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Cancer and the Microenvironment: Myeloma-Osteoclast Interactions as a Model

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ABSTRACT

We have investigated the interaction between tumor cells and specific cells in their microenvironment using myeloma as a model. The role of myeloma-induced osteoclastogenesis in the disease was studied *ex vivo*. Myeloma plasma cells freshly purified from patients' bone marrow attracted committed osteoclast (OC) precursors ($n = 9$; $P < 0.01$) and in 22 experiments directly induced their differentiation to multinucleated, bone-resorbing OCs ($P < 0.00002$) in a receptor activator of nuclear factor-κB ligand-mediated mechanism that was inhibited by the receptor activator of nuclear factor-κB (RANK-Fc) in 13 experiments by 71 ± 12% ($P < 0.008$). In contrast, myeloma cells did not induce differentiation of peripheral blood mononuclear cells. Myeloma plasma cells cocultured with OCs retained their viability and proliferative activity for >13 weeks. After 14 days in coculture, the plasma cells from 29 patients had higher viability ($P < 2 \times 10^{-6}$), fewer apoptotic cells ($P < 4 \times 10^{-15}$), and a higher bromodeoxyuridine labeling index ($P < 0.0006$) than controls. Physical contact between OCs and myeloma cells was required for these effects to take place. No differences were observed between OCs from healthy donors and those from myeloma patients. Blocking interleukin 6 activity, while reducing survival of myeloma cells, had no effect on their proliferative activity. These results support data obtained from animal models and clinical observations on the essential role of the microenvironment in tumor sustenance and progression.

INTRODUCTION

The microenvironment is now recognized as a principal facilitator of the malignant disease process. Tumors have been shown to induce changes in their microenvironment and in turn require these changes for continued survival. Various malignant tumors have been shown to depend on the neoangiogenesis they induce (1, 2). Similarly, inhibitors of changes in the bone marrow (BM) microenvironment induced by tumors that metastasize to bone also had antitumor activity (3–6). We have investigated the relationship between tumor-induced osteoclastogenesis and the malignant process using multiple myeloma (MM) as a model.

MM plasma cells (PCs) typically reside in and disseminate through the hematopoietic BM. Among the most frequent consequences of accumulation of myeloma cells in the BM is increased osteoclast (OC) activity resulting in lytic bone disease in ≥80% of patients. In studies using the severe combined immunodeficiency (SCID)-hu model, in which primary human myeloma cells grow exclusively in and interact with the human microenvironment (7, 8), we have demonstrated that in medullary but not extramedullary disease, survival and growth of myeloma cells depend on OC activity (9). These studies highlighted the interdependence of myeloma growth and lytic bone disease, also seen in the 5T murine myeloma (10) and observed in clinical trials (11, 12).

Under physiological conditions, osteoblasts play a major role in recruitment of OC precursors (pOC) and induction of OC formation via production of chemokines and osteoclastogenic cytokines (13–15). Lytic bone disease in myeloma is characterized by reduced number of osteoblasts on bone surfaces adjacent to myeloma cells (16, 17), suggesting that tumor cells may play a direct role in increasing OC activity through production of key osteoclastogenic factors. Whereas recent studies indicated that MM PCs indirectly induce the formation of OCs through disruption of the balance of osteoprotegerin and the receptor activator of nuclear factor-κB ligand (RANKL) in the BM (18–21), other studies suggested that myeloma cells express RANKL (22–24) or macrophage inflammatory protein-1α and can directly induce differentiation of OC progenitors into OCs (25). This may explain how myeloma cells induce osteoclastogenesis in bone areas highly infiltrated by myeloma cells with reduced number of osteogenic cells. However, a direct effect of primary human myeloma cells on recruitment and differentiation of pOC has not been demonstrated.

Even though the most prominent changes in the cellular makeup of the BM microenvironment associated with myeloma are decreases in osteogenic cells and increases in OC numbers and in microvessel density (16, 26), numerous studies focused instead on the interactions between myeloma cell lines or primary MM PCs with BM-derived fibroblastoid stromal cells of mesenechymal origin. These reports demonstrated that myeloma-stroma cell interactions result in modulation of the expression of cytokines (27, 28) and adhesion molecules (29–31) in both cell types, disruption of the balance of bone remodeling (18, 19, 32), and protection of myeloma cells from spontaneous and drug-induced apoptosis (33–35).

To study the relationship between myeloma cells and OCs, we established an *ex vivo* culture system and investigated the consequences of freshly purified MM PC interaction with pOC and with active OCs.

MATERIALS AND METHODS

Reagents and Kits. Neutralizing polyclonal antibodies against human interleukin (IL)-6, human IL-6 receptor, monoclonal antibody to RANKL, and ELISA kit for human IL-6 were obtained from R&D (Minneapolis, MN). Antihuman bromodeoxyuridine (BrdUrd) was obtained from DAKO Corp. (Carpinteria, CA). Ficoll-Paque and [^{3}H]thymidine [thymidine (dThd)] were from Amersham Pharmacia Biotech (Piscataway, NJ). Monoclonal antibodies to human CD38 (PE) and CD45 (FITC) for fluorescence-activated cell-sorting analysis were from BD Biosciences (San Jose, CA). α-MEM and antibiotic mixture containing penicillin, streptomycin, and neomycin were from Gibco (Grand Island, NY). Fetal bovine serum was from Hyclone (Logan, UT). Recombinant human macrophage colony-stimulating factor (M-CSF) and RANKL were from RDI (Flanders, NJ). Dexamethasone, BrdUrd, 5-fluoro-2′-deoxyuridine (FrdU), and leukocyte acid phosphatase kit for tartrate-resistant acid phosphatase (TRAP) were from Sigma (St. Louis, MO). Antihuman CD138 antibody for immunomagnetic bead separation was from Miltenyi-Biotec (Auburn, CA). Monoclonal antibody to human vitronectin receptor was from Biosource International (Camarillo, CA). Human RANK-Fc was a kind gift from Immunex (Seattle, WA). Cell culture plates were from Becton Dickinson (Franklin Lakes, NJ). Chamber slides were from Nalge Nunc International (Naperville, IL), Transwell inserts were from Costar (Corning, NY), annexin V/propidium iodide detection kit for flow cytometry was from Caltag Laboratories (Burlingame, CA), and immunoperoxidase detection kits were from Vector (Burlingame, CA).

