Journal of Biomolecular Techniques
13:62–71 © 2002 ABRF

ARTICLES

PDGF BB Purified from Osteoclasts Acts as Osteoblastogenesis Inhibitory Factor (OBIF)

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The functions of bone-forming osteoblasts and bone-resorbing osteoclasts are intimately linked. Recently it has been revealed that osteoblasts regulate differentiation of osteoclasts by two factors: as a stimulator of osteoclastogenesis, the receptor activator of NF-κB ligand (RANKL); and as an inhibitor, osteoprotegerin (OPG). However, no signaling factors from osteoclasts to osteoblasts have yet been identified. In this study, we found that the conditioned medium of mouse osteoclast-like RAW264.7 cells treated with RANKL contains activity that inhibits differentiation of mouse osteoblast-like MC3T3-E1 cells. We named this factor osteoblastogenesis inhibitory factor (OBIF). We partially purified OBIF with successive three-step chromatography by heparin affinity, anion exchange, and reverse-phase columns. This inhibitory activity appeared as one peak in each chromatography step, indicating that the factor was a single entity. Active fractions were loaded on SDS-PAGE, digested *in gel* by trypsin, and analyzed by liquid chromatography equipped with tandem mass spectrometry (LC/MS/MS). Subsequently, we found platelet-derived growth factor BB homodimer (PDGF BB) to be an OBIF candidate protein, and neutralization of the inhibitory activity of the medium with anti-PDGF antibody confirmed this identification. These results demonstrate, for the first time, that osteoclasts regulate osteoblasts directly and suggest that PDGF BB is a key factor in bone remodeling.

KEY WORDS: platelet-derived growth factor BB, receptor activator of NF-κB ligand, differentiation, protein purification, mass spectrometry.

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The most remarkable breakthrough in protein chemistry in these past 10 years has been protein identification and characterization by mass spectrometry.[1] Now we can immediately identify proteins from discrete bands on SDS-PAGE at the silver stain level (1–10 ng on gel). This progress includes the invention of new ionization methods (matrix-assisted laser desorption/ionization[2] and electrospray ionization[3]) and the accumulation of data in protein sequence databases accompanied by massive genome and cDNA sequencing projects. On the other hand, protein purification with column chromatographies has been used to discover new proteins or new functions of proteins for some time. Some researchers were convinced that this technique would be rendered useless in a few decades because it was old, time-consuming, low-tech, and dependent on each

researcher's handling, which is sometimes hard to reproduce. However, recent progress in separation science has greatly facilitated high-throughput reproducible purification. We used conventional purification coupled with mass spectrometry to discover a new functional protein, putative osteoblast inhibitory factor (OBIF), from the osteoclastic cell line RAW264.7.

Bone is an active tissue in which bone formation by osteoblasts is followed by bone resorption by osteoclasts in a recurring cycle called bone remodeling.[4–7] Osteoclasts are multinucleated cells derived from hematopoietic cells of monocyte/macrophage lineage, whereas osteoblasts are derived from mesenchymal lineage and have characteristics similar to fibroblasts. As shown in Figure 1, osteoblasts regulate osteoclast differentiation with osteoprotegerin (OPG) and the receptor activator of NF-κB ligand (RANKL). OPG and RANKL both belong to the tumor necrosis factor superfamily. OPG is a secreted protein and inhibits osteoclastogenesis, or osteoclastic differentiation from osteoclast precursors to mature osteoclasts which can resorb bone.[8,9] RANKL, on the other hand, is a membrane protein and induces osteoclastogenesis.[10–12]

OPG and RANKL led us to speculate that an analogous substance secreted by osteoclasts, such as hypothetical OBIF or osteoblast differentiation factor (OBDF), could regulate osteoblastic differentiation. Galvin et al.[13] showed that authentic osteoclasts and osteoclast-like cells produce a soluble heat-labile factor with a molecular weight greater than 3500 that inhibits osteoblast alkaline phosphatase (ALP) activity and collagen synthesis, which are known as osteoblast differentiation markers. However, so far it has not been reported whether this soluble factor is a protein or not. To understand the process of bone remodeling at the molecular level, identification of such osteoblastogenesis regulation factors, if they exist, is essential.

In this study, we attempted to identify the regulatory factor involved in the differentiation of osteoblasts from osteoclasts/osteoclast precursors using the mouse osteoclastic cell line RAW264.7 and the mouse osteoblastic cell line MC3T3-E1. We found OBIF activity in the conditioned medium of osteoblastic cells induced to differentiate to osteoclasts with sRANKL. We purified this activity partially by chromatographic techniques and identified the candidate protein, platelet-derived growth factor BB homodimer (PDGF BB), by mass spectrometry. PDGF BB as OBIF was then confirmed by anti-PDGF neutralization experiment.

