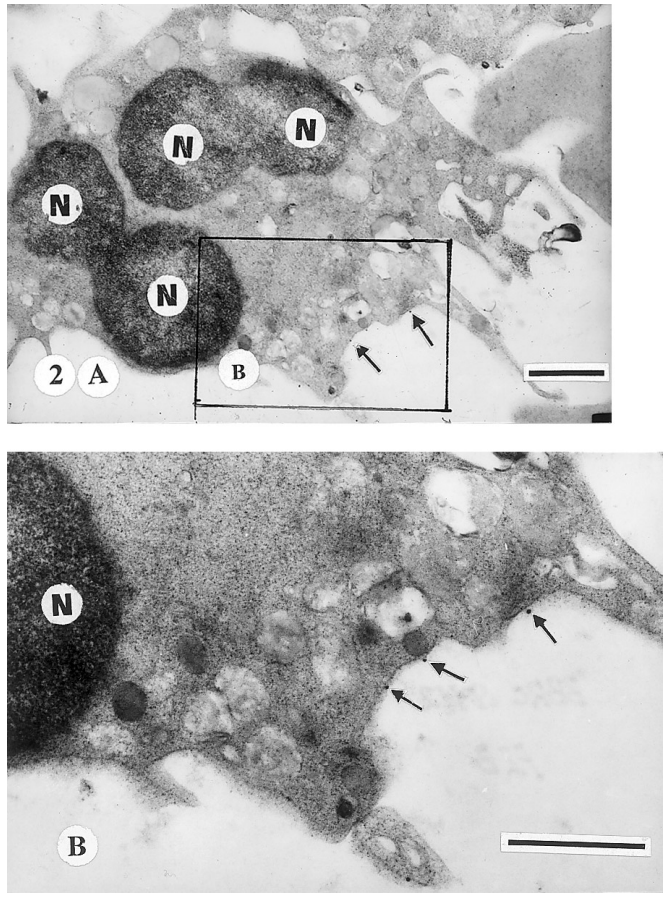

FIG. 2. (A) The cell had multiple nuclei and the pseudopodia was irregular. (B) Fewer gold particles (arrowheads) were present on the osteoclastic membrane after the gold-labeled IgG was added 5 min. Scale bar = 1 μm.

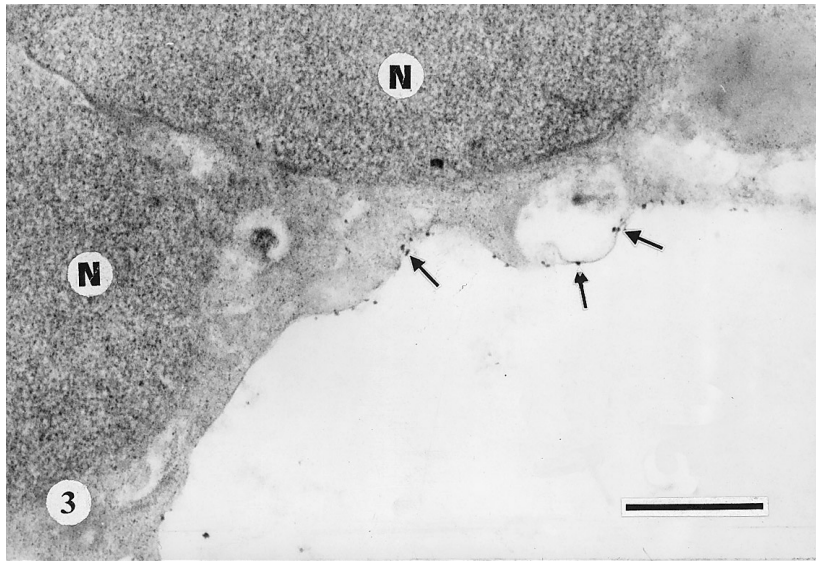

FIG. 3. Much more gold particles were located on the cellular membrane (arrows) at 10 min of incubation, but the distribution was irregular. Scale bar = 500 nm.

1.43 to 2.75U l^{-1}(p = 0.008) at the concentration of PDGF-BB of 40 ng/ml. Obviously, more than 80% of the ACP activity was resistant to the tartrate (Table 1).

Resorption pits analysis. The resorption lacuna were readily stained by toludine blue. Compared with the scanning electron microscopy, the toludine blue staining and the light microscopy would serve as a simple method for the quantitative assessment of bone resorption by osteoclasts (12). From the results, we can find both the area and the number of the Howship's lacuna were affected by the dose of PDGF-BB (Table 2).

DISCUSSION

Osteoclasts are multinucleated cells that derive from the mononuclear preosteoclasts of hematopoietic tissues. In the culture, the multinucleated cells attached to the culture flasks faster than that of osteoblasts and other cells. Therefore, the purity of osteoclasts after the unattached cells were washed away is very high, on the basis of image analysis of tartrate-resistant acid phosphatase stained multinucleated cells (4).

