Microenvironment for myeloma growth and drug resistance

Masahiro ABE

Bone provides a unique microenvironment for myeloma (MM) cell growth and survival, including niches to foster clonogenic MM cells. MM cells stimulate bone resorption by enhancing osteoclastogenesis, while suppressing bone formation by inhibiting osteoblastic differentiation from bone marrow stromal cells, leading to extensive bone destruction with rapid loss of bone. MM cells alter the microenvironment through bone destruction in bone where they colonize, which in turn favors tumor growth and survival, thereby forming a vicious cycle between tumor progression and bone destruction. MM is still difficult to be cured despite the recent implementation of new agents, and its bone disease also remains a significant clinical problem. Further elucidation of the molecular mechanisms of tumor-bone interactions and tumor growth in the bone microenvironment will provide us with new approaches that have a real impact on both bone disease and tumor progression.

Key words: myeloma niche, Pim-2, Glycolysis, clonogenic myeloma cells, side population

1. Introduction

Multiple myeloma (MM) has a unique propensity to expand almost exclusively in bone, and develop devastating bone destruction. MM cells stimulate bone resorption by enhancing osteoclastogenesis, and suppress bone formation by inhibiting osteoblastic differentiation from bone marrow stromal cells, leading to extensive bone destruction. Cytokines aberrantly over-produced by MM cells, including macrophage inflammatory protein (MIP)-1α and MIP-1β as well as receptor activator of nuclear factor-κB (RANK) ligand up-regulated in bone marrow stromal cells play a major role in the enhancement of osteoclastogenesis and bone resorption in MM. In contrast to enhanced osteoclastogenesis in MM, osteoblastic differentiation from bone marrow stromal cells is suppressed to cause extensive bone destruction with rapid loss of bone. Although a canonical Wingless-type (Wnt) signaling pathway plays a critical role in osteoblast differentiation, MM cells have been demonstrated to secrete soluble inhibitors of the canonical Wnt signaling pathway, including Dickkopf-1 (DKK-1) and secreted Frizzled-related protein (sFRP)-2. DKK-1 is also secreted by stromal cells and osteoblasts in the presence of MM cells. The levels of DKK-1 in sera and bone marrow plasma are increased in MM patients and correlated with the presence of osteolytic lesions. Besides these Wnt inhibitors, several factors derived from MM cells and/or their surrounding microenvironment in bone such as IL-3, IL-7, TNFα, and TGF-β appear to suppress osteoblastic differentiation. More recently, activin A and sclerostin, potent inhibitors of osteoblastogenesis, have been demonstrated to be over-produced in bone lesions in MM. Therefore, multiple factors act together to eventually develop extensive bone destruction in MM.

Bone provides a unique microenvironment for normal hematopoietic and cancer cell growth, including niches to foster cancer as well as hematopoietic stem cells. The fact that MM cells grow and expand almost exclusively in the bone marrow suggests the importance of the bone marrow microenvironment in supporting MM cell growth and survival. While MM cells perturb a normal bone metabolism to develop bone destruction, a crosstalk between MM cells and the microenvironment in bone lesions leads to the progressive vicious cycle phase of tumor growth and bone destruction. In addition to the growth advantage that MM cells have acquired through genetic alterations and clonal selection, the cellular microenvironment skewed in MM endows MM cells with an additional growth and survival potential. In this review, current understanding of the mechanisms of MM progression and drug resistance in bone lesions and therapeutic approaches based on these mechanisms will be discussed.
Autocrine activation of VLA-4 via VLA-4 or VLA-5 confers cell adhesion-mediated drug resistance to enhance osteoclastogenesis. Importantly, the adhesion of MM cells and their MIP-1 production has been suggested to promote MM cell homing or colonization in the bone marrow, which up-regulates the production by stromal/pre-osteoblastic cells of growth and anti-apoptotic factors and CAM-DR for MM cells. In contrast to the above factors over-produced in the bone marrow in MM, a murine model permissive for MM growth revealed decreased levels of host-derived adiponectin. Interestingly, adiponectin appears to be decreased in the serum of patients with MGUS that subsequently progressed to MM. Adiponectin was found to increase MM cell apoptosis through activation of the AMP kinase pathway, and tumor burden and bone disease were enhanced in MM models using adiponectin-deficient mice.

