Introduction

Tumor-induced bone disease is a major cause of morbidity and mortality. Many cancers either grow predominantly in bone, such as multiple myeloma, or metastasize to bone, such as breast and prostate cancer. Irrespective of the organ of origin tumor cells induce significant changes in bone compromising skeletal integrity and resulting in bone pain, hypercalcemia and an increased likelihood of fracture. The cellular and molecular mechanisms responsible for the development of tumor-induced bone diseases are poorly understood, although increased osteoclast formation and/or activity appears to play a critical role, whether tumors are predominantly osteolytic or osteosclerotic. The identification of new signalling pathways that regulate osteoclast formation in cancers that grow in bone is a major research goal. The successful identification of such systems would provide new approaches to treating this aspect of cancer. The discovery of the ligand for receptor activator of NFkB (RANKL) system represents one such opportunity.

Mechanisms of tumor-induced bone disease

The RANKL system plays a critical role in normal osteoclast formation and activity and it is likely that abnormal regulation of this system may contribute to osteoclast formation in tumor-induced bone disease. The presence of tumour cells in the local bone marrow microenvironment make them ideally placed to alter the balance between RANKL, RANK and OPG, in favour of RANKL, to promote osteoclast formation and bone resorption (Figure 1). This could occur in a number of ways. First, the tumor cells may up-regulate expression of RANKL in cells of the local bone marrow microenvironment to promote osteoclast formation indirectly. Secondly, tumor cells may express RANKL themselves and promote osteoclast formation directly, in a bone marrow microenvironment independent manner. Alternatively, tumor cells may inhibit the production of OPG, the soluble decoy receptor, within the bone marrow microenvironment to allow bone resorption to go unchecked. In tumors that are characterized by increased osteoclastic bone resorption and the development of osteolytic bone disease such as multiple myeloma or breast cancer bone disease there is now evidence to support a role for each of these mechanisms. However, in tumors that give rise to predominantly osteosclerotic disease, such as prostate cancer metastasis to bone, our understanding is less clear. Increased osteoclast formation and bone resorption can occur when these tumors grow in bone and the RANKL system may contribute to the increase in osteoclast formation, using the mechanisms outlined above. Alternatively, tumor cells may inhibit bone resorption by inhibiting RANKL expression or promoting OPG production to allow bone formation to exceed bone resorption (Figure 1). It is possible that both mechanisms operate at different stages of disease development.

The RANKL system in the development of multiple myeloma bone disease

The bone disease in multiple myeloma is characterized by the presence of lytic bone lesions, an increased propensity to fracture, hypercalcemia and bone pain, making this a source of considerable morbidity. Histomorphometric studies have shown this to be mediated by an increase in osteoclastic bone resorption1-3, although it is not clear whether this is due to
increased osteoclast recruitment, increased resorative activity, or both. Myeloma cells are found closely associated with sites of active bone resorption, and bone resorption is correlated with tumor burden, suggesting that myeloma cells may promote osteoclast formation and bone resorption directly. Although early studies reported the production of an osteoclast activating activity by human myeloma cells the specific identity of the factor(s) responsible remains unclear. A number of cytokines, including interleukin-1β, lymphotoxins, tumor necrosis factor, beta and more recently macrophage inflammatory protein-1 alpha (MIP1α) have been implicated. However, with the exception of MIP1α there are little data to suggest that inhibiting these factors can prevent the development of myeloma bone disease in either animal models or the clinical setting. RANKL has also been studied and implicated in the direct induction of osteoclastic bone resorption in myeloma.