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Myeloma Cells. Myeloma PCs were obtained from heparinized BM aspirates from 48 patients with active myeloma during scheduled clinic visits, from peripheral blood of one patient with PC leukemia, and from one plural effusion. Signed institutional review board-approved informed consent forms are kept on record. Pertinent patient information is provided in Table 1. The BM, peripheral blood, and plural effusion samples were separated by density centrifugation using Ficoll-Paque (specific gravity, 1.077 g/ml), and the proportion of MM PCs in the light-density cell fractions was determined by CD38/CD45 flow cytometry (36). PCs were isolated using CD138 immunomagnetic bead selection and the autoMACs automated separation system (Miltenyi-Biotec, Auburn, CA). PC purity was determined by CD38/CD45 flow cytometry to be routinely ≥94%. ANBL-6 cells were obtained from the American Type Culture Collection (Manassas, VA). ARP-1 and CAG myeloma cell lines were established in our laboratory. Myeloma cell viability was determined by trypan blue exclusion, and apoptotic cells were enumerated using an annexin V/propidium iodide kit.

Preparation of Committed pOC and OCs. Peripheral blood mononuclear cells (PBMCs) were obtained from 30 patients with myeloma and 2 healthy donors by leukopheresis and from 3 additional healthy volunteers by Ficoll centrifugation. Signed institutional review board-approved informed consent forms are kept on record. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air at 2.5×10^6 cells/ml α-MEM supplemented with 10% fetal bovine serum, antibiotics, RANKL (50 ng/ml), human M-CSF (25 ng/ml), and 10 nM dexamethasone (OC medium). After 2–4 days, the cultures were washed three times with fresh media to remove the nonadherent cells. The remaining adherent cells were mononucleated, expressed TRAP and vitronectin receptors, and were considered committed pOC. To generate OCs, OC medium was added, and the cultures were continued for an additional 6–10 days, at which time they contained large numbers of multinucleated OCs with bone-resorbing activity.

Migration Assays. Chemotaxis assays were performed in duplicates using 5-μm pore size Transwell inserts in 24-well plates. The upper chamber contained 1×10^5 pOC in 0.3 ml of OC medum. The bottom chambers contained 0.7 ml of conditioned media from 48-h cultures of purified myeloma cells (10^6 cells/ml) or fresh medium in control wells. After 3 h at 37°C, the filters were removed, fixed in 10% phosphate-buffered formalin for 10 min, and stained for TRAP. The number of pOC in five nonoverlapping 0.5-mm^2 areas of the lower surface of the filter was determined microscopically. In four experiments, 1×10^6 purified MM PCs in 1 ml of M-CSF-containing media were placed in the lower chambers. After 7 days in culture, the inserts were removed, 1 ml of fresh M-CSF-containing media was added, and culture was continued for an additional 7 days, after which the number of multinucleated (≥3 nuclei) TRAP-positive OCs was determined.

OC Precursor Differentiation Assay. Experiments were performed in duplicates in 24-well plates. Freshly obtained unstimulated MM PBMCs (1×10^6 cells/well) or committed pOC were cultured alone or with 0.5–1×10^6 purified MM PCs (cocultures) for 7–14 days in α-MEM supplemented with human M-CSF (25 ng/ml) but without RANKL. The number of TRAP-positive multinucleated OCs in each well was counted using a phase-contrast microscope.

Table 1 *Characterization of myeloma patients whose multiple myeloma plasma cells were studied*

Patient characteristic		No. of patients
Stage[a]	IA	7
	II	1
	IIA	5
	III	1
	IIIA	28
	IIIB	8
Prior therapy	Yes	23
	No	27
PC (%)[b]	<10%	7
	10–50%	32
	51–93%	10
	PE	1
Isotype	IgA	9
	IgG	30
	Free light chain	11

[a] Stage at diagnosis, according to the Durie-Salmon staging system.
[b] PC, plasma cell. Percentage of plasma cells in bone marrow or peripheral blood, determined by CD38/CD45 flow cytometry.

Myeloma PCs and OC Cocultures. OCs were washed three times with PBS to detach and remove the nonadherent cells. Purified MM PCs (0.5×10^6 cells/ml in OC medium supplemented with M-CSF and RANKL but lacking dexamethasone) were cultured alone or added to OCs in duplicates in 24-well plates (1 ml/well) for 14 days. In 12 experiments, MM PCs were placed in 0.45-μm pore size Transwell inserts in wells containing OCs (noncontact cultures). In seven additional experiments, MM PCs were cultured with media conditioned by OCs or by MM PC and OC cocultures. At the end of each experiment, MM PCs were counted, and the media were removed and kept frozen at −20°C for additional studies as indicated below. Portions of myeloma cells from one replicate were processed for assessing BrdUrd labeling index (LI) and annexin V binding, and the other replicate was used for measuring [^{3}H]dThd incorporation.

In other experiments, MM PCs were cultured alone or with OCs in 30-mm-diameter culture plates (3 ml/plate). After each 3–4 weeks of culture, the myeloma cells were transferred into plates containing freshly prepared OCs, and cultures were continued for up to 13 weeks. Cell numbers, viability, and annexin V binding were determined at weekly intervals.

OC Activity Assays. The ability of OCs generated from PBMCs and MM PBMCs to resorb bone and form resorption pits was tested using bovine bone slices (a kind gift from Dr. R. L. Jilka; University of Arkansas for Medical Sciences) and BD BioCoat Osteologic (BD Biosciences, Bedford MA) bone resorption assay. Briefly, pOC were collected by incubation on ice with cold PBS for 10 min, followed by incubation with 10 mM EDTA in PBS for 10 min at 37°C. Cells (1×10^5) in 100 μl of α-MEM supplemented with OC media were added onto the bone slices, and mineralized discs were placed in a 24-well plate and allowed to adhere to the bone slices and discs at 37°C for 1 h. After 7–14 days of continuous incubation with OC media, resorption pits on mineralized discs were visualized according to the manufacturer's instructions. Bone slices were treated with bleach solution for 5 min, washed with water, and lightly stained with 1% toluidine blue. Images of resorption pits were taken using Nikon eclipse 450 microscope. For testing the ability MM PCs to induce formation of functional OCs, pOC were incubated with or without MM PCs on mineralized discs in 1 ml of fresh media containing M-CSF but lacking RANKL and dexamethasone or with complete OC media containing M-CSF, RANKL, and dexamethasone (positive controls).

