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PROSPECTS

Role of Osteoblast Suppression in Multiple Myeloma

James Peter Stewart and John D. Shaughnessy, Jr.*

Donna D. and Donald M. Lambert Laboratory of Myeloma Genetics, Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Abstract Multiple myeloma is the most common form of plasma cell dyscrasia and virtually all cases of myeloma exhibit osteolytic lesions, which result in bone pain, pathological fractures, spinal cord compression, and hypercalcaemia. Malignant plasma cells disrupt the delicate balance between bone formation and bone resorption, which ultimately leads to the debilitating osteolytic lesions. This review focuses principally on mechanisms of osteoblast inhibition by malignant plasma cells with emphasis placed on our experimental findings, which support a model for abnormal Wnt signaling in osteoblast suppression. We describe how excessive amounts of soluble Wnt inhibitors secreted by malignant plasma cells in multiple myeloma could promote osteolytic lesions, tumor growth, suppress hematopoiesis, prevent proper engraftment, and expansion of transplanted stem cells. Finally, we detail current therapies shown to disrupt the interaction between the myeloma cell and the microenvironment, leading to activation of osteoblasts. J. Cell. Biochem. 98: 1–13, 2006.

Key words: osteoblast; osteoclast; multiple myeloma; DKK1; FRZB; Wnt

Multiple myeloma is the most common form of plasma cell dyscrasia, affecting B-cells that have traversed the postgerminal center. Plasma cells in multiple myeloma first enter blood vessels in the periphery, from where they eventually migrate to, survive in, and expand exclusively in the bone marrow [Asosingh et al., 2002]. At advanced stages, myeloma cells can grow independently of the bone marrow microenvironment, causing a more aggressive presentation of the disease. Although complete responses can be obtained with high-dose therapy in more than 40% of patients, survival varies widely—from a few months to more than 15 years.

Multiple myeloma causes a constellation of disease manifestations, including anemia and immunosuppression, due to suppression of normal hematopoietic stem cell function, and monoclonal immunoglobulin secretion, which can lead to end-stage organ damage. A cardinal clinical symptom of myeloma is the presence of osteolytic lesions, resulting in bone pain, pathological fractures, spinal cord compression, and hypercalcaemia.

This review will focus principally on mechanisms of osteoblast inhibition by malignant plasma cells leading to the perturbation in the coupling between bone formation and bone resorption, which ultimately leads to the debilitating osteolytic lesions evident in virtually all cases of multiple myeloma. Emphasis is placed on our experimental findings that support a model for abnormal Wnt signaling in osteoblast suppression.

OVERVIEW OF BONE FORMATION AND TURNOVER

To understand the molecular pathways exploited by plasma cells in creating osteolytic lesions in multiple myeloma, an understanding of normal bone formation and turnover is of benefit. Coupled bone turnover is a process that is active throughout most adult life and is brought about by the opposing actions of osteoblasts and osteoclasts.

Osteoblasts originate from mesenchymal stem cells (MSCs) and are responsible for bone matrix synthesis by secreting collagen, which

*Correspondence to: John D. Shaughnessy, Jr., PhD, Donna D. and Donald M. Lambert Laboratory of Myeloma Genetics, Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR 72205. E-mail: shaughnessyjohn@uams.edu

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forms strands called osteoids [Franz-Odendaal et al., 2005]. Osteoblasts cause calcium salts and phosphorus to precipitate from the blood and bond with the newly formed osteoid to mineralize the bone tissue. As new bone layers form, osteoblasts become trapped in the osteoids and differentiate into osteocytes.

The exact role of osteocytes has yet to be determined, but it is believed that osteocytes are involved in sensing mechanical stresses in the bone matrix. It has also recently been reported that osteocytes are constitutive negative regulators of osteoclast activity and may also play a role in osteoblast viability [Takai et al., 2004; Gu et al., 2005]. Osteoblasts can also regulate osteoclast activity through expression of cytokines, such as receptor activator of nuclear factor-κB ligand (RANKL), which activates osteoclast differentiation, and osteoprotegerin (OPG), which inhibits RANKL by acting as a decoy receptor [Glass et al., 2005].

Osteoclasts are large multinucleated cells that originate from the monocyte lineage and are responsible for bone resorption. They secrete bone-reabsorbing enzymes from their characteristic ruffled border, and these enzymes digest bone matrix. Bone lesions are formed when the regulation of bone mass, which is maintained by a balance between bone-forming osteoblasts and bone-reabsorbing osteoclasts, is perturbed (Fig. 1).

BONE LESIONS IN MALIGNANCY

Hematological malignancies, such as multiple myeloma and adult T-cell leukemia (ATL),

