Introduction

The majority of human metabolic bone diseases are caused by an excessive extent of bone resorption that exceeds the rate of bone formation, resulting in loss of bone mass. Osteoclasts are multinucleated cells that are specialized in resorbing bone. Enhanced bone resorption may be due to accelerated osteoclastogenesis from precursor cells, enhanced fusion and activation of osteoclasts, and prolonged life span due to an inhibition of osteoclast apoptosis \(^1^2\). The discovery and characterization of the essential cytokines for osteoclast biology, receptor activator of nuclear factor (NF)-\(\kappa\)B ligand (RANKL), its receptor RANK, and its decoy receptor osteoprotegerin (OPG) have led to a novel concept of bone metabolism \(^2^5\). With accumulating evidence of the role of the OPG/RANKL/RANK system in normal skeletal physiology, it became clear that many clinically relevant metabolic bone diseases in humans, including inflammatory bone diseases (e.g., rheumatoid arthritis), malignant bone tumors (e.g., myeloma or osteolytic metastases) and different forms of osteoporoses are related to, or caused by, alterations of the OPG/RANKL/RANK system \(^2\).

In this review, we will summarize the current knowledge of the paracrine role of the OPG/RANKL/RANK system in postmenopausal, glucocorticoid-induced, transplantation-associated, immobilization-induced and senile osteoporosis as well as hyperparathyroidism. We will discuss the role of the OPG/RANKL/RANK system in the pathogenesis of these disorders based on \textit{in vitro} studies, animal models, and clinical observations, provide an overview of the clinical relevance and potential limitations using serum levels of RANKL and OPG as biochemical markers of bone metabolism in these disorders, and highlight the use of RANKL blockade as a therapeutic strategy.

Abstract

The OPG/RANKL/RANK cytokine system is essential for osteoclast biology. Various studies suggest that human metabolic bone diseases are related to alterations of this system. Here we summarize OPG/RANKL/RANK abnormalities in different forms of osteoporoses and hyperparathyroidism. Skeletal estrogen agonists (including 17\(\beta\)-estradiol, raloxifene, and genistein) induce osteoblastic OPG production through estrogen receptor-\(\alpha\) activation \textit{in vitro}, while immune cells appear to over-express RANKL in estrogen deficiency \textit{in vivo}. Of note, OPG administration can prevent bone loss associated with estrogen deficiency as observed in both animal models and a small clinical study. Glucocorticoids and immunosuppressants concurrently up-regulate RANKL and suppress OPG in osteoblastic cells \textit{in vitro}, and glucocorticoids are among the most powerful drugs to suppress OPG serum levels \textit{in vivo}. As for mechanisms of immobilization-induced bone loss, it appears that mechanical strain inhibits RANKL production through the ERK 1/2 MAP kinase pathway and up-regulates OPG production \textit{in vitro}. Hence, lack of mechanical strain during immobilization may favor an enhanced RANKL-to-OPG ratio leading to increased bone loss. As for hyperparathyroidism, chronic PTH exposure concurrently enhances RANKL production and suppresses OPG secretion through activation of osteoblastic protein kinase A \textit{in vitro} which would favor increased osteoclastic activity. In sum, the capacity for OPG to antagonize the increases in bone loss seen in many rodent models of metabolic bone disease implicates RANKL/OPG imbalances as the likely etiology and supports the potential role for a RANKL antagonist as a therapeutic intervention in these settings.

Keywords: Estrogen, Glucocorticoids, Osteoporosis, Osteoprotegerin, Parathyroid Hormone, RANK Ligand

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**Postmenopausal osteoporosis**

**Cellular and molecular mechanisms**

Osteoblastic cells express both estrogen receptor (ER)-α and -β and transactivation of ERs modulates the expression of various cytokines and peptide growth factors. Osteoblast-derived cytokines have long been implicated as paracrine mediators of estrogens, and alterations of some of these cytokines have been documented in estrogen deficiency with partial reversal after estrogen replacement therapy. Of note, 17β-estradiol enhanced OPG gene expression and protein secretion through a transcriptional mechanism in mesenchymal cells and mature osteoblastic cells that had been stably transfected with the ER-α, while no such effect was observed in cells transfected with the ER-β.

Interestingly, phytoestrogens such as genistein are also able to enhance osteoblastic OPG production through ER-α-dependent mechanisms and concurrently suppress RANKL gene expression which is associated with an inhibition of osteoclastogenesis. Of considerable clinical interest are the recent findings that raloxifene lowers the osteoblastic RANKL-to-OPG ratio by stimulating OPG production and inhibiting RANKL production. Using knock out mice lacking either ER-α, ER-β or both, Lindberg et al. reported that male mice with targeted deletion of ER-α or ER-α and ER-β had an increased OPG-to-RANKL mRNA ratio within vertebral bone, while ER-β knock out mice did not differ in that respect from wild type mice. These data indicate an important role of the ER-α in mediating the effects of estrogen agonists on skeletal OPG production.