The PDGF R-β contains 1067 amino acid residues and a characteristic feature of the receptor is the split tyrosine kinase domain. Binding of PDGF-BB to the specific cell surface PDGFR-coinduces receptor dimerization and activation of their intrinsic tyrosine kinase activities. This leads to receptor autophosphorylation and the tyrosine phosphorylation of numerous other cellular proteins, which then initiate activation of diverse transduction, cascades culminating in DNA synthesis and cell

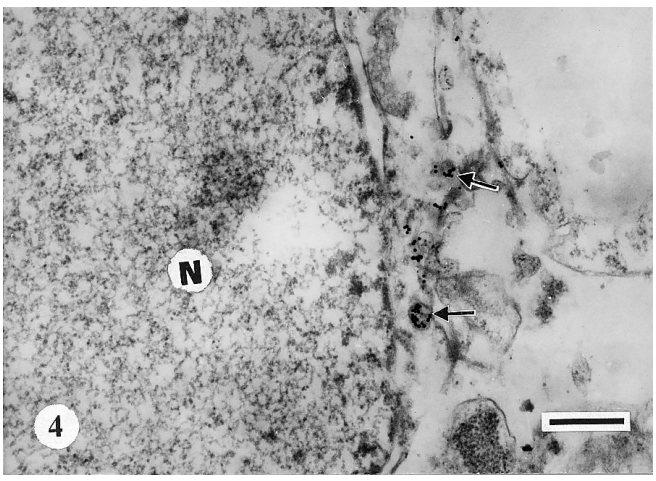

FIG. 4. Incubated for 15 min, gold particles were capsulated to form coated pits (arrows). Scale bar = 500 nm.

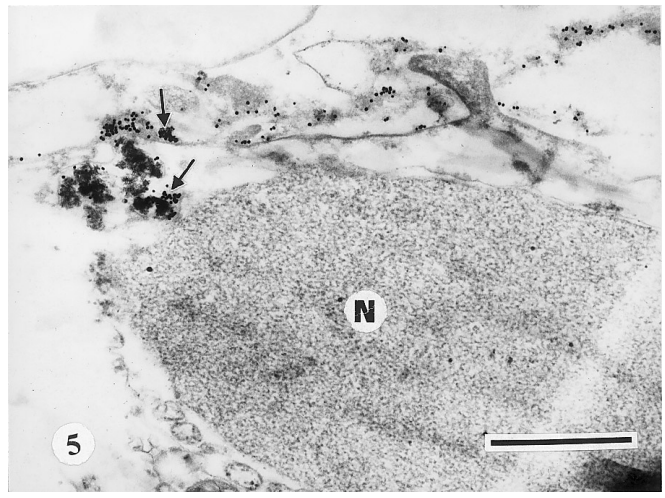

FIG. 5. Gold particles were endocytosed (arrows). Scale bar = 1 μm.

division(13). This process results in many kinds of enzymes to be synthesized and secreted, especially the ACP. ACP is an essential enzyme in the bone resorption process, and Zaidi et al found that osteoclastic bone resorption can be abolished after the ACP was inhibited by antiserum that had been shown to cross-react completely with human TRAP (14). TRAP, isoenzyme of ACP, had been located in ruffled boarder of osteoclasts (15). In present study, it is easy to find that more than 80% of ACP released from osteoclasts is TRAP. It coincides with previous study that the serum TRAP mainly derives from the osteoclasts. In most cases, such as children, postmenopausal women, patients with hyperparathyroidism and hyperthyroidism, the serum TRAP activity significantly rise, which means that the bone metabolism is very active (16). Therefore, the TRAP could be looked as a sensitive and specific marker for bone turnover (16–18).

According to the results, we can find that the number of the Howship's lacuna agreed with the dose of PDGF-BB. It is known that mononuclear cells fuse into the mature osteoclasts (19, 20). But the humoral stimulus is independent in the fusion procedure. As a potent mitogen, PDGF-BB promotes cellular proliferation and differentiation. Therefore, we believe that the increase of resorption pits is in part the result of the fusion of mononuclear cells into the osteoclasts. It is speculated that the binding of PDGF-BB to the receptor β is involved in regulation of PDGF-induced cytoskeletal rearrangements and cellular movement (13). As the result, that the PDGF-BB may promote the osteoclasts to attach the bone slices will be partly responsible for the increase in Howship's lacuna.

There is positive correlation between the volume and the area of the resorption pits, and the pit area can reflect the resorptive degree of osteoclasts on the bone slices. Definitely, the contact area of large cells to the bone is greater than that of the small one. It is easy to understand that the larger cells can secret more enzymes and acid substances, too. That the fusion of cells and more enzymes and acid result in the augment of the volume of the resorption pits.

