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REVIEW

Skeletal and extraskeletal actions of denosumab

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Abstract Osteoclasts and osteoblasts define skeletal mass, structure and strength through their respective actions in resorbing and forming bone. This remodeling process is orchestrated by the actions of hormones and growth factors, which regulate a cytokine system comprising the receptor activator of nuclear factor κB ligand (RANKL), its receptor RANK and the soluble decoy receptor osteoprotegerin (OPG). Bone resorption depends on RANKL, which determines osteoclast formation, activity and survival. Importantly, cells of the osteoblastic lineage mainly provide RANKL and therefore, are central in the regulation of osteoclast functions. Catabolic effects of RANKL are inhibited by OPG, a TNF receptor family member that binds RANKL, thereby preventing the activation of its receptor RANK, which is expressed by osteoclast precursors. Because this cytokine network is pivotal for the regulation of bone mass in health and diseases, including osteoporosis, rheumatoid arthritis and malignant bone conditions, it has been successfully used for the generation of a targeted therapy to block osteoclast actions. The clinical approval of denosumab, a fully monoclonal antibody against RANKL, provides a novel option to treat bone diseases with a potent, targeted and reversible inhibitor of bone resorption. Although RANKL is also expressed by endothelial cells, T lymphocytes, synovial fibroblasts and various tumor cells, no meaningful clinical extraskeletal effects have been reported after administration of denosumab. This article summarizes the molecular and cellular basis of the RANKL/RANK/OPG system and presents preclinical and clinical studies on the skeletal actions of denosumab.

Keywords RANKL · RANK · OPG · Denosumab · Osteoporosis · Bone metastases

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Introduction

The adult skeleton consists of trabecular and cortical bone that is continuously resorbed and formed in a process called bone remodeling. This process regulates calcium homeostasis, keeping extracellular calcium levels within a tightly controlled physiological range and is responsible for the repair of bone microdamage. Bone remodeling is well balanced in young adults. In elderly people,

postmenopausal women and patients with inflammatory or malignant bone diseases, bone resorption is commonly enhanced, leading to reduced bone mass, impaired bone quality and skeletal fractures.

The discovery and characterization of the RANKL/RANK/OPG pathway has contributed to a better understanding of bone biology and paved the way for developing novel therapies for bone diseases characterized by excessive osteoclastic activity [1, 2]. The principal regulator of bone resorption is RANKL, a transmembrane protein that is highly expressed by osteoblasts [3], periosteal cells [4] and osteocytes [5]. RANKL binds to its receptor RANK, which is mainly expressed by osteoclasts and preosteoclasts [6], but has also been shown to be expressed by endothelial cells, smooth muscle cells, T and B lymphocytes, dendritic cells and various malignant cells [7–9]. RANKL- and RANK-knockout mice are severely osteopetrotic, display defects in tooth eruption, lack lymph nodes and fail to develop a lactating mammary gland [10–12] (Table 1). After binding to RANK, RANKL stimulates the formation, activity and survival of osteoclasts, resulting in increased bone resorption [13]. Osteoprotegerin (OPG) is a soluble 'decoy receptor' that is also expressed by osteoblasts and binds to RANKL with high affinity [14]. Because OPG directly competes with RANK for the binding sites of RANKL, osteoclastogenesis and subsequent bone resorption can be prevented by OPG [15, 16]. During physiological bone remodeling, the ratio of RANKL to OPG is balanced. However, an excess of RANKL is found in estrogen deficiency [17], systemic glucocorticoid exposure [18], active inflammatory process in rheumatoid arthritis [19], skeletal malignancies such as multiple myeloma [20], bone metastases [21] and vascular calcification [22], resulting in exacerbated bone loss. Detailed understanding of the RANKL/RANK pathway and its clinical implications led to the development of potential therapeutic targets. The first compounds to be tested were OPG analogues, such as OPG–Fc fusion proteins [23]. These had several limitations concerning half-life and specificity. Thus, a fully human monoclonal antibody against RANKL was developed. A significantly longer circulating half-life allowed a reduction in dosing frequency. The low potential for immune responses [24] and a higher specificity for RANKL preventing interactions with other TNF ligands [25] were other favorable characteristics of this antibody and led to its approval in 2010 (Fig. 1).