Immunohistochemistry. Cytospin slides of myeloma cells (40,000 cells/slide) or chamber slides with pOC and OCs were fixed with 10% phosphate-buffered formalin for 20 min, thoroughly washed, and boiled in citrate buffer in a microwave oven for antigen retrieval. After peroxidase quenching with 3% hydrogen peroxide for 10 min, the slides were incubated with monoclonal antibodies against human vitronectin receptor (5 μg/ml) or RANKL (5 μg/ml) for 60 min. The assays were completed using Vector's immunoperoxidase kit and counterstaining with hematoxylin.

BrdUrd LI. BrdUrd and FrdU (20 and 2 μM final concentrations, respectively) were added to cultures of myeloma cells alone or to cocultures for 2 h. Myeloma cells were then collected, and cytospin slides were prepared (40,000 cells/slide) and fixed in 10% phosphate-buffered formalin for 20 min. Duplicate slides were treated with 5 N HCL for 15 min and with 3% hydrogen peroxide for 10 min before incubation with monoclonal antibody against BrdUrd (5 μg/ml). BrdUrd incorporation was detected using Vector's immunoperoxidase kit, and cells were lightly counterstained with hematoxylin. BrdUrd LI was calculated as the percentage of positive cells out of 500 cells in each duplicate slide.

Cell Proliferation Assay. Cell proliferation was assessed by the addition of 0.5 μCi [^{3}H]dThd/well during the last 6 h of a 14-day culture. The cells were then harvested onto glass fiber filters with an automatic cell harvester (Molecular Devices Corp., Sunnyvale, CA), and incorporation was measured using a TRI-CARB 1600TR scintillation analyzer (Packard Instrument Co., Meriden, CT). Media taken from culture of OCs alone were used as background.

Phagocytosis by OCs. CAG and ARP1 myeloma cell lines were incubated with 10^{-6} M dexamethasone for 5 days to induce cell death. The cells were then stained with trypan blue (diluted 1:1 with PBS) for 10 min and washed five times with 30 ml of PBS, resuspended in OC media, and added onto OC cultures in 8-well chamber slides (1×10^5 cells/well). Phagocytosis of dead myeloma cells by OCs was observed under a light microscope 4–24 h later.

Statistical Analysis. Unless indicated otherwise, all values were expressed as mean ± SE. The effect of myeloma cell conditioned media on migration of pOC was evaluated using the nonparametric Mann-Whitney test. Student's

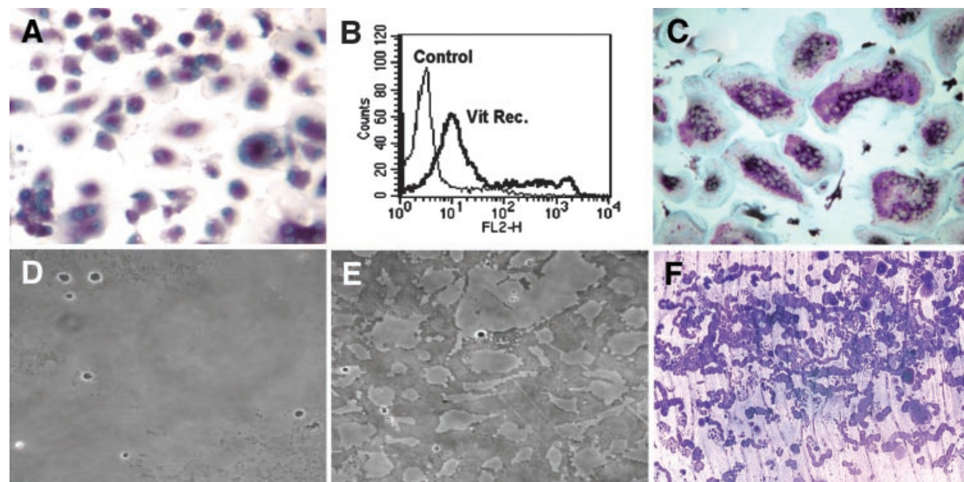

Fig. 1. Characterization of human osteoclast precursors (pOC) and osteoclasts (OCs) generated from peripheral blood mononuclear cells. *A* and *B*, pOC cultured for 2–4 days with complete OC media are mostly mononucleated and express tartrate-resistant acid phosphatase (*A*; original magnification, ×100) and vitronectin receptor (*B*) as determined by flow cytometry. *C*, after 6–10 additional days, pOC differentiate into multinucleated, tartrate-resistant acid phosphatase-expressing OCs (original magnification, ×200). *D*, pOC cultured on mineralized disk with OC media lacking receptor activator of nuclear factor-κB ligand (RANKL) did not form resorption pits. *E* and *F*, when pOC are incubated on mineralized disk (*E*) and bone slices (*F*) with complete OC media, high numbers of resorption pits are detected.

paired *t* test was used to test the effect of MM PCs on differentiation of freshly obtained MM PBMCs and pOC and to evaluate the effect of different culture conditions and treatments on myeloma cell numbers, viability, apoptosis, and proliferation.

RESULTS

Characterization of OC Cultures. Cultures of pOC and mature active multinucleated OCs were prepared from PBMCs from healthy adults and from myeloma patients' MM PBMCs. After 2–4 days of incubation with osteoclastic media, the nonadherent cells were removed, leaving mostly adherent cells of the myelomonocytic lineage. More than 95% of the remaining adherent cells expressed TRAP and vitronectin receptor (Fig. 1, *A* and *B*). Of these, >90% were mononucleated cells, and the rest were small, multinucleated cells. Furthermore, pOC whose media were changed after 2–4 days of culture in complete osteoclastic media to osteoclastic media lacking RANKL and then cultured for an additional 6–21 days remained largely mononucleated and did not form resorption pits on thin mineralized discs (Fig. 1*D*). However, when cultured for an additional 6–10 days in RANKL-containing media, pOC differentiated into large, multinucleated OC-like cells (Fig. 1*C*) that similarly formed resorption pits on thin mineralized discs (Fig. 1*E*) and bone slices (Fig. 1*F*). In a preliminary study, inoculation of pOC into irradiated human bone in our SCID-hu model (7) resulted in loss of human bone density, confirming that the pOC injected indeed differentiate to mature, multinucleated, functional OCs (data not shown). In seven randomly selected experiments, the number of TRAP-positive multinucleated OCs was 426 ± 156 (mean ± SD)/well in 24-well culture plates. All OC cultures were devoid of fibroblast-like cells. The OCs remained viable for more than 30 days. The pOC were used to test the effect of myeloma cells on migration and differentiation, whereas cultures of mature OCs were used to test their effect on myeloma cell growth and survival.

