PERSPECTIVES

Recent Insights into Myeloma Bone Disease

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Multiple myeloma, a clonal neoplasm of plasma cells, is the second most common adult hematological malignancy and the most common cancer with the skeleton as its primary site. It has a worldwide prevalence of about 145,000 cases, affects 70,000 people in the United States, accrues 15,000 new cases yearly, and accounts for 1% to 2% of cancer-related deaths (1). The average life span after diagnosis is less than three years, and this has not changed significantly over the past three decades. The beneficial effects of conventional therapeutic regimens on myeloma tumor burden are modest, and relapse is invariable. Although newer treatment modalities, like stem cell transplantation, have the potential to increase life span modestly, they do not in general avert relapse or cure the disease. Myeloma thus remains a uniformly fatal cancer, and novel and effective therapies are badly needed.

Myeloma is unique in its propensity to cause osteolysis, with 80% of patients suffering from devastating and progressive bone destruction resulting in severe and unremitting bone pain, pathological fracture, hypercalcemia, and spinal cord compression. The development of myeloma bone disease is characterized by increased bone resorption and markedly impaired bone formation. Myeloma bone disease is a significant clinical problem for which there is, as yet, no effective cure.

Myeloma Bone Disease

Bone resorption in myeloma is dependent on osteoclast stimulation. Although it has been known for more than 30 years that osteoclasts are hyperstimulated by cytokines in myeloma, identification of the cytokines responsible has proven elusive. The problem has been that the usual approach of identifying such factors by the use of cultured tumor cell lines, followed by protein purification, has not been successful, as it is now apparent that cell-cell interactions are required for the production of cytokines. Cell-cell interactions involve myeloma cells and other cells in the marrow microenvironment, including stromal cells, cells in the osteoblast lineage, and possibly osteoclasts themselves. Currently, it seems that the cytokines most likely responsible for bone resorption in myeloma are receptor activator of NF-κB ligand (RANKL) (2-6) and macrophage inflammatory protein (MIP)-1α (7-9). However, a number of other cytokines, including lymphotoxin and parathyroid hormone-related protein (PTHrP), have been implicated in the disease (10;11). There is considerable debate as to whether these bone-resorbing cytokines arise from myeloma cells, accessory cells involved in cell-cell interactions, or both. There is evidence for both notions. RANKL is a crucial mediator of osteoclast formation and activity, which are increased in multiple myeloma. RANKL is expressed by bone marrow stromal cells and osteoblasts, and expression is increased when these cells are cultured in the presence of myeloma cells, demonstrating the importance of myeloma cell-stromal cell interactions (4-6). In addition, increasing evidence suggests that myeloma cells may also express RANKL, thus providing a mechanism for

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direct stimulation of osteoclast formation by myeloma cells (5;6).

Normally, bone formation occurs at sites of prior bone resorption in the skeleton. However, in patients with myeloma, osteoclastic bone resorption becomes uncoupled from bone formation, which results in a reduced number of osteoblasts, reduced cancellous bone volume, and impaired activity of the remaining osteoblasts (12;13). Data from recent studies measuring bone formation markers in patients with myeloma provide support for early observations (14;15). The impaired bone formation response further compounds the bone deficit and accentuates complications, such as pathological fractures. Over the last several years, our understanding of the pathogenesis of myeloma-induced osteolysis and the osteoclastogenic and osteoblastic factors involved has greatly increased. Elucidation of the underlying mechanisms is imperative to develop new and effective therapies that both target the tumor and enhance bone formation to correct the bone deficit.