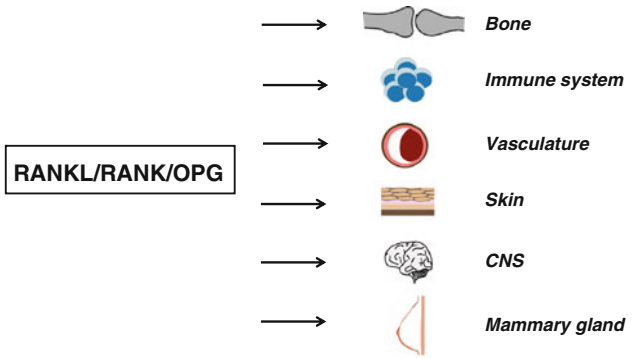

Fig. 1 Target organs for the RANKL/RANK/OPG pathway. *CNS* central nervous system

Table 1 Preclinical animal studies of RANKL inhibition using OPG

Disease	Animal model	Outcome
Cancer	Mouse model of myeloma metastasis [89]	Bone metastasis ↓
	Intratibial/intracardial injection of prostate cancer cells into immunodeficient mice [90, 91]	
	Intracardial injection of breast cancer cells into immunodeficient mice [86]	
Arterial calcification	$LDLr^{-/-}$ mice [104]	Atherosclerosis ↔
		Calcified lesions ↓
Rheumatoid arthritis	Human TNF transgenic mice [52, 120]	Bone destruction ↓
	Collagen-induced arthritis in rats [50, 51, 54]	Inflammation ↔
	Adjuvant-induced arthritis in rats [46, 48–51]	
Inflammatory bowel disease	Il-2-deficient mice [122]	Gastrointestinal inflammation ↓
Fever	RANKL injection into the lateral ventricle of mice [133]	Fever response ↓

Skeletal actions of denosumab

Animal studies

Several animal studies highlight the essential role of RANKL/RANK/OPG in the regulation of bone remodeling. The suppression of bone remodeling by OPG in healthy rats leads to increased bone volume and density, whereas stimulation of bone remodeling in normal mice or rats by RANKL leads to reduced bone volume [26, 27]. Furthermore, injection of recombinant RANKL affects bone quality parameters such as matrix mineralization, cortical geometry and trabecular microarchitecture [28]. Conversely, RANKL inhibition through OPG–Fc in rats resulted in a profound inhibition in the number of functioning osteoclasts and a progressive increase in bone volume and bone mineral density [29]. Furthermore, 12 months of denosumab treatment in eugonad cynomolgus monkeys was associated with significant increases in cortical and cancellous bone strength [30].

In addition to regulating physiological bone remodeling, the RANKL/RANK/OPG cytokine network has also been found to be dysregulated in several animal models of human disease. Ovariectomy is associated with increased bone turnover and changes in qualitative bone parameters in rodents. An increase in RANKL levels [31] as well as increased [32] or decreased [33] OPG levels has been reported in this setting. In ovariectomized rats, OPG treatment was associated with reductions in osteoclast surfaces while increasing biomechanical strength parameters and bone mineral density at various sites [34, 35].

Preclinical orchiectomy models imitate androgen ablation therapy on bone [36, 37]. Androgens directly suppress the formation and bone-resorbing activity of osteoclasts [38, 39]. OPG expression in osteoblasts can be both upregulated [40] and downregulated [41] by androgens, and an increase of RANKL expression within the bone marrow could be shown in androgen-deficient rats [42]. OPG treatment prevented the deleterious effects of orchiectomy on bone microarchitecture when applied to orchiectomized mice [43]. Androgen replacement therapy can partially reverse the periosteal formation deficit when applied to rats after orchiectomy [44].