Studies have shown that myeloma cells themselves may express RANKL and drive bone resorption, directly. The murine myeloma cell line, 5T2MM, expresses RANKL, and induces osteoclast formation and the development of osteolytic bone lesions in C57BL/KaLwRij mice. Murine ST2 stromal cells also up-regulate expression of RANKL in 5TGM1 murine myeloma cells via an interaction between the αβ integrin and vascular cell adhesion molecule. Flow cytometric and immunohistochemical studies have also demonstrated expression of RANKL on CD138+ myeloma cells isolated from the bone marrow of patients with multiple myeloma. Furthermore, Farrugia et al. have shown that CD38++ CD45+ myeloma cells express RANKL and promote osteoclast formation in vitro. CD138+ myeloma cells have also been shown to promote osteoclast formation in vitro. These data suggest that myeloma cells may promote bone resorption directly in a RANKL dependent, microenvironment independent manner. However, not all studies have been able to detect expression of RANKL in myeloma cells. Immunohistochemical and in situ hybridization studies have shown increased expression of RANKL in biopsies from patients with multiple myeloma. In these studies expression appeared to be localized to stromal cells. Expression was not detected in the myeloma cells raising the possibility that cells in the bone microenvironment are an important source of RANKL. In support of this 5TGM1...
murine myeloma cells have been shown to up-regulate expression of RANKL in ST2 stromal cells via an interaction between the αb1 integrin on 5TGM1 cells and vascular cell adhesion molecule on ST2 cells. Human myeloma cells have also been reported to increase expression of RANKL in bone marrow stromal cells and osteoblasts, in a contact-dependent manner. Furthermore, the ARP-1 myeloma cell line also increases RANKL expression in stromal cells and these stromal cells have a greater ability to support the formation of osteoclasts in vitro. In addition to bone marrow stromal cells and osteoblasts, other cells in the bone marrow may also express RANKL. In support of this, myeloma cells have been shown to promote T cell expression of RANKL via interleukin-7 production by the myeloma cells. Endothelial cells also express RANKL and may support osteoclast formation in vitro. Furthermore, B9/BM1 myeloma cells promote RANKL expression in endothelial cells and increase the osteoclast-inducing activity of these cells.

Myeloma cells not only express RANKL or up-regulate expression in the bone marrow but also down-regulate expression of OPG. OPG expression has been shown to be decreased in biopsies taken from patients with myeloma when compared to control and myeloma cells inhibit OPG production in stromal cells and osteoblasts. Furthermore, endothelial cells produce OPG (22) and ST33MM murine myeloma cells down-regulate OPG production in these cells. Serum concentrations of OPG are decreased in patients with myeloma and inversely correlated with the number of lytic bone lesions. In contrast, serum soluble RANKL concentrations are significantly elevated in patients with myeloma and the ratio between RANKL and OPG is also increased, associated with extent of bone disease and predicts survival.

The ability of antagonists of RANKL to prevent the development of myeloma bone disease has been examined in a number of murine models. These antagonists include Fc.OPG, which is the ligand binding domain of OPG fused to the Fc domain of human IgG, and RANK.Fc, a soluble form of the receptor RANK also fused to the Fc domain of the human IgG. Both of these constructs will bind RANKL and prevent association with RANK, and inhibit osteoclast formation. Injection of ST2MM murine myeloma cells, via the tail vein, into C57BL/Ka/J mice leads to the development of a myeloma bone disease characterized by the presence of lytic bone lesions and cancellous bone loss. This bone loss is mediated by increased osteoclast formation. ST2MM cells are restricted to the bone marrow and, occasionally, the spleen. Treatment of myeloma-bearing mice, from the time of paraprotein development, with recombinant Fc.OPG, at 30mg/kg i.v., 3 times per week, prevents the ST2MM-induced bone loss and the development of lytic bone lesions. Fc.OPG treatment completely prevented osteoclast formation. The ability of OPG to prevent tumor-induced bone loss has also been examined following lentiviral delivery of OPG to ARH-77 cells. Although these cells do not have the same immunophenotype as myeloma cells, they cause an osteolytic bone disease, similar to myeloma bone disease, when injected into SCID mice. Parental ARH-77 cells induce the development of lytic bone lesions in SCID mice, whereas those cells engineered to over-express OPG did not induce bone loss.