MATERIALS AND METHODS

Materials

Mouse osteoblastic cell line MC3T3-E1 was purchased from Riken Cell Bank (Tsukuba, Japan), and mouse myeloid cell line RAW264.7 was from the American Type Culture Collection (Rockville, MD). Recombinant human bone morphogenetic protein-4 (BMP-4), recombinant rat platelet-derived growth factor BB (rrPDGF BB), anti-human PDGF antibody, anti-PDGF BB antibody, and anti-transforming growth factor-β3 (TGF-β3) antibody were purchased from R&D Systems (Minneapolis, MN). Modified trypsin (sequencing grade) was purchashed from Promega (Madison, WI). Alpha-minimal essential medium (α-MEM), Dulbecco's minimum essential medium (DMEM), and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA).

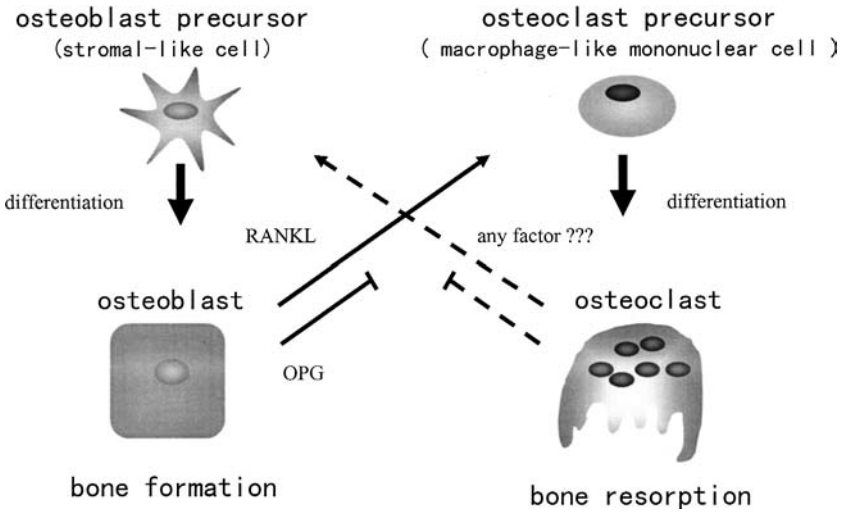

FIGURE 1

A schematic model of osteoblast/osteoblast precursor—osteoclast/osteoclast precursor interaction. Osteoblasts regulate osteoclastogenesis by expressing RANKL and OPG. It was not previously known whether osteoclasts express any factor that regulates osteoblastogenesis.

Recombinant mouse RANKL extracellular region (137-316) fused to glutathione-S-transferase (sRANKL) was expressed in *Escherichia coli* using the vector pGEX-3X (Amersham Biosciences, Uppsala, Sweden), and purified by affinity chromatography using a glutathione Sepharose column (Amersham Biosciences).

Cell Culture

MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS and antibiotics (50 U/mL penicillin, 100 μg/mL streptomycin) in humidified 5% CO_2/95% air at 37°C.

Conditioned Medium of RAW264.7 Cells

RAW264.7 cells were plated at 1.5 × 104 cells/cm² on plastic 500 cm² culture trays (Sumitomo Bakelite, Tokyo, Japan) in 75 mL DMEM supplemented with 10% FBS and antibiotics in humidified 5% CO_2/95% air at 37°C. Starting the next day, cells were treated with or without 100 ng/mL sRANKL for 1 to 4 days, and one third of the culture medium (25 mL) was changed every day with fresh medium with or without sRANKL, respectively. One day before collection, cells were washed with serum-free DMEM (DMEM supplemented with only antibiotics) twice, and the medium was replaced with 75 mL serum-free DMEM.

Immediately after collection, the conditioned media were filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA). A portion of the conditioned medium buffer (1.5 mL) was changed to Dulbecco's phosphate buffered saline (PBS) by gel filtration (HiTrap Desalting, Amersham Biosciences), and the culture medium was then tested for ALP activity.

Purification of OBIF

OBIF activity in the samples obtained from each purification step was determined by observing the suppression of MC3T3-E1 differentiation, using ALP activity as a differentiation marker. One unit per milliliter of OBIF activity was defined as the amount required for 50% inhibition of ALP activity induced by BMP-4.

