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Osteoactivin is a novel osteoclastic protein and plays a key role in osteoclast differentiation and activity

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Abstract **This study presents gene expression, protein expression, and in situ immunohistochemical evidence that osteoclasts express high levels of osteoactivin (OA), which had previously been reported to be an osteoblast-specific protein in bone. OA expression in osteoclasts was up-regulated upon receptor activator of NFκB ligand-induced differentiation. Suppression of functional activity of OA with neutralizing antibody reduced cell size, number of nuclei, fusion, and bone resorption activity of osteoclasts. OA was co-immunoprecipitated with integrin β_3 and β_1, indicating that OA co-localizes with integrin β_3 and/or β_1 in a hetero-polymeric complex in osteoclasts. These findings indicate that OA is a novel osteoclastic protein and plays a role in osteoclast differentiation and/or activity.**

Keywords: Osteoactivin; Osteoclast; Bone resorption; Fusion; Spreading; Integrin

1. Introduction

Osteoactivin (OA), discovered by the mRNA differential display approach in the mouse bone [1], was initially cloned from a murine dendritic cell cDNA library as *Dchil* (dendritic cell-associated, heparan sulfate proteoglycan-dependent integrin ligand) [2]. It is a homologous to human *Gpnmb* (glycoprotein non-metastatic melanomal protein B) [3]. OA plays a regulatory role in endothelial cell adhesion [2] and is linked to development of retinal pigment epithelium and iris [4]. The nervous system, basal layer of the skin, germinal cells of hair follicles, and the forming nephrons of the kidney of late mouse embryos show high OA protein expression [5]. It is also found to be highly expressed in various malignant tumors [3,6,7]. OA up-regulates expression of matrix metalloproteinase (MMP)-3 and -9 in the infiltrating fibroblasts into denervated skeletal muscle [8]. Overexpression of OA protects skeletal muscle from severe degeneration caused by long-term denervation in mice, suggesting that an OA-mediated increase in MMPs in skeletal muscle might protect injured muscle from fibrosis, leading to better regeneration after denervation [9]. Transgenic expression of OA in the liver also reduces hepatic fibrosis [10]. OA expression is increased in the injured or diseased liver [11]. Because of its suggestive functions in cell adhesion, migration, and differentiation, it was postulated that OA is involved in the pathophysiological cascade of tissue injury and repair [11].

The OA gene, located on human chromosome 7p15.1 or on mouse chromosome 6 [5], has 11 exons with an open reading frame of 1,716 bp, encoding a protein of 572 amino acid residues. It has 13 N-linked glycosylation sites, a heparin binding domain and an integrin binding arginine-glycine-aspartic acid motif (RGD) at its extracellular domain [1,2,11]. RGDS tetramer completely blocked the OA-induced endothelial cell adhesion, suggesting that the OA-mediated endothelial cell adhesion involves integrin binding [2].

In bone, an in situ hybridization analysis showed that OA expression was restricted to mature active osteoblasts [1,5]. The differentiation and maturation of osteoblasts are associated with the temporal expression of OA in primary rat osteoblasts [5,12]. Blocking the function of OA with an antibody reduces the differentiation and functional activity of rat osteoblasts without affecting cell proliferation and viability [12]. A recent study indicated that the OA expression in osteoblasts was up-regulated by bone morphogenetic protein (BMP)-2 and that OA acted as a downstream mediator of the BMP2-mediated activation of osteoblast differentiation [13]. Accordingly, it was concluded that OA is an osteoblast-specific gene in bone and plays an essential role in osteoblast differentiation [5,12]. In osteoblasts, OA exists as two isoforms: a 65-kDa type I transmembrane protein, and a highly glycosylated 115-kDa secreted glycoprotein [3]. However, the functional role of each form has not been determined.

Our preliminary comparative microarray analysis of the murine RAW264.7 cells and their derived osteoclast-like cells indicated that osteoclast-like cells expressed high levels of OA/*Dchil*/*Gpnmb* mRNA transcript, and that the OA/*Dchil*/*Gpnmb* expression was enhanced 3.25-fold by the soluble receptor activator of NFκB ligand (sRANKL) treatment. This

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Abbreviations: OA, osteoactivin; *Dchil*, dendritic cell-associated, heparan sulfate proteoglycan-dependent integrin ligand; *Gpnmb*, glycoprotein non-metastatic melanomal protein B; MMP, matrix metalloproteinase; RGD, arginine-glycine-aspartic acid motif; BMP, bone morphogenetic protein; RANKL, receptor activator of NFκB ligand; DMEM, Dulbecco's modified Eagle's medium; α-MEM, minimum essential medium α; CM, conditioned medium; TRACP, tartrate-resistant acid phosphatase; RT-PCR, reverse transcriptase-polymerase chain reaction; C_T, cycle threshold

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gene expression result indicates that OA gene expression in bone may not be restricted to osteoblasts and that cells of osteoclast lineage may also express this gene. Congruent with this possibility, macrophages, which share the same cell lineage with osteoclasts, also express substantial OA protein levels and its expression in macrophages is enhanced by IFN-γ and lipopolysaccharide [14]. A very recent report has also shown a high expression level of the OA/*Dchil*/*Gpnmb* gene in the RAW264.7 cell-derived osteoclast-like cells [15].

