Inhibitory effects of osteoblasts and increased bone formation on myeloma in novel culture systems and a myelomatous mouse model

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Background and Objectives. Multiple myeloma (MM) growth in the bone marrow is associated with increased osteoclast activity and a reduced number of osteoblasts. Experimental studies suggest that bone disease drives the progression of MM. Whereas those studies focused on the critical role of myeloma-induced osteoclastogenesis in disease progression, little is known about the impact of osteoblasts and increased bone formation on MM.

Design and Methods. We investigated the effect of isolated osteoblasts and osteoclasts on survival and proliferation of primary MM plasma cells (PC) in co-cultures and triple-cultures, and tested the effect of mesenchymal stem cells (MSC) on bone mineral density and MM growth in myelomatous human bones of SCID-hu mice.

Results. Whereas osteoclasts promoted survival and proliferation of MM PC, osteoblasts supported or inhibited MM PC, depending on the source of the MM cells. In triple-cultures osteoblasts attenuated the effect of osteoclasts on MM PC in 18 of 24 experiments. The anti-MM response to osteoblasts correlated with advanced clinical stage. Injection of MSC into myelomatous bones resulted in marked inhibition of tumor growth in three of nine experiments and stabilization of disease in two additional experiments. The anti-MM response of MSC was associated with increased human bone mineral density. Immunohistochemical analysis indicated that the MSC were well engrafted and, in responding mice, differentiated into osteogenic cells.

Interpretation and Conclusions. MM PC from the majority of patients are susceptible to growth inhibition by osteoblasts; however, growth of MM PC from certain patients is accelerated by osteoblasts. *In vivo*, increased bone formation is associated with reduced myeloma burden.

Key words: myeloma, osteoblasts, osteoclasts, mesenchymal stem cells, bone formation.

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Multiple myeloma (MM) is typically associated with induction of osteolytic bone disease in more than 80% of patients. Histomorphometric studies in patients suggest that changes in bone remodeling precede the progression of MM.[1,2] Coupled increases in the number of osteoclasts and osteoblasts were seen in patients with monoclonal gammopathy of undetermined significance and in those with early-stage MM. In more progressive stages, the numbers of osteoclasts increased whereas that of osteoblasts decreased, resulting in an uncoupling of the processes of osteoclastic resorption and osteoblastic bone formation.[3,4] Whereas numerous studies have focused on the cellular and molecular mechanisms of myeloma-induced osteoclastogenesis,[5-9] the processes by which osteoblast activity is reduced in myelomatous bones have just been unveiled. Silvestris *et al.* demonstrated *in vitro* that myeloma cells induce apoptosis in osteoblasts through direct physical contact and via production of soluble factors.[10,11] Tian *et al.* reported that myeloma cells from patients with bone disease produce the Wnt signaling inhibitor, dickkopf-1 (DKK1), and that DKK1 inhibits osteoblast differentia-

tion.[12] A recent study also demonstrated the important role of interleukin-3 as a myeloma growth factor and the ability of this cytokine to promote osteoclastogenesis and inhibit osteoblast differentiation.[9,13] Experimental data suggest that myeloma bone disease and tumor growth are interdependent. Inhibition of osteoclast activity with bisphosphonates and inhibitors of receptor activator of the NF-κB ligand (RANKL) in primary human myeloma-bearing SCID-hu mice[5,6] and a 5T murine myeloma model[14,15] was associated with inhibition of myeloma growth. We and others also demonstrated the ability of isolated osteoclasts to support survival and proliferation of MM cells through a process requiring cell-cell contact.[7,16] Whereas those studies focused on the critical role of myeloma-induced osteoclastogenesis in disease progression, little is known about the impact of osteoblasts on myeloma progression. In this study we report on the effects of osteoblasts on myeloma cells in co-cultures of primary purified MM cells with osteoblasts and osteoclasts (co- and triple-cultures) and on the impact of osteoblasts on myeloma progression and bone formation in our *in vivo* SCID-hu model.

Table 1. Characterization of myeloma patients.

Patients' characterization	No. of patients
Stage*	
IA	4
IB	2
IIA	3
IIIA	14
IIIB	3
PCL°	1
Prior Therapy	
Yes	6
No	21
Cytogenetic abnormalities	
Yes	14
No	13
Bone disease#	
Yes	17
No	10
Isotype	
IgA	7
IgG	15
Free light chain	5

*Stage at diagnosis, according to the Durie-Salmon staging system; °plasma cell leukemia; #detected by magnetic resonance imaging and radiography.

