INVESTIGATING THE INTERACTION BETWEEN OSTEOPROTEGERIN AND RANKL OR TRAIL: EVIDENCE FOR A PIVOTAL ROLE FOR OSTEOPROTEGERIN IN REGULATING TWO DISTINCT PATHWAYS

Srdjan Vitovski, Jennifer S. Phillips, Jon Sayers and Peter I. Croucher.

From the Academic Unit of Bone Biology, Section of Musculoskeletal Science and the Section of Infection, Inflammation and Immunity, University of Sheffield School of Medical and Biomedical Sciences, Sheffield, Yorkshire, S10 2RX, UK

Address correspondence to: Peter I. Croucher, Academic Unit of Bone Biology, Section of Musculoskeletal Science, University of Sheffield Medical School, Beech Hill Road, Sheffield, Yorkshire, S10 2RX, UK Tel: 44 114 271 2414, Fax: 44 114 271 1711, E-mail: p.croucher@sheffield.ac.uk

Running Title: Binding affinity of OPG for RANKL and TRAIL

SUMMARY

Osteoprotegerin (OPG) binds the ligand for receptor activator of nuclear factor kappa B (RANKL) to prevent association with its receptor RANK and inhibit osteoclast-mediated bone resorption. OPG has been reported, recently, to inhibit TNF-related apoptosis-induced ligand (TRAIL)-induced tumor cell apoptosis. This raises the possibility that OPG may play a unique role in regulating these two signalling pathways. However, there are little data on the interactions between OPG, RANKL and TRAIL and the relative affinity of OPG for these two ligands is unknown. In the present study we examined the ability of OPG to bind native human TRAIL and RANKL under physiological conditions.

Native TRAIL was expressed in E. coli, purified to homogeneity and shown to induce human myeloma cell apoptosis. OPG inhibited native TRAIL from binding the TRAIL-R1 at 37°C in vitro. Similarly, OPG prevented RANKL from binding to RANK. TRAIL also prevented OPG-mediated inhibition of RANKL from binding RANK. The affinity of OPG for native TRAIL and RANKL at 37°C was determined by plasmon surface resonance analysis. OPG had a binding affinity for TRAIL of 45 nM, whereas, the affinity of OPG for RANKL was 23 nM. These data suggest that OPG can bind both RANKL and TRAIL and that the affinity of OPG for these two ligands is of a similar order of magnitude. Furthermore, OPG prevented TRAIL-mediated reductions in cell viability, whereas TRAIL inhibited OPG–mediated inhibition of osteoclastogenesis in vitro. This highlights the pivotal role of OPG in regulating the biology of both RANKL and TRAIL.

INTRODUCTION

Bone is a complex tissue that is constantly being replaced through the process of bone remodelling. This process is mediated by the removal of bone by osteoclasts and its subsequent replacement by osteoblasts. Disruptions to the normal, coupled, activity of these cell types leads to skeletal disorders. The activity of osteoclasts and osteoblasts is regulated by the concerted actions of specific messengers including hormones, adhesion molecules, local growth factors and cytokines. One family of cytokines that play a central role in regulating osteoclast cell function is the tumour necrosis factor superfamily (1). Although a number of TNF family members have been shown to regulate osteoclast formation and function, one system has been shown to play a central role. This is the osteoprotegerin (OPG) / ligand for receptor activator of NFκB (RANKL) / receptor activator of NFκB (RANK) pathway (2).

Osteoprotegerin was first identified through screening a fetal rat intestinal cDNA library, searching an expressed sequence tag database, and
by direct purification from the supernatant derived from human fibroblasts (3-5). Analysis of the protein sequence identified structural motifs, which were similar to those seen in members of the TNF receptor (TNFR) family and thus OPG was identified as a new member of the TNFR family (3,6). However, OPG lacks a transmembrane domain and unlike the majority of members of this family is secreted into the extracellular space (7). Subsequently, a ligand for osteoprotegerin was identified using an expression cloning strategy (8,9). The ligand was shown to be identical to two previously described proteins that regulate dendritic cell and mature T cell survival, the receptor activator of nuclear factor kappa B ligand (RANKL) and TNF-related activation-induced cytokine (TRANCE) (10,11). The agreed nomenclature for this molecule is now RANKL. RANKL is expressed as a membrane-bound protein; however this molecule can be cleaved by a specific protease to produce a soluble form that retains biological activity (12). Finally, a cognate receptor that mediates RANKL activity was identified by genomic analysis of a primary osteoclast precursor cell cDNA library (13). This molecule was shown to be identical to the receptor activator of nuclear factor kappa B (RANK) (10). RANKL has been shown to stimulate osteoclast formation in vitro, whereas, both recombinant and purified native OPG have been shown to inhibit osteoclast formation and activity. Hepatic over-expression of OPG in mice results in osteopetrosis, whereas, inactivation of the OPG gene leads to osteoporosis. Furthermore, inactivation of RANKL in mice leads to osteopetrosis. These studies have highlighted the critical nature of this system in regulating normal skeletal biology. Within this system OPG plays a critical role in regulating osteoclast formation and preventing uncontrolled bone resorption.

