REVIEW

Osteoprotegerin ligand and osteoprotegerin: novel implications for osteoclast biology and bone metabolism

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Introduction

The integrity of the skeleton depends upon bone remodeling, the permanent and controlled resorption of bone by osteoclasts (OC) and its subsequent replacement by bone-forming osteoblasts. An imbalance between bone resorption and bone formation results in osteoporosis (if bone resorption exceeds bone formation) or osteopetrosis (if bone formation exceeds bone resorption). The regulation of the balance between osteoblastic and osteoclastic activity is critical for the understanding of normal bone cell biology and forms the cellular basis of the pathogenesis of metabolic bone diseases.

A variety of local growth factors and cytokines as well as systemic peptide and steroid hormones regulate the differentiation of immature precursor cells and the activity of mature cells in both the osteoblastic and the osteoclastic lineage (1–7). The differentiation of OC precursors from a macrophage-like stem cell into the mature multinucleated OC (osteoclastogenesis) depends on various local factors secreted by bone marrow-residing pluripotent stromal cells and mature osteoblasts (1–3, 7). Regulation of osteoclastogenesis (and OC activity) by osteoblastic factors may therefore couple osteoclastic bone resorption to osteoblastic bone formation, and may constitute a local regulatory mechanism for bone remodeling.

During the last decade evidence has emerged that osteoclastogenesis requires the presence of stromal cells, $1\alpha,25\text{-dihydroxyvitamin D}_3$ ($1\alpha,25\text{-(OH)}_2\text{D}_3$) and glucocorticoids *in vitro* (1–3, 7). Although extensive *in vitro* studies and animal experiments using knock-out or transgenic mice have identified various candidate cytokines that are capable of regulating osteoclastogenesis (and are variably produced by stromal cells treated with $1\alpha,25\text{-(OH)}_2\text{D}_3$ and/or glucocorticoids), until recently no single cytokine has been identified that is both necessary and sufficient to induce osteoclastogenesis. The recent discovery of osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) (8, 9), a soluble member of the tumor necrosis factor (TNF) receptor (TNFR) superfamily, and the subsequent identification of its cognate ligand, osteoprotegerin ligand (OPG-L)/osteoclast differentiation factor (ODF)

(10, 11) (Table 1), has provided new insights into the regulation of osteoclastogenesis. TNF-related ligands and their receptors are critical for various aspects of cell survival and cell death (12).

This review summarizes the rapidly evolving progress made in our understanding of OPG/OCIF and OPG-L/ODF, including their protein and gene organization, their expression and regulation, and their effects both *in vitro* and *in vivo*. These novel findings have led to a new paradigm of osteoclast biology with major novel implications for bone metabolism and the pathogenesis of metabolic bone diseases.

Osteoprotegerin/osteoclastogenesis inhibitory factor

Identification of OPG/OCIF

OPG/OCIF was identified independently by several groups (8, 9, 13–16). Based on sequence homology, Simonet *et al.* (8) identified OPG as a novel TNFR superfamily member during a sequencing project of rat intestinal cDNA. This was followed by sequence analysis of the rat, the mouse and the human OPG cDNA (8). Using a protein purification approach of medium from the human lung fibroblast cell line IMR-90, Tsuda *et al.* (15) initially identified OCIF as a heparin-binding basic glycoprotein. The same group subsequently cloned the human OCIF cDNA by screening an IMR-90 cDNA library (9). In addition, OPG/OCIF was also identified by Tan *et al.* (13) by screening various cell line libraries and Kwon *et al.* (14) by screening an expressed sequence tag database for homologous regions for the cysteine-rich motifs of the TNFR, and was named TNF receptor-related molecule-1 (TR1). Yun *et al.* (16) cloned OPG/OCIF from a follicular dendritic cell (FDC)-like cell line FDC-1 and named it FDC receptor-1 (FDCR-1).

OPG/OCIF protein and gene organization

Functional characterization of the OPG/OCIF protein The OPG/OCIF propeptide from human, mouse and rat consists of 401 amino acids (8, 9, 13, 14). The human and the mouse OPG/OCIF proteins are 85% and 94% identical to the rat OPG/OCIF protein

Table 1 Glossary of abbreviations used

Abbreviation	Definition	Reference
DC	Dendritic cell(s)	(16)
FDCCR-1	Follicular dendritic cell receptor-1 (syn. OPG)	
OC	Osteoclast(s)	
OCIF	Osteoclastogenesis inhibitory factor (syn. OPG)	(9)
ODAR	Osteoclast differentiation and activation receptor (syn. RANK)	(58)
ODF	Osteoclast differentiation factor (syn. OPG-L)	(11)
OPG	Osteoprotegerin (syn. OCIF)	(8)
OPG-L	Osteoprotegerin ligand (syn. ODF)	(10)
RANK	Receptor activator of NF- κ B (syn. ODAR)	(47, 57)
RANKL	Receptor activator of NF- κ B ligand (syn. OPG-L)	(47)
TNF	Tumor necrosis factor	
TNFR	Tumor necrosis factor receptor	
TR1	TNF receptor-related molecule (syn. OPG)	(13, 14)
TRAF	TNF receptor-associated factor	(64)
TRAIL	TNF-related apoptosis-inducing ligand	(28)
TRANCE	TNF-related activation-induced cytokine (syn. OPG-L)	(46)

Syn, synonym.

which indicates a high degree of conservation during evolution (8). Post-translationally the 21 amino acid signal peptide is cleaved, generating the mature OPG/OCIF peptide of 380 amino acids (8, 14). Based on its hydrophobicity profile, OPG/OCIF lacks transmembrane and cytoplasmic domains, and is a secreted protein (8, 9, 13, 14). Alignment analysis of the amino acid sequence of TNFR superfamily members revealed that the N-terminal portion of OPG/OCIF is most homologous to TNFR-2 and CD40 (8). The N-terminal end has four repeats of cysteine-rich motifs (8, 9, 13, 14). These cysteine-rich motifs which consist of four to six cysteine pairs within the domains form intra-chain disulfide bridges which in CD40 have been implicated in the formation of 'tethered-loops' at both ends of each domain (13). In contrast, the C-terminal portion shares no homology with any other TNFR superfamily members except for two death domain homologous (DDH) tandem regions in the 204 C-terminal residues which have a high homology to Fas and TNFR-1 (9).

OPG/OCIF is a glycoprotein with four (8) or five (14) potential N-glycosylation sites. OPG/OCIF is synthesized as a monomer and assembled as a homodimer within the cell, and then secreted mainly as a disulfide-linked homodimer into the extracellular compartment, although small amounts of the OPG/OCIF monomer are present in the cell medium (8). Of note, Yun *et al.* (16) recently also reported the presence of membrane-bound OPG/OCIF on the surface of transfected kidney cells and a non-transfected dendritic cell (DC) line.

The OPG/OCIF monomer migrates at 55–62 kDa (8, 14, 15) under reducing conditions and at 40 kDa following removal of the N-glycosylation sites (8, 14, 15), while the OPG/OCIF homodimer migrates at 110–120 kDa under non-reducing conditions (8, 15). Functional analysis of recombinant human OPG/OCIF expressed in CHO cells indicated that both the OPG/

OCIF monomer and the homodimer had similar heat and acid stabilities, sialic acid contents, and biological activities as assessed by their ability to inhibit osteoclastogenesis *in vitro* (15, 17). The OPG/OCIF homodimer displayed a higher heparin-binding ability and a higher calcium-lowering potency in normal rats while the monomer had a longer half-life following intravenous administration and a higher area under the curve value (17).