Design and Methods

Reagents and kits

Anti-human bromodeoxyuridine (BrdU) was obtained from DAKO Corp. (Carpinteria, CA, USA). Ficoll-Paque was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Monoclonal antibodies to human CD38 (phycoery-thin) and CD45 (fluorescence isothiocyanate) for FACS analysis were from BD Biosciences (San Josè, CA, USA). Anti-human osteocalcin was from BioTrend (Cologne, Germany), anti-human bone morphogenetic protein (BMP)-2 was from Biogenesis (Kingston, NH, USA). Anti-CD166 was from Antigenix America (Huntington Station, NY, USA). αMEM and an antibiotic cocktail containing penicillin, streptomycin and neomycin were from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, Utah, USA). Recombinant human macrophage colony-stimulating factor (M-CSF) and RANKL were from RDI (Flanders, NJ, USA). Dexa-methasone, sodium β-glycerophosphate (βGP), trypsin-ethylenediaminetetraacetic acid (EDTA), BrdU, FrdU, silver nitrate and a leukocyte acid phosphatase kit for tartrate-resistant acid phosphatase (TRAP), alkaline phosphatase diagnostic kit #85 and polybrene were all from SIGMA (St. Louis, MO, USA). L-ascorbic acid-2-phosphate (ascorbate) was from WAKO Chemicals (Richmond, VA, USA). Anti-human CD138 antibody for immunomagnetic bead separation was from Miltenyi-Biotec (Auburn, CA, USA). Monoclonal antibody to enhanced green fluorescent protein (EGFP) was from Invitrogen (Carlsbad, CA, USA). Cell culture plates were from Becton Dickinson (Franklin Lakes, NJ, USA) and the transwell inserts were from Costar (Corning, NY, USA).

The annexin V/PI detection kit for flow cytometry was from Caltag Laboratories (Burlingame, CA, USA), and the immunoperoxidase detection kits were from Vector (Burlingame, CA, USA).

Myeloma cells

Myeloma plasma cells (MM PC) were obtained from heparinized bone marrow aspirates from 27 patients with active myeloma during scheduled clinic visits. Signed Institutional Review Board–approved informed consent forms are kept on record. Pertinent information about the patients is provided in Table 1. The bone marrow samples were separated by density centrifugation using Ficoll-Paque (specific gravity 1.077 g/mL) and the proportion of MM PC in the light-density cell fractions determined by CD38/CD45 flow cytometry.[17] Plasma cells were isolated using CD138 immunomagnetic bead selection and the autoMACs automated separation system (Miltenyi-Biotec, Auburn, CA, USA). Plasma cell purity was determined by CD38/CD45 flow cytometry to be routinely ≥94%. Myeloma cell viability was determined by trypan blue exclusion and apoptotic cells were enumerated using an annexin V/PI kit.[7]

Preparation of mesenchymal stem cells and osteoblasts

Mesenchymal stem cells (MSC) were prepared as described by Pittenger.[18] Briefly, bone marrow mononu-cleated cells (2×10^6 cells/mL) from patients with MM and human fetal bone were cultured in DMEM-LG supplemented with 10% FBS and antibiotics (MSC media). Half of the media was replaced every 4-6 days and adherent cells allowed to reach 80% confluency before they were sub-cultured with trypsin-EDTA. The adherent MSC expressed CD166[19] (Figure 1) but not CD45 and CD34. For differentiation into osteoblasts, MSC were incubated with DMEM-LG supplemented with 10% FBS, dexamethasone (100 nM), βGP (10 mM) and ascorbate (0.05 mM). The MSC formed aggregates or nodules, had increased expression of alkaline phosphatase within 1-2 weeks, followed by calcium deposition, lost CD166 expression and increased expression of osteocalcin and BMP-2 (Figure 1).