OPG has recently been shown to bind another ligand, the TNF-related apoptosis-induced ligand (TRAIL) (14). TRAIL (Apo2L) is also a member of the tumour necrosis factor superfamily (1). TRAIL functions as a homotrimer and mediates its biological activity through four TRAIL receptors (15). Two of these receptors, death receptor 4 (DR4 or TRAILR1), and death receptor 5 (DR5 or TRAILR2) contain death domain motifs and promote apoptosis. In contrast, the other two receptors, DcR1, which lacks the cytoplasmic domain, and DcR2, which contains only a partial death domain, are unable to induce apoptosis and function as decoy receptors. TRAIL binding to DR4 or DR5 results in signalling through the death inducing signalling complex and induces apoptosis in tumour cells but appears to lack activity against normal cells. Blocking TRAIL activity in vivo has been shown to promote tumour development and increase liver metastasis, and mice deficient in TRAIL have increased metastasis and tumour growth (16-19). These data suggest that one of the normal physiological roles of TRAIL is in immune surveillance. More recently TRAIL has been shown to prevent OPG from inhibiting RANKL-induced osteoclast formation in vitro (20). Furthermore, OPG produced by tumour cells or osteoblasts is able to prevent TRAIL-induced apoptosis of tumour cells (21,22). These data are consistent with OPG playing a pivotal role in regulating these two TNF pathways, the RANKL/RANK system and the TRAIL/TRAILR system. However, there are only limited data investigating the binding of OPG to RANKL and TRAIL and these studies utilise non-native proteins and at non-physiological temperatures. Indeed, binding studies have suggested that the affinity of OPG for RANKL may be as much as 200 times greater than for TRAIL (23). This has been used to argue that the OPG:TRAIL interaction is of limited biological importance. However, this conclusion is not supported by data generated in complex cell based systems in vitro (21,22).

Thus, the nature of the binding of native OPG with RANKL and TRAIL at physiological temperature is poorly understood and there are no data directly comparing the binding affinity of OPG for RANKL and TRAIL. Therefore, in the present study we have investigated the ability of OPG to bind to both RANKL and TRAIL and prevent association with their cognate receptors. We have also investigated the ability of TRAIL to prevent OPG from inhibiting RANKL binding RANK and determined the binding affinity of OPG for RANKL and TRAIL under physiological conditions.
EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. - *E. coli* strain XL1Blue MRF: D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 Thi-1 recA1 gyrA96 relA1 lac [F proAb lacIqZDM15 Tn10 (Tetr)], (Stratagene) was used for cloning, stable propagation and preparation of plasmid DNA. *E. coli* BL21 (F, ompT, hsdSB, gal, dcm), an OmpT negative, protease deficient strain was used as a plasmid host for all TRAIL expression experiments (24). *E. coli* strains were grown in LB medium supplemented with 100μg/ml ampicillin.

Cloning of the TRAIL gene. - I.M.A.G.E. Consortium human TRAIL cDNA clone 5504670 (Geneservice Ltd Cambridge, UK), was used as a source for cloning. The TRAIL gene was amplified by PCR using *Pfu* polymerase (25) (Stratagene). The forward primer carried an *EcoRI* restriction site and overlapped the coding sequence for amino acids 114-119. The reverse primer was designed to overlap the sequence coding for amino acids 277-281 and contained a *BamHI* restriction site. After amplification the PCR product was gel-purified, incubated with the appropriate restriction enzymes and cloned into the vector pUC18 (26) under the control of the IPTG inducible lac promoter. The ligation mixture was electroporated into competent *E. coli* XL1BlueMRF cells and transformants selected for ampicillin resistance. Plasmid DNA was isolated from individual clones using the alkaline extraction method (27) and analysed for the presence of the TRAIL insert. The representative plasmid was designated pTRAIL1. All other general DNA manipulations were performed as previously described (28).

