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Review

Update on the pathogenesis of osteolysis in multiple myeloma patients

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Abstract. Multiple myeloma (MM) is a plasma cell malignancy characterized by the high capacity to induce osteolytic bone lesions that mainly result from an increased bone resorption related to the stimulation of osteoclast recruitment and activity. Although it is known that myeloma cells induce osteoclastic bone resorption, the biological mechanisms involved in the pathophysiology of MM-induced bone resorption have been unclear for several years. Recently, new data seem to elucidate which mechanism is critically involved in the activation of osteoclastic cells in MM. The critical osteoclastogenetic factor RANKL and its soluble antagonist osteoprotegerin (OPG) are the major candidates in the pathophysiology of MM bone disease. Human MM cells induce an imbalance in the RANKL/OPG ratio in the bone marrow environment that triggers the osteoclast formation and activation leading to bone destruction. The role or RANKL/OPG system and other osteoclast stimulating factors in the pathophysiology of MM bone disease are summarized in this update.

Key words: Myeloma, osteoclast, osteolysis

Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by the high capacity to induce bone destruction (1-2). Almost all patients with MM have osteolytic bone lesions more frequently localized at the spine, ribs, skull and pelvis even if any site of the skeletal can be involved. Osteolytic bone lesions in MM patients mainly result from an increased bone resorption related to the stimulation of osteoclast recruitment and activity (2-4).

The histomorphometric studies, performed in MM patients, have demonstrated that the increase of osteoclastogenesis and osteoclast activity is an early event that occurs in close contact with myeloma cells (2, 5-6) suggesting that local factors rather than systemic mechanisms are involved in the pathogenesis of osteolytic bone lesions. An increase of osteoblastogenesis has been also observed in the early phase of disease or in patients with a low myeloma cell burden. On the contrary, MM patients with high plasma cell infiltrate or active disease are characterized by a lower number of osteoblasts and a decreased bone formation that contributes together with the increased osteoclast activity in the development of bone lesions (2, 5).

Although it is known that myeloma cells induce osteoclastic bone resorption, the biological mechanisms involved in the pathophysiology of MM-induced bone resorption have been unclear for several years.

Osteoclast activating factors

First, Mundy et al. (7) demonstrated that the conditioned media of human myeloma cells stimula-

ted osteoclast activity but, despite many suggested candidates, the critical osteoclast activating factors (OAFs) involved have resisted to be identified. It has been suggested that myeloma cells are able to produce in vitro several osteoclastogenic cytokines such as IL-6, IL-1β, TNF-α, HGF and PTHrP (Figure 1) but most of the studies on MM samples in vivo have been inconclusive because of the presence of other contaminating cells (stromal cells or lympho-monocytes) as demonstrated by the lack of IL-6 and IL-1β production or the rarity of PTHrP expression by high purified myeloma cells (3-4, 8-9). It has been also reported that myeloma cells induce the release of osteoclastogenic cytokines such as IL-6 or IL-11 by stromal/osteoblastic cells underlining the potential role of the microenvironment in the activation of osteoclastic cells (9-12).

However, none of all these cytokines has been demonstrated to be critical in the induction of the bone destruction in vivo and in MM patients or correlated with the extension of the MM bone disease.

Osteoprotegerin (OPG)/RANKL/RANK system

Recently, two molecules belonging to the TNF receptor-ligand superfamily, the osteoprotegerin (OPG) and its ligand OPGL namely the receptor activator of NF-kB ligand (RANKL) also known as TNF-related activation-induced cytokine (TRANCE) have been identified as critical in the regulation of osteoclast activity leading to a new paradigm in the bone biology (13-14). Extensive studies have shown that OPG and RANKL exert a coupled control of bone resorption (13-22) (Figure 2).