In contrast to when grown together with osteoclasts,[7] MM PC often firmly adhered to MSC and osteoblasts, preventing complete recovery of tumor cells after co-culture. To overcome this obstacle, we designed a novel co-culture system using transwell inserts with 1-μm pores. In this system, osteoblasts were generated on the backside of the inserts' membranes and MM PC were cultured in the upper chamber of the inserts (Figure 2). Histologic examination demonstrated that osteoblast cytoplasmic villi pass through the membrane pores, allowing contact with tumor cells (Figure 2) as previously reported for a similar culture system.[20] For culturing osteoblasts in our culture system, 6-well inserts were flipped upside down and placed in a sterile deep dish. MSC were collected with trypsin-EDTA and resuspended in MSC media (approximately 0.5×10^6/mL). Approximately 600 μL of cells were placed onto the center of the inverted insert. The dish was then covered with parafilm and placed in the incubator for 1 hour, allowing cells to adhere to the
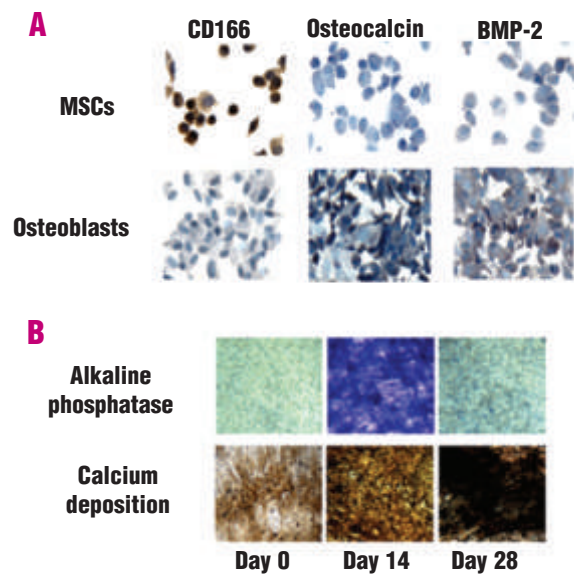
membrane. Following incubation, the inserts were flipped back and placed in 6-plate wells. When MSC were approximately 80% confluent, they were cultured with osteoblastic media for 2-3 weeks.

Preparation of osteoclasts

Highly purified bone-resorbing osteoclasts were prepared as previously described[7] and generated on upper chambers of 6-well transwell inserts with 1-µm pores (Figure 2). Briefly, peripheral blood mononuclear cells were obtained from MM patients and healthy subjects. Signed informed consent forms are kept on record. The cells were cultured at 2.5×10^6 cells/mL in α-MEM supplemented with 10% FBS, antibiotics, RANKL (50 ng/mL) and M-CSF (25 ng/mL) (osteoclast media) for 10–14 days, at which time they contained large numbers of multinucleated osteoclasts with bone-resorbing activity.[7] Prior to co- and triple-culturing with MM cells and osteoblasts, the osteoclasts were washed three times with phosphate-buffered saline (PBS) to detach and remove non-adherent cells.

Preparation of osteoclasts and osteoblasts for triple-cultures

Using the same transwell inserts, for most experiments, MSC were first cultured on the backside of the insert membrane with osteoblastic media while osteoclast precursors were incubated thereafter on the upper chamber. To allow differentiation of osteoblasts and osteoclasts simultaneously, the inserts were then incubated with α-MEM supplemented with 10% PBS, RANKL (50 ng/mL), M-CSF (25 ng/mL), dexamethasone (100 nM), ascorbate
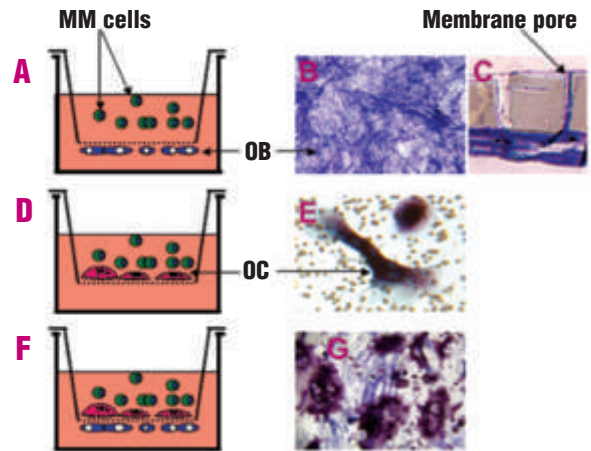
(0.05 mM) and βGP (10 mM) for approximately 2 weeks. This procedure resulted in simultaneous growth of TRAP-expressing multinucleated osteoclasts and alkaline phosphates-expressing osteoblasts (Figure 2).