An additional mechanism how estrogens interact with the OPG/RANKL/RANK system is related to the repression by 17β-estradiol of c-jun N-terminal kinase (JNK) activity, a critical down-stream signal in the RANK pathway. This may blunt the responsiveness of osteoclastic RANK receptors, thus diminishing the biological effects of RANKL.

**In vivo studies**

Compared to premenopausal women or postmenopausal women on estrogen replacement therapy, postmenopausal women without estrogen replacement therapy displayed a 2- to 3-fold higher RANKL expression index (as determined by FACS analysis) on marrow stromal cells, B- and T-lymphocytes. Furthermore, RANKL expression was positively correlated with biochemical markers of bone resorption (serum C-terminal telopeptide of type I collagen and urine N-telopeptide of type I collagen) and inversely with serum levels of 17β-estradiol.

Single nucleotide polymorphisms in the OPG gene have been implicated to confer an increased risk of developing postmenopausal osteoporosis. In a cohort of postmenopausal Slovenian women with osteoporosis, polymorphisms 209 G→A and 245 T→G in the promoter region of OPG were negatively correlated with bone mineral density (BMD) at the lumbar spine but not at the femoral neck. Another genetic study demonstrated that polymorphisms 163 A→G and 245 T→G in the OPG gene promoter were more common in Danish patients with vertebral fractures. Other studies evaluating OPG polymorphisms in cohorts from different ethnic backgrounds did not show an unambiguous association between OPG genotypes and BMD, osteoporotic fractures, or markers of bone turnover.

**RANKL and OPG serum levels in postmenopausal osteoporosis**

With the use of commercially available, sensitive assay systems, soluble RANKL and OPG serum levels have been assessed and related to clinical data in postmenopausal osteoporosis. Important limitations of such studies include that (I) RANKL and OPG are not bone-specific and are produced by various non-skeletal tissues, (II) systemic cytokine levels may not necessarily reflect local cytokine production or action within the bone microenvironment, (III) changes of the RANKL-to-OPG serum ratio may be a cause or consequence of estrogen deficiency.

Despite these potential limitations, Rogers et al. found a significant weak positive relationship between circulating OPG levels and serum estradiol, a weak inverse association between serum levels of OPG and bone turnover markers, and a significant positive relationship between OPG serum levels and BMD at total body, total hip, and femoral neck in a population-based cohort of 180 postmenopausal women. A positive correlation between serum levels of estradiol and OPG was also reported in two studies on women and men. However two studies in postmenopausal women revealed a negative correlation between BMD and OPG serum levels. This was teleologically interpreted as a counter-regulatory mechanism in order to prevent further bone loss.

**RANKL blockade in osteoporosis associated with estrogen deficiency**

In animal models, ovariectomy-induced bone loss was prevented by parenteral administration of a recombinant OPG-IgG-Fc fusion protein or by OPG gene delivery using an adenoviral expression vector. It appears that OPG and PTH-(1-34) have additive effects on BMD and mechanical strength in osteopenic ovariec- tomized rats. Detailed light and electron microscopy analysis of bone from ovariec- tomized mice treated with OPG revealed significantly increased trabecular bone area which was mainly due to disappearance of ruffled borders in osteoclasts.

A small controlled study in 52 women with postmenopausal osteoporosis demonstrated that a single subcutaneous injection of a recombinant OPG-IgG-Fc fusion protein (3 mg/kg) markedly reduced biochemical markers of bone resorption and formation.
### Glucocorticoid-induced osteoporosis

#### Cellular and molecular mechanisms

Systemic glucocorticoid exposure results in rapid bone loss due to a combined effect of decreased bone formation and enhanced bone resorption. While increased osteoblastic apoptosis and decreased osteoblastic synthesis of bone matrix proteins largely account for decreased osteoblastic activity, an enhanced RANKL-to-OPG ratio may be a crucial paracrine mechanism for increased bone resorption. In several studies, glucocorticoids were shown to suppress OPG mRNA expression and protein secretion and to concurrently up-regulate RANKL mRNA expression in various osteoblastic cell models independently of the stage of differentiation and the baseline level of expression. Glucocorticoid response elements have been detected in the mouse RANKL gene, suggesting a regulation through enhanced transcriptional activity. A transcriptional mechanism of OPG inhibition by glucocorticoids is also suggested on the basis of molecular studies in human osteoblasts.