The 5T Radl Murine Model of Myeloma

A major problem with the identification of factors and mechanisms for impaired bone formation has been the lack of a confirmatory *in vivo* model to validate the results. The most commonly used models are: the SCID-ARH77 model, in which ARH-77 human B-lymphoblastoid cells derived from a patient with myeloma are injected into irradiated SCID mice; the SCID-hu model, in which freshly isolated human myeloma cells are implanted into human fetal bone xenografts in SCID mice; and the 5T Radl murine model of myeloma (16-18). An important distinction is that the 5T Radl model enables the study of myeloma cell growth, development of myeloma bone disease, and interactions between tumor cells and cells of the bone marrow microenvironment in animals that have not been immuno-compromised or irradiated. In the 5T Radl model, myeloma occurs spontaneously in elderly in-bred C57BL/KaLwRijHsd mice and is propagated by intravenous, subcutaneous, and peritoneal transplantation into young

recipients of the same substrain. The 5T Radl model shares several clinical, histological, immunological, and cytogenetic features with human multiple myeloma (17-20). When inoculated intravenously into naïve C57BL/KaLwRijHsd mice, 5T myeloma cells home specifically to the skeleton and spleen, where they localize and proliferate. Titers of the monoclonal paraprotein can be assayed in serum to monitor increasing tumor burden. Foci of osteolytic bone destruction are evident around proliferating tumor cells, and these bone lesions can be followed and quantified radiographically. Increased osteoclastic bone resorption in these lesions can be further documented by bone histomorphometry (18;19;21;22). A stroma-independent variant (5TGM1) that gives identical results when inoculated into syngeneic naïve C57BL/KaLwRijHsd or bg-nu-xid mice has also been established (21). The 5TGM1 model has proved to be a convenient *in vivo* model in which to study the biology of myeloma bone disease to establish the significance of putative myelomagenic and osteoclastogenic factors, as has been recently shown with MIP-1α (22), and to determine pre-clinical efficacy of novel therapies for myeloma (21). Although tumor progression and tumor-induced osteolysis have been extensively characterized in the 5TGM1 model, no *in vivo* model of myeloma bone disease is available in which dysregulation of osteoblast function has been characterized. Myeloma is associated with a systemic bone disease with progressive generalized cancellous bone loss, consistent with what has been observed on [^{99m}Tc]-MDP skeletal scans in 5TGM1 tumor-bearing mice (23). In addition, recent dynamic histomorphometric studies of bones from 5TGM1 tumor-bearing mice showed that cancellous bone volume and bone formation rates were reduced, similar to the findings in patients with myeloma (24).

Proteasome Inhibitors in Myeloma Bone Disease

An entirely new approach has recently been suggested for the treatment of multiple myeloma based on the exquisite sensitivity of myeloma cells to the activity of the

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proteasome, the main non-lysosomal intracellular machinery in eukaryotic cells for proteolytic degradation of the cytoplasmic and nuclear proteins involved in key biological processes, including cell cycle progression, transcriptional activation, signal transduction, and apoptosis (25). This is exemplified by the discovery and development of the anticancer agent bortezomib (Velcade; PS-341), a novel boronated dipeptide that works by reversibly inhibiting the effects of the multimeric, multi-catalytic proteasome. Bortezomib, originally shown to be cytotoxic against a panel of tumor cells, is a potent inhibitor of myeloma cell growth and survival *in vitro* (26). In fact, proteasome inhibitors are much more effective in myeloma than in other tumors, indicating that myeloma cells are especially dependent on proteasome function, possibly because of proteasomal inhibition of the ubiquitous transcription factor NF-κB, whose transcriptional activity is regulated via proteasomal degradation (27). NF-κB has been implicated in the growth and survival of myeloma cells *in vitro* (28), but its role in myeloma tumor progression *in vivo* is unknown. Importantly, bortezomib inhibits NF-κB activity *in vitro* and *in vivo* (29).

Bortezomib is the first, and to date only, proteasome inhibitor to be used in patients (30). Data accrued from phase I, multi-center phase II, and ongoing phase III clinical trials indicate that, when administered intravenously, bortezomib has remarkable therapeutic efficacy and an acceptable toxicity profile in patients with advanced or refractory myeloma (30). This notable advance has led to its fast-tracked approval by the United States Food and Drug Administration for use in the treatment of patients with myeloma (31).

