Osteoprotegerin Is a Receptor for the Cytotoxic Ligand TRAIL*

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Osteoprotegerin (OPG) is a secreted TNF receptor family member that increases bone density and causes splenomegaly when overexpressed in transgenic mice (14). OPG inhibits osteoclastogenesis in vitro, suggesting that increased bone density results from decreased numbers of mature osteoclasts. It has been suggested that OPG may neutralize a TNF-related ligand that promotes osteoclast differentiation or bind a membrane-anchored TNF-related ligand that regulates osteoclastogenesis via reverse signaling (14). To identify interactions between novel TNF ligand and receptor family members, we screened a panel of receptor-Fc fusion proteins for binding to TRAIL and have identified a novel interaction between OPG and TRAIL. We demonstrate that OPG binds to TRAIL in vitro and can block TRAIL-induced apoptosis. Conversely, TRAIL appears to block OPG-mediated inhibition of osteoclastogenesis in vitro. These results suggest that OPG and TRAIL may function to inhibit each other.

EXPERIMENTAL PROCEDURES

Preparation of Proteins and Antibodies—All cDNAs were identified by homology searches of an assembled Expressed Sequence Tag data base, and the 5′-most cDNA clones were obtained. The cDNAs encoding the native leader peptides and extracellular domains of OPG (amino acids 1–401) (14), herpes virus entry mediator (HVEM) (amino acids 1–199) (11, 18–20), DR5 (amino acids 1–401) (14), herpes virus entry mediator (HVEM) (amino acids 1–199) (11, 18–20), DR5 (amino acids 1–192) (15–17), DR3 (amino acids 1–199) (11, 18–20), DR5 (amino acids 1–133 of Ref. 7) (7–12), and TRID (amino acids 1–240) (7–10) were polymerase chain reaction-amplified and subcloned upstream of an in-frame Factor Xa protease cleavage site and the hinge-Fc region of a human IgG1 heavy chain in COSFelix (21, 22). The ILSR-Fc construct was described previously (22). OPG-Fc, HVEM-Fc, DR3-Fc, and TRID-Fc were purified from conditioned medium of Chinese hamster ovary stable transfectants by protein G affinity chromatography (Amersham Pharmacia Biotech). Samples contaminated with Fc dimer were further purified on Superdex 200 (Amersham). DR5-Fc protein was purified from the conditioned medium of COS cell transient transfectants by affinity chromatography on Prosep A (Bioprocessing Ltd.). Soluble receptors without the Fc domain were prepared by digesting with 1/80 v/v Factor Xa (Hemotologic Technologies, Inc.) at 4 °C for 72 h. Fc dimer was removed by protein A affinity chromatography (Amersham).

Soluble TRAIL tagged at the N terminus with the FLAG epitope (TRAIL-FLAG, amino acids 95–281) (4) was constructed in the pCDN mammalian expression vector (23) that had been modified to contain an in-frame tissue plasminogen activator signal sequence upstream of the FLAG-TRAIL coding region. Soluble TRAIL-Flag was purified from 30 liters of conditioned medium from a Chinese hamster ovary stable transfectant by protein G affinity chromatography (Amersham). TRAIL-Flag eluted at ~90 kDa, slightly larger than expected for a trimer, but appeared to be predominantly trimeric by analytical ultracentrifugation analysis.2 Polyclonal antibodies to TRAIL were raised in rabbits by injection of TRAIL (amino acids 41–281) that contained an epitope tag and hexa-
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Receptor Precipitations—TRAIL-Flag (250 ng) was added to 2 μg of receptor-Fc in binding buffer (25 mM HEPES, pH 7.2, 0.25% bovine serum albumin, 0.01% Tween in RPMI 1640) and incubated for 2 h on ice. Protein A-Sepharose 4B (Amersham, 30 μl of a 75% slurry) was used in receptor precipitation with 2 μg of receptor-Fc proteins, and complexes were precipitated with protein A-Sepharose. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western analysis with an anti-TRAIL polyclonal antiserum.

Expression System (24), solubilized in guanidine HCl, and purified by nickel nitrotriacetic acid chromatography.

Receptor was added immediately before the Fc fusion. DR5-Fc (250 ng) was added to 2 μg of soluble TRAIL-Flag before addition of OPG-Fc. Samples without competitor are indicated by '●'.