DNA sequence determination. - The TRAIL insert in plasmid pTRAIL1 was sequenced using the dye termination method with an ABI Big Dye sequencing kit (Applied Biosystems). DNA sequence analysis was performed using MacVector and AssemblyLIGN (International Biotechnologies, Cambridge, U.K.) software packages.

TRAIL protein purification. - Cells from an overnight 3 ml culture were used to inoculate 400 ml of LB medium. The culture was grown to an A<sub>550nm</sub> of 0.5 (mid-log phase). TRAIL expression was induced by addition of IPTG (final concentration 0.5 mM). Incubation was continued for 3 hr and bacterial cells pelleted by centrifugation. The bacterial cell pellet was resuspended in 50 mM Tris-HCl pH 8, 200 mM NaCl buffer containing lysosome, sodium deoxycholate and PMSF (Sigma) and a crude lysate obtained by sonification. The lysate was centrifuged to remove cell debris and the supernatant treated with polyethyleneimine to remove DNA. Protein was recovered by precipitation with ammonium sulphate and dialysed against loading buffer (25 mM potassium phosphate pH 6). The dialysed sample was then loaded onto an SP Sepharose cation exchange column (GE Healthcare) and eluted with a 0-1 M NaCl gradient. Fractions containing TRAIL protein were pooled, dialysed against 50 mM Tris-HCl pH 8, 200 mM NaCl buffer and loaded on a Ni-chelating HiTrap column (GE Healthcare). The elution was performed using a 1-250 mM linear imidazole gradient. Fractions containing TRAIL were concentrated using a VIVASPAN concentration device (Vivascience) and the protein purified to homogeneity by gel filtration using a Superdex 200 matrix column (GE Healthcare). A Detoxi-Gel<sup>®</sup> AffinityPak<sup>®</sup> Pre-packed Column (Pierce) was used to remove bacterial endotoxin.

Protein electrophoresis and western blot analysis. - Protein fractions were subjected to SDS-PAGE (29) using a 12% polyacrylamide separating gel and blotted onto BioTrace<sup>®</sup> PVDF membrane (PALL Life Sciences) using a Trans-blot SD semidry transfer cell (BIO-RAD) according to manufacturers instructions. After staining and drying, the excised bands were used for N-terminal amino acid sequencing. Unstained membrane was probed by Western blot analysis using an anti-TRAIL antibody (R&D Systems) and detected with a peroxidase-conjugated rabbit anti mouse antibody (DAKO) and o-phenylenediamine dihydrochloride as a substrate (Sigma).

N-terminal protein sequence determination and Maldi-TOF analysis. - N-terminal sequence was determined by Dr J. N. Keen in the BioMolecular Analysis Facility, School of Biochemistry and Molecular Biology, University of Leeds, UK.
Maldi-Tof analysis was performed by the Aberdeen Protein Facility, Department of Medical Sciences, Aberdeen University, UK.

Assessment of TRAIL bioactivity. - TRAIL bioactivity was assessed on the human myeloma cell line RPMI-8226 using a colorimetric cytotoxicity assay (30). Briefly, serial two-fold dilutions of TRAIL (200-50 ng/ml in 50 μl), prepared in RPMI-1640 culture media, were added to the wells of 96-well tissue culture plate. RPMI 8226 human myeloma cells (50 μl of 10^5/ml) were added and incubated for 24 hours at 37°C. 20 μl of CellTiter96® AQueous One Solution Reagent containing MTS and PES (Promega) were added to each well. Absorbance at 490 nm was measured after two hours. Wells containing cells only in the absence of TRAIL and wells containing medium only were used as controls.

The ability of TRAIL to induce apoptosis of RPMI-8226 cells was determined by assessing changes in nuclear morphology and using a fluorescence in situ nick translation assay as described previously (22).

Assessment of OPG-RANKL and OPG-TRAIL interactions using solid phase assay. - Measurement of the direct interaction between the RANK, RANKL, OPG and TRAIL proteins was performed using a modified ELISA technique. The capacity of OPG to directly inhibit RANKL binding to RANK was investigated using the following approach. RANK (R&D Systems), 50 ng in 0.1 ml of carbonate/bicarbonate coating buffer pH9.0 was bound to 96 well microtitre plates (ImmunoL 2, Dynex) by incubation overnight at 37°C. Wells were washed with washing buffer (0.005% TWEEN 20 in PBS) and blocked for 2 h with 2% skimmed milk in PBS. Plates were incubated with 0.1 ml of buffer containing 25 ng/ml RANKL (R&D Systems) and increasing concentration of OPG (R&D Systems) (range 25-800 ng). Control wells contained RANKL protein only. For comparison, wells containing a constant concentration of RANKL and OPG (25 ng each per well) and variable concentrations of TRAIL (range 25-800 ng per well) were prepared. RANKL bound to RANK was detected with an anti-RANKL antibody. All assays were performed in triplicate.