Induction of OC Formation by Myeloma Cells. We initially tested whether MM PCs can affect differentiation of OCs from the precursors in a stromal cell-free environment. Unstimulated MM PBMCs or committed pOC were cultured alone or cocultured with purified MM PCs for 7–14 days in OC media without dexamethasone and RANKL. Whereas MM PCs did not induce formation of OCs from MM PBMCs ($n = 6$), myeloma cells from 22 patients increased the formation of multinucleated, TRAP-positive cells from pOC by 6-fold from 50 ± 8 in control cultures ($P < 0.00002$; Fig. 2, *A*−*C*). To test whether these were functional OCs, in three experiments (pOC alone, MM PCs alone, and pOC plus MM), PCs were cultured on thin mineralized discs in OC media without dexamethasone and RANKL. Only the multinucleated cells induced by MM PCs in these cultures formed resorption pits (Fig. 2*F*) and hence were functional OCs, whereas myeloma cells alone

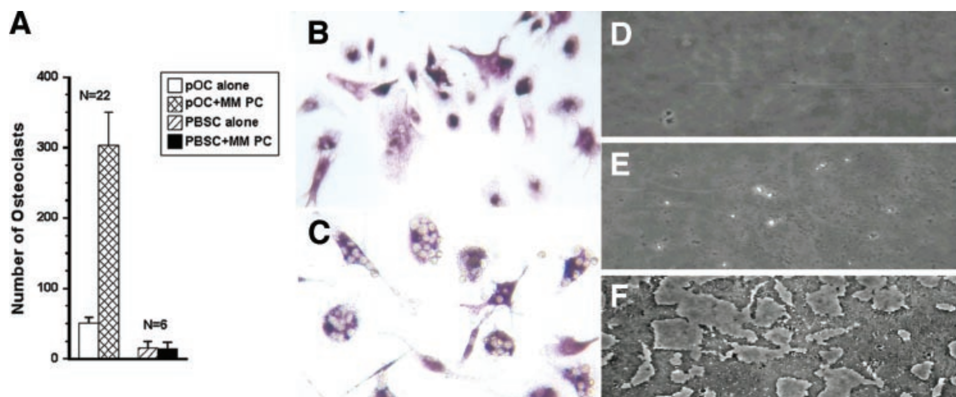

Fig. 2. Myeloma plasma cells (PCs) directly induce differentiation of committed osteoclast precursors (pOC) but not unstimulated multiple myeloma (MM) peripheral blood mononuclear cells to mature, functional, multinucleated osteoclasts (OCs). *A*, MM peripheral blood mononuclear cells ($n = 6$) and pOC ($n = 22$) were cultured alone or cocultured with MM PCs in OC media lacking receptor activator of nuclear factor-κB ligand (RANKL) and dexamethasone. Numbers of multinucleated OCs were increased only in cocultures with pOC ($P < 0.00002$). *B* and *C*, tartrate-resistant acid phosphatase staining of pOC alone (*B*) and pOC + MM PCs (*C*) demonstrating MM PC-induced formation of multinucleated OCs in *C* (original magnification, ×200). *D–F*, whereas purified myeloma PCs (*D*) and pOC (*E*) cultured alone did not form resorption pits on mineralized discs, coculture of the same purified myeloma PCs and pOC resulted in formation of large resorption pits (*F*), indicating that myeloma cells directly induce differentiation of active OCs.

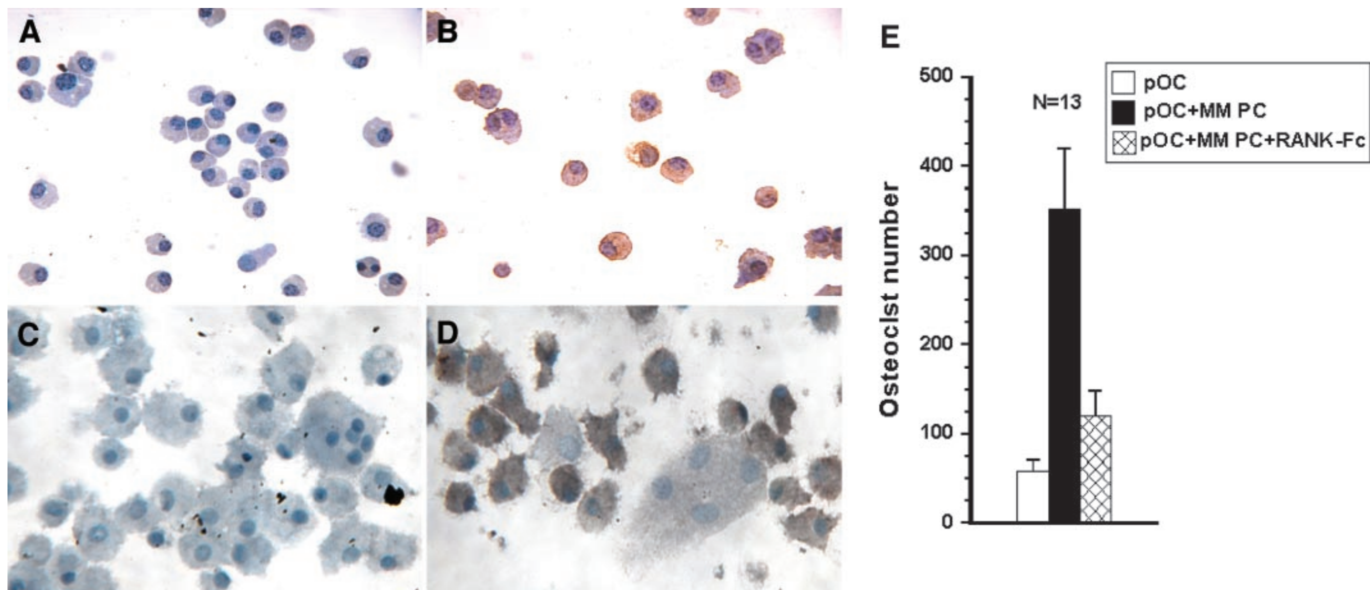

Fig. 3. Myeloma plasma cell (PC)-induced osteoclast (OC) formation is receptor activator of nuclear factor-κB ligand (RANKL) mediated. *A–D,* myeloma PCs express RANKL after coculture with OC precursors. Cytospin slides of multiple myeloma (MM) PC recovered from two different coculture experiments were reacted with control antibody (*A* and *C*) and antibody to RANKL (*B* and *D*). Note surface and cytoplasmic expression of RANKL by MM PCs but not by multinucleated OCs. *E,* OC precursors were cultured alone or cocultured with MM PCs ($n = 13$) in the presence and absence of RANK-Fc. Addition of RANK-Fc to cocultures inhibited MM PC-induced OC formation by 71 ± 12% ($P < 0.008$).

