Cell–cell contact between marrow stromal cells and myeloma cells via VCAM-1 and α₄β₁-integrin enhances production of osteoclast-stimulating activity

Toshiki Michigami, Nobuaki Shimizu, Paul J. Williams, Maria Niewolna, Sarah L. Dallas, Gregory R. Mundy, and Toshiyuki Yoneda

Myeloma is a unique hematologic malignancy that exclusively home in the bone marrow and induces massive osteoclastic bone destruction presumably by producing cytokines that promote the differentiation of the hematopoietic progenitors to osteoclasts (osteoclastogenesis). It is recognized that neighboring bone marrow stromal cells influence the expression of the malignant phenotype in myeloma cells. This study examined the role of the interactions between myeloma cells and neighboring stromal cells in the production of osteoclastogenic factors to elucidate the mechanism underlying extensive osteoclastic bone destruction. A murine myeloma cell line 5TGM1, which causes severe osteolysis, expresses α₄β₁-integrin and tightly adheres to the mouse marrow stromal cell line ST2, which expresses the vascular cell adhesion molecule-1 (VCAM-1), a ligand for α₄β₁-integrin. Co-cultures of 5TGM1 with primary bone marrow cells generated tartrate-resistant acid phosphatase-positive multinucleated bone-resorbing osteoclasts. Co-cultures of 5TGM1 with ST2 showed increased production of bone-resorbing activity and neutralizing antibodies against VCAM-1 or α₄β₁-integrin inhibited this. The 5TGM1 cells contacting recombinant VCAM-1 produced increased osteoclastogenic and bone-resorbing activity. The activity was not blocked by the neutralizing antibody to known osteoclastogenic cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor, or parathyroid hormone-related peptide. These data suggest that myeloma cells are responsible for producing osteoclastogenic activity and that establishment of direct contact with marrow stromal cells via α₄β₁-integrin/VCAM-1 increases the production of this activity by myeloma cells. They also suggest that the presence of stromal cells may provide a microenvironment that allows exclusive colonization of myeloma cells in the bone marrow. (Blood. 2000;96:1953-1960)

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Materials and methods

Cells

The 5TGM1 myeloma cells were initially derived from a myeloma called 5T33 that arose spontaneously in aged C57BL/KaLwRij mice. 2-5 5TGM1 is a subclonal cell line we established from 5T33 multiple myeloma that was originally described by Radl and colleagues 18 and expresses α₄β₁-integrin. 19 We found that cell–cell interactions between 5TGM1 myeloma cells and marrow stromal cells that are mediated through α₄β₁-integrin/VCAM-1 increased the production of osteoclastogenic activity by the myeloma cells. We propose that this cell–cell interaction is critical to the development and progression of myeloma-induced osteolysis.

From the Division of Endocrinology and Metabolism, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX; Department of Biochemistry, Osaka University Faculty of Dentistry, Suita, Osaka, Japan

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Reprints: Toshiyuki Yoneda, Division of Endocrinology and Metabolism, Department of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr, San Antonio, TX 78284-7877; e-mail: yoneda@uthscsa.edu.

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liver and kidney. The STG1M cells produce interleukin (IL)-6, but their growth is not promoted by IL-6. Human myeloma cell line IM-9, U266B1, and mouse B-cell lymphoma cell line RAW8.1 were purchased from American Type Culture Collection (Manassas, VA). ARH-77 human plasma cell leukemia cells, which are shown to develop myeloma bone disease, were obtained from Dr Roodman in our institute. These cells were grown in IMDM supplemented with 10% FBS. The ST2 bone marrow stromal cells purchased from RIKEN Cell Bank (Tsukuba, Japan) were cultured in IMDM or α-minimum essential medium (MEM) supplemented with 10% FBS. Chinese hamster ovary (CHO) cells expressing human α₃-integrin were generously provided by Dr Takada (The Scripps Research Institute). They were cultured in Dulbecco modified Eagle minimal essential medium (DMEM) (Hazleton Biologics, Lenaxa, KS) supplemented with 10% FBS.

**Antibodies**
Neutralizing monoclonal antibodies against murine VCAM-1 (M/K-2.7, IgG1k), α₃β₁ integrin (9428), and intracellular adhesion molecule-1 (ICAM-1, YN1/1.7), were kindly given by Dr Kensuke Miyake (Saga Medical University, Saga, Japan). Neutralizing monoclonal antibodies to murine osteoclast-activating cytokines were purchased from R & D Systems (Minneapolis, MN). Parathyroid hormone-related protein (PTH-rP) monoclonal antibody was described by Ratclife and coworkers. Control monoclonal antibody (MOPC-1, mouse IgG1) was purchased from Sigma (St Louis, MO). Isocyanate-conjugated α₃-integrin antibody for flow cytometric analysis was purchased from Chemicon International (Temecula, CA).

