Osteoclast-dependent and-independent roles of the RANKL/RANK/OPG pathway in tumorigenesis and metastasis

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ABSTRACT

RANKL is a TNF ligand superfamily member that is essential for the formation, activation, and function of osteoclasts. RANKL functions via its cognate receptor RANK and is inhibited by the soluble decoy receptor osteoprotegerin (OPG). In skeletal metastases, the ratio of RANKL:OPG is upregulated which leads to increased osteoclast-mediated bone destruction. These changes in the bone microenvironment not only compromise the structural integrity of bone, leading to severe clinical morbidities, but have also been implicated in establishment of de novo bone metastasis and the progression of existing skeletal tumors. Evaluation of RANKL inhibitors, including the fully human anti-RANKL antibody denosumab, in cancer patients has demonstrated reductions in tumor-induced bone resorption activity and successful management of skeletal complications of bone metastases. RANKL also functions as a major paracrine effector of the mitogenic action of progesterone in mouse mammary epithelium and has a role in ovarian hormone-dependent expansion and regenerative potential of mammary stem cells (MaSC). RANKL inhibition attenuates mammary tumorigenesis and pulmonary metastases in mouse models. These data suggest that the contribution of progesterone to increased mammary cancer incidence is mediated at least in part by RANKL-dependent changes in the mammary epithelium and RANKL also directly promotes distant metastases. In summary, the anti-tumor and anti-metastatic effects of RANKL inhibition can occur by at least two distinct mechanisms, one in the bone via osteoclast-dependent effects and the second via direct effects on the tumor cells of various origins and/or mammary epithelium.
BACKGROUND

CONTROL OF NORMAL BONE REMODELING BY THE RANK/RANKL PATHWAY

The cells that control bone remodeling include the osteoblast, which deposits new bone, and the osteoclast, a specialized cell of hematopoietic origin which resorbs the inorganic and organic matrix of the bone. Osteoblast and osteoclast function are coordinately regulated in normal bone remodeling. Identification of the RANK, RANKL, and osteoprotegerin (OPG) pathway in the mid-1990’s revealed a key molecular axis for osteoclast formation, function, and survival and provided crucial insights into the regulation of normal, physiological bone remodeling. Functional genomics in mice demonstrated that RANKL (TNFSF11), a member of the TNF ligand superfamily, and RANK (TNFRSF11a), the cognate TNFR family receptor for RANKL, were essential for osteoclastogenesis in vivo. A functional interaction between RANKL, expressed by bone stromal cells of the osteoblast lineage, and RANK, expressed by osteoclast precursors of hematopoietic myeloid lineage, is necessary for osteoclast differentiation, survival and activation. Knockout mice lacking either RANK or RANKL develop significant osteopetrosis resulting from a lack of osteoclasts and absence of bone resorption (1, 2). OPG (TNFRSF11b), another member of the TNFR superfamily, can also bind RANKL and functions as a soluble decoy receptor for RANKL. The critical role of OPG in osteoclastogenesis and bone remodeling was first demonstrated by the increased bone mass as a result of reduced osteoclast numbers observed in transgenic mice overexpressing OPG (3) and conversely, the osteopenia observed in OPG knockout mice (4). Given that the precise and balanced interaction of RANK, RANKL, and OPG is critical for osteoclastogenesis and therefore the maintenance of homeostatic bone remodeling and bone mass, it was hypothesized that this pathway may become dysregulated within the bone during disease states and contribute to pathologic bone loss such as post-menopausal osteoporosis or cancer-induced bone destruction (Figure 1).

A unique feature of skeletal metastases and multiple myeloma is the active involvement of the host, particularly osteoclasts, in the pathophysiological process leading to bone destruction and skeletal tumor growth. Accelerated osteoclast action and subsequent increased bone resorption...
have been operatively linked to a full spectrum of skeletal complications (eg, hypercalcemia, pathologic fracture, bone pain, spinal cord compression, surgery or radiation treatment to the bone, and etc.) that result from bone metastases and multiple myeloma. Osteoclast involvement is observed in all types of cancer-induced bone destruction, regardless of the lesions’ radiographic appearance as either osteolytic or osteoblastic, and irrespective of the tumor type of origin (5-7).