(Fig. 2*D*) or pOC cultured without MM PCs (Fig. 2*E*) on mineralized discs did not form resorption pits.

To investigate whether MM PC-induced OC differentiation is RANKL mediated, we tested whether MM PCs expressed RANKL and whether antagonists of RANKL would inhibit this differentiation process. Myeloma cells from eight patients were recovered from the cocultures and immunohistochemically reacted with anti-RANKL and control antibodies. MM PCs from seven examined patients expressed RANKL on their surface (Fig. 3, *A*−*D*). Addition of RANK-Fc to 13 cultures inhibited MM PC-induced OC formation in 12 of the 13 experiments by 16–100% (median, 90%) and had no inhibitory effect in one experiment ($P < 0.008$; Fig. 3*E*).

Effect of Myeloma Cell Conditioned Media on Migration of pOC. We then tested whether myeloma cells actively attract pOC in a Transwell migration assay. OC precursors from three different patients were cultured in the upper chamber of the Transwell, and myeloma cell conditioned media were placed in the lower chamber. The number of TRAP-positive cells that migrated across the 5-μm pore-size membrane in 3 h was determined microscopically. As shown in Fig. 4*A*, myeloma cell conditioned media from nine patients increased migration of pOC by 1.6–4.2-fold over that seen in control wells [127 ± 37 (mean ± SD); $P = 0.01$].

To demonstrate that myeloma cells induced both migration of pOC and their differentiation to OCs, in four experiments MM PCs were placed in the bottom compartment of Transwell plates, and pOC were placed in the upper compartment. After 14 days, the cells in the bottom of the wells containing MM PCs had large numbers of multinucleated TRAP-positive cells compared with very few cells in control wells, indicating that MM PCs not only attract pOC but also affect their survival and differentiation to OCs ($P < 0.03$; Fig. 4*B*).

Growth of Myeloma Cells in Coculture with OCs. To investigate whether OCs affect survival and growth of myeloma cells, freshly purified MM PCs from 29 patients were cocultured with cultured OCs from 17 myeloma patients for 14 days. Because of logistic considerations, the myeloma cells and OCs in each coculture were not from the same patient. Purity of the myeloma cells used for these experiments was ≥94%, and their viability was ≥94%. Six

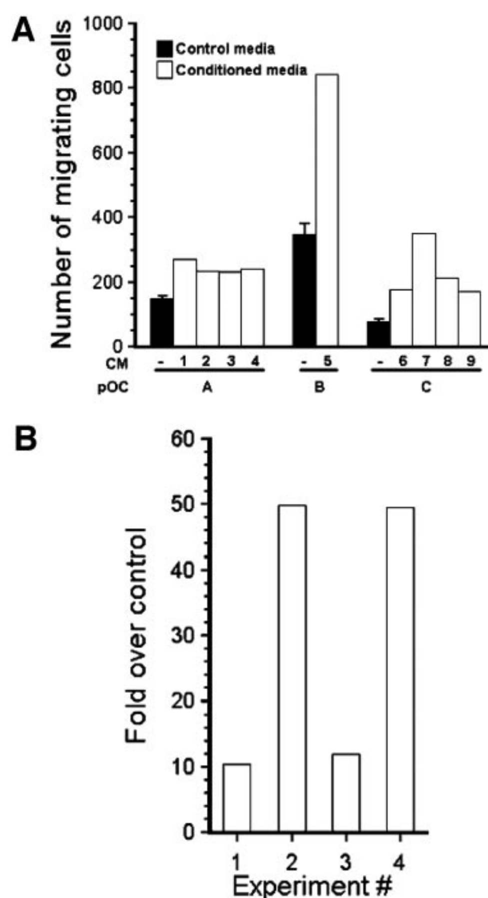

Fig. 4. Myeloma plasma cells induce migration of osteoclast precursors (pOC) and their differentiation into osteoclasts. *A,* myeloma plasma cell (PC) conditioned media from 9 (*CM 1–9*) patients induce migration of pOC, prepared from three patients' multiple myeloma peripheral blood mononuclear cells (*pOC A, B,* and *C*), in a Transwell migration system ($P < 0.01$). *B,* myeloma PCs from four patients were cultured in the bottom chambers of Transwells while pOC from four different patients were cultured in the upper chambers. After 14 days, the numbers of multinucleated osteoclasts in the bottom of multiple myeloma PC-containing wells were increased over controls ($P < 0.03$).

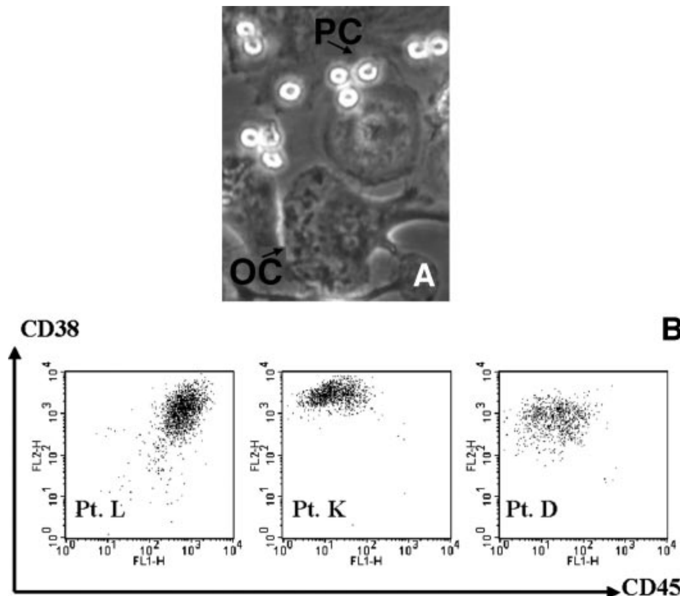

Fig. 5. Recovery of multiple myeloma (MM) plasma cells (PCs) from coculture with osteoclasts. *A,* phase-contrast microphotograph of MM PCs cocultured with osteoclasts. MM PCs remained in suspension and were easily recovered (original magnification, ×200). *B,* nonadherent cells recovered from cocultures by gentle pipetting consisted of ≥95% MM PCs as determined by CD45/CD38 flow cytometry.