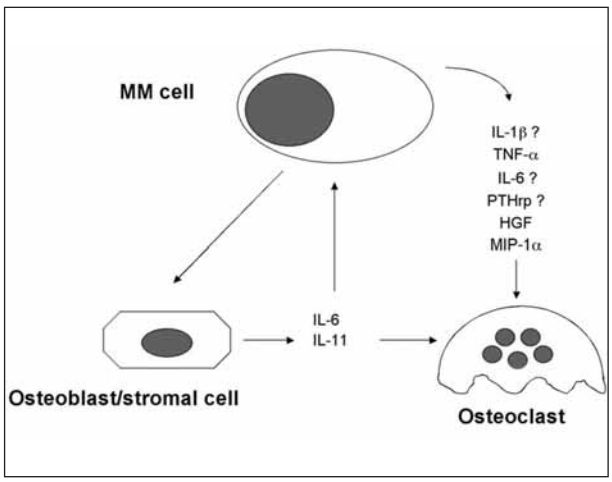

Figure 1. Osteoclast activating factors in MM. Myeloma cells activate osteoclasts directly or through the interaction with stromal/osteoblastic cells. Several osteoclast activating factors (OAFs) can be involved in MM-induced bone destruction

RANKL is a polypeptide of 217 aminoacids that exerts its biological activity both in a trans-membrane form of about 40-45 kD and in soluble one of 31 kD. It has been demonstrated that stromal/ osteoblastic cells express RANKL in response either to systemic factors such as PTH, dexamethasone and vitamin D_3 or local osteoclastogenic cytokines IL-1, TNF and IL-11 (13-16). RANKL directly induces osteoclastogenesis together with M-CSF and inhibits osteoclast apoptosis by binding to its specific receptor (RANK) present on osteoclast progenitors and mature osteoclasts (15, 16, 18-19) (Figure 2). More recently, it has been suggested that activated T lymphocytes, other than stromal/osteoblastic cells, produce RANKL and may maintain bone homeostasis through the cross-talk between RANKL production and interferon-γ (IFN-γ) secretion (23). In physiopathological conditions, such as arthritis, activated T cells are capable of regulating bone loss through the expression of RANKL (24-25) (Figure 2).

OPG is a soluble decoy receptor of about 100-110 kD, produced by stromal/osteoblastic cells, that antagonizes the effects of RANKL on osteoclastic cells inhibiting bone resorption (13-14, 17) (Figure 2). It has been shown that OPG binds RANKL and prevents the interaction between RANKL and its receptor RANK blocking the osteoclast formation (13-14, 17). The critical role of OPG/RANKL system in the regulation of bone resorption has been confirmed in mouse models (13-17, 19-22). The presence of severe osteoporosis with fractures has been reported in OPG knockout mice (17, 20) and the development of osteopetrosis is observed in transgenic mice overexpressing OPG (16) or in RANKL and RANK knockout mice (13-15, 21-22). Moreover, RANKL administration in mice induces a dose dependent hypercalcemia and consistently OPG is able to block malignant hypercalcemia in mouse models (26).