The objectives of this study were twofold: (1) to confirm the gene expression data by demonstrating OA protein expression in primary osteoclasts in vitro and in vivo and (2) to assess the role of OA in osteoclast differentiation by determining the effects of neutralizing anti-OA antibody on the formation and function of murine osteoclasts in vitro.

2. Materials and methods

2.1. Cell cultures

Primary bone marrow cells, flushed out of long bones of adult C57BL/6J (B6) mice, were cultured in minimum essential medium α (α-MEM) supplemented with 10% fetal bovine serum (FBS). After 24-hour cultures, the adherent stromal cells and the non-adherent cells, which contained osteoclast precursors, were separated. Osteoblasts were isolated from calvaria of adult B6 mice by 90 min crude collagenase digestion and were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc., Herndon, VA) containing 10% FBS.

2.2. Osteoclast differentiation

To generate marrow-derived osteoclasts, the non-adherent marrow osteoclast precursors were plated at a density of 4×10^5 cells/cm^2 in α-MEM and 10% FBS in the presence of 50 ng/ml sRANKL (PeproTech, Rocky Hill, NJ), 50 ng/ml mCSF (CalBiochem, San Diego, CA), with or without 100–200 pg/ml TGF-β_1 (R&D Systems, Minneapolis, MN). Under this condition, identifiable tartrate-resistant acid phosphatase (TRACP)-positive, multinucleated osteoclasts began to appear after 3 days of treatment. After 4 or 6 days, >60% or >90%, respectively, of the cells converted to large, TRACP-positive, multinucleated osteoclasts that resorbed bone on dentine slices (data not shown).

2.3. Neutralizing anti-OA antibody treatment

To assess the effects of a neutralizing anti-OA antibody on osteoclast differentiation, the non-adherent marrow osteoclast precursor cells were plated at a density of 2×10^5 cells/cm^2 and treated with sRANKL, mCSF, and TGF-β_1 for 4 days. During the final 24 h of the RANKL treatment, normal goat IgG (20 μg/ml) or goat anti-mouse OA-neutralizing polyclonal antibody (R&D Systems) at 5, 10, 20 μg/ml was each added to a group of three replicate wells. Cells were then fixed in 5% formalin for 30 min and stained for TRACP. The cell size and number of nuclei of the derived osteoclast in six randomly selected areas were measured with the OsteoMeasure system (Osteometrics Inc., Atlanta, GA) equipped with the manufacturer's software and a digitizing tablet under a microscope.

2.4. Real-time RT-PCR

Total RNA was isolated from osteoclasts, pre-osteoclasts, osteoblasts, and stromal cells, with the RNeasy® Mini Kit (Qiagen, Valencia, CA) and each reverse-transcribed to cDNA using the ThermoScript™ reverse transcriptase-polymerase chain reaction (RT-PCR) System (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using gene-specific PCR primers [synthesized by Integrated DNA Technologies (Coralville, IA)], and the QuantiTect™ SYBR® Green detection system (Qiagen) in the ABI PRISM 7900 Cycler (Applied Biosystems, Foster City, CA). The PCR amplification was performed for 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min each, after an initial hot start at 95 °C for 10 min. The relative level of gene expression was determined by the cycle threshold (ΔC_T) method.

2.5. Western blotting

Total cellular protein was extracted in a commercially-made RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) containing the Sigma protease inhibitor cocktail (St. Louis, MO), supplemented with phenylmethylsulfonyl fluoride, NaF, and Na_3VO_4. Western immunoblots for OA protein were performed using both the rat anti-mouse OA monoclonal antibody (R&D Systems) and the goat anti-mouse OA polyclonal antibody, followed by enhanced chemiluminescence.

2.6. Immunohistochemical staining of OA

Serial paraffin-embedded mouse femoral bone sections of a transgenic mouse with targeted overexpression of an osteoclastic protein-tyrosine phosphatase in cells of osteoclastic origin using a TRACP exon 1C promoter, which showed elevated osteoclastic resorption [16] were used for in vivo identification of OA protein in osteoclasts. After removal of paraffin wax, the first serial section was stained for TRACP [17] for identification of osteoclasts. The second section was stained immunohistochemically for OA, using the polyclonal goat anti-mouse OA antibody; while the third section was stained with non-immune rat IgG as a negative control. Detection of immunoreactive OA protein was performed with biotinylated anti-goat IgG antibody, streptavidin-horse radish peroxidase (Vector Labs, Burlingame, CA), and 3-3′-diaminobenzidine-peroxide. Double TRACP histochemical and OA immunohistochemical stainings on the same section were not performed, since the pinkish stained TRACP products was not readily identifiable in the presence of brownish stained OA product in osteoclasts.