Analysis of the human OPG/OCIF protein has demonstrated the presence of seven distinct protein domains, the N-terminal domains 1 to 4, each of which contains one of the four cysteine-rich motifs, domains 5 and 6, each containing one DDH region, and the C-terminal domain 7 (18). Analysis of the structure–function relationship of the OPG/OCIF protein revealed that the integrity of all four cysteine-rich motifs at the N-terminus is necessary and sufficient for the inhibition of osteoclastogenesis, whereas truncation of the C-terminal portion to residue 194 does not alter it (8, 18). The C-terminal domains 5 to 7 have the potential to mediate cytotoxicity when fused with the transmembrane region of Fas (18); however, it is unclear whether the genuine OPG/OCIF that lacks a transmembrane membrane is involved in cytotoxicity. Domain 7 harbors the heparin-binding site which does not correlate with the bioactivity of OPG/OCIF and a cysteine residue (position 400) which is required for homodimer formation (18).

OPG/OCIF gene organization The human and mouse OPG/OCIF genes are single copy genes containing five exons which are distributed over 29 kb (18–20). The human OPG/OCIF gene has been localized to chromosome 8q23–24 (8, 14), a gene locus harboring a cluster of bone-related genes, including genes implicated in hereditary multiple exostoses, certain types of

chondrosarcoma, and the gene encoding bone morphogenetic protein (BMP)-1 (8). The exon/intron organization has recently been reported for the human (18, 19) and the mouse OPG/OCIF gene (20). The human OPG/OCIF gene has one major transcription-initiation site located 67 nucleotides up-stream of the ATG codon and two minor transcription-initiation sites at positions 646 and 667, suggesting the presence of two promoters (19). The mouse OPG/OCIF gene has one major transcription-initiation site located 200 nucleotides up-stream of the ATG codon and two minor transcription-initiation sites at positions 237 and 182 (20).

In the human OPG/OCIF gene, exon 1 encodes ten amino acid residues of the signal peptide, exon 2 encodes the first two cysteine-rich domains and 73% of the third, while exon 3 encodes the remainder of the third and the fourth cysteine-rich domain (19). Coding of more than one cysteine-rich motif by a single exon has been previously reported for human nerve growth factor receptor, but not for other TNFR members (19). Exon 4 encodes the majority of the first DDH, whereas exon 5 encodes the remainder of the first and the second DDH, and contains the translation-termination codon (19). Of note, exon 4 is in part a duplication of exon 5 (19). Compared with the human OPG/OCIF cDNA (19), the untranslated region of the mouse OPG/OCIF cDNA contains an additional 529 bp (20).

The most abundant OPG/OCIF mRNA species has a size between 2.2 and 3.0 kb (8, 9, 13, 14). The variability is due to the alternative use of transcription-initiation sites (19). Two additional OPG/OCIF mRNA species, a 6.5–6.6 kb mRNA and a 4.2–4.4 kb mRNA are present in some tissues and are usually less abundant in all tissues or cell lines examined (9). These longer transcripts contain the 3'-half of intron 2 (4.2–4.4 kb fragment) or the entire intron 2 (6.5–6.6 kb fragment) (19).

Tissue and cellular expression of OPG/OCIF

OPG/OCIF is expressed in a variety of tissues and cell systems and is not restricted to bone (8, 9, 13, 14). Among human fetal tissues, OPG/OCIF mRNA is highly expressed in the lung, the kidneys, the liver and the brain (9). In adult human tissues, high levels of OPG/OCIF mRNA have been detected in the heart, the lung, the kidneys, and bone (8, 9, 13, 14). Other adult tissues that express high OPG/OCIF mRNA levels include the placenta, the liver, the thyroid gland, the spinal cord, and the brain (8, 9, 14). In addition, OPG/OCIF mRNA is expressed in various immune and hematopoietic tissues, and mesenchymal organs (8, 9). In mice, OPG/OCIF mRNA levels are high in the heart, lung, kidney, liver, brain, placenta, stomach, intestine, skin, and calvaria (8). Analysis of a whole mouse embryo demonstrated increased OPG/OCIF gene expression on day 7, which decreased by day 11, and increased again by day 15 (8). A detailed study by Mizuno *et al.* (20)

demonstrated that OPG/OCIF mRNA in the mouse fetus is expressed at day 8.5, while it is expressed in the maternal decidua as early as day 5.5. As assessed by *in situ* hybridization in a day-15 mouse embryo, OPG/OCIF mRNA was localized to the cartilaginous aspects of developing bone, the aorta and other large arteries, the skin, and the gastrointestinal tract (8).

OPG/OCIF mRNA levels have been detected in various human and murine primary cells and cell lines which, in part, reflect the tissue expression of OPG/OCIF, including various osteoblastic lineage cell lines such as the marrow stromal cell lines ST-2 (9), MC3T3-E1 (9), TF274 (13), and hMS (21), the osteosarcoma cell lines MG-63 (13, 14, 22, 23) and SaOS-2 (23), and various normal human osteoblastic cells lines (hFOB, hFOB/ER-9) (21, 24) as well as primary human trabecular osteoblasts (21, 23). Of interest, some of the conditionally immortalized cell lines express tenfold higher OPG/OCIF mRNA levels when cultured under differentiating conditions (21, 24). Among osteoblastic lineage cells, the osteosarcoma cell line MG-63 produces the most abundant amounts of OPG/OCIF (22). Other cells that express high OPG/OCIF mRNA levels include primary endothelial cells (13, 14), human aorta smooth muscle cells (14), the embryonic pulmonary fibroblastic cell line IMR-90 (9, 15) and primary fibroblasts (14), the monocytic leukemia cell line THP-1 (13, 14), the ovarian cancer cell line CAOV-3 (14), the breast cancer cell line MCF7 (13), and several lymphoma cell lines (13). In the immune system, OPG/OCIF expression is restricted to lymphoid cells, and is most abundant in B cells, DC, and follicular DC-enriched fractions.

Biological effects of OPG/OCIF

Genetic models

Overexpression of OPG/OCIF in transgenic mice An important role of OPG/OCIF in the regulation of bone metabolism has been derived from the phenotype of transgenic mice overexpressing OPG/OCIF (8). Overexpression of OPG/OCIF resulted in early, progressive osteopetrosis at various skeletal sites, including the long bones, vertebrae, and the pelvis (8). In contrast to other animal models of osteopetrosis, these transgenic mice displayed no other skeletal or extraskeletal abnormalities, except for splenomegaly which was due to compensatory extramedullary hematopoiesis (8).

Histologically, a systemic increase in mineralized trabecular bone was noted in mice overexpressing OPG/OCIF, including areas where hematopoiesis normally occurs (8). The most intriguing histological finding was a marked decrease in the number of trabecular OC, whereas the number of other hematopoietic cells of the monocyte-macrophage lineage was normal (8). This indicates that overexpression of OPG/OCIF increased bone mass mainly by inhibiting the terminal stage(s) of OC differentiation.

Deletion of OPG/OCIF in knock-out mice Two recent studies have demonstrated that OPG/OCIF knock-out mice develop severe, early onset osteoporosis (25, 26). Using a targeting vector which replaced the portion of exon 2 encoding the first two cysteine-rich domains of OPG/OCIF, Bucay *et al.* (25) and Mizuno *et al.* (26) generated viable and fertile OPG/OCIF-deficient mice (25, 26). OPG/OCIF-deficient mice had an increased postnatal mortality associated with an increased incidence of vertebral and enchondral bone fractures. OPG/OCIF-deficient mice were normal at birth, suggesting that OPG/OCIF deficiency did not affect embryogenesis or organogenesis (25, 26). Postnatally, OPG/OCIF-deficient mice had a decreased bone mineral density with thin cortical bone in the femur and the pelvis, and destruction of the femoral growth plates. They also suffered from multiple fractures, particularly involving the long bones as early as the first (25) or second month of life (26). Older mice had severe deformities of the vertebral column due to serial compression fractures of the vertebral bodies, leading to marked kyphosis. They also developed collapsed epiphyses of the distal femur (25).

Trabecular bone mineral density at 2 months of age was decreased in OPG/OCIF-deficient mice by 45% compared with wild-type littermates whereas cortical bone density was reduced by 19% (25). Of note, total body bone density of 6-month-old heterozygous mice was 6% lower than that of wild-type animals, suggesting that even the loss of a single allele of OPG/OCIF results in significant bone loss (25). In the second study, the femurs of 13-week-old OPG/OCIF-deficient male mice had a 28% lower bone mineral density and both bone strength and stiffness were reduced by more than 50% (26).