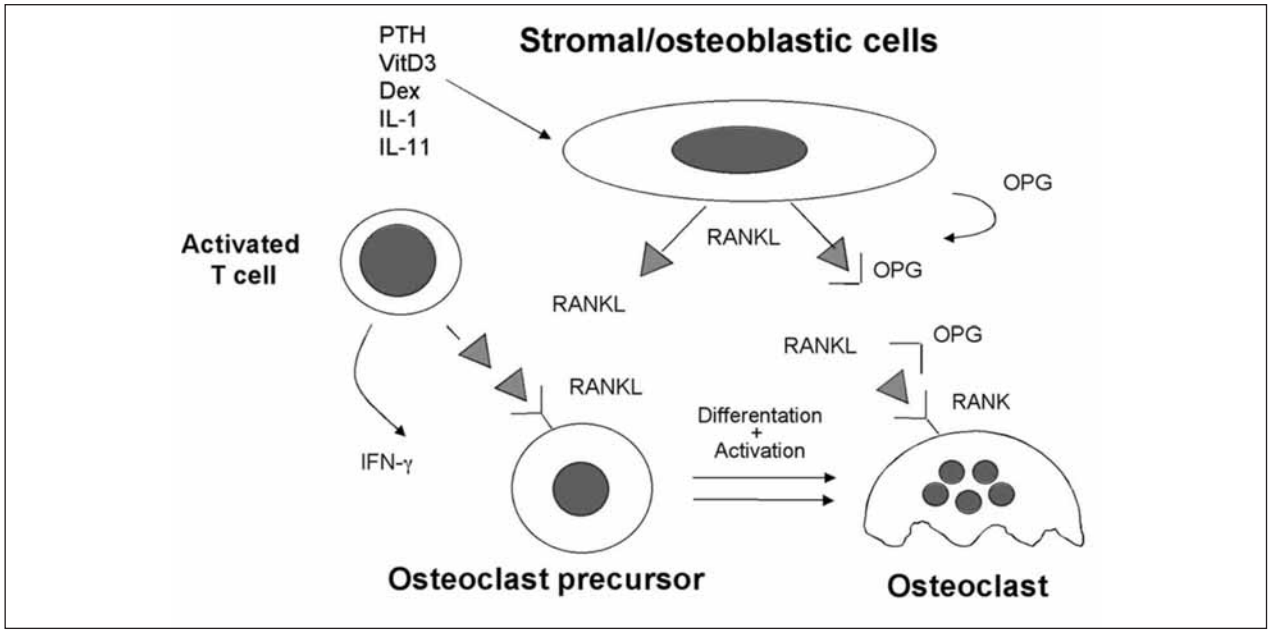

Figure 2. Role of OPG and RANKL in bone biology. RANKL and its soluble antagonist OPG are new factors identified as critical in the regulation of osteoclast formation and activation. RANK/OPG system exerts a coupled control of bone resorption. RANKL is expressed either by stromal osteoblastic cells in response to local and systemic factors or by activated T lymphocytes and it induces osteoclast differentation and activation. OPG is a soluble factor that blocks the stimulatory effect of RANKL on osteoclasts and inhibits bone resorption

Role of RANKL/OPG in the pathophysiology of MM-induced bone disease

Because RANKL and its soluble antagonist OPG are likely to play a critical role in the regulation of bone resorption and the OPG/RANKL ratio in stromal/osteoblastic cells determines the level of osteoclast formation we as well as other authors have investigated the potential involvement of this system in MM-induced bone destruction.

The direct RANKL expression by human myeloma cells is controversial. RANKL mRNA and protein have not been found in several human myeloma cell lines (HMCLs) (27-28). Consistently, high-purified $CD138^+$ MM cells obtained from MM patients failed to express RANKL mRNA. RANKL was not also detected in the conditioned medium of fresh MM cells and HMCLs (29). Moreover, RANKL immunostaining, performed by different groups on bone marrow (BM) biopsies of MM patients using a specific anti-RANKL mAb has shown that myeloma cells are negative for RANKL expression (27-28, 30). More recently, Shaughnessy et al. (31), using the microarray technology, confirmed that RANKL has not been detected in any MM cells of either 83 osteolytic or 87 non-osteolytic MM patients as well as in normal BM plasma cells. All these experimental observations clearly demonstrate that human myeloma cells do not express or directly produce the critical osteoclastogenic factor RANKL. In contrast, other authors found that human myeloma cells produce RANKL (32-35). In particular, Farrugia et al. (32) have shown that sorted $CD38^{+++}$ subpopulation expressed RANKL protein by flow cytometry and RANKL mRNA as well as its receptor RANK mRNA by RT-PCR. However they found that also $CD38^+$ cells that are not myeloma cells express RANKL suggesting that the direct RANKL production by myeloma is not a critical determinant in myeloma-induced osteoclast formation.

On the contrary, strong evidence suggests that human MM cells induce RANKL expression in stro-