2.7. Resorption pit formation assay

The non-adherent marrow osteoclast precursor cells were plated on dentine slices at a density of 2×10^5 cells/cm^2 and treated with sRANKL, mCSF, and TGF-β_1 for 4 days. During the final 24 h of treatment, normal goat IgG (10 or 20 μg/ml) or the goat anti-mouse OA-neutralizing polyclonal antibody at 2, 10, or 20 μg/ml was added. Dentine slices were then trypsinized and sonicated to remove the attached osteoclasts, and the resorption pits were stained with acid-hematoxylin (Sigma–Aldrich, St. Louis, MO). The size of individual pits was determined using the OsteoMeasure system.

2.8. RGD peptide treatment

Primary murine marrow cells (at 2.5×10^5 cells/cm^2 in 24-well plates) were pre-treated with sRANKL, mCSF, and TGF-β_1 for 3 days as described above. At the beginning of day 4, a RGD blocking peptide [c(RGDyK), an $\alpha_v\beta_3$ integrin binding peptide] or the corresponding negative control peptide [c(RADyK)] [both were obtained from Peptides International Inc. (Louisville, KY)], each at 30 μM, was added to the cells for an additional 48 h. Cells were then fixed with 5% formalin and stained for TRACP. The average cell size was determined as described above.

2.9. Statistical analysis

Statistical significance was determined with one-way ANOVA followed by the Tukey post-hoc test or with two-tailed Student's *t*-test. The difference was considered significant, when $P < 0.05$.

3. Results and discussion

Past research on the RANKL pathway has enormously advanced our understanding of the regulation of osteoclast formation and activity at the molecular level but also underscored its complexity [18,19]. While a number of genes and signaling pathways essential for osteoclast formation and activation have been identified, there are likely additional regulatory genes and pathways. In this regard, our preliminary microarray survey to compare the gene expression profile of RAW264.7 cells and their derived osteoclast-like cells has identified a novel osteoclastic gene, OA/*Dchil*/*Gpnmb*, that was highly expressed in mature osteoclasts and was up-regulated 3.25 ± 1.22-fold ($P < 0.001$) after the RANKL-induced osteo-

clast differentiation. In this study, we measured the expression of OA/*Dchil*/*Gpnmb* mRNA in primary marrow-derived osteoclasts by real-time RT-PCR to confirm the microarray results (Table 1). Marrow pre-osteoclasts, stromal cells, and osteoblasts were included for comparison. The relative TRACP mRNA transcript level was included as a marker gene for osteoclasts. As expected, the TRACP mRNA expression level was the highest in osteoclasts ($P < 0.001$) and different from the other test cell types (ANOVA: $P < 0.001$). The expression level of OA/*Dchil*/*Gpnmb* transcript was similarly greater in osteoclasts than that in marrow pre-osteoclasts and in osteoblasts by 8.6- and 28.7-fold, respectively ($P < 0.001$ for each). Interestingly, the relative OA/*Dchil*/*Gpnmb* mRNA level of stromal cells was not significantly different from that of osteoclasts. The high OA/*Dchil*/*Gpnmb* expression level in stromal cells was presumably due to the contaminating cell types that are known to express high levels of OA/*Dchil*, such as fibroblasts, chondrocytes, and dendritic cells.

To confirm that OA protein is expressed in functionally active osteoclasts in vivo, we performed in situ immunocytochemical staining of OA in mouse femoral sections. Fig. 1A shows multiple resorption sites containing active TRACP-positive osteoclasts (stained in pink) on the trabecular bone surface. These osteoclasts in a serial section were stained positively (in brown) for OA (Fig. 1B). A number of cuboidal shaped osteoblasts on the bone surface also stained positively for OA. As a negative control, another serial section stained with control non-immune IgG showed no positive OA staining in the TRACP-positive osteoclasts (Fig. 1C). These findings confirm that mature osteoblasts express the OA protein, but they also clearly demonstrate that active osteoclasts on bone surfaces also express substantial levels of the OA protein. These results contradict the previous conclusion that OA is an osteoblast-exclusive protein in bone [1,5,12].

Our gene expression data indicate that OA expression is up-regulated by RANKL during osteoclast differentiation. To characterize the temporal relationship between OA expression and the sRANKL-induced osteoclast differentiation, we assessed the time-dependent change in OA protein levels by Western blot analyses during the 6-day sRANKL-induced differentiation of pre-osteoclasts into osteoclasts. Two commercially available anti-mouse OA antibodies (a polyclonal neutralizing antibody and a monoclonal antibody) were used for this work. OA in osteoblasts has been reported to exist as two forms: the un-glycosylated 65-kDa form and a 115-kDa highly glycosylated secreted form [3]. Thus, osteoblasts from two inbred mouse strains (C3H/HeJ and C57BL/6J) [treated with an osteogenic medium containing 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate for 6 days to promote differentiation] were included for comparison. Fig. 1D shows that the polyclonal anti-mouse OA antibody recognized both the glycosylated and unglycosylated forms of OA in both osteoblasts and osteoclasts. In contrast, the monoclonal antibody recognized only the 65 kDa, presumably the unglycosylated form, of OA (Fig. 1E). We confirmed that OA in mouse osteoblasts existed as the 65 kDa (unglycosylated) and the 115 kDa (presumably glycosylated) forms (Fig. 1D). While murine osteoclasts also expressed glycosylated and unglycosylated forms of OA proteins, there was a major difference between the glycosylated forms of OA in osteoclasts and those in osteoblasts. Murine osteoclasts expressed at least three glycosylated forms (i.e., 139 kDa, 100 kDa, and 80 kDa) of OA in addition to the 65-kDa unglycosylated form. The 115-kDa glycosylated form of OA was not found in osteoclasts. It appears that the majority of the OA protein in osteoclasts is glycosylated, but the glycosylation process may be different in osteoclasts than that in osteoblasts. The significance of these multiple glycosylated forms of OA in osteoclasts is not clear and needs further investigation. Nevertheless, there was an obvious time-dependent increase in the expression of both glycosylated and unglycosylated OA proteins upon the sRANKL-induced differentiation, confirming that the OA protein expression in pre-osteoclasts is temporally associated with the sRANKL-induced differentiation.