Binding reactions with full-length TRAIL were performed essentially as described above, except that serial dilutions of cleaved competitor OPG (2 μg/ml to 5 ng/ml, 100 μl/well) were added after washing and incubated for 1 h at room temperature. The Fc fusions were detected with a biotinylated goat anti-human IgG antibody (Southern Biotechnology Associates) (1/4000 dilution, 100 μl/well), horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates) (1/4000 dilution, 100 μl/well), and ABTS peroxidase substrate (100 μl/well, Kirkegaard and Perry Laboratories). The

| Receptor | kₐ (μM⁻¹ s⁻¹) | kᵋ (s⁻¹) | Calculated Kᵋ⁻¹ |
|----------|---------------|-----------|----------------|
| OPG-Fc   | 6.6 × 10⁴     | 2.0 × 10⁻⁴ | 3.0            |
| TRID-Fc  | 2.9 × 10³     | 3.2 × 10⁻⁴ | 1.1            |
| DR5-Fc   | 3.4 × 10³     | 2.6 × 10⁻⁴ | 0.76           |

The Fc fusions were detected with a biotinylated goat anti-human IgG antibody (Southern Biotechnology Associates) (1/4000 dilution, 100 μl/well), horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates) (1/4000 dilution, 100 μl/well), and ABTS peroxidase substrate (100 μl/well, Kirkegaard and Perry Laboratories). The

Fig. 1. OPG binds to TRAIL. A, immunoprecipitation of TRAIL-Flag by receptor-Fc proteins. TRAIL-Flag was incubated with the indicated receptor-Fc proteins, and complexes were precipitated with protein A-Sepharose. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western analysis with an anti-TRAIL polyclonal antiserum. B, cleaved OPG, TRID, and DR5 compete with Fc fusions for binding to TRAIL. Binding reactions were essentially as described above, except that a 10-fold molar excess of cleaved receptor was added immediately before the Fc fusion. C, OPG-Fc binds to cell-associated full-length TRAIL (FL-TRAIL). 293 cells were transiently transfected with a full-length TRAIL expression construct and labeled with [35S]methionine, and cell lysates were subjected to immunoprecipitation. Unlabeled soluble TRAIL-Flag was added as above where indicated. D, OPG- and DR5-Fc bind to TRAIL-Flag in ELISA. Microtiter plates were coated with an anti-Flag M2 monoclonal antibody and allowed to bind TRAIL-Flag. The plates were incubated with serial dilutions of OPG-Fc (●) and DR5-Fc (○) proteins, and binding was detected with a biotinylated goat anti-human IgG antibody followed by streptavidin-horseradish peroxidase and ABTS peroxidase substrate. E, OPG and DR5 compete with OPG-Fc for binding to TRAIL-Flag. The assay was performed essentially as described above, except that serial dilutions of cleaved competitor OPG (●) or DR5 (○) were incubated with the anti-Flag-captured TRAIL-Flag before addition of OPG-Fc. Samples without competitor are indicated by △. s, soluble; KD, kilodaltons.
FIG. 2. OPG blocks TRAIL-induced apoptosis. Jurkat cells were treated with increasing amounts of OPG (A), DR5-Fc (B), or DR3-Fc (C) in the presence of TRAIL-Flag plus anti-FLAG M2 monoclonal antibody (Kodak) or the anti-Fas agonist monoclonal antibody CH-11 (MBL) for 3 h. Cytotoxicity was assessed by determining the percentage of propidium iodide (PI)-permeable cells (mean ± S.D., n = 2) by fluorescence-activated cell sorter analysis. D and E, cytoplasts of Jurkat cells treated with TRAIL-Flag as described above in the absence (D) or presence (E) of OPG (5 μg/ml) were stained with 4′,6-diamidino-2-phenylindole dihydrochloride, and the nuclei were examined by fluorescence microscopy. Note the dramatic reduction in nuclear fragmentation in OPG-treated cells.

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To identify interactions between novel TNF ligand and receptor family members, we screened a panel of receptor-Fc fusion proteins for binding to TRAIL by immunoprecipitation. TRAIL bound to TRID-Fc and DR5-Fc proteins as expected (Fig. 1A). TRAIL also bound to OPG-Fc. TRAIL did not bind to DR3-Fc and HVEM-Fc (Fig. 1A), two additional members of the TNF receptor superfamily, indicating that the OPG-TRAIL interaction is not Fc-mediated. To confirm that binding of OPG-Fc to TRAIL was not to the Fc region, a 10-fold molar excess of OPG cleaved of its Fc domain was incubated with TRAIL and the Fc fusion proteins before immunoprecipitation. Cleaved OPG, TRID, and DR5 effectively competed away the binding of OPG-Fc to TRAIL (Fig. 1B). To determine if OPG could bind to cell-associated TRAIL, HEK293 cells were transiently transfected with a full-length TRAIL expression vector, labeled with [35S]methionine, and the cell lysate was subjected to immunoprecipitation by the OPG-Fc fusion protein. OPG-Fc precipitated a protein of the expected size in TRAIL but not in control cells, indicating that OPG-Fc binds to cell-associated TRAIL. Unlabeled soluble TRAIL-Flag effectively competed with the [35S]-labeled full-length TRAIL for binding to OPG-Fc.

