Critical Role of AKT Protein in Myeloma-induced Osteoclast Formation and Osteolysis*

Huiling Cao‡, Ke Zhu§§, Lugui Qiu‡, Shuai Li‡, Hanjie Niu‡, Mu Hao‡, Shenyong Yang§, Zhongfang Zhao‡, Yumei Lai¶, Judith L. Anderson**, Jie Fan†, Hee-Jeong Im†, Di Chen‡, G. David Roodman**; and Guozhi Xiao‡

Background: Myeloma cells cause abnormal osteoclast formation and osteolysis.

Results: Myeloma cells up-regulate AKT in osteoclast precursors and promote osteoclast formation. Systemic AKT inhibition blocks the myeloma-induced osteolysis and tumor growth in bone.

Conclusion: AKT is critical for the myeloma promotion of osteoclast formation and osteolysis.

Significance: AKT could be a useful target for treating patients with myeloma bone disease.

Abnormal osteoclast formation and osteolysis are the hallmarks of multiple myeloma (MM) bone disease, yet the underlying molecular mechanisms are incompletely understood. Here, we show that the AKT pathway was up-regulated in primary bone marrow monocytes (BMM) from patients with MM, which resulted in sustained high expression of the receptor activator of NF-κB (RANK) in osteoclast precursors. The up-regulation of RANK expression and osteoclast formation in the MM BMM cultures was blocked by AKT inhibition. Conditioned media from MM cell cultures activated AKT and increased RANK expression and osteoclast formation in BMM cultures. Inhibiting AKT in cultured MM cells decreased their growth and ability to promote osteoclast formation. Of clinical significance, systemic administration of the AKT inhibitor LY294002 blocked the formation of tumor tissues in the bone marrow cavity and essentially abolished the MM-induced osteoclast formation and osteolysis in SCID mice. The level of activating transcription factor 4 (ATF4) protein was up-regulated in the BMM cultures from multiple myeloma patients. Adenoviral overexpression of ATF4 activated RANK expression in osteoclast precursors. These results demonstrate a new role of AKT in the MM promotion of osteoclast formation and bone osteolysis through, at least in part, the ATF4-dependent up-regulation of RANK expression in osteoclast precursors.

Multiple myeloma bone disease is characterized by progressive osteolysis associated with abnormally increased osteoclast formation and activation. Up to 90% of patients with multiple myeloma (MM) develop osteolytic lesions (1), which cause major clinical problems such as bone pain, pathological fractures, spinal cord compression, and hypercalcemia of malignancy (2–4). Studying the underlying molecular mechanisms will help to develop new therapeutic targets to treat osteolytic lesions and related complications in patients with MM.

Osteoclasts originate from cells in the monocyte/macrophage lineage (5). Osteoclast formation and maturation are tightly regulated by osteoblast/stromal cell/osteocyte/hypertrophic chondrocyte-derived factors such as M-CSF, RANKL, and OPG. OPG is a soluble decoy receptor that blocks RANKL binding to RANK and thereby inhibits osteoclast differentiation (6, 7). M-CSF binds to its receptor CSF1R on early macrophage lineage cells and activates RANK expression (8). This is a critical step for generating osteoclast precursors and early osteoclast differentiation. RANKL then binds to RANK on osteoclast precursors and recruits TRAF6, which results in the activation of multiple signaling pathways, including IκB (IKK) complexes (IKKα, -β, and -γ and NIK-IKKα) and MAPKs (ERK1/2, p38, and JNK) (9, 10), leading to activation of critical transcription factors such as NF-κB, c-FOS, and NFATc1 and eventually osteoclast formation (10–12).