Histologically, the bones of OPG/OCIF-deficient mice were markedly osteoporotic and almost completely lacked trabecular bone after 2 months of life (25, 26). Loss of cancellous bone in OPG/OCIF-deficient mice became apparent even by the first week of life, while cortical bone became osteoporotic by 4 weeks of age (25). Further histological findings included the presence of numerous OC (25, 26) and osteoblasts (25) surrounding an increased number of cortical vessels, replacement of lamellar bone with woven bone in the cortex, and increased osteocyte density, consistent with increased bone turnover (25). This was confirmed by bone histomorphometry, which revealed increased osteoclastic and osteoblastic activity in OPG/OCIF-deficient mice, and serum alkaline phosphatase levels were more than fourfold higher than in wild-type mice (25). These data are consistent with an enhanced osteoclastic bone resorption (due to unopposed effects of OPG-L/ODF which would normally be neutralized by OPG/OCIF) with compensatory increased osteoblastic bone formation.

Surprisingly, OPG/OCIF-deficient mice had arterial calcification in the large arteries by 2 weeks of age. This was followed by profound calcification by 2 months of

life in as many as 60% of the OPG/OCIF-deficient mice (25). Arterial calcification in 6-month-old OPG/OCIF-deficient littermates affected the aorta and the renal arteries, was mainly limited to the media of the artery, and was associated with intimal and medial proliferation and partial aortic dissection (25). Of interest, OPG/OCIF had previously been localized by *in situ* hybridization of a mouse embryo to the media of these arteries (8). No vascular abnormalities were reported in the second study (26).

Exogenous administration of OPG/OCIF

In vitro effects of OPG/OCIF As shown in Fig. 1, the major effects of OPG/OCIF include inhibition of the terminal stages of osteoclastogenesis from OC precursors (8, 9, 14, 15, 18, 27–29) and the activity of mature OC (10, 14, 30, 31). Simonet *et al.* (8) were the

Figure 1 Birth and death of an osteoclast. Differential regulation by OPG/OCIF, OPG-L/ODF, and M-CSF. Following initial commitment from pluripotent stem cells, osteoclast precursor cells undergo sequential differentiation, fusion, and activation. Finally, activated osteoclasts are inactivated by the induction of apoptosis. OPG-L/ODF and OPG/OCIF regulate these processes in opposite directions.

first to demonstrate an inhibition of the development of tartaric acid-resistant acid phosphatase (TRAP)-positive OC and TRAP activity in an osteoclastogenesis assay using a spleen cell/bone marrow stromal cell co-culture system following the administration of an OPG-Fc fusion protein. At a concentration of 10–100 ng/ml, OPG-Fc fusion protein completely prevented the OC formation (8). TRAP activity was inhibited by 50% at a concentration of 1 ng/ml (8). Of interest, the bioactivity of OPG/OCIF required the presence of residues N-terminal to cysteine 185 which encodes a TNFR-like domain (8). Tsuda *et al.* (15) using OPG/OCIF purified from cultured medium of fetal lung fibroblasts reported inhibition of osteoclastogenesis at concentrations ranging from 1 to 40 ng/ml (ED_{50} at 4 to 6 ng/ml). Both the monomer and the homodimer of OPG/OCIF had comparable bioactivities (15). The sensitive period for OPG/OCIF actions during *in vitro* osteoclastogenesis over 11 days appears to be days 5 through 11, whereas addition of OPG/OCIF from days 0 to 3 or on days 7 and 8 did not affect the numbers of OC formed (32). A similar time-course with a slightly different result was also reported by Yasuda *et al.* (9). One recent study suggested that OPG/OCIF also decreased OC survival by inducing apoptosis (Fig. 1), which could be prevented by a specific inhibitor of caspase-3, which is critical for apoptosis (32). OPG/OCIF has subsequently been shown to inhibit osteoclastogenesis induced by $1\alpha,25(OH)_2D_3$ (14, 15), prostaglandin (PG) E_2 (14, 29), parathyroid hormone (PTH) (14, 15), interleukin (IL)-1 (14), and IL-11 (14, 15). The potent inhibitory effect of OPG/OCIF on osteoclastogenesis has recently been demonstrated in the preosteoclastic macrophage-like cell line C7, in which OPG/OCIF is the single most potent inhibitory factor (27).

In addition, OPG/OCIF was also found to inhibit the pit-forming activity of mature OC (14, 31, 33). Moreover, OPG/OCIF also inhibited ^{45}Ca release from a mouse fetal long bone organ culture model stimulated by $1\alpha,25(OH)_2D_3$ (14, 30), PGE_2 (30), PTH (30), and IL-1 α (30). Bone resorption by OC was inhibited by OPG, and stimulation of OC by OPG-L/ODF was blocked by OPG/OCIF (33). These effects, however, required higher concentrations of OPG/OCIF ranging from 50 to 1000 ng/ml (14, 30, 31). Treatment of isolated mature OC with OPG/OCIF reduced or disrupted the formation of the F-actin ring which is correlated with their bone-resorbing capacity (31). The inhibition by OPG/OCIF of bone resorption by mature OC is explained by the biological effects of the OPG-L/ODF to directly stimulate the activity of mature OC which are counteracted by OPG/OCIF (10).

Regulation by OPG/OCIF of proliferation and differentiation of non-osteoclastic lineage cells has been extensively studied by Tsuda *et al.* (15). OPG/OCIF had no effect on marrow stromal cell lines (ST-2, MC3T3-E1) and various cell lines of mesenchymal origin. In contrast, Kwon *et al.* (14) recently reported a threefold

increase in proliferation of human foreskin fibroblasts following treatment with OPG/OCIF (200 ng/ml) which could be prevented by co-treatment with a monoclonal antibody against OPG/OCIF. In addition, OPG/OCIF which has been demonstrated to be a soluble receptor for TNF-related ligand (TRAIL), inhibits TRAIL-induced apoptosis of the Jurkat T cell line (28).

In vivo effects of OPG Treatment of 4-week-old mice with daily subcutaneous injections of recombinant murine OPG/OCIF (10 mg/kg per day) for 7 days resulted in a threefold increase in the trabecular bone mass at the proximal tibial metaphysis (8). This increase following exogenous administration of OPG/OCIF was even higher than that following pamidronate administration, and occurred at a serum steady-state OPG/OCIF level of 320 ± 176 ng/ml (8). Similar effects have also been reported by Yasuda *et al.* (9) who treated 5-week-old rats with recombinant OPG/OCIF (24 mg/kg per day) for 2 weeks and demonstrated a 1.5-fold increase of bone mineral density and a 1.8-fold increase of bone volume at the proximal tibial metaphysis (9). In addition, the administration of recombinant murine OPG/OCIF (5 mg/kg per day) for 2 weeks following ovariectomy increased bone volume and decreased OC number, suggesting that OPG/OCIF prevented estrogen deficiency-induced osteoclastic bone resorption (8).

OPG/OCIF has been reported to inhibit osteoclastic bone resorption in various states of normal (17, 36) and increased bone resorption, including during normal linear bone growth (34), following the administration of IL-1 (34) or OPG-L/ODF (10), after thyroparathyroidectomy and subsequent challenge with PTH- and $1\alpha,25(OH)_2D_3$ (35), and in humoral hypercalcemia of malignancy (34, 36). The rapid onset of the hypocalcemic effect in these studies suggests a direct effect of OPG/OCIF on OC activity (35).