RANKL and OPG serum levels in patients on systemic glucocorticoid therapy

In keeping with the profound inhibition of osteoblastic OPG production by glucocorticoids in vitro, several clinical studies have documented that systemic glucocorticoid therapy resulted in decreased OPG serum levels. In 12 patients with renal diseases, short-term administration of glucocorticoids resulted in a 30% reduction of circulating OPG levels along with a reduction of osteocalcin serum levels. A similar, more detailed study by the same group on 13 patients treated with glucocorticoids (33 mg prednisolone per day) for chronic glomerulonephritis reported decreased BMD at the lumbar spine after 6 months of treatment that was paralleled by decreased OPG serum levels and increased bone resorption markers as early as 2 weeks following initiation of therapy. In this study, serum levels of OPG were positively and independently correlated with BMD. In a study on 25 patients with active Crohn’s disease, von Tirpitz et al. evaluated BMD and biochemical markers of bone metabolism (including serum levels of OPG and soluble RANKL) before and during a 3-month period of high-dose glucocorticoid treatment (60 mg prednisolone per day). OPG (and osteocalcin) serum levels decreased after the first 2 weeks of treatment and returned to baseline levels after 3 months, while soluble RANKL serum levels tended to increase during steroid treatment. Of note, another study demonstrated 24% higher OPG serum levels in 34 patients with Cushing’s syndrome compared to healthy controls, and OPG serum levels were significantly correlated with serum cortisol levels. This may reflect a general difference between exogenous vs. endogenous glucocorticoid exposure or represent a compensatory mechanism to prevent further bone loss in long-standing cortisol excess. Another possibility is that exogenous glucocorticoid treatment in other studies was shorter and better defined.

### Transplantation-associated osteoporosis

#### Cellular and molecular mechanisms

Immunosuppressants that are commonly used after allogeneic transplantation have been implicated in the pathogenesis of transplantation-associated osteoporosis. Different immunosuppressive agents (cyclosporine A, tacrolimus, and sirolimus) concurrently enhance RANKL mRNA expression and suppress OPG mRNA levels and OPG protein secretion in undifferentiated human mesenchymal bone marrow stromal cells which appear to be rather susceptible to the effects of these drugs. By contrast, mature osteoblasts are relatively resistant against these drugs.

RANKL and OPG serum levels in transplant recipients

Several studies have assessed OPG and sRANKL serum levels in patients following kidney, liver, and heart transplantation. In patients receiving cyclosporine and glucocorticoids after renal transplantation, OPG serum levels decreased significantly on day 14 and day 28 compared to baseline levels while creatinine clearance increased steadily in this time span. A more detailed study conducted in renal transplant recipients assessed sRANKL and OPG levels, and found no significant difference between a group of 48 kidney transplant recipients and 25 healthy volunteers, while other markers of bone turnover were significantly higher in the transplant group. In this study, OPG levels were related to age, time on dialysis before transplantation, renal function, cyclosporine and azathioprine dose, and 25-hydroxycholecalciferol levels, whereas RANKL was related to leukocyte counts, cyclosporine dose, and β2 microglobulin levels.

In a study on 15 patients with orthotopic liver transplantation, OPG serum levels were not found to be different from a healthy control group, and were not different between osteoporotic vs. non-osteoporotic patients. Serum levels of OPG were positively correlated with serum levels of crosslaps, osteocalcin, parathyroid hormone, and creatinine. In a cross-sectional study on 57 cardiac transplant recipients from the same group, multiple regression analysis revealed OPG levels to be independently correlated to the BMD at the femoral neck. After adjustment for other determinants, serum OPG levels were the only significant predictor of prevalent vertebral fractures. The small number of patients (less than 100 in each of these studies) and the heterogeneity of etiologies and co-morbidities and simultaneous glucocorticoid therapy in the majority of patients which by itself lowers OPG serum levels are potential limitations of these studies.

### Immobilization-induced osteoporosis

#### Cellular and molecular mechanisms

Several recent studies have implicated the OPG/RANKL/RANK system as a paracrine mediator of mechanical strain on bone metabolism. Mechanical strain...
applied to murine primary stromal cells decreased RANKL mRNA levels by approximately 40% which was paralleled by a 50% reduction of osteoclast formation46. More detailed follow-up studies indicated that RANKL inhibition following mechanical strain critically depends on the activation of the ERK 1/2 MAP kinase pathway50,56. Application of tensile force to the distal alveolar bone surface of the maxillary molar in growing rats, enhanced mRNA signals of OPG and transforming growth factor-β1 (another inhibitor of osteoclast functions) as shown by in situ hybridization and increased the number of apoptotic osteoclasts51. Taken together, mechanical strain enhances the RANKL-to-OPG ratio by differentially modulating both components of this cytokine system, and lack of mechanical strain during periods of immobilization may lead to an imbalance of this ratio.