Abnormal osteoclast formation and activation are mediated by factors produced and induced by tumor cells (13). For example, breast cancer cell-produced parathyroid hormone-related protein induces osteoclast formation through up-regulation of RANKL by osteoblasts (14, 15). Furthermore, the NF-κB pathway in breast cancer cells promotes osteolytic bone metastasis by activating osteoclast formation via up-regulation of granulocytic-macrophage colony-stimulating factor (GM-CSF) (16). Monocyte chemotactic protein-1 (MCP-1) mediates prostate cancer- and lung cancer-induced osteoclast formation and bone resorption (17, 18). Macrophage inflammatory protein.
Myeloma Activation of Osteoclastogenesis

TABLE 1

Human qPCR primers

| Name | 5’ primer | 3’ primer |
|------|-----------|-----------|
| ATF4 | GGCTCCCATCGGGCTGGCCTG | CCATTCTCCAACATCTCACTGCC |
| β3  | GCAATGGAAGCTTGGAGTGT | AGATCGAGGGTGAGATGAG |
| CATK | ACCGGGGATTACCTGCCAAG | GAGGGCGGCTTCTCCTAGAT |
| DAP-12 | GAGCCGAGTCGCCTATATC | ATACGGGGTCTTGTGTTG |
| c-FMS | CAGACCCAGCTATCACTGGC | CTGGCCTGACACGACATC |
| C-FOS | AAAGGAGATTCGCAGGGAAGAATAGGCT | AGACGAGGAAAGCAGTTAAGCAGTCAGC |
| GAPDH | TCCACACCACTGGTGCTGTTA | ACCAGTCAGGACCTGGA |
| MMP-9 | CTTCTCTCTTCCCTGGTAG | CTAATCTACTTGGTGG |
| NFATc1 | GCATCACAGGGAAACGGCTGTC | GAAGTTCATGTCGAGTTGAG |
| PL1 | CAGAAGACCTGGTGCCATCGTAT | GGAGCTCCGTGAAGTTGTTC |
| RANK | ATGCCGGTTCAGCCTCTCTCTC | AACTCCCTATCTTCTCTC |
| TRAF6 | TTGTGCTAGTGCTGTCGAG | CGTGAGGAAACAAAAGCAG |

(MIP)-1α promotes the development of osteolytic lesions in patients with MM (19, 20). Enhanced RANKL expression plays an important role in tumor-induced osteoclast formation and bone destruction (13, 21). However, molecular mechanisms whereby the MM cells promote osteoclast formation and activation and osteolysis are not completely understood.

AKT, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a critical role in multiple cellular processes such as cell proliferation, survival, and migration, glucose metabolism, and gene transcription (22, 23). Accumulative evidence shows that the PI3K/AKT pathway plays a critical role in activating osteoclast differentiation (24, 25). However, if and how AKT mediates the MM-induced osteoclast formation and osteolysis have not been addressed.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture media, fetal bovine serum, and horse serum were obtained from Thermo Scientific HyClone (Logan, UT). LY294002 was purchased from LC Laboratories (Woburn, MA); ascorbic acid and DMSO were from Sigma; human recombinant M-CSF, RANKL, and TNF-α were from R&D Systems Inc. (Minneapolis, MN). All other chemicals were of analytical grade.

Human Bone Marrow Samples—Bone marrow aspirates were collected in heparin from normal donors (ND) and patients with MM. Protocols were approved by the respective Institutional Review Board committees of the University of Pittsburgh and the Institute of Hematology and Blood Diseases, Chinese Academy of Medical Sciences.

DNA Constructs, transfection, and Adenoviral Infection—pCMV/β-gal, pCMV/ATF4, pCMV/AKT-CA, and wild-type and mutant p40SE1-luc plasmids (an ATF4 reporter plasmid) were previously described (26, 27). Rank-luc reporter plasmid was constructed by PCR subcloning of a −1073/+79 mouse Rank gene promoter into the pGL3-luc vector (Promega, Madison, WI) in the project laboratory. For transfection experiments, the amounts of plasmid DNAs were balanced as necessary with β-galactosidase expression plasmid such that the total DNA was constant in each group. Adenoviruses expressing ATF4 or EGFP were described previously (28, 29). The amount of adenovirus was balanced as necessary with a control adenovirus expressing EGFP such that the total amount was constant in each group.

