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MULTIPLE MYELOMA BONE DISEASE: PATHOPHYSIOLOGY OF OSTEOBLAST INHIBITION.

Short title: Multiple myeloma and osteoblasts

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Abstract

Multiple myeloma (MM) is a plasma cell malignancy characterized by a high capacity to induce osteolytic bone lesions. Bone destruction in MM results from increased osteoclast formation and activity that occur in close proximity to myeloma cells. However, histomorphometric studies have demonstrated that MM patients with osteolytic bone lesions have lower numbers of osteoblasts and decreased bone formation. This impaired bone formation plays a critical role in the bone destructive process. Recently, the biological mechanisms involved in the osteoblast inhibition induced by MM cells have begun to be elucidated. In this article, the pathophysiology underlying osteoblast inhibition in MM is reviewed.

Introduction

Bone destruction is a hallmark of multiple myeloma (MM).[1] Almost all MM patients develop osteolytic bone lesions that can cause pathologic fractures and severe bone pain. Osteolytic lesions result from increased bone resorption due to stimulation of osteoclast formation and activity that occurs in close proximity to myeloma cells.[2-4] Histomorphometric studies have demonstrated that, in MM patients with bone lesions, there is uncoupled or severely imbalanced bone remodeling with increased bone resorption and decreased or absent bone formation. In contrast, MM patients without bone lesions display balanced bone remodeling with increased osteoclastogenesis and normal or increased bone formation rates.[5,6] These histomorphometric studies are supported by clinical studies showing that MM patients with bone lesions have reduced bone formation markers, such as alkaline phosphatase and osteocalcin, together with the increased bone resorption markers.[7] Similarly, marked osteoblastopenia and reduced bone formation have also been reported in murine models of MM that develop bone lesions.[8] These data suggest that myeloma cells suppress osteoblasts and thereby inhibit bone formation. This inhibition of osteoblast activity plays a critical role in the pathogenesis of MM bone disease. In the last few years several potential mechanisms involved in this process have been reported, which will be discussed below. Increased knowledge of the signaling pathways involved in the regulation of osteoblast formation have provided us a better understanding of the pathophysiological mechanisms involved in MM-induced osteoblast inhibition and have identified several potential therapeutics targets for the treatment of MM bone disease.

Pathophysiology of osteoblast and bone formation inhibition in MM

Runx2/Cbfa1 pathway and its involvement in MM-induced osteoblast suppression

The formation and differentiation of osteoblastic cells from mesenchymal stem cells require the activity and function of the transcription factor Runx2/Cbfa1.[9-13] Runx2/Cbfa1 acting together with other transcription factors such as Osterix, induces bone formation.[9] Runx2/Cbfa1 deficient mice (Runx2-/-) completely lack osteoblasts and bone formation, demonstrating a critical role for this factor in osteoblastogenesis.[9,14] However, Runx2 overexpression also impairs bone formation, indicating that, depending on the stage of osteoblast differentiation, Runx2/Cbfa1 can have different effects on bone.[15] Human osteoblast differentiation is primarily associated with increased Runx2/Cbfa1 activity without a change in protein levels.[16,17] The activation of Runx2/Cbfa1 in human bone marrow stromal and osteoblastic cells induces the expression of the osteoblast markers collagen I, alkaline phosphatase and osteocalcin during the different stages of the osteoblast maturation.[12,13,16,17] Multiple signal pathways converge to interact with Runx2/Cbfa1 to regulate osteoblast differentiation, including binding with AP-1.[9,11-13] Runx2/Cbfa1 itself is regulated by phosphorylation, and Runx2/Cbfa1 interacts with other transcription factors including Smad proteins or Hey-1 that may affect its function.[9,11-13]

The potential involvement of Runx2/Cbfa1 mediated transcription in MM induced osteoblast inhibition has recently been reported.[18] When human MM cells were co-cultured with osteoprogenitor cells, MM cells inhibited osteoblast differentiation in long term bone marrow cultures, reducing the number of both early osteoblast precursors, fibroblast colony-forming units (CFU-F), and the more differentiated osteoblast precursor, the colony-forming osteoblast units (CFU-OB), and decreasing the expression of osteoblast differentiation markers, alkaline phosphatase, osteocalcin and collagen I.[18] This effect was mediated by blocking Runx2/Cbfa1 activity in human osteoprogenitor cells. In addition, because Runx2/Cbfa1 also stimulates the secretion of the major osteoclast inhibitor, osteoprotegerin, (OPG) in osteoprogenitor cells,[19]

it is possible that the inhibition of Runx2/Cbfa1 activity in MM could also increase osteoclastogenesis.

