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Bone-Resorbing Cells in Multiple Myeloma: Osteoclasts, Myeloma Cell Polykaryons, or Both?

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ABSTRACT

Myeloma bone disease (MBD) leads to progressive destruction of the skeleton and is the most severe cause of morbidity in multiple myeloma. Its pathogenetic mechanisms are not fully understood, though the current evidence points to osteoclast (OC) hyperactivity coupled with defective osteoblast function unable to counteract bone resorption. OCs are generated in bone marrow by myeloid progenitors through increased levels of receptor activator of nuclear factor κB ligand and M-CSF, whose intracellular pathways propagate signals that activate sequential transcription factors, resulting in the production of major OC enzymes that drive specific functions such as acidification and degradation of the bone matrix. Osteolytic lesions, however, are not characterized by massive OC content, whereas malignant plasma cells, which are usually present in a high number, may occur as large multinucleated cells. The possibility that myeloma cells fuse and generate polykaryons in vivo is suggested by the in vitro formation of multinuclear cells that express tartrate-resistant acid phosphatase and produce pits and erosive lacunae on experimental osteologic substrates. Further, the detection in vivo of polykaryons with chromosome translocations typical of myeloma cells lends support to the view that myeloma polykaryons may act as functional OCs and participate in the skeletal destruction by resorbing bone. *The Oncologist* 2009;14:264–275

Editor's note: This review addresses the main evidence provided by the authors' studies and the literature for the view that malignant plasma cells exert osteoclast-like activity. See also the article by Sezer in this issue of the Journal.

INTRODUCTION

Bone destruction is a hallmark of multiple myeloma (MM), a B-cell neoplasm characterized by the marrow expansion of a plasma cell clone that leads to both resorption of surrounding bone and formation of typical osteolytic lesions [1, 2]. Myeloma bone disease (MBD), indeed, is the major cause of morbidity because of pathological fractures, spinal cord compression, chronic bone pain and extreme disability [3]. Its progression is often unimpeded by antimyeloma treatments, including chemotherapy, autologous stem cell transplantation, or biological drugs, even in responding patients, whereas bisphosphonates may temporarily control skeletal involvement [4].

   The pathogenetic mechanisms underlying MBD are unclear, though malignant plasma cells are believed to exert a

major osteoclastogenic effect through recruitment, differentiation, and activation of osteoclast (OC) progenitors within the bone marrow. This process is mediated by stromal cells that are structural components of the marrow niches housing the myeloma cells [5]. Following their interaction with the malignant cell clone, these cells, which physiologically differentiate into osteoblasts (OBs), increase their production of osteoclastogenic factors, including receptor activator of nuclear factor κB ligand (RANKL) and interleukin (IL)-6. They remain deregulated under the influence of myeloma cells and hence result in persistently high levels of those factors within the marrow milieu [6, 7].

Hyperactive osteoclastogenesis, however, is not accompanied by enhanced OB activity to replace the bone loss [8]. This imbalance is particularly evident in patients with advanced MBD and illustrated by histomorphometric studies showing the extreme paucity of newly formed bone near osteolytic lesions [9]. Patients with multiple bone lesions also display a reduction in their serum levels of bone-formation markers, such as alkaline phosphatase, osteocalcin, and procollagen type-I propeptides, coupled with a moderate to severe increase in markers of bone resorption, including ionized calcium levels [10]. Similarly to OC deregulation, defective bone remodeling is also related to negative regulation of mesenchymal stem cell differentiation by myeloma cells through a number of inhibitory mechanisms [11].

OCs are primarily involved in bone resorption and are currently thought to originate from marrow progenitors of the myeloid cell lineage. Marrow macrophages, indeed, are sensitive to the increased levels of inflammatory cytokines within the myeloma microenvironment and promptly undergo OC differentiation in response to high RANKL levels directly reinforced by the malignant myeloma cell clone [12, 13]. Once polarized in their function, marrow OCs release considerable amounts of proteolytic enzymes such as tartrate-resistant acid phosphatase (TRAcP), carbonic anhydrase, cathepsin K, and metalloproteinases that promote the progressive degradation of both the organic and inorganic bone matrix. The major detrimental role in skeletal destruction may thus be supposed to be directly related to mobilization of marrow OCs under the compelling influence of myeloma cells.

Recent studies from our group and others, however, suggest that marrow OCs are not solely responsible for gross bone devastation in MBD because of the apparent discrepancy between the severity of skeletal involvement and the number of OCs in the osteolytic lacunae observed in several histomorphometric studies [14]. Furthermore, patients with advanced or terminal MM are usually pancytopenic as a result of repeated chemotherapy and have a small marrow reserve of hematopoietic progenitors able to differ-

entiate into OCs. Because they are also frequently refractory to hematopoietic growth factors, such as erythropoietin, G-CSF, and M-CSF, their few remaining macrophages would not be able to produce the adequate amounts of OCs necessary to produce extensive bone resorption.

New pathogenetic studies on myeloma osteoclastogenesis, however, indicate that malignant plasma cells can transdifferentiate to functional OC-like cells in the marrow microenvironment and directly participate in bone resorption [15–17]. This would reconcile the severe progression of MBD with the paucity of marrow OC activity in patients with a defective marrow matrix.

This review addresses the main evidence provided by our studies and the literature for the view that malignant plasma cells exert OC-like activity.