Gene Expression Studies—RNA isolation and reverse transcription (RT) were previously described (30). Quantitative real-time RT-PCR analysis was performed to measure the relative mRNA levels using the SYBR Green kit (Bio-Rad). Melting curve analysis was used to confirm the specificity of the PCR products. Four to six samples were run for each primer set. The levels of mRNA were calculated by the ΔΔCt method. Samples were normalized to Gapdh expression. The DNA sequences of human and mouse primers used for qPCR were summarized in Tables 1 and 2. Western blot analysis was performed as described previously (26, 30). Antibodies used were from the following sources: antibodies against NFATc1, c-FOS, RANK, ATF4, and anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); antibodies recognizing phosphorylated and total AKT and PL1 were from Cell Signaling Technology Inc. (Beverly, MA); and mouse monoclonal antibody against β-actin was from Sigma.

In Vitro Osteoclast Assays—For osteoclast studies using bone marrow cells from humans, nonadherent BMMs were isolated from patients with MM and NDs. For undifferentiated cultures, cells were cultured in the proliferation media (α-MEM containing 20% horse serum and 10 ng/ml human recombinant M-CSF) for 10 days. For differentiation studies, cells were cultured in the differentiation media (proliferation media containing 50 ng/ml human recombinant RANKL) for 21 days, followed by TRAP or 23C6 staining or gene expression studies. The anti-23C6 antibody, which recognizes the αβ3 integrin, a receptor for vitronectin on osteoclasts, was described previously (31–33). The TRAP- or 23C6-positive multinucleated cells (MNCs) (≥3 nuclei) were scored using an inverted microscope. For osteoclast studies using primary mouse bone marrow cells (BMMs), nonadherent BMMs were isolated from total bone marrow cells and cultured on tissue culture dishes for 48 h. For differentiation, cells were first cultured in the proliferation media (α-MEM containing 10% FBS and 10 ng/ml M-CSF) for 3 days and switched to differentiation media (proliferation media plus 50 ng/ml RANKL) for 5–7 days, followed by TRAP staining or gene expression studies. The TRAP-positive MNCs (≥3 nuclei) were scored using an inverted microscope.

Mouse Model and Histological Analysis and Bone Histomorphometry—For intratibial injection, both the left and right tibiae of 4-week-old male SCID mice (10 mice/group or 20 tibiae/group) were injected with 1 × 10⁶ 5TGM1 cells in 20 μl of PBS or 20 μl of PBS alone (control group without MM injec-
MM Activates Osteoclast Formation in Primary BMM Cultures

To study the mechanisms whereby MM cells activate osteoclast formation, we measured osteoclast differentiation in primary BMM cultures from patients with MM in vitro compared with BMM cultures from ND with or without the addition of exogenous RANKL. We measured the numbers of TRAP-positive multinucleated osteoclasts (MNCs) generated by each. Results showed that the number of TRAP+ MNCs (≥3 nuclei) in the RANKL-differentiated MM BMM cultures was dramatically increased compared with that in the ND BMM cultures (Fig. 1A, A and B). The MNCs formed in the RANKL-differentiated MM BMM cultures were much larger than those from the ND BMM cultures (Fig. 1B). Surprisingly, we found a number of TRAP+ MNCs in the MM BMM cultures in the absence of exogenously added RANKL, which were absent from the ND BMM control cultures (Fig. 1A and C). This could result from potential contamination of osteoblasts and stromal cells and bone marrow lymphocytes, which produce RANKL and OPG (36). For this reason, we measured the expression levels of both factors by qPCR analysis. Results showed that, although both mRNAs were detected in both cultures, the RANKL/OPG ratio was actually reduced in the MM versus ND cultures (Fig. 1D). Therefore, the enhanced osteoclast formation in the MM cultures is not due to increased RANKL and/or reduced OPG expression produced by cells in the cultures. qPCR analysis showed that the mRNA levels of osteoclast differentiation marker genes, including those encoding cathepsin K, integrin β3 (β3), NFATc1 (a master regulator of osteoclast differentiation), and matrix metalloproteinase-9 (MMP-9) were all significantly increased in the differentiated MM relative to ND BMM cultures (Fig. 1E). The mRNA level of c-FMS, the gene that encodes the receptor for the macrophage colony-stimulating factor (CSF-1R), was slightly but significantly increased in the RANKL-differentiated MM versus ND BMM cultures (Fig. 1E). Western blot analysis revealed that the levels of NFATc1 protein were increased in the RANKL-differentiated BMM cultures from five patients with MM compared with those in five NDs (Fig. 1F). Collectively, these results suggest that osteoclast differentiation capacity of the MM BMMs is increased in both RANKL-dependent and RANKL-independent manners.