2. MM niche

By analogy with the formation of a cancer pre-metastatic niche by the migration and seeding of hematopoietic cells to metastatic sites, MM cell-induced cell types in MM bone lesions, namely osteoclasts, vascular endothelial cells, and bone marrow stromal cells with defective osteoblastic differentiation create suitable microenvironment for MM cell growth and survival as a feeder for MM cells. Such a microenvironment can be construed as the “MM niche” (Figure 1).

2.1. Bone marrow stromal cells

Among cell components in the bone marrow, the roles of bone marrow stromal cells in MM cell growth and survival have been most extensively studied. The interaction between MM cells and stromal cells confers MM cell homing, growth, survival, and resistance to chemotherapy. MM cells stimulate stromal cells to produce various growth and anti-apoptotic factors for MM cells, including IL-6, IGF-1, SDF-1α, IL-21, B-cell-activating factor (BAFF), and VEGF, while inducing RANK ligand to enhance osteoclastogenesis. Importantly, the adhesion of MM cells to stromal cells and their extracellular matrices (ECM) via VLA-4 or VLA-5 confers cell adhesion-mediated drug resistance (CAM-DR) on MM cells. Autocrine activation of VLA-4 on MM cells by MM cell-derived MIP-1 and up-regulation of MIP-1 production by MM cells through VLA-4-VCAM-1 interactions appear to form a positive feedback loop between the adhesion of MM cells and their MIP-1 production. In addition to osteoclastogenesis, MIP-1 has been suggested to promote MM cell homing or colonization in the bone marrow, which up-regulates the production by stromal/pre-osteoblastic cells of growth and anti-apoptotic factors and CAM-DR for MM cells. In contrast to the above factors over-produced in the bone marrow in MM, a murine model permissive for MM growth revealed decreased levels of host-derived adiponectin. Interestingly, adiponectin appears to be decreased in the serum of patients with MGUS that subsequently progressed to MM. Adiponectin was found to increase MM cell apoptosis through activation of the AMP kinase pathway, and tumor burden and bone disease were enhanced in MM models using adiponectin-deficient mice.

2.2. OCs

Other than stromal cells, OCs induced by MM cells are also among the major cellular components of the bone marrow microenvironment. Intriguingly, OCs enhance MM cell growth and survival via an interaction between MM cells and OCs. The effects of OCs on MM cell growth seem more potent than those of primary bone marrow stromal cells, while MM cells more firmly adhere to stromal cells and confer drug resistance. Similar to bone marrow stromal cells, OCs protect MM cells from apoptosis induced by anti-cancer drugs, including doxorubicin. BAFF and a proliferation-inducing ligand (APRIL), members of the tumor necrosis factor (TNF) family, have been implicated as survival factors for myeloma cells. OCs are a predominant cell type producing these factors in the bone marrow in MM. Treatment with TACI-Fc, a decoy receptor for both BAFF and APRIL, markedly enhanced apoptosis of MM cells in the presence of OCs, indicating a supporting role for BAFF and APRIL in OC-mediated MM cell growth and survival. Ge et al. reported that the serine protease fibroblast activation protein (FAP) is up-regulated in OCs after coculture with MM cells. Knockdown of FAP expression reduced MM cell survival in cocultures. In addition, FAP is also expressed in osteogenic cells, bone marrow stromal cells, adipocytes, and vascular endothelial cells in the bone marrow. Hexokinase II (HKII), a key enzyme of glycolysis, is widely over-expressed in cancer cells. HKII has been demonstrated to be over-expressed in most MM cells. The inhibitor of HKII 3-bromopyruvate (3BrPA) promptly and substantially suppresses ATP production and induces cell death in MM cells. Interestingly, cocultures with OCs but not bone marrow stromal cells enhanced the phosphorylation of Akt along with an increase in HKII levels and lactate production in MM cells. The PI3K-Akt signaling has been demonstrated to phosphorylate HKII to facilitate its association with a mitochondrial membrane, and thereby stabilize HKII to post-translationally increase intracellular HKII levels. Consistently, the enhancement of HKII levels and lactate production in MM cells by OCs...
were mostly abrogated by the PI3K inhibitor LY294002, suggesting activation of glycolysis in MM cells by OCs via the PI3K-Akt-HKII pathway. Treatment with 3BrPA was able to markedly induce cell death in MM cells even in cocultures with OCs.