In addition to causing destructive bone loss and associated clinical sequelae, tumor-induced osteoclasts contribute to the establishment, growth, and survival of tumors; products of osteoclastic-bone resorption including matrix-derived growth factors (eg TGF-β, IGF1) and elevated calcium levels increase tumor cell proliferation and survival and induce further production of osteolytic and osteoblastic factors, thereby creating a positive feedback loop known as the vicious cycle (7). As osteoclasts are highly related to macrophages, it is perhaps not surprising that many pro-tumorigenic factors can be produced by osteoclasts directly, including growth- (PDGF-α, IL-1, TNF, IL-6, IGF-1, BAFF, APRIL) or angiogenic-factors (VEGFα, VEGFc, bFGF) in addition to the production of matrix metalloproteinases (eg, MMP-7, -9, -14) (8, 9). Thus, the osteoclast may indirectly provide factors which contribute to skeletal tumor establishment and progression as a consequence of bone matrix turnover in addition to cellular production, independent of bone resorption. Initial studies of breast cancer by Stephen Paget in 1889 identified the bone as an optimal site (or “soil”) for distant metastases (“seed”) (10). It is now clear that many other tumors, including prostate, lung, renal, melanoma, thyroid, etc. have a high propensity to metastasize to bone, highlighting the skeleton as a fecund metastatic site capable of harboring early tumor colonization and actively promoting the growth of skeletal tumors.
THE RANKL, RANK, OPG PATHWAY IN BONE AND CANCER INDUCED BONE DISEASE

In each of these aforementioned pathologic processes (bone destruction, metastatic establishment, and tumor progression), the tumor has co-opted the host by upsetting the balance of two key factors that normally govern physiological osteoclast formation and bone remodeling: RANKL and OPG (Figure 1). The cancer-induced signals capable of altering the normally well-balanced RANKL:OPG ratio can be extremely diverse, reflecting the different sources of RANKL and OPG, the assortment of tumor types which affect bone and the heterogeneity of individual tumor types (7, 11). More importantly, these diverse signals and mechanisms which elevate osteoclastogenesis and bone destruction converge on the RANKL pathway. Many different cytokines or factors produced by skeletal tumors (e.g. IL-1β, IL-6, IL-8, IL-11, IL-17, MIP1α, TNFα, PTHrP, PGE2) cause increased RANKL production by stromal cells in the bone microenvironment, including cells of the osteoblast lineage. OPG, the normal decoy receptor for RANKL, can also be down regulated by tumors via various mechanisms; reduced synthesis or active degradation of OPG in the bone is observed as a consequence of tumor-derived factors (11). Certain factors in metastatic cancer have dual effects on the RANKL:OPG ratio. For instance, PTHrP, IL-1, and PGE2 have been shown to act on the bone stroma and stimulate osteoclast activity by increasing RANKL and decreasing OPG simultaneously (7). T-lymphocytes, including activated T-cells (12) and CD4+CD25+ T-regulatory cells (T-regs) (13), may be another source of RANKL in bone metastases or other extraskeletal cancer settings in which RANKL may function (see below). Multiple myeloma cells can increase RANKL production in T-lymphocytes (14), however functional evidence for T-cell contribution of RANKL in cancer/bone interactions is lacking largely because most preclinical bone cancer models are studied in immunocompromised hosts.

Increases in RANKL that lead to cancer-induced osteoclastogenesis are not limited to infiltrating immune cells or reactive changes in the bone stroma caused by the tumor, but can be contributed by the tumor cells themselves. RANKL expression has also been reported on some tumor types, including breast cancer, prostate cancer, multiple myeloma and renal carcinoma (as will be described below). RANKL produced by tumor cells can increase osteoclastogenesis in vitro (15),
suggesting that the tumor cell localized in the skeleton may also directly contribute an osteoclastogenic signal. It is certainly possible that the bone microenvironment will provide signals that can increase RANKL on metastatic tumor cells which, in turn, enhances further osteoclast activity and creates a feed-forward loop. For instance, RANKL increases have been observed in prostate tumor cell lines after treatment with TGF-β (16), a product of bone resorption, or upon stimulation of the epithelial-to-mesenchymal transition (EMT) by TGF-β plus EGF treatment, or transfection with SNAIL (17). Intriguingly, EMT changes in tumor cells residing in the bone have been linked to a more invasive phenotype and development of overt bone metastases from dormant micrometastases (17, 18), although a functional role for RANKL in this transition has not yet been defined. Consistent with the hypothesis that RANKL production by tumor cells accelerates osteoclast formation and bone metastasis is the observation that a high expression level of RANKL in primary renal cell carcinoma is associated with a shorter bone metastasis-free survival (19). A similar relationship between high RANKL expression levels in primary tumors and development of bone metastases has also been described for hepatocellular carcinoma patients (20).

There is currently no definitive data correlating circulating levels of RANKL in serum with bone metastases. This may reflect the preponderance of the membrane-bound vs. soluble form of RANKL, the relatively low levels of RANKL detected in the serum, or the technical limitations of such assays (21). It is also likely that increases in RANKL remain localized to the bone lesion where focal activation of bone remodeling and increased osteoclast activity are closely juxtaposed to tumor infiltration (22). In contrast, a relationship between the higher ratio of serum RANKL:OPG and a greater extent of bone lesions has been observed in multiple myeloma (23), which may reflect either the extensive tumor cell production of RANKL or the systemic nature of this hematologic tumor.