PATHOGENESIS OF MBD

OC hyperactivity is the predominant event of MBD. Accelerated osteoclastogenesis is primarily dependent on locally acting resorptive factors produced by both OBs and stromal cells in response to multiple interactions between these and the malignant plasma cells. Cell-to-cell interactions lead to formation of a neoplastic unit releasing powerful inflammatory cytokines, namely, IL-1β, IL-3, IL-6 as a survival factor for myeloma cells, IL-11, tumor necrosis factor (TNF)-α, parathyroid hormone-related protein, hepatocyte growth factor, basic fibroblast growth factor, metalloproteinases, and macrophage inflammatory protein (MIP)-1α [6]. Increased cytokine concentrations drive the inflammatory state in the marrow milieu that enhances the sensitivity of OC precursors to functional ligands, although additional factors concur by different mechanisms, as summarized in Table 1, to accelerate OC maturation [18, 19]. The next step is closely related to the presence of high local concentrations of RANKL and M-CSF.

The sequential events promoting this scenario are an integral part of a number of molecular interactions between malignant plasma cells and the stroma, such as the binding of very late antigen 4 and $\alpha_v\beta_3$ integrin molecules expressed by malignant plasma cells to vascular cell adhesion molecule 1 on stromal cells. These reciprocal interactions lead to functional suppression of stromal cells whose maturation to OBs is severely affected by the inhibition of major osteogenic factors, such as Runx2 and β-catenin. Furthermore, the result of the high concentration of inflammatory cytokines is that stromal cells and immature OBs are persistently activated and hence responsible for the chronic release of RANKL and M-CSF. Thus, accelerated recruitment and differentiation of OC progenitors result in cell fusion, inhibition of apoptosis, and OC hyperactivity.

Table 1. Incomplete list of marrow soluble factors promoting bone resorption in myeloma

	Cellular origin	Biological role
Major osteoclastogenic factors		
RANKL	Stromal and myeloma cells	Differentiation of macrophages to OCs
M-CSF	Stromal cells	Induction of OC differentiation
Chemokines		
MIP-1α	Macrophages and myeloma cells	Chemotaxis activation in OC precursors
VEGF	Myeloma cells	OC activation; IL-6 induction by stromal cells
Cytokines		
IL-1β	Myeloma cells	Inflammatory inducer
IL-3	Marrow T cells	Stromal cell activation
IL-6	Stromal and myeloma cells, OCs	Myeloma cell growth
IL-11	Stromal and myeloma cells	OC activation through RANKL/OPG pathway
TNF-α	Myeloma cells	Apoptosis inducer
TNF-β	Myeloma cells	Apoptosis inducer
HGF	Myeloma cells	Inflammatory inducer
Other factors		
MMP-7	Myeloma cells	Protein digestion
MMP-13	OCs	Bone matrix degradation
MMP-14	OCs	Bone matrix degradation
PTHrP	Myeloma cells	Stromal cell stimulation
bFGF	Stromal cells	Stromal cell proliferation

Abbreviations: bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; IL, interleukin; MIP, macrophage inflammatory protein; MMP, metalloproteinase; OC, osteoclast; OPG, osteoprotegerin; PTHrP, parathyroid hormone-related protein; RANKL, receptor activator of nuclear factor κB ligand; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Osteoprotegerin (OPG), a soluble decoy receptor of RANKL released by marrow stromal cells to counterbalance excessive osteoclastogenesis, is also suppressed in patients with active MBD [20]. The reason for this is unclear, though functional deregulation of stromal cells following their assimilation within the myeloma cell population may lead to a major transcriptional defect. Soluble OPG can be neutralized in the marrow by the syndecan-1 (CD138) molecule expressed by myeloma cells. Syndecan-1 comprises a transmembrane proteoglycan containing heparin sulphate groups that binds OPG through its heparin domain and activates its degradation within the lysosomal compartment of the malignant plasma cells [21]. The overall outcome is a transcriptional and post-translational decrease in OPG that strongly reduces the neutralizing potential for RANKL, thus reinforcing OC progenitor differentiation and hyperactivation.

The role of OPG can also be deduced from the observation that patients with minimal skeletal involvement and inactive MBD display normal RANKL/OPG ratios that increase with the severity of MBD [22] and may predict survival in MM [23]. In contrast, monoclonal gammopathy of undetermined significance patients show lower values of RANKL/OPG ratios and of other bone resorption markers, such as MIP-1α, TRAcP, and N-telopeptide of collagen type-I, than patients with advanced MM [24]. Higher marrow RANKL levels and a reverse RANKL/OPG ratio thus indicate that OC hyperactivation is its major cause. Recent studies emphasize that RANKL released by malignant plasma cells increases its marrow levels and accelerates OC differentiation and maturation [12].

Defective osteogenesis to compensate for the bone loss induced by OC hyperactivity also contributes to the development of MBD. OB function is severely impaired, as shown by the minimal occurrence of osteocalcin and bone morphogenetic proteins as new bone products in osteolytic lesions. The inability of OBs to repair erosions is also a result of mechanisms induced by malignant plasma cells, such as the direct OB cytotoxicity induced by myeloma cells that is caused by their presentation of Fas-L and tumor necrosis factor receptor apoptosis-inducing ligand as apoptosis inducers [25]. They also release inhibitory factors within the stromal niches, namely Dkk1 (dickkopf1), whose RNA levels correlate with both serum protein and MBD extent [26], the Frizzled related proteins sFRP-1 to sFRP-4 that block the maturation of pre-OBs in the mouse, as well as noggin, gremlin, IL-3, and IL-7 [8]. Further OB inhibitors, including IL-11 and insulin growth factor binding protein 4, are variably enhanced in the sera of MBD patients [27, 28].

These inhibitors render immature OBs permanently ineffective and bone erosions are rarely repaired, even during the prolonged remission induced by chemotherapy or following treatments with new biological drugs, when osteolytic lesions are apparently dormant for the low levels of

myeloma cells. However, although this may account for a lower production of OB inhibitors, no bone formation occurs in osteolytic nests [29].