Several lines of evidence have indicated that the osteoblasts may regulate the osteoclasts function. The prostaglandin may be produced when osteoblasts are stimulated by PDGF and promotes bone resorption (21). But the highly purified osteoclasts stimulated by PDGF were not studied before. According to our results, the PDGF-BB is very potent for osteoclastic bone resorption directly through the PDGF R-β. Due to its replication and differentiation effects on osteoblasts, we believe that the PDGF-BB may exert important effects on the bone metabolism, especially bone repair and reconstruction.

TABLE 1

The Supernatant Acid Phosphatase Activity (Total, Tartrate Resistant)

Concentration (ng/ml)	Total ACP activity ($U\ l^{-1}$)	TRAP activity ($U\ l^{-1}$)
0	1.65 ± 0.13	1.43 ± 0.15
10	2.55 ± 0.17	2.40 ± 0.25
20	2.58 ± 0.40	2.35 ± 0.24
30	2.80 ± 0.78*	2.68 ± 0.80*
40	2.86 ± 0.15*	2.75 ± 0.24**

*$p < 0.05$, **$p < 0.01$.

TABLE 2

The Effects of PDGF-BB on the Osteoclasts

Concentration (ng/ml)	Area of pits (μm^2)	Number of pits
0	435.08 ± 237.50	14.00 ± 1.41
10	560.09 ± 228.26*	19.00 ± 2.00
20	590.32 ± 285.57*	23.75 ± 2.50*
30	616.94 ± 241.73**	24.75 ± 0.95**
40	630.26 ± 240.64**	26.00 ± 2.00**

*$p < 0.05$, **$p < 0.01$.

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REFERENCES

1. Rao, C. D., Igaraghi, H., Chiu, I. M., Robbins, K. C., and Aaronson, S. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2392–2396.
2. Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urden, M. S., Eddy, R., Shows, T. B., Philpott, K., Meller, A. L., Knott, T. J., and Scott, J. (1986) *Nature* **320,** 695–699.
3. Matsui, T., Heidaran, M., Miki, T., Popescu, N., LaRochelle, W., Kraus, M., Pierce, J., and Aaronso, S. (1989) *Science* **243,** 800–804.
4. Claesson-Welsh, L., Eriksson, A., Westermark, B., and Heldin, C.-H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4917–4921.
5. Claesson-Welsh, L., Eriksson, A., Moren, A., Severinsson, L., Ek, B., Ostman, A., Betschultz, C., and Heldin, C.-H. (1988) *Mol. Cell. Biol.* **8,** 3476–3486.
6. Lambrecht, J. T., and Marks, S. C., Jr. (1996) *Clin. Anat.* **9,** 41–45.
7. Gay, C. V., Kief, N. L., and Bekker, P. J. (1993) *Biochem. Biophys. Res. Commun.* **192,** 1251–1259.
8. Berthois, Y., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2496–2500.
9. Piao, Y. J.(1993) *in* The Techniques of Electron Microscopy in Biology (Zhang, J. Q., and Piao, Y. J., Eds.), pp. 84–108, Sun Yat-Sen Univ. Press, Guangzhou.
10. May, L. G., and Gay, C. V. (1996) *In Vitro Cell. Biol. Anim.* **32,** 269–278.
11. Vyberg, M., and Nielson, S. (1998) *Appl. Immunohistochem.* **6,** 3–10.
12. Arnett, T. R., and Dempster, D. W. (1987) *Endocrinology* **120,** 602–608.
13. Claesson-Welsh, L. (1994) *J. Biol. Chem.* **269,** 32023–32026.

14. Zaidi, M., Mooga, B., Moss, D. W., and MacIntype, I. (1989) *Biochem. Biophys. Res. Commun.* **159,** 68–71.
15. Minkin, C. (1982) *Calcif. Tissue Int.* **34,** 285–290.
16. Chung, C. K., Panesar, N. S., Haines, C., Masarei, J., and Swaminathan, R. (1995) *Clin. Chem.* **41,** 679–686.
17. Nakanishi, M., Yoh, K., Uchida, K., Maruo, S., and Matsuoka, A. (1998) *Clin. Chem.* **44,** 221–235.
18. Janckila, A. J., Cardwell, E. M., and Yam, L. T. (1997) *Hybridoma* **16,** 175–182.
19. Rodman, G. D. (1996) *Endocrine Rev.* **17,** 308–322.
20. Athanason, N. A. (1996) *J. Bone Joint Surg. (Am).* **78,** 1096–1112.
21. Tashjian, A. H., Jr., Hohmann, E. L., Antoniades, H. N., and Levin, L. (1982) *Endocrinology* **111,** 118–124.