Co- and triple-cultures of MM cells with osteoblasts and osteoclasts

Osteoclasts and osteoblasts in co- and triple-cultures were washed with PBS and resuspended in α-MEM supplemented with 10% PBS, RANKL (50 ng/mL), M-CSF (25 ng/mL), ascorbate (50 nM) and βGP (10 µM) (triple-culture media). For all experiments the upper chamber contained 1 mL of triple-culture media containing 0.5×10^6 MM PC while the bottom chamber contained 2 mL of triple-culture media. MM PC were cultured in duplicates for 7-10 days. At the end of each experiment, MM PC were collected, viable cells counted with trypan blue and processed for annexin V binding and BrdU staining as previously reported.[7]

Establishment of MSC stably transduced with enhanced green fluorescent protein (EGFP)

The pLEGFP retroviral vector containing EGFP (Clontech, Palo Alto, CA, USA) was made to transiently transfect the packaging cell line Phoenix Eco using SuperFect (QIAGEN Inc., Valencia, CA, USA). Supernatants containing retroviral particles were collected 24-48 hours after transfection. MSC from two patients with bone disease and from human fetal bones, at approximately 60% confluency, were infected with the retroviral particles in the presence of 8 µg/mL polybrene for 12 hours at which time the media were replaced with fresh

culture medium. In some experiment, cells were exposed to the supernatants containing the viral particles once more before being selected by culturing them in the presence of 200-400 μg/mL of G418 for 2-3 weeks.

Engraftment of MSC in myelomatous SCID-hu mice

Myelomatous SCID-hu mice were produced as previously described.[6,21-23] Upon establishment of myeloma growth, assessed by increased levels of human monoclonal immunoglobulins (hIg) in the mice sera using ELISA and by radiographic evaluation of lytic bone lesions, $0.5×10^6$ EGFP-expressing MSC were collected with the use of trypsin-EDTA and resuspended in 50 μL PBS. The MSC and PBS were injected directly into the implanted bones in SCID-hu mice. Experiments were continued for 8-16 weeks post-injection. Changes in the bone mineral density of the implanted bones were determined using a PIXImus DEXA (GE Medical Systems LUNAR, Madison, WI, USA).

Immunohistochemistry

Decalcified bone sections from primary myeloma-bearing SCID-hu mice were deparaffinized in xylene, rehydrated with ethanol, rinsed in PBS, and subjected to antigen retrieval using microwave as previously described.[6,23] Cultured MSC and osteoblasts were trypsinized and then cytospin slides were prepared and fixed with 10% phosphate-buffered formalin for 20 min. After peroxidase quenching with 3% hydrogen peroxide for 10 min, the slides were incubated with monoclonal antibodies against EGFP, and human CD166, osteocalcin and BMP-2 (5-10 μg/mL) for 30-60 min and developed using Dako's immunoperoxidase kit and counterstaining with hematoxylin.

von Kossa staining

For detection of calcium deposition (von Kossa staining), MSC and osteoblasts were fixed in 10% phosphate-buffered formalin for 10 min. Freshly prepared 5% silver nitrate was added and the specimens left in the dark for 10 min, rinsed with distilled water and then exposed to UV light for 15 min while covered with water. The reaction was stopped by rinsing thoroughly with distilled water.

Statistical analysis

Unless indicated otherwise, all values are expressed as mean±SEM. Student's paired t-test was used to test the effect of different culture conditions on myeloma cell numbers, viability, apoptosis, and proliferation, and to test the effect of MSC on tumor growth and human bone mineral density in SCID-hu mice. Fisher's exact test was used to compare response to osteoblasts and detection of bone disease and clinical stage of patients whose MM PC were tested.

Results

Effects of primary osteoblasts and osteoclasts on survival and proliferation of myeloma plasma cells