ORIGIN OF OCS AND THEIR ROLE IN MBD

OCs are polynuclear giant cells with bone-resorbing capability. Under normal conditions, differentiation of OC progenitors and bone modeling mainly occur in the marrow niches of the trabecular bone. The origin of OCs was undefined before the 1980s because the major hypothesis postulated that both bone-resorbing and bone-forming cells were derived from a common progenitor [30]. The macrophage origin of OCs was suggested by experiments in osteopetrotic mice whose bone-resorbing function was restored by infusion of spleen or marrow cells from wild-type mice [31], while later work showed that resorptive polykaryons are generated by cells of the monocyte/macrophage lineage [32]. A crucial point of these studies, however, was the inability of marrow macrophages to generate OCs in the absence of OBs. The role of RANKL as a key osteoclastogenic molecule expressed by these cells and presented by stromal cells or OBs to the OC progenitors was thus confirmed when OPG was shown to block OC formation and induce osteopetrosis in OPG transgenic mice [33].

RANKL has now been widely investigated. Initially characterized as a T-cell–specific protein [34], it was subsequently detected on marrow stromal cells and OBs [35], and in other cell types including endothelial cells and chondrocytes [36]. Its function in osteoclastogenesis has been demonstrated in RANKL-deficient mice, which, like transgenic OPG-overexpressing mice, develop severe osteopetrosis as a result of defective OC differentiation, whereas RANKL transgenic mice develop severe osteoporosis as a result of hyperactive bone resorption [35]. The RANK pathway activated by RANKL involves many transcription factors that, starting with the adaptor protein TNF-receptor-associated factor 6 (Fig. 1), act through mitogen-activated protein kinase, phosphoinositol-3-kinase (PI3K), and nuclear factor (NF)kB to activate calcineurin. This then dephosphorylates nuclear factor–activated T cell (NFATc)1, promotes its nuclear translocation, and leads to activation of major osteoclastogenic factors and phosphokinases, with the final expression of enzymes that degrade the bone matrix, such cathepsin K and vATPase.

M-CSF is a cytokine that activates a parallel pathway for the differentiation of resorptive cells by macrophages. It promotes both proliferation and survival of the precursors of the OC lineage through the tyrosine kinase receptor c-Fms, which interferes with the $\alpha_v\beta_3$ integrin pathway. Downstream proliferation and survival signals from c-Fms propagate the PI3K–Akt pathway and ultimately prime the

phosphorylation of extracellular signal–related kinase (ERK)-1 and ERK-2. Although M-CSF is expressed by other cells, such as the endothelium, it is primarily produced in OBs and its pivotal role in osteoclastogenesis has been clearly established by the development of typical OC-deficient osteopetrosis in mice bearing a mutated *M-CSF* gene [37].

The third pathway involved in OC differentiation is promoted by the immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors DAP12 and FcRγ [38]. These receptors interact with the c-Fms pathway and are costimulators, by cell-to-cell contact, with a number of undefined ligands reciprocally expressed by OC precursors and OBs. The signaling pathway downstream from ITAMs includes Syc, phospholipase C (PLC)γ2, and NFATc2 for differentiation of OC precursors [39]. Discovery of the role of ITAMs in OC differentiation has promoted new studies on the relationship between osteoclastogenesis and the immune system, because several molecules that regulate bone resorption, namely DAP12, FcRγ, RANK, RANKL, PLCγ2, NFATc2, and NFκB, are critical for lymph node and immune cell differentiation as well. TNF-α may also activate OC precursors. Data from independent groups, in fact, show that TNF-α–induced OCs display efficient resorptive ability in vitro. This may also be true in vivo [40, 41].

Besides these pathways, other molecules participate in the activation of bone resorption. Following their adhesion to the bone surface, OCs undergo typical polarization that includes a modified shape of the cytoskeleton with a sealing zone enriched in fibrillar actin, whereas the nuclei are prevalently localized in the direction of the antiresorptive surface. The ruffled membrane forming the actin ring is actually the resorptive organelle. It is formed by OCs after their contact with the bone surface through the direct transport of acidified cytoplasmic vesicles toward the bone-apposed cell membrane. The actin ring seals a distinct extracellular microenvironment between cell and bone surface, in which organic and inorganic components of the bone matrix are degraded by the major proteolytic enzymes released by the OCs.