To address the functional contribution of RANKL to cancer-induced bone disease, pharmacologic RANKL inhibitors such as OPG and RANK-Fc have been tested in rodent models and these studies have been the subject of recent reviews (11). Preclinical models of cancer bone metastasis and multiple myeloma typically develop osteolytic, osteoblastic, or mixed lesions after systemic or intratibial injection in rodents. RANKL inhibition has been tested
in models representing many different tumor types including multiple myeloma, breast cancer, prostate cancer, lung cancer, colon cancer, etc. and in each case has been shown to effectively reduce tumor-induced bone lesions (11). Given the specific mechanism of RANKL inhibition, the observed broad activity across lesion types suggests that osteoclastic activity may be a requisite element for both osteolytic and osteoblastic lesions. In addition to the beneficial effect on bone lesions, RANKL blockade has also reduced other tumor- associated sequelae, including bone pain (24) and hypercalcemia of malignancy (25).

Based on the observed reciprocal feedback (the vicious cycle) occurring between bone and tumor, one would predict that reduction of bone resorption would not only reduce the tumor-induced bone destruction but also slow skeletal tumor progression. Studies using preclinical cancer/bone models representing different solid tumors and multiple myeloma have demonstrated a substantial reduction in tumor growth in the bone as a consequence of the potent osteoclast blockade achieved with RANKL inhibition (6, 7, 26). The reductions in skeletal tumor burden upon RANKL inhibition are dependent on the bone microenvironment and are associated with increased tumor cell apoptosis, decreased tumor cell proliferation, and increased survival of tumor-bearing mice (27-30). RANKL inhibition effectively reduces skeletal tumor growth as a monotherapy, but as would be predicted by an approach which targets the bone microenvironment and reduces local growth factor and calcium production, the reduction in skeletal tumor burden is additive when combined with chemotherapy, hormonal therapy or targeted therapies (28, 31-33).

If the “seed and soil” observations and vicious cycle hypothesis postulate that the fertile nature of the bone microenvironment actively contributes to the processes of early tumor colonization and metastatic outgrowth, then skeletal metastases might be prevented or delayed through pharmacologic blockade of bone turnover (34), with RANKL inhibition as a potential approach. In order to test this hypothesis in preclinical models, however, one must consider limitations of experimental bone metastases models including the observation that skeletal growth of xenografted human tumor cells may ultimately achieve a critical mass capable of self-maintenance independent of the bone microenvironment. To address this limitation, it has been necessary to model tumor/bone interactions at early stages of bone metastasis and colonization.
using high-sensitivity, small-animal imaging techniques. Prophylactic treatment of mice with the RANKL inhibitor OPG-Fc was employed to reduce baseline osteoclast activity and bone resorption prior to inoculation of breast tumor MDA-231 cells. This strategy subsequently delayed de novo formation of metastatic skeletal tumors as monitored by bioluminescent imaging, presumably by arresting the vicious cycle supporting initial tumor growth (27).

THE RANKL PATHWAY IN NORMAL MAMMARY BIOLOGY AND CANCER

In addition to severe osteopetrosis, RANK and RANKL knockout mice manifested a lactation defect, revealing an intrinsic functionality of the pathway in mammary epithelium that is also relevant to mammary tumorigenesis and metastases. The failure of RANK or RANKL knockout mice to lactate was due to a marked inability to develop lobulo-alveolar mammary structures which normally undergo a massive expansion under hormonal control during pregnancy and eventually differentiate into milk-secreting tissue. Proliferation and survival of the mammary epithelium are reduced in the absence of a productive RANKL/RANK signal and transplantation experiments demonstrated this defect to be autonomous to mammary epithelial tissue (35). RANKL expression is greatly increased in luminal mammary epithelial cells at mid-gestation pregnancy and can be induced by factors such as prolactin, PTHrP, and progesterone (35). In mice, RANK expression is observed at low levels in luminal and basal cells of the mammary epithelium and becomes more highly expressed at mid-gestation pregnancy, most selectively at ductal branch points (36).