Regulation of OPG/OCIF by systemic hormones and factors

Given the phenotype of osteopetrosis in transgenic mice overexpressing OPG/OCIF (8) and of osteoporosis in OPG/OCIF knock-out mice (25, 26), OPG/OCIF has been considered an obvious candidate cytokine receptor which mediates the stimulatory or inhibitory effects of various systemic hormones and cytokines on osteoclastogenesis (Table 2). OPG/OCIF mRNA and protein levels were stimulated in various human osteoblastic cell systems by $1\alpha,25(OH)_2D_3$ (21), BMP-2 (21), IL-1 α (37) and $-\beta$ (21), and TNF- α (21, 38) and TNF- β (38). Other studies using murine osteoblastic/stromal cells demonstrated down-regulation of OPG/OCIF mRNA levels in marrow stromal cell lines (ST-2, tsj2) by $1\alpha,25(OH)_2D_3$ (9, 39), but not in primary osteoblasts (39) as well as down-regulation of OPG/OCIF mRNA levels in primary osteoblasts by $1\alpha,25(OH)_2D_3$, IL-1 α , and PTH (40). Some of these discrepancies may be due to species differences or may reflect the

Table 2 Regulation of OPG/OCIF

Stimulation	Cells	Reference
BMP-2	hFOB	(21)
Calcium chloride	IMR-90, ST-2, MC3T3-E1	(9)
1 α ,25-(OH) $_2$ D $_3$	hFOB, hOB	(21)
17 β -Estradiol	hFOB/ER-9, hOB	(24)
IL-1 α	MG-63, hOB	(37)
IL-1 β	hFOB, human fibroblasts	(21, 14)
Phorbol 12-myristate 13-acetate	Kidney cell lines	(13)
Transforming growth factor- β	MC3T3/G2/PA6, ST-2, MC3T3-E1, KS483, murine primary osteoblasts	(40, 44)
TNF- α	hFOB, MG-63, human fibroblasts	(21, 38, 14)
TNF- β	MG-63	(38)
Inhibition	Cells	Reference
Dexamethasone	hFOB, hOB, hMS, ST-2	(22, 23, 40)
1 α ,25-(OH) $_2$ D $_3$	ST-2, tsJ2, murine primary osteoblasts	(39, 40)
Hydrocortisone	hOB, MS, MG-63	(23)
IL-1 α	Murine primary osteoblasts	(40)
PGE $_2$	MS, murine primary osteoblasts	(45, 40)
PTH	Murine primary osteoblasts	(40)

Abbreviations: hFOB/ER-9, human fetal osteoblastic cell line stably transfected with the estrogen receptor; hOB, primary human trabecular osteoblasts; hFOB, human fetal osteoblastic cell line; hMS, human marrow stromal cell line; IMR-90, human embryonic lung fibroblasts; ST-2, tsJ2, and MC3T3-E1, marrow stromal cell lines; MG-63, human osteosarcoma cell line; MS, primary marrow stromal cells.

variability of the cell systems regarding their stage of osteoblastic differentiation.

OPG/OCIF mRNA levels have also been shown to be stimulated by increasing concentrations of calcium chloride in murine marrow stromal cell lines (9). At the local bone level calcium is mainly released from bone at sites of active osteoclastic activity (9), and an increase of OPG/OCIF with inhibition of osteoclastogenesis in response to released calcium ions may constitute an autocrine negative feedback loop to limit the bone-resorbing effects of OC. The biological significance of OPG/OCIF induction by IL-1 and TNF in human osteoblasts is less clear (21, 37, 38), although this has recently also been reported for human foreskin fibroblasts (14). Teleologically, up-regulation of OPG/OCIF production by proinflammatory, bone-resorbing cytokines IL-1 β and TNF- α (which also stimulate OPG-L/ODF production) may represent a mechanism by which the action(s) of these cytokines are counterbalanced (21). Similarly, up-regulation of TNFR family members by TNF ligands has been reported for other family members (41).

Transforming growth factor- β (TGF- β) which inhibits osteoclastic bone resorption (42, 43) was also found to stimulate the production of OPG/OCIF by various bone marrow stromal cell lines and primary osteoblasts, and subsequently to inhibit osteoclastogenesis (40, 44). Moreover, the inhibitory effects of TGF- β on osteoclastogenesis were prevented by treatment with an OPG/OCIF neutralizing antibody, suggesting direct mediation of the anti-resorptive effects of TGF- β by OPG/OCIF (40, 44). By contrast, the insulin-like growth factor system which may act as an anabolic growth factor system in bone cells was not found to regulate osteoblastic OPG/

OCIF production (L C Hofbauer, B L Riggs & S Khosla, unpublished observation).

Of interest, treatment of estrogen-responsive osteoblasts (hFOB/ER-9) with 17 β -estradiol stimulated OPG/OCIF mRNA and protein concentrations by fourfold in a dose- and time-dependent fashion (24). These effects could be abrogated by treatment with the specific estrogen receptor antagonist ICI 182,780. In addition, the concentration of OPG/OCIF produced by hFOB/ER-9 cells (2–8 ng/ml) was in the range where OPG/OCIF had its ED $_{50}$ (24). As mentioned earlier, the administration of OPG/OCIF completely prevented ovariectomy-induced bone resorption and bone loss (8).

OPG/OCIF production is inhibited by glucocorticoids (22, 23, 40) and PGE $_2$ (40, 45). Inhibition of OPG/OCIF mRNA levels and protein concentrations in human cell systems was robust (by 90%) and occurred in all human osteoblastic cell systems assessed, including marrow stromal cells, osteoblasts, and the osteosarcoma cell line, MG-63 (22, 23). In murine cell systems, dexamethasone decreased OPG/OCIF mRNA levels in the stromal cell line, ST-2, while it had a biphasic effect with an initial decrease and subsequent increase in primary osteoblasts (40). OPG/OCIF mRNA levels were also inhibited by PGE $_2$ in primary human bone marrow stromal cells (45) and primary murine osteoblasts (40).

Osteoprotegerin ligand/osteoclast differentiation factor

Identification of OPG-L/ODF

The ligand for OPG/OCIF has recently been identified by two groups (10, 11). Using an OPG-fusion protein as an

immunoprobe, Lacey *et al.* (10) identified OPG-binding sites on the surfaces of a myelomonocytic cell line, expression cloned murine OPG ligand (OPG-L) cDNA from a myelomonocytic cell library, and subsequently cloned human OPG-L cDNA from a lymph node library. Similarly, Yasuda *et al.* (11) expression cloned ODF cDNA from a cDNA library of the murine marrow stromal cell line ST-2 following treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ and dexamethasone. Alignment analysis revealed the identity of OPG-L/ODF to two independently cloned members of the TNF ligand family, TNF-related activation-induced cytokine (TRANCE) (46) and receptor activator of nuclear factor (NF)- κB ligand (RANKL) (47). TRANCE was identified as an immediate early gene which is induced by stimulation of the T cell receptor (46), while RANKL was identified as an important factor that stimulates the function of DC (47).

OPG-L/ODF protein and gene organization

Functional characterization of the OPG-L/ODF protein The human and murine OPG-L/ODF are polypeptides of 317 and 316 amino acid respectively (10, 11, 46, 47). Human OPG-L/ODF has an 83–87% homology to murine OPG-L/ODF, indicating high conservation during the evolution (10, 46, 47). Analysis of OPG-L/ODF revealed the absence of a signal peptide and the presence of a 21- to 24-residue hydrophobic domain which predicts a transmembrane domain (10, 11, 46, 47). OPG-L/ODF is a type II transmembrane protein. Human OPG-L/ODF has a cytoplasmic domain at the N-terminus (residues 1–48), a transmembrane domain (residues 49–69), and an extracellular region at the C-terminus (residues 70–317) (10, 46, 47). The C-terminal region of OPG-L/ODF contains two functional domains, a stalk region (residues 71–157), and the active ligand site (residues 158–317) (10). The active ligand site is predicted to contain ten β -sheet-forming sequences that are present in all TNF-related proteins (10, 46). OPG-L/ODF is most closely related to TNF-related apoptosis-inducing ligand (TRAIL, 20–34% homology (10, 46); CD40 ligand, 28% homology (47); Fas ligand, 19% homology (46)). OPG-L/ODF contains two potential N-glycosylation sites (46, 47).