Sustained High Expression of RANK Is a Major Feature of the BMMs from MM Patients—To study the early molecular events in the MM BMM cultures, we measured the expression levels of genes that are known to regulate early osteoclast differentiation in undifferentiated BMM cultures from patients with MM and NDs. Results showed that the level of RANK mRNA was increased by more than 10-fold in undifferentiated BMM cultures from patients with MM compared with that from NDs (Fig. 2A). In contrast, the levels of c-FMS, PLI, c-FOS, TRAF6, and DAP-12 mRNAs were not significantly increased in the undifferentiated MM versus ND BMM cultures (Fig. 2A). Western blot analysis confirmed that the level of RANK protein was increased in the undifferentiated MM relative to ND BMM cultures (Fig. 2B). Importantly, the levels of RANK mRNA were significantly increased in the uncultured BMMs from 11 patients with MM compared with those from the uncultured BMMs from six NDs (Fig. 2C). Because RANK expression is a critical step for generating osteoclast precursors, these results suggest that MM cells greatly promote early osteoclast differentiation. Surprisingly, although RANK is a marker of osteoclast precursors, we found that its expression continued to increase even in the terminally differentiated osteoclast cultures from patients with MM (i.e. in the presence of RANKL for 21 days) (Fig. 2D). Western blot analysis confirmed that the level of RANK protein was increased in the RANKL-differentiated MM relative to ND BMM cultures (Fig. 2E).

Critical Role of AKT in RANK Expression and Osteoclast Formation in BMM Cultures from MM Patients—Although the above results clearly showed that RANK expression was upregulated in osteoclast precursors from patients with MM, the underlying mechanisms remain unknown. Recent studies showed that AKT plays a role in promotion of early osteoclast differentiation (24, 25). We investigated whether AKT is upregulated in the MM BMM cultures. As shown in Fig. 3A, in the RANKL-differentiated MM BMM cultures, the levels of both

### Table 2: Mouse qPCR primers

| Name | 5’ primer | 3’ primer |
|------|-----------|-----------|
| Atf4 | GAGCTTCTGAAACAGCGAAGTG | TGGCCACCTCCAGATGTCATC |
| Dap12 | GCCGTGGTCCGCGAGGTCAAG | TCTGGTCTCTGACCCTGAAGCTCC |
| Gapdh | AGATGCCAGCTGGTCTGAGTAGA | CTCGAAATGGCACGCTCTGTGAGAC |
| Rank | AGAGGGGAGGCTCAGGGTCC | AAGTTTCATCACCTGGCCGCTAGA |