All purification steps except the reverse-phase chromatography were performed at 4°C. A quantity (1.8 L) of the conditioned medium stimulated with sRANKL for 4 days was supplemented with a protease inhibitor cocktail (Complete, Roche, Basel, Switzerland), and was divided into nine equal portions. The conditioned medium was applied to a heparin column (5 mL, HiTrap Heparin, Amersham Biosciences) equilibrated with 10 mM sodium phosphate, pH 7.5. After washing the column with the same buffer, proteins were eluted from the column with a 20-column volume (CV) linear gradient from 0 to 2 M NaCl. The buffer of the fractions was changed to PBS-C (PBS containing 0.05% CHAPS) by HiTrap Desalting, and the fractions were then tested for OBIF activity. The active fractions from nine chromatography runs were pooled and dialyzed against 30-fold volume of Tris-CHAPS buffer (20 mM Tris-Cl, pH 7.0, containing 0.05% CHAPS) for 18 h using a dialysis tube with a molecular weight cut-off of 12,000 D (Sanko Junyaku, Tokyo, Japan), and centrifuged at 10,000 × *g* for 20 min. The supernatant was applied to an anion exchange column (1 mL, Resource Q, Amersham Biosciences) equilibrated with 10 mM sodium phosphate, pH 7.5. After washing the column with the same buffer, proteins were eluted from the column with a 40-CV linear gradient from 0 to 1 M NaCl. The active fraction was adjusted to 5% acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (TFA), and applied to a reverse-phase column (1 mm ID × 150 mm, Develosil 300 C4-HG-5, Nomura Chemical, Tokyo, Japan) equilibrated with 5% CH_3CN containing 0.1% TFA. After washing the column with the same buffer, proteins were eluted from the column with a 40-CV linear gradient from 5% to 50% CH_3CN. Four microliters of each fraction was immediately diluted with 400 μL PBS-BC (PBS containing 0.05% CHAPS and 1 mg/mL bovine serum albumin), and tested for OBIF activity. Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as the standard protein.

Identification of Purified OBIF by Mass Spectrometry

The active fractions were evaporated to 2–3 μL, supplemented with Laemmli sample buffer (Bio-Rad, Cambridge, MA) containing 5% 2-mercaptoethanol, and loaded onto SDS-PAGE (10–20 gradient gel, Bio-Rad). The gel was visualized with zinc stain (Bio-Rad). Bands excised from the gel were destained with 2.5 mM Tris containing 19.2 mM glycine; reduced with 20 mM NH_4HCO_3, pH 8.0, containing 10 mM dithiothreitol; alkylated with 20 mM NH_4HCO_3, pH 8.0, containing 55 mM iodoacetamide; washed with 20 mM NH_4HCO_3, pH 8.0; and dried in an evaporator. Proteins in the gels were digested for 15 h at 37°C by 20 μL modified trypsin (10 ng/μL) in 20 mM NH_4HCO_3, pH 8.0. The

resulting peptides were extracted once with 50 μL FA/H_2O buffer (0.05% formic acid) and twice with 50 μL FA/CH_3CN buffer (0.05% formic acid in CH_3CN), 5 min each. After the collected extracts were evaporated to 2–3 μL, 10 μL FA/H_2O buffer was added, and the extracts were then analyzed by liquid chromatography equipped with tandem mass spectrometry (LC/MS/MS). Five microliters of each sample was applied to a capillary reverse-phase chromatography column (75 μL ID × 150 mm, PepMap C18 3 μm, LC Packings, San Francisco, CA, USA) equilibrated with FA/H_2O. After washing the column with the same buffer, peptides were eluted from the column with a 20-CV linear gradient from 0% to 40% FA/CH_3CN buffer, and introduced directly into an ion trap mass spectrometer (LCQ, ThermoQuest, San Jose, CA, USA) in triple scan mode (full MS, zoom scan, and MS/MS) with a fused silica PicoTip (New Objective, Woburn, MA). The resulting spectra were searched against the SWISS-PROT database (Release 38) using the SEQUEST program (University of Washington, licensed to ThermoQuest).

Neutralization of OBIF Activity with Anti-PDGF Antibody

The buffer of the conditioned medium of RAW264.7 cells treated with or without sRANKL for 4 days was changed to PBS by HiTrap Desalting, and the culture medium was then tested for ALP activity with the same volume of anti-human PDGF antibody (final 20 μg/mL), anti-PDGF BB antibody (final 20 μg/mL), anti-TGF-β3 antibody (final 20 μg/mL) as a control antibody, or PBS. rrPDGF BB (final 5 ng/mL) in PBS was used as the control.

Assay for ALP Activity

MC3T3-E1 cells were plated at 5000 cells/well in 96 well plates (Corning, Acton, MA) in 100 μL/well α-MEM. After incubation for one day, 20 μL of each sample and 2 μL BMP-4 (1 μg/mL, final 50 ng/mL) were added to each well. After incubation for two more days, all the medium was discarded and 100 μL/well ALP buffer (600 mM diethanolamine containing 0.6 mM $MgCl_2$, 0.1% Triton X-100, and 30 mM *p*-nitrophenyl-phosphate) was added. After reaction for 5 min at room temperature, absorbance at 405 nm was measured. The obtained absorbance was normalized by linear fitting using absorbance of wells containing no cells as zero and wells treated with BMP-4 and vehicle of the sample as one.