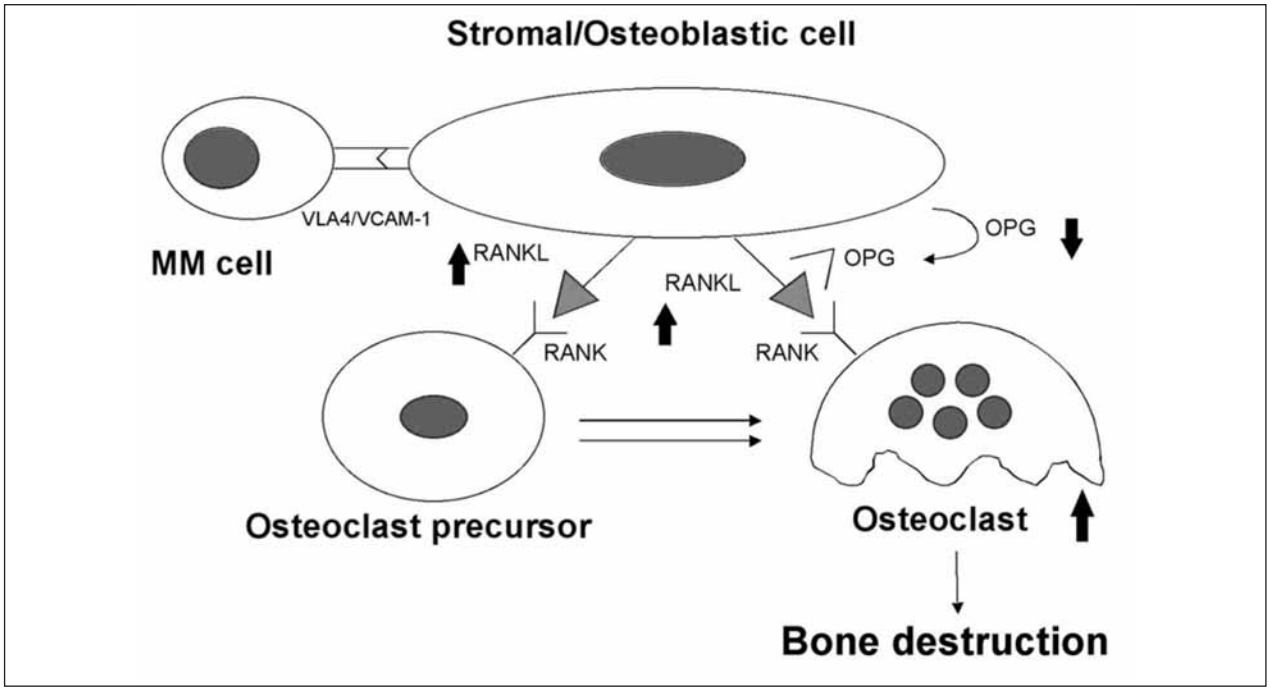

Figure 3. MM-induced imbalance of RANKL and OPG in BM stromal/osteoblastic cells. MM cells upregulate RANKL and downregulate OPG in stromal/osteoblastic cells through the cell to cell contact. The RANKL/OPG imbalance in favor of RANKL induces osteoclast activation and bone destruction

mal cells and they decrease OPG expression and secretion by osteoblastic cells inducing an imbalance of OPG/RANKL ratio in favor of RANKL (27-28, 30) (Figure 3). RANKL up-regulation has been observed at both mRNA and protein level in a co-culture system with HMCLs and either human BM stromal cells (BMSC)/pre-osteoblastic cells (28) or primary murine stromal cells (27). MM cells also inhibit OPG expression and secretion by osteoblastic cells in a co-culture system (27-28). The cell-to-cell contact is critical in the induction of RANKL in BMSC by MM cells as demonstrated by the lack of effect on RANKL expression in transwell system without cellular contact. In particular the cell adhesion between MM cells and BMSC is mediated by VLA-4/VCAM-1 integrin system (9-10). It has been reported that blocking antibody anti-VLA-4 completely blunted the induction of RANKL by MM in human BMSC (28). The role of cell adhesion and VLA-4/VCAM-1 interaction in the activation of osteoclastic cells by myeloma cells has been also reported in a murine model of MM-induced bone disease showing that the blocking of VLA-4 binding of myeloma cells to VCAM-1 on stromal cells decreases the release of bone by resorbing factors by stromal cells and suppresses the development of osteolytic bone lesions (36).

RANKL induction in BMSC by MM cells is critical in the formation of osteoclastic cells as demonstrated by the capacity of the RANK-Fc, a molecule made by fusing the Fc portion of immunoglobulin to a soluble form of the RANK receptor that antagonizes RANKL/RANK interaction, to block in vitro osteoclastogenesis in a co-culture system with murine stromal cells and MM cells (27).

Growing evidence suggests that T lymphocytes may also regulate bone resorption and maintain bone homeostasis through the cross-talk between RANKL and IFN-γ, a cytokine that strongly suppresses osteoclastogenesis (23). In physiopathological conditions, such as arthritis, activated T cells are capable of regulating bone loss through the expression of RANKL (24-25). In addition, recent data highlight the invol-

