EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 34, No. 5, 347-352, November 2002

Osteoprotegerin is present on the membrane of osteoclasts isolated from mouse long bones

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Accepted 2 October 2002

Abbreviations: OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; IL-1α, interleukin-1α; TNF-α, tumor necrosis factor-α; HBSS, Hank's balanced salt solution; α-MEM, α-modified minimal essential medium

Abstract

Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor superfamily, is known to inhibit osteoclastogenesis by acting as a soluble decoy receptor for the receptor activator of NF-κB ligand (RANKL). We report the presence of OPG on the membrane of osteoclasts and the possibility of the direct action of OPG on them. Highly pure osteoclast precursors were isolated from mouse long bones and induced to differentiate into mature osteoclasts by M-CSF and soluble RANKL (sRANKL). The presence of OPG on the membrane of these cells was confirmed by western blotting and immunostaining. Furthermore, sRANKL was found to be bound to the OPG on the osteoclast precursors. These results suggest that OPG might have a new role during the differentiation of osteoclasts beyond its role as a soluble decoy receptor. The mechanism of the existence of OPG on osteoclast precursors remains to be found.

Keywords: bone and bones, cell membrane, cytokine receptors, mice, osteoclast, tumor necrosis factor

Introduction

Osteoclasts are responsible for bone resorption, and their formation is precisely regulated (Roodman, 1999). Osteoprotegerin (OPG, also named as osteoclastogenesis inhibitory factor, OCIF and TNF receptor-like molecule 1, TR1) is secreted by osteoblasts, bone marrow stromal cells, fibroblasts and T-lymphocytes, and is suggested to be an important regulator in osteoclastic bone resorption (Simonet *et al*., 1997; Tsuda *et al*., 1997; Kwon *et al*., 1998; Yasuda *et al*., 1998a; Kong *et al*., 1999).

The hepatic over-expression of OPG in transgenic mice has resulted in profound but non-lethal osteopetrosis (Simonet *et al*., 1997), and systemic OPG administration has increased the bone mass (Simonet *et al*., 1997; Akatsu *et al*., 1998; Yamamoto *et al*., 1998; Yasuda *et al*., 1998a). OPG inhibited the formation of osteoclasts in a dose-dependent manner *in vitro* (Simonet *et al*., 1997; Tsuda *et al*., 1997; Kwon *et al*., 1998; Yasuda *et al*., 1998a), and severe osteoporosis was induced as a result of the deletion of the endogenous OPG gene (Bucay *et al*., 1998; Mizuno *et al*., 1998). All these activities have been attributed to OPG acting as a soluble decoy receptor for the receptor activator of NF-κB ligand [RANKL, also known as osteoprotegerin ligand (OPGL), osteoclast differentiation factor (ODF), and TNF-related activation-induced cytokine (TRANCE)] (Lacey *et al*., 1998; Yasuda *et al*., 1998b; Takahashi *et al*., 1999).

However, there are some conflicting reports on the role of OPG as a soluble decoy receptor. First, serum concentration of OPG was found to be higher in women with postmenopausal osteoporosis than in normal women (Yano *et al*., 1999), which is contradictory to expectation. Second, interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α), which induce osteoclastogenesis, have been found to up-regulate the level of OPG mRNA in several osteoblastic cell lines (Brandstrom *et al*., 1998; Vidal *et al*., 1998). The relative abundance of OPG and RANKL has been suggested as a possible explanation of this discrepancy. However, it was found that the expression of RANKL was consistently up-regulated by pro-resorptive hormones and cytokines, while OPG expressions were quite variable (Shalhoub *et al*., 2000). Considering the profound inhibitory effects of OPG on bone resorption were demonstrated *in vivo* and *in vitro,* these patterns and the variable nature of OPG expression are quite questionnaire.

In search of other regulatory mechanism, the presence of OPG in highly pure osteoclast precursors from mouse long bones was examined. Now we report the presence of OPG on the cell membrane of osteoclasts, suggesting that OPG may have a different mode of action.