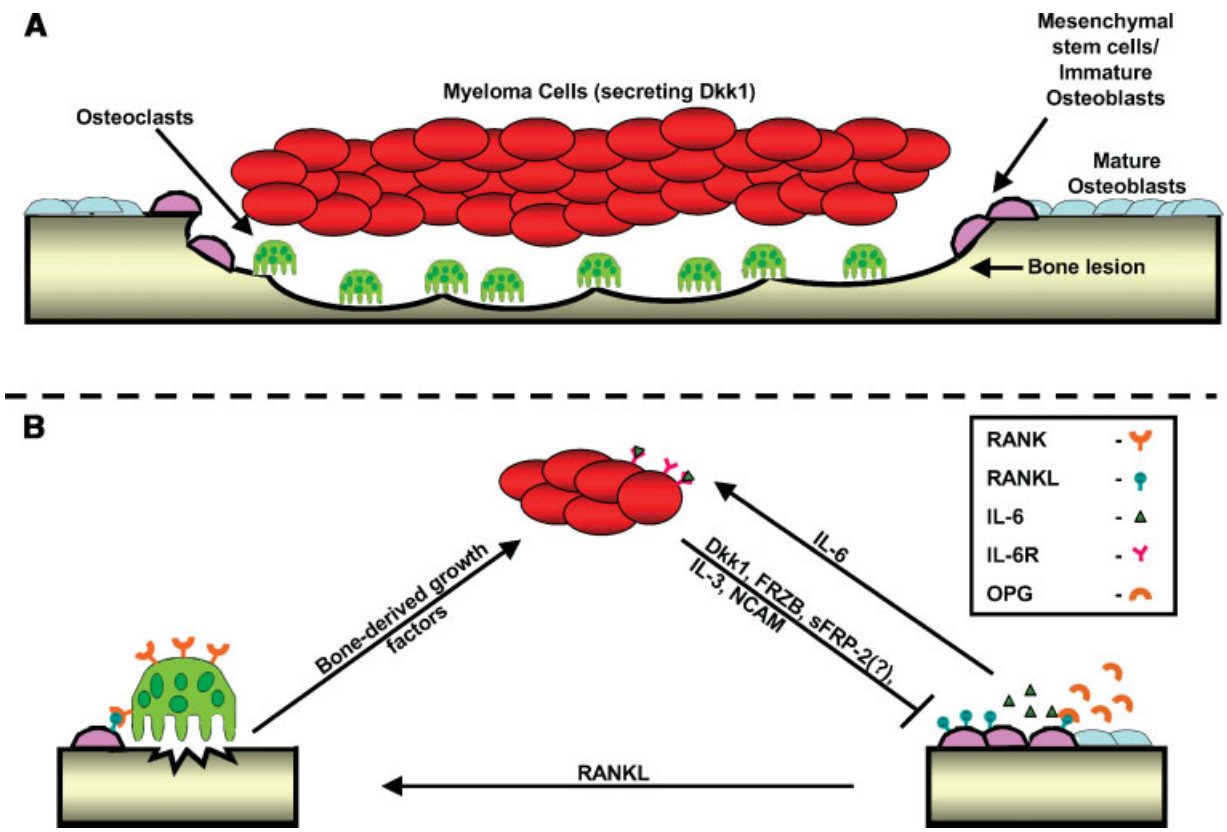

Fig. 1. Model of osteoblast suppression in multiple myeloma. **A**: Simplified diagram depicting the main cell types involved in bone lesions in multiple myeloma. These are (DKK1 secreting) multiple myeloma cells, osteoblasts (bone forming), undifferentiated osteoblasts (mesenchymal stem cells), and osteoclasts (bone resorption). **B**: Factors leading to osteoblast suppression in multiple myeloma and the subsequent effect on osteoclast and myeloma cells. Multiple myeloma cells can secrete or express a number of factors (DKK1, sFRP-2, sFRP-3/FRZB, NCAM, and IL-3), which inhibit osteoblast differentiation. sFRP family members inhibit Wnt signaling by binding to Wnt proteins directly whereas DKK1 forms a complex with Kremen and LRP6 leading to removal of LRP6 from cell membrane. Immature osteoblasts express IL-6 and RANKL, which would aid in multiple myeloma proliferation and osteoclast differentiation, respectively. Increase in osteoclast activity would lead to increased growth factors being released from the degraded bone.

and solid tumors, such as lung, breast, and prostate cancer, have a great affinity for bone, where they cause painful bone lesions by altering elements involved in bone formation and turnover. Prostate cancer frequently causes osteoblastic (bone-forming) lesions, whereas lung and breast cancer, ATL, and multiple myeloma are often associated with osteolytic (bone-degrading) lesions [Roodman, 2004]. Breast and lung cancer metastases are usually characterized as osteolytic, but approximately 15% are osteoblastic or mixed while multiple myeloma is almost exclusively osteolytic [Kozlow and Guise, 2005]. Osteolytic lesions in multiple myeloma are only found adjacent to intramedullary plasma cell foci or plasmacytomas, suggesting myeloma plasma cells may secrete factors that promote activation of osteoclasts, inactivation of osteoblasts, or both. Although very rare, osteosclerotic bone lesions in multiple myeloma have been documented [Lacy et al., 1997].

Investigation of the reduction of osteoblast activity in multiple myeloma has been overshadowed by the volume of work focused on the role of hyperactivated osteoclasts. However, a functional defect of osteoblasts is also important in the osteolytic process in myeloma, evidenced by the fact that treatment with bisphosphonates, which block bone resorption and prevent further bone destruction, does not result in repair of the osteolytic lesions already present [Djulbegovic et al., 2002]. Furthermore, histomorphometric studies performed over a decade ago indicated that multiple myeloma patients with osteolytic lesions were characterized by an unbalanced bone remodeling, and it was suggested that the number and function of osteoblasts were decreased in these patients while osteoclasts were hyperactivated [Bataille et al., 1989]. Indeed, multiple myeloma patients without bone lesions have increased bone resorption, but this is counterbalanced by increased bone formation [Abildgaard et al., 2000], whereas myeloma patients with evidence of bone lesions have an inhibition of osteoblasts along with increased bone resorption, thereby experiencing a disruption in the fine balance between bone formation and resorption.