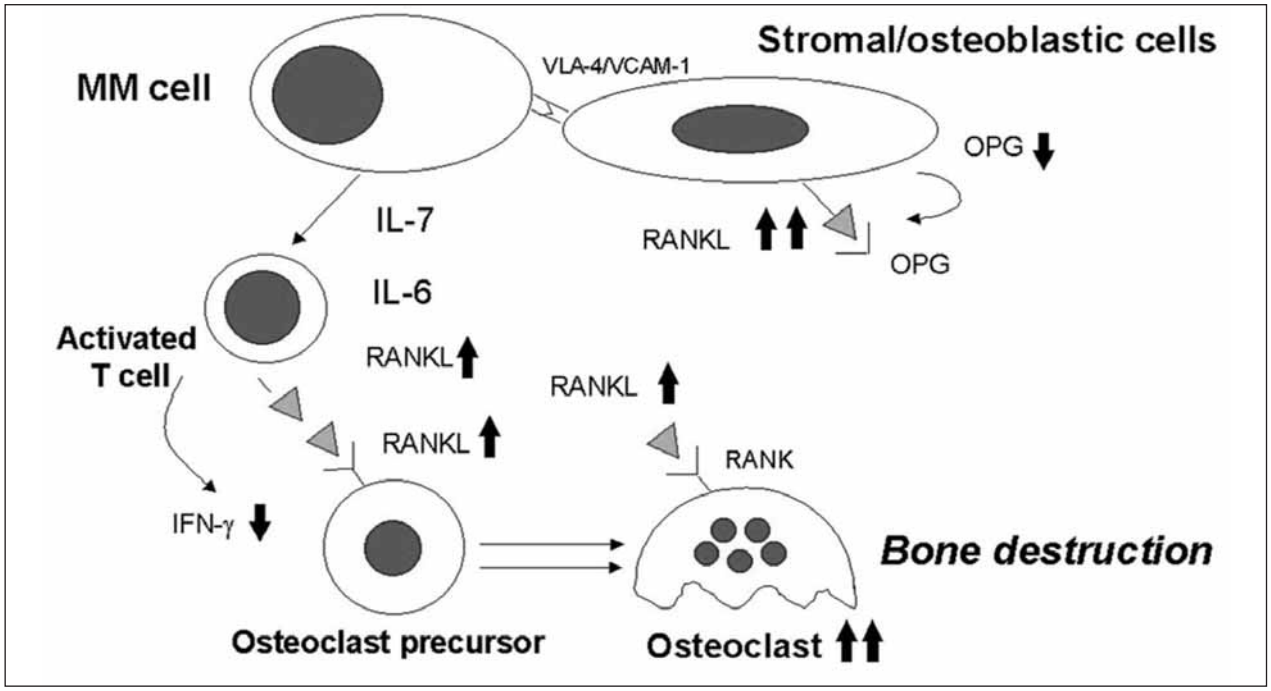

Figure 4. Model for MM-induced osteoclastogenesis through RANKL induction. Myeloma cells induce an imbalance in the OPG/RANKL ratio in stromal/osteoblastic cells through the cell-to-cell contact. In addition myeloma cells stimulate RANKL and down-regulate IFN-γ secretion by T cells at least in part through the direct release of IL-7 or indirect involvement of the high IL-6 levels induced by myeloma cells in bone environment. The high BM expression and level of the critical osteoclastogenic factor RANKL associated with lower levels of OPG induce the activation of osteoclasts and trigger the bone destruction in MM patients

vement of RANKL expressed by T lymphocytes in the mechanism of hypercalcemia in adult T-cell leukemia (37). These observations prompted us to investigate whether MM cells could also affect RANKL expression by T lymphocytes. We found that HMCLs upregulate RANKL expression and secretion in activated T cells in a transwell co-culture system and similarly fresh purified MM cells induce RANKL in autologous T cells (29). In the same system a down-regulation of IFN-γ was also observed. The up-regulation of RANKL in T lymphocytes by MM cells seems to be mediated by the release of soluble factors. Among the molecules that could be responsible for the stimulation of RANKL, we focused our attention on IL-7. Recently it has been postulated that IL-7 might be involved in osteoclast activation because IL-7 stimulates RANKL production by T cells in vitro (38) and induces bone loss in vivo by induction of RANKL (39). We found that HMCLs and fresh MM cells secrete IL-7 in the presence of IL-6 (29). The role of IL-7 on RANKL stimulation in T lymphocytes by myeloma cells was confirmed by the inhibitory effect exerted by an antibody anti -IL-7 in the co-cultures; furthermore we found that IL-7 neutralization inhibited MM-induced in vitro osteoclastogenesis (29). An inhibitory effect on RANKL in the co-cultures was also observed in the presence of anti-IL-6 mAb. It is likely that IL-6 is indirectly involved in the mechanism underlying RANKL stimulation by HMCLs because no evidence indicates that IL-6 stimulates RANKL in T lymphocytes or in other cell systems (40).