CD138-enriched MM PC were cultured alone, co-cultured with osteoclasts or osteoblasts, and cultured in triple-cultures with osteoblasts+osteoclasts (n=24) (Figure 3). As previously reported by us,[7] osteoclasts consistently supported MM PC survival and proliferation: MM PC in co-cultures with osteoclasts had more viable cells, fewer annexin V positive cells, and higher BrdU labeling indices (LI) than in cultures of MM PC alone and in co-cultures with osteoblasts. In contrast to osteoclasts, osteoblasts slightly promoted MM PC survival as indicated by a lower percentage of annexin V positive cells, but the number of viable cells and the BrdU LI were not significantly different from those obtained with MM PC cultured alone. To reflect *in vivo* conditions more closely and since MM PC often succumb to spontaneous apoptosis when cultured without appropriate cellular and cytokine support, we also tested the effect of osteoclasts and osteoblasts on the survival and proliferation of MM PC in our triple-cultures. MM PC in the triple-cultures had fewer viable cells ($p<0.01$), more annexin V positive cells ($p<0.04$), and a lower BrdU LI ($p<0.04$) than in co-culture with osteoclasts. As shown in Figure 4, although the overall number of viable myeloma cells was reduced by 23±8% in the triple-cultures as compared with MM PC/osteoclast co-cultures ($p<0.01$), osteoblasts had diverse effects on MM PC survival and proliferation in this culture system; whereas in 14 of 24 experiments (patients 1-14) the number of viable tumor cells was reduced by more then 30% compared with MM PC/osteoclasts co-cultures, it increased by more than 20% in three other experiments (patients 22-24). The response of MM PC to osteoblasts did not correlate with patients' parameters such as M protein isotype, serum levels of β2 microglobulin, or cytogenetic abnormalities. However, 69% of patients whose MM PC were responsive to osteoblasts, had lytic bone disease and focal lesions, detected by X-ray radiographs and/or magnetic resonance imaging.[12] Seventy-seven percent of those patients were also in clinical stages IIIa or IIIb. In contrast, only 33% of patients whose MM PC were not suppressed by osteoblasts (patients 19-24) had bone disease or were at advanced clinical stages. Overall, MM PC from patients in clinical stage III had a significantly higher incidence of suppression by osteoblasts ($p=0.04$).

To test heterogeneity among osteoblasts and MM PC, in seven experiments osteoblasts from different sources were triple-cultured with the same MM PC and osteoclasts (Figure 5A). In five of those seven experiments, different osteoblasts had similar effects (inhibitory or stimulatory) on MM PC from individual patients. In six additional experiments, MM PC from two or three patients were used in triple-cultures with the same osteoblasts (Figure 5B). In five of the six sets of experiments the effect of osteoblasts on MM PC from different patients varied considerably. These results suggest that the anti-myeloma effect of osteoblasts is not osteoblast-source-specific but rather reflects inter-patient heterogeneity of MM cells.

Effects of MSC on bone mineral density and myeloma progression in myelomatous SCID-hu mice

Myeloma cells from nine patients were injected into the human bones of SCID-hu hosts, and the effect on myeloma burden was assessed by the level of monotypic hIg in murine serum as previously described.[21,22] Growth of cells
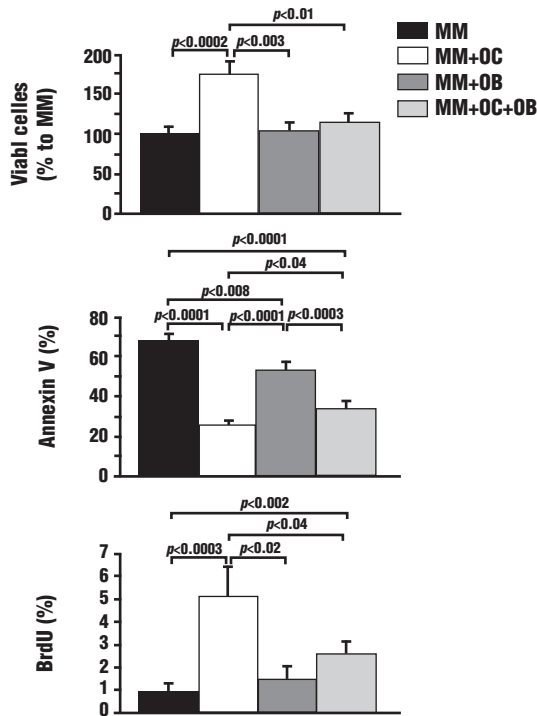
Figure 3. Survival and proliferation of MM cells in co- and triple-cultures with osteoblasts and osteoclasts. Primary CD138-selected MM cells were cultured alone, co-cultured with osteoblasts (MM + OB) and osteoclasts (MM + OC), or triple-cultured (MM + OC + OB) for 7-10 days. **A.** Number of viable MM cells. **B.** Percent apoptotic MM cells detected by annexin V flow cytometry. **C.** Proliferation level in MM cells as determined by the BrdU labeling index.
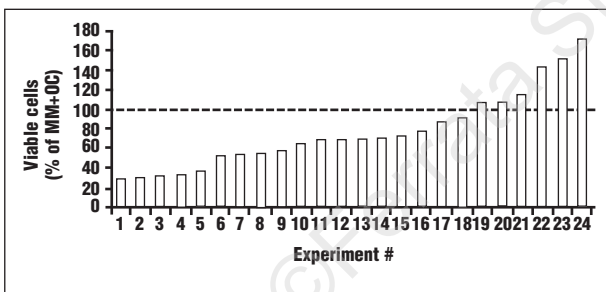