DKK1 EXPRESSION IN MULTIPLE MYELOMA

Microarray studies have provided key insights into the biology and clinical behavior of multiple myeloma. Previously, we sought to identify genes that were overexpressed and associated with the presence of bone lesions in patients with multiple myeloma by comparing microarray data for patients who had bone lesions with data for those who did not [Tian et al., 2003]. We were able to identify four genes significantly overexpressed by plasma cells from patients with osteolytic lesions: dihydrofolate reductase (*DHFR*), proteasome activator subunit (*PSME2*), CDC28 protein kinased 2 (*CKS2*), and Dickkopf-1 (*DKK1*).

We focused on *DKK1* as it was a secreted factor in a signaling pathway previously linked to the function of osteoblasts. A low expression of DKK1 mRNA was observed in patients with monoclonal gammopathy of undetermined significance (MGUS), a benign plasma cell dyscrasia that is a precursor to myeloma, and in multiple myeloma patients who had no focal lesions by MRI, whereas a high level of expression of both DKK1 mRNA and protein was observed in multiple myeloma patients who had one or more osteolytic lesions by MRI. We also observed a statistically significant correlation between DKK1 mRNA levels and protein levels in both bone marrow biopsy specimens, using immunohistochemistry, and bone marrow plasma, using ELISA.

DKK1—a Wnt Signaling Antagonist

Wingless/ints (Wnts) are a family of 19 secreted glycoproteins that have a well-characterized role in maintenance and development of stem cells. Dysregulation of the Wnt signaling pathway has been reported in both degenerative disorders and malignancies [Nusse, 2005]. Wnt signaling is mediated by the frizzled family of receptors, comprising seven transmembrane molecules with a long amino-terminal extension. Binding of Wnt to the frizzled receptor often requires recruitment of the low-density lipoprotein receptor-related protein (LRP) 5/6. Following binding of Wnt to the receptor, intracellular signaling can cascade along one of three pathways: the β-catenin/TCF pathway, which activates target genes in the nucleus, the Wnt/Ca^{2+} pathway, or the planar cell polarity pathway, which involves the jun N-terminal kinase (JNK) pathway and cytoskeletal modifications. The β-catenin/TCF pathway is a canonical pathway, and the latter two are non-canonical. Activation of the canonical Wnt signaling pathway leads to β-catenin

stabilization, which subsequently can translocate to the nucleus and form a transcriptionally active β-catenin/TCF/LEF DNA binding complex [Kawano and Kypta, 2003].

DKK1 is one of a number of proteins that regulates the Wnt pathway (Fig. 2). DKK1 specifically binds to the Wnt coreceptors LRP5/6, thereby preventing a functional frizzled/LRP Wnt receptor complex from forming [Kawano and Kypta, 2003]. There is some evidence that DKK family members DKK1 and DKK2 do have functions that are independent of the Wnt-antagonistic effect, but this work is at an early stage [Li et al., 2005]. Another family of Wnt signaling inhibitors includes the secreted frizzled related proteins (sFRPs) [Kawano and Kypta, 2003]. This family consists of five members that act by binding directly to Wnt proteins, thus preventing their association with the frizzled family of receptors.

DKK1—Role in Osteoblast Suppression in Myeloma

Recent studies show that Wnt signaling is critical for the differentiation of progenitor cell lines into osteoblasts [Day et al., 2005] and activation of OPG production in differentiated osteoblasts, which is a major inhibitor of osteoclastogenesis [Glass et al., 2005]. The fact that Wnt signaling plays a central role in human bone metabolism came from studies showing that osteoporosis-pseudoglioma is caused by loss-of-function mutations in the DKK1 receptor LRP5 [Gong et al., 2001]. Remarkably, a couple of years after this finding, two separate groups of researchers showed that a gain-of-function