Fig. 1F compares the effects of several known effectors of osteoclasts (mCSF, TNF-α, TGF-β_1, IL-1, IL-6, and sRANKL) on the expression of OA protein during osteoclastic differentiation. With the exception of sRANKL, none of the test effectors had appreciable effects on the OA protein expression, suggesting that the up-regulation of OA expression in osteoclasts may be specific for RANKL. Since none of the test effectors alone was able to produce appreciable numbers of TRACP-positive, multinucleated osteoclasts without sRANKL in vitro, it is conceivable that OA gene expression in osteoclasts is associated with osteoclast differentiation. In osteoblasts, the expression of OA is up-regulated by BMP-2 [13], and in macrophages, it was enhanced by IFN-γ and lipopolysaccharide [14]. We have not tested whether these effectors could also stimulate OA expression in osteoclasts.

The polyclonal anti-mouse OA antibody used in this study is a neutralizing antibody that has been shown by the supplier to block the bioactivity of OA to induce SVEC4-10 cell adhesion. Thus, we next used this neutralizing antibody to assess the functional role of OA in osteoclast differentiation and/or activ-

Table 1
Comparison of the relative TRACP and OA/*Dchil*/*Gpnmb* mRNA transcript levels in primary murine marrow-derived osteoclasts with those in other marrow cells and osteoblasts by real-time RT-PCR

Cell type	TRACP mRNA transcript		OA/*Dchil*/*Gpnmb* mRNA transcript	
	ΔC_T	Relative fold changes	ΔC_T	Relative fold changes
Marrow-derived osteoclasts	2.67 ± 0.84	1045.5 ± 1.79	1.88 ± 0.73	8.6 ± 1.6
Non-adherent marrow cells	12.7 ± 1.8***	1.0 ± 3.4***	4.98 ± 0.56***	1.0 ± 1.4***
Adherent stromal cells	9.50 ± 0.49***	9.2 ± 1.40***	1.72 ± 0.54	9.6 ± 1.4
Osteoblasts	10.1 ± 0.2***	6.1 ± 1.1***	6.87 ± 0.43***	0.3 ± 1.3***
ANOVA	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

The identity of each PCR product was confirmed by DNA sequencing. The data are in mean ± S.D. (n = 3–4 per group) and are shown as ΔC_T (C_T of the gene-of-interest − C_T of β-actin) and fold-changes compared to non-adherent marrow cells.
***$P < 0.001$ in comparison with osteoclasts by Tukey post-hoc test.