A critical role in OC polarization is apparently exerted by $\alpha_v\beta_3$ integrin [42]. OC motility, adhesion to bone, and polarization of the resorptive machinery, in fact, all require $\alpha_v\beta_3$ integrin, a transmembrane heterodimer with low or high binding activity in relation to its basal or activated state. OCs and the placenta are particularly rich in $\alpha_v\beta_3$ integrin. It is also expressed by other cells, in particular the endothelium, in both inflammation and neoangiogenesis, as well as by several tumors, such as melanoma. The β_3 chain is essential for crosstalk between OCs and bone, because it
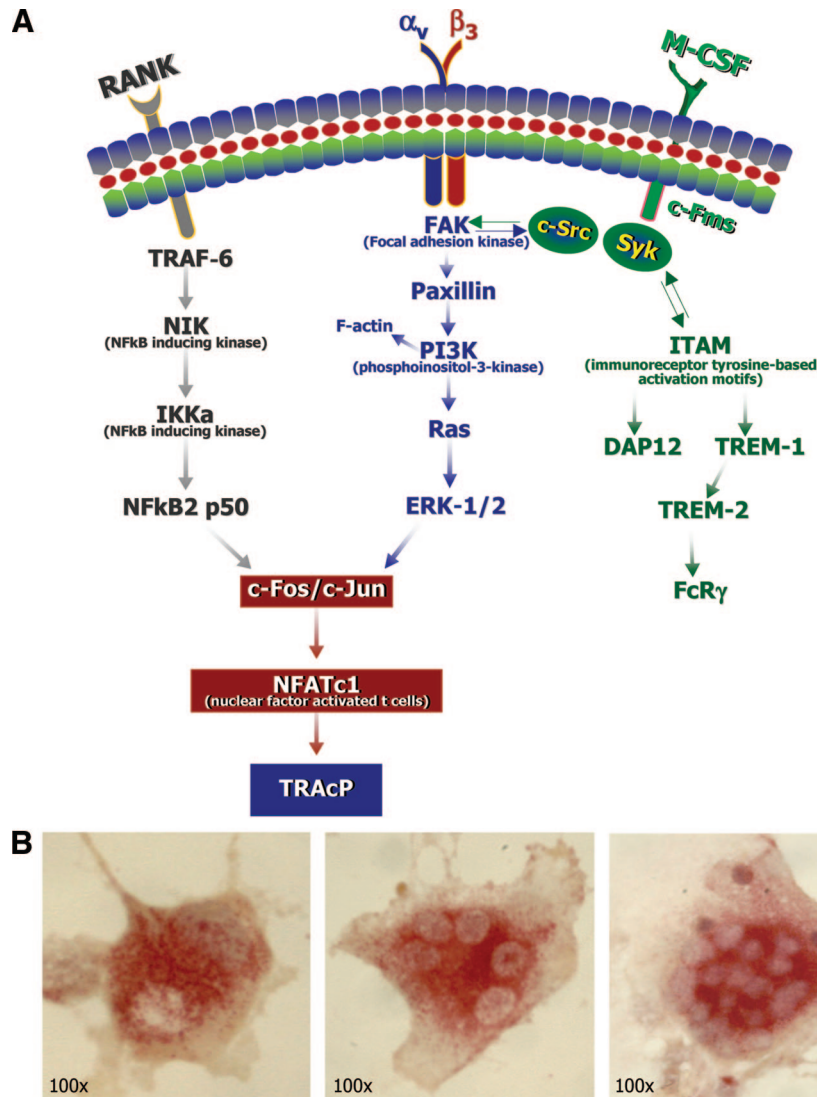
Figure 1. Molecular pathways of OC differentiation and activation. **(A):** OCs originate in marrow from myeloid progenitors through the activation of intracellular signaling pathways. These primarily include RANKL secreted by stromal cells, whereas the $\alpha_v\beta_3$ integrin pathway helps to propagate signals that ultimately drive the phosphorylation of specific kinases, namely c-Fos and c-Jun, resulting in the expression of TRAcP. The c-Fms pathway also interacts with the $\alpha_v\beta_3$ integrin intracellular signals and activates the ITAM molecules that regulate the expression of markers largely exposed by cells of the myeloid lineage. **(B):** Progressive differentiation of peripheral macrophages treated in vitro with RANKL and M-CSF leads to the formation of multinuclear giant cells within 2 weeks. The differentiation to OCs of these polykaryons was confirmed by the intracytoplasmic expression of TRAcP as an OC enzyme, which occurs as a brown granulation under the light microscope (magnification, 100×).

Abbreviations: ERK, extracellular signal–related kinase; NFκB. nuclear factor κB; OC, osteoclast; RANKL, receptor activator of nuclear factor κB ligand; TRAcP, tartrate-resistant acid phosphatase; TRAF-6, tumor necrosis factor receptor–associated factor 6; TREM, triggering receptor expressed by myeloid cells.

is recognized by the arginine-glycine-aspartic acid (RGD) amino acid motif exposed by bone matrix proteins, such as osteopontin or bone sialoprotein, and propagates, through PI3K, the intracellular signals that activate cytoskeleton rearrangement and formation of the ruffled border. These $\alpha_v\beta_3$ integrin–induced functions in OCs are also regulated by a molecular signaling pathway that activates other kinases and interacts with both ITAM and c-Fms (Fig. 1). After integrin ligation by the RGD domain, in fact, c-Src is primarily phosphorylated and activates Syk, which is recruited within the ITAM pathway, whereas c-Fms promotes the phosphorylation of Vav3, with subsequent activation of Rac, and enhances OC migration and bone resorption. The role of the β_3 chain in propelling OC polarization has been definitively proven in mice lacking the integrin subunit, which develop severe OC dysfunction resulting in the defective formation of the actin ring and the ruffled border, as well as an increase in bone mass. Re-

expression of wild-type β_3 chain restores the osteoporotic response [43].

OC polykaryons are large polynuclear cells formed by the fusion of macrophages. This fusion is needed to activate the molecular mechanisms inducing the acidification of the bone sealed by the ruffled border as well as its resorption. Its regulatory mechanisms, however, have not been established, though several membrane molecules play a functional role. Besides major regulation by RANKL in inducing expression of the functional OC receptors CD44, CD47, ADAM12, and CD9 [44], much interest has been directed to monocyte chemoattractant protein 1 [45] and, more recently, to dendritic cell–specific transmembrane protein (DC-STAMP) [46]. This seven transmembrane receptor has been described on dendritic cells and is highly expressed by OCs, but not by macrophages. Its role in the formation of OC polykaryons has been demonstrated in DC-STAMP homozygote mice, whose macrophage fusion and generation of OCs were completely abrogated despite the normal expression of other functional OC markers and cytoskeletal structures. Once DC-STAMP was restored in these mice by retroviral transfection, OC polykaryons were generated [47].