Statistical Analysis

Results are expressed as means ± SEM. Although the data are expressed and illustrated as normalized activities or OBIF activity, all statistical significance was analyzed by two-tailed unpaired Student's *t*-test using the original numerical data. Statistical differences of $p < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Osteoblastogenesis Inhibition of RAW264.7 Conditioned Medium

Until recently, there had been no homogenous osteoclast experimental system, and many studies were performed by co-culturing with stromal cells.[14] Looking for a signaling molecule from osteoclast to osteoblast had been extremely difficult; in other words, though such an activity was found, it was very difficult to say whether that factor was from the osteoclasts or from the co-culturing cells. Recently, however, it was reported that mouse myeloid RAW264.7 cells could be differentiated into osteoclasts by stimulation with RANKL only.[15] With this in mind, we chose RAW264.7 cells as an osteoclast cell model.

We observed that 100 ng/mL sRANKL stimulation caused RAW264.7 cells to develop into tartrate-resistant acid phosphatase (TRAP) positive cells after 1 day. TRAP positive is one of the known markers of osteoclast differentiation. The stimulated RAW264.7 cells formed multinucleated cells after 3–4 days, and finally started to make bone-resorbing pits after 7 days of stimulation (data not shown). To examine whether RAW264.7 cells secreted a certain factor that regulates differentiation of osteoblasts, we chose MC3T3-E1 cells as an osteoblast cell model and their ALP activity as the osteoblast differentiation marker, since MC3T3-E1 has been well studied and has the capacity to form calcified bone tissue in vitro.[16]

The conditioned media of RAW264.7 cells treated or untreated with sRANKL for 1 to 4 days were tested for MC3T3-E1 ALP activity. Media was replaced with serum-free media to decrease the influence of the serum one day before collection, and the buffer of the assay samples was changed to PBS to remove small molecules and adjust the pH.

As shown in Figure 2, the conditioned media of the cells treated with sRANKL strongly inhibited MC3T3-E1 differentiation induced by BMP-4, and inhibition activity plateaued after 3 days. On the other hand, the media of untreated cells showed

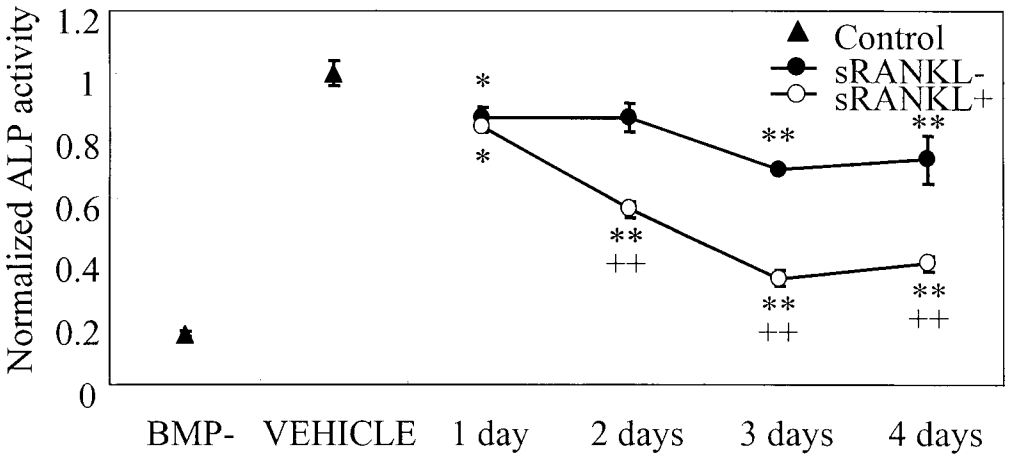

FIGURE 2

Effects of conditioned media of RAW264.7 cells on ALP activity of MC3T3-E1 cells. Normalized alkaline phosphatase activity of osteoblast precursor-like cell line MC3T3-E1. Cells were cultured with BMP-4 and RAW264.7 conditioned media treated *(open circles)* or untreated *(closed circles)* with sRANKL for 2 days. *Closed triangles* are control samples. BMP– was cultured without BMP-4, and vehicle was cultured with BMP-4 and PBS. Results are expressed as means ± SEM (n ± 3–6). *$p < 0.05$, **$p < 0.01$ vs. vehicle. ++$p < 0.01$ vs. corresponding cultures treated without sRANKL. Experiments were performed three times with similar results as shown.

weak inhibition activity. Uncultured media incubated for 1 to 4 days with or without sRANKL showed no influence on ALP activity (data not shown).

When we first found OBIF activity in the conditioned medium, we suspected TGF-β was the molecule responsible for this activity, because TGF-β is well known to be an important factor for bone formation and resorption[17, 18] and can inhibit osteoblastic differentiation of MC3T3-E119. However, western blot analysis and neutralizing anti-TGF-β antibody revealed that TGF-β was not the OBIF substance secreted from RAW264.7 (data not shown). Therefore, we named this factor osteoblastogenesis inhibitory factor (OBIF) and attempted to identify it.