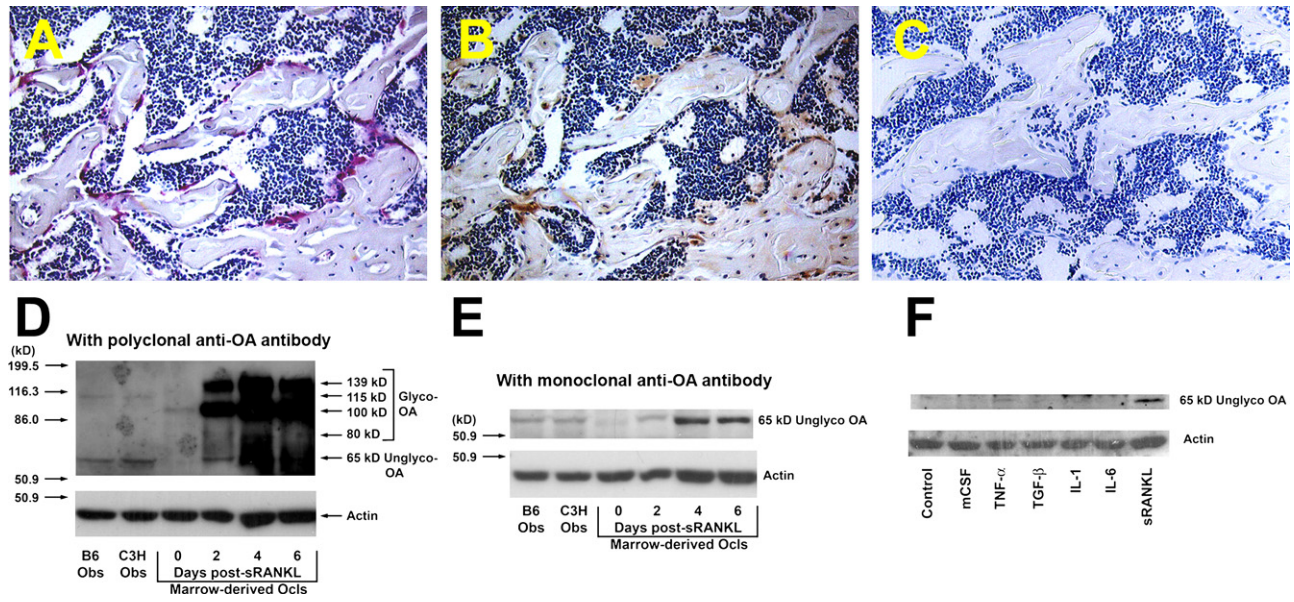

Fig. 1. Murine osteoclasts express substantial OA protein levels, which is up-regulated by RANKL. (A) The histochemical staining of TRACP activity (in pink) as a marker of osteoclasts in the first serial femoral section. (B) The in situ immunocytochemical staining of OA (in brown) in osteoclasts stained with the goat anti-mouse OA polyclonal antibody in the second serial femoral section. (C) The third serial section, in which in situ immunocytochemical staining of OA was performed using a non-immune anti-goat IgG antibody. (D) Western immunoblot analysis of cellular OA protein in marrow-derived osteoclasts before and after 2, 4, or 6 days of sRANKL treatment using the goat polyclonal anti-murine OA antibody. Differentiated osteoblasts of both C57BL/6J (B6 Obs) and C3H/HeJ (C3H Obs) inbred mouse strains were included for comparison. (E) Western immunoblot analysis of the duplicate blot of (D) using the rat anti-OA monoclonal antibody. (F) Shows a Western immunoblot of the 65-kDa unglycosylated OA (identified with the rat anti-OA monoclonal antibody) after 4 days of treatment with indicated effectors of osteoclasts. Similar results were seen with anti-OA polyclonal antibody (data not shown).

ity. We treated marrow-derived pre-osteoclasts with various amounts of the neutralizing antibody during the final 24 h of the 4-day sRANKL treatment and then determined the effects on osteoclast differentiation by measuring the average size and number of nuclei per osteoclast of the derived osteoclasts. This time point was chosen, because no significant osteoclast-like cells were seen before 3 days and because OA expression in osteoclasts appeared to be maximally up-regulated after 4 days of sRANKL treatment (Fig. 1D and E). Fig. 2A shows that the neutralizing antibody yielded a dose-dependent decrease in the average cell size of the derived osteoclasts, while the non-immune goat IgG treatment had no detectable effect. The neutralizing antibody, not only decreased the average cell size of the derived osteoclasts, but also significantly and dose-dependently reduced the average number of nuclei per osteoclast (Fig. 2B). Because the average cell size and number of nuclei per osteoclast are determined by cell fusion and/or cell spreading, these findings suggest that OA may have regulatory functions in osteoclast fusion and/or spreading. Because the polyclonal anti-OA antibody, but not the monoclonal antibody that recognizes only the un-glycosylated form, blocked the sRANKL-induced osteoclast formation, these results suggest that the glycosylated forms, and not the un-glycosylated form, mediate the sRANKL-induced osteoclast differentiation, spreading, and/or fusion.

Increases in the degree of multinucleation enhance the capacity of osteoclasts to resorb bone [20], and cell spreading is essential for the osteoclastic resorption process [21]. We next tested the possibility that the neutralizing anti-OA antibody would also have an inhibitory effect on bone resorption activity of the osteoclast, by measuring the effects of the final 24-hour treatment with neutralizing anti-OA antibody during the 4-day sRANKL treatment on the bone resorption activity of the derived osteoclasts with a resorption pit formation assay. The neutralizing anti-OA antibody produced a significant and dose-dependent decrease in the average size of the resorption pits (Fig. 3A), supporting our interpretation that OA has a functional role in the overall osteoclastic resorption process. Because we did not measure the average depth of the resorption pits, which is relevant to the overall resorption capacity of the osteoclast, we do not know if the suppression of the functional activity of OA would also reduce the bone resorption capacity of the osteoclast by reducing the average pit depth. Nevertheless, because TRACP expression and secretion are markers of osteoclastic resorption, our findings that the neutralizing antibody also significantly suppressed the cellular and conditioned medium (CM) levels of TRACP activity in osteoclasts (Fig. 3B) are consistent with an inhibitory effect of the neutralizing antibody on osteoclastic resorption. Together, our results have clearly demonstrated that suppression of the functional activity of OA in osteoclasts not only reduces the fusion and/or spreading of osteoclasts, but also inhibits their overall bone resorption activity.