The ability of TRAIL to bind OPG was also examined by ELISA. Both OPG-Fc and DR5-Fc proteins were capable of binding to anti-Flag-immobilized TRAIL-Flag (Fig. 1D). In blocking assays, cleaved DR5 prevented OPG-Fc from binding to TRAIL-Flag (Fig. 1E), indicating that the DR5 and OPG binding sites overlap. Cleaved OPG was more effective than cleaved DR5 in blocking TRAIL-Flag binding (Fig. 1E), possibly reflecting the fact that the cleaved OPG is a dimer, whereas

plates were read at 405 nm on a UV max microplate reader (Molecular Devices). For competition assays, serial dilutions of OPG or DR5 without Fc domains (50 μg/ml to 30 ng/ml, 100 μl/well) were added to the plate for 1 h at room temperature before the addition of OPG-Fc (10 μg/ml, 10 μl/well).

Determination of the Affinity of TRAIL for Its Receptors—The association and dissociation rates of the interaction of TRAIL with captured receptor fusion proteins were determined by surface plasmon resonance using a BIAcore 1000 (BIAcore Inc.). The capture surface was a protein A (Pierce) modified CM5 sensor chip (26). The sensor surface was equilibrated with a buffer of 20 mM sodium phosphate, 150 mM sodium chloride, and 0.005% Tween 20, pH 7.4, and analyses were performed at 30 μl/min at 25 °C. The receptor-Fc fusion protein was diluted into the above buffer to 1 μg/ml, and a 20 μl injection was passed over the capture surface, followed by a 150-μl injection of the TRAIL-Flag. After the association phase, 500 s of dissociation data was collected. The surface was regenerated after each cycle. Sets of 3–4 analyte concentrations, 500 20 nM, were collected and analyzed by nonlinear regression analysis (27) using the BIAevaluation software 2.1. The dissociation data was fitted on the basis of the AB = A + B model. The association data was fitted to A + B = AB using the type 1 model.

Apoptosis Assays—Jurkat cells (ATCC) (5 × 10⁶ cells/ml) were treated with increasing concentrations of OPG (0, 0.2, 1.0, or 5.0 μg/ml), DR5-Fc (0, 0.008, 0.04, and 0.2 μg/ml), or DR3-Fc (0, 0.2, 1.0, or 5.0 μg/ml), and apoptosis was induced with an anti-Fas CH-11 monoclonal antibody (0.1 μg/ml, Medical and Biological Laboratories) or TRAIL-Flag (0.1 μg/ml) with anti-FLAG M2 monoclonal antibody (3 μg/ml, Kodak) for 3 h. The anti-Flag M2 antibody was added to all treatments to normalize for potential nonspecific effects. Cells were stained with propidium iodide (6.5 μg/ml, Boehringer Mannheim) and quantitated by fluorescence-activated cell sorter analysis. Cytoplasts of Jurkat cells were fixed for 30 min in ice-cold 1% paraformaldehyde in phosphate-buffered saline, washed in phosphate-buffered saline, and stained for 30 min with 4′,6-diamidino-2-phenylindole dihydrochloride (1 μg/ml, Boehringer Mannheim).
cleaved DR5 is a monomer (Ref. 14 and data not shown). Although overlapping, the binding sites of OPG-Fc and DR5-Fc are not identical, since only OPG-Fc recognized directly immobilized TRAIL-Flag (i.e. without an anti-Flag antibody, data not shown).

The affinity of each receptor for TRAIL was determined by measuring the kinetics of TRAIL binding to each by surface plasmon resonance (Table 1). OPG-Fc binds to TRAIL-Flag with an affinity of 3.0 nM, which is slightly weaker than the affinities of TRID-Fc and DR5-Fc for TRAIL-Flag (1.1 nM and 0.76 nM, respectively). The affinities of each receptor are within the range of affinities reported for other physiologically relevant TNF-like ligand and receptor pairs (30–34).