A vicious loop between IL-6 and IL-7 in MM can be hypothesized because it has been recently demonstrated that IL-7 stimulates IL-6 secretion by BM stromal cells (41) and our data indicate that IL-6 induces IL-7 in MM cells. Thus, high levels of IL-6 in the BM environment could induce IL-7 production by MM that in turn contributes to maintain high IL-

6 levels and to stimulate RANKL in T cells. The potential involvement of IL-7 is supported by the "in vivo" finding of higher IL-7 levels in peripheral serum and BM plasma of MM patients than in normal subjects (29).

RANKL/OPG imbalance in the BM environment of MM patients

Different groups have shown that MM patients have an imbalance in the OPG/RANKL expression in the BM environment confirming the "in vitro" experimental data. Giuliani et al. (28) showed that ex vivo BM specimens obtained from MM patients over-express RANKL mRNA in comparison with those from healthy donors. Moreover, immunostaining on BM biopsies has demonstrated an increase of the number of RANKL positive stromal cells together with a reduction of OPG expression in trabecular osteoblasts of MM patients with osteolytic lesions as compared to healthy subjects. Roux et al. (30) tested RANK and RANKL expression by immunohistochemistry in 15 MM patients, 6 patients with MGUS and in 10 normal subjects. They found that RANKL is expressed by endosteal bone surface, vessels and by vimentin positive stromal cells but not by plasma cells. Moreover, they confirmed that stromal cells of MM patients presented significantly higher levels of RANKL expression as compared to normal and MGUS subjects. Finally, Pearse et al. (27) confirmed with both immunohistochemistry and *in situ* hybridization that MM patients have an increased RANKL staining with a decreased OPG expression in the BM as compared to healthy subjects or non-MM B cell malignancies.

They have also found that, other than stromal cells, $CD3^+$ activated T cells in the BM biopsies express RANKL (27), indicating that RANKL expression is increased in MM-infiltrated environment by the interaction of malignant plasma cells with both stromal cells and activated T cells. In line with this observation Giuliani et al. (29) found that purified $CD3^+$ T lymphocytes obtained from MM patients with extensive skeletal destruction are activated and they express RANKL mRNA in contrast with those without bone lesions (29). The presence of activated T cells in MM patients was previously demonstrated by other authors (42), confirming the potential involvement of T lymphocytes in MM-induced bone disease.

The imbalance of RANKL/OPG system observed in the BM environment has been confirmed by the finding of high RANKL serum levels and reduced OPG levels in MM patients as compared to normal subjects (28,43-44). Seidel et al. (43) showed that OPG levels were decreased to a greater extent in patients with osteolytic lesions as compared to patients without bone disease. More recently, Terpos et al. (44) have measured soluble RANKL and OPG in 121 newly diagnosed MM patients showing that serum RANKL levels and RANKL/OPG ratio were elevated in MM and correlated with bone disease and with markers of bone resorption. Moreover, RANKL/OPG ratio, together with β_2-microglobulin and C-reactive protein were independent prognostic factors predicting survival in MM patients.

Effect of RANKL system inhibition in murine models of human MM bone disease

The critical role of RANKL in the MM-induced bone disease has been further confirmed in murine models of human MM bone using RANKL specific inhibitors OPG or RANK-Fc. Pearse et al. (27) found that RANK-Fc completely blocks bone destruction reducing the number of osteoclastic cells in severe combined immunodeficiency (SCID) /ARH-77 mouse model and in the SCID-hu-MM mice model injected with primary MM cells. Administration of RANK-Fc also caused a marked reduction of tumor burden assessed histologically and serum paraprotein in the SCID-hu-MM mice accompanied by restoration of OPG and RANKL expression within the human xenograft, further supporting the causal association between MM bone destruction and RANKL/OPG deregulation. Other authors have demonstrated that also OPG inhibits the development of osteolytic lesions in 5T2 MM murine models (35) and decreases both osteolytic lesions and tumor burden in 5T33 MM murine models (45). All these data suggest that blocking bone resorption induced by RANKL may decrease tumor burden as well as bone destruction in MM patients supporting the critical role of the environment in MM cells growth.

