Development of an In Vivo Model of Human Multiple Myeloma Bone Disease

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Osteolytic bone destruction and its complications, bone pain, pathologic fractures, and hypercalcemia, are a major source of morbidity and mortality in patients with multiple myeloma. The bone destruction in multiple myeloma is due to increased osteoclast (OCL) activity and decreased bone formation in areas of bone adjacent to myeloma cells. The mechanisms underlying osteolysis in multiple myeloma in vivo are unclear. We used a human plasma cell leukemia cell line, ARH-77, that has disseminated growth in mice with severe combined immunodeficiency (SCID) and expresses IgGκ, as a model for human multiple myeloma. SCID mice were irradiated with 400 rads and mice were injected either with 10^6 ARH-77 cells intravenously (ARH-77 mice) or vehicle 24 hours after irradiation. Development of bone disease was assessed by blood ionized calcium levels, x-rays, and histology. All ARH-77, but none of control mice that survived irradiation, developed hind limb paralysis 28 to 35 days after injection and developed hypercalcemia (1.36 to 1.46 mmol/L) a mean of 5 days after becoming paraplegic. Lytic bone lesions were detected using x-rays in all the hypercalcemic mice examined. No lytic lesions or hypercalcemia developed in the controls. Controls or ARH-77 mice after developing hypercalcemia, were then killed and bone marrow plasma from the long bones was obtained, concentrated, and assayed for bone-resorbing activity. Bone marrow plasma from ARH-77 mice induced significant bone resorption in the fetal rat long bone resorption assay when compared with controls (percentage of total 40Ca released = 22% ± 2% v 11% ± 1%). Histologic examination of tissues from the ARH-77 mice showed infiltration of myeloma cells in the liver and spleen and marked infiltration in vertebrae and long bones, with loss of bony trabeculae and increased OCL numbers. Interestingly, cultures of ARH-77 mouse bone marrow for early OCL precursors colony-forming unit–granulocyte–macrophage (CFU-GM) showed a threefold increase in CFU-GM from ARH-77 marrow versus controls (185 ± 32 v 40 ± 3 per 2 x 10^6 cells plated). Bone-resorbing human and murine cytokines such as interleukin-6 (IL-6), IL-1α or β, TGFα, lymphotixin, and TNFα were not significantly increased in ARH-77 mouse sera or marrow plasma, compared with control mice, although ARH-77 cells produce IL-6 and lymphotixin in vitro. Conditioned media from ARH-77 cells induced significant bone resorption in the fetal rat long bone resorption assay when compared with untreated media (percentage of total 40Ca released = 22% ± 2% v 11% ± 1%). This effect was not blocked by anti–IL-6 or antilymphotoxin (percentage of total 40Ca released = 19% ± 1% and 22% ± 1%, respectively). Thus, we have developed a model of human multiple myeloma bone disease that should be very useful to dissect the pathogenesis of the bone destruction in multiple myeloma.

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The mechanisms responsible for the extensive bone destruction in multiple myeloma are not well understood due to lack of a good human cell model of multiple myeloma bone disease. Bone destruction is a prominent clinical feature of almost all patients with multiple myeloma. In addition to infections, it is responsible for many of the most debilitating clinical features of the disease, including intractable bone pain, fractures occurring either spontaneously or after trivial injury, and hypercalcemia with its attendant signs and symptoms.

The bone disease in multiple myeloma is mainly osteolytic with increased osteoclastic bone resorption in areas of bone adjacent to myeloma cells. In addition, bone formation is also decreased in patients with high tumor burdens. These data suggest that locally acting factors produced by myeloma cells play an important role in the extensive bone destruction seen in these patients. To date, these factors have not been clearly identified in vivo.

Therefore, as an initial step to clarify the mechanisms responsible for the osteolytic bone destruction in multiple myeloma, we developed an in vivo model of human myeloma bone disease that mimicked the disease in humans and could allow identification of the factors responsible for the extensive bone destruction in vivo based on the studies of Huang et al. They previously reported that the human myeloma cell line (ARH-77) had disseminated growth in SCID mice and documented the presence of human IgG in the ARH-77 mice serum. We have used this cell line to successfully develop an in vivo model of human myeloma bone disease, as will be described in this report.