Plasmon Surface Resonance (BIAcore) analysis of OPG-RANKL and OPG-TRAIL interactions. - The affinity of OPG for RANKL and TRAIL was determined using a BIAcore 2000 optical biosensor (BIACORE AB) and resonance equilibrium method (31). A streptavidin sensor chip SA5 was conditioned with 1M NaCl, 50 mM NaOH. Biotinylated anti-OPG antibody (R&D Systems) was injected over the flow cell until the response reached 1000 response units. OPG (100 nM) was injected at a flow rate of 5 μl/min until 500 response units were immobilised. Increasing concentrations of RANKL or TRAIL (range 1-200 mM) were injected at 5 μl/min for 4 minutes and the response recorded. The chip surface was regenerated to baseline after each cycle with 5 mM HCl. The resonance signal measured on the reference cell (containing anti-OPG antibody only) was subtracted from the signal measured on the experimental flow cell. All experiments were performed at 20°C and 37°C and all sensograms corrected for buffer injection. Results were fitted into 1 to 1 binding model using BIAevaluation (peroxidase conjugated rabbit anti-mouse antibody diluted 1:500, 0.1 ml per well, DAKO). Wells were incubated with the chromogenic substrate o-phenylenediamine dihydrochloride (Sigma), and colour measured at 490 nm.

In separate experiments, the capacity of OPG to inhibit TRAIL binding to its receptor R1 was investigated using an identical approach. TRAILR1 (R&D Systems), 50 ng in 0.1 ml of carbonate/bicarbonate buffer, was bound to a microtiter plate. After washing and blocking, wells were incubated with a fixed concentration of TRAIL (0.1 ml of 25 ng/ml) and increasing concentrations of OPG or soluble TRAILR1 (range 25-800 ng per well). The concentration of bound TRAIL was assessed using an anti-TRAIL antibody (R&D Systems).

The potential of TRAIL to inhibit OPG from binding to RANKL was investigated using an identical protocol. RANK (50 ng in 0.1 ml) was bound to wells and incubated with a fixed concentration of RANKL and OPG (25 ng each per well) and variable concentrations of TRAIL (range 25-800 ng per well). RANKL bound to RANK was detected with an anti-RANKL antibody. All assays were performed in triplicate.
Assessing the ability of TRAIL to block OPG induced inhibition of osteoclast formation -
Mouse mononuclear cells were isolated from whole blood using Histopaque-1077 (Sigma) and seeded onto glass coverslips in a 96-well plate at a density of $1 \times 10^5$ per well in α-MEM (Life Technologies) containing 10% FCS. After 2 h the coverslips were washed and further incubated in medium (0.2 ml per well) containing macrophage colony-stimulating factor (MCSF; 25ng/ml; R&D Systems), and recombinant human RANKL (30 ng/ml) in the presence or absence of OPG (50ng/ml) or OPG in combination with TRAIL (500ng/ml). The cells were incubated for 7 days at 37°C and osteoclasts stained for tartrate-resistant acid phosphatase (TRAP) activity using an acid phosphatase kit (Sigma). The number of TRAP-positive multinuclear osteoclasts was counted under light microscopy.

Statistical analysis. - All data are represented as mean±SEM. Concentration response relationships were analysed by analysis of variance with a Scheffe post hoc test.

RESULTS

Soluble extracellular domain of the TRAIL protein, expressed in E. coli, is biologically active –
An high fidelity PCR amplified extracellular segment of the TRAIL gene was cloned into a pUC18 based vector under the control of inducible lac promoter. After induction, the bacterial pellet was successfully processed to obtain protein. Following cell lysis, insoluble particles and DNA were removed and the cell lysate subjected to cation exchange, affinity and gel filtration chromatography to obtain pure soluble TRAIL (Fig. 1A). Western blot analysis confirmed that the purified protein reacted with a specific anti-TRAIL antibody (Fig. 1B). The authenticity of the protein was confirmed by N-terminal amino acid analysis which showed the sequence Val-Arg-Glu-Arg-Gly-Pro-Gln, and was confirmed to be identical to the published sequence. Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-ToF) analysis further confirmed the expressed protein to be TRAIL. Biological activity was assessed by measuring the cytotoxic activity of purified TRAIL on the human myeloma cell line RPMI 8226. Increasing concentrations of TRAIL were shown to reduce cell viability (Fig. 2A). At concentrations of 50 to 100 ng/ml TRAIL cell viability was reduced by more than 90%. To confirm that the reduction in viability is a consequence of increasing apoptosis RPMI-8226 cells were treated with TRAIL and nuclei examined following staining with DAPI. Nuclei were shown to have condensed chromatin and the presence of apoptotic bodies, all features consistent with apoptosis (Fig. 2B). The in situ nick translation assay confirmed that the TRAIL protein promoted an increase in tumour cell apoptosis (Fig. 2C and D).