Purification and Identification of OBIF Candidate Protein

At first we subjected the conditioned media of RAW264.7 cells cultured with or without sRANKL to two-dimensional electrophoresis (2DE) followed by mass spectrometry, since we expected that one of the spots specific for sRANKL could be OBIF. Although there are limitations to the use of 2DE, such as the detection of low-abundance, hydrophobic, and alkaline proteins,[20, 21] secreted proteins are soluble and considered to be "well-behaving proteins" on 2DE. However, from their annotation (data not shown) the identified proteins were not likely to be OBIF, and since cytokines act at very low concentrations (ED_{50}: 0.1–100 ng/mL), we speculated that the sensitivity of 2DE was not sufficient for the analysis of OBIF. In addition, a large number of spots were detected by 2DE differential display analysis. Therefore, instead of using the "comprehensive approach" with 2DE, we turned to classical protein purification.

OBIF was purified by successive chromatography by heparin affinity (Fig. 3A), anion exchange (Fig. 3B), and reverse-phase (Fig. 3C) columns from 1.8 L of conditioned medium of RAW264.7 cells stimulated with RANKL for 4 days. The use of CHAPS to prevent OBIF activity from adsorption to the plasticware was indispensable after the first step of heparin affinity chromatography. Although purification with a reverse-phase column can result in the loss of OBIF activity, we decided to use it since many secreted cytokines are resistant to the drastic conditions used in reverse-phase chromatography, and since the resolution of reverse-phase chromatography is far superior to that of other chromatographic techniques.

As shown in Figure 3, the appearance of OBIF activity as a single peak in each chromatography step suggested that OBIF was a single substance. The data of the purification steps are summarized in Table 1. Protein concentration of fractions from reverse-phase chromatography could not be determined because

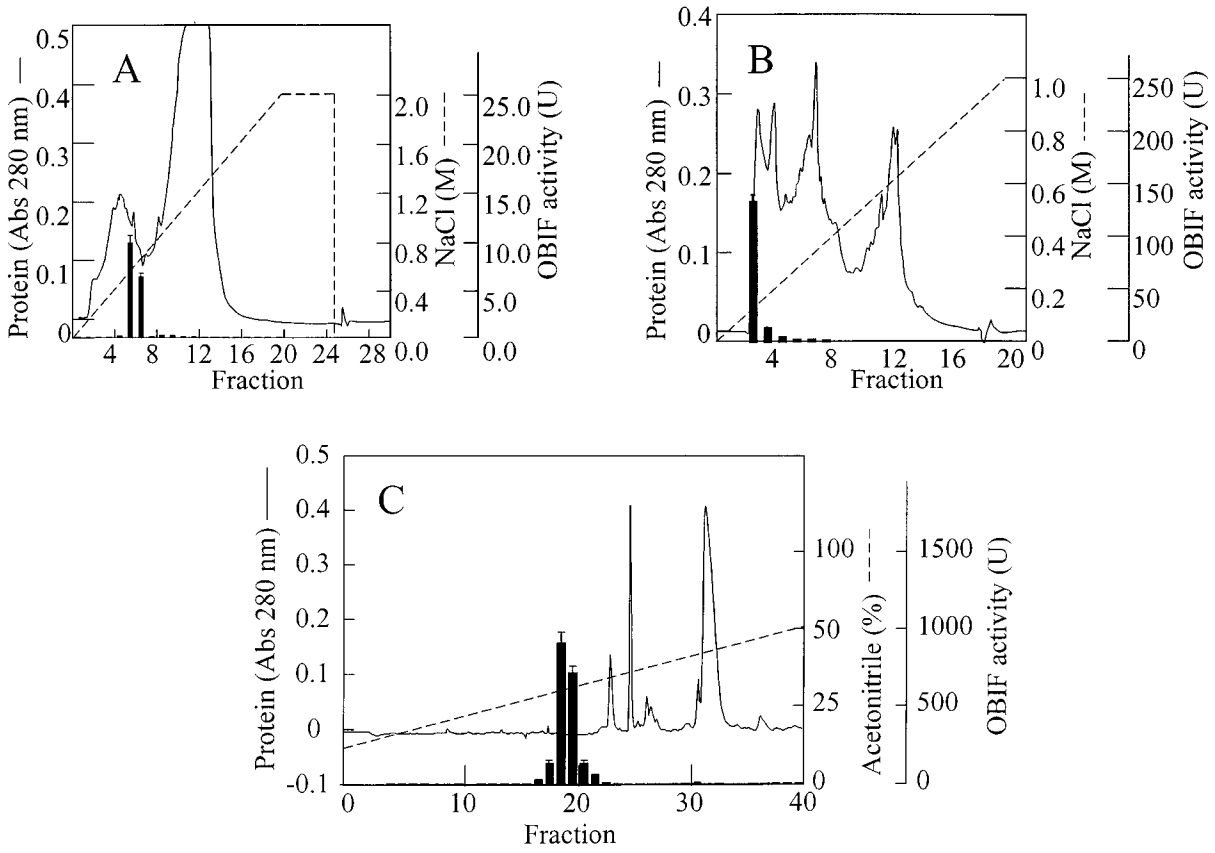

FIGURE 3

Purification of OBIF from conditioned medium of RAW264.7. **A:** HiTrap heparin affinity chromatography. **B:** Resource Q anion-exchange chromatography. **C:** C4 reverse-phase chromatography. One unit per milliliter of OBIF activity was defined as the amount required for 50% inhibition of ALP activity induced by BMP-4.