Fusion events that give rise to multinucleated osteoclasts can occur at different stages: fusion of mononucleated osteoclast precursors will initially form small osteoclasts with few nuclei, and fusion between multinucleated osteoclasts will then give rise to large osteoclasts with large numbers of nuclei. If OA is involved in the fusion of mononucleated osteoclast precursors, blocking the function of OA by the neutralizing antibody would reduce the number of both the small and large multinucleated osteoclasts. In contrast, if OA is involved only in the formation of large multinucleated osteoblasts by promoting the fusion process of smaller multinucleated cells, the neutral-

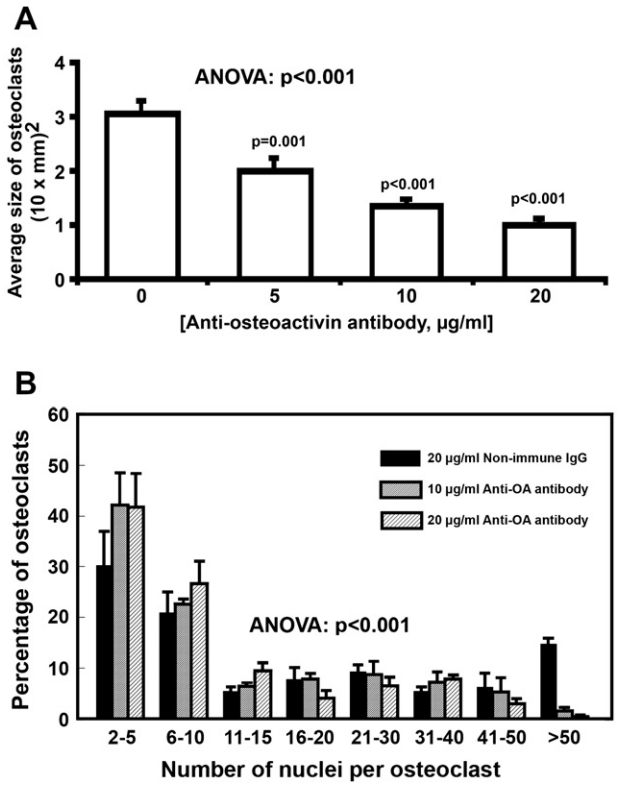

Fig. 2. Neutralizing antibody-mediated suppression of OA functional activity reduced the cell area (A) and number of nuclei (B) of the marrow-derived osteoclasts. Marrow-derived osteoclasts were treated with 0 (i.e., 20 μg/ml normal goat IgG), 5, 10, or 20 μg/ml of goat anti-OA neutralizing antibody during the last 24 h of the sRANKL treatment. Because of the significant difference in the relative size of the derived osteoclasts in the normal IgG and anti-OA-treated cultures, the actual number of osteoclasts analyzed in the control and treated group was different, ranging from ~50 per well (in the normal IgG-treated control group) to ~200 per well (in the group treated with 20 μg/ml anti-OA). To adjust for the difference in the number of cells analyzed, the results are shown as the percentage of total number of osteoclasts counted. Mean ± S.D., $n = 3$. Significance was assessed by ANOVA followed by Tukey post-hoc test.

izing anti-OA antibody treatment should primarily reduce the number of large multinucleated osteoclasts that have multiple nuclei clusters. Consistent with the concept that formation of large osteoclasts involved fusion between smaller multinucleated osteoclasts, the large multinucleated osteoclasts derived from the sRANKL-treated murine marrow osteoclast precursors contained multiple clusters of nuclei (left panel of Fig. 4). However, the number of nuclei clusters in the anti-OA-treated marrow-derived osteoclasts was greatly reduced (right panel of Fig. 4); a finding that is consistent with the possibility that the function of OA, in the context of osteoclast fusion, acts primarily through promotion of fusion of smaller multinucleated cells into large multinucleated cells. To assess if OA also has a regulatory function in earlier events, we have also performed an additional experiment to determine the effect of addition of the neutralizing antibody on day 2 (for 24 h) of the RANKL treatment on formation of osteoclast-like cells. We did not see a statistically significant effect (data not shown). Because RANKL treatment has already up-regulated OA expression after 2 days (Fig. 1D), the lack of an obvious effect of the neutralizing antibody at day 2 of RANKL treatment suggests that OA might not have an essential regulatory function in early fusion events.

Little is known about the molecular mechanism of action of OA. However, OA contains an integrin-recognition RGD motif in both its extracellular and intracellular domains [1,2], and it has been suggested that integrin binding is involved in the OA-mediated endothelial cell adhesion [2]. In this regard, some of the processes essential for osteoclast differentiation and activation, such as the re-organization, polarization, and construction of osteoclast-specific structures (e.g., sealing zone and ruffled borders), involve the integrin-dependent cytoskeleton reorganization [22,23]. There is compelling evidence that the RANKL-induced osteoclast differentiation [24,25], adhesion, spreading, and re-organization of cytoskeleton in osteoclasts [24] and resorption activity of osteoclasts [26] are all in part mediated through the integrin (especially $\alpha_v\beta_3$ integrin) signaling pathway. However, the requirement of β_3 integrin can be compensated by β_1 integrin [23]. We postulate that OA may