OPG-L/ODF is present in two forms, a cell-associated form (as mentioned above) and a soluble form which is derived from the cell-associated form by post-translational processing at positions 139 (murine OPG-L/ODF) or 140 and 145 (human OPG-L/ODF) (10). The cell-associated form migrates at 40–45 kDa (10, 11, 46) while the soluble form migrates at 31 kDa (10). Expression and functional analysis of truncated OPG-L/ODF variants revealed that truncated forms that contained residues 126, 137, and 158 all bound to OPG/OCIF with a similar affinity, suggesting that the soluble OPG-L/ODF is biologically active (10). Future studies are required to assess whether the soluble OPG-

L/ODF form is released by enzymatic cleavage, and, if so, to determine the expression, tissue distribution, and regulation of this putative protease.

OPG-L/ODF gene organization The human OPG-L/ODF gene has been localized to chromosome 13q14 (46, 47). The exon/intron organization of OPG-L/ODF has not been reported to date. The murine full-length OPG-L/ODF cDNA consists of 2237 bp with a Kozak consensus sequence at 137 bp of the 5' end (46). All studies have confirmed the presence of a single OPG-L/ODF mRNA species at 2.2–2.4 kb (10, 11, 46–48).

Of note, the murine OPG-L/ODF gene promoter contains a binding site for OSF2/Cbfa-1, an essential transcription factor for osteoblastogenesis (49), and the expression of OPG-L/ODF mRNA depends on the functional activity of Cbfa-1 (50). OPG-L/ODF mRNA levels were absent in Cbfa-1-deficient mice which also had impaired osteoclastogenesis (50). Thus, it is likely that the presence of the OSF2/Cbfa-1-responsive element within the OPG-L/ODF gene promoter may represent a mechanism whereby osteoblastogenesis and osteoclastogenesis are coupled (49, 50). Clearly, further studies are needed to define the role of OSF2/Cbfa-1 and OPG-L/ODF as molecular mediators of bone remodeling.

Tissue and cellular expression of OPG-L/ODF

OPG-L/ODF gene expression is most abundant in the skeleton (bone, bone marrow) and in lymphoid tissues (lymph node, spleen, thymus, Peyer's patches, fetal liver) (10, 11, 46, 47). Other sites of significant OPG-L/ODF mRNA expression include heart, skeletal muscle, lung, stomach, placenta, the thyroid gland, and peripheral blood leukocytes (11, 47). Consistent with the tissue distribution in humans, high OPG-L/ODF mRNA levels have been detected in lymph node-derived T cells but not in spleen-derived B cells (46). OPG-L/ODF mRNA was also found to be expressed by stromal cells and osteosarcoma cell lines (SaOS-2, MG-63) (10, 22, 46, 48).

In rodents, OPG-L/ODF mRNA is highly expressed in lymph node, spleen, thymus, and the cerebellum (10). Furthermore, OPG-L/ODF mRNA has been detected in two murine marrow stromal cell lines (ST-2, MC3T3-E1), the rat osteosarcoma cell line ROS (10), primary murine calvarial osteoblasts (11), and a variety of primary and transformed murine lymphoid cell lines, and was positively correlated with the content of T lymphocytes (47). *In situ* hybridization analysis of a 15.5-day-old mouse embryo localized high OPG-L/ODF mRNA expression to the white splenic pulp, the medulla of the thymus, and the cortical areas of developing lymph nodes, and intestinal lymphoid patches, suggesting that T cell-rich tissues are a major source of OPG-L/ODF (10). In fetal murine bone, OPG-L/ODF is mainly expressed in the primitive mesenchymal cells adjacent

to the cartilaginous Anlagen and in hypertrophic chondrocytes within the Anlagen as well as in regions of primary ossification and modeling (10). In adult murine bone, OPG-L/ODF was mainly found in the growth plate and the metaphyseal periosteum, which are areas of high osteoclastic bone resorption and active bone remodeling during growth (10).

Biological effects of OPG-L/ODF

Genetic models: deletion of OPG-L/ODF in knock-out mice

Consistent with the effects of OPG-L/ODF (10) and the phenotypes of OPG/OCIF transgenic (8) and knock-out mice (25, 26), OPG-L/ODF knock-out mice have severe osteopetrosis and lack mature OC (51). OPG-L/ODF knock-out mice are viable and normal at birth (51). There is generalized increased bone mineral density (osteopetrosis) throughout life and the long bones contain large amounts of cartilage and mineral leading to compensatory extramedullary hematopoiesis (51). Exogenous administration of OPG-L/ODF to OPG-L/ODF knock-out mice can rescue bone abnormalities and induces the differentiation of OC, suggesting that OC precursor cells are present, but (in the absence of OPG-L/ODF) fail to differentiate towards mature OC (51). In contrast to OPG/OCIF transgenic mice (8), OPG-L/ODF knock-out mice lack teeth, display shorter broad and flat bones, and have immune system abnormalities, including B and T cell maturation defect, thymus hypoplasia and lymph node agenesis and hypoplasia (51). The target tissues of OPG-L/ODF deficiency (skeleton, immune system) are identical to those with the most abundant OPG-L/ODF gene expression (10, 11).

Exogenous administration of OPG-L/ODF

In vitro effects of OPG-L/ODF on bone metabolism As shown in Fig. 1, the major *in vitro* effects of OPG-L/ODF include promotion of differentiation, fusion, activation, and survival of OC as well as inhibition of OC apoptosis (10, 11, 33, 52–55). In fact, the combination of OPG-L/ODF and the permissive factor M-CSF is able to induce the differentiation of mature OC from pluripotent OC precursors *in vitro* in the absence of additional hormones, cytokines, or stromal cells, implying that these two cytokines are both necessary and sufficient for osteoclastogenesis (10, 11, 52, 53). In a murine marrow stromal cell culture, OPG-L/ODF increased TRAP activity and the number of OC identified by positive TRAP cytochemistry (10, 11). The ED₅₀ of OPG-L/ODF for these effects was 1 ng/ml (10). Treatment of OC progenitors with OPG-L/ODF also increased the expression of other OC differentiation markers, including β_3 -integrin, TRAP, cathepsin K, c-src, as well as calcitonin and vitronectin receptors (10, 11, 52, 53). Recently, the stimulation of osteoclastogenesis by OPG-

L/ODF was also demonstrated in human monocytes derived from peripheral blood (52, 53), indicating that OPG-L/ODF is active in promoting osteoclastogenesis across species. However, it was shown that the time required for the complete differentiation of OC in human cells is longer (11 days) than that of murine cell systems (7 days) (52). The stimulatory effects of OPG-L/ODF on osteoclastogenesis were dose-related (10, 11, 52, 53), blocked by co-administration of excess OPG/OCIF (10, 11), and resulted in functionally active OC, as assessed by their ability to form large resorption lacunae on bone or dentine slices (10, 11, 52, 53).

The rapid onset of changes induced by OPG-L/ODF and OPG/OCIF on bone metabolism indicated that OPG-L/ODF may also regulate OC activity. Lacey *et al.* (10) demonstrated that OPG-L/ODF, at a concentration ranging from 1 to 10 ng/ml, activates mature rat OC to form resorption pits using bovine bone slices in the absence of M-CSF, thus demonstrating that OPG-L/ODF has the potential to stimulate OC activity. This was recently confirmed by Tsukii *et al.* (30) who demonstrated that OPG-L/ODF dose-dependently increased ⁴⁵Ca release by mature OC in a fetal mouse long bone culture system. Fuller *et al.* (33) reported that bone resorption was inhibited by soluble OPG-L/ODF receptor and OPG, and stimulation of the OC activity by OPG-L/ODF was blocked by OPG.

In vivo effects of OPG-L/ODF on bone metabolism The subcutaneous administration of recombinant OPG-L/ODF to normal mice caused early onset (within 1 day), severe hypercalcemia in a dose-dependent fashion, suggesting that OPG-L/ODF directly stimulates the activity of mature OC (10). In further support of this, administration of OPG/OCIF to normocalcemic animals (17, 36), OPG-L/ODF-treated mice (10), thyroparathyroidectomized rats challenged with PTH or 1 α ,25-(OH)₂D₃ (35), and mice with humoral hypercalcemia of malignancy (34, 36) rapidly lowered serum calcium levels. Radiographic and histomorphometric analysis of OPG-L/ODF-treated mice (3 days of treatment) demonstrated a dose-related profound bone loss associated with the presence of larger and higher nucleated mature OC and doubling of the OC area while the number of OC was not changed (10).