In addition to Fc.OPG, the ability of a soluble RANK construct (RANK.Fc) to inhibit the development of myeloma bone disease has also been examined. RANK.Fc treatment of mice bearing 5TGM1 murine myeloma cells, in a syngeneic system similar to the ST2MM model, has been shown to prevent the development of myeloma bone disease. Treatment of SCID mice bearing ARH-77 cells with RANK.Fc also prevents the development of ARH-77-induced bone disease. Furthermore, RANK.Fc treatment of SCID mice bearing human fetal bone explants, implanted in the flanks and injected with primary myeloma cells, prevented the myeloma cell-induced bone loss. Thus, targeting RANKL with Fc.OPG or lentiviral delivery of OPG, or with RANK.Fc is effective in preventing the development of myeloma bone disease in a number of experimental models.

The RANKL system and breast cancer-induced bone disease

Breast cancer cells also promote the development of osteolytic bone disease. Parathyroid hormone-related protein (PTHrP) has been reported to be one of the principal mediators of this bone loss, however, the precise molecular mechanisms responsible remain unclear, raising the possibility that other signalling systems may also be involved.

Studies have reported that breast cancer cell lines, including MDA-MB-231, MCF-7 and T47D do not express RANKL and cannot support osteoclast formation in vitro. Primary breast tumor samples have also been reported not to express RANKL. However, breast cancer cells expressing PTHrP have been shown to increase RANKL expression in osteoblasts and increase their osteoclast forming potential in vitro. Intra-cardiac injection of MCF-7 cells, over-expressing PTHrP, stimulate an increase in osteoclast formation and increase the rate of development of lytic bone disease. Injection of MDA-MB-231 breast cancer cells also promotes increased osteoclast formation and the development of osteolytic bone lesions in vivo. Analysis of RANKL mRNA expression in the tibia and femora of mice with MDA-MB-231 induced lytic lesions demonstrated little RANKL in bones without metastases but strong RANKL expression in bones with evidence of lytic bone lesions. RANKL was not detected in the MDA-MB-231 cells suggesting that expression, in vivo, was not from the tumor cells. MDA-MB-231 cells were also reported to be able to induce osteoclast formation in cultures of murine bone marrow cells and osteoblasts, an effect that was inhibited by OPG. These data suggest that breast cancer cells may promote RANKL expression in osteoblasts and/or stromal cells, which stimulates osteoclast formation. PTHrP may be a key mediator of the induction of RANKL.

In contrast to myeloma, few studies have examined the effect of inhibiting RANKL on the development of breast
cancer bone disease in vivo. However, the ability of a Fc.OPG construct has been examined in the MDA-MB-231 model. Treatment of mice bearing MDA-MB-231 cells with Fc.OPG, 25mg/kg, administered three times per week for four weeks was associated with the prevention of radiographically evident osteolytic bone lesions at 4 weeks. Fc.OPG treatment was also associated with a significant reduction in osteoclast numbers. This would be consistent with RANKL playing a role in the development of breast cancer-induced bone lesions.

The RANKL system and prostate cancer bone disease

Prostate cancer cells commonly metastasize to bone but unlike myeloma and breast cancer typically give rise to osteosclerotic bone disease. Although the cellular and molecular mechanisms responsible are unclear, there is increasing evidence to suggest that osteoclastic bone resorption may accompany the development of the osteosclerotic disease. This is based upon histological studies that demonstrated that bone surfaces opposed to prostate cancer cells in bone may have increased numbers of osteoclasts. Furthermore, bisphosphonates have been shown to be effective in preventing prostate cancer bone disease in experimental models of osteolytic and osteosclerotic bone disease induced by prostate cancer cells. Since osteoclastic bone resorption may contribute to the development of prostate cancer bone disease, a number of studies have examined the role of RANKL in promoting osteoclast formation in this disease.