The effect of MM cells on Runx2/Cbfa1 activity appears to be primarily mediated by cell-to-cell contact between MM and osteoprogenitor cells. This cell-to-cell contact involves interactions between VLA-4 on MM cells and VCAM-1 on osteoblast progenitors, as demonstrated by the capacity of a neutralizing anti-VLA-4 antibody to reduce the inhibitory effects of MM cells on Runx2/Cbfa1 activity.[18] Similarly, it has been previously reported that human myeloma cells also inhibit osteocalcin secretion by osteoblast-like cells through the cell-to-cell contact.[20] The role of the cell-to-cell contact via VLA-4/VCAM-1 interaction in the development of bone lesions and osteoclast activation and osteoblast inhibition in MM has been recently demonstrated using in vivo mice models.[21,22] When the human myeloma cell line, JJN3, which strongly expresses VLA-4,[23] is implanted in irradiated mice with severe combined immunodeficiency (SCID), the mice develop lytic lesions and marked osteoblastopenia with a significant reduction of bone formation.[8] In addition to VLA-4/VCAM-1, other adhesion molecules appear to be involved in the inhibition of osteoblastogenesis by human myeloma cells. For example, NCAM-NCAM interactions between myeloma cells and stromal/osteoblastic cells can decrease bone matrix production by osteoblastic cells, and may contribute to the development of bone lesions in MM patients.[20,24]

Soluble factors may also contribute to the inhibitory effects of MM cells on osteoblast differentiation and Runx2/Cbfa1 activity.[18] Interleuluin-7 (IL-7) decreases Runx2/Cbfa1 promoter activity in osteoblastic cells and the expression of osteoblast markers.[25] Moreover IL-7 can inhibit bone formation in vivo in mice. [25] We have demonstrated that IL-7 inhibited both CFU-F and CFU-OB formation in human bone marrow cultures, and reduced Runx2/Cbfa1 activity in human osteoprogenitor cells.[18] The potential involvement of IL-7 in MM has been supported by the demonstration of higher IL-7 plasma levels in MM patients

compared to normal subjects[26] and by the capacity of blocking antibodies to IL-7 to partially blunt the inhibitory effects of MM cells on osteoblast differentiation.[18] These studies suggest that MM cells block Runx2/Cbfa1 activity and osteoblast differentiation either by cell-to-cell contact or by secreting IL-7, which leads to a reduction in the number of more differentiated osteoblastic cells. This decreased osteoblast activity contributes to the development of MM bone lesions. These in vitro observations have been confirmed in vivo by immunohistochemical studies performed in MM patients. These studies showed that patients with bone lesions had a reduced number of osteoblastic cells and a reduction in Runx2/Cbfa1 expressing stromal/osteoblastic cells compared to patients without bone disease.[18] These results suggest that increasing Runx2/CBfa1 activity or blocking IL-7 could be potential therapeutic targets in MM. In support of this hypothesis, Zhao et al have reported that transfer of the Runx2 gene enhances osteogenic activity of bone marrow stromal cells in vivo in mice,[27] and blocking IL-7 in ovariectomized mice stimulated bone formation.[25] These observations will need to be confirmed in vivo in MM models.

Several mouse models of MM bone disease have been developed, which demonstrate osteoblast suppression in MM.[8,28,29] These include the SCID-hu model, in which human MM cells are inoculated into human fetal bone grafts implanted in SCID mice, and the 5T murine model in which a spontaneous MM, which developed in elderly mice, is transplanted into syngenic young mice. Both models show suppression of osteoblast activity and bone formation together with osteoclast activation in the bone marrow infiltrated by MM cells. These models have demonstrated the critical role of RANKL in osteoclast activation and bone lesion development in MM.[28,29] These models will be useful to investigate whether modulation of Runx2/Cbfa1 and/or IL-7 may affect bone lesions induced by MM, and if they are potential therapeutic approaches to increase bone formation in MM patients.