OC polykaryons are deeply involved in the bone devastation associated with MBD. As in other bone-homing tumors, such as breast, prostate, and lung cancers, myeloma cells produce major osteoclastogenic factors. One key factor is MIP-1α. Because this chemokine is largely secreted by malignant plasma cells, its serum levels correlate with both the extent of osteolytic lesions and survival in MM patients [48]. It acts as a RANKL-independent OC activator in vitro, whereas in the myeloma marrow it enhances OC precursor differentiation in conjunction with RANKL and IL-6. RANKL levels are usually increased in the myeloma microenvironment because of secretion by both malignant plasma cells and other cells of the immune system. RANKL is, in fact, produced by activated T and B cells to regulate bone cell functions and take part in the triggering of OC formation, activation, and survival [49]. Thus, the myeloma bone environment and myeloma cells by themselves stimulate OCs. OC hyperactivity ultimately primes the tumor cells to release more OC-stimulating factors and generates a reciprocal cycle of tumor growth and bone destruction. Animal models have demonstrated that myeloma-induced osteolysis is mainly ascribable to OCs recruited near bone sites in which the malignant clone is growing (Fig. 2), where these cell-to-cell interactions may result in enhancement of myeloma cell proliferation in response to specific cytokines released by OCs, such as IL-6 [23, 50].

However, while the upregulated OC activity in MBD is revealed by increased bone resorption marker levels [10],
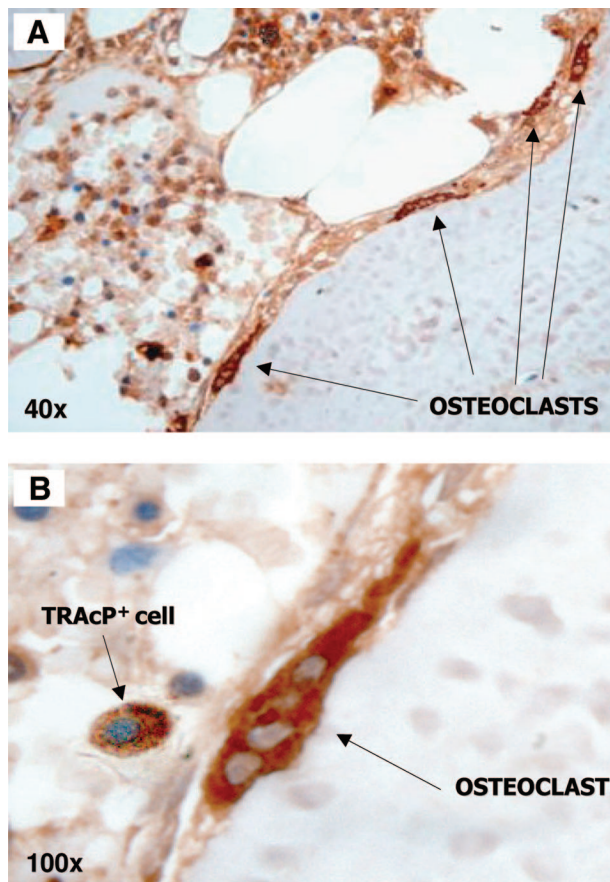
Figure 2. Detection of OCs in myeloma bone biopsies. **(A):** OCs in trabeculae facing the calvarian bone were detected as TRAcP$^+$ cells that displayed typical elongation of the cytoskeleton and multinuclearity. **(B):** In several instances, there were TRAcP$^+$ mononuclear cells close to OCs with apparent plasma cell morphology. Their ontogenetic derivation from the macrophage or myeloma B cell lineage is unclear (magnifications are in each panel).

Abbreviations: OC, osteoclast; TRAcP, tartrate-resistant acid phosphatase.

histomorphometric studies suggest that OC accumulation close to or within the osteolytic lesions is not high enough to explain the bone destruction.

MYELOMA CELL POLYKARYONS

MBD is not usually regarded as the product of malignant plasma cell activity. Instead, it is thought to reflect a severe imbalance between OC and OB functions, whereby resorption prevails over formation because of predominant OC hyperactivity. As already mentioned, however, OCs located near or inside the lesions may not account for the severe devastation in advanced MBD. In contrast, conglomerates of plasma cells with highly malignant morphologic features usually occur in these sites, as well as in marrow, as myeloma cell nests.
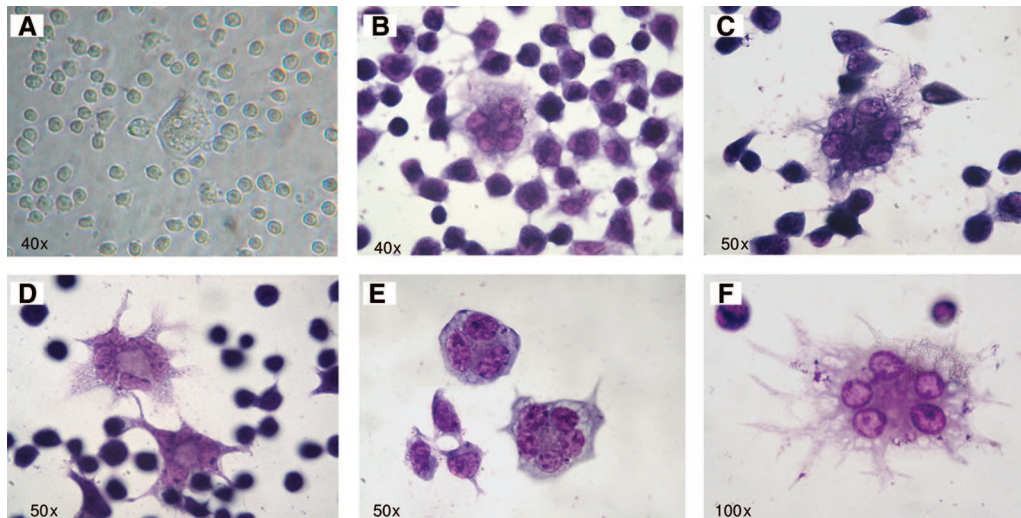
Figure 3. In vitro formation of polykaryons from long-term cultures of U-266 myeloma cells. The myeloma cell line, cultured for up to 4 weeks, spontaneously generated adherent multinuclear cells that were observed after 2 weeks with a phase contrast microscope **(A)** and subsequently stained with May-Grunwald **(B, C, D:** 3 weeks of culture). These cells acquired the appearance of typical OC polykaryons after 4 weeks **(E, F)** (magnifications are in each panel).