Analysis of biopsy samples from patients with prostate cancer has demonstrated expression of RANKL in primary prostate cancer cells, with expression being increased in prostate cancer cells that metastasize to bone. A number of prostate cancer cell lines, including PC-3, C4-2B and LuCaP 35, are able to produce a soluble form of RANKL. Media conditioned by C4-2B cells, have been shown to promote osteoclast formation in cultures of murine bone marrow cells and LuCaP 35 cells have been shown to promote the formation of TRAP+ve, multi-nucleated, RAW 264.7 cells. In vivo, injection of C4-2B or PC-3 cells into the tibia of SCID mice promotes osteoclast formation and the development of osteolytic bone lesions. In contrast, injection of LuCaP 35, which also produces sRANKL, into human fetal bone explants, implanted into the flanks of SCID mice, results in evidence of palpable tumors and a detectable serum PSA in 70% of mice. In those mice with evidence of tumor growth there was radiographic evidence of osteosclerosis in the implanted bone with an increase in bone mineral density and cancellous bone area. Both osteoclast perimeter and osteoblast perimeter were increased in tumor-bearing bone when compared to control. Treatment of mice with RANK.Fc reduced osteoclast numbers to the control, non-tumor-bearing level and prevented the tumor-induced increase in bone mineral density. However, RANK.Fc had little effect on osteoblast perimeter. RANK.Fc also reduced biochemical markers of both bone resorption (urinary NTX) and bone formation (serum bone specific alkaline phosphatase and osteocalcin).

RANKL and other experimental models of tumor-induced bone disease

Tumors other than myeloma, breast and prostate cancer cells can also stimulate the development of osteolytic bone disease. For example intra-cardiac injection of the murine colon adenocarcinoma cell line, Colon-26, results in the development of lytic bone disease and this is mediated by increased osteoclast formation. Treatment of mice bearing Colon-26 cells with Fc.OPG inhibited osteoclast formation.
Neuroblastomas can also metastasize to bone and this is associated with poor prognosis. The neuroblastoma cell line NB-19 has been reported to promote the formation of TRAP+ve cells in vitro. Osteoclast-like cell formation was associated with increased RANKL expression and could be blocked with OPG. Subcutaneous injection of these cells into nude mice resulted in the development of osteolytic bone lesions; however, it is not established whether these lesions could be prevented with OPG.

In addition to tumors that grow in bone or metastasize to bone, some tumors can affect bone from distant sites. Tumors such as squamous cell carcinoma of the lung or renal carcinomas are often associated with the development of humoral hypercalcemia of malignancy (HHM). This is caused by an increase in osteoclast formation and resorptive activity, and is mediated by the release of osteoclast stimulatory factors by the primary tumor. These mediators also promote increased renal tubular re-absorption of calcium, which contributes significantly to the development of HHM. One of the most common mediators of hypercalcemia of malignancy is PTHrP. Given the evidence that PTHrP can regulate RANKL expression locally, studies have investigated whether targeting the RANKL system in experimental models of HHM can prevent the development of hypercalcemia. In early in vivo studies recombinant OPG was shown to prevent the hypercalcemia and bone resorption induced by a number of stimulators of bone resorption including PTHrp. Studies have also shown that inoculation of RWGT2 cells, which are derived from a patient with squamous cell carcinoma of the lung, into the subcutaneous tissue of athymic mice results in the development of hypercalcemia. This is associated with a marked induction in osteoclast formation and a decrease in cancellous bone volume. Treatment of mice with established hypercalcemia with murine RANK.Fc reduced blood ionised calcium concentrations, prevented the tumor-induced decrease in bone volume and completely inhibited osteoclast formation.

The bone marrow microenvironment plays a key role in regulating the growth and survival of tumor cells. Factors

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*The 5T33MM variant used in this study does not develop myeloma bone disease

**Table 1.** Summary of the effect of targeting the RANKL system on the development of tumor-induced bone disease and tumor development in pre-clinical models.
produced by osteoclasts or released from bone matrix during the process of bone resorption may provide critical growth and survival signals for tumor cells. Inhibiting the independence between tumor cells and osteoclasts by preventing osteoclastic bone resorption would be predicted to slow or prevent tumor growth in bone. Increasingly, studies have begun to examine this hypothesis (Table 1).