Role of Wnt signaling and Wnt inhibitors in MM bone disease

The Wnt signaling pathway plays an important role in the regulation of osteoblast formation either through its canonical pathway, mediated by β-catenin nuclear translocation, or in part through the non-canonical pathway.[30] The canonical Wnt signaling pathway is activated by Wnt 1/3a, which interacts with the Lrp5-6/Fzd receptor complexes.[30] This interaction induces the phosphorylation of the cytoplasmatic tail of the receptor, blocking the binding and the formation of the complex GSK3β, Axin, APC and β-catenin that normally promotes the phosphorylation and degradation of β-catenin. The increased levels of free β-catenin in the cytoplasm induces its translocation into the nucleus, where β-catenin affects gene expression, mainly through the activation of the transcription factor Lef1/TCF.[30] Recent evidence indicates that in murine systems, Wnt signaling promotes the proliferation, expansion and survival of pre and immature osteoblastic cells.[30] In particular, bone morphogenic protein (BMP)-2 or other osteogenic molecules induce osteoblastic differentiation of murine mesenchymal stem cells by stimulating Wnt signaling through the modulation of Wnt stimulators and/or inhibitors.[30,31] Several molecules negatively regulate canonical Wnt signaling. Dickkopfs (DKKs) including DKK-1, the secreted frizzled related proteins (sFRPs) such as sFRP1-4 and Wnt inhibitory factor (Wif-1) are the major soluble Wnt inhibitors present in murine osteoblasts, which block early osteoblast formation and induce the death of immature cells.[30] These factors also stimulate terminal osteoblast differentiation,[32] indicating a dual effect of these factors on Wnt signaling and osteoblastogenesis. A relationship between the Wnt signaling and Runx2 pathways has been recently shown in murine osteoblasts, suggesting that Wnt signaling and activation may promote osteogenesis by directly stimulating Runx2/Cbfa1 gene expression.[33]

In vivo mouse models support the role of Wnt signaling in the regulation of bone mass.[34] Transgenic mice overexpressing DKK-1 develop osteopenia while mice expressing a mutant LRP5 gene with low affinity for DKK-1 develop increased bone mass.[34]

The role of Wnt signaling in human osteoblastogenesis is under active investigation. Inactivating mutations of the LRP5 Wnt co-receptor cause osteoporosis,[35] indicating that Wnt activation may have an important role in human bone formation. In contrast, other workers have demonstrated that Wnt activation in human bone marrow cells suppresses osteogenic differentiation,[36,37] suggesting that Wnt signaling is required to maintain stromal cells in an undifferentiated state.

The potential involvement of Wnt signaling in the suppression of osteoblast formation and function in MM has been recently reported. Tian et al[38] found that primary $CD138^+$ MM cells overexpress DKK-1 as compared to plasma cells from MGUS patients and normal plasma cells. Further, using gene expression profiling, they showed a tight relationship between DKK-1 expression by MM cells and the occurrence of focal lytic bone lesions in MM patients.[38] High DKK-1 levels were also observed in bone marrow and peripheral sera in MM patients correlated with the presence of bone lesions.[38] Interestingly, patients with advanced disease, as well as human myeloma cell lines did not express DKK-1, suggesting that DKK-1 may mediate bone destruction in the early phases of disease.[38] In line with this hypothesis others failed to find a correlation between DKK-1 serum levels and the presence of bone lesions evaluated by conventional RX scan in stage II and III MM patients.[39]

A potential correlation between DKK-1 expression by MM cells, the presence of specific genetic abnormalities and the occurrence of focal bone lesions has been also investigated. In this study, MM patients were classified into five molecular subtypes on the basis of cyclin D1 expression and the presence of translocation in the immunoglobulin heavy-chain gene (TC). A higher incidence of focal bone lesions was observed in patients in the TC1 (11q13 or 6p21; cyclin D1 or cyclin D3) and TC2 (hyperdiploid but not 11q13; cyclin D1) groups as compared to TC4 (4p16; MMSET and FGFR3) and TC5 (16q23 or 20q11; c-maf or maf-b).[40] Among the patients with bone lesions, significantly higher DKK-1 expression levels were observed in

the TC1 and TC2 groups in comparison with the other groups, suggesting a potential link between the genetic abnormality and DKK-1 expression.