TABLE 1

Purification Table of Osteoblastogenesis Inhibitory Factor from sRANKL-Treated RAW264.7 Conditioned Medium

Step	Protein conc. (mg/mL)	Activity conc. (U/mL)	Volume (mL)	Total protein (mg)	Total activity (U)	Specific content (U/mg)	Step recovery (%)	Recovery (%)
Conditioned medium	0.16	0.8	1800	288	1440	5	—	—
Heparin Sepharose CL-6B	0.1	10.7	60	5.84	640	110	44.4	44.4
Resource Q	0.088	133	2	0.176	265	1506	41.4	18.4
Develosil 300 C4-HG-5	n.d.	911	0.1	n.d.	91.1	n.d.	34.4	6.33

Osteoblastogenesis inhibitory factor was purified from sRANKL-treated RAW264.7 conditioned medium (see Fig. 2). One unit per milliliter of OBIF activity was defined as the amount required for 50% inhibition in ALP assay (see Materials and Methods).

n.d., not determined.

protein was below the detection limit of the BCA protein assay. We obtained 1100-fold concentrated OBIF protein in 6% yield.

To identify OBIF, the fractions eluted from the reverse-phase column with significant OBIF activity were subjected to SDS-PAGE under reducing conditions, and visualized by zinc stain (Fig. 4). Nine bands in fr. 19 were excised, reduced, alkylated, and digested with trypsin according to a modified version of the protocal used by Wilm et al.[22] The resulting peptides were separated by a nano-flow capillary column equipped with on-line MS/MS. The obtained MS/MS spectra were analyzed using the SEQUEST program. The results are shown in Table 2. Eight bands out of nine were identified, and the same protein, PDGF B chain, was identified from band 5, band 7, and band 8, which had good correlation with the OBIF activity. A peptide coverage map obtained from these three bands is shown in Figure 5.

PDGF is not uniform but has three isoforms. They are disulfide-linked dimers made of A and B polypeptide chains. All three isoforms (AA, AB, and BB) exist in nature and have mitogenic activity.[23] No observation of peptides from the PDGF A chain or from the removed part of the B chain suggests that PDGF exists as the secretory form of the BB homodimer. The molecular weight, 30 kDa, of the PDGF BB secretory form agrees with the estimated molecular weight, 15–45 kDa, by gel filtration (data not shown). Shimokado et al.[24] demonstrated that activated macrophages secrete PDGF. With respect to its effects on bone formation, there is a discrepancy between in vitro studies and in vivo studies, but at least in in vitro studies it has been reported that PDGF inhibits differentiated function in osteoblastic cells. In addition, identified proteins from

TABLE 2

List of Identified Proteins from SDS-PAGE of the Most Active OBIF Fraction (fr. 19)

	Species	Identified protein	MW (kDa)
Band 1	n.i.	n.i.	n.i.
Band 2	Bovine	Serum albumin	67k
Band 3	Bovine	Serum albumin	67k
Band 4	Bovine	Plasminogen (fragment)	89k
Band 5	Mouse	PDGF B chain	12k
Band 6	Mouse	Translation initiation factor 3A (fragment)	162k
Band 7	Mouse	PDGFb B chain	12k
Band 8	Mouse	PDGFb B chain	12k
Band 9	Mouse	ATPase inhibitor	10k

n.i., not identified; PDGF, platelet-derived growth factor.