The significance of excessive RANKL activity in an inflammatory bone loss setting has been demonstrated by ample preclinical data in rheumatoid arthritis (RA) models. Animal models of RA are characterized by increased RANKL levels in inflamed joints [45] and the serum [46]. A significant rise in the RANKL–OPG ratio that is caused by a T cell-mediated release of RANKL has been shown in a murine model of RA [47] and this ratio further correlates with joint erosions and osteoclast activity [48]. Conversely, RANKL inhibition through OPG treatment suppressed the formation of bone erosions and downregulated the activity of osteoclasts in rodent models of adjuvant arthritis [49, 50], collagen-induced arthritis [50, 51] and TNF-mediated arthritis [52]. Furthermore, OPG also prevented systemic bone loss [53] and provided adequate cartilage preservation in arthritic mice [54].

RANKL/OPG abnormalities have also been implicated in glucocorticoid-induced bone loss. Glucocorticoids exert several direct and indirect adverse effects on bone, primarily through a reduction of osteoblasts and osteocyte activity and lifespan [55]. Evidence exists that OPG and RANKL are involved in the pathogenesis of glucocorticoid-induced bone loss. Glucocorticoids suppress OPG expression in osteoblast cell lines [56] while upregulating RANKL expression [57]. In murine models, glucocorticoids decrease bone mineral density mainly through suppression of bone formation [58, 59], whereas treatment with OPG prevented these changes [60].

Table 2 Clinical studies using denosumab

Name	Reference	BTM	BMD	Fracture
Single-dose placebo controlled in post-menopausal women	Bekker et al. [61]	+	−	−
Efficacy and safety in postmenopausal women with low BMD	McClung et al. [62]	+	+	−
Treatment of postmenopausal osteoporosis (FREEDOM)	Cummings et al. [70]	+	+	+
Prevention of postmenopausal osteoporosis	Miller et al. [65]	+	+	−
Comparison with alendronate in post-menopausal women with low BMD (DECIDE)	Brown et al. [72]	+	+	−
Treatment of bone loss in men on androgen-deprivation treatment for non-metastatic prostate cancer (HALT)	Smith et al. [97]	−	+	+
Treatment of bone loss in women on aromatase inhibitors for non-metastatic breast cancer	Ellis et al. [94]	−	+	−

BMD bone mineral density, *BTM* bone turnover markers

Adapted from "Osteoporosis: now and the future"; *Lancet* 377(9773):1276–87

Clinical data

Denosumab in osteoporosis

The antiresorptive activity and safety of denosumab were first shown in a phase I trial in postmenopausal women

(Table 2). A rapid decrease of the bone resorption marker N-telopeptide by 81 % with denosumab at a dose of 3 mg/kg was demonstrated [61]. Subsequently, a phase II randomized placebo-controlled study was conducted in 412 postmenopausal women with low bone mass. In this study, 60 mg denosumab every 6 months during 1 year increased the lumbar spine bone mineral density (BMD) by 6.7 % [62]. The study was extended to cover a total of 4 years using different treatment protocols. While discontinuation of denosumab led to a decrease of BMD by 6.6 % at the lumbar spine within 12 months and an increase of bone turnover markers above baseline, resuming therapy increased BMD levels. This points towards a reversible action of denosumab on bone, which is in contrast to alendronate treatment where discontinuation resulted in a minor decrease of lumbar spine BMD and unchanged values of bone resorption markers [63–66].