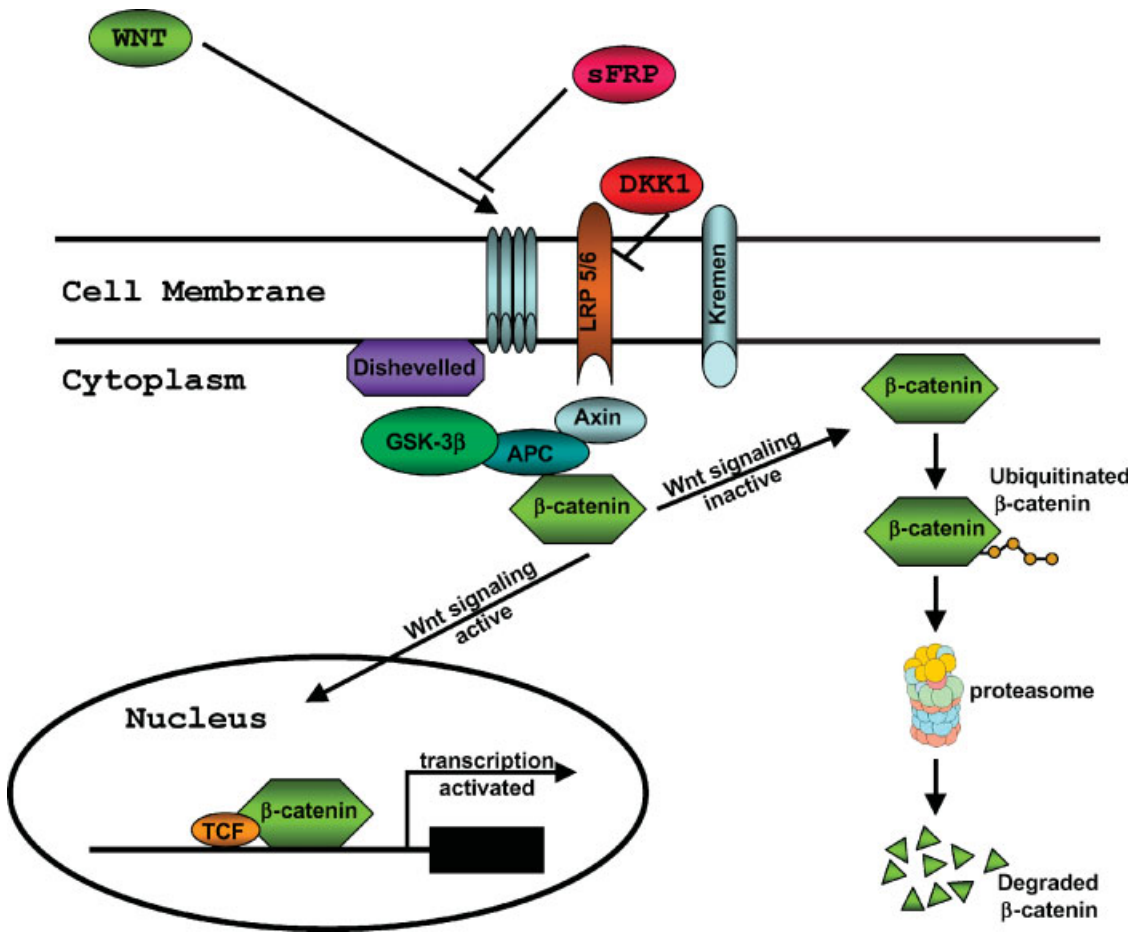

Fig. 2. The Wnt signaling pathway. The canonical wnt signaling pathway has been simplified to include only the main components of the pathway. Wnt proteins bind to the Frizzled receptor leading to recruitment of the low-density lipoprotein receptor-related protein (LRP) 5/6. Wnt signaling disrupts the complex of GSK3β, Axin, APC, and β-catenin resulting in inhibition of the proteasomal degradation mediated by the β-TRCP ubiquitin ligase, which results in increased β-catenin levels and translocation to the nucleus. Upon entry to the nucleus β-catenin can interact with TCF to regulate gene expression.

mutations in LRP5 results in high bone mass (osteopetrosis) syndrome [Boyden et al., 2002; Little et al., 2002].

These seminal observations led us to determine the functional significance of the observed upregulation of DKK1 mRNA in plasma cells derived from patients with osteolytic lesions. We examined the differentiation of the uncommitted mesenchymal progenitor cell line C2C12 into osteoblasts through a Wnt/β-catenin-dependent pathway when stimulated with 50 ng/ml bone morphogenetic protein type 2 (BMP-2) by measuring increases in alkaline phosphatase, a specific marker of osteoblast differentiation. We observed that BMP-2 induced differentiation of C2C12 cells into osteoblasts and that this effect was inhibited by either 50 ng/ml of recombinant human DKK1 or by bone marrow plasma with a DKK1 level of more than 12 ng/ml (obtained from myeloma patients). The inhibition by DKK1 was specifically reversed by the anti-DKK1 antibody. How DKK1 is able to inhibit BMP-2-induced osteoblast differentiation is not completely clear, but it is thought to be due to the fact the BMP-2 induces an autocrine activation of Wnt signaling [Rawadi et al., 2003]. Whether DKK1 directly inhibits BMP signaling is not known.

DKK1—Role in Osteoblast Suppression in Malignancy

The role of DKK1 in promoting the development of bone lesions is not limited to multiple myeloma but has recently been expanded to prostate and breast cancer. The osteolytic prostate cancer line PC-3, when transfected with shRNA targeting *DKK1*, reverted to an osteoblastic phenotype [Hall et al., 2005]. In addition, transfection of *DKK1* into the osteoblastic prostate cancer cell line C4-2B, which normally induces a mix of osteoblastic and osteolytic lesions, caused the cells to develop highly osteolytic tumors in SCID mice. Prostate cancer cell lines have also been found to express a variety of Wnt mRNAs, and Wnt mRNA expression was increased in primary prostate cancer compared with non-neoplastic prostate tissue [Hall et al., 2005].

We have shown that the breast cancer cell line MDA-231 expresses very high levels of DKK1 (unpublished data), and others have shown that this cell line is also highly osteolytic when transplanted into mice [Guise et al., 2002]. Whether *DKK1* plays a role in the bone metastatic phenotype of primary prostate and breast cancer is not known. It is interesting to note that the prostate is one of the most abundant sources of DKK1 in adult humans [Fedi et al., 1999]. Finally, transgenic mice overexpressing DKK1 under the control of the mouse alpha1 collagen promoter exhibit dwarfism, short limbs, and osteoporosis [Guo et al., 2004]. All these data reinforce the hypothesis that regulation of bone turnover by modulation of the Wnt signaling pathway may be perturbed in several human malignancies.