Genetic studies revealed that both RANKL and progesterone receptor (PR) function at similar stages in lactational morphogenesis and these proteins are co-localized in luminal mammary epithelial cells. Recent experiments have shown that the major mitogenic effect of progesterone occurs via increases in RANKL within PR⁺ luminal epithelial cells. RANKL, acting as a paracrine factor, then induces mitogenesis of neighboring ER-/PR- mammary epithelial cells (37-39). Moreover, other recent studies have shown that RANKL can also mediate the non-proliferative expansion of the mouse mammary gland, via increases in the number and regenerative capacity of mammary stem cells (MaSC). Despite their ER-/PR- phenotype, MaSC are profoundly responsive to ovarian hormone signaling, and RANKL was identified as a
paracrine effector of the progesterone-dependent effects on MaSC occurring during either gestation or estrous cycles (40, 41).

RANKL PROMOTES MAMMARY TUMORIGENESIS

The fundamental roles of the RANKL pathway in the normal physiology of the mammary gland have significant implications in cancer and tumorigenesis. RANKL-driven hormone (progesterone)-dependent proliferation, survival, and non-proliferative expansion of MaSC could each contribute to mammary cancer initiation, progression, and recurrence (Figure 2). During normal lactational morphogenesis, it is well characterized that RANK and RANKL adhere to strict yet overlapping spatial-temporal expression patterns within the mammary epithelium under control of lactation hormones. However, when RANKL or RANK are overexpressed in the mammary epithelium (via mouse transgenic models using the MMTV promoter) in the absence of strict hormonal control, inappropriate mammary proliferation is observed (36, 42). Despite the aberrant proliferation (including hyperplasia) induced by an overactive RANKL pathway in these two models, spontaneous mammary tumors were not observed in aged virgin mice. However, accelerated preneoplasias and increased mammary tumor formation were observed in MMTV-RANK mice after multiparity or treatment with the carcinogen DMBA in combination with a synthetic progestin hormone (medroxyprogesterone acetate; MPA) (38); settings in which mammary epithelial RANKL expression is increased due to increased sex hormone levels. Pharmacologic blockade of RANKL decreased incidence and delayed onset of mammary tumors induced by DMBA and MPA in both MMTV-RANK transgenic mice and wildtype mice (38). The reduction in mammary tumor formation was preceded by a reduction in preneoplasias and correlated with rapid and sustained reductions in hormone-induced mammary epithelial proliferation and cyclin D1 expression as well as increased apoptosis, suggesting that RANKL affects early stages of tumor formation. A similar reduction in mammary tumor formation (also induced by MPA/DMBA) was observed by Schramek et al. (43) in mice where RANK had been selectively deleted from the mammary epithelium by a tissue specific Cre recombinase-mediated approach. This genetic approach also revealed that the RANKL pathway protects against DNA damage-induced cell death and expands the putative stem cell component of mammary tumors. In a model of mammary cancer induced by the c-neu oncogene in the absence of exogenous...
hormone (MMTV-neu transgenic mice), inhibition of RANKL (beginning at 5 months of age) did not affect median time to spontaneous mammary tumor formation, but did decrease the number of preneoplastic lesions and mammary tumors (38). Mammary tumor formation was not reduced with another inhibitor of bone resorption (ie the nitrogen-containing bisphosphonate, zoledronic acid [ZA]) (38). In line with the observed reductions in mammary epithelial biomarkers upon RANKL inhibition and the protection against tumors observed in the mammary-selective knockout of RANK, these data confirm a direct, osteoclast-independent, functionality of the RANKL pathway in cancer.

A DIRECT ROLE OF THE RANKL PATHWAY IN DISTANT METASTASES

There is also preclinical evidence that blockade of RANKL will reduce distant metastasis, potentially via mechanisms distinct from the bone-intrinsic, anti-osteoclast effect (Figure 2). Treatment of transgenic MMTV-neu mice with RANK-Fc significantly decreased spontaneous lung metastases (38). Consistent with this observation, crossing the MMTV-neu mice with RANK heterozygotes reduced the incidence of spontaneous lung metastases (44). Reductions in bone metastases or lung metastases have been achieved with pharmacologic blockade of RANKL in animals bearing RANK-positive melanoma cells (45) or transplanted primary MMTV-neu tumor cells (44). Reciprocally, enhanced lung metastases have been observed upon systemic RANKL exposure in mice bearing orthotopically-transplanted tumor cells, originating either from a RANK-positive human breast tumor cell line or tumor cells derived from MMTV-neu mice (44).