**Reverse transcription polymerase chain reaction (RT-PCR)**
Total RNA was prepared from STG1M, primary culture of bone marrow stromal cells, and ST2 marrow stromal cell line by the single-step RNA isolation method using TRizol reagent (GIBCO). RNA (3 μg) was incubated with 50 ng of random hexamer at 70°C for 10 minutes and chilled on ice, then converted to first-strand complementary DNA (cDNA) using reverse transcriptase (Perkin-Elmer, Branchburg, NJ) according to the manufacturer’s instruction. The primers used for PCR were as follows: murine VCAM-1 5'-primer, 5'-OH-GTCTGCCTCATTGTTCCTC-3'; murine VCAM-1 3'-primer, 5'-OH-GCTGCGGTCCACCAACATT-3'; murine α₃ integrin 5'-primer, 5'-OH-ACCACCCCTTGACGGCTTTG-3'; murine α₃ integrin 3'-primer, 5'-OH-GGTCTTGCCTTAGCAACACTGC-3'; murine α₃ integrin 5'-primer, 5'-OH-GATCCCATACGCAACTACAAGG-3'; murine α₃ integrin 3'-primer, 5'-OH-GATCCCATACGCAACTACAAGG-3'. PCR products were separated on 2.5% agarose gels containing ethidium bromide and visualized under UV light. The size of the fragments was confirmed by reference to molecular weight markers. GAPDH served as a control.

**Flow cytometric analysis of VCAM-1 and α₃β₁-integrin expression**
Cell suspensions containing either ST2 stromal cells or STG1M myeloma cells (1 × 10⁶ cells/mL) were treated with 10% normal mouse serum and subsequently incubated with either the anti-VCAM-1 antibody (M/K-2.7, 1:10 dilution), followed by the treatment with fluorescent isocyanate-conjugated goat antirat IgG or isocyanate-conjugated anti–α₃-integrin antibody (1:1 dilution). Expression of the VCAM-1 or α₃-integrin on cell surface was determined using a flowcytometer (FACStar plus; Becton Dickinson, San Jose, CA).

**Attachment of STG1M myeloma cells onto ST2 mouse bone marrow stromal cells**
The ST2 cells were cultured in α-MEM supplemented with 10% FBS until confluency in 48-well culture plates (Coster, Cambridge, MA). Growing STG1M cells were labeled with 10 μCi [methyl-³H] thymidine (New England Nuclear, Boston, MA) for 24 hours. ³H-labeled STG1M cells (20 000 cells, 8654 ± 244 pmu) were then incubated on the ST2 cell monolayer in the absence or presence of antibodies to VCAM-1 or α₃β₁-integrin for 1 hour. Nonadherent cells were removed by washing with 5% trichloroacetic acid twice and phosphate-buffered saline (PBS) twice, and adherent cells were solubilized in 300 μL of 0.25 mMol/L NaOH and neutralized with the same volume of 0.25 mMol/L HCl; the radioactivity was determined in a liquid scintillation counter.

**Double-staining of the co-cultures for tartrate-resistant acid phosphatase (TRAP) and VCAM-1 expression**
Co-cultures were fixed with 3.7% formaldehyde and stained first for TRAP as described below. TRAP-stained cultures were next treated with 0.6% hydrogen peroxide for 15 minutes and then with 0.8% rabbit serum for 1 hour. Subsequently, the cultures were incubated with anti-VCMA-1 antibody (M/K-2.7, 1:10 dilution) at room temperature for 45 minutes, washed with PBS containing 1% rabbit serum (×4), incubated with secondary antibody (rabbit antirat IgG, Vector Laboratories, Burlingame, CA) at room temperature for 45 minutes, washed with PBS containing 1% rabbit serum (×4), and visualized using a commercial kit (Vectastain Elite ABC kit, Vector Laboratories).