Statistical Analysis—Data were analyzed with a GraphPad Prism software (4.0) (San Diego). A one-way analysis of variance was used followed by the Tukey test. Results were expressed as means ± S.D. Differences with a p < 0.05 was considered as statistically significant. All experiments were repeated at least two times, and similar results were obtained.
phospho-AKT and total AKT proteins were up-regulated compared with those in the ND BMM cultures. Because RANK and AKT were similarly up-regulated in the MM BMM cultures, we determined whether AKT is required for the up-regulation of RANK expression in the MM BMM cultures. Results showed that LY294002, a specific AKT inhibitor, dose-dependently decreased RANK mRNA expression in the MM BMM cultures (Fig. 3B). In contrast, the inhibitor did not reduce the expression of PU.1 mRNA in the MM BMMs (Fig. 3B). LY294002 similarly decreased RANK protein expression in the MM BMM cultures in a dose-dependent manner (Fig. 3C). Importantly, the AKT inhibitor blocked formation of the MNCs in the MM BMM cultures (Fig. 3, D–F). Furthermore, overexpression of a constitutively active form of AKT (AKT-CA) increased the mouse Rank promoter activity in COS-7 cells (Fig. 3F). Taken together, these results suggest that MM may activate RANK expression and osteoclast formation through, at least in part, up-regulation of AKT in osteoclast precursors.

Soluble Factors Produced by MM Cells Increase AKT Phosphorylation, RANK Expression, and Osteoclast Formation—To determine whether MM cells produce soluble factors responsible for the increased AKT and RANK expression and osteoclast formation, primary mouse BMMs were treated with the conditioned media (CM) from human MM1.S or mouse 5TGM1 MM cell lines. Western blot analysis showed that AKT phosphorylation was increased by CM from both MM cell lines (Fig. 4A). The MM1.S-CM increased RANK expression at both the mRNA and protein levels in dose- and time-dependent manners (Fig. 4, B and C). The MM1.S-CM dose-dependently promoted formation of both mononuclear and multinucleated osteoclasts (Fig. 4, D and E). However, the MM1.S-CM did not alter the formation of colony-forming units-granulocyte-macrophage (CFU-GM) (Fig. 4, F and G). The MM1.S-CM dose-dependently promoted formation of both mononuclear and multinucleated osteoclasts (Fig. 4, D and E). However, the MM1.S-CM did not alter the formation of colony-forming units-granulocyte-macrophage (CFU-GM) (Fig. 4, F and G), which contains the earliest osteoclast precursors (37).

ATF4 Protein Is Up-regulated in MM Osteoclast Cultures and by STGm1-CM and TNF-α—Our recent study demonstrates that loss of ATF4 impaired M-CSF induction of RANK expression in osteoclast precursors (25). Interestingly, we found that the level of ATF4 protein was increased in the RANKL-differentiated BMM cultures from four patients with MM compared with that of four NDs (Fig. 5A). The level of ATF4 mRNA was not increased in MM versus ND cultures (Fig. 5B), suggesting a
post-transcriptional regulation. The 5TGM1-CM and TNF-α, a well known osteoclastogenic factor produced by many cancer cells, including MM cells, increased the ATF4 protein levels in the BMM cultures (Fig. 5, C and E). In contrast, 5TGM1-CM and TNF-α did not increase the levels of Atf4 mRNA (Fig. 5, D and F). Interestingly, slower migrating bands indicated by an arrow were observed in the MM BMM cultures (Fig. 5 A) and increased by treatments with 5TGM1-CM (Fig. 5 C) or TNF-α (Fig. 5E). These bands were probably phosphorylated forms of ATF4 because similar bands in primary mouse BMMs disappeared with calf intestinal phosphatase treatment (25).