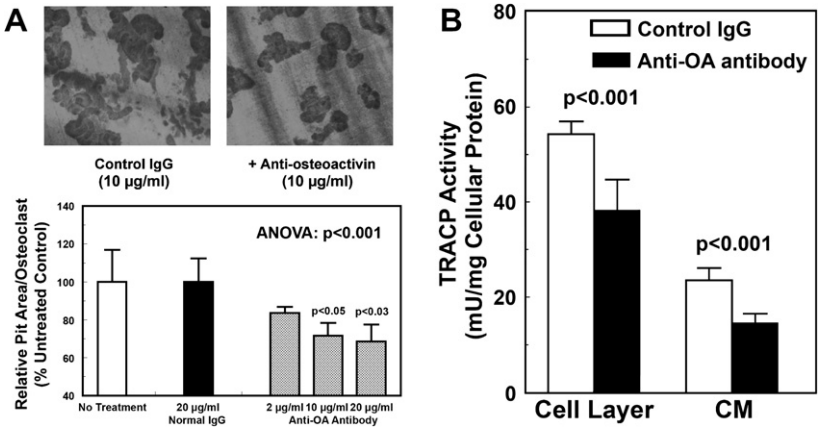

Fig. 3. Suppression of OA functional activity by the neutralizing antibody reduced bone resorption activity (A) and cellular and secreted TRACP activity (B) of marrow-derived osteoclasts. Top panel of A shows representative resorption pits formed by murine osteoclasts treated with normal goat IgG (left) and by those treated with goat anti-OA neutralizing antibody, and bottom panel of A summarizes dose-dependent effects of anti-OA antibody on the average size of resorption pits (mean ± S.D., $n = 3$) as % of the IgG-treated control osteoclasts. B summarizes the TRACP activity of the cellular layer and conditioned medium (CM) of marrow-derived osteoclasts after 24-hour treatment with goat IgG (control IgG) or the neutralizing anti-OA antibody (anti-OA antibody). Results are shown as mean ± S.D., $n = 6$.

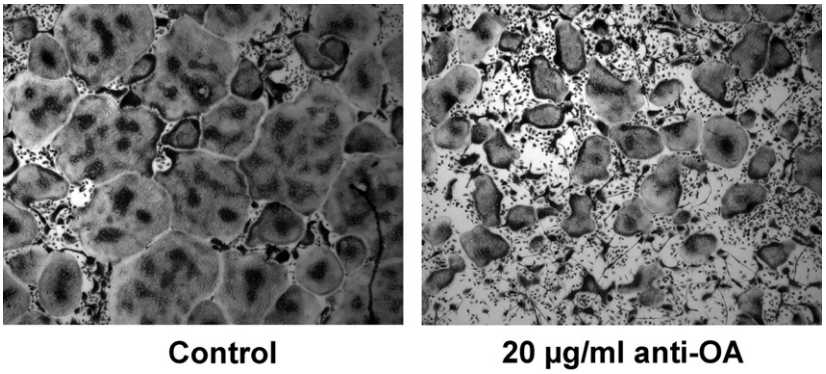

Fig. 4. The neutralizing anti-OA antibody treatment reduced the size and the number of nuclei clusters in marrow-derived osteoclasts. Left panel is a photomicrograph of osteoclasts derived from sRANKL-treated marrow osteoclast precursors, many of which are large and contains multiple nuclei clusters. Right panel is a photomicrograph of osteoclasts derived from the neutralizing anti-OA antibody treated marrow osteoclast precursors.

be involved in the integrin signaling of β_3 and/or β_1 through binding to the hetero-polymeric complex of integrins on the cell surface of osteoclasts and as such, functions as a "co-integrin receptor" to activate the integrin signaling. As an initial test of our hypothesis of an association between OA and the integrin signaling complex, we tested if integrin β_3 or β_1 in osteoclasts would be co-immunoprecipitated with OA. Consistent with our hypothesis, a significant amount of OA was found to be co-immunoprecipitated with integrin β_3 and β_1 (Fig. 5). If the action of OA to regulate osteoclast fusion and/or spreading is in part mediated through the integrin signaling, blocking the action of integrin $\alpha_v\beta_3$ with an inhibitory RGD peptide should mimick the action of the neutralizing anti-OA antibody on cell size of the marrow-derived osteoclasts. As predicted, addition of an inhibitory RGD peptide [c(RGDyK)], but not that of the control RGD peptide [c(RADyK)], to the marrow-derived osteoclast precursors during the final 48 h of the total of 5-day sRANKL treatment significantly reduced the average size of the derived osteoclasts by >40% (Fig. 6). However, it is highly likely that integrin signaling mediates a number of mechanism and pathways to regulate cell spreading and/or fusion, in addition to that of OA. Thus, we can not rule out the possibility that at least some of the observed effects of the inhibitory RGD peptide may be unrelated to blocking of the OA action in osteoclasts. In this regard, there appeared to be slight differences in the morphology of RGD peptide-treated osteoclasts as opposed to that of anti-OA-treated osteoclasts, in that the inhibition of osteoclast spreading by the RGD peptide was more pronounced than that by anti-OA neutralizing antibody (data not shown). At any rate, these findings provide circumstantial support for the hypothesis that OA may act in part through an interaction with the integrin β_3 and/or β_1 pathway to regulate osteoclast fusion, migration, and/or resorption. However, much additional work is needed to confirm this interesting hypothesis.