MATERIALS AND METHODS

Preparation of ARH-77 multiple myeloma cell line conditioned media. ARH-77 cells, an IgGκ-secreting human plasma cell leukaemia cell line, were generously provided by Dr E. Vittela (University of Texas Southwestern Medical Center, Dallas, TX). ARH-77 cells were plated at 2.5 x 10^6 cells/mL in RPMI-1640 (GIBCO, Grand Island, NY) containing 20% fetal calf serum (FCS; HyClone Laboratory, Logan, UT) and cultured for 5 days. Conditioned media from the cultures was collected and concentrated 4X using a Microconcentrator Centriprep 3 (Amicon, Danvers, MA).

Transplantation of ARH-77 cells in SCID mice. Female SCID mice (6 to 8 weeks old) were obtained from the University of Wisconsin Gnomobi Laboratory. Mice were irradiated with 400 rads using a 60Co source and 24 hours after irradiation were injected in the tail vein with 10^6 ARH-77 cells (ARH-77 mice) intravenously. Mice that were irradiated but injected with vehicle rather than cells

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served as a control group. Mice were then followed-up weekly by measurement of serum calcium levels and whole body X-rays. When the ARH-77 mice became hypercalcemic (whole blood Ca++, >1.35 mmol/L), they were anesthetized with methoxyflurane (Pitman Moore, Mundelein, IL) and killed by cervical dislocation. Marrow cells and marrow plasma were then isolated from long bones as described below. Vertebral bones were dissected free of surrounding tissue and used for bone histomorphometry studies.

Collection of bone marrow plasma and assay of early osteoclast precursors (colony-forming unit-granulocyte-macrophage [CFU-GM]). Femurs were removed aseptically and dissected free of adhering tissue. The ends of the femurs were cut with a scalpel blade and the marrow was flushed with 5 mL of α-minimal essential medium (α-MEM) containing 0.1% (vol/vol) penicillin-streptomycin (GIBCO) using a 25-gauge needle. The cell suspension was centrifuged at 400g for 10 minutes and bone marrow plasma was collected and concentrated 5× using a Microconcentrator (Amicon, Danvers, MA). Bone marrow cells (5 × 10^6/mL) were resuspended in α-MEM containing 15% FCS (Hyclone Laboratory) and incubated for 2 hours at 37°C in a humid atmosphere of 5% CO2-air to remove cells adherent to plastic. The nonadherent bone marrow cells (10^6/mL) were plated on 35-mm tissue culture dishes (Falcon, Lincoln Park, NJ) in 1 mL of 0.8% methylcellulose (MC; Aldrich Co, Milwaukee, WI), supplemented with 20% FCS, 1% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), and 1.25 ng/mL of recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF; Immunex Co, Seattle, WA) as the source of colony-stimulating activity. Each assay was performed in triplicate. Cultures were incubated at 37°C in a humid atmosphere of 5% CO2-air for 7 days, at which time colonies (>40 cells) and clusters (>10 and <400 cells) were counted with an inverted microscope.

Bone resorption assays. Timed-pregnant rats were injected with 250 μCi of 45CaCl2 at day 18 of gestation. One day later, the rats were killed by cervical dislocation and the embryos were removed. The explanted radii and ulnae were cultured on circles of mixed ester membrane filters (0.45 μm; Whatman, Hillsboro, OR) on stainless steel grids in 0.5 mL of chemically defined medium (Sigma) supplemented with 1 mg/mL BSA (Sigma) and penicillin-streptomycin (50 U/mL and 50 mg/mL, respectively) in 5% CO2 in air at 37°C, as modified by Raiz and Niemann. The radii and ulnae were incubated for 24 hours in control media to allow for the removal of the exchangeable 45Ca before transferring to equilibrated control or experimental media. Experimental media contained varying concentrations of either bone marrow plasma from ARH-77 mice or control mice, media conditioned by ARH-77 cells in vitro, or untreated culture media. Control or experimental media were then changed after 72 hours. The bone explants were incubated for a total of 5 days. Bone-resorbing activity was measured as the percentage of total 45Ca released from the bone into the media over the 5 days of incubation.