OPG prevents TRAIL binding TRAIL receptor R1 and RANKL binding cognate receptor RANK. - A competitive ELISA technique was developed to measure the effect of OPG on TRAIL-TRAILR1 binding. TRAIL receptor was bound to a microtitre plate and incubated with a fixed amount of TRAIL and increasing concentrations of OPG or soluble TRAILR1. An anti-TRAIL antibody was used to establish the amount of bound TRAIL. Soluble TRAILR1 inhibited TRAIL from binding immobilised receptor in a concentration dependent manner (p<0.001) (Fig. 3A). OPG was also able to dose-dependently inhibit TRAIL from binding to the TRAILR1 at 37°C (p<0.005), suggesting that OPG could partially compete with the TRAILR1 for TRAIL (Fig. 3B). The magnitude of inhibition was reduced when compared to soluble TRAILR1; however inhibition was still seen at concentrations of 25 ng/ml and higher.

In parallel experiments the ability of OPG to prevent the RANK-RANKL interaction was investigated. Microtitre plate wells were coated with RANK and incubated with a fixed amount of RANKL and increasing concentrations of soluble RANK or OPG. Soluble RANK prevented RANKL binding immobilised RANK in a concentration dependent manner (p<0.001) (Fig. 4A). OPG also dose-dependently inhibited RANKL from binding RANK (p<0.001) (Fig. 4B).
The level of inhibition was similar for sRANK and OPG, which is consistent with the two proteins having a similar affinity for RANKL.

Since OPG was shown to bind TRAIL we also investigated whether TRAIL could prevent OPG from inhibiting RANKL binding to RANK. Microtitre plates coated with RANK were incubated with a fixed amount of RANKL and OPG and increasing concentrations of TRAIL. TRAIL dose-dependently prevented OPG from blocking RANKL-RANK binding (p<0.001) (Fig. 5). TRAIL was shown not to bind RANK directly (data not shown). This reversal of the potential of OPG to inhibit RANKL-RANK binding is a direct consequence of the capacity of TRAIL to bind OPG.

OPG has a similar affinity for TRAIL as RANKL at physiological temperature - Quantitative measurement of OPG-TRAIL and OPG-RANKL binding was performed by BIAcore analysis. OPG was captured on a streptavidin-coated chip via a biotinylated anti-OPG antibody. Increasing concentrations of TRAIL or RANKL were injected over the chip surface and the response recorded in the form of a sensogram (Fig. 6). The steady-state response, in response units (RU), was obtained for each analyte concentration and data fitted to the Hill equation to generate an equilibrium dissociation constant. The binding affinity of OPG for TRAIL at 37°C was 45 nM whereas the affinity for RANKL was 23 nM. The influence of temperature and molecular structure of interacting proteins were also examined. Binding experiments were performed at both 20°C and 37°C. Two forms of OPG protein, native OPG and OPG fused to the Fc fragment of the human heavy chain were also used as capturing ligands. Two forms of TRAIL were also examined, the first produced in E. coli as a non-glycolysated form (detailed above) and the second produced in mammalian cells as glycosylated protein (R&D Systems). Temperature had little effect on OPG binding TRAIL. Equally the two forms of OPG had a limited effect on binding TRAIL (Fig. 6C). In contrast, temperature did effect OPG binding RANKL. The binding affinity of OPG for RANKL was lower at 20°C than at 37°C. Furthermore, the native OPG had a lower affinity for RANKL than the OPG-Fc form of the protein (Fig. 6C).

OPG prevents TRAIL-induced apoptosis and TRAIL prevents OPG inhibition of osteoclast formation in vitro - To determine the consequences of the TRAIL-OPG interaction we performed two different in vitro assays. The potential of OPG to inhibit TRAIL mediated apoptosis of the human myeloma cell line RPMI8226 was performed using an MTS based cytotoxic assay. Increasing concentrations of TRAIL reduced cell viability (Fig 7A). This was reversed by incubating with increasing concentrations of OPG (Fig 7).