Materials and Methods

Isolation of osteoclast precursors and induction of differentiation into mature osteoclasts

To separate the purest osteoclast precursors, we first removed other bone cells from the bone tissues and then collected the osteoclast precursors, because osteoclasts adhere more strongly to the bone matrix. The long bones were aseptically removed from five-week-old female mice (ICR) and the soft tissues were cleaned off. The marrow was flushed out using 22-gauge needle. The bones were placed in ice-cold Hank's balanced salt solution (HBSS) and minced into small pieces. The minced bone pieces were digested five times with enzyme solution [1 mg/ml type II collagenase (Gibco BRL, Gaithersburg, MD), 0.05% trypsin (Gibco BRL), 4 mM EDTA (Gibco BRL) in HBSS] for 15 min each time to remove cells other than the osteoclast precursors. After enzyme-digestion, the bone pieces were washed three times with HBSS and transferred to 50-ml with ice-cold α-modified minimal essential medium (α-MEM). After 15 min, the bone pieces were shaken vigorously for 1 min to collect the osteoclast precursors and the cell suspension was passed through a 40-μm cell strainer (Falcon). The isolated cells were induced to differentiate into mature osteoclasts by 20 ng/ml M-CSF (R&D systems, Minneapolis, MN) and soluble RANKL (sRANKL, Peprotech, England).

Western blot analysis

The isolated osteoclast precursors were cultured for two days in the presence of M-CSF and sRANKL. The cultured cells were lysed in a RIPA buffer (1$\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) on ice for 15 min. The cell lysate was passed through the 22-gauge needle and centrifuged at 14,000$\times g$ at 4°C for 20 min. The supernatant containing 20 μg of protein was separated on a 10% SDS-polyacrylamide gel under reducing and non-reducing conditions as previously reported (Tomoyasu *et al.*, 1998). After being transferred to the PVDF membrane (Sigma, St. Louis, MO), the proteins were blotted with anti-OPG monoclonal antibodies (Kwon *et al.*, 1998).

Immunostaining

The cells cultured for two or seven days were washed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS. The immunostaining procedures were performed using a Vectastain ABC kit (Vector, Burlingame, CA) according to the manufacturer's instructions.

Immunoprecipitation of biotinylated surface proteins by anti-OPG antibody

The cells cultured for two days were incubated with 1 mg/ml biotinylation reagent (EZ-LinkTM Sulfo-NHS-LC-Biotin, Pierce, Rockford, IL) in PBS at 4°C for 2 h with constant rocking. After biotinylation, the cells were lysed in a RIPA buffer on ice for 15 minutes. The lysate was centrifuged at 14,000$\times g$ at 4°C for 20 min. The supernatant was precleared with mouse IgG_1, an isotype control antibody, at a dilution of 1 μg/ml of lysate and 15 μl of protein A/G agarose (Calbiochem, Cambridge, MA) at 4°C for 4 h with constant rocking.

After centrifugation of the precleared lysate, the supernatant was incubated with 1 μg/ml of anti-OPG antibody or mouse IgG_1 and 15 μl of protein A/G agarose at 4°C overnight with constant rocking. The beads were washed three times with a RIPA buffer and boiled for 5 min. The proteins were separated on a SDS 10% polyacrylamide gel, and transferred to a PVDF membrane. The blots were incubated with 0.5 μg/ml of streptavidin-horseradish peroxidase at room temperature for 1 h. The biotinylated surface proteins were visualized with a luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

Cross-linking of sRANKL to surface proteins of osteoclast precursors

The cells were cultured for two days, and incubated with 200 ng/ml sRANKL in PBS at 4°C for 1 h with constant rocking. After washing with PBS, the cells were incubated with a cross-linking agent, BS3 (1 mM in PBS, Pierce), which is impermeable to cell membranes, at room temperature for 15 minutes. The cells were lysed with a lysis buffer (1% Triton X-100, 10% glycerol in PBS) on ice for 15 min. The lysates were centrifuged at 14,000 xg at 4°C for 20 min. The supernatant was precleared and immunoprecipitated with an anti-OPG antibody as described above. A possible sRANKL-membranous OPG complex was detected in the blot with anti-RANKL antibodies (Peprotech).