Assay of bone-resorbing cytokines. The human and murine cytokines that induce bone resorption (human IL-1β, human TGFβ, PTHrP, human IL-6, human TNFβ, murine IL-6, murine TNFβ, and murine IL-1α) were measured in the peripheral blood sera and bone marrow plasma of ARH-77 or control mice and the ARH-77 cell conditioned media using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Boston, MA). The lower limit of detection for cytokines in these assays was approximately 10 pg/mL.

Processing of specimens for histology. Bones from all animals were fixed in 10% phosphate-buffered formalin for 24 to 48 hours, decalcified in 14% EDTA for 2 to 3 weeks, processed through graded alcohols, and embedded in paraffin wax. Serial sections (3-μm thick) of vertebral bodies were cut at various levels and stained with hematoxylin, eosin, orange G, and phloxine for histologic analysis. Consecutive sections (2-μm thick) were also taken at various levels to allow us to examine the same cell for expression of tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts. These sections were deparaffinized in xylene and immersed in acetone for 5 minutes. They were then placed for 1 hour in the substrate solution that contained 0.09 mmol/L naphthol-AS-Bi phosphate (Sigma) in 0.2 mol/L acetate and 0.4 mol/L L-(-) tartaric acid (Sigma) at pH 4.9. Sections were then placed for 30 minutes in hexazonized paraarsonilin in 0.2 mol/L acetate buffer with 0.4 mol/L tartaric acid, rinsed, and counterstained with methyl green and light green SF yellowish (Sigma).

Statistical analysis. Results are expressed as the mean ± the standard error of the mean (SEM) and are presented for typical experiments. Results were similar in two or more independent experiments. Differences were compared using Student’s t-test or ANOVA and were considered significant for P values <.05.

RESULTS

ARH-77 mice. All ARH-77 mice that survived irradiation developed hind limb paralysis 28 to 35 days after the injection of the cells and lost 10% of their lean body mass by the time they become paraplegic. Figure 1 shows the whole blood calcium levels in ARH-77 or control mice over the course of the experiments. All the ARH-77 mice developed hypercalcemia approximately 5 days after becoming paraplegic, with a mean whole blood ionized calcium of 1.43 mmol/L (range, 1.35 to 1.46 mmol/L). Multiple lytic lesions and diffuse osteopenia were detected in the hypercalcemic mice by X-rays (Fig 2). Neither hypercalcemia nor lytic bone lesions developed in the controls (P = .0002).

Cytokines. Levels of known bone-resorbing human and murine cytokines were measured in the peripheral blood sera and bone marrow plasma of ARH-77 or control mice, as well as in media conditioned by ARH-77 cells. Although ARH-77 cells secrete IL-6 (120 pg/mL) and TNFβ (800 pg/mL) in vitro, these cytokines were undetectable in the bone marrow plasma or sera from the ARH-77 mice. Human IL-
Fig 2. Radiologic examination of ARH-77 mice. Lytic lesions are present in ARH-77 mice throughout axial skeleton (arrows). Lytic lesions are present on left femur, right tibia, and right sacrum.

1β was detected in the bone marrow plasma of ARH-77 mice at a concentration of 20 pg/mL, a level incapable of stimulating bone resorption. Murine IL-6 and IL-1 were detected in both the ARH-77 mice and control bone marrow plasma and serum, respectively, but the levels did not differ significantly.

Effects of bone marrow plasma from ARH-77 mice and ARH-77 cells conditioned media on bone resorption. Bone marrow plasma from ARH-77 mice induced significant bone resorption in the fetal rat long bone resorption assay when compared with bone marrow plasma from controls (percentage of total 45Ca released = 35% ± 4% v 11% ± 1%), as shown in Fig 3. Conditioned media from ARH-77 cells induced significant bone resorption in the same assay when compared with untreated media (percentage of total 45Ca released = 22% ± 2% v 11% ± 1%), as shown in Fig 4. Antibodies against TNF and lymphotoxin failed to block this effect significantly (percentage of total 45Ca released = 22% ± 1% and 19% ± 1%, respectively; Fig 4). A similar pattern of bone resorption activity was seen in two independent experiments.