Figure 4. Osteoblasts attenuate the stimulatory effect of osteoclasts on myeloma cells. MM cells were co-cultured with osteoclasts and triple-cultured with osteoclasts and osteoblasts. Results are expressed as percent viable MM cells in triple-cultures versus co-cultures. Note, that although osteoblasts had diverse effect on MM cells, overall, the number of viable MM cells in triple-cultures was reduced by 23% (*p*<0.01).
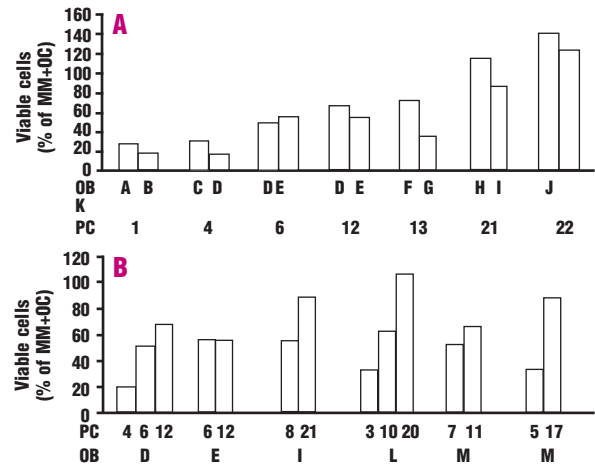

Figure 5. Anti-myeloma efficacy of osteoblasts depends on the source of the MM cells. **A.** Osteoblasts from different patients were triple-cultured with the same MM PC and osteoclasts. In five of seven experiments (PC 1, 4, 6, 12 and 22) MM PC from individual patients responded similarly to different osteoblasts. **B.** MM PC from two to three patients were triple-cultured with the same osteoblasts (OB) and osteoclasts. Note that but for one experiment (OB E) the effect of osteoblasts on MM PC from different patients varied greatly.

Because treatment was initiated when myeloma was already established, various degrees of bone loss were apparent on radiographs at the time of MSC and PBS inoculation.[6] Injection of MSC into SCID-hu mice resulted in inhibition of tumor growth in three experiments by 86%, 77% and 34% from pre-treatment levels and a retardation of growth in two additional experiments (responsive mice).

Injection of patients' MSC (3 of 5 experiments) and fetal MSC (2 of 4 experiments) resulted in inhibition of myeloma growth. The levels of hIg in the five responsive mice decreased from 492±206 to 205±68 μg/mL (*p*<0.04, pre- versus post-treatment), whereas in the non-responders hIg levels increased from 310±114 to 909±271 μg/mL (*p*<0.05, pre- versus post-treatment). Levels of hIg expressed as percent change from pre-treatment levels are shown in Table 2.

We also examined the relationship between the effect of MSC on myeloma growth and on bone metabolism. In the three of five responding experiments, bone mineral density increased by 2%, 13% and 18% from pre-treatment levels. Overall, bone mineral density in responding mice was increased by 5%±4 compared with a loss of 32%±12 and 23%+18 in the non-responders and control animals, respectively (*p*<0.02, Table 2). As demonstrated in Figure 6, the increased human bone mass caused by MSC as compared with PBS was also evident on X-ray radiographs.

Engraftment of EGFP-transduced MSC in myelomatous bones of SCID-hu mice was assessed microscopically and by immunohistochemical staining for EGFP (Figure 7). The injected MSC in responding mice differentiated into osteogenic cells such as osteoblasts and osteocytes, indicating that reduced bone loss in these mice was, in part, due to increased bone formation. A lesser number of cells
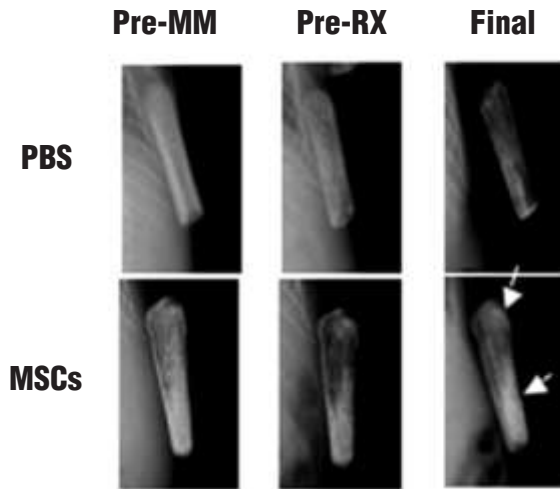
was restricted to the human microenvironment as determined post-mortem by CD38/CD45 flow cytometry and by cIg immunohistochemistry. Myeloma growth was associated with an increased number of osteoclasts and reduced number of osteoblasts in the human bones, and frequently with osteolysis of the human bones as seen on X-ray radiographs. To assess the effect of MSC on bone mineral density and myeloma growth, myelomatous SCID-hu hosts, each engrafted with myeloma cells from a different patient (n=9), were inoculated with EGFP-transduced MSC (fetal MSC n=4, MSC from first patient n=3 and second patient n=2) or with PBS (controls, n=5).