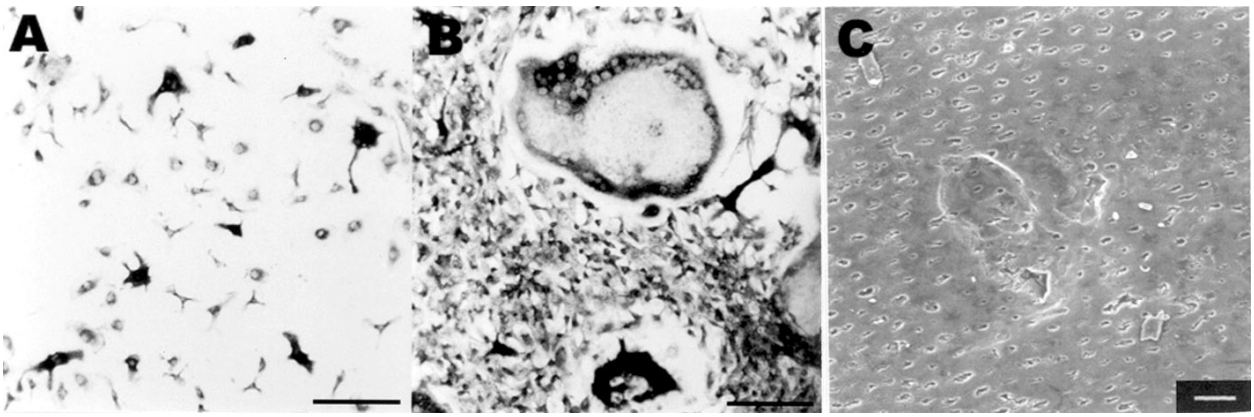

Figure 1. Morphology of highly purified osteoclast precursors isolated from the long bones of mouse. The isolated cells were cultured for 2 days (A) and 7 days (B) in the presence of 20 ng/ml M-CSF and 30 ng/ml sRANKL and stained for TRAP. Numerous mature, multinucleated TRAP-positive cells were attained within the 7 day-culture, which were engaged in extensive resorption of dentin slices (C) as revealed by scanning electron microscopy. Bars: A, B, 50 μm; C, 10 μm.

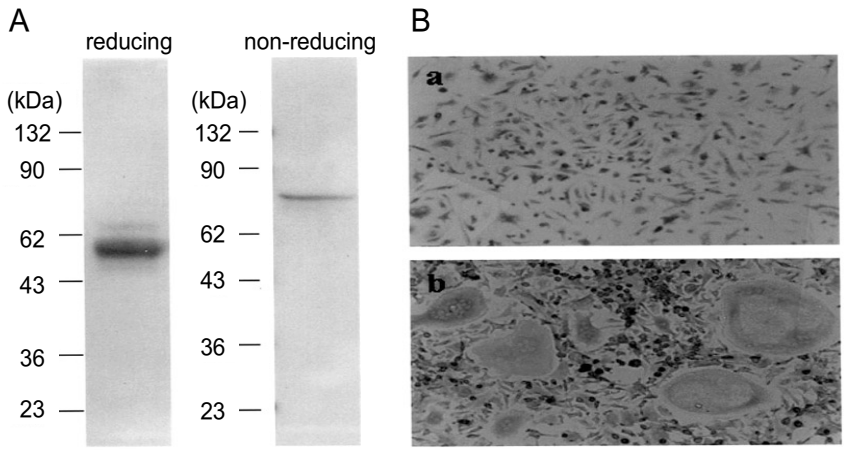

Figure 2. Expression of OPG in cultured osteoclasts. The OPG expression of the cultured osteoclasts was confirmed by western blot analysis (A), and immunostaining (B). The isolated osteoclast precursors were cultured for 2 days in the presence of M-CSF and sRANKL. Anti-OPG mAb detected a band with M_r of 55,000-60,000 under the reducing condition and 75,000-80,000 under the nonreducing condition (A). Both the precursors (B-a) and mature osteoclasts (B-b) were immunostained by anti-OPG mAb; however, the precursors were stained more deeply. The result shown is a representative of three independent experiments.