Of interest, the formation of osteoclasts in mice following administration of soluble OPG-L/ODF was limited to bone, although other tissues also produce significant concentrations of M-CSF (10). This finding suggests that intrinsic factors (bone matrix proteins, adhesion molecules, and others) may be produced in the bone/bone marrow microenvironment that facilitate osteoclastogenesis.

Effects of OPG-L/ODF on immune function As noted earlier, the OPG-L/ODF was initially identified as TRANCE (46) or RANKL (47) and was demonstrated to have important effects on immune function. RANKL

induced cluster formation by DC and stimulated allostimulatory capacity of DC on T cells as well as cytokine-activated T cell growth (47). TRANCE, initially identified as an immediate early gene upon T cell receptor activation, induced c-Jun N-terminal kinase (JNK) activation in T cells, but not B cells (46). Subsequent studies demonstrated that TRANCE binds to high-affinity sites located on the surface of mature DC (56). TRANCE inhibited apoptosis of both bone marrow- and monocyte-derived DC, thus increasing DC survival, possibly by increasing the expression of the anti-apoptotic gene Bcl-x_L, while it had no effect on the proliferation or survival of T or B cells (56). These studies demonstrate that TRANCE/RANKL, in addition to its important role in osteoclastogenesis, represents a paracrine mediator for the communication between T cells and DC. Moreover, the two receptors of OPG-L/ODF, osteoclast differentiation and activation receptor (ODAR)/receptor activator of NF- κ B (RANK) (47) and OPG/OCIF (16), are up-regulated by CD40 stimulation in immune cells. These data suggest that TRANCE/RANKL may act as a pleiotropic cytokine within the bone marrow microenvironment to regulate the immune system and bone metabolism.

Regulation of OPG-L/ODF expression

Hormones and cytokines that modulate the production of OPG-L/ODF (Table 3) are likely to be major regulators of osteoclastogenesis, because OPG-L/ODF (in combination with M-CSF) is both necessary and sufficient to induce osteoclastogenesis, and replaces the need for co-culture with stromal cells or the presence of conditioned medium from stromal or osteoblastic cells. Among the factors that up-regulate OPG-L/ODF mRNA steady-state levels in marrow stromal and osteoblastic cells are dexamethasone, 1 α ,25-(OH)₂D₃, IL-11, PTH, and PGE₂ (11, 23, 39). In addition, the pro-inflammatory cytokines IL-1 β and TNF- α have recently been demonstrated

to stimulate OPG-L/ODF mRNA steady-state levels in primary and immortalized marrow stromal cells and the osteosarcoma cell line MG-63 (48). The concept that some of the bone-resorbing hormones and cytokines regulate bone resorption indirectly by increasing the production of OPG-L/ODF is further supported by a recent study by Tsukii *et al.* (30). While OPG-L/ODF dose-dependently stimulated bone resorption in a fetal mouse long bone culture system as assessed by ⁴⁵Ca release, bone resorption induced by 1 α ,25-(OH)₂D₃, PTH, PGE₂, and IL-1 α was prevented by co-treatment with OPG/OCIF and by OPG-L/ODF neutralizing antibodies (30). By contrast, Takai *et al.* (44) showed that TGF- β suppressed OPG-L/ODF mRNA steady-state levels (and concurrently stimulated OPG/OCIF mRNA levels) by murine stromal cells (ST-2) (Table 3) which resulted in an inhibition of osteoclastogenesis.

Receptor for OPG-L/ODF

Identification and organization of ODAR/RANK

The receptor for OPG-L/RANKL, RANK, was identified by Anderson *et al.* (47). The human RANK is a polypeptide of 616 amino acids and belongs to the type I transmembrane protein class. RANK mapped to the human chromosome 18q22.1 (47). Its hydropathy analysis predicts a signal peptide of 28 amino acids, an N-terminal extracellular domain of 184 amino acids, a short transmembrane domain of 21 amino acids and a large C-terminal cytoplasmic domain of 383 amino acids (47). Similar to OPG, the extracellular domain of RANK contains four cysteine-rich pseudorepeats and two N-glycosylation sites (47, 57). The human RANK is 70% homologous to the murine RANK (47) and when compared with other TNFR family members is 40% identical to CD40 (47). More recently, Hsu and colleagues (58) characterized RANK which they named ODAR (58).

Table 3 Regulation of OPG-L/ODF

Stimulation	Cells	Reference
Dexamethasone	Primary marrow stromal cells	(23)
	hFOB	(23)
1 α ,25-(OH) ₂ D ₃	Primary murine osteoblasts ST-2, tsJ2 cells, KUSA/O	(11, 39)
IL-1 β	Primary human marrow stromal cells, hMS, MG-63 cells	(48)
IL-11	Primary murine osteoblasts	(11, 39)
PTH	Primary murine osteoblasts	(11, 39)
PGE ₂	Primary mouse calvaria cells	(11)
TNF- α	Primary human marrow stromal cells, hMS, MG-63 cells	(48)
Inhibition		
TGF- β	ST-2	(44)

Abbreviations: ST-2, tsJ2, and KUSA/O, murine marrow stromal cell lines; hMS, human marrow stromal cell line, hFOB, human fetal osteoblastic cell line.

Expression and regulation of ODAR/RANK

ODAR/RANK mRNA is expressed in many tissues, including skeletal muscle, liver, small intestine and colon, thymus and adrenal gland (47). In addition to OC (58, 59), ODAR/RANK mRNA is expressed in various lymphocytic and non-lymphocytic cell lines (47). The most abundant ODAR/RANK mRNA species is a 4.5 kb mRNA fragment in humans (47) and a 5.5 kb mRNA fragment in mice (59); however, there are additional ODAR/RANK mRNA species (3.0 kb, 3.5 kb) as a result of alternative polyadenylation signals. Although ODAR/RANK mRNA is expressed in various cell systems, the expression of ODAR/RANK protein is restricted to OC, DC, B and T cell lines, and fibroblasts (47), suggesting that ODAR/RANK expression may be post-transcriptionally regulated (46, 47). Among bone cells, ODAR/RANK mRNA is exclusively expressed in OC precursor cells isolated by FACS analysis using fluorescein labeled OPG-L/ODF (58). Regulation of ODAR/RANK expression in OC has not been studied. In immune cells, ODAR/RANK on the surface of T cells is up-regulated by IL-4 and TGF- β (47), while CD40 ligand induces the expression of ODAR/RANK on DC (47).

Biological effects of ODAR/RANK

Two studies have established that OPG-L/ODF binds specifically and with high affinity to ODAR/RANK located on osteoclastic lineage cells, and that activation of ODAR/RANK by OPG-L/ODF is necessary and sufficient for osteoclastogenesis (58, 59). Of interest, transgenic mice overexpressing soluble ODAR/RANK-Fc fusion protein (which acts as a neutralizing receptor for OPG-L/ODF) had osteopetrosis, similar to transgenic mice overexpressing OPG/OCIF (58). Consistent with this, administration of soluble ODAR/RANK-Fc fusion proteins blocked OC differentiation and activation *in vivo* (58) and *in vitro* (58, 59) similar to the effects of OPG/OCIF.

In an elegant study, Nakagawa *et al.* (59) employed two different RANK antibodies, a polyvalent divalent antibody (ODAR/RANK-Ab) and a monovalent version of ODAR/RANK-Ab (Fab-RANK). While the ODAR/RANK-Ab which is able to cross-link and cluster antigens mimicked OPG-L/ODF action and stimulated osteoclastogenesis, the monovalent Fab-RANK which lacks the ability to cross-link and to cluster antigens behaved as an OPG-L/ODF antagonist and inhibited osteoclastogenesis (59). This study suggests that the clustering of ODAR/RANK is required for ODAR/RANK activation during osteoclastogenesis (59).