These observations collectively suggest the contribution of OCs to the aggressiveness or drug resistance of MM cells. Thus, MM cells and OCs directly interact to augment their growth and activity, thereby forming a vicious cycle that leads to extensive bone destruction and MM expansion (Figure 1). These observations provide a rationale for therapeutic strategies targeting the OC-MM interaction.

### 2.3. Angiogenesis

Similar to osteoclastogenesis, angiogenesis is enhanced in the bone marrow in MM in parallel with tumor progression. Bone marrow stromal cells as well as MM cells secrete angiogenic factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). In vascular endothelial cells, the VEGF receptor VEGF-R2 mutually interacts with αvβ3 integrin after the binding of their respective ligands to efficiently transduce their downstream signaling and induce angiogenesis. Of note, OCs constitutively secrete a large amount of the proangiogenic factor osteopontin, a ligand of αvβ3 integrin, which cooperates with VEGF secreted from MM cells to enhance angiogenesis and induce the production of osteoclastogenic activity by vascular endothelial cells. Osteopontin is subject to enzymatic cleavage, and its fragments become functionally active. OCs also produce matrix metalloproteinase (MMP)-9, which has been demonstrated to be responsible for angiogenesis induced by OCs. MMP-9 may affect the activity of other factors elaborated in bone lesions in MM including osteopontin. Therefore, a close link between MM cells, OCs, and vascular endothelial cells can be established in MM bone lesions, thereby forming a vicious cycle involving bone destruction, angiogenesis, and MM expansion (Figure 1).

### 2.4. Niches for MM cancer stem cell (CSC)-like cells

The presence of CSCs or cancer-initiating cells have been demonstrated in various types of cancers, and regarded as a predominant cause of drug resistance. Likewise, MM CSCs have been postulated, and are considered to contribute to disease relapse through their drug-resistant nature. Thus, MM CSCs become among the most important targets in the treatment for MM. However, MM CSCs are still conceptual in many senses, because of the lack of sensitive and reliable methods to identify them. Besides clonotypic B cells, phenotypically distinct MM plasma cell fractions have been demonstrated to possess a clonogenic capacity, leading to long-lasting controversies regarding the cells of origin in MM or MM-initiating cells. It is hard to explain the clonogenic capacity of differentiated MM cells only with the notion of a one-way hierarchical model from MM CSCs to differentiated MM cells. MM CSCs may not be a static population but survive as phenotypically and functionally different cell types via the transition between stem-like and non-stem-like states in local microenvironments, as observed in other types of cancers.

Recently, Chaidos et al. elegantly addressed a phenotypic and functional interconvertible state between CD138+ and CD138− cells. They identified a distinct subpopulation with CD19−CD38+CD319−CD138+ immunophenotype in clonotypic cells in both the bone marrow and circulation in patients with MM, and termed as pre-plasma cell. They identified a CD138+ plasma cell to pre-plasma cell transition within clonotypic subpopulations in the bone marrow samples of 30 patients with MM by dynamic mathematical models. Together with the observations with the forward transition of clonotypic CD19+ cells and/or CD138low cells to differentiated CD138+ MM plasma cells, bidirectional transitions between differentiated and undifferentiated MM clonotypic cells are suggested in patients with MM (Figure 3). Because the inoculation of phenotypically differentiated MM cells alone could form MM in xenograft models, which is transplantable to secondary and tertiary recipients, MM-initiating cells seem to arise from MM cell population in vivo. Collectively, these findings
imply the existence of phenotypic as well as functional plasticity between CSCs and non-CSC populations in MM cells. As reported for SCID-hu or SCID-rab models in which human MM cells were injected into bone marrow cavities of subcutaneously implanted human or rabbit bones, MM growth was restricted to the implanted bones, suggesting that human or rabbit bones serve niches for MM CSCs in these experimental models.40,45 Yaccoby demonstrated that in cocultures with osteoclasts, differentiated MM cells became plasmablastic along with reduced expression of CD38 and CD138, and developed resistance to dexamethasone-induced apoptosis.46 Interestingly, Chaidos et al. found that when human CD138+ mature MM cells were injected into immunodeficiency mice via tail veins, the spleen and liver contained predominantly immature clonotypic plasma cells, including pre-PCs, rather than CD138+ mature MM cells, although CD138+ mature MM cells composed the majority of clonotypic cells in the bone marrow. Thus, extra-osseous tissues are thought to harbor rather immature clonotypic cells egressed from the bone marrow, and provide niches for clonogenic MM cells to foster them. Immature clonotypic cells or clonogenic MM cells colonized in extra-osseous may egress again and home to the bone marrow.