randomly selected samples contained 25 ± 6% annexin V-positive MM PCs. Unlike their tight adherence to stromal cells, MM PCs did not adhere to the OCs and were easily recovered form cocultures by gentle pipetting (Fig. 5*A*). The nonadherent cells recovered after 14 days of coculture with OCs contained ≥95% myeloma cells (Fig. 5*B*). Although the total number of myeloma cells recovered from cocultures was 33% lower than that from cultures of myeloma cell alone ($P < 0.000002$), their viability was significantly higher (95% *versus* 38%; $P < 0.0000002$), and their apoptotic rate was significantly lower (22% *versus* 70% by annexin V; $P < 4 \times 10^{-15}$) than those of myeloma cells cultured alone (Table 2; Fig. 6*A*). Myeloma cells in cocultures with OCs also had higher proliferation rates as indicated by BrdUrd LI (1.3 ± 0.2 *versus* 0.3 ± 0.1; $P < 0.0006$; Fig. 6*B*) and by [^{3}H]dThd incorporation (5502 ± 1822 *versus* 1878 ± 883 cpm; $P < 0.03$). In all experiments, MM PCs in the cocultures produced a higher amount of monotypic immunoglobulins (data not shown). OCs generated from healthy donors were equally supportive of MM PC growth and survival as those generated from PBMCs from myeloma patients. These results are summarized in Table 2. In some experiments, cocultures were maintained for up to 13 weeks. Although the total numbers of myeloma cells in the cocultures were gradually decreased over time, their viability was consistently >95% throughout the experimental period (data not shown).

We investigated whether the reduced numbers of myeloma cells, their high viability, and low apoptotic rate reflected phagocytosis of apoptotic/dying cells by OCs. Fig. 6*C* demonstrated that OCs indeed removed apoptotic and dying myeloma cells by phagocytosis, confirming previous reports (37, 38). The phagocytic activity of OCs did not affect their viability or their ability to support myeloma cell growth and survival.

To capture the heterogeneity among OCs and MM PCs from different patients, we cocultured myeloma cells from several patients with OCs from one patient (Fig. 7*A*) and OCs from different patients with the same MM PCs (Fig. 7*B*). Whereas the effects of OCs on MM PCs from different patients varied widely, myeloma cells from individual patients responded similarly to different OCs, suggesting between-patient heterogeneity in MM PCs but not in OCs.

Role of Myeloma Cell/OC Contact on Myeloma Cell Growth and Survival. To test whether direct physical contact between MM PCs and OCs is required for the observed effects of OCs on survival and proliferation of myeloma cells, a noncontact coculture system was used in 12 experiments (Table 3). Viability in the noncontact culture was reduced by 52% compared with cocultures ($P < 0.001$), annexin V binding was increased 3.7-fold ($P < 0.0001$), BrdUrd LI was reduced by 46% ($P < 0.05$), and [^{3}H]dThd uptake was reduced by 58% ($P < 0.002$) in the noncontact cultures. The number of viable tumor cells in the noncontact cultures was 33% lower than that in cocultures ($P < 0.003$). These values were not significantly different from those obtained from myeloma cells cultured alone. Similar results were obtained when myeloma cells were cultured with OC conditioned media or with OC-myeloma cell coculture conditioned media. These results indicate that soluble factors alone are not sufficient to support the survival and proliferation of myeloma cells in the cocultures.

Role of IL-6 in OC-Induced Myeloma Cell Growth and Survival. The IL-6 concentration in coculture conditioned media was 2768 ± 1686 pg/ml and was not significantly different from the combined IL-6 levels in cultures of OCs alone (2966 ± 1853 pg/ml) and MM PCs alone (140 ± 57 pg/ml). To determine the role of IL-6 in the effects of OCs on myeloma cells, we used a combination of blocking antibodies against IL-6 and IL-6 receptor (5 μg/ml each) in 15 experiments. In a preliminary experiment, treatment of IL-6-dependent ANBL6 cells cocultured with OCs with similar concentrations of IL-6 + human IL-6 receptor neutralizing antibodies for 4 days reduced viable cell numbers from 313 × 10^3 in control to 181 × 10^3 in treated cultures, reduced BrdUrd LI from 17.6 to 3.7, and reduced [^{3}H]dThd incorporation from 36,719 to 15,451 cpm, whereas annexin V binding cells increased from 13% to 26% in treated cultures,

Table 2 *Effects of osteoclasts on survival and proliferation of myeloma plasma cells*

	OCs[a] from myeloma patients		Patient *vs.* donor OCs	
	PCs alone	Cocultured PCs	Patient OCs	Donor OCs
N	29	29[b]	6[c]	6
Total no. of PCs (×10^3)	448 ± 26	302 ± 22 ($P = 2 \times 10^{-6}$)	291 ± 43	297 ± 47
No. of viable PCs (×10^3)	166 ± 18	287 ± 2 ($P = 12 \times 10^{-7}$)	278 ± 41	265 ± 36
Cell viability (%)	38.3 ± 3.7	94.5 ± 0.9 ($P = 4 \times 10^{-15}$)	95.6 ± 0.2	95.6 ± 1.2
Annexin V PCs (%)	70 ± 3	22 ± 1 ($P = 2 \times 10^{-12}$)	25 ± 3	36 ± 9
BrdUrd (%)	0.3 ± 0.1	1.3 ± 0.2 ($P = 0.0006$)	0.9 ± 0.3	1.5 ± 0.4
[^{3}H]Thymidine (cpm)	1878 ± 883	5502 ± 1822 ($P = 0.03$)	3169 ± 634	2649 ± 897

[a] OC, osteoclast; PC, plasma cell; BrdUrd, bromodeoxyuridine.
[b] Myeloma plasma cells from 29 patients were cultured alone (PCs alone) or cocultured with osteoclasts prepared from peripheral blood stem cells from 17 myeloma patients (cocultured PCs).
[c] Myeloma plasma cells from six patients were each cocultured with osteoclasts prepared from peripheral blood stem cells from six myeloma patients (Patient OCs) and six healthy donors (Donor OCs).