Recent observations suggest that malignant plasma cells take part in bone destruction. This OC-like activity has been shown by ourselves [17] and is indicated by other findings [16] that support the capability of these cells to form in vitro polykaryons with functional properties similar to OCs.

Multinuclearity of malignant plasma cells is a rare event observed in <3% of patients. The morphology of multinuclear giant myeloma cells has been associated with different types of myeloma, including the nonsecretory [51], Bence-Jones [52], IgAκ [53], and other [54] phenotypes. These studies provide no clear explanation for this multinuclearity. They do, however, emphasize its association with a poor prognosis and severe progression of MBD. Other observations have linked multinuclearity with the monocytoid pattern determined by both morphologic and phenotypic criteria [55]. Extension of the investigation to a number of myeloma cell lines, in fact, disclosed a broad spectrum of myelomonocytic, natural killer (NK), and T-cell markers, including the CD5 molecule, a T-cell antigen expressed by a subset of B cells involved in autoimmune and immunoproliferative diseases [56].

The heterogeneity of myeloma cells was recently assessed using molecular approaches documenting, in U-266 cells (an IgEλ myeloma line), the expression of markers belonging to either the myelomonocytic, NK cell, neuronal cell, or dendritic cell lineage and then downregulation by the autocrine control exerted by IL-6, as well as the lack of *Pax-5* as a master gene of the B-cell lineage [57]. The observation of myelomonocytic nuclei in the presence of multilineage markers confirmed the tendency of these cells to undergo variable nuclear transformation.

The nuclear heterogeneity of myeloma cells is also illustrated by the results of combined cytogenetic and immunohistochemical studies demonstrating a few chromosomal translocations typical of malignant plasma cells in marrow polykaryons with OC morphology. Nuclei with t(4,14) and t(11,14) were of malignant origin, transcriptionally active, and fully integrated with the other nuclei in OC-like cells. These OC–myeloma hybrids occurred in >30% of the OC population and were detectable together with mononucleated plasma cells, near osteolytic lesions. Similar hybrids were also generated in cocultures of OCs with myeloma cells. Malignant plasma cells may thus be supposed to "corrupt" normal OCs by fusing with normal OCs, transferring their malignant DNA, and affecting their function.

Involvement of malignant plasma cells in the generation of bone erosion has long been disputed. Animal studies point to their direct involvement, but this has not been demonstrated in humans. We have shown that U-266 and MCC-2 myelomas [58] undergo functional OC-like transformation in vitro when stimulated with several osteoclastogenic cytokines [17]. On prolonged cultures, we found that a number of adherent cells from both lines displayed a typical OC-like phenotype, including the polykaryon morphology associated with the expression of TRAcP and other OC markers. Polykaryon transformation may also occur in the absence of such stimulation in the case of normal OCs that emerge in cultured peripheral monocytes, even in the absence of RANKL and M-CSF [59]. Figure 3 illustrates the morphological pattern of adherent U-266 cells we cultured for up to 3–4 weeks in complete medium only. Several polykaryons with extended pseudopodes and
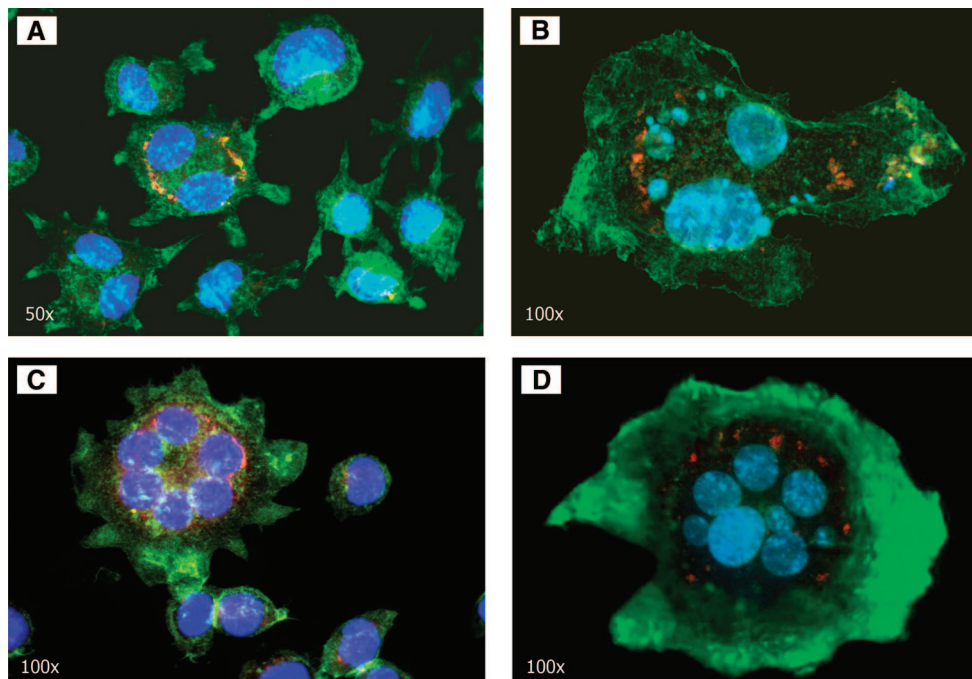
Figure 4. Morphology of myeloma cell polykaryons. Triple fluorescence analysis of adherent U-266 polykaryons disclosed multinuclearity, revealed by 4′,6-diamidino-2-phenylindole, the enrichment in actin of the ruffled border (particularly in **B**, **C**, and **D**) by falloidin green, and the cytoplasmic λ chains detected by a phycoerythrin-conjugated anti-λ rabbit antiserum (red) (magnifications are in each panel).

condensed chromatin in their nuclei observed among the mononuclear cells are characterized by the cytoplasmic expression of λ chains that were revealed as red agglomerates by a phycoerythrin-conjugated antiserum (Fig. 4). The nuclei per cell increased in number with time, and up to 20 were present after 4 weeks.