However, the mechanism by which DKK-1 production by MM cells is related to bone destruction is still unclear. Neutralizing anti-DKK-1 antibody can block the inhibitory effect of bone marrow plasma of MM patients on BMP-2 induced alkaline phosphatase expression and osteoblast formation by a murine mesenchymal cell line, but failed to block the inhibitory effects of MM cells on human bone marrow osteoblast formation.[18] In addition, only very high concentrations of DKK-1 are able to inhibit CFU-F and CFU-OB formation and to block β-catenin signaling in human bone marrow osteoprogenitor cells.[18]

Other mechanisms could be involved in DDK-1 mediated bone destruction in MM. For example, a link between cell adhesion and the Wnt pathway was recently reported. Wnt inhibitors such as DKK-1 are triggered by cell contact and modulate adhesion of leukemia cells to osteoblasts.[41] Possibly, DKK-1 production by MM cells could be involved in the adhesion of stromal cells and MM cells, which is critical for osteoclast activation[4] and Runx2/Cbfa1 mediated osteoblast inhibition.[18] Furthermore crosstalk between MM cells and the microenvironment can stimulate both DKK-1 and IL-6 production in human bone marrow cultures.[42]

MM cells may also produce other Wnt inhibitors, including SFRP-3/FRZB. FRZB is highly expressed by $CD138^+$ MM cells from patients as compared to MGUS patients and may contribute together with DKK-1 to the development of osteolytic lesions. sFRP-2 has been also reported to be produced by some human myeloma cell lines and by patients with advanced MM bone disease, and can inhibit osteoblast differentiation.[43] However, other investigators have not detected expression of sFRP-2 as well as sFRP-1 and sFRP-4 in MM patients.[18]

Data obtained in mouse models of MM suggest that the Wnt/β-catenin signaling pathway may be a target for the treatment of bone disease. Recent studies indicate that a pharmacological orally bio-available GSK-3α and 3β inhibitor increases markers of bone formation and bone mass in ovariectomized rats.[34,44] Studies in the SCID-hu mice model of MM[28] have shown that anti-DKK-1 increases bone mineral density and the number of osteocalcin positive osteoblasts compared to control mice.[45] Interestingly, a reduction of the number of osteoclastic cells was also observed, suggesting that Wnt signaling could be involved in the regulation of bone resorption. These studies suggest that the Wnt signaling pathway is a promising potential target for treatment of MM bone disease. However, further studies are necessary to clarify the involvement of Wnt signaling in MM induced bone destruction and the potential use and safety of Wnt inhibitors in MM, since β-catenin and the other molecules of the canonical Wnt signaling cascade have been linked to tumorigenesis.[34] Further, active Wnt signaling has been demonstrated in MM cells, suggesting that stimulation of Wnt signaling may increase MM cell proliferation.[46] Thus the potential utility of neutralization of DKK-1 in MM must be evaluated carefully.

Role of IL-3 in osteoblast inhibition by MM cells

IL-3 has been reported as a potential osteoblast inhibitor in MM patients.[47] In both murine and human system, IL-3 inhibited basal and BMP-2 stimulated osteoblast formation in a dose-dependent manner, without affecting cell growth. IL-3 blocked differentiation of pre-osteoblasts to mature osteoblasts in vitro, at concentrations comparable to those seen in bone marrow plasma from patients with MM.[47] IL-3 levels in bone marrow plasma from patients with MM were increased in approximately 70% of patients compared to normal controls or MGUS patients.[48] IL-3 is also produced by T lymphocytes in the myeloma bone microenvironment.[49] Importantly, bone marrow plasma from patients with MM with high levels of IL-3 inhibited OBL formation in human cultures, and this inhibition was partially