Figure 6. Effect of exogenous MSC on human bone mass in myelomatous SCID-hu mice. X-ray radiographs of the myelomatous human bone before MM cell engraftment (Pre-MM), MSC or PBS injection (Pre-Rx) and at the end of the experiment (Final). Whereas bone mass in PBS-treated bone decreased during the experimental period, injection of MSC resulted in increased bone mass (pointed by arrows).

Figure 7. Mesenchymal stem cells are capable of engraftment and differentiation into the mesenchymal lineage in myelomatous SCID-hu mice. MSC isolated from bone marrow of patients with myeloma were retrovirally labeled with EGFP and injected directly into the myelomatous bone in SCID-hu mice. **A-C.** Using florescent microscopy, EGFP-expressing osteoblasts (OB), osteocytes (OS), adipocytes (AD) and fibroblast-like cells (FB) were identified. **D, E.** Immunohistochemical staining for EGFP confirmed the fluorescent microscopy observation. Note the existence of positive and negative EGFP-expressing OB, AD and FB in these bones.

also differentiated into adipocytes and fibroblast-like cells (Figure 7). The injected MSC and their progeny in non-responding mice were rarely identified, although these MSC were capable of engraftment in non-myelomatous implanted human bones (*data not shown*).

Discussion

We have developed novel co- and triple-culture systems and employed our established *in vivo* SCID-hu model to study the effect of osteoblasts on myeloma growth and the association between bone metabolism and tumor progression *in vivo*. Our study revealed that osteoblasts inhibited survival and proliferation of myeloma cells from a subset of patients while they had no impact or even stimulated survival in another subset. The effects of osteoblasts on myeloma cells was also observed in the presence of osteoclasts (triple-cultures), which promoted survival and proliferation of myeloma cells from all patients studied.[7] Furthermore, in five of nine experiments injection of patients' MSC into myelomatous bones in SCID-hu mice resulted in inhibition or retardation of myeloma growth, an effect that was associated with increased bone mass. Our study suggests that increased osteoblast activity in myelomatous bones via direct injection of exogenous osteoblast precursors is feasible and that this procedure may not only improve bone density but also inhibit myeloma growth.

Previous studies in murine models showed a close association between myeloma progression and increased osteoclast activity.[5,6,14,15,24] To our knowledge, no studies have demonstrated the impact of osteogenic cells on long-term survival and proliferation of myeloma cells. The *ex vivo* experiments confirmed our previous observations of
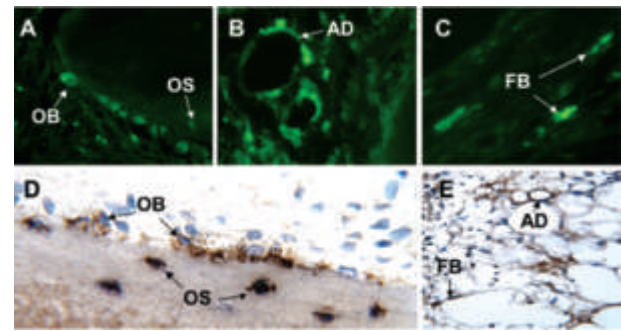
Table 2. The anti-myeloma effect of MSC is associated with increased bone mineral density in myeloma-bearing SCID-hu mice.*

	PBS (n=5)	MSC Responders (n=5)	Non-responders (n=4)
Human Ig (% change from pre-treatment)	421±208	-35±20°	406±148
Human BMD (% change from pre-treatment)	-32±12	5±4#	-23±18