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3. Targeting MM cell-MM niche interaction

MM remains incurable even with the implementation of novel anti-MM agents, high-dose chemotherapies, and immunotherapies, and thus there is a need to target and disrupt the MM niche to improve the efficacy of the present therapeutic modalities against MM progression as well as MM bone disease.

3.1. Inhibition of osteoclastogenesis

The administration of inhibitors of OC activity, including bisphosphonates, RANK-Fc, and osteoprotegerin, not only prevents MM-induced bone destruction but also interferes with tumor progression in animal models of MM in some reports.47-49 The repeated administration of bisphosphonates has also been reported to reduce the tumor burden without chemotherapy in a portion of MM patients.50,51 These observa-
tions are consistent with the notion that an interaction between OCs and MM cells may play an important role in MM expansion in the bone marrow. In addition, the MM-OC interactions create an acidic milieu by protons produced by OCs and lactate by proliferating MM cells (the Warburg effect), which blunts cytotoxic effects of various chemotherapeutic agents as well as the activity of cytotoxic immune cells. Therefore, potent anti-resorptive therapy together with tumor reduction is needed to effectively suppress osteoclastogenesis, thereby disrupting the vicious cycle between osteoclastogenesis and MM expansion.

OCs produce the anti-apoptotic factors BAFF and APRIL, as noted above. BAFF is also produced by bone marrow stromal cells. Gene expression of TACI, a receptor for these factors, in MM cells can distinguish tumors with a signature of microenvironment dependence. To target these factors, TACI-Ig (atacicept) and anti-BAFF neutralizing antibody have been developed and shown to exert anti-MM effects in preclinical animal models. Denosumab, a fully human monoclonal antibody against RANK ligand that inhibits the RANK ligand-RANK system, is under worldwide clinical trials with patients with MM.

### 3.2. Induction of bone formation

A reverse correlation between osteoblastic differentiation and MM tumor growth has been reported in patients with MM treated with the proteasome inhibitor bortezomib and carfilzomib. Serum levels of bone-specific alkaline phosphatase were found to be elevated after treatment with bortezomib, which were inversely correlated with a reduction in the tumor burden. Such bone anabolic effects of bortezomib and their correlation with tumor regression were further demonstrated in MM animal models. MM growth inhibition associated with osteoblastic differentiation has also been observed in MM animal models treated with other anabolic agents such as anti-DKK1 antibody, lithium chloride, and activin A inhibitor, as well as with enforced expression of Wnt3a within bone. These observations suggest that anti-MM activity emerges with osteoblastic differentiation, and that MM cells may protect themselves from such osteoblast-mediated growth suppression by inhibiting the terminal differentiation of osteoblasts. The pro-anabolic agent BHQ880, an anti-DKK-1 monoclonal antibody, is currently being evaluated in clinical trials to test the promising preclinical results.

Transforming growth factor-β (TGF-β) is a potent inhibitor of terminal osteoblastic differentiation and mineralization. It is produced by osteoblasts and osteocytes, and deposited in bone matrices as its latent form. In MM bone destructive lesions, TGF-β appears to be abundantly released from bone matrices through enhanced bone resorption and activated by acids and MMPs secreted from OCs. We have demonstrated that blockade of TGF-β action releases stromal cells from their differentiation arrest by MM, and that terminally differentiated osteoblasts inhibit MM cell growth and survival and potentiate responsiveness to anti-MM agents. These results support the idea that the suppression of osteoblastic differentiation by MM both accelerates bone loss and creates an MM niche to provide favorable conditions for MM growth and survival (Figure 1). Thus, the induction of osteoblastic differentiation through TGF-β inhibition may provide a novel approach to ameliorating bone destruction and tumor progression in MM.