In addition to their multinuclearity, myeloma cell polykaryons have also been reported to produce TRAcP in extended cultures [17]. Its expression is apparently correlated with the duration of the culture. Figure 5 shows a polykaryon as well as TRAcP$^+$ mononuclear cells belonging to the U-266 myeloma cell line, after 4 weeks of culture. The enzyme was uniformly distributed as diffuse or granular staining of intracellular components.

Functional similarities between myeloma polykaryons and OCs also involve other functional aspects of these cells. Similar functions are also displayed by these polykaryons and OCs. A functional form of the calcitonin receptor (CTR) expressed by myeloma cells, including U-266, belongs to the molecular variant expressed by OCs [60]. In addition, adherent polykaryons rearrange the cytoskeleton to form a ring of fibrillar actin identical to the sealing zone required by OCs to define the bone area to be resorbed. This ring determines the ability of myeloma polykaryons to produce bone erosions on experimental osteologic substrates resembling either the inorganic or the organic matrix, as

provided by both calcium phosphate and dentine disks [17, 61]. As shown in Figure 6, after a few days, the lacunae and erosion pits produced by U-266 myeloma cells on both substrates are identical to those produced by OCs. This pattern is to be referred to as true resorption exerted by these polykaryons because parallel experiments using Daudi cells as control lymphoblasts were unable to produce similar erosions [17]. Interestingly, both the actin ring and erosion are prevented when calcitonin is included in the culture medium [60], as in the case of OCs.

These studies strongly support the role of malignant plasma cells in MBD. Myeloma polykaryons have occasionally been detected in marrow aspirates. Because their in vitro morphologic and functional properties are highly similar to those of OCs, they may participate in osteolysis in vivo and be indistinguishable from normal OCs.

BONE-RESORBING CELLS IN MBD

The nature of the cells responsible for the dramatic devastation associated with MBD is uncertain. As already stated, the paucity of OCs in or near osteolytic lesions has been demonstrated histomorphometrically. Substantial numbers of malignant plasma cells are usually detected, though their direct participation in bone destruction has not been demonstrated.

Investigation of direct degradation of the bone matrix by cancer cells in other bone tumors [15, 62, 63], too, has failed to
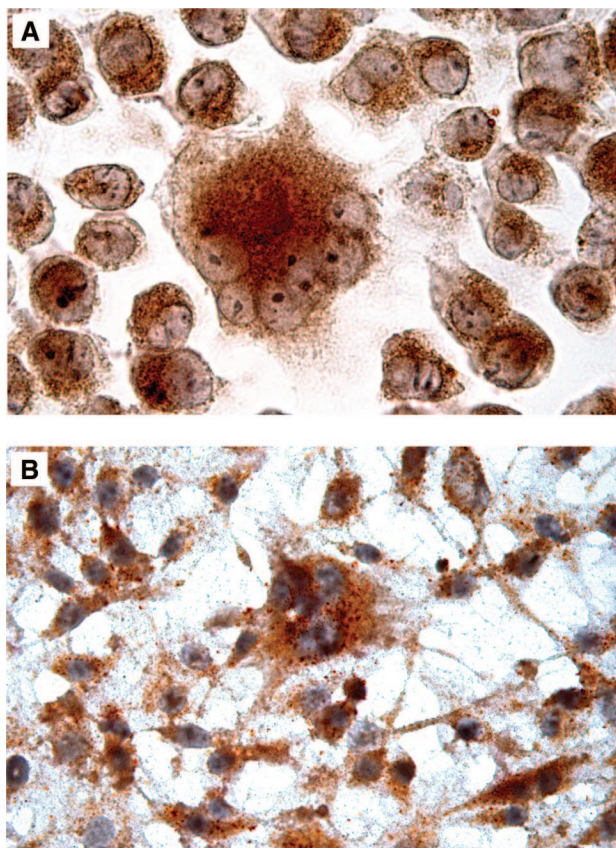
Figure 5. Expression of TRAcP by U-266 myeloma cells. TRAcP detection in a polykaryon as well as in surrounding mononuclear U-266 cells, after 4 weeks of incubation (**A**) in a similar fashion as in GCT cells (**B**), an in vitro established osteoclast-like cell line deriving from a giant cell tumor of bone [72] (magnification, 100×).

Abbreviation: TRAcP, tartrate-resistant acid phosphatase.