*PBS: phosphate-buffered saline; MSC: mesenchymal stem cells; BMD: bone mineral density; *Control PBS- and non-responding MSC-treated mice had increased tumor burden levels and marked reduction of BMD compared with pre-treatment levels. Responding MSC-treated mice had reduced tumor burden and increased BMD levels. °p<0.001 responders vs. non-responders (MSCs- and PBS-treated); #p<0.02 responders vs. non-responders (MSC- and PBS-treated).*

the vital role of osteoclasts on maintaining the disease process.[6,7] Osteoblasts, in contrast, had diverse effects on myeloma cells, these effects being dependent on the source of the myeloma cells. Interestingly, the majority of patients whose myeloma cells were suppressed by osteoblasts were in clinical stage IIIa/IIIb and had severe bone disease. This suggests that increased osteoblast activity may help control tumor growth even in patients with advanced myeloma. We speculate that in these patients, myeloma cells reduce osteoblast activity, either via induction of osteoblast apoptosis[11] or by inhibition of their differentiation,[12,13] as part of mechanisms by which myeloma cells alter the bone marrow microenvironment for their advantage.

The mechanisms by which osteoblasts interfere with myeloma cell growth are still unclear. Osteogenic cells produce great amount of different members of the transforming growth factor-β superfamily including BMPs and activin A, which have been shown to induce growth arrest of B lymphocytes and myeloma cells *in vitro*.[25-29] Osteoblasts secrete osteonectin, a matrix cellular protein that inhibits survival and growth of epithelial tumor

cells.[30] Circulating levels of osteonectin seem to be inversely correlated with myeloma stage.[31] Osteoblasts express high level of connexins - transmembrane proteins that regulate osteoblast differentiation and apoptosis and mediate intercellular communications between osteogenic cells.[32] Connexins are also tumor suppressor genes and their expression is reduced or lost in many tumors. It has been suggested that Cx43 and Cx26 induce their tumor-suppressing properties by a mechanism that is independent of significant gap junctional intercellular communication and possibly through the down-regulation of key genes involved in tumor growth. For instance, transfection of the MDA-231 breast cell line with Cx43 reduced these cells' growth potential and down-regulated FGFR3 and CXCR4, both of which are involved in myeloma pathology.[33] In addition, osteoblasts produce high levels of osteoprotegerin, a factor that indirectly impedes myeloma growth *in vivo* through inhibition of osteoclast differentiation.[15] Thus, it is also possible that osteoblasts affect myeloma cells, as shown in triple-culture experiments and *in vivo*, indirectly through interference with the interaction of myeloma cells with osteoclasts.

Repair of lytic bone lesions is rarely seen, even in myeloma patients with prolonged complete remissions. We hypothesized that prevention of bone repair is caused, at least in part, by reduced numbers and impaired activity of MSC in focal bone lesions. *In vitro*, MSC have the ability to differentiate into various cell lineages, including osteoblasts, chondrocytes, adipocytes, skeletal myoblasts and endothelial cells.[18,34,35] The osteogenic potential of MSC has been demonstrated in animal models[36,37] and in patients with osteogenesis imperfecta.[38] We tested the effect of MSC on bone remodeling and tumor growth in myelomatous SCID-hu mice using EGFP-transduced MSC. Histological and immunohistological examinations revealed that patients' MSC were capable of engraftment and differentiation into various cells of the mesenchymal lineage, including osteoblasts and osteocytes. The differentiated MSC stimulated bone formation in five of nine experiments, an effect that was associated with inhibition of tumor burden in these mice. In non-responding mice, MSC failed to affect either bone mass or myeloma growth.

In summary, we demonstrated that growth of myeloma cells from a subset of patients was restrained by osteoblasts *ex vivo* and by MSC injection in myelomatous SCID-hu mice. Further studies to unravel the molecular mechanisms by which osteoblasts affect myeloma cells are warranted. We conclude that increased osteoblast activity via exogenous cytotherapy and/or endogenous approaches, such as treatment with bone anabolic agents, will benefit patients with myeloma for various reasons. First, it will increase bone formation, and relieve skeletal complications. Secondly, it may help control myeloma progression, particularly when combined with specific inhibitors of osteoclast activity. Third, osteoblasts play an important role in maintaining the hematopoietic stem cell niche,[39,40] and thus, an increased osteoblast pool may improve hematopoietic recovery, and the ability to mobilize hematopoietic stem cells. Finally, increased bone formation in patients with monoclonal gammopathy of undetermined significance, smoldering myeloma and patients in remission may prevent transformation into active myeloma.

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