To determine whether TRAIL could prevent the OPG induced inhibition of RANKL regulated osteoclast formation we assessed its effects on murine osteoclastogenesis. RANKL and MCSF induced the formation of TRAP+ve multinucleated osteoclasts (Fig 7B) with capacity to resorb mineralised substrates (data not shown). OPG prevented RANKL induced osteoclast formation (Fig 7B and C). However, TRAIL blocked the OPG induced inhibition of osteoclast formation (Fig 7, C).

DISCUSSION

In the present study we have investigated the nature of the binding of OPG with two ligands, RANKL and TRAIL. An important pre-requisite in our approach was to use native proteins devoid of any additional modifications, which could influence their binding properties and lead to inaccurate conclusions regarding the precise physiological role of individual receptor/ligand interactions. To achieve this we expressed an extra-cellular form of TRAIL (aa114-281 only) in E. coli. The authenticity of the TRAIL protein was confirmed by western blot analysis with a TRAIL specific antibody, N-terminal amino acid sequencing and MALDI-Tof analysis. Treatment of the human myeloma cell line, RPMI-8226 cells, with the TRAIL protein demonstrated a significant reduction in cell viability. This was comparable to a reduction in viability seen with a recombinant form of soluble TRAIL expressed in mammalian cells. The decrease in viability was associated with changes in nuclear morphology, including condensed chromatin and the formation of apoptotic bodies, consistent with apoptosis. Induction of apoptosis by the TRAIL protein was
also seen using an in situ nick translation assay. These data confirm that this form of TRAIL retained appropriate biological activity.

The induction of apoptosis is consistent with previous reports demonstrating that different forms of TRAIL protein can induce apoptosis in a range of cell types. A FLAG-tagged extra-cellular domain of TRAIL protein (aa95-281) was reported to have limited activity, although oligomerisation with an anti-FLAG antibody enhanced biological activity (32). Furthermore, a polyhistidine tagged soluble form (aa114-281) was demonstrated to be functional but was cytotoxic to human hepatocytes (33,34). However, this form of TRAIL was shown to contain a substantial fraction of dimers and trimers of a non-naturally polymerised protein characterised by the existence of intermolecular disulfide bonds (35). Importantly, it is the FLAG-tagged and BIOTIN-tagged TRAIL proteins that have previously been used for the measurement of binding affinity of TRAIL for OPG (14,23). A similar form of TRAIL to that expressed in the present study (aa114-281) was shown to be fully active (36,37). Subsequently, it has been shown that this form of protein undergoes appropriate polymerisation to form biologically active homo-trimers containing an internal zinc atom. This supports the suggestion that native TRAIL devoid of additional modifications is more appropriate for binding studies than tagged variants.

In the present study, using a solid phase assay, we demonstrated that OPG prevents RANKL from binding to RANK. In a similar experimental system OPG was shown to prevent TRAIL from binding the TRAIL-R1. We also demonstrated that TRAIL has the capacity to reverse the inhibitory action of OPG on RANKL:RANK binding by interacting with OPG. TRAIL, when present in excess over OPG, directly enhanced the binding of RANKL to its receptor RANK by titrating out the inhibitory molecule. This contrasts with a previous report demonstrating that OPG could not inhibit TRAIL-TRAIL-R1 binding (23). The reason for the difference between the two studies is unclear but may be explained by the nature of the proteins used to demonstrate inhibition of binding. In the previous study an OPG-Fc construct and a biotin-tagged form of TRAIL were used (23), whereas, in the present study, proteins devoid of these additions were used. These protein modifications could mask the relevant epitopes involved in recognition of binding, therefore preventing interaction. Alternatively, they could introduce conformational constrains, which may affect the position and availability of the reacting epitopes for binding.