The efficacy of denosumab in the treatment of postmenopausal osteoporosis was determined in the FREEDOM (Fracture Reduction Evaluation of Denosumab in Osteoporosis every 6 months) trial and was summarized in several reviews [67–69]. In this phase 3 study, patients received either subcutaneous injections of 60 mg denosumab twice a year or placebo for 3 years. In patients receiving denosumab, vertebral fractures, hip fractures and non-vertebral fractures were decreased by 68, 40 and 20 %, respectively [70]. Especially those patients with a moderate-to-high fracture risk benefited from denosumab as shown with FRAX analysis, a computer-based algorithm that assesses fracture probability from clinical risk factors [71]. Kidney functions were not affected and the appearance of other side effects was comparable to the placebo group. The DECIDE (The Determining Efficacy: Comparison of Initiating Denosumab versus Alendronate) study compared denosumab to alendronate treatment in postmenopausal women during a period of 12 months and revealed that denosumab was more effective in increasing BMD than alendronate at all skeletal sites measured (total hip, femoral neck, trochanter, lumbar spine and distal radius) [72]. Extension of the FREEDOM trial corroborated the efficacy and safety of denosumab. After two additional years of denosumab treatment, annual fracture incidences were still less than the rates observed in the FREEDOM placebo group, the BMD further increased at the lumbar spine and total hip BMD up to 13.7 and 7 %, respectively and no increase in adverse events were reported [73]. Furthermore, in the DAPS (Denosumab Adherence Preference Satisfaction) study, it was shown that postmenopausal women with osteoporosis who received subcutaneous injections of denosumab showed better adherence, compliance and persistence than women who were treated with alendronate once a week [74].

Denosumab in malignant conditions

Bone metastases are frequent complications especially of human prostate, breast and lung cancer [75], renal cell carcinomas [76] as well as multiple myeloma [77] mostly causing osteolysis and hypercalcemia. The RANKL/RANK/OPG signaling pathway has been implicated in regulating cancer cell migration to bone and progression of bone metastases [78–82]. Myeloma cells have been shown to upregulate RANKL expression [83–85] and to downregulate OPG protein production via sequestration and lysosomal inactivation [86]. In a murine model of bone metastasis, higher levels of RANKL were measured and were found to be inhibited by a recombinant OPG–FC protein [87]. Furthermore, elevated RANKL and RANK expressions have been observed in clear renal cell carcinomas compared with non-neoplastic renal tissue. In vitro experiments revealed that adding recombinant RANKL to clear renal cell carcinomas, Caki-1, increased cell migration, which could be inhibited by the administration of recombinant OPG [88]. Furthermore, stimulation of RANK-positive human breast cancer cells with recombinant RANKL induced actin polymerization, which resembles chemokine receptor signaling in cancer cell lines and increased cell migration. Neutralization of RANKL by OPG in a mouse model of myeloma metastasis resulted in a marked reduction in tumor burden in bones, while bisphosphonate treatment with zoledronic acid did not change the tumor burden of cancer metastasis [89]. In a murine prostate cancer model, OPG treatment resulted in reduction of osteoclast numbers and skeletal tumor burden [90, 91], and applying OPG to mice after injection of prostate cancer cells into the tibia and subcutaneously completely prevented the development of tibial tumors, but had no effect on subcutaneous tumor growth [92]. Another complication of bone metastasis, humoral hypercalcemia, was also shown to be reduced with OPG. In a murine model of hypercalcemia, OPG administration (5 mg/kg) significantly suppressed hypercalcemia compared with high-dose bisphosphonate treatment (pamidronate/zoledronic acid, 5 mg/kg) [93].

Targeting RANKL with a recombinant OPG construct in humans was initially examined on a small cohort of patients with breast cancer and multiple myeloma [23]. Receiving a single dose of denosumab reduced bone resorption up to 84 days compared with pamidronate (90 mg), which exhibited suppressive bone resorption effects for 3–4 weeks.

Beneficial effects of denosumab on BMD and the reduction of vertebral fractures were further demonstrated in two phase 3 trials with either non-metastatic breast cancer patients receiving aromatase inhibitors or non-metastatic

prostate cancer patients receiving androgen-deprivation therapy [94, 95].