To gain mechanistic insight into the pro-metastatic activity of RANKL, considerations of in vitro experiments, in vivo expression patterns of RANK and RANKL, and other potential biological activities of RANKL are necessary. Expression analyses and treatment of cells in vitro with RANKL have shown functional RANK protein expression on the surface of prostate, breast, osteosarcoma, melanoma, and lung cancer cell lines. In most studies, RANKL does not appear to increase proliferation of RANK-expressing tumor cells (46, 47), although increased cell number and protection against DNA damage-induced cell death, activated by either chemotherapy or γ-irradiation, has been observed in certain cell lines (43). In addition, RANKL will affect a
variety of other tumor cell behaviors potentially relevant to tumor progression and metastasis, including increased tumor cell growth in semi-solid media, loss of apical-basal polarity, and stimulated migration and invasion (38, 43, 45, 46, 48, 49). RANKL treatment has been documented to induce a variety of factors potentially involved in migration, angiogenesis, and invasion, including MMP1, MMP9, the matrix metalloproteinase inducer EMMPRIN/CD47, ICAM-1, IL-6, IL-8, VEGF (47) and can decrease expression of the metastasis suppressor serpin 5b/maspin (50).

RANKL-dependent promotion of tumor cell migration and invasion (as defined in in vitro experiments described above) may certainly contribute to increased distant metastases observed in vivo. RANKL would be predicted to be highly expressed in normal (or reactive normal) tissues at distant sites of metastases, including peripheral lymph nodes and bone (51), in addition to any RANKL potentially expressed within the primary tumor. In tumor cell lines, RANK expression on tumor cells is not strictly required for bone metastasis in experimental metastases models; however, two in vivo studies are supportive of the skeletal source of RANKL enhancing metastases of RANK-expressing tumors directly: 1) the selective prevention of RANK-expressing tumor cells homing to the bone by RANKL inhibition but not by bisphosphonates (45) and 2) the increased skeletal growth rate of tumor cells with high RANK expression compared with tumor cell controls with low RANK expression (52). By the same mechanism, RANKL present within lymph nodes or other metastatic sites could then enhance metastatic outgrowth of RANK-positive tumors. Consistent with the above experimental results, a recent analysis reported that high RANK expression in primary breast tumors was associated with lymph node involvement and a higher risk to develop bone metastases (53).

Precise mechanisms for the observed reduction in lung metastases achieved with RANKL inhibition may depend upon the model employed. RANKL is not detected within the primary tumor or inflammatory infiltrate in the spontaneous transgenic MMTV-neu mammary tumor model (38), which contrasts with the RANKL expression within tumor-infiltrating T-regs reported after orthotopic transplantation of primary MMTV-neu tumor cells (or a cell line derived from MMTV-neu tumors) (44). Thus, the clear reduction in pulmonary metastases by RANKL inhibition observed in the transgenic MMTV-neu model is not due to RANKL from
infiltrating T-lymphocytes and may instead be explained by the overall reduced tumor burden observed in this model (38) and/or other mechanism influencing survival (44) or colonization of metastatic cells. The potential for a direct contribution of RANKL to tumor progression and metastases by tumor-infiltrating T-lymphocytes observed in orthotopically-transplanted tumors (44) is intriguing given the earlier observations that RANKL is frequently observed (> 65%) in the infiltrating monocytic cells within the stroma of human primary breast tumors (38) and is upregulated in inflammatory, relative to non-inflammatory, human breast cancers (54).

Interestingly, while there is no evidence (either from genetics or pharmacologic inhibition) that RANKL inhibition is immunosuppressive in vivo (55), RANKL has been shown to promote the activity of T-regs (56) and macrophages (57), cell types which are capable of enhancing tumor progression and metastases. There are currently no studies directly addressing any function of RANKL on immune components of the tumor microenvironment; but this hypothesis should be considered as additional potential pro-tumorigenic and prometastatic mechanisms along with the direct effects on tumor cells and/or normal mammary epithelium outlined above.

**CLINICAL-TRANSLATIONAL ADVANCES**

Targeting RANKL in order to inhibit tumor-induced osteoclasts was shown in pre-clinical proof-of-concept experiments to be a rational approach for the prevention and treatment of skeletal complications of malignancy, including metastatic colonization of the bone. Pharmacodynamic bone resorption biomarkers, including the N-telopeptide of type I collagen (NTX), are useful in the translational evaluation of an osteoclast inhibitor. NTX is a product of bone degradation and a marker of elevated bone resorption, which is measured as the ratio of urinary NTX to creatinine (uNTX/Cr). Elevated levels of uNTX/Cr have been associated with increased risk for experiencing skeletal complications, disease progression, and death in patients with bone metastases (58).