The tremendous impact of proteasome inhibition as a therapeutic strategy in the clinical management of myeloma is all the more relevant because of the recent demonstration that several structurally-unrelated inhibitors of the ubiquitin-proteasome pathway (including bortezomib) have beneficial anabolic effects on the skeleton *in vivo* (32). Retrospective analysis of a cohort of patients with myeloma treated with bortezomib also revealed increases in serum bone-specific alkaline phosphatase activity, a marker of bone formation (33). Although an anti-tumor effect of bortezomib has been demonstrated in a human xenograft model in SCID mice (34), myeloma is primarily a skeletal disease, and there is increasing recognition of the role of the bone marrow microenvironment, and specifically of cell-cell interactions between tumor cells and osteoblast/bone marrow stromal cells, in myelomagenesis and disease progression.

The mechanisms by which proteasome inhibitors mediate these important effects on the "seed" (*i.e.*, tumor cells) and "soil" (*i.e.*, the bone microenvironment) in myeloma are only just beginning to be unraveled. Ubiquitination of several cytoplasmic and nuclear regulatory proteins, governed by specific enzymes (E1, E2, and E3) in a conjugation cascade, targets them for degradation by the proteasome and thus has profound effects on their functions. Several classes of E3 ubiquitin ligases, including multi-complex SCF (Skp1, Cullin Cul 1) ligases have been described that catalyze the attachment of ubiquitin to proteins. These SCF complexes, in turn bind a variable F-box protein that recruit specific phosphorylated substrates for ubiquitination through specific protein–protein interaction domains. E3 ubiquitin ligases thus serve as the substrate-targeting and rate-limiting component of the ubiquitin-proteasome system. Recent studies have shown that the profound anti-myeloma effect of bortezomib *in vivo* is mimicked by expression in myeloma cells of a dominant-negative (ΔF) mutant of SCF$^{\beta\text{-TrCP1/FWD1}}$ (35), the IκB E3 ligase that targets molecular components of the NF-κB signaling cascade for proteasomal processing and/or degradation (27).

Dickkopf1, Bone Formation, and Myeloma Bone Disease

The Wnt family of secreted glycoproteins plays essential roles during cell growth and differentiation in both embryos and adults. There are two Wnt signaling pathways, canonical (β-catenin-dependent) and non-canonical (β-catenin-independent). Canonical Wnt signaling is transduced by

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two transmembrane receptor families, frizzled proteins and lipoprotein receptor-related proteins 5 and 6 (LRP5/6), resulting in stabilization of cytoplasmic β-catenin levels. A novel and unexpected role for the canonical Wnt signaling pathway (and specifically for the LRP5 receptor) in regulating bone mass in humans and rodents was recently uncovered, with the association of a "loss of function" mutation in the *LRP5* gene with profound osteopenia in the osteoporosis-pseudoglioma syndrome (36) and generation of *Lrp5* null mutant mice, which develop osteopenia and multiple fractures (37). A unique *G*171*V* "gain of function" point mutation in *LRP5* has also been associated with a dramatic "high bone mass" syndrome with evidence of resistance to fractures (38). Polymorphisms have also been identified in the *LRP5* gene locus that are associated with BMD (39;40). Lastly, ectopic expression of a non-degradable form of β-catenin induces osteoblastic differentiation in C3H10T1/2 cells (41) and transgenic mice (expressing a constitutively active β-catenin allele targeted to osteoblasts), in which canonical Wnt signaling is directly activated, express a high bone mass phenotype, which completely rescues the osteopenic phenotype of the *Lrp5*-null mice (42).

In mutant mice expressing a human LRP5 *G171V* transgene (which has the activating *LRP5* mutation that is associated with high bone mass and constitutive β-catenin activation), the osteoprotegerin (OPG) to RANKL mRNA ratio is increased, compared with that of normal wild-type littermate controls (43). This relative increase in OPG inhibits osteoclastogenesis. β-catenin overexpression has also recently been linked to increased OPG expression in other situations (42;44). In *ApcMin/+* mutant mice, there is constitutive transcriptional activity of the β-catenin/Tcf4 complex in the nuclei of intestinal epithelial cells, which is the consequence of inefficient degradation of β-catenin caused by defective adenomatous polyposis coli (APC) protein. In cells from such mice, expression of OPG is enhanced (44). More recently, it was reported that *OPG* was one of the genes upregulated in microarray experiments on Wnt-3a-treated C3H10T1/2 cells (45). Taken together, these

data suggest that Wnt signaling may not only regulate osteoblastic differentiation and bone formation, but may also modulate osteoclastogenesis and osteoclastic bone resorption.