Activin A is a TGF-β superfamily member associated with a variety of fundamental biological processes, including embryogenesis, organ development, gonadal hormone signaling, and erythropoiesis. It also affects bone remodeling, and plays a crucial role in the skeleton. It has been reported that bone marrow plasma levels of activin A are increased in MM patients with bone lesions. MM cells induce the release of activin A from bone marrow stromal cells in part via activation of their JNK pathway (Figure 1). Furthermore, activin A inhibits osteoblastic differentiation and bone formation by stimulating intracellular signaling protein Smad2 activity and inhibiting distal-less homeobox (DLX)-5 expression. The soluble activin type IIA (ActRIIA)-Fc fusion protein is now under clinical development for patients with MM.

A large amount of ECM proteins is accumulated with osteoblastic differentiation. TGF-β-binding small leucine-rich proteoglycans, biglycan and decorin, were found to be among the most up-regulated proteins produced by terminally differentiated osteoblasts. These proteoglycans bind various growth factors and modulate their actions. Decorin has been identified among osteoblast-derived factors responsible for the suppression of MM cell growth and survival. Modification of the sulfation status of extracellular proteoglycan chains markedly regulated the binding and signal transduction of a number of growth factors and cytokines. Forced expression of extracellular heparan sulfate 6-O-endosulfatas, which are down-regulated in MM cells, was reported to markedly promote the deposition of ECM within the tumor microenvironment, along with a marked reduction in MM tumor growth. These findings suggest that modification of the sulfation status of proteoglycans can have a considerable impact on MM cell growth and survival. A variety of ECM proteins and enzymes affecting the sulfation status, including sulfatases and sulfotransferases, have been found to be targets of Runx-2, an essential osteogenic transcription factor, and up-regulated in mature osteoblasts.
3.3. Targeting MM cell signaling pathways up-regulated in bone lesions

A number of intracellular signaling mediators, including NF-κB, Akt, p38MAPK, and the adaptor protein p62, have been well demonstrated to be activated in both MM cells and their surrounding environmental cells and regulate the pathological bone metabolism and tumor growth in MM.

In pursuing factors responsible for MM tumor survival in bone lesions, we have found that the serine/threonine kinase Pim-2 is constitutively over-expressed as a critical pro-survival mediator in MM cells, and its expression is further up-regulated in cocultures with bone marrow stromal cells or OCs. Pim-2 has been identified using cDNA microarrays as one of the 34 most highly over-expressed genes in MM cell lines in comparison with lymphoma and AML cell lines. Although Pim-2 appears to be the most up-regulated member of the Pim family, MM cells also expressed Pim-1, but rarely Pim-3, at various levels. Pim-1 and Pim-2 were first identified as oncogenic factors based on observations of the development of lymphomas in transgenic mice over-expressing these kinases. In addition to enhancing oncogenic and survival signals, Pim kinases have also been shown to be involved in the expression and activation of drug efflux transporters.

Pim-1 phosphorylates the ATP-binding cassette (ABC) transporter ABCG2, also known as breast cancer resistance protein (BCRP), and thereby promotes its multimerization with its stable membrane expression, leading to drug resistance. Because ABCG2 is preferentially expressed in MM cells, the up-regulation of Pim kinases may involve a drug efflux in MM cells to reduce the efficacy of drug treatment. Sensitization of MM cells to chemotherapeutic agents by Pim inhibition warrants further study on combinatory treatment of anti-MM agents such as bortezomib and melphalan with Pim inhibitors. Inhibition of the IGF-1/PI3K/Akt pathway by Akt inhibitors has drawn considerable attention as a new therapeutic modality against MM. However, because the survival pathways elicited by Pim kinases seem independent of the PI3K/Akt pathway, Pim kinases should be targeted together to improve the anti-MM efficacy of PI3K/Akt pathway inhibitors. As Pim-2 expression is not increased in normal bystander cells, Pim-2 seems to be a specific target for the treatment of MM. Specific inhibitors for Pim-2 are under development at several pharmaceutical companies as anti-cancer agents, and may be available in the near future.