**Formation of TRAP-positive multinucleated osteoclasts and resorption pits in co-culture of STG1M myeloma cells and primary mouse bone marrow cells**
Mouse bone marrow cells were obtained from 5-week-old male C57BL mice as described previously. Femurs and tibiae were dissected aseptically, cut off both ends. Bone marrow cells were flushed out, collected, and incubated in α-MEM supplemented with 10% FBS (HyClone, Logan, UT) in 100-mm culture dishes (Becton Dickinson Labware, Bedford, MA) for 2 hours and nonadherent cells containing hemopoietic osteoclast precursors and stromal cells were harvested. Bone marrow cells (1 × 10⁶/dish) and STG1M cells (1000 cells/well) in 300 μL of the culture medium were plated onto 48-well culture plates (day 1). On day 2, 300 μL of spent medium was replaced with the same volume of fresh medium. As a positive control, a potent osteoclastogenic agent, 1α,25-dihydroxyvitamin D₃ (1,25-D₃, Biomol, Plymouth Meeting, PA), was added to bone marrow cultures. The co-cultures were conducted in the absence of 1,25-D₃ unless indicated.

To confirm that these TRAP-positive multinucleated osteoclasts have the capacity to resorb bone, STG1M cells and marrow cells were co-cultured on 5 × 5-mm whale dentine slices in the same condition, and resorption pits formed on these dentine slices were examined by scanning electron microscopy as described. In some experiments, co-cultures of STG1M myeloma cells and marrow cells were performed using transwell inserts (24-well, Becton Dickinson Labware) to prevent the direct contact between these 2 types of cells.

**Conditioned medium**
The ST2 marrow cells (5 × 10⁶/dish) and STG1M myeloma cells (5 × 10⁵/dish) were plated together onto 60-mm culture dishes (Becton Dickinson) in IMDM supplemented with 10% FBS and cultured overnight, washed with serum-free IMDM twice, and incubated in 5 mL of serum-free IMDM with 0.1% bovine serum albumin (BSA). After 48 hours, conditioned media were harvested, centrifuged to remove cell debris, and stored at −20°C until use. Conditioned medium was also harvested from the co-cultures in
which 5TGM1 myeloma cells were cultured with monolayer of ST2 stromal cells that had been fixed with 2.5% paraformaldehyde.

In some experiments, 5TGM1 mouse myeloma cells and IM-9, U266B1, and ARH-77 human myeloma cells (1 × 10⁶/mL/24-well) were cultured for 24 hours in IMDM with 5% FBS in plates coated with or without 1µg/mL recombinant soluble VCAM-1 (rsVCAM-1) that lacks transmembrane and cytoplasmic domains (kindly provided by Dr Lobb, Biogen, Cambridge, MA). Conditioned media were then harvested and assessed for bone-resorbing activity in fetal rat long bone assay as described below and for the capacity to stimulate osteoclastogenesis in mouse marrow cultures. IMDM incubated without cells in the presence and absence of rsVCAM-1 served as controls.

In other experiments, ST2 mouse bone marrow cells (2 × 10⁶/24 well) were cultured for 24 hours on a monolayer of CHO cells that had been transfected with αv-integrin or empty vector and fixed with 2.5% paraformaldehyde. Conditioned media of these cultures were assayed for osteoclastogenic and bone-resorbing activity in mouse bone marrow assay and fetal rat long bone assay, respectively.

Organ cultures of ⁴⁵Ca-labeled fetal rat long bones

Conditioned media harvested as described above were assayed for bone-resorbing activity by organ cultures of ⁴⁵Ca-labeled fetal rat long bones as described previously.²³ Pregnant rats were injected with 250 µCi of ⁴⁵Ca (New England Nuclear) on the 18th day of gestation. Radius and ulna bone shafts were excised from 19-day-old fetuses under the dissecting microscope and precultured for 24 hours in Biggers-Gwatkin-Jackson medium (Sigma) supplemented with 0.1% BSA between air and liquid phase on stainless mesh grids. Bones were then cultured in the presence of conditioned media (50% v/v) or in control medium for 120 hours. The media were changed once at 48 hours of the culture. At the end of the culture, bones were harvested and treated in ice-cold 5% trichloroacetic acid for 2 hours; ⁴⁵Ca radioactivity in bones and media was determined in a liquid scintillation counter. Bone resorption was expressed as the percentage of ⁴⁵Ca released into the medium from bones as calculated by ⁴⁵Ca count in medium/⁴⁵Ca count in medium and bone × 100.

Statistical analysis

All data were presented as the mean ± SEM and analyzed by analysis of variance, followed by a paired t test.