Analysis of early osteoclast precursors in ARH-77 mice. To determine if osteoclast precursors were increased in marrow of ARH-77 mice compared with controls, cultures for early osteoclast precursors (CFU-GM) were performed. CFU-GM colonies were increased threefold in ARH-77 mice versus controls (185 ± 32 v 40 ± 3 per 2 x 105 cells plated; Fig 5).

Histology. The bone marrow of the ARH-77 mice was infiltrated by ARH-77 cells (Fig 6). Immunostaining of the bone marrow cells for human κ and λ light chains showed plasmablasts that expressed κ light chains and not λ light chains. Histologically, the ARH-77 mice showed infiltration of myeloma cells in the liver, spleen, and bones. The vertebral and long bones had marked infiltration by the tumor with loss of bony trabeculae and increased osteoclast numbers without an osteoblastic response (Fig 7A). Deep resorption pits were associated with the osteoclasts adjacent to myeloma cells. In contrast, the bone next to normal bone marrow had a smooth contour. This increase in osteoclast numbers was even more dramatic when the bone sections were stained for TRAP, a marker enzyme for osteoclasts (Fig 7B). There was a marked increase in osteoclast numbers.
in areas of bone adjacent to myeloma cells, but not in areas of bone adjacent to normal bone marrow.

**Human κ and λ light chain immunostaining.** Cytospin slides were prepared with $10^5$ ARH-77 cells/slide and dried overnight. The slides were then fixed with acetone, dried, and rehydrated with phosphate-buffered saline (PBS). After suspending the slides in blocking solution of 10% ovalbumin, the peroxidase conjugated rabbit anti-human κ or rabbit human λ light chain (Dako, Carpinteria, CA) were applied. Aminoethylcarbazide chromogen was applied as substrate, and the slides were then counterstained with Bio-media hematoxylin. Cells positive for κ or λ light chains showed intense red staining of the cytoplasm.

**DISCUSSION**

Bone destruction is one of the most prominent features of multiple myeloma and is present in about 80% of patients.

The precise molecular mechanisms responsible for the bone destruction in multiple myeloma remain unclear, but observations over time have shown a number of facts. The mechanism by which bone is destroyed in myeloma is via the osteoclast, the normal bone-resorbing cell. Osteoclasts accumulate on bone-resorbing surfaces in myeloma only adjacent to collections of myeloma cells and not in areas adjacent to normal bone marrow. Thus, it appears that the mechanism by which osteoclasts are stimulated in myeloma is a local one. In addition, bone formation is inhibited when tumor burden is high, resulting in uncoupling of normal bone remodeling. It is therefore likely that interactions between myeloma cells and bone cells play an important role in the development of bone disease. Myeloma cells may produce factors that affect osteoclasts, osteoclast precursors, and/or osteoblasts, thereby uncoupling normal bone remodeling. Osteoblasts may be stimulated to produce factors that enhance osteoclastic bone resorption, and/or osteoblasts themselves may produce factors that may stimulate the growth of myeloma cells, acting to amplify the effects of myeloma on bone resorption.

Since the initial description of a myeloma-derived osteoclast activating factor in 1974 by Mundy et al., several cytokines have been identified that are produced by human myeloma cell lines and induce bone resorption in fetal rat long bone resorption assays in vitro, including IL-1, IL-6, lymphotoxin, and transforming growth factor α. Among these, a leading candidate for the cytokine responsible for the bone destruction associated with myeloma is IL-6. IL-6 has been shown to be a myeloma growth factor in vivo and is known to stimulate osteoclastic bone resorption and osteoclast formation, and osteoblasts, which have been reported to be recruited to areas of bone marrow involvement in early multiple myeloma, secrete IL-6.
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Fig 6. Bone marrow involvement by ARH-77 cells in vivo. (A) Wright-Giemsa stain of ARH-77 mouse bone marrow cells showing plasmoblasts with basophilic cytoplasm, high nuclear-cytoplasm ratio, and prominent nucleoli. (B) Human κ light chain immunostaining of bone marrow cells from ARH-77 mice. Plasmoblasts stained positively for cytoplasmic κ light chains.