Early versions of RANKL antagonists included recombinant forms of OPG (e.g. Fc-OPG or OPG-Fc [AMGN 0007]). The first demonstration of biological activity of RANKL inhibition in cancer patients was observed with multiple myeloma and breast carcinoma patients who had radiographic evidence of lytic or mixed bone disease. OPG-Fc produced rapid, dose-related
declines in bone resorption biomarkers (including uNTX/Cr levels) (59). However, clinical development of OPG forms by Amgen was terminated due to the relatively short half-life of OPG-Fc in cancer patients and questions regarding the potential safety risk of a neutralizing immune response against endogenous OPG. Another variant of OPG (CEP-37251; Cephalon) was also being developed; however the phase 1 clinical study in healthy post-menopausal women was apparently terminated (60). A RANKL antibody form derived from camelidae (i.e. llamas) termed ALX-0141, has been tested in a phase 1 study of healthy post-menopausal women (61). Amgen has developed a fully-human antibody against RANKL (AMG 162; denosumab) which has demonstrated greater selectivity to human RANKL, favorable pharmacokinetics, and ease of manufacturing as compared to OPG molecules. Denosumab is a fully human, IgG2 monoclonal antibody that binds human RANKL with high affinity ($K_D = 3\cdot10^{-12}$ M) and as a fully human protein, would not be predicted to engender an immune response in patients (62). It binds to soluble and membrane-bound human RANKL and non-human primate RANKL, but not mouse RANKL. It does not cross-react with other TNF ligand family members. Similar to OPG, denosumab functions as a reversible RANKL antagonist, preventing RANKL interaction with RANK and inhibiting osteoclast differentiation, activation, and survival. Denosumab is administered via a subcutaneous (SC) route.

A phase 1 evaluation in patients with multiple myeloma or breast cancer metastatic to bone demonstrated a pharmacodynamic effect of denosumab within 24 hours after a single SC dose (59). Compared with a single intravenous (IV) dose of the antiresorptive bisphosphonate, pamidronate 90 mg, the decrease in bone turnover markers (including uNTX/Cr levels) produced by denosumab was similar in magnitude, but more sustained. In these patients, denosumab was generally well tolerated with no serious adverse events or anti-denosumab antibodies detected.

Phase 2 studies evaluated the safety and efficacy of different dosing regimens of denosumab in cancer patients and informed dose and schedule selection for subsequent phase 3 trials. Also, phase 2 and phase 3 studies established the unique mode of action of denosumab relative to other bone-targeted drugs, specifically nitrogen-containing bisphosphonates (nBPs) such as pamidronate and ZA. Bisphosphonates bind to hydroxyapatite bone mineral surfaces where they inhibit mature osteoclasts locally, at sites of bone resorption (63). Intravenous nBPs have been
effective in prevention of skeletal complications in cancer, but safety and tolerability issues exist, including renal toxicity and acute-phase reactions. High levels of uNTX/Cr persist in a substantial proportion of patients with breast and prostate cancers and other solid tumors while on IV nBP treatment. As a result, these patients may experience skeletal-related events (SREs; including pathologic fracture, radiation or surgery to bone, or spinal cord compression) (58), indicating the need for improved therapies.

Denosumab reduced bone turnover markers in cancer patients in two phase 2 studies. One study in women with bone metastases from breast cancer concluded that more patients treated with denosumab had bone turnover suppression compared with IV nBP (64). In a second randomized phase 2 study, denosumab was tested in cancer patients with bone metastasis from prostate, breast, or other solid tumors, or multiple myeloma, who had persistent elevated levels of uNTx/Cr levels despite being treated with IV nBP (65). In these patients, 71% who received denosumab had reduced levels of uNTx/Cr as compared to 29% of patients who continued to receive IV nBP. Furthermore, suppression of serum TRAP5b, an osteoclast marker, was 2.5-fold greater in patients treated with denosumab compared with patients continuing treatment with IV nBP. In both studies, the safety profile of denosumab was consistent with a cancer population receiving systemic antineoplastic treatment.

Evaluation of the phase 2 results, using a population pharmacokinetic/pharmacodynamic model, suggested that a dose of 120 mg denosumab given every 4 weeks would suppress uNTX/Cr levels by more than 90% in most patients (64) and pharmacokinetic analyses of serum denosumab levels indicate a mean half-life of approximately 30 days (66). Altogether, these phase 2 studies clearly defined the efficacy of denosumab in cancer patients, independent of the tumor type, and established the ability of denosumab to control hyperactive bone resorption.