ATF4 Is Phosphorylated and Up-regulated by AKT—To determine whether ATF4 is downstream of AKT, we performed several experiments. First, we found that AKT inhibitor LY294002 decreased the ATF4 protein levels in primary BMM cultures from patients with MM in a dose-dependent manner (Fig. 6A). Second, overexpression of a constitutively active form of AKT (AKT-CA) increased the level of ATF4 protein in COS-7 cells (Fig. 6B). There was a slower migrating ATF4 band on Western blots that was observed in samples from the AKT-CA-transfected cells (Fig. 6B). Third, transfection assays revealed that AKT-CA increased ATF4-dependent transcriptional activity (Fig. 6C), which was abolished by introduction of a 3-bp point mutation in the ATF4-binding core sequence (Fig. 6D). Fourth, we tested whether ATF4 can be directly phosphorylated by AKT enzyme by performing in vitro kinase assays using purified GST-ATF4 protein and AKT1 enzyme in the presence of [γ-32P]ATP. As shown in Fig. 6E, purified GST-ATF4 but not GST protein was directly and strongly phosphorylated by the AKT1 enzyme in vitro. Finally, adenoviral overexpression of ATF4 increased the level of Rank mRNA by 10-fold in osteoclast precursors (Fig. 6F). In contrast, Dap-12 (DNA-activating protein 12), a membrane protein expressed in both macrophages and osteoclasts, was not increased by ATF4 in BMMs (Fig. 6E). Blocking AKT Reduces Tumor Burden in Bone Marrow Cavity and Inhibits the MM-induced Osteoclast Formation and Osteolysis in SCID Mice—To determine whether AKT plays a role in the MM-induced osteoclast formation and osteolysis in vivo, we injected 5TGM1 MM cells into the tibial marrow cavity of SCID mice. One week after the injection, animals were subcutaneously injected with LY294002 (40 mg/kg body weight) or vehicle (DMSO) twice per week for 3 weeks. This LY294002 dose is in the range used by other investigators to efficiently block the PI3K/AKT pathway in mice (38, 39). We examined whether the MM cells stimulated osteoclast formation by staining histological sections for the osteoclast enzyme TRAP. Results showed that 5TGM1 cells increased osteoclast surface/
Bone surface (Oc.S/BS) and osteoclast number/bone perimeter (Oc.Nb/BPm) of tibiae by 2.3- and 1.8-fold, respectively ($p < 0.05$ for both PBS/DMSO versus 5TGM1/DMSO) (Fig. 7, A–C). Strikingly, the MM-induced increases in Oc.S/BS and Oc.Nb/BPm were completely inhibited by LY294002 (Fig. 7, A–C). We further determined whether blocking AKT affects the MM-induced osteolysis by examining histological sections. Results showed that, in DMSO-injected mice, the majority of the trabecular bone of the proximal tibial metaphysis was destroyed and that the marrow cavity was replaced by tumor tissues. In contrast, in those LY294002-injected mice, the bone destruction was largely prevented (Fig. 7, D and E). The formation of tumor tissues in bone marrow cavity was suppressed by AKT inhibition (Fig. 7D). It should be noted that although AKT was reported to regulate osteoclast and osteoblast function in mice (24), the LY294002 treatment under our experimental conditions only slightly decreased the basal level of the Oc.S/BS ($p = 0.087$, PBS/DMSO versus 5TGM1/LY). In addition, LY294002 did not cause any reductions in the Oc.Nb/BPm and trabecular bone area ($p > 0.12$ for both parameters, PBS/DMSO versus 5TGM1/LY).

**AKT Is Critical for the MM Cell Growth and Promotion of Osteoclast Formation in Vitro**—We next performed in vitro experiments to examine whether the AKT pathway in the MM cells is required for the tumor cell growth and promotion of osteoclast formation. Results showed that the LY294002 treatment significantly decreased the growth of MM1.S cells (Fig. 8A). Western blot analysis confirmed that the level of phospho-AKT but not total AKT protein was decreased by the AKT inhibitor (Fig. 8B). Finally, osteoclast formation induced by the CM from MM1.S cells was significantly reduced by treatment of the tumor cells with LY294002 (Fig. 8, C and D).