Results

Isolation of highly pure osteoclast precursors

About 96% of the isolated cells were tartrate-resistant acid phosphatase (TRAP)-positive and mononuclear (Figure 1A). Numerous multinucleated TRAP-positive cells were attained within a seven-day culture by M-CSF and sRANKL (Figure 1B), and were engaged in resorption of dentin slice (Figure 1C). In this study, we used the cells cultured for two days as osteoclast precursors, and those for seven days as mature osteoclasts.

Presence of OPG in osteoclasts

The cell lysates of osteoclast precursors and mature osteoclasts that were induced by M-CSF and sRANKL were found to contain OPG molecules. The anti-OPG antibody detected a band with a molecular weight of 50-55 kDa under reducing conditions and a band with a molecular weight of 75-80 kDa under non-reducing conditions (Figure 2A). This suggests that OPG molecules might be bound to other molecule(s) and form a hetero-complex in the osteoclasts. Or it might be a result from a conformational change. Immunostaining also showed that almost all osteoclast precursors and mature osteoclasts were containing OPG molecules (Figure 2B). The precursors contained more

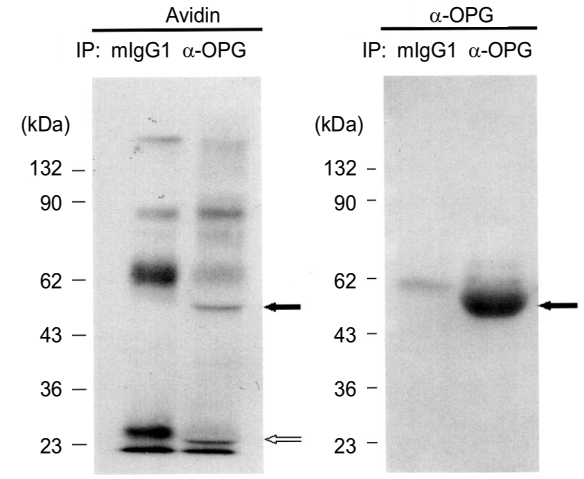

Figure 3. Membrane form of OPG expressed in the cultured osteoclasts. The surface proteins of the two-day cultured osteoclasts were biotinylated and immunoprecipitated with anti-OPG mAb. The precipitates were fractionated in a 10% SDS-PAGE, transferred to PVDF membrane, and visualized by avidin-HRP conjugate (left panel). A protein band with M_r of ~55,000 corresponding to OPG molecules (filled arrow) was detected under the reducing condition, and confirmed by the Western blot analysis with anti-OPG mAb (right panel). Another protein band with M_r of ~25,000 (open arrow) did not react with the anti-OPG mAb. A representative result of two independent experiments is shown.

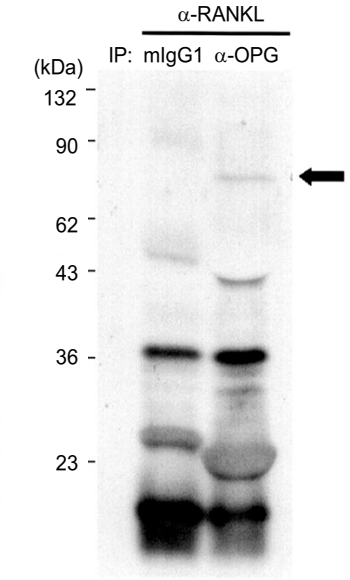

Figure 4. Binding of soluble RANKL to membranous OPG. Soluble RANKL was bound and cross-linked to surface proteins of the osteoclast precursors cultured for 2 days, and the cell lysate was prepared. A band with M_r of 75,000-80,000 (arrow) was detected, which corresponds to a calculated M_r of sRANKL-OPG complex. A representative result of two independent experiments is shown.

OPG than the mature cells.

OPG is present on the membrane of osteoclast precursors

To localize OPG on the cell membrane, immunoprecipitation of the biotinylated surface proteins was carried out. From the lysate of surface-biotinylated osteoclast precursors, a 55-kDa protein could be immunoprecipitated with an anti-OPG antibody and visualized with avidin-HRP conjugate (Figure 3). We confirmed the protein band as that of OPG by western blot analysis using the anti-OPG antibody in the same blot. Another protein of about 25 kDa in the immunoprecipitate was also detected by avidin-HRP. However, it did not react to the anti-OPG antibody in subsequent western blot analyses. It is likely that this protein exists in conjunction with OPG on the osteoclast membrane.