The observation that OPG binds TRAIL in vitro suggests that OPG may inhibit the cytotoxic activity of TRAIL. To examine this possibility, Jurkat cells were treated with anti-Flag-aggregated TRAIL-Flag in the presence or absence of OPG. Anti-Flag-aggregated TRAIL-Flag exhibited significant cytotoxicity within 3 h (Fig. 2A). OPG demonstrated a dose-dependent inhibition of TRAIL-induced cytotoxicity and blocked the appearance of apoptotic nuclei in Jurkat cells treated with TRAIL-Flag (Fig. 2, D and E). However, OPG did not prevent killing by an agonist anti-Fas monoclonal antibody, another potent inducer of apoptosis, indicating that OPG does not activate a nonspecific survival pathway (Fig. 2A). DR5-Fc exhibited a similar but more potent inhibition of TRAIL-induced cytotoxicity (Fig. 2B), likely due to its higher affinity for TRAIL. DR3-Fc, which does not bind TRAIL, did not inhibit TRAIL-induced killing (Fig. 2C). This data suggests that OPG, which is secreted and detectable in the circulation (14), may be a soluble antagonist receptor for TRAIL. OPG may negatively regulate TRAIL activity, similar to the inhibition of TNFα by secreted TNF receptors of poxviruses (35). Furthermore, TNF receptors are shed from the cell surface and found in the circulation following certain stimuli. It has been suggested that soluble TNF receptors may inhibit or clear circulating TNFα (36). A similar role for OPG might also underlie the splenomegaly in mice that overexpress OPG (14). In mice lacking the anti-apoptotic Bcl-2 gene, there is a decrease in the size of the spleen, suggesting that spleen cell homeostasis is regulated by apoptotic mechanisms (37). Thus, if TRAIL is involved in regulation of spleen cell homeostasis by inducing apoptosis, then constitutive overexpression of OPG might result in decreased apoptosis and splenomegaly.

OPG inhibits osteoclastogenesis in vitro (14). To determine the significance of the OPG-TRAIL interaction in osteoclastogenesis, we examined the effects of TRAIL in an in vitro osteoclastogenesis assay. Human stromal cells were co-cultured with murine bone marrow cells for 7 days in the presence of OPG, TRAIL, or TL4, another TNF-like ligand that does not bind OPG.4 OPG inhibited the formation of multinucleate TRAP positive osteoclasts as expected, with an IC₅₀ of approximately 60 pM (Fig. 3). TRAIL alone had no effect on osteoclastogenesis. However, TRAIL completely blocked the inhibitory effect of OPG (Fig. 3), suggesting that soluble TRAIL may function to regulate the activity of OPG. TL4 had no effect on osteoclastogenesis in the presence or absence of OPG.

It has been suggested that OPG may inhibit osteoclastogenesis by binding to a pro-osteoclastogenic TNF-related ligand (14). Our data suggest that this ligand may not be TRAIL. First, soluble TRAIL alone had no effect on osteoclastogenesis, and the IC₅₀ of OPG is approximately 50-fold lower than the affinity of OPG for soluble TRAIL. Secondly, anti-Flag-aggregated TRAIL, which may mimic membrane TRAIL, induced massive apoptosis in these co-cultures, which is the opposite activity to what one would expect for the OPG ligand (data not shown). However, further experiments will be necessary to fully define the role of TRAIL in osteoclastogenesis and the existence of other OPG ligands.

The identification of a fifth receptor for TRAIL suggests that complex regulatory mechanisms control its activity. A soluble antagonist receptor, OPG, and two decoy receptors, DcR1/TRID/TRAIL-R3 and DcR2, may prevent the transduction of apoptotic signaling through the death receptors DR4 and DR5/TRICK2/TRAIL-R2. It has also been suggested that the generation of two DR5/TRICK2/TRAIL-R2 isoforms by alternate splicing may regulate cellular responses to TRAIL (11). Addi-
tionally, secreted TRAIL itself may antagonize OPG activity or prevent signaling by membrane-anchored TRAIL through its death receptors, as has been described for FasL and TNFα (reviewed in Ref. 3). A complete understanding of these regulatory circuits awaits the determination of soluble TRAIL and OPG levels in vivo and an analysis of knockout mutants of TRAIL and its receptors.

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Note Added in Proof—Recently two groups (H. Yasuda et al. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3597–3602 and D. L. Lacey et al. (1998) Cell 93, 155–176) reported the identification of a different OPG ligand that is identical to TRANCE/RANK-L. TLA was recently described as LIGHT (D. N. Mauri et al. (1998) Immunity 8, 21–30), another member of the TNF family.

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