produce any convincing evidence. The contribution of malignant plasma cells to bone destruction is currently attributed to multiple interactions with other cells of the marrow milieu whose promotion of the secretion of soluble factors accelerates the differentiation of myeloid precursors to OC polykaryons [64, 65]. However, their ability to produce severe bone destruction is rendered unlikely by the fact that leukopenia is usually associated with the conventional management of myeloma and the bone marrow is commonly depleted of the myeloid progenitors needed to form high numbers of OCs, and even myeloid growth factors are often unable to induce absolute neutrophil counts compatible with chemotherapy. The myeloid cells are thus insufficient to supply functional amounts of OCs, whose richness in nuclei reflects their dynamic maturation and correlates with resorptive activity [66, 67]. Marrow OCs may act as aggressive bone-resorbing cells during early MBD when the myeloid marrow matrix is not restrained by chemotherapy.
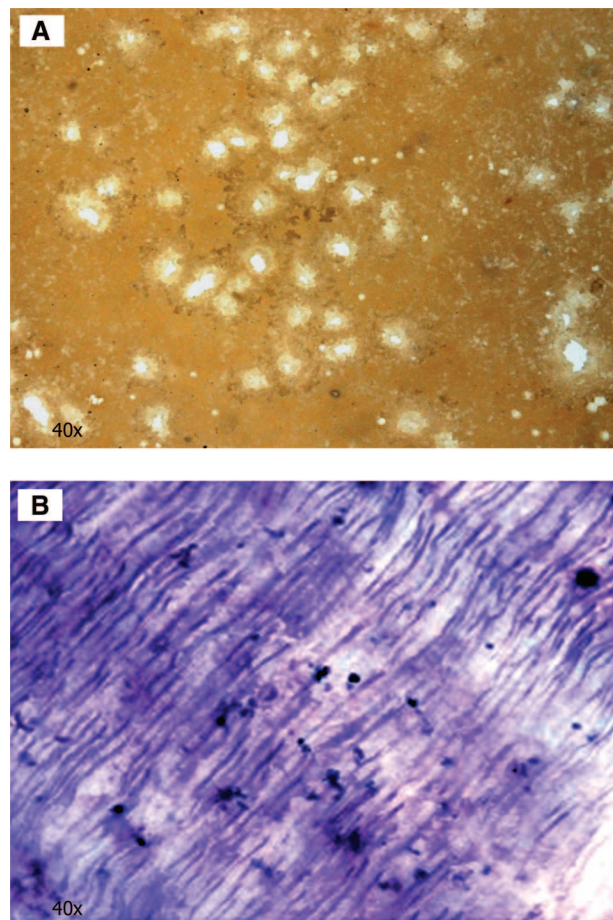
Generation of myeloma polykaryons in vitro also raises

Figure 6. Experimental bone resorption by U-266 polykaryons. Resorption by adherent U-266 cells of calcium phosphate (**A**) and dentine (**B**) to resemble inorganic and organic bone substrates. The polykaryons were removed after 2 weeks and the substrates were inspected by light microscopy after von Kossa (**A**) and toluidine blue (**B**) staining. The erosive lacunae on calcium phosphate produced by each U-266 polykaryon illustrated their resorption ability, whereas the dark spots on dentine slices, obtained by toluidine blue after cell removal, substantiated their ability to resorb the organic substrate (magnification, 40×).

the question of whether or not malignant plasma cells are major effectors in MBD. These polykaryons are present in bone erosion sites. Their contribution to bone destruction, however, implies that B cells from the malignant plasma cell clone also generate bone-resorbing cells. It would thus seem that myeloma cells can undergo a program of myeloid transdifferentiation that is presumably regulated by multiple molecular events within the intracellular transcriptional machinery and may drive malignant plasma cells to acquire new properties, including adhesion, multinuclearity, the expression of peculiar OC markers, such as the CTR [60], and the ability to resorb the bone substrate. However, these effects of myeloma polykaryons have been detected in vitro using established myeloma cell lines, whereas major phe-

notypic studies of primary myeloma cells have not described the expression of myeloid markers. Myeloid transdifferentiation has not been confirmed in vivo. Even so, experimental work indicates that specific genes can drive the differentiation of B cells into macrophages [68]. On the other hand, myeloid transdifferentiation of malignant plasma cells may occur after activation of specific molecules of cell adhesion. For instance, as for OCs, $\alpha_v\beta_3$ integrin, which is greatly expressed by malignant myeloma cells [69], is thought to trigger intracellular activation signals that interact with ITAM molecules and regulate the expression of myeloid transcription factors. Work in progress in our lab supports this hypothesis [70].

The demonstration that OC–myeloma cell hybrids are also detectable in vivo [16] is of interest and emphasizes the basic mechanisms of cell fusion in vivo, multinuclearity, and the ability to resorb the bone matrix. These cells are bone-resorbing polykaryons and have been postulated to have derived from macrophages originated by dedifferentiated clonal B cells that are able to fuse their membrane and generate multinuclear cells. Another explanation for the in vivo occurrence of polykaryons with myeloma chromosome translocation is that they originate through fusion between mature OCs and mature myeloma cells that could result in the transfer of whole myeloma nuclei into OCs, including the genes that regulate the expression of specific markers and integrins driving OC functions, both in vivo and in vitro. In this context, other heterotypic fusions between monocytoid cells and cancer cells or stem cells are apparently involved in tumor biology [71].

CONCLUSIONS

Emphasis has recently been placed on the role of malignant plasma cells in the induction of MBD, though it has not been shown that they act as deregulated OCs in vivo and promote progressive derangement of the skeleton. There is, however,

much to suggest that they originate polykaryons both in vivo, near osteolytic lesions, and in vitro, where these multinuclear cells, like native OCs, resorb experimental bone substrates.

Studies of OC pathophysiology have identified the pathways by which OCs come from myeloid progenitors, fuse their membranes, and drive bone resorption. These pathways are apparently active in myeloma cells and may trigger their myeloid differentiation to OC-like cells.

Native OCs are undoubtedly involved in the pathogenesis of MBD as cellular targets of the altered cytokine network in marrow and are probably supported by bone-resorbing myeloma polykaryons in the devastating effects on the skeleton, especially in patients with severe MBD and a poor marrow matrix. The concomitance of malignant plasma cells and myeloma polykaryons in erosive sites provides indirect evidence of their role in MBD pathophysiology, as observed in vitro. It seems likely that marrow OCs are primarily activated in their resorbing ability by myeloma cells in early disease, whereas the formation of myeloma polykaryons may increase as disease progresses and the myeloid matrix is depleted. Further work is needed to define the molecular events that regulate the myeloid reprogramming of myeloma cells and enable their polykaryon transformation.

AUTHOR CONTRIBUTIONS

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