Evidence of binding of sRANKL to OPG on osteoclast membrane

The anti-RANKL antibody detected a 75-80-kDa protein in the anti-OPG-immunoprecipitate from the lysates of the osteoclast precursors that had been treated with sRANKL and cross-linked afterwards. This 75-80 kDa band corresponds to the calculated molecular weight of the sRANKL-OPG complex. This strongly suggests that RANKL may also bind to OPG on the osteoclast membrane in addition to its genuine receptor, RANK.

Discussion

In this study, we demonstrate that OPG is present on the cell membrane of osteoclast precursors and osteoclasts, suggesting that OPG may have a different mode of action in addition to its role as a soluble decoy receptor for RANKL. This result may provide a new ground for the explanation to the previous contradicting reports on OPG. The findings from western blot and immunostaining suggest that osteoclast itself could produce OPG that may be more important than the soluble OPG secreted by osteoblasts or stromal cells, which is also supported by demonstration of OPG mRNA by RT-PCR from our highly purified osteoclast precursor cell preparation (data not shown), although it is admitted that our osteoclast precursor cell preparation is not absolutely free from other cell types.

OPG is known to be a secreted glycoprotein without a transmembrane domain, although it is one of the TNF receptor family members (Simonet *et al.*, 1997; Yasuda *et al.*, 1998a). We observed that OPG in the osteoclasts exists as a membrane bound form,

while its cDNA sequence was identical to that previously reported which has no transmembrane region (data not shown). The membrane form of OPG was once reported in dendritic cells (Yun *et al*., 1998), although no other reports have mentioned it to our knowledge.

We speculate two possibilities in OPG's mode of existence on the cell membrane: it might exist either simply as a membrane-bound form or as an authentic transmembrane form. The OPG molecule is known to have a heparin-binding domain in its C-terminal, which may confer OPG molecule stickiness (Yamaguchi *et al*., 1998). This membrane-bound OPG on the osteoclasts might still serve as a decoy receptor for RANKL. This speculation is supported by our result that sRANKL did bind to OPG on the osteoclast membranes. The other possibility is that OPG exists as a transmembrane protein and transduces the extracellular signals such as RANKL or TRAIL. Considering the fact that the C-terminal portion of OPG contains death domain homologous regions, OPG has strong potential to mediate cytotoxic signals (Yamaguchi *et al*., 1998). Apoptosis of the osteoclast precursors might be mediated by the OPG that is transducing the extracellular signals from their ligands. TRAIL, a cytotoxic ligand for which OPG is currently a well-known decoy receptor, is a member of the tumor necrosis factor family and exhibits potent cytotoxic activity when bound to its surface receptor, TRAIL-Rs (Emery *et al*., 1998). Lastly, we would like to mention on our finding of a 25-kDa protein. From the data shown Figure 2 and 3, it is most likely a membranous protein and speculated to form a complex with OPG in osteoclasts. Characterization of this protein may provide a clue to elucidate the mode of existence of OPG and its function.

In summary, we demonstrated that OPG is present on the membrane of osteoclast precursors and osteoclasts. Furthermore, sRANKL is able to bind to the OPG on the membrane of osteoclast precursors. These results suggest that OPG present on the osteoclast precursors might have a new role in the differentiation of osteoclasts in addition to its role as a soluble decoy receptor. The mode of existence and function of OPG on osteoclast precursors, which has no transmembrane region, remains to be found.

Amendment

During the preparation of this manuscript, Standal *et al.* (Blood First Edition Paper, prepublished online June 21, 2002; DOI 10.1182/blood-2002-04-1190) reported that OPG is bound, internalized and degraded by multiple myeloma cells, supporting our results.

Acknowledgements

We thank Drs. Z.-H. Lee and J.-H. Baek for helpful discussion. K.M. Woo was supported by grant KRF-99-003-F00337 F4109 from the Korea Research Foundation. K.-K. Kim was supported by SRC fund to IRC (the Immunomodulation Research Center), University of Ulsan, from KOSEF and Ministry of Korean Sciences and Technology.

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