Nevertheless, IL-6 has not been found to be consistently elevated in sera of patients with multiple myeloma and the level of IL-6 has not been correlated with the extent of multiple myeloma bone disease. Furthermore, IL-6 is a weak bone-resorbing factor. Therefore, the role that IL-6 plays in the pathogenesis of myeloma bone disease in vivo is not clearly understood. In addition, there may be other cytokines that are active in myeloma bone disease, but, to date, they have not been clearly identified.

To help clarify the mechanisms of bone destruction in multiple myeloma, it is necessary to develop an in vivo model of human myeloma bone disease that mimics the bone disease in humans and allows identification of the factors playing a role in the bone destruction in vivo. Radl et al. reported that aging C57BL/KalwRij mice developed spontaneous multiple myeloma with bone marrow involvement and diffuse osteolytic bone lesions. Transplantation of the bone marrow cells of these animals to younger animals of the same strain successfully induced disseminated multiple myeloma. Even though the disease in these animals resembles that in humans in many aspects, including the presence of osteolytic bone destruction, its usefulness to study the pathogenetic mechanisms of multiple myeloma bone disease in humans is limited by the fact that it represents a model of mouse...
multiple myeloma and that the cytokines involved may not necessarily represent those involved in human multiple myeloma. For example, IL-6 is a potent stimulator of human but not murine osteoclast formation and bone resorption. Therefore, other investigators have tried to develop animal models of multiple myeloma using human myeloma cells. Feo-Zuppardi et al. reported long-term engraftment of freshly isolated primary human myeloma cells after intraperitoneal injection in SCID mice. Even though they documented circulating levels of human IgG in these mice for more than 30 days after injection of the cells, these mice did not develop disseminated disease or bone marrow involvement, but only had collections of plasma cells in the peritoneal cavity.

Other investigators have also used the intraperitoneal route to inject a murine plasmacytoma into Balb/c mice as a model for human multiple myeloma, and again the mice failed to develop the disseminated disease or bone involvement that characterizes human multiple myeloma.

Recently, Huang et al. successfully transplanted and showed engraftment of a human multiple myeloma cell line, ARH-77, in SCID mice. The mice developed disseminated disease with bone marrow involvement and some microscopic osteolytic lesions in the vertebrae and bones of the skull. Because these cells showed disseminated growth in SCID mice, we have used transplantation of ARH-77 cells into SCID mice to develop an in vivo model of human myeloma bone disease. This model is unique in that all the...
mice injected with the human myeloma cell line developed hypercalcemia and osteolytic bone lesions, as assessed radiographically. Histologically, the ARH-77 mice developed osteolytic bone destruction with increased osteoclastic bone resorption in areas of bone adjacent to myeloma cells but not in areas of bone adjacent to normal bone marrow.

To determine if early osteoclast precursors were increased in ARH-77 mice as well, we performed CFU-GM cultures from the bone marrow cells of the ARH-77 mice and controls. The bulk of recent evidence supports CFU-GM as the earliest identifiable osteoclast precursor. CFU-GM colony formation was significantly elevated in the ARH-77 mice as compared with the control mice, suggesting that the myeloma cells induce not only mature osteoclast recruitment to the areas of bone adjacent to the tumor, but also new osteoclast formation.

Furthermore, bone marrow plasma from these ARH-77 mice and conditioned media from ARH-77 cells induced bone resorption in the fetal rat long bone resorption assay. Levels of either human or murine cytokines known to induce bone resorption were not significantly elevated in the bone marrow plasma or serum of these mice, although ARH-77 cells produce significant amounts of IL-6 and TNFβ in vitro. Furthermore, neutralizing antibodies against IL-6 and lymphotoxin failed to block the bone resorption induced by ARH-77 conditioned media in the fetal rat long bone resorption assay. These data suggest that other factors produced by myeloma cells or the marrow microenvironment may play an important role in the bone disease in multiple myeloma. This model should be very helpful in the identification and characterization of these factors, which may lead to novel therapeutic strategies that improve the quality of life and survival of these patients.

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