Skeletal-related events are serious, irreversible complications of tumors that metastasize to the bone. To evaluate the ability of denosumab to prevent SREs, three large phase 3 clinical studies were performed in patients who had bone metastases from breast cancer (67), castration-resistant prostate cancer (CRPC) (68), and any other advanced cancer (excluding breast and prostate) or multiple myeloma (69). Each trial was a randomized, double-blind, double-dummy, active
controlled comparison of SC denosumab 120 mg with IV ZA 4 mg (adjusted for creatinine clearance) every 4 weeks. The primary endpoint for each study was time to first on-study SRE. In the breast cancer trial (n = 2046), denosumab significantly delayed the time to first SRE by 18% versus ZA ($P = 0.01$, superiority) and reduced the risk of multiple SREs by 23% versus ZA ($P = 0.001$, superiority), demonstrating the durability of the positive effect on skeletal complications (67). In men with CRPC and bone metastases (n = 1904), denosumab significantly delayed the time to first on-study SRE by 18% as compared with ZA ($P = 0.008$, superiority) and the time to first-and-subsequent SRE ($P = 0.008$, superiority) (68). Finally, Henry et al. (69) reported that denosumab delayed time to first on-study SRE (HR 0.84; $P = 0.0007$, noninferiority) compared with ZA in patients with bone metastases from a variety of solid tumors (excluding prostate and breast cancer, but representing over 50 different tumor types) or multiple myeloma. For all three studies, time to disease progression and overall survival rates were similar between the denosumab- and ZA-treated cohorts. The overall rate of on-study adverse events was also similar between the two cohorts, including infrequent observations of osteonecrosis of the jaw typically associated with previously reported risk factors. Rates of adverse events potentially associated with renal toxicity and acute-phase reactions were elevated in patients treated with ZA, while a greater incidence of hypocalcemia was observed in the denosumab cohorts. Hypocalcemia was manageable, not associated with significant clinical sequelae, and was consistent with denosumab’s mechanism of action. These 3 identically-designed pivotal clinical studies were the basis upon which denosumab achieved approval by the FDA in November 2010 for prevention of SREs in patients with bone metastases from solid tumors.

CONCLUSIONS AND FUTURE DIRECTIONS

The early findings of an essential role of RANKL in physiological osteoclastogenesis was the fundamental rationale for targeting this pathway as a treatment for skeletal complications in cancer, and a therapeutic antibody, denosumab, has been successfully developed in the clinic for this application in patients with solid tumor bone metastases. As preclinical experiments have demonstrated, the interference with the bone microenvironment and potent reduction in bone resorption with a RANKL inhibitor also has potential utility in the inhibition of early metastatic
colonization. Consistent with this hypothesis, denosumab was recently shown to be effective in delaying development of bone metastasis in men with CRPC (70) and this study represents the first large randomized study to show that targeting the bone microenvironment has this effect in cancer patients. This hypothesis is also currently being addressed in women with early-stage breast cancer at high risk of disease recurrence to determine if denosumab in combination with standard of care adjuvant/neoadjuvant cancer treatment will improve bone metastasis-free and disease-free survival compared with standard of care treatment alone (71).

While more recent preclinical basic research in rodents has indicated that RANKL can promote a spectrum of effects relevant to cancer initiation, progression, and metastasis via direct effects on either tumor cells or the mammary epithelium, significant gaps remain in the translation of these more recent findings to the clinic. The prominent role of RANKL as a paracrine effector of progesterone action in the mouse mammary epithelium clearly has implications in humans, given the potential role for progesterone specifically as a risk factor in human breast cancer as supported by its well established mitogenic effects in the breast (72) and by large epidemiologic studies (73). However, there are fundamental differences in the anatomy and hormone responsiveness of the rodent mammary gland as compared with the primate which may limit the utility of the preclinical findings. In order to show relevance of the progesterone and RANKL pathway association, data linking the RANKL pathway (RANKL, RANK, and OPG) expression with hormone exposure and breast density and proliferation in humans, or a suitable non-human primate model, is needed. To address any association of the RANK/RANKL pathway with cancer risk or progression, genome-wide association studies or analysis of mRNA or protein expression may be helpful, but each approach has its limitations. Using a candidate gene approach, a recent genetic association study has associated a single nucleotide polymorphism (SNP) in the RANK gene with breast cancer risk (74). In addition to progesterone, multiple other potential stimuli of RANKL in cancer may exist (eg PTHrP, prolactin, tumor stromal cells, infiltrating T-lymphocytes) and further studies are necessary not only to verify the many diverse sources of RANKL with well-validated methodologies but also to determine if any of these RANKL sources are associated with disease outcome. Likewise, it will be crucial to define any tumor subpopulations that express RANK, again using well-validated and specific approaches, and to relate RANK expression with additional disease outcomes. Recent analysis of RANK
mRNA expression in human breast cancer biopsies indicates relatively increased expression in the basal tumor subtype and an association of higher RANK mRNA expression with poor survival (53). Finally, the identification of tumor-specific (or mammary epithelial-specific) biomarkers of a RANKL response would aid in the translational evaluation of this drug in extraskeletal tissues and, ultimately, the testing of the current preclinical hypotheses in patients.

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FIGURE LEGENDS

Figure 1. Bone-intrinsic functionality of the RANKL pathway in normal and pathological osteoclastogenesis.