DKK1—Possible Effect of HSC Maintenance

The effect of altered Wnt signaling may not be limited to osteoblasts within the bone marrow of patients with myeloma. Anemia and immunosuppression are two common clinical symptoms associated with multiple myeloma, but the mechanisms leading to these symptoms are poorly understood. It has been suggested that crowding out of normal hematopoietic cells in the bone marrow by the increasing number of multiple myeloma cells may play a role [Barlogie and Beck, 1993]. Hematopoietic stem cell (HSC) proliferation and self-renewal is dependent on extracellular factors in the microenvironment and occurs through adhesion in a bone marrow niche (the so-called HSC niche). It has been previously shown that components of the Wnt signaling pathway are involved in enhancing the self-renewal and proliferation of HSCs [Reya et al., 2003; Duncan et al., 2005]. The effect of DKK1 secretion by multiple myeloma cells on the HSC population may be worth studying to determine if the immunosuppression and anemia associated with multiple myeloma may be due, in part, to increased levels of DKK1 in the bone marrow microenvironment.

It is of interest to note that adenoviral-mediated DKK1 overexpression promoted loss of stem cells in the colon crypt, thereby enforcing the hypothesis that DKK1 may affect stem cell maintenance [Kuhnert et al., 2004]. In our published study of a cohort of patients with multiple myeloma, we did not observe a difference in the percentage of patients with a hemoglobin value <10 g/dl between patients with ≥ 1 lesions compared with patients with no evidence of osteolytic lesions [Tian et al., 2003]. Furthermore, a functioning osteoblast population creates the HSC niche [Calvi et al., 2003; Zhang et al., 2003], but multiple myeloma cells

secreting DKK1 may reduce the functional capacity of osteoblasts to provide this niche. Since stem cell transplantation is the standard of care for multiple myeloma and stem cells need to home to and expand in the bone marrow in the context of this niche, excessive amounts of DKK1 could prevent proper engraftment and expansion of the transplanted stem cells.

DKK1—Direct Effects on Myeloma Cells

It is therefore possible that secretion of DKK1 by malignant plasma cells has pleiotropic effects on the microenvironment; however, it is currently unknown if DKK1 functions in an autocrine fashion on these plasma cells. It is noteworthy that Qiang et al. [2003] have previously shown that myeloma cell lines exhibit a functional Wnt signaling pathway, whereas cell lines derived from patients with B-cell lymphoma, a malignancy that represents an earlier stage of B-cell development, do not express any of the numerous Wnt receptors [Qiang et al., 2003]. In these studies, the investigators showed that myeloma cells signal through both canonical and noncanonical pathways and that noncanonical signaling induced dramatic morphological changes [Qiang et al., 2003]. Derksen and colleagues showed that myeloma cells signal through Wnt and that this induces proliferation [Derksen et al., 2004], and recent studies have shown that Wnt signaling is functional in primary myeloma cells and induces migration [Qiang et al., 2005]. DKK1, which blocks signaling specifically through the canonical Wnt pathway, could not inhibit Wnt-induced changes in morphology and motility in either myeloma cell lines or primary myeloma samples as this was dependent on activation of the Wnt/RhoA pathway [Qiang et al., 2005].

Regulation of DKK1 Expression in Multiple Myeloma Cells

Newly diagnosed multiple myeloma patients with lytic lesions often have high DKK1 mRNA expression, but *DKK1* is rarely detected when gene expression is performed at an early stage, for example, MGUS, or at advanced stages of the disease. In addition, *DKK1* expression is rarely detectable in myeloma cell lines. Therefore, an important goal for future research is to determine why malignant plasma cells turn DKK1 on and why *DKK1* expression is lost in more advanced stages of the disease. The finding that a subgroup of patients who have myeloma with lytic lesions but do not express *DKK1* may be explained by the possibility these patients present at a more advanced stage of disease. It is noteworthy that osteoclast numbers increase as myeloma progresses and that loss of *DKK1* expression temporally follows the increase in osteoclasts. Evidence that osteoclasts play a direct role in negatively regulating *DKK1* in myeloma cells comes from in vitro data showing that co-culturing myeloma plasma cells with osteoclasts uniformly results in a downregulation of *DKK1* (S. Yaccoby and J. Shaughnessy, unpublished data). *DKK1* is a direct transcriptional target of p53 [Wang et al., 2000], and p53 loss or mutation is frequently seen in late-stage multiple myeloma [Neri et al., 1993]; therefore, loss of DKK1 may be due, in part, to loss of p53.

It is also interesting to note that the *DKK1* gene has recently been shown to be a downstream target of the Wnt signaling pathway and, therefore, may be involved in a negative feedback loop [Gonzalez-Sancho et al., 2005]. Furthermore, patients with multiple myeloma can be divided into at least seven distinct subgroups based on gene expression profiling [Zhan et al., 2003, 2004; Bergsagel and Kuehl, 2005]. One group, defined by hyperdiploidy, has been shown to have the highest incidence of bone lesions [Zhan et al., 2004; Bergsagel and Kuehl, 2005]. Taken together, it is tempting to speculate that loss of *DKK1* in late-stage disease may occur, in part, through increased interaction of myeloma cells with osteoclasts and accumulation of genetic abnormalities.