Results

Expression of VCAM-1 and αv-integrin in stromal cells and 5TGM1 myeloma cells

Using RT-PCR, we examined the expression of αv-integrin and VCAM-1 in myeloma cells and bone marrow stromal cells, respectively. The 5TGM1 myeloma cells expressed αv-integrin as reported previously,¹⁹ whereas ST2 stromal cells did not show αv-integrin expression (Figure 1Ai). Both ST2 stromal cells and primary bone marrow stromal cells expressed VCAM-1, whereas 5TGM1 did not (Figure 1Aii). Expression of VCAM-1 on ST2 cells (Figure 1Bi) and αv-integrin expression on 5TGM1 cells (Figure 1Biv) was also demonstrated by flow cytometric analysis.

In a separate experiment, we also examined the expression of β3-integrin, because αvβ3-integrin is a receptor for VCAM-1 as well. However, we did not detect β3 expression in 5TGM1 cells by RT-PCR (data not shown).

Attachment of 5TGM1 myeloma cells to ST2 cell monolayer in the absence or presence of antibodies to VCAM-1 and αvβ3-integrin

We then examined whether VCAM-1 and αv-integrin play a role in the attachment between ST2 stromal cells and 5TGM1 myeloma cells. The 5TGM1 cells grow in suspension. In contrast, almost 100% 5TGM1 cells adhered to ST2 cell monolayer (Figure 2). The anti-VCAM-1 antibody (10 µg/mL) significantly and anti-αvβ3-integrin antibody (10 µg/mL) more profoundly inhibited the attachment of 5TGM1 myeloma cells to ST2 monolayer (Figure 2), suggesting that the attachment of 5TGM1 myeloma cells to ST2 stromal cells is mediated via αv-integrin and VCAM-1. The results also demonstrate that VCAM-1 and αvβ3-integrin expressed on these cells are biologically functional and that these antibodies have neutralizing activity. Increase in the concentration of these antibodies to 20 µg/mL did not show further inhibition of the attachment (data not shown). Moreover, combined treatment with anti-VCAM-1 antibody (10 µg/mL) and anti-αvβ3-integrin antibody (10 µg/mL) did not further inhibit the attachment of 5TGM1 myeloma cells to ST2 monolayer (Figure 2).
multinucleated osteoclasts formation (Figure 3A). Cultured with bone marrow cells did not cause TRAP-positive stromal cells. Nonmyeloma RAW8.1 B-cell lymphoma cells co-positive for VCAM-1 (Figure 3Bii), suggesting that these cells are cells surrounding TRAP-positive multinucleated osteoclasts were capable of resorbing bone and possessed the osteoclast phenotype. When these TRAP-stained co-cultures were subsequently immunostained with anti–VCAM-1 antibody, we observed fibroblast-like cells surrounding TRAP-positive multinucleated osteoclasts were positive for VCAM-1 (Figure 3Biii), suggesting that these cells are stromal cells. Nonmyeloma RAW8.1 B-cell lymphoma cells co-cultured with bone marrow cells did not cause TRAP-positive multinucleated osteoclasts formation (Figure 3A).

To confirm that a direct contact between STG1 myeloma cells and bone marrow stromal cells is necessary for TRAP-positive multinucleated osteoclast formation, we carried out co-culture experiments using trans-well inserts in which 5TGM1 cells were cultured to confluency in 48-well culture plates. 3H-Thymidine-labeled bone marrow cells and 5TGM1 myeloma cells, many TRAP-positive multinucleated osteoclasts formed (Figure 3A). In contrast, bone marrow cells alone did not form TRAP-positive multinucleated osteoclasts (Figure 3A). In contrast, bone marrow cells cultured in the presence of 10 nmol/L 1,25-D$_3$ formed numerous TRAP-positive multinucleated osteoclasts after 6 days of culture, as previously described.24,26 In the co-cultures of bone marrow cells and STG1 myeloma cells, many TRAP-positive multinucleated osteoclasts formed (Figure 3A,Bi). These TRAP-positive multinucleated cells exhibited resorption pit formation on dentine slices (Figure 3Bii), demonstrating that these cells were capable of resorbing bone and possessed the osteoclast phenotype. When these TRAP-stained co-cultures were subsequently immunostained with anti–VCAM-1 antibody, we observed fibroblast-like cells surrounding TRAP-positive multinucleated osteoclasts were positive for VCAM-1 (Figure 3Biii), suggesting that these cells are stromal cells.