Left panel: Normal bone turnover/remodeling observed in healthy physiological systems. Bone stromal cells, including cells of the osteoblast lineage, provide a limited amount of RANKL, which leads to osteoclast differentiation, survival, and activation and subsequent bone resorption. Resorption is balanced by osteoblast-dependent new bone formation. If occult micrometastases were to exist, they would remain dormant due to the low level of bone turnover (37).

Right panel: Tumor-induced high bone turnover/remodeling. 1) A variety of tumor-derived factors will cause increases in RANKL and/or decreases in OPG within the bone stroma. 2) Some tumor cells can also directly produce RANKL. 3) The increased RANKL: OPG ratio leads to increased osteoclast formation, survival, and activity which increases the rate of bone remodeling/turnover. 4) Pathological bone remodeling, characterized by increased osteoclast and osteoblast activities, causes a spectrum of bone lesions in bone metastasis patients, ranging from predominately osteoblastic to predominately osteolytic. Multiple myeloma gives rise to purely lytic bone lesions. 5) As a result of the increased osteoclast function, many changes occur within the reactive bone microenvironment. These changes include increases in local levels of calcium, release of activated growth factors from the bone matrix, and increased production of growth and/or angiogenic factors by the osteoclast. 6) Increased tumor cell growth and survival, including the outgrowth of dormant micrometastases, occurs as a result of these bone matrix- and osteoclast-derived factors. 7) Skeletal tumor cells respond to these bone signals with further production of additional pro-resorptive factors, generating a feed-forward loop known as the ‘vicious cycle’.
Figure 2. Direct pro-tumorigenic and pro-metastatic activities of RANKL.

Using mammary transformation as a model, this figure indicates several different sources of RANKL which may impact multiple steps in tumor progression. For instance, hormonal influences (eg, progesterone, PTHrP, prolactin) may increase RANKL not only in the normal mammary epithelium, but also in preneoplastic, tumor, and metastatic lesions. RANKL has also been observed within preneoplastic and neoplastic cells independent of hormone influences. RANKL expressed on T-lymphocytes may potentially be important at multiple stages in cancer, from preneoplasias through metastases. Certain metastatic sites, such as the lymph node and bone are rich sources of RANKL. Different RANKL-dependent activities relevant to cancer formation and progression are summarized above. RANKL may function at multiple steps in tumor initiation, progression and recurrence, including to the increased proliferation and survival of normal mammary epithelium and tumor cells, as well as the enhanced numbers and regenerative potential of mammary stem cells (MaSC) or the putative stem cell component of tumors. Other RANKL-dependent activities on tumor cells potentially relevant to distant metastatic spread are reflected by the in vitro observations of enhanced migration and invasion or increased growth in a semi-solid medium.
Physiological bone turnover/remodeling (normal)

- Tumor, dormant micro-metastasis

Tumor cell expressing RANKL

Bone destruction (spectrum of lesions)

Mixed osteolytic/osteoblastic

Osteoblastic → Osteolytic

Homeostatic maintenance of bone mass

Bone resorption → Bone formation

Tumor-induced pathologic bone turnover/remodeling (high)

- Variety of tumor-derived factors

- Tumor cell expressing RANKL

- Expanded tumor mass

- Tumor

- Variety of tumor-derived factors

- Osteoblasts

- Osteoclasts

- Bone resorption

- Osteoblasts

- Bone formation
RANKL by hormones (Progesterone, PTHrP, Prolactin)

Potential sources of RANKL

RANKL in preneoplastic or neoplastic cells
RANKL in T-cells
RANKL at metastatic sites (lymph node, bone)

RANKL-induced effects in tumorigenesis and metastasis

Mechanism of RANKL-dependent effects (in vivo and in vitro)

↑ Proliferation
↑ Survival
Loss of apical/basal polarity

Expansion and regenerative capacity of stem cell component

↑ Migration/invasion
↑ Anchorage-independent growth

Normal mammary epithelium
Preneoplasia
Adenocarcinoma

Mammary stem cell (MaSC)

Bone
Lung

Metastasis

Survival

Loss of apical/basal polarity

Adenocarcinoma

Mechanism of RANKL-dependent effects (in vivo and in vitro)

Potential sources of RANKL

RANKL by hormones (Progesterone, PTHrP, Prolactin)

Mammary stem cell (MaSC)

↑ Proliferation
↑ Survival
Loss of apical/basal polarity

Expansion and regenerative capacity of stem cell component

↑ Migration/invasion
↑ Anchorage-independent growth
Osteoclast-dependent and-independent roles of the RANKL/RANK/OPG pathway in tumorigenesis and metastasis

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