DKK1 in Therapy

We have performed baseline and 48-h follow-up gene expression profiling on multiple myeloma patients before and after therapy with a single chemotherapeutic drug. The compounds studied were dexamethasone ($n = 20$), thalidomide ($n = 18$), the immunomodulatory agent Revlimid ($n = 15$), and the proteasome inhibitor PS-341 (Velcade, bortezomib) ($n = 11$) [Shaughnessy et al., 2002]. We found that six genes were consistently activated after 48-h treatment with either thalidomide or Revlimid (IMiD), a potent thalidomide analog. One of the six genes, *DKK1*, was hyperactivated to a median of 125% in 14 out of 18 patients treated with thalidomide and upregulated to a median of 315% in 13 out of 15 cases treated with IMiD. *DKK1* was upregulated by a median value of

23% by dexamethasone, and a change was essentially undetectable in patient samples treated with Velcade. Therefore, the in vivo effects of thalidomide and IMiD have powerful upregulating effects on *DKK1*, whereas dexamethasone and the proteasome inhibitor Velcade have an intermediate to little effect on *DKK1* expression, respectively. Virtually all patients treated with thalidomide or IMiD who did not show a hyperactivation of *DKK1* after 48-h treatment had little or no detectable expression in the baseline sample, suggesting these patients had an inherent inability to activate *DKK1* expression.

INVOLVEMENT OF OTHER WNT SIGNALING PROTEINS IN OSTEOLYTIC LESIONS

FRZB/sFRP-3 and sFRP-2

The secreted Wnt signaling antagonist FRZB/sFRP-3 has also been implicated in the pathogenesis of multiple myeloma and was shown to be upregulated in newly diagnosed patients, whereas plasma cells from 45 samples harvested from healthy bone marrow done donors and from 10 patients with Waldenstrom's macroglobulinemia, a plasma cell malignancy lacking bone disease, showed no *FRZB* expression [De Vos et al., 2001; Zhan et al., 2002 and unpublished data]. In another study, *FRZB* was shown to be the most upregulated gene during the transition of MGUS to multiple myeloma [Davies et al., 2003]. We have shown that myeloma plasma cells express high levels of the FRZB protein and that FRZB mRNA and protein levels are correlated with osteolytic lesions, albeit at a lower significance than DKK1 (unpublished data).

Oshima et al. [2005] observed that most multiple myeloma cells from patients with advanced bone destructive lesions expressed sFRP-2. They determined the biological significance of this by showing that exogenous sFRP-2 suppressed osteoblast differentiation induced by BMP-2 and that immunodepletion of sFRP-2 significantly restored mineralized nodule formation in vitro. Upon examination of our microarray data from a panel of 351 newly diagnosed multiple myeloma patients, we were able to show elevated expression of sFRP-2 mRNA in only eight cases, in agreement with Giuliani et al. [2005], who were unable to detect expression of sFRP-2 mRNA by RT-PCR in purified plasma cells. Therefore, although the role of sFRP-2 in osteoblast biology is unquestionable, the conflicting data regarding sFRP-2 expression warrants further research to determine the significance of sFRP-3 and sFRP-2 in osteoblast suppression in the context of multiple myeloma.

Runx2/Cbfa1/AML3

MSCs are a pluripotent cell type that can differentiate into several distinct lineages, including osteoblasts and adipocytes. A transcription factor called Runx2 (also known as Cbfa1) has been shown to be key in driving MSCs to differentiate into osteoblasts. Interestingly, Giuliani et al. [2005] showed a significant reduction of Runx2-positive cells in the bone marrow of multiple myeloma patients exhibiting osteolytic lesions compared to bone marrow samples where lesions were not detected. This would suggest that MSCs would be less inclined to differentiate into osteoblasts. Intriguingly, a recent publication showed that two components downstream of the Wnt signaling pathway, TCF1 and β-catenin, can bind to and regulate *Runx2* gene expression in MSCs [Gaur et al., 2005]. This may indicate that the significant reduction of Runx2-positive cells observed in the bone marrow of multiple myeloma patients exhibiting osteolytic lesions is actually the result of DKK1-secreting plasma cells inhibiting the Wnt signaling pathway in the surrounding microenvironment. This must be taken into consideration when determining targets for future pharmaceutical compounds; for example, an inhibitor of DKK1 may be as functionally effective as an inhibitor of Runx2 if further research confirms a negative correlation between DKK1 and Runx2 expression in the bone marrow of multiple myeloma patients.

Similar to studies of Wnt signaling, it may prove beneficial to examine other components of the pathway involving Runx2. The recently identified TAZ (transcriptional coactivator with PDZ-binding domain) protein was found to coactivate Runx2-dependent gene transcription and promote osteoblastogenesis [Hong et al., 2005]. Therefore, a reduction of TAZ-positive cells in the bone marrow of multiple myeloma patients may lead to osteolytic lesions.

The Role of Interleukins

It has been observed that there is a significant increase in osteoclast recruitment and activity in the vicinity of myeloma cells, suggesting myeloma cells secrete an osteoclast activating

factor (OAF) or induce other cell types within the bone marrow stroma to do so. Also, if one were to look at factors known to promote differentiation of osteoclasts from MSCs and/or osteoclast bone resorption, one would find a number of cytokines that are produced by lymphocytes. Furthermore, bone resorption can release large amounts of growth factors from the mineralized matrix of bone, such as TGF-β and BMPs [Fox and Lovibond, 2005].