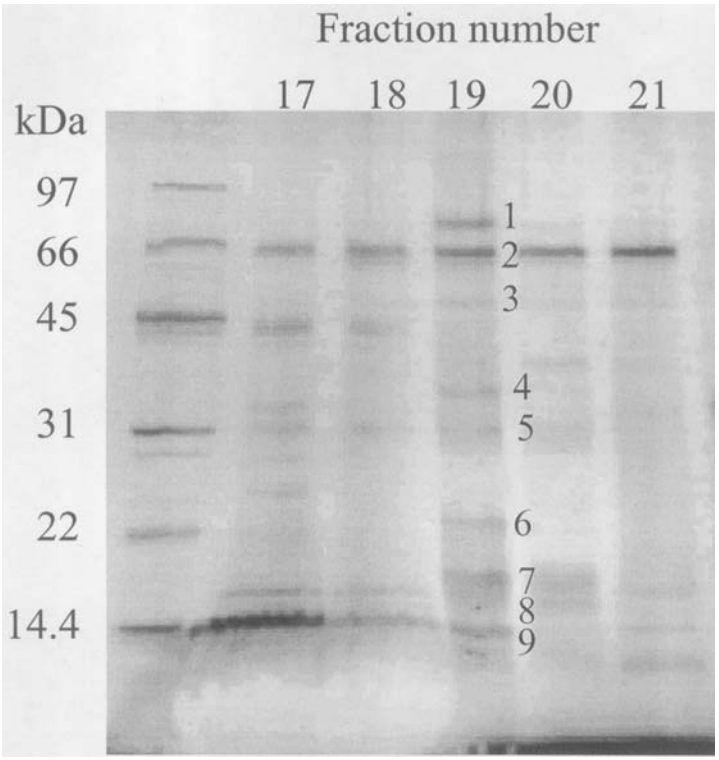

FIGURE 4

SDS-PAGE and mitogenic activity of purified OBIF. Reverse-phase chromatography fractions around the most active fraction, fr. 19, were loaded on polyacrylamide 10–20% gradient gel under reduced conditions. After electrophoresis, protein bands were visualized with Zn stain.

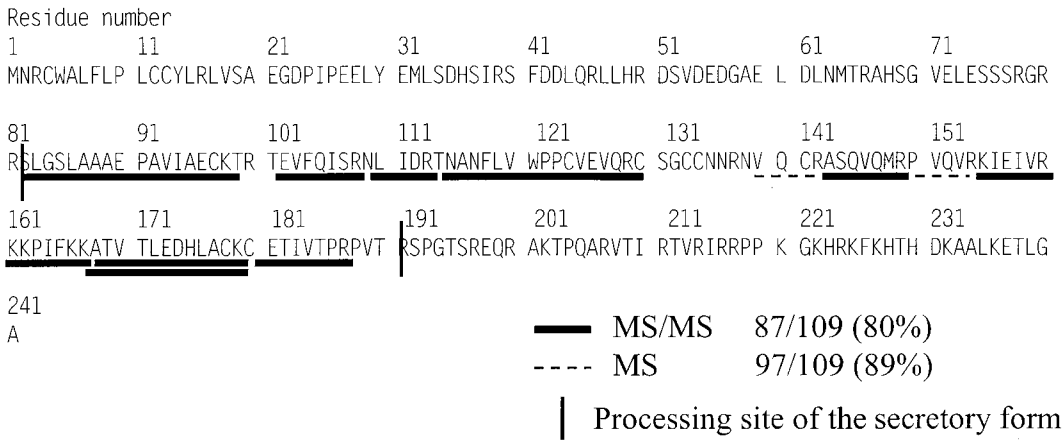

FIGURE 5

Peptide coverage map of PDGF in the LC/MS/MS analysis of band 5, band 7, and band 8. *Underlined* sequences were fitted to tryptic peptides observed in the MS/MS spectra and *dashed-lined* sequences were observed in the MS spectra. *Vertical lines* indicate the processing site of the secreted form of the PDGF BB homodimer.

other bands have not been reported to have differentiation or growth activities. All of the results and reports are consistent with identification of OBIF as PDGF BB.

Confirmation of PDGF BB as OBIF by Neutralizing Antibody

In order to see if PDGF BB was the substance responsible for osteoblastogenesis inhibition, neutralization experiment using anti-human PDGF antibody and anti-PDGF BB antibody was performed. Anti-human PDGF antibody and anti-PDGF BB antibody neutralized 83% and 86% of OBIF activity of conditioned medium cultured with sRANKL, and neutralized 70% and 61% of OBIF activity of conditioned medium cultured without sRANKL, respectively (Fig. 6). Anti-human PDGF antibody can suppress the effect of all three isoforms of PDGF, but anti-PDGF BB antibody can suppress only the effect of PDGF BB. Comparable suppression by the two antibodies indicates that there is neither an AA nor an AB isoform of PDGF. In addition, the same degree of suppression for conditioned medium with or without sRANKL indicates that the substance responsible for relatively weak OBIF activity of conditioned medium without sRANKL is also PDGF BB. Therefore, we concluded that PDGF BB is the major substance responsible for OBIF activity produced by RAW264.7 cells. Further studies are required to elucidate the role of PDGF BB in bone remodeling.

Classic Protein Purification as a Post-Genome Approach

Over the last several years, scientists have made great efforts to discover novel protein functions from the relationship between disease and massive genome databases. Studying comprehensive patterns of gene/protein expression in various normal and disease states has been widely used to discover novel drug target proteins. For protein, expression profiling analysis or differential display analysis using 2DE has been widely examined. Because 2DE is rather time-consuming and biased toward "well-behaving proteins in 2DE," several researchers turned their attention to direct peptide analysis using stable isotopes[25–27] or 2-dimensional liquid chromatography/mass spectroscopy (2D-LC/MS)[28,29] protocols. Although these approaches could uncover the proteins involved in a disease state, it would be very difficult to determine a protein that is actually important in the disease state. Functional analysis of all proteins involved in a disease could lead to finding the key proteins of the disease. However, there are a great number of proteins involved in a specific disease and there is no practical method for high throughput functional analysis of proteins. Functional analysis from comprehensive profiling analysis would be time-intensive, inefficient, and impractical. Protein interaction maps[30–33] and bioinformatics would help decrease the number of the candidate proteins of biological importance in the future. There is a long and winding road from genome to function.