Having established that OPG could bind both RANKL and TRAIL and compete for their respective receptors we determined the binding affinity of OPG for RANKL and TRAIL, using a surface plasmon resonance technique. OPG was bound to a biotinylated sensor chip via a specific OPG antibody. This approach has previously been shown to retain flexibility of the immobilised molecule, more accurately reflecting physiological conditions (38). At 37°C we demonstrate that OPG has a binding affinity of 23 nM for RANKL. Although this is in the same order as the affinity reported previously (from 1.7 nM to 10 nM; 8,39,40) the value in the present study is higher. The reason for this is unclear but may reflect differences in the mechanisms used to couple molecules to the sensor chip. Equally in the present study affinity was determined at 37°C. In contrast, in previous studies affinity was determined at 25°C. In the present study we demonstrated that temperature influenced affinity, with lower temperatures being associated with reduced affinity. We also demonstrated that OPG binds to native TRAIL with an affinity of 45 nM. This is of a similar order of magnitude to OPG binding RANKL and is consistent with data obtained using the competitive solid phase assays. However, these data contrast with previous studies that have reported binding affinities ranging from 3 nM to 400 nM (14,23). The reason for the broad range and the differences between studies is unclear. Emery et al reported the affinity of OPG-TRAIL complex to be 3 nM using plasmon surface resonance measurement (14). However, affinity was determined at 25°C using modified forms of proteins (OPG-Fc and TRAIL-Flag). Although we have demonstrated that temperature has a limited effect on OPG binding to TRAIL it is possible that the FLAG tag will influence binding. An affinity constant of 400nM for OPG-Fc binding TRAIL-biotin has been reported. However, this study used
isothermal titration calorimetry rather than BIAcore analysis and modified proteins (23).

In the present study the data were all generated at physiological temperature using native proteins, and within the same experiment, allowing direct comparison. The data demonstrate that OPG has a similar affinity for RANKL and TRAIL. This is entirely consistent with the data generated using the solid phase assay and that has been reported in more complex in vitro culture conditions (21,22,41). The mechanism responsible for the OPG binding RANKL and TRAIL is unclear. Although the crystal structures of RANKL and TRAIL have been solved there is no published structure for OPG. However, modelling studies have allowed OPG peptidomimetics to be designed (42). These peptides have been shown to block RANKL activity in a functional osteoclast assay but not the ability of TRAIL to induce tumour cell apoptosis (43). This is in contrast to native OPG, which is able to inhibit both processes. The solid phase assays used in the present studies did demonstrate that OPG was less effective at preventing TRAIL binding TRAILR1, than RANKL binding RANK. However, equilibrium dissociation constants were similar. This suggests that OPG can bind both RANKL and TRAIL but there are different structural determinants of binding. In the former case OPG may be able to completely prevent RANKL binding RANK, whereas OPG may only be partly able inhibit binding of TRAIL to TRAILR1.

Finally, we demonstrated the potential importance of the OPG-TRAIL interaction using live cells and tissue culture based assays. OPG was able to prevent TRAIL-mediated reductions in cell viability, confirming our previous reports (21-22). Furthermore, the opposite was also observed in that TRAIL was shown to prevent OPG inhibition of RANKL induced osteoclastogenesis.

These data are likely to have important biological consequences as it places OPG in a key position to regulate the function of these two important signalling pathways (Fig. 8). OPG clearly plays a critical role in regulating osteoclastic bone resorption. However, the present data strongly suggests that OPG will also play an important role in regulating TRAIL biology. Since TRAIL has been shown to play an important role in immunesurveillance it is likely that OPG may also regulate this activity. This may be particularly important in tissues in which OPG is highly expressed, for example in bone. In particular this may have implications for tumors that colonise bone such as multiple myeloma, breast or prostate cancer metastasis. The importance of OPG in regulating these different pathways in these locations is likely to be dependent upon the relative concentrations of the different components of the pathways, their location and the timing of expression of these molecules.
REFERENCES


**FIGURE LEGENDS**

**Fig 1. Expression and purification of recombinant human TRAIL protein.** (A) TRAIL was cloned and expressed using the bacterial expression system outlined in the Experimental Procedures Section and purified by a series of chromatography steps. M, molecular weight protein standards (size in kDa is shown); 1, un-induced culture; 2, induced culture; 3, cell lysate after cation chromatography; 4, following Ni²⁺ affinity chromatography; 5, following gel filtration. The arrow indicates the position of TRAIL protein. (B) Western blot analysis of purified protein (PP) demonstrated immunoreactivity with an anti-TRAIL antibody (arrowed). The authenticity of the pure protein was confirmed by N-terminal amino acid sequencing and MALDI-ToF analysis.
Fig 2. Cloned TRAIL is biologically active. Recombinant human TRAIL expressed in E. coli reduces cell viability and induces apoptosis in the human myeloma cell line RPMI 8226. (A) Reduction in cell viability following treatment with purified TRAIL as measured using the MTS based cromogenic cytotoxic assay. (B) Apoptotic cell death was apparent upon treatment of RPMI-8226 cells with purified TRAIL. Cell nuclei were stained with DAPI and examined by fluorescence microscopy – original magnification 40X. Apoptotic nuclei are arrowed. (C) Apoptosis was assessed using the fluorescence nick translation assay. TRAIL was shown to increase fluorescently label cells (arrowed). (D) The effect of increasing concentrations on the proportion of the apoptotic cells as determined using the fluorescence nick translation assay. Con = recombinant TRAIL expressed in mammalian cells (R&D Systems). For details see Experimental Procedures.