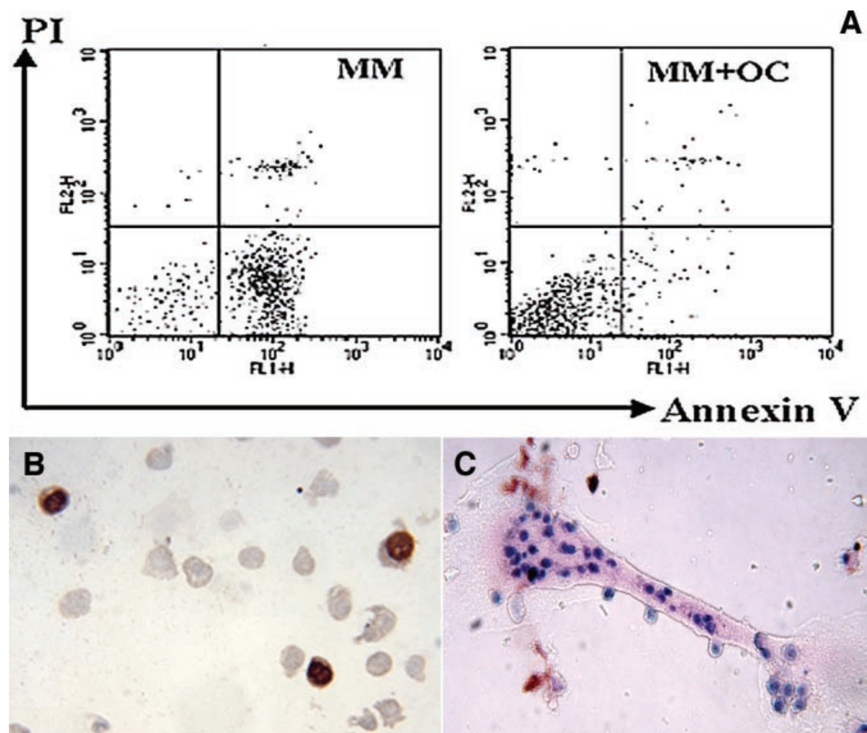

Fig. 6. Osteoclasts (OCs) support myeloma cell survival and proliferation and phagocytose dying tumor cells. *A* and *B,* representative experiment of annexin V/propidium iodide flow cytometric analysis of multiple myeloma (MM) plasma cells (PCs) after 14 days in culture alone (*A, left panel*) or with OCs (*A, right panel*). *B,* sustained proliferative activity of MM PCs after 14 days of coculture with OCs. *Dark brown nuclei* denote cells that incorporated bromodeoxyuridine (original magnification, ×200). *C,* phagocytosis of dying MM PCs (stained with trypan blue) by OCs (lightly stained with Nuclear fast red; original magnification, ×400).

confirming the validity of the assay. As shown in Table 4, blocking IL-6 activity reduced the number of viable primary MM PCs by 30% ($P < 0.0001$) and reduced [^{3}H]dThd incorporation, although not reaching significance, by 22%. Whereas blocking IL-6 activity had no effect on the BrdUrd LI, annexin V binding increased in treated cells by 78% from 18 ± 2% in control cells, suggesting that IL-6 acts as an antiapoptotic rather than a growth factor in this long-term coculture system.

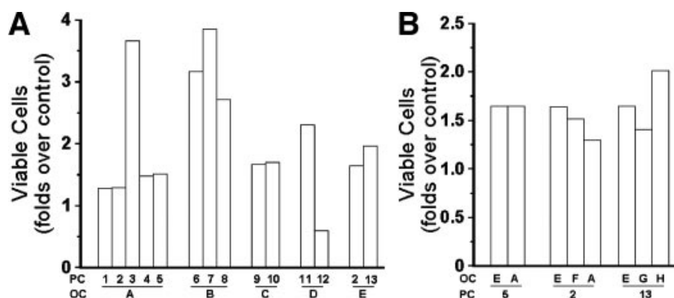

Fig. 7. Effect of osteoclasts (OCs) on numbers of viable multiple myeloma plasma cells (PCs). *A,* myeloma PCs from different patients were cocultured with the same OCs. *B,* myeloma PCs from one patient were cocultured with different OCs. Note the heterogeneity in multiple myeloma response in *A* as opposed to similar effects of different OCs on myeloma cells in *B*.

DISCUSSION

Observations from clinical trials and studies on animal models of myeloma demonstrated that inhibitors of bone resorption also have an antimyeloma effect and that this effect is indirect and mediated through inhibition of OC activity (9–12, 18). In *ex vivo* cocultures of highly purified pOC and MM PCs, myeloma cells recruited committed pOC and directly induced their differentiation into mature, functional OCs in a stromal cell-free environment. Mature OCs, in turn, supported long-term survival and proliferation of primary myeloma PCs. In this system, primary MM PCs did not induce differentiation of unstimulated progenitors, suggesting that pOC but not uncommitted precursors up-regulate RANKL expression by myeloma cells and that initial commitment of myelomonocytic progenitors to the osteoclastic lineage is mediated by other cells or factors in the microenvironment. In addition to osteoblasts whose number in myelomatous bones is reduced, we hypothesize that cells such as vascular endothelial cells, whose numbers are increased in myeloma, are involved in this process. Indeed vascular endothelial cells produce chemokines that attract

Table 3 *Effect of culture conditions on the survival and proliferation of myeloma cells*[a]

	Contact *vs.* noncontact		Contact *vs.* CM[b]		
	Cocultured	Noncontact	Cocultured	OC CM	OC + PC CM
n	12	12	7	6	7
Total no. of PCs (×10^3)	313 ± 33	411 ± 40 (P = 0.007)	314 ± 33	440 ± 44 (P = 0.04)	493 ± 44 (P = 0.002)
No. of viable PCs (×10^3)	294 ± 35	197 ± 35 (P = 0.003)	308 ± 31	213 ± 45 (P = 0.01)	250 ± 30 (P = 0.04)
Cell viability (%)	92.8 ± 1.6	44.5 ± 5.2 (P = 0.001)	96.8 ± 0.7	50.8 ± 7.9 (P = 0.0001)	52.8 ± 7.3 (P = 0.0001)
Annexin V PCs (%)	19.2 ± 2.1	70.8 ± 5.5 (P = 0.0001)	30 ± 3	55 ± 6 (P = 0.01)	61 ± 4 (P = 0.001)
BrdUrd (%)	0.70 ± 0.21	0.38 ± 0.23 (P = 0.05)	ND	ND	ND
[^{3}H]Thymidine (cpm)	4895 ± 2798	2072 ± 914 (P = 0.002)	2483 ± 462	1133 ± 250 (P = 0.03)	1769 ± 585 (NS)

[a] Myeloma plasma cells were cultured together with osteoclasts (Cocultured), cultured separated from osteoclasts in transwells (Noncontact), with media conditioned by osteoclasts (OC CM), or with media conditioned by cocultures of myeloma cells with osteoclasts (OC + PC CM).