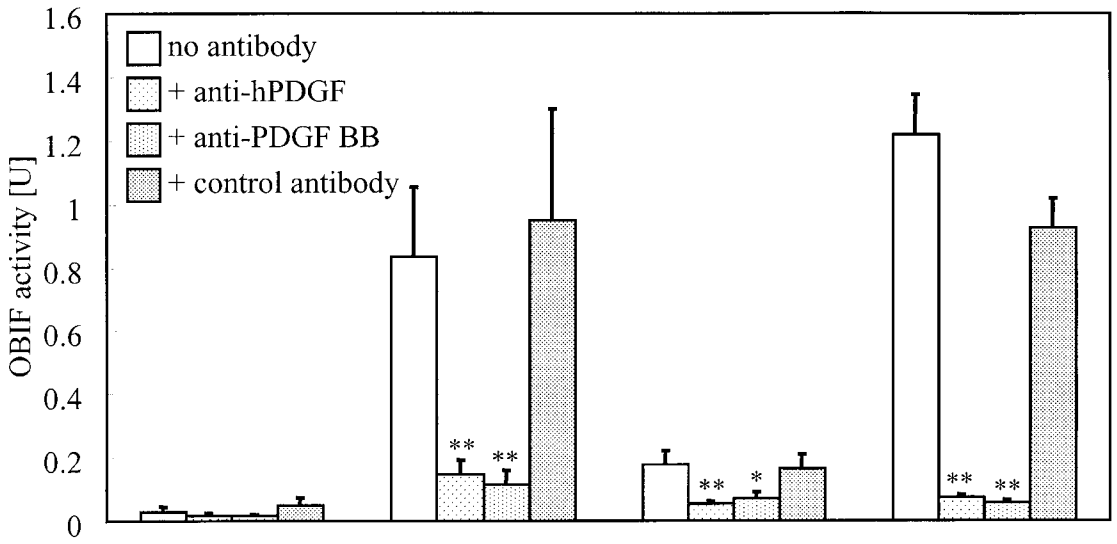

FIGURE 6

Anti-PDGF antibodies neutralize OBIF activity of osteoclast conditioned medium. OBIF activity of vehicle (PBS), rat recombinant PDGF (5 ng/mL), and RAW264.7 conditioned medium treated with or without sRANKL, was determined by MC3T3-E1 ALP assay in the absence or presence of anti-hPDGF antibody (20 μg/mL), anti-PDGF BB antibody (2 μg/mL), or control antibody (20 μg/mL). Results are expressed as means ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$ vs. corresponding cultures without antibody. Experiments were performed three times with similar results as shown.

Distinguishing a gene or protein from its function could overcome the problem with the current comprehensive genomic/proteomic approach to finding biologically important proteins because the bottleneck—functional analysis—is not necessary. In that sense, protein purification using activity-based assays is a rather old but valuable approach. On the other hand, identification of proteins by mass spectrometry is a fast and sensitive approach. This method can identify target proteins from a mixture as long as each band is discrete on the SDS-PAGE. Recent direct peptide analysis enabled us to identify a number of proteins at the same time without prior SDS-PAGE separation. Therefore, complete purification is not required for the identification of a protein. This technological breakthrough as well as development of various chromatography media and new affinity gels has resulted in a less amount of starting material and fewer steps for purification of proteins being required, which means protein purification takes much less time than ever before. Once a new functional protein is isolated, it opens up new avenues to the elucidation of related proteins, such as interacting proteins or small molecules, using recent techniques. A purification approach requires the detection of biological activity and few cases appear to exist. Nevertheless, we believe that purification of proteins based on their activity, although seemingly rather old-fashioned, is still very important by virtue of the development of modern protein chemistry.

CONCLUSION

We found osteoblastogenesis inhibitory activity in the conditioned medium of osteoclast precursor-like cells, and determined that this activity is inducible by sRANKL. The factor, named osteoblast inhibitory factor, was purified by chromatography, identified as PDGF BB by mass spectrometry, and confirmed by neutralizing antibodies. While PDGF has been known to have an effect on bone metabolism, these results demonstrate, for the first time, that osteoclasts/osteoclast precursors stimulated by RANKL directly regulate osteoblastic differentiation with PDGF BB and indicate that PDGF BB is an important factor in bone remodeling.

ACKNOWLEDGEMENTS

This work was supported by Sankyo Co., Ltd. The authors acknowledge the American Society for Bone and Mineral Research for granting us permission to reproduce figures and tables from *J Bone Miner Res* 2002;17:257–265. The authors also acknowledge S. Hayashi for sRANKL expression and purification, T. Takazawa for technical help of mass spectrometry, and Dr. T. Matsuoka for helpful discussion of the manuscript.

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