The role of interleukins in the development of bone lesions is not limited to an effect on osteoclasts as studies have recently looked at the consequence of particular interleukins on osteoblast number and activity. In normal bone development, where the processes of bone formation and bone resorption are coupled, it would be beneficial for a system to produce a single factor to promote activation of bone resorption while simultaneously having a negative effect on bone formation. This has been shown to be the case for interleukin-3 (IL-3), which leads to increased osteoclast formation but is also an inhibitor of osteoblast differentiation [Lee et al., 2004; Erhlich et al., 2005]. Erhlich et al. [2005] reported that IL-3 is a potential inhibitor of osteoblast differentiation in multiple myeloma. Bone marrow plasma from multiple myeloma patients, which exhibited high IL-3 levels, blocked osteoblast formation in human cultures, and this inhibition was partially reversed by the addition of a neutralizing antibody to human IL-3 [Erhlich et al., 2005].

Histomorphometric analysis of multiple myeloma patients with evidence of bone lesions is indicative of an inhibition in osteoblast activity [Abildgaard et al., 2000]. Although there is an increase in osteoblast recruitment, the activity of individual osteoblasts appears to be reduced. Karadag et al. [2000] employed an in vitro method to allow MSCs to differentiate into osteoblasts. Co-culture of the MSCs with myeloma cell lines caused an increase in the number of osteoblast colonies; the researchers subsequently identified IL-6 and soluble IL-6 receptor as being critical in this process. They also showed that when a phorbol ester, PMA, which promotes IL-6 shedding, was introduced to the co-culture, the number of osteoblast colonies was enhanced even further.

Remarkably, recent studies have shown that MSCs secrete DKK1 and that this promotes their proliferation at the expense of differentiation [Gregory et al., 2003]. Gunn and colleagues have shown that conditioned media from MSCs can induce multiple myeloma cells lines to produce DKK1 [Gunn et al., 2005, in press]. Moreover, this group showed that MSC cultures producing DKK1 also produce high levels of IL-6, that IL-6–dependent cell lines survive and proliferate in MSC conditioned media, and that this can be inhibited by a neutralizing antibody to IL-6 [Gunn et al., 2005, in press].

IL-6 is a central growth and survival factor for myeloma [Kishimoto, 2005]. It could therefore be hypothesized that myeloma cells, through secretion of DKK1 and IL-6R, lead to recruitment of more immature osteoblasts to the microenvironment surrounding the multiple myeloma foci. In addition, the secretion of DKK1 would prevent the increased numbers of immature osteoblasts recruited to the immediate vicinity from terminally differentiating, keeping them in an immature stage where their secretion of IL-6 is known to be highest. The higher levels of IL-6 would promote growth and resistance to apoptosis. Moreover, immature, but not mature, osteoblasts are rich sources of RANK ligand [Atkins et al., 2003]. Thus, secretion of DKK1 by myeloma cells, leading to inhibition of osteoblast differentiation and ultimately an increase in an immature osteoblast population, may contribute to the development of osteolytic lesions by stimulating osteoclast activation through increased IL-6 and RANKL production and reduced OPG production.

Another possible explanation for the imbalance between osteoclasts and osteoblasts that has been recently raised is that, over time, myeloma cells can undergo morphologic, phenotypic, and functional transformation into osteoclast-like cells [Calvani et al., 2005].

The Possible Role of NCAM

The mechanisms described above are related to factors secreted by plasma cells in multiple myeloma that act in a paracrine fashion on osteoblasts. It has also been hypothesized that direct cell–cell interactions may also directly influence osteoblast suppression. The neural cell adhesion molecule (NCAM/CD56) may be one such molecule that supports this hypothesis. NCAM is known to be overexpressed by multiple myeloma cells, mainly of the kappa subtype, and is correlated with the presence of bone lesions [Ely and Knowles, 2002]. It is worth noting here that we have shown that *DKK1* and *NCAM* gene expression are highly correlated in

patients with multiple myeloma (unpublished data).

CURRENT AND EMERGING THERAPIES

Traditionally, the focus of cancer chemotherapy has been to target the tumor cell directly. Given that survival of multiple myeloma is intimately dependent on an interaction with the bone marrow, a larger emphasis on targeting the microenvironment could prove beneficial. Some of the current therapies that were also thought to target the myeloma cell have been shown to disrupt the interaction between the myeloma cell and microenvironment. In addition, there are a number of novel compounds being investigated that target the microenvironment, which may add to the much needed list of compounds that are already employed in combating multiple myeloma. Finally, harnessing the knowledge of perturbations that occur in the Wnt signaling pathway in multiple myeloma, and other malignancies in general, has led to a number of possible therapeutic targets.

Proteasome Inhibitors

Garrett et al. [2003] have shown that treatment of osteoblasts with proteasome inhibitors (targeting the chymotryptic component of the proteasome) led to increased osteoblast differentiation and bone formation in vitro and in vivo through the regulation of BMP-2 transcription and protein expression [Garrett et al., 2003]. Recently, Velcade^{TM} (bortezomib), the first clinically approved proteasome inhibitor for treatment of multiple myeloma, was shown to stimulate new bone formation in the neonatal mouse, to inhibit IL-1-stimulated bone resorption, and to inhibit *DKK1* expression in three human cell lines of mesenchymal origin [Oyajobi et al., 2004]. Consistent with these preclinical data, our group has recently shown that response to Velcade is associated with increases in serum markers of osteoblast differentiation [Zangari et al., 2005].