Dkk1 is the prototypic member of a family of cysteine-rich proteins that act as extracellular antagonists of the canonical Wnt signaling. Dkk1, originally identified as an inducer of head formation in amphibian embryos, acts by binding directly to one of the subunits (LRP5/6) of the Wnt receptor complex (46;47). Dkk1 also interacts with two other single transmembrane proteins (Kremen-1/-2), which act as high-affinity receptors (48). There are data demonstrating that Kremen proteins form a ternary complex with Dkk1 and LRP5/6, inducing rapid endocytosis and removal of the latter Wnt co-receptor from the plasma membrane thereby terminating or attenuating canonical Wnt-induced signaling. In addition to previous reports that Dkk1 expression is spatially restricted to the eye in post-natal mice, Dkk1 expression has also been found in osteoblasts, osteocytes and bone marrow stromal cells (24;49).

A recent report has implicated DKK1 in the impaired osteoblast activity in myeloma bone disease. DKK1 was identified on cDNA microarrays as being preferentially and abundantly expressed in bone marrow aspirates from patients with myeloma (50). Specifically, DKK1 overexpression was reported to be associated with the presence of focal lesions detected on magnetic resonance imaging. In addition, osteoblast differentiation, assessed by a bone morphogenetic protein (BMP)-2-induced increase in alkaline phosphatase activity in pre-osteoblastic C2C12 cells, was blocked by marrow plasma from patients with myeloma and recombinant Dkk1 (50). Consistent with these observations, an earlier report also demonstrated that Dkk1 significantly reduced alkaline phosphatase activity in osteoblastic MC3T3-E1 cells and that Dkk1 overexpression in these cells blocked BMP-2-dependent mineralized matrix formation (51). Together, these data and the compelling impact of Wnt-induced signaling in regulating bone mass *in vivo* have led to the speculation that Dkk1 (an

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antagonist of Wnt signaling) is a mediator of the osteoblast dysfunction in myeloma bone disease.

In recent studies, bortezomib has been found to inhibit Dkk1 gene expression in rodent bone as well as in human and rodent cells of mesenchymal origin, including osteoblasts (24). Glucocorticoids have recently been shown to enhance Dkk1 expression in human osteoblasts (52). It is well established that glucocorticoids including dexamethasone, which is widely used in the treatment of myeloma, have a negative impact on the skeleton. Thus, it is clear that not all classes of drugs with anti-myeloma activity *in vivo* will enhance bone formation, and this beneficial effect may be unique to inhibitors of the ubiquitin-proteasome pathway.

Conclusions

Multiple myeloma remains an incurable cancer, and its unique propensity to cause bone destruction is responsible for the associated high morbidity and mortality rates. Myeloma-associated bone loss is characterized by intense local osteoclast stimulation accompanied by impaired osteoblast function, with a marked inability of osteoblasts to respond appropriately to replace lost bone. The detailed molecular mechanisms underlying myeloma bone disease remain unknown. Most attention has been focused on the osteoclast, but recently, the importance of osteoblast dysfunction has also been recognized, and its potential mediators have been examined. Bisphosphonates, potent inhibitors of osteoclastic bone resorption, have become the mainstay for the treatment of myeloma and other cancer-induced bone diseases; however, these drugs do not restore bone loss. None of the currently available chemotherapeutic regimens correct myeloma-induced osteoblast dysfunction or reverse bone loss. Understanding the role of Dkk1 in the development of myeloma bone disease and the mechanisms that are directly involved in the profound anti-tumor and bone anabolic effect of proteasome inhibition in myeloma will help to define novel molecular targets for the development of new therapies for the treatment of myeloma bone disease.

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