Role of MIP-1α in MM-induced bone destruction

MIP-1α is a chemokine recently suggested as a new OAF in MM induced bone disease. MIP-1α is chemoattractant for human osteoclasts (46) and induces osteoclast formation in vitro in rat marrow cultures (47). It has been demonstrated that MIP-1α is produced directly by several HMCLs and fresh MM cells in the majority of MM patients (48-50). Higher levels of MIP-1α protein and mRNA have been observed in BM plasma and isolated BM cells of MM patients as compared to normal subjects (48). Moreover blocking anti-MIP-1α or anti its receptor CCR5 Abs reduce MM-induced in vitro osteoclast formation (48,50). Anti sense anti-MIP-1α is also able to block bone destruction in SCID/ARH-77 mouse model (51). A strong association between MIP-1α mRNA expression by purified MM cells and active bone disease has been demonstrated in MM patients by microarray technology (52). All these evidences suggest that MIP-1α is a potential candidate as OAF in MM involved in the osteoclast activation in MM patients, even if MIP-1α is not a critical factor for the osteoclast formation and activation such as RANKL. The potential relationship between MIP-1α and RANKL has been evaluated in human and murine system with controversial results. It has been demonstrated that MIP-1α induces RANKL in murine stromal cells (50) and requires RANKL for its effects on osteoclastic cells as demonstrated by the lack of the effect of MIP-1α in knock-out RANK $^{-/-}$ mice (53). These evidences suggest that RANK/RANKL signaling pathway seems to be essential in mice for the osteoclastogenic effect of MIP-1α in vivo leading further to the notion that RANKL is the final critical factor in the osteoclast activation. On the contrary, Han et al. have demonstrated that the osteoclastogenic effect of MIP-1α is independent of RANKL in showing that MIP-1α directly stimulates the osteoclast progenitors without increasing the expression of RANKL in human nonadherent BM mononuclear cells (54). This notion supports the hypothesis that MIP-1α, produced by myeloma cells, independently in combination with RANKL that is produced by BM stromal cells in response to myeloma cells enhances the osteoclast formation in MM patients.

Osteoblast function in MM patients

Histomorphometric studies and biochemical indicators of bone turnover in MM have shown that although osteoclast number and function are increased in MM, the key difference in vivo between the presence and absence of lytic lesions is that osteoblasts are fewer and less active in patients with lytic lesions. In the early stages of MM, bone formation is increased reflecting the coupling of resorption to formation. However, as the disease progresses, bone formation is decreased and this leads to an uncoupling resorption and formation and rapid bone loss (5). This suggests that myeloma cells could first stimulate osteoblastic function during the early stages of the disease then inhibit it or even be toxic for these cells during overt expansion of the tumor. Few inhibiting interactions between osteoblasts and MM have been described so far. Recently, Shaughnessy et al (55) reported the production of the potential osteoblast inhibitor DKK1 by myeloma cells. Actually, DKK1 can block Wnt signaling, an important pathway involved in osteoblast differentiation and function, and its overexpression in MM is associated with lytic bone disease. However, further experiments need to be done to confirm these data. Other potential means for the interplay between osteoblasts and myeloma cells could be through homophilic binding by the neural cell adhesion molecule NCAM/CD56 (56). NCAM-NCAM homophilic binding between CD56+/NCAM-positive MM cells and osteoblasts may induce a decrease in osteoblast function as previously described for osteocalcin production (10).

Conclusions

In conclusion, we can now propose a new pathophysiological model of the MM bone destruction that highlights the critical role of the OPG/RANKL system. Myeloma cells induce an imbalance in the OPG/RANKL ratio in stromal/osteoblastic cells through the cell-to-cell contact. In addition myeloma cells can stimulate RANKL and downregulate IFN-γ secretion by T cells at least in part through the direct release of IL-7 or the indirect invol-

vement of the high IL-6 levels induced by myeloma cells in bone environment (Figure 4).

The high BM expression and level of the critical osteoclast activating factor RANKL associated with lower levels of the osteoclastogenic inhibitor OPG induce osteoclast activation and bone destruction in MM patients.

Other OAFs produced directly by MM cells, in particular the chemokine MIP-1a, contribute in the activation of osteoclastic cells through the final common mediator RANKL.

The recognition of the critical role of RANKL/OPG system in the pathophysiology of MM-induced osteoclastogenesis gives the rational design for future new therapeutical approach using RANKL specific inhibitors OPG or RANK-Fc for the treatment of MM-induced bone disease as suggested by a recent phase I study with recombinant OPG in MM patients (55).

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