In the 5TMM murine model of myeloma Fc.OPG treatment was associated with a 25% decrease in serum paraprotein concentration. Furthermore, in the more aggressive 5T33MM model, Fc.OPG, administered from the time of tumor cell injection, also caused a significant decrease in serum paraprotein and this was associated with a decrease in tumour burden and an increase in survival. RANK.Fc treatment has also been reported to decrease tumor burden in the 5TGM1 model and the SCID/Hu models. However, lentiviral delivery of OPG to ARH-77 cells, or RANK.Fc treatment of ARH-77 bearing mice was not associated with an effect on serum paraprotein concentrations. The difference between these studies is likely to reflect the model used. In the 5T series of models, and the SCID/Hu model, the tumor cells are confined to the bone marrow microenvironment and dependent upon this environment for their growth and survival. These cells do not grow in vitro in the absence of appropriate stromal cell feeder layers. In contrast, ARH-77 cells grow in vitro, and in vivo are not bone marrow microenvironment dependent and can be found in other organs. Thus, measurement of serum paraprotein concentrations as a marker of tumor burden in this model may be masked by production at sites other than bone. Since Fc.OPG has no direct anti-myeloma effect in vitro, these studies point towards the effect of antagonising the RANKL system on tumor burden being an indirect anti-tumor effect, mediated by the inhibition of osteoclastic resorption.

In the MDA-MB-231 breast cancer model, Fc.OPG treatment was associated with a reduction in both the number and size of tumor foci in the bone marrow. This translated into an 80% decrease in total skeletal tumor burden. In the C4-2B prostate cancer model, treatment of tumor-bearing mice with murine OPG.Fc for 4 weeks following tumor cell injection prevented the growth of PSA-positive tumor cells in the tibia at 4 and 16 weeks. However, the volume of subcutaneous tumor implants, measured every 4 weeks for 16 weeks, was not affected by OPG.Fc treatment. OPG also had no direct effect on tumor cell proliferation or rates of apoptosis in vitro in this study. Treatment of SCID/Hu mice bearing LuCaP 35 prostate cancer cells with RANK.Fc also resulted in a significant reduction in the proportion of bone marrow occupied by tumor cells and a decrease in serum PSA concentration. As in the C4-2B model, RANK.Fc treatment had no effect on the growth of LuCaP 35 cells implanted subcutaneously. In the Colon-26 model Fc.OPG was also associated with an inhibition of tumor growth in bone.

Conclusions

The importance of osteoclastic bone resorption in tumors that induce lytic bone lesions is well recognised. However, more recent studies have shown that tumors associated with osteosclerotic bone disease also have an element of osteoclastic bone resorption. These observations have prompted extensive investigations into the role of the RANKL system in the development of tumor-induced bone disease, particularly in multiple myeloma and in breast and prostate cancer bone metastases. These studies have provided strong evidence to suggest that irrespective of the tumor type, tumor cells have the ability to influence expression of the RANKL system in the bone marrow microenvironment. This may be by expressing components of this system themselves or by influencing expression in cells of the bone marrow microenvironment. However, the critical nature of the RANKL system in the development of tumor-induced bone disease has been best demonstrated in murine models. Targeting RANKL in tumor-bearing animals with either Fc.OPG or RANK.Fc is able to prevent the development of tumor-induced bone disease. This provides compelling evidence that RANKL is likely to play a causal role in the development of tumor bone disease. Furthermore, by inhibiting osteoclastic bone resorption and changing the local microenvironment, inhibitors of the RANKL system are also able to slow tumor growth in bone. These studies provide strong support for the idea of interdependence between tumor cells and bone, with tumor cells promoting bone resorption and in return the resorbing bone microenvironment supporting the growth and survival of tumor cells. This provides us with new opportunities for treating not only the bone disease but indirectly affecting the growth of tumor cells in bone. Targeting the RANKL system with molecules such as Fc.OPG or RANK.Fc represents one such approach.

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