[b] CM, conditioned media; OC, osteoclast; PC, plasma cell; BrdUrd, bromodeoxyuridine; ND, not done; NS, not significant.

Table 4 *Role of interleukin 6 in osteoclast-induced myeloma cell survival and proliferation*[a]

	Cocultured PCs	Anti-IL-6 + anti-IL-6R[b]
N	15	15
No. of viable PCs ($\times 10^3$)	297 ± 33	200 ± 34 (P = 0.0001)
Cell viability (%)	94 ± 1	84 ± 5 (P = 0.04)
Annexin V PCs (%)	18 ± 2	32 ± 4 (P = 0.005)
BrdUrd (%)	0.96 ± 0.2	0.86 ± 0.2 (NS)
[^{3}H]Thymidine (cpm)	6082 ± 2750	4722 ± 2447 (NS)

[a] Myeloma plasma cells from 15 patients were cocultured with osteoclasts in the absence (Cocultured PCs) or presence (Anti-IL-6 + anti-IL-6R) of neutralizing antibodies to interleukin 6 + interleukin 6 receptor (5 μg/ml each) for 14 days.

[b] IL-6, interleukin 6; IL-6R, interleukin 6 receptor; PC, plasma cell; BrdUrd, bromodeoxyuridine; NS, not significant.

monocytes (39); express key osteoclastogenic cytokines such M-CSF (40), RANKL, and osteoprotegerin; and can directly induce formation of active multinucleated OCs (41). The RANKL/osteoprotegerin system on vascular endothelial cells is highly regulated by inflammatory cytokines that are involved in myeloma pathogenesis including tumor necrosis factor α, transforming growth factor β, and IL-1 (41, 42). Preliminary results from the SCID-hu model show high expression of RANKL, IL-6, stromal cell-derived factor-1 (SDF-1), and hepatocyte growth factor (HGF) by myeloma-associated microvessels (data not shown).

MM PC-induced differentiation of pOC was RANKL mediated, as evidenced by the inhibitory effects of RANK-Fc. Whether myeloma cells express RANKL has been controversial. Whereas some studies failed to detect RANKL expression by myeloma cells in patients' BM biopsies and aspirates using reverse transcription-PCR, *in situ* hybridization, and immunohistochemistry (18–20), others demonstrated that patients' myeloma PCs (23, 24) and murine 5T2 cells (22) do express RANKL. We found expression of RANKL by myeloma PCs after coculture with OCs in seven of eight experiments by immunohistochemistry. These results demonstrate that in addition to sequestering osteoprotegerin (21), affecting osteoclastogenesis via macrophage inflammatory protein-1α production (25, 43), and modulating expression of these cytokines by cells in the BM microenvironment (18, 19), myeloma cells act directly on pOC via RANKL to promote OC formation (24).

The close association between myeloma cell growth and OC activity has been gleaned clinically (11, 12) and has been demonstrated in our SCID-hu model for primary myeloma (9, 18), in the ARH77 xenograft model (18, 44), and in the 5T2 murine model (10). The OC/myeloma cell coculture experiments clearly demonstrate the ability of OCs to support long-term survival and proliferation of primary MM PCs. OCs from myeloma patients and from healthy donors were equally as effective and similarly supported myeloma cells from all patients. Although myeloma cells did not firmly adhere to OCs, cell-cell contact was essential, as demonstrated in noncontact experiments and using media conditioned by OCs or OC-myeloma PC cocultures. This indicates that soluble factors release by OCs alone or after their direct interaction with MM PCs are insufficient to promote myeloma cell growth and survival. Taken together with the clinical reports and the data from the experimental models, these results underscore the critical role that OCs play in sustaining the myeloma disease process.

Previous studies reported that BM stromal cells supported survival and growth of primary human myeloma cells (45, 46) and that BM mesenchymal cells from patients with myeloma abnormally expressed adhesion molecules and cytokines, suggesting that these changes are required to promote myeloma (47). Our study differs from these reports in several key features; in contrast to BM stroma that consists of an ill-defined mixture of differentiated and undifferentiated cells of mesenchymal and hematopoietic origin (48), we demonstrate the ability of a single cell lineage to support sustained survival and proliferation of myeloma PCs. Additionally, we report that OC cultures from healthy donors are equally as effective in sustaining myeloma cells as those derived from myeloma patients. In contrast to cultures with BM stromal cells, myeloma PCs do not adhere tightly to OCs, the MM PC:OC ratio is very high (estimated ratio, 1000:1), probably more akin to the ratio *in vivo*, suggesting that some intercellular communication between myeloma PCs in contact with OCs and other myeloma PCs might occur. The OCs actively phagocytized dying myeloma cells, explaining the lower number of MM PCs recovered from cocultures compared with control cultures.

Whereas we find IL-6 production by both MM PCs and OCs, IL-6 production in MM PC/OC cocultures was not up-regulated, in contrast to MM PC/stromal cell cocultures (27, 49, 50). IL-6 has been reported to be a central myeloma growth factor (51–54) and to protect MM PCs from spontaneous, drug-induced (33, 55, 56), and Fas-induced apoptosis (57). Inhibition of IL-6 signaling for 14 days resulted in a 78% increase in apoptotic cells and a 33% reduction in viable cell number but had no effect on myeloma cell proliferation, indicating that IL-6 has antiapoptotic activity in myeloma with no effect on MM PC proliferation. The large number of viable MM PCs in the anti-IL-6 + anti-IL-6 receptor-treated cultures may suggest that IL-6-mediated gp130 signaling was not completely inhibited or that other gp130 activating cytokines are present. Alternatively, it is possible that other survival mechanisms, such as the Ras/mitogen-activated protein kinase pathway, have been activated (58).

The data presented here demonstrate that myeloma PCs directly induce osteoclastogenesis through RANKL. Osteoclasts, in turn, support survival and proliferation of myeloma cells in a mechanism that is still under investigation. Our findings lend support to observations from clinical trials and from animal models on the central role of OCs in sustaining the disease.

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