Wnt Inhibitors

Similar to Velcade and Imatinib, it appears that compounds targeting components of the Wnt signaling pathway could be used to treat not only multiple myeloma, but also a range of malignancies and disorders that are associated with bone destruction. Below we briefly mention some of the promising Wnt inhibitors that have been reported.

Examination of the mechanisms regulating *DKK1* gene expression is an area our laboratory is actively investigating. The knowledge of how *DKK1* expression is downregulated in later stages of multiple myeloma could be harnessed to develop novel chemotherapeutic agents. Early reports suggest that neutralizing antibodies raised against DKK1 or small peptides can inhibit DKK1 activity. We have shown that blocking *DKK1* activity in SCID-rab mice implanted with primary myeloma cells, which express high levels of *DKK1* by microarray, reduced osteolytic bone resorption, increased bone mineral density, and ultimately controlled myeloma progression in a significant fraction of cases [Yaccoby et al., 2005]. Furthermore, Gregory et al. [2005a] reported that three peptides corresponding to residues 217–269 in *DKK1* were each found to enhance the proliferation of hMSCs in culture over 2 days, with the most active peptide being able to reduce endogenous *DKK1* expression. Therefore, there may be an application for antibodies to DKK1 in preventing osteolytic lesions in multiple myeloma and metastatic breast cancer.

DKK1 is activated by many of the drugs used to treat myeloma (e.g., dexamethasone, thalidomide). The role of *DKK1* activation as a result of chemotherapy treatment of multiple myeloma is currently unknown and a focus of intense study. It could be envisaged that an antibody to DKK1 could be applied during the induction phase of treatment, when these drugs are first administered and prior to the stem cell transplantation, to enhance engraftment and growth of the stem cells. Recent studies have shown that dexamethasone treatment of osteoblasts results in a time- and dose-dependent increase in *DKK1* expression [Ohnaka et al., 2005]. It is also known that long-term exposure to dexamethasone and steroids for treatment of chronic inflammatory diseases has the unwanted side effect of inducing osteoporosis [Maricic, 2005]. We and others have shown that *DKK1* is activated by the glucocorticoid dexamethasone [Shaughnessy et al., 2002; Ohnaka et al., 2005]. Taken together, these data suggest that *DKK1* may mediate these osteoporotic effects and that antibodies to DKK1 may prevent the development of steroid-induced osteoporosis without hindering the immunomodulatory and beneficial effects of the drugs.

There is also evidence in the literature that sFRP may be negatively regulated by estrogen

[Das et al., 2000]. Thus, antibody therapy to *DKK1* may aid in prevention of bone loss in postmenopausal women. In addition, Caricasole et al. [2004] have shown that upregulation of *DKK1* may be implicated in Alzheimer's disease (AD) and suggested that DKK1 antagonist molecules could act as neuroprotective agents in AD.

The mesenchymal stem cell requires Wnt signaling to differentiate into mature bone-forming osteoblasts. In addition, Wnt signaling in osteoblasts is required for the production, by these cells, of the potent osteoclast inhibitor OPG. Thus, inhibition of Wnt signaling by *DKK1* may promote both features by inhibiting osteoblast differentiation and promoting osteoclast development via reduced production of OPG by ostoeblasts. Depleting or inhibiting *DKK1* and *FRZB* function could restore osteoblast differentiation, which would increase OPG production, reduce RANKL and IL-6 production, and, hence, decrease osteoclast activity, thus preventing tumor progression.

Activation of the Wnt pathway leads to inhibition of the serine/threonine kinase glycogen synthase kinase 3β (GSK-3β), which phosphorylates and promotes the degradation of β-catenin. Dexamethasone activates GSK-3β, leading to inhibition of the osteoblast cell cycle [Ohnaka et al., 2005]. Therefore, another target of the Wnt pathway that is being actively investigated is inhibition of GSK-3β. One potential problem with this therapy would be systemic hyperactivation of Wnt signaling, increasing the risk for transformation of other cell types. Treatment with lithium chloride, a pharmacological Wnt activator that elicits its function by inactivating GSK-3β, may well lead to activation of Wnt signaling, even in the presence of *DKK1*, as its effects would be downstream of *DKK1* inhibition of Wnt signaling and the level of receptor ligand interaction [Gregory et al., 2005b]. Further studies are required to determine the effects of long-term exposure as Vaes et al. [2005] reported that continuous activation of Wnt signaling by LiCl treatment decreased osteoblast extracellular calcium deposition and osteoblast differentiation.

SUMMARY

In this review, we have examined the importance of understanding the suppression of osteoblast differentiation and activity and bone defects within the context of multiple myeloma. We have described how malignant plasma cells in multiple myeloma secrete excessive amounts of soluble Wnt inhibitors and detailed how this could promote osteolytic lesions and tumor growth, suppress hematopoiesis, and prevent proper engraftment and expansion of transplanted stem cells. It is apparent that osteoblast suppression in multiple myeloma can lead to broad pathological effects, including bone lesions, and that there are multiple mechanisms involved. In what is often termed a "vicious cycle," perturbation of the pathways described above results in the decrease of osteoblast activity/number while often leading to a concurrent increase in osteoclast activity/number, or vice versa. Elucidation of the molecular signals leading to regulation of the coupled bone formation and bone resorption process is a prerequisite for the design of novel pharmaceutical agents that will prevent further bone destruction, and, importantly, repair existing damage.

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