reversed by addition of a neutralizing antibody to human IL-3.[47] The inhibitory effect of IL-3 was increased in the presence of TNFα, a cytokine induced in the MM marrow microenvironment.[47] Interestingly, the effects of IL-3 were indirect and were mediated by $CD45^+/CD11b^+$ monocyte/macrophages in both human and mouse primary culture systems. IL-3 increased the number of $CD45^+$ hematopoietic cells in stromal cell cultures, and depletion of the $CD45^+$ cells abolished the inhibitory effects of IL-3 on osteoblasts.[47] Importantly, reconstitution $CD45^+$ depleted cultures with $CD45^+$ cells, restored the capacity of IL-3 to inhibit osteoblast differentiation. IL-3 is also involved in osteoclast activation in MM patients and can stimulate osteoclast formation in vitro.[48] These results suggest that IL-3 plays a dual role in the bone destructive process in MM, by both stimulating osteoclast and indirectly inhibiting osteoblast formation. Clearly these in vitro data should be confirmed in vivo MM models to better understand whether IL-3 modulation could be potentially useful in the treatment of MM bone disease.

Other mechanisms involved in the inhibition of bone formation in MM

In addition to blocking osteoblast formation, several studies suggest that MM cells may directly act on mature osteoblastic cells. Myeloma cells have been reported to inhibit osteoblast proliferation.[50] and up-regulate osteoblast apoptosis when co-cultured with osteoblast.[51] Moreover, osteoblasts obtained from MM patients with extensive bone lesions appear to be highly prone to undergoing apoptosis as compared to osteoblasts from patients without bone lesions.[51] Recently, Tinhofer et al[52] reported that MM cells induce apoptosis of osteoblasts when co-cultured with human osteoblastic cells. In addition to killing osteoblasts, human MM cells sensitized osteoblastic cells to cell death mediated by recombinant TRAIL, and in turn osteoblastic cells protected MM cells from TRAIL-mediated apoptosis.[52]

Effects of the ubiquitin-proteasome pathway and proteasome inhibitors in osteoblasts and bone formation: a potential therapeutic target in MM bone disease.

The ubiquitin-proteasome pathway is the major cellular degradative system for several proteins involved in cell proliferation and survival in MM cells.[53] Recently, Garrett et al demonstrated that this pathway may regulate osteoblast differentiation and bone formation in vitro and in vivo in mice.[54] The ubiquitin-proteasome pathway can modulate the expression of BMP-2,[54] which can induce osteoblast differentiation through the Wnt signaling[31] and regulates the proteolytic degradation of the osteoblast transcription factor Runx2/Cbfa1.[55] Different proteasome inhibitors that bind the catalytic β subunits of the 20S proteasome and block its activity are able to stimulate bone formation in neonatal murine calvarial bones.[54] A strong correlation between the capacity of these compounds to inhibit proteasomal activity in osteoblasts and their bone forming activity was also demonstrated.[54] Consistent with these in vitro observations, the administration of the natural proteasome inhibitors, PS1 and epoximicin, to mice increases bone volume and bone formation rate over 70% after 5 days,[54] indicating a potent stimulatory effect of these drugs on osteoblastic cells. The potential involvement of the ubiquitin-proteasome pathway in MM-induced osteoblast suppression is not known. However, these studies suggest a potential use of these drugs as anabolic agents in MM bone disease. This hypothesis is strongly supported by the in vivo observations obtained in MM patients treated with Bortezomib,[56,57] the first representative of this class of drugs with a potent anti-myeloma activity actually recommended for the treatment of MM patients in relapse or non-responder ones and under investigation as first line therapy. An increase of total alkaline phosphatase and in parallel of bone specific alkaline phosphatase has been reported in MM patients that respond to the treatment with Bortezomib, but not in non-responders.[56,57] No data are available on the potential effect of Bortezomib on other markers of bone formation or osteolytic lesions in MM. However, based on results obtained in mice,

Bortezomib may directly stimulate osteoblastic cells or osteoblast differentiation. Alternatively, the rapid apoptosis of MM cells induced by Bortezomib and their removal from the bone marrow could result in the recovery of osteoblast differentiation. Further studies will be necessary to further clarify the role of proteasome antagonists in bone formation in myeloma.