TRAP-positive multinucleated osteoclast formation in the co-cultures of STG1 myeloma cells and mouse bone marrow cells

We next examined whether the cell-cell interaction between myeloma cells and stromal cells caused a generation of osteoclasts. To determine this, STG1 myeloma cells were co-cultured with bone marrow cells that contain both stromal cells and hematopoietic osteoclast progenitor cells. In preliminary experiments, we found that the co-culture consisting of 1 million bone marrow cells and 1000 STG1 myeloma cells produced the greatest number of TRAP-positive multinucleated osteoclasts. Increase in number of STG1 myeloma cells rather decreased osteoclast formation due to the overgrowth of STG1 myeloma cells during 6-day culture.

Bone marrow cells alone did not form TRAP-positive multinucleated osteoclasts (Figure 3A). In contrast, bone marrow cells cultured in the presence of 10 nmol/L 1,25-D$_3$ formed numerous TRAP-positive multinucleated osteoclasts after 6 days of culture, as previously described.24,26 In the co-cultures of bone marrow cells and STG1 myeloma cells, many TRAP-positive multinucleated osteoclasts formed (Figure 3A,Bi). These TRAP-positive multinucleated cells exhibited resorption pit formation on dentine slices (Figure 3Bii), demonstrating that these cells were capable of resorbing bone and possessed the osteoclast phenotype. When these TRAP-stained co-cultures were subsequently immunostained with anti–VCAM-1 antibody, we observed fibroblast-like cells surrounding TRAP-positive multinucleated osteoclasts were positive for VCAM-1 (Figure 3Biii), suggesting that these cells are stromal cells. Nonmyeloma RAW8.1 B-cell lymphoma cells co-cultured with bone marrow cells did not cause TRAP-positive multinucleated osteoclasts formation (Figure 3A).

To confirm that a direct contact between STG1 myeloma cells and bone marrow stromal cells is necessary for TRAP-positive multinucleated osteoclast formation, we carried out co-culture experiments using trans-well inserts in which STG1 cells were separated from bone marrow cells by a membrane. In this co-culture condition, there were very few osteoclasts formed (data not shown). The result indicates that the direct contact is essential.

Effect of antibodies to VCAM-1 and α$_i$β$_i$-integrin on TRAP-positive multinucleated osteoclast formation in the co-culture of STG1 myeloma cells and marrow cells

We subsequently determined the role of VCAM-1 and α$_i$β$_i$-integrin in osteoclast formation in the co-cultures using the neutralizing antibody to VCAM-1 or α$_i$β$_i$-integrin. Both anti–VCAM-1 antibody (VCAM-1 Ab, 10 μg/mL) and anti–α$_i$β$_i$-integrin antibody (α$_i$β$_i$ Ab, 10 μg/mL) dramatically inhibited TRAP-positive multinucleated osteoclast formation (Figure 4). On the other hand, antibody against ICAM-1 (ICAM-1 Ab, 10 μg/mL) and control IgG had no effect on the osteoclast formation (Figure 4).

To determine whether this inhibition by VCAM-1 Ab and α$_i$β$_i$ Ab was specific for the STG1-induced osteoclast formation, effects of these antibodies were examined on the 1,25-D$_3$-induced osteoclast formation in the mouse bone marrow cell cultures. Neither VCAM-1 nor α$_i$β$_i$ Ab inhibited osteoclast formation induced by 10$^{-8}$ mol/L 1,25-D$_3$ (data not shown). The result is consistent with the notion that the interaction between VCAM-1 and α$_i$β$_i$-integrin is specifically critical to the osteoclast formation seen in the co-cultures of STG1 myeloma cells and bone marrow cells.

Feuerbach and coworkers27 have recently demonstrated that a neutralizing antibody to VCAM-1 inhibits 1,25-D$_3$-induced osteoclast formation using a unique stromal cell line of which VCAM-1

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**Figure 2. Attachment of 5TGM1 myeloma cells to monolayer of mouse stromal cell line ST2 and effect of neutralizing antibodies to VCAM-1 and α$_i$β$_i$-integrin.**

<table>
<thead>
<tr>
<th>% attached/inoculated</th>
<th>Untreated</th>
<th>IgG</th>
<th>VCAM-1 Ab</th>
<th>α$_i$β$_i$ Ab</th>
<th>VCAM-1 Ab + α$_i$β$_i$ Ab</th>
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*Significantly different from IgG control (P < .01).

**Figure 3. Number of TRAP-positive multinucleated osteoclasts formed in the co-cultures of STG1 myeloma cells and primary mouse bone marrow cells.**

(A) STG1 myeloma cells (1 × 10$^7$) were plated together with primary mouse bone marrow cells (BMC, 1 × 10$^6$) in 48-well plates, and cultured for 6 days. Cells were fixed and stained for TRAP activity as described in text. TRAP-positive cells with more than 3 nuclei were manually counted under a microscope as described.24,26 Data are expressed as mean ± SE (n = 6). *Significantly different from BMC alone (P < .01).