3.4. Targeting glycolysis in drug-resistant and clonogenic MM cells

The most important property of CSCs is that they are resistant to chemotherapeutic agents. Targeting MM CSCs is clinically relevant, and various approaches have been proposed to target molecular, metabolic and epigenetic signatures and the self-renewal signaling pathway characteristic of MM CSC-like cells.

The side population (SP) phenotype is characteristic of stem cells in various normal tissues. CSCs also exhibit high levels of ABC transporter activity and confer drug resistance besides their clonogenic or tumor-initiating capacity. SP cells are identified by their ability to efflux Hoechst33342 dye, a substrate for ABCG2, suggesting that SP cells have high ABC transporter activity. SP cells have been also demonstrated as a drug resistant fraction in many cancers, and are considered to contain their CSCs. In MM, the distinct fraction of SP cells has been detected in both MM cell lines and primary MM cells.

Malignant cells increase their expression of glycolytic enzymes and glucose uptake to markedly enhance glycolysis (aerobic glycolysis; the Warburg effect), which leads to the production of a large amount of ATP and biomass such as nucleic acids and lipids essential for cell survival and division. In parallel with enhanced glycolysis, ATP production by oxidative phosphorylation in the tricarboxylic acid (TCA) cycle in mitochondria is suppressed through oncogenic alterations including the mutation of p53. Glycolysis in MM cells has been demonstrated to be further up-regulated in cocultures with OCs. Because ABC transporter activity is dependent on ATP, and because ATP production in cancer cells is largely dependent on enhanced glycolysis, inhibition of glycolysis by 3BrPA was found to promptly and effectively suppress ATP production and ABC transporter activity in ABC transporter-expressing malignant cells and restored their drug sensitivity.
susceptibility to anti-cancer drugs [29]. Although the relationship between ABCG2 expression and SP phenotype has been established, other ABC transporters are also involved in the SP phenotype with high ABC transporter activity and drug resistance. Therefore, the efficacy of strategies targeting a single transporter appears to be limited. In contrast, inhibition of glycolysis is able to simultaneously inactivate all types of ABC transporters in cancer cells including SP cells, because each transporter is dependent on ATP generated largely through enhanced glycolysis. The SP cells exhibited the increased expression of genes involved in the glycolytic pathway including GLUT1, GLUT3, PDK1 and PFK2; and generated a larger amount of ATP and lactate per cell compared to MP cells [29]. Thus, glycolysis appears to be highly accelerated in SP cells. Furthermore, the ability of RPMI8226 cells to form colonies was completely abolished by relatively low concentrations of 3BrPA. From these results, it is plausible to assume that inhibition of glycolysis can disrupt their clonogenic capacity and drug resistant nature in SP cells.

4. Perspectives

MM cells and their surrounding cells in the bone marrow microenvironment closely interplay with each other to create an MM niche in the bone marrow to promote MM cell growth and protect MM cells from spontaneous and drug-induced apoptosis (Figure 1). In addition to cytotoxic agents for MM cells, novel agents targeting bone disease appear to be promising therapeutic strategies for the treatment of MM tumor expansion. However, because MM remains incurable even after effectively debulking the tumor mass, the idea of MM CSCs has been proposed to represent functional properties of small numbers of cells that have innate resistance to chemotherapeutic agents and the ability to live long and regrow in local microenvironments. Given the interconversion between MM CSC-like cells or undifferentiated clonotypic cells and MM cells, we should target all types of clonotypic cells from MM CSCs to mature MM cells. Although MM cells preferentially grow within the bone marrow, we also need to clarify where MM CSCs localize and how they self-renew upon interaction with MM microenvironment. The establishment of sophisticated, refined and humanized methods to recapitulate MM microenvironment, which supports MM CSC-like cell survival and self-renewal, is urgently needed. Further elucidation of the molecular mechanisms that keep MM clonogenicity and drive the transition between stem-like and non-stem-like states in local microenvironments will provide us with new approaches with crucial impact on the therapeutic paradigm in MM.

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