Signal transduction of ODAR/RANK

Anderson *et al.* (47) were the first to report that ODAR/RANK overexpression is sufficient to activate NF- κ B. Several recent studies have since provided important insights into the intracellular signal transduction

following activation of the ODAR/RANK (47, 58). ODAR/RANK interacts with TNFR-associated factor (TRAF) family members to activate the transcriptional factor NF- κ B and the protein kinase JNK (47, 57, 60). TRAFs constitute a family of six cytoplasmic adapter proteins which contain zinc fingers and are known to regulate cell survival, proliferation, and apoptosis by activating NF- κ B and other transcriptional factors (61). The intracellular domain of the human ODAR/RANK contains three putative TRAF-binding domains which strongly bind TRAF1, TRAF2, TRAF3, and TRAF6, and weakly bind TRAF5 (57, 60, 62).

Functional analysis of the human ODAR/RANK revealed that the C-terminal 85 amino acids which bind TRAF2, TRAF5, and TRAF6 are essential for NF- κ B activation (57). In a similar manner, the C-terminal 93 amino acids which bind TRAF2, TRAF5, and TRAF6 are essential for the murine ODAR/RANK (60, 62). Recent studies suggest that NF- κ B and JNK activation by ODAR/RANK are differentially regulated by TRAFs (56, 57, 60, 62, 63). Interestingly, ODAR/RANK-induced JNK and NF- κ B activation are induced in cells overexpressing TRAF2, TRAF5, and TRAF6 (63), while NF- κ B activation is suppressed in cells with dominant negative forms of TRAF2, TRAF5, and TRAF6 (60). A TRAF6-binding site (amino acids 340–421) appears to be specifically essential for NF- κ B, but not for JNK activation (62). Similarly, an intact zinc finger of the TRAFs is required only for NF- κ B (63). Of note, TRAF2 has been reported to be a gene target of NF- κ B transcriptional activity (64). The most compelling evidence for the TRAF proteins as regulators of osteoclast function was recently derived from the generation of TRAF6-deficient mice which had osteopetrosis and impaired tooth eruption (65). At the cellular level, osteoclasts from these TRAF6 knock-out mice failed to form ruffled borders, suggesting an essential role for TRAF6 in osteoclast activation (65).

New basic science and clinical implications

OPG-L/OPG and the cytokine network

Genetic manipulation of the OPG-L/OPG system resulted in both extremes of skeletal phenotype. Both targeted ablation of OPG-L/ODF in knock-out mice (51) and overexpression of OPG/OCIF in transgenic mice (8) resulted in impaired osteoclastogenesis and osteopetrosis, while both the administration of OPG-L/ODF (10) and targeted ablation of OPG/OCIF in knock-out mice (25, 26) resulted in enhanced osteoclastogenesis and severe osteoporosis (Table 4). Because of these phenotypes, and because OPG-L/ODF, in the presence of M-CSF, is necessary and sufficient for osteoclastogenesis, and OPG/OCIF represents its endogenous antagonist (Fig. 1), it has been suggested that the interactive network of bone-resorbing and anti-resorptive cytokines

Table 4 Skeletal phenotypes associated with alterations of the OPG-L/ODF-OPG/OCIF system

Phenotype	Intervention	Other abnormalities	Reference
Osteoporosis	OPG/OCIF knock-out	Arterial calcification	(25)
		—	(26)
Osteopetrosis	OPG-L/ODF administration	Hypercalcemia	(11)
	OPG/OCIF overexpression	Extramedullary hematopoiesis	(8)
	OPG/OCIF administration	—	(8)
	OPG-L/ODF knock-out	Extramedullary hematopoiesis, delayed tooth eruption, short, flat bones, defects in B and T cell maturation	(51)
	sODAR overexpression	—	(58)
	TRAF6 knock-out	Delayed tooth eruption, hypophosphatemia, defects in IL-1, CD40 and lipopolysaccharide signaling	(65)

sODAR, soluble osteoclast differentiation and activation receptor.

and hormones converges at the levels of the OPG-L/OPG system (Fig. 2). OPG-L/OPG would then serve as the final common effector system to regulate the formation of OC from precursors in the bone marrow and its subsequent activation. Thus, the various calcitropic cytokines and hormones would indirectly regulate bone resorption by regulating either OPG-L/ODF or OPG/OCIF, or both (Fig. 2). In support of this concept are the findings that bone resorption induced by $1\alpha,25-(\text{OH})_2\text{D}_3$, PTH, PGE_2 , and IL-1 α was prevented in a fetal mouse long bone culture system by OPG/OCIF and by OPG-L/ODF-neutralizing antibody (30). Moreover, IL-1 and TNF up-regulate OPG-L/ODF gene expression (48) and OPG/OCIF production (21, 37, 38), the latter of which may represent a counter-regulatory protective mechanism. Conceptually, convergence of various calcitropic cytokines, peptide and steroid hormones to two final effectors of osteoclast formation and activation, OPG-L/ODF and OPG/OCIF, may provide a highly

efficient and balanced regulatory mechanism of bone resorption.

Of note, IL-6, a cytokine implicated in bone loss associated with sex hormone deficiency (5–7), has no effect on the OPG-L/OPG system (21, 37, 38). In fact, a recent study indicated that IL-6 may regulate osteoclastogenesis independent of OPG-L/ODF (66). In mice with targeted ablation of NF- κ B1 and NF- κ B2, transcription factors required for OPG-L/ODF signaling, no osteoclastogenesis occurred in cultured spleen cells following treatment with OPG-L/ODF, M-CSF, IL-1 β , and TNF- α , suggesting that both OPG-L/ODF and NF- κ B are essential for osteoclastogenesis. However, IL-6 in the presence of IL-6 soluble receptor induced OC formation in the NF- κ B double knock-out mice (6% of that induced by OPG-L/ODF in wild-type mice), indicating an OPG-L/ODF-independent signaling pathway (66).

Thus, osteoclastogenesis appears to be regulated by two parallel pathways, a major NF- κ B-dependent

Figure 2 Balance of osteoclast differentiation and activation by OPG-L/ODF and OPG/OCIF. Regulation by hormones and cytokines. $17\beta\text{-E}_2$, 17β -estradiol.

pathway which requires OPG-L/M-CSF and ODAR/RANK activation, and a minor NF- κ B-independent pathway which involves IL-6 and gp130 activation. Both systems may be differentially modulated by factors.

OPG-L/OPG and estrogen deficiency

Postmenopausal osteoporosis is associated with increased osteoclastogenesis and bone resorption which results in rapid bone loss within the first 4 to 8 years after menopause (67). A stimulation of the production and action of proinflammatory and bone-resorbing cytokines (IL-1, TNF- α , IL-6) by bone marrow stromal cells and osteoblasts elicited by estrogen deficiency has been previously suggested as a major mechanism of postmenopausal osteoporosis (5–7).

Recent studies have shown that estrogen (24) and TGF- β (40, 44), a peptide growth factor which is induced in osteoblastic cells by estrogen (68), increases the production of OPG/OCIF by osteoblasts and stromal cells respectively. TGF- β concurrently also decreases OPG-L/ODF mRNA expression by stromal cells, and these effects are associated with an inhibition of osteoclastogenesis (44). A previous study has suggested that the effects of estrogen on apoptosis of murine OC are mediated by TGF- β (69). These data indicate that estrogen may act by increasing OPG/OCIF which then would neutralize the OPG-L/ODF and prevent it from stimulating osteoclastogenesis, and these effects may be potentiated by increased production of TGF- β (Fig. 2). Whether estrogen also regulates the production of the OPG-L/ODF production is not known.

Recently, Yano *et al.* (70) reported a threefold increase in serum OPG/OCIF levels with aging in humans and a small increase in osteoporotic compared with age-matched non-osteoporotic postmenopausal women, and hypothesized that these increases reflect a compensatory response to the increase in bone resorption. In contrast, Arrighi *et al.* (71) failed to find major increases in serum levels of OPG/OCIF with age in a large cohort of women. Further studies are needed to examine these controversial issues in more detail.