Fig 3. OPG prevents TRAIL binding TRAIL receptor R1. Microtitre plates containing bound TRAIL receptor R1 were incubated with samples containing a constant amount of TRAIL (25 ng) and an increasing amount of TRAILR1 (A) or OPG (B). After washing the plates were incubated with biotinylated anti-TRAIL antibody and bound antibody visualised with avidin-conjugated horseradish peroxidase and the chromogenic substrate, o-phenylenediamine dihydrochloride. The amount of bound TRAIL in individual wells is expressed as a percentage of the TRAIL bound in control wells in the absence of OPG or TRAILR1 (100%). * p<0.05 when compared to control.

Fig 4. OPG prevents RANKL binding the receptor RANK. Microtitre plates containing bound RANK were incubated with samples containing a constant amount of RANKL (25 ng) and an increasing amounts of RANK (A) or OPG (B). After washing the plates were incubated with biotinylated anti-RANKL antibody and visualised with avidin-conjugated horseradish peroxidase and the chromogenic substrate, o-phenylenediamine dihydrochloride. The amount of bound RANKL in individual wells is expressed as a percentage of the RANKL bound in control wells in the absence of OPG or RANK (100%). * p<0.001 when compared to control.

Fig 5. TRAIL reverses the potential of OPG to inhibit RANKL binding to RANK. Microtitre plates containing bound RANK were incubated with a constant amount of RANKL and OPG (25 ng each) and an increasing amount of TRAIL. After washing the plate was incubated with biotinylated anti-RANKL antibody and visualised with avidin-conjugated horseradish peroxidase and the chromogenic substrate, o-phenylenediamine dihydrochloride. The amount of bound RANKL in individual wells is expressed as a percentage of the RANKL bound in control wells in the absence of TRAIL (100%). * p<0.001 when compared to control.

Fig 6. OPG has a similar affinity for TRAIL and RANKL at 37°C. Human OPG or OPG-Fc was captured onto a streptavidin–coated SA chip via a biotinylated anti-OPG antibody and increasing concentrations of TRAIL or RANKL protein passed over. The change in surface plasmon resonance as a consequence of binding to immobilised OPG was recorded in the form of a sensogram (A). An example overley plot of sensograms showing (from bottom to top) increasing concentrations of TRAIL (1, 10, 2x20 40, 80, 150 nM) passed over immobilised OPG. Only the regions used in determining equilibrium dissociation constants are shown. (B) The equilibrium dissociation constant K_d was calculated by curve fitting to data points generated from the sensograms (C). Equilibrium dissociation constants for OPG and OPG-Fc binding to RANKL, TRAIL in bacteria and TRAIL expressed in mammalian cells (TRAIL (Glyc)) at 20°C and 37°C are shown.
Fig 7. OPG prevents TRAIL mediated reductions in cell viability, and TRAIL reverses the ability of OPG to inhibit osteoclastogenesis. A. Human myeloma RPMI8226 cells were incubated with increasing amounts of TRAIL alone (hatched columns) or in combination with OPG (black columns). Cell viability was measured using the MTS cytotoxic assay. Data are expressed as a percentage of control in the absence of TRAIL or OPG (white column). B. Mouse peripheral blood monocytes were incubated in the presence of RANKL and MCSF to induce osteoclastogenesis. Cultures were incubated with or without OPG and/or TRAIL. Osteoclasts are identified following TRAP staining as TRAP+ve multinucleated cells (arrowed). C. The relative number of TRAP+ve multinucleated osteoclasts is illustrated.

Fig 8. Diagramatic representation of the central role that OPG plays in regulating the RANKL/RANK and TRAIL/TRAILR1 pathways.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
Figure 7
Figure 8
Investigating the interaction between osteoprotegerin and rankl or trail: Evidence for a pivotal role for osteoprotegerin in regulating two distinct pathways
Srdjan Vitovski, Jennifer S. Phillips, Jon R. Sayers and Peter I. Croucher

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