Role of osteoblasts in MM cell growth

The relationship between MM cells and osteoblasts is complex. Several studies indicate that osteoblastic cells may regulate MM cells growth and survival. Osteoblasts secret IL-6 in co-culture systems with MM cells,[20,58] contributing to the high IL-6 levels present in the bone marrow microenvironment that support MM cell growth. In addition, osteoblastic cells may also stimulate MM survival by blocking TRAIL-mediated apoptosis of MM cells through the secretion of osteoprotegerin (OPG), a decoy receptor or both RANKL and TRAIL.[59] Recently Yaccoby et al,[60] using a triple co-culture system with osteoclasts, osteoblasts and MM cells, reported that osteoblastic cells may attenuate the stimulatory effect of osteoclastic cells on MM cell survival, suggesting a potential dual role of osteoblastic cells on the growth of MM cells, dependent of the experimental conditions. Interestingly in the SCID-hu MM model, the same authors found that the stimulation of bone formation by the injection of mesenchymal stem cells resulted in an inhibition of MM growth.[61] These results suggest that increasing bone formation in MM patients could result in a reduction in tumor burden.

Summary

Multiple factors appear to be involved in the osteoblast suppression in MM. Wnt antagonists, blockade of Runx2 and soluble factors such as IL-3 and IL-7 have recently been identified as osteoblast inhibitors in MM (Figure 1). Further, myeloma cells may directly induce osteoblast

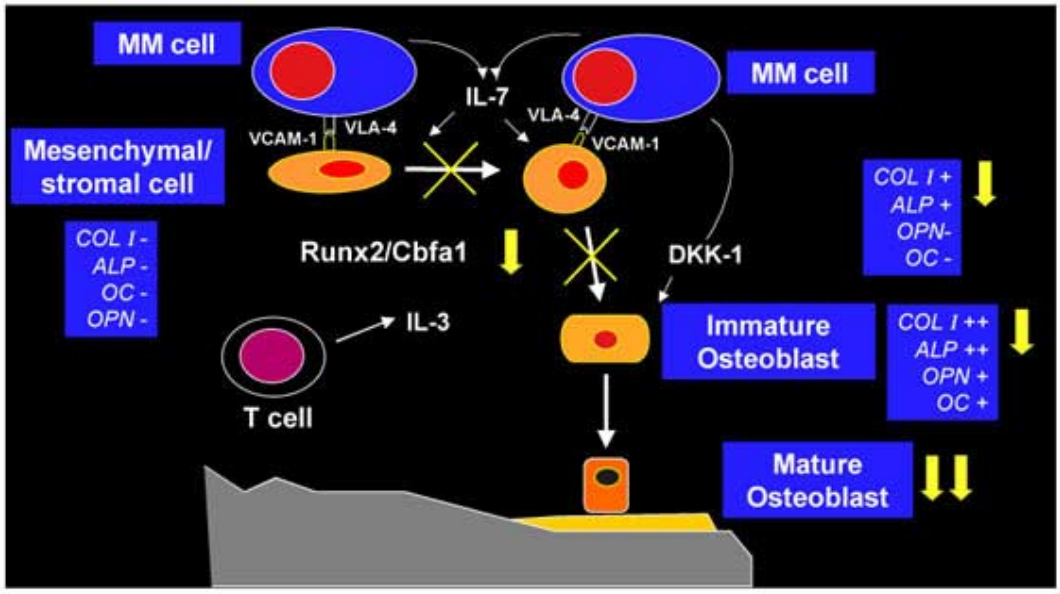
apoptosis via TRAIL. Emerging data also indicate that the ubiquitin-proteasome pathway may regulate osteoblast formation and differentiation, and that proteasome inhibitors may stimulate bone formation in vivo.

The identification of the pathophysiologic mechanisms responsible for osteoblast inhibition in MM should provide new therapeutic targets for this devastating complication of MM.

Figure legend

Several mechanisms are potentially involved in myeloma-induced inhibition of osteoblast formation and differentiation. Myeloma cells inhibit osteoblastogenesis by blocking Runx2 activity in mesenchymal and osteoprogenitor cells through the direct cell-to-cell contact with the involvement of VLA-4/VCAM-1 interaction. Soluble factors as IL-7 may contribute to the suppression of Runx2 activity by myeloma cells. The direct production of the Wnt inhibitor DKK-1 by myeloma cells could inhibit osteoblast formation. Finally, IL-3 overproduction in the myeloma microenvironment may be involved in the inhibition of osteoblast formation and differentiation.

Figure 1



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