**DISCUSSION**

Abnormal osteoclast differentiation is a major contributor to osteolysis caused by major cancers such as MM, breast, lung, and prostate cancers. Bone-residing cancer cells promote osteoclast formation and activation (16, 40–45). Increased osteoclast formation and activity and bone resorption increase releases of growth factors from the bone matrix that stimulate cancer cell growth (40–46). Therefore, there is a vicious cycle between osteoclast-mediated osteolysis and tumor growth and...
progression in bone marrow. Breaking the vicious cycle is of major clinical significance because osteolytic lesions and related complications such as severe bone pain, pathological fractures, spinal cord compression, and hypercalcemia of malignancy are common causes of morbidity and sometimes mortality (42, 47).

In this study, we found that AKT in osteoclasts and their precursors plays an important role in the MM promotion of osteoclast formation and activation and osteolytic lesions. Both AKT expression and phosphorylation are up-regulated in primary bone marrow osteoclast cultures from patients with MM. Blocking AKT inhibits the MM-induced osteoclast formation in vitro and abolishes the MM-induced increase in osteoclast differentiation and osteolysis in bone. We demonstrate that AKT promotes osteoclast formation through, at least in part, up-regulation of RANK in osteoclast precursors. The expression of AKT and RANK are both increased in primary bone marrow osteoclast cultures from patients with MM. AKT inhibition reduces the level of RANK mRNA and osteoclast formation in the MM BMM cultures. Overexpression of the consti-
tutively active form of AKT increases the RANK gene promoter activity. We further demonstrate that ATF4, which is up-regulated by AKT, TNF-α/H9251, and soluble factors produced by the MM cells, is a new upstream transcriptional activator of RANK gene expression in osteoclast precursors.

Increased RANK expression in osteoclast precursors in the MM bone marrow could greatly increase the sensitivity for RANKL to induce osteoclast formation and maturation, suggesting that, even under low concentrations of RANKL, osteoclast differentiation can be enhanced in the bone marrow of patients with MM. This notion is supported by the following evidence. (i) Osteoclast formation is greatly enhanced in the MM BMM cultures in the absence of exogenously added RANKL, in which the RANKL/OPG ratio is reduced (Fig. 1D). (ii) The levels of RANK from many tumor cells that can activate osteoclast formation are very low (17). Furthermore, sustained high expression of RANK even in...
highly differentiated osteoclast cultures (i.e. 21 days in the presence of RANKL) from patients with MM could also suggest that part of the BMM cells in the MM bone marrow are maintained at the osteoclast precursor state because RANK expression is a marker of osteoclast precursors. These precursor cells can actively proliferate and increase the osteoclast numbers in bone marrow, which could further contribute to the increased osteoclast formation and osteolysis in patients with MM.
Although results from this study demonstrate that AKT is critical for the MM-induced osteoclast formation, how MM cells activate AKT remains unclear. Cancer cells can produce and induce factors that can promote osteoclast formation and activity, including granulocyte-macrophage colony-stimulating factor (GM-CSF) (16), MCP-1 (17), MIP-1α and MIP-1β...
(19, 20), TNF-α (48), and parathyroid hormone-related protein (14, 15). Future studies will determine whether any of those factors are responsible for MM cell activation of AKT in osteoclast precursors. It will be interesting to determine whether AKT also mediates the osteoclast formation and osteolysis caused by other cancers such as breast, lung, and prostate in the future.

It should be noted that blocking AKT in MM cells decreases their growth in vitro and that systemic inhibition of AKT blocks the formation of tumor tissues in the bone marrow cavity in SCID mice. Interestingly, our in vitro studies show that AKT inhibition in the MM cells also reduces the ability of tumor cells to activate osteoclast formation, which should contribute to the reduced osteoclast formation induced by the tumor cells. Finally, although the AKT inhibitor essentially abolishes the MM-induced osteolysis and bone loss, it does not significantly affect the basal level osteoclast formation and bone mass under our experimental conditions. Collectively, these results suggest that AKT could be a potential target for inhibiting the MM-induced osteoclast oestolotic lesions as well as tumor growth and progression, thus breaking the vicious cycle in the patients with MM.

Acknowledgment—We thank Noriyoshi Kurihara (Indiana University School of Medicine) for technical assistance.

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