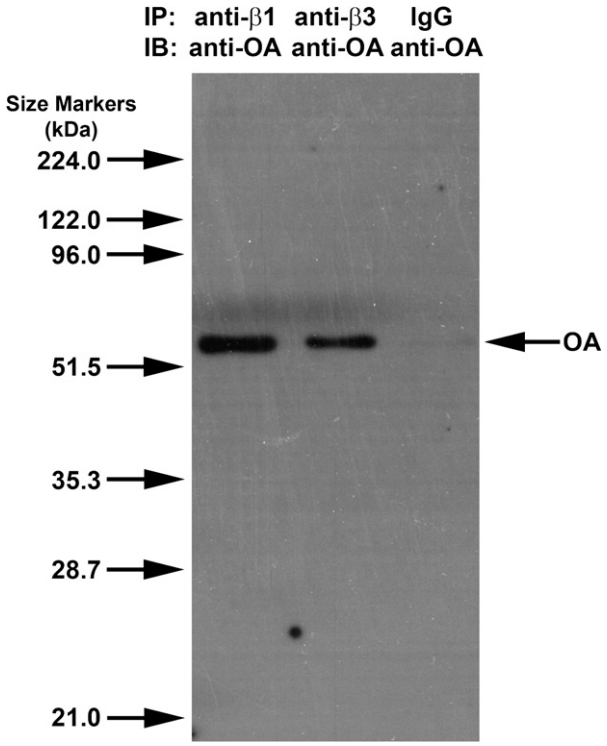

Fig. 5. OA co-immunoprecipitated with integrin β_3 or β_1 in osteoclasts. Cell extract of marrow-derived osteoclasts were immunoprecipitated (IP) with an anti-murine integrin β_1, anti-murine integrin β_3 antibody, or non-immune IgG. The co-immunoprecipitated proteins were resolved on 10% SDS–PAGE, and the presence of OA was identified by Western blot (IB) using the rat anti-OA monoclonal antibody.

With respect to subcellular localization of OA, a recent study using immunofluorescence confocal microscopy in RAW264.7 cells [15] has shown that OA is primarily at a perinuclear location during the first three days of RANKL treatment. However, after 5–7 days of RANKL treatment, the localization of OA is restricted to late endosomes and lysosomes. The localization of OA to these compartments is consistent with the presence of a predicted endosomal/lysosomal-sorting signal located immediately after the transmembrane domain of OA [27]. These findings suggest that OA resides in the endocytic pathway of osteoclasts and is probably targeted to the plasma membrane or extracellular space, where it exerts its biological function, upon osteoclast differentiation and maturation. In this regard, intracellular membrane trafficking and endocytic pathways are regulated by RANKL and are important for osteoclast function [28]. These findings are also consistent with our conclusion that OA is involved in primarily the late rather than early osteoclast differentiation processes.

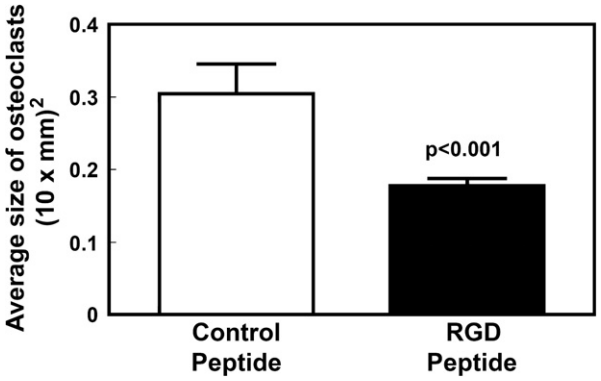

Fig. 6. The inhibitory RGD peptide for integrin $\alpha_v\beta_3$ reduced the average size of marrow-derived osteoclasts. Mouse marrow-derived osteoclast precursors were treated with 30 μM of either the c(RGDyK) peptide, an inhibitory peptide for integrin $\alpha_v\beta_3$ (RGD peptide), or the inactive c(RADyK) negative control peptide (control peptide) during the final 48 h of the 5-day sRANKL treatment. The average size of osteoclasts was measured at the end of the treatment.

In summary, we have demonstrated for the first time that osteoclasts express substantial amounts of OA and that OA plays an important functional role in the regulation of osteoclast formation and/or activity, presumably in part through mediating the RANKL-dependent fusion and/or spreading of osteoclasts. This study also provides evidence that OA may act in part through the signaling pathway of integrin β_3 and/or β_1 in osteoclasts and raises the interesting possibility that the OA/integrin signaling interaction may be essential in the RANKL-induced osteoclast formation, spreading, fusion, and bone resorption activity.

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