RANKL blockade in animal models of immobilization

RANKL blockade using parenteral administration of OPG has been used to mitigate bone loss associated with immobilization in tail-suspension and sciatic nerve damage experiments. In the murine tail suspension model, OPG treatment at a dose of 0.3 mg/kg per day mitigated bone loss by decreasing femoral endocortical resorption by 24%55. In another murine model where immobilization was caused by sciatic nerve crush, femoral bone loss was reduced from 3.8% in the placebo group to 1.4% in the treatment group receiving OPG at a dose of 0.3 mg/kg per day53. In a rat model of sciatic nerve crush, OPG administration similarly mitigated femoral bone loss in a dose-dependent manner54.

Senile osteoporosis

Age-related skeletal OPG expression

Data on age-related skeletal OPG expression are controversial. Bone marrow cells obtained from 18 subjects (age range 38-84 years) displayed an age-related decrease of OPG mRNA levels as assessed by quantitative RT-PCR55. By contrast, analysis of skeletal protein content of 60 postmenopausal women (age range 47-74 years) revealed an age-related increase in OPG and insulin-like growth factor-binding proteins 3 and 5 protein content, and each of these proteins was inversely correlated with BMD at the femoral neck and the lumbar spine56.

Age-related changes of OPG serum levels

All studies conducted to date have clearly demonstrated that OPG serum levels increase with aging in women and men with or without osteoporosis25-27,57,58. Because OPG serum levels are positively correlated with biochemical markers of bone formation or resorption in most of these studies, the age-related increase of OPG serum levels is considered a counter-regulatory mechanism to prevent further bone loss. Alternative explanations are decreased OPG serum clear-

RANKL blockade in hyperparathyroidism

In a murine model of experimental hypercalcemia and enhanced bone resorption following challenge with administration of PTH or PTH-related peptide, treatment with a recombinant chimeric Fc fusion form of human OPG with enhanced biological activity (2.5 mg/kg per day) was capable of preventing hypercalcemia and bone resorption72. The role of OPG or RANKL antibody treatment for untreatable or recurrent hyperparathyroidism has not been evaluated in humans.

Hyperparathyroidism

Cellular and molecular mechanisms

After activation of its osteoblastic receptors, parathyroid hormone (PTH) concurrently stimulates RANKL expression and inhibits OPG production by osteoblasts, and thus promotes osteoclastogenesis59-63. The enhancement of the osteoblastic RANKL-to-OPG ratio depends on the activation of protein kinase A and subsequent signalling pathways36,64-67. A potential molecular basis for the dual clinical effects of PTH (catabolic on continuous exposure; anabolic on intermittent exposure) was recently suggested by an elegant in vitro model. While continuous PTH exposure enhanced the RANKL-to-OPG ratio by up to 25-fold and stimulated osteoclastogenesis, intermittent PTH exposure stimulated IGF-1 mRNA, an anabolic skeletal growth factor68. Of note, treatment of rats with PTH also decreased OPG mRNA levels in rat femur metaphyseal and diaphyseal bone in vivo69.

The RANKL/RANK/OPG system and parathyroid hormone function in humans

In a careful study, Seck et al. reported that PTH serum levels were negatively correlated with OPG mRNA levels in bone tissue of 169 women who underwent surgery for early breast cancer70. Surprisingly, PTH serum levels were also negatively correlated with RANKL mRNA levels in this study. In a small study on 20 patients undergoing parathyroidectomy for primary hyperparathyroidism, OPG serum levels did not correlate with PTH before surgery and were not affected by parathyroidectomy as assessed 12 months later, while bone turnover markers decreased and BMD increased during this period71.
Summary

RANKL and OPG represent osteoblast-derived paracrine cytokines that are essential for osteoclast functions. An imbalance of these two factors with an enhanced RANKL-to-OPG ratio favors osteoclast differentiation and activation and promotes bone loss. Systemic factors that enhance the RANKL-to-OPG ratio include glucocorticoids, immunosuppressants, estrogen deficiency, and continuous exposure of PTH. By contrast, the RANKL-to-OPG ratio is reduced by estrogens (17β-estradiol, raloxifene, genistein) and mechanical strain. Restoring a balanced RANKL-to-OPG by modulating endogenous RANKL and OPG production or by RANKL blockade (with OPG-like molecules or RANKL antibodies) may represent a future therapeutic strategy to prevent bone loss.

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