Although prevention of bone loss in oophorectomized rats has also been reported when other candidate cytokines such as IL-1 β , TNF- α , and IL-6 were antagonized or neutralized (5–7), the OPG-L/OPG system is unique. In contrast to other candidate cytokines, OPG/OCIF prevented bone loss when administered to oophorectomized rats (8) and resulted in both phenotypic extremes of osteopetrosis (OPG/OCIF overexpression) (8) or (OPG-L/ODF deletion) (51), and osteoporosis (OPG/OCIF deletion) (25, 26).

Finally, in further support of the OPG-L/OPG/M-CSF system as the mediator of the anti-resorptive effects of estrogen on bone are the findings that the production of the permissive factor M-CSF by human bone marrow cultures is suppressed by estrogen (72), and that

estrogen deficiency induced by ovariectomy results in an increased production of M-CSF (73).

OPG-L/OPG and glucocorticoid excess

Glucocorticoid-induced osteoporosis represents the most common form of secondary osteoporosis and is a serious complication of systemic glucocorticoid use (74, 75). It is generally accepted that glucocorticoids decrease bone formation (74–77) and increase bone resorption *in vitro* (78–80) as well as *in vivo* (81–83). Recent studies have clearly identified the OPG-L/OPG system as a potential glucocorticoid target and potential mediator for the detrimental skeletal effects of glucocorticoids (22, 23). In these two studies, glucocorticoids are the single most potent inhibitors of OPG/OCIF production (by up to 90%) by various human osteoblastic cell systems regardless of their stage of differentiation, phenotype, and absolute OPG/OCIF mRNA steady levels (22, 23). Dexamethasone also decreased OPG/OCIF mRNA levels in the murine stromal cell line, ST-2, while it had a biphasic effect with an initial decrease and a subsequent increase in primary murine osteoblasts (40). Concurrently, glucocorticoids also stimulated OPG-L/ODF mRNA steady levels by two- to fourfold (23) and enhanced the stimulatory effect of $1\alpha,25\text{-(OH)}_2\text{D}_3$ on OPG-L/ODF mRNA steady levels (11). Thus, glucocorticoids markedly increased the relative OPG-L/OPG ratio in human osteoblastic cell systems (Fig. 2), which could explain the rapid and severe bone loss following initiation of systemic glucocorticoid therapy (74, 75).

OPG-L/OPG and osteopetrosis

Osteopetrosis is a rare polygenic disorder of bone metabolism characterized by a generalized increase of bone mass due to decreased osteoclastogenesis and bone resorption (84). The mechanisms underlying its pathogenesis have obvious implications for the understanding of the development of metabolic bone diseases. In view of the new paradigm of osteoclastogenesis in which OPG-L/ODF, OPG/OCIF and M-CSF are key determinants, the pathogenesis of osteopetrosis can be revisited.

Blocking of the actions of OPG-L/ODF by overexpression of the neutralizing receptor OPG/OCIF results in osteopetrosis (8). In addition, OPG-L/ODF knock-out mice (51) and transgenic mice overexpressing the soluble ODAR/RANK (58) also have osteopetrosis. Moreover, mice lacking both forms of the transcription factors NF- κ B1 and NF- κ B2, which are involved in intracellular signaling by ODAR/RANK (47), also have osteopetrosis (85). Consistent with this are the findings in NF- κ B1- and NF- κ B2-deficient mice, in which OPG-L/ODF and M-CSF failed to induce osteoclastogenesis (66). Osteopetrosis is also present in mice with a deficiency of TRAF6, an intracellular adapter protein activated by ODAR/RANK (65). In addition, a mutation in the

coding region of the M-CSF gene which leads to the absence of M-CSF protein has been detected in the *op/op* mouse, a murine model of spontaneous osteopetrosis, which can be corrected by exogenous administration of M-CSF (86). These data collectively indicate that various forms of experimental models of osteopetrosis can be attributed to disruption of the production, action, intracellular signal pathway, or nuclear activation of either OPG-L/ODF or M-CSF. These different defects ultimately converge at the level of OC differentiation and/or activation and lead to impaired bone resorption.

OPG-L/OPG and other metabolic bone diseases

Other important metabolic bone diseases in which the OPG-L/OPG system may be involved in mediating increased bone resorption or in which exogenous administration of OPG/OCIF may be potentially useful include humoral hypercalcemia of malignancy, rheumatoid arthritis, and primary hyperparathyroidism. The hypocalcemic effect of OPG/OCIF has been demonstrated in various animal models, including normocalcemic animals (17, 36) and hypercalcemic animals due to treatment with OPG-L/ODF (10), IL-1 (34), thyro-parathyroidectomy and subsequent challenge with PTH and $1\alpha,25\text{-(OH)}_2\text{D}_3$ (35), and nude mice with humoral hypercalcemia of malignancy (34, 36). In these studies, administration of OPG/OCIF had a rapid onset, profound, and dose-dependent hypocalcemic effect, was well tolerated and had no adverse extraskeletal effects. Thus, the administration of recombinant OPG/OCIF may be a useful tool in the treatment of severe hypercalcemia in the clinical setting.

Recently, activated CD4⁺ helper cells were found to produce OPG-L/ODF in the presence of proinflammatory cytokines (87). Since CD4⁺ helper cells also account for the majority of inflammatory cells infiltrating the synovium in rheumatoid arthritis, abnormal OPG-L/ODF production may also be involved in the pathogenesis of periarticular bone loss adjacent to affected joints in rheumatoid arthritis (87). Interestingly, OPG/OCIF levels in the synovial fluid are lower in patients with rheumatoid arthritis than in patients with other forms of arthropathy (gout, osteoarthritis, trauma) (88). Thus, the combination of increased OPG-L/ODF production by inflammatory cells and the decreased OPG/OCIF production may contribute to the increased periarticular bone resorption in patients with rheumatoid arthritis.

Finally, the observations that PTH increased OPG-L/ODF mRNA levels (11, 33, 39) and decreased OPG/OCIF mRNA levels (40) in osteoblastic cells, thus increasing the OPG-L/OPG ratio (39), suggest that the OPG-L/OPG system may also contribute to the increased bone loss in primary hyperparathyroidism. Clearly, additional studies are required to directly assess the role of the OPG-L/OPG system as a paracrine mediator of bone resorption in hyperparathyroidism.

Conclusions

Enhanced osteoclastogenesis is a hallmark of various forms of osteoporosis. The recent discovery of OPG-L/ODF has led to a paradigmatic shift of osteoclastogenesis. Based on extensive studies, it has become clear that OPG-L/ODF (in the presence of M-CSF) is the essential cytokine for osteoclastogenesis, and that no other cytokines are required. OPG-L/ODF is produced by marrow stromal cells and osteoblasts, and stimulates the differentiation and activation of OC by binding to its high-affinity receptor, ODAR/RANK located on the surface of OC and activating NF- κ B. The bone-resorbing effects of OPG-L/ODF are physiologically counterbalanced by the endogenous, naturally occurring soluble receptor OPG/OCIF which competes with ODAR/RANK for OPG-L/ODF. Most of the calcitropic cytokines, peptide and steroid hormones have been shown to regulate the expression of OPG-L/ODF or OPG/OCIF, or both, indicating a gatekeeping and integrating function of these two factors (Fig. 2). Importantly, some of the commonly used therapeutic agents (glucocorticoids, estrogen, PTH, $1\alpha,25\text{-(OH)}_2\text{D}_3$) differentially modulate the production of OPG-L and/or OPG/OCIF. These discoveries raise the hope that therapeutic strategies aimed at decreasing the OPG-L/ODF to OPG/OCIF ratio may suppress osteoclast formation and activation, and prevent bone loss. In light of the recent accumulation of synonyms (Table 1), a revised unitary nomenclature for the components of this cytokine system appears to be mandatory.

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