In a phase III randomized double-blind clinical trial in 1,904 men with bone metastases from castration-resistant prostate cancer, denosumab was compared with zoledronic acid. Denosumab at a dose of 120 mg once a month was found to be superior to placebo or zoledronic acid in preventing tumor-related bone lesions or skeletal-related events (SREs) as measured with the time between first diagnosis of prostate cancer and first-documented event of bone metastasis [96, 97]. These results were reproducible in a double-blind controlled study of 2,049 women with advanced breast cancer randomized to receive either monthly denosumab (120 mg) or zoledronic acid (4 mg) [98], where denosumab was superior in delaying the time to first and subsequent SRE per month by 18 and 23 % respectively. In a phase III randomized double-blind study in 1,779 patients with advanced cancer involving bone metastases or multiple myeloma, denosumab treatment was found to be non-inferior to zoledronic acid treatment regarding the median time of first on study SRE (21 vs. 16 months, respectively) [99].

Extraskeletal actions of denosumab

Denosumab in the treatment of arterial calcification

Osteoporosis and arterial calcification are prevalent diseases in Western society that may coincide. Especially in postmenopausal women, bone loss is associated with the progression of vascular calcification [100], and has been attributed to abnormalities of the RANKL/RANK/OPG pathway [101]. This was first suspected based on the phenotype from $OPG^{-/-}$ mice, where two thirds developed severe osteoporosis as well as marked calcified lesions in the aorta and renal arteries, sites of endogenous OPG expression, 2 months after birth [102]. Transgenic overexpression of OPG in these mice inhibited the development of those calcified lesions, whereas an injection of recombinant OPG to adult $OPG^{-/-}$ mice was not able to rescue arterial calcification, indicating that a lifelong deficiency prevents arterial calcification but OPG administration is not able to fully reverse this process [103]. However, treatment of an atherogenic mouse model with recombinant OPG significantly reduced calcified lesions, implying that OPG inhibits vascular calcification by blocking RANKL [104]. Similarly, RANKL induced calcification of vascular smooth muscle cells in vitro, which was inhibited after coincubation with OPG [8]. The induction of osteogenesis in the vasculature was shown to be dependent on a RANKL-mediated increased expression of bone morphogenic protein-2 and matrix Gla protein, two important regulators of the calcification process [105]. Furthermore, a direct effect of denosumab on RANKL was demonstrated in human RANKL knock-in mice expressing a chimeric murine/human RANKL protein, where denosumab reduced calcium deposition in the aortic wall of glucocorticoid-treated human RANKL knock-in mice [106].

Given this preclinical evidence, it seems reasonable that treating osteoporotic patients with denosumab could also have beneficial effects on arterial calcification. However, thus far, there is no evidence that denosumab reduces arterial calcification in osteoporosis patients. Several phase II studies showed no differences concerning the incidence of vascular disorders compared with placebo or bisphosphonate treatment, respectively [63, 107, 108]. Similarly, in the FREEDOM study, where 7,868 women between 60 and 90 years received either 60-mg denosumab or a placebo for 36 months, no differences regarding cardiovascular events were detected [70].

Denosumab in the treatment of inflammatory conditions

Inflammatory diseases such as rheumatoid arthritis (RA) [109] inflammatory bowel disease (IBD) [110], chronic obstructive pulmonary disease (COPD) [111] or systemic lupus erythematosis [112] are accompanied with systemic and local bone loss. The RANKL/RANK/OPG circuit and its function in the immune system is considered to be responsible for the connection between inflammation and bone destruction [113]. RANKL is expressed by activated CD4+ T cells [47], which activate osteoclasts [47, 114] and further stimulate other RANKL-expressing cells, such as dendritic cells or monocytes/macrophages [115, 116], promoting a catabolic milieu in the bone. In the event of COPD, it was shown that the level of RANKL and the RANKL/OPG ratio in the serum were significantly higher in patients with low BMD probably caused by the systemic inflammation [117]. But although inflammation and bone destruction are accompanied, several studies indicate that the RANKL/RANK/OPG pathway is only responsible for the situation in the bone and has no influence on the inflammatory component. In the case of collagen-induced arthritis in rats, a suitable model for RA, treatment with Fc–OPG prevented joint destruction had no effects on the inflammation [54]. Similarly, systemic OPG treatment of type II collagen-immunized mice overexpressing IL-17, a T cell cytokine that was shown to promote RANKL expression in vitro [118], prevented bone erosion [119]. In addition, in human TNF transgenic mice, which spontaneously develop inflammatory arthritis, OPG treatment increased BMD about 89 %; however, levels of inflammatory cytokines were not affected [120]. Finally, $RANKL^{-/-}$ mice remained sensitive to arthritis following administration of an arthritis-inducing serum [121].

However, although these studies propose no immunomodulatory function of RANKL, studies in IL-2-deficient mice, a model of autoimmunity that exhibits hyperactivation of CD4+ T cells leading to IBD, showed that treatment with Fc–OPG not only increased bone density but also reduced gastrointestinal inflammation. The authors assumed that these diverse results are because of significant differences of the underlying mechanisms involved in T cell-mediated inflammatory colitis, where RANK+ dendritic cells are the major antigen presenting cells and bone disease [122].

In humans, denosumab treatment is limited to RA so far. As is in preclinical studies, denosumab only improved bone parameters without any anti-inflammatory action. In a phase II clinical trial, injections of 180-mg denosumab twice a year to RA patients receiving methotrexate treatment significantly inhibited structural damage [123]. Other investigations showed that not only structural damage was inhibited but also denosumab treatment increased bone mineral density in the cortical bone [124] as well as the hands and wrists [125], but did not affect inflammation.

Immune system and thermoregulation

Apart from a T cell-mediated inflammatory response elicited by RANKL, other potential immune effects exist. For instance, RANKL and RANK-deficient mice completely lack lymph nodes and have a hypoplastic thymus [10, 11, 126], whereas OPG-deficient mice exhibit hypertrophic thyme accompanied with an increased number of mature medullary thymic epithelial cells [127]. Simultaneously, some RANK mutations in humans are associated with hypogammaglobulinemia [128–130], a disorder caused by a lack of B lymphocytes leading to an impaired antibody response to antigens.

Another immune mechanism for RANKL and RANK, which are also expressed in the central nervous system [131, 132], was reported in the context of central fever response in inflammation. In mice and rats, RANKL injections into the lateral ventricle caused severe fever with no effects on osteoclasts and which could be inhibited with OPG [133]. On the contrary, peripheral intraperitoneal injections of RANKL had no impact on body temperature, indicating that only central RANKL/RANK signaling is required for fever response. Furthermore, two children with RANK mutations leading to autosomal-recessive osteopetrosis could not become febrile during pneumonia identifying RANKL/RANK as key thermoregulators.

It further has been suggested that RANKL/RANK also might regulate immunity via the control of epidermal immune response. It was reported that RANKL is inducible on keratinocytes upon UV exposure and induces RANK-expressing Langerhans cells to regulate CD4+ CD25+ regulatory T cells, which show immunosuppressive effects. In line with that study, RANKL expression was strongly induced in psoriatic lesions, whereas in the epidermis of patients with cutaneous lupus erythematosus, an autoimmune disorder triggered by sun exposure, no RANKL expression was detected indicating its involvement in the suppression of autoimmunity [134].

Despite these preclinical findings, extension of the FREEDOM trial and pooled analysis of other clinical trials revealed that the overall susceptibility to infections or autoimmune diseases after denosumab treatment is not different from placebo treatment [70, 135].

Conclusions

Denosumab, a fully human monoclonal antibody to RANKL, displays an effective therapy to reduce fractures in osteoporosis, SREs in patients with bone metastases because of breast and prostate cancer as well as bone erosions in inflammatory disorders such as RA. Although several preclinical studies also indicate extraskeletal functions for the RANKL/RANK/OPG pathway, e.g., in the process of arterial calcification, fever regulation or immunosuppression in the skin, there is no evidence from clinical phase 3 studies to support that RANKL blockade by denosumab interferes with these processes.

Conflict of interest LCH has received honoraria from Amgen, Merck, Novartis, and Nycomed. KS, ET, MR, TDR and CH have no conflict of interest.

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