(Bi) Double staining for TRAP and VCAM-1 of the co-cultures. Note that TRAP-positive large multinucleated osteoclasts (red) were surrounded by fibroblast-like VCAM-1-positive cells (black), which are most likely stromal cells. STG1 myeloma cells were washed away during processing for staining. (Bii) Pit formation on dentine slices by TRAP-positive multinucleated osteoclasts formed in the co-culture. Co-culture was carried out on sperm whale dentine slices. After 6 days of culture, these dentine slices were fixed and examined by scanning electron microscopy for resorption pit formation as described.24,25
expression is up-regulated by 1,25-D$_3$. However, in our hands, there was no change in VCAM-1 expression in primary bone marrow stromal cells and ST2 cells used in the present study even in the presence of 1,25-D$_3$ (data not shown). This apparent discrepancy might be due to the different biologic properties of the stromal cells used in both studies.

Effect of conditioned medium harvested from the co-cultures of 5TGM1 myeloma cells and ST2 stromal cells on bone resorption

The results obtained in these co-culture experiments suggested to us that the direct contact between 5TGM1 myeloma cells and bone marrow stromal cells mediated via $\alpha_\beta_1$-integrin and VCAM-1 produces a soluble factor that stimulates osteoclast formation and function. Accordingly, we tested the effects of conditioned medium of the co-cultures of 5TGM1 myeloma cells and ST2 stromal cells on osteoclastic bone resorption. The conditioned medium at 40% (v/v) from the co-cultures showed increased bone-resorbing activity. Conditioned medium at 40% of the co-cultures of 5TGM1 myeloma cells and ST2 stromal cells produced a soluble factor that stimulates osteoclast formation and bone resorption. The rsVCAM-1 itself had no effects on bone resorption (data not shown).

The 5TGM1 myeloma cells did not attach to rsVCAM-1–coated plates in the presence of anti–VCAM-1 Ab (10 $\mu$g/mL) or anti–$\alpha_\beta_1$-integrin Ab (10 $\mu$g/mL) during the entire culture period. Conditioned medium of 5TGM1 cells cultured in rsVCAM-1–coated plates in the presence of these antibodies showed marked decrease in the osteoclastogenic activity (Figure 6C).

Of note, several human myeloma cells also showed the production of osteoclastogenic activity when they were cultured in rsVCAM-1–coated plates (Figure 6A). Among these cells, ARH-77 cells have been shown to induce myeloma bone disease.20

Production of osteoclastogenic activity in ST2 stromal cells in contact with $\alpha_\beta_1$-integrin

To exclude the possibility that ST2 bone marrow cells produce osteoclastogenic activity when they contact $\alpha_\beta_1$-integrin expressed on 5TGM1 myeloma cells in the co-cultures, ST2 cells were cultured on monolayer of fixed CHO cells expressing $\alpha_\beta_1$-integrin. We observed that attachment of ST2 cells to fixed CHO cells expressing $\alpha_\beta_1$-integrin was increased compared with fixed CHO cells with empty vector or noncoated plates (data not shown). However, none of conditioned media harvested from these cultures showed osteoclastogenic activity in the bone marrow culture assay (Figure 7). These data strongly suggest that 5TGM1 myeloma cells are responsible for the production of osteoclastogenic and bone-resorbing activity in the co-cultures.

Effect of neutralizing antibodies to known osteoclastogenic cytokines on bone-resorbing activity producer by 5TGM1 cells

In an attempt to identify 5TGM1-derived cytokine responsible for osteoclastogenic activity and bone resorption, conditioned media from the co-cultures were treated with a saturating concentration of neutralizing antibodies to several known bone resorption-stimulating cytokines including IL-1$\alpha$, IL-1$\beta$, IL-6, tumor necrosis factor- $\alpha$ (TNF-$\alpha$), and IL-6.

Production of osteoclastogenic and bone-resorbing activity in 5TGM1 myeloma cells in contact with rsVCAM-1

From the results described above, it seemed likely that 5TGM-1 cells were the producer of the bone-resorbing activity. To clarify this, 5TGM1 myeloma cells were cultured in rsVCAM-1–coated plates in the absence of stromal cells. Of note, 5TGM1 myeloma cells became tightly attached to rsVCAM-1–coated plates. Conditioned medium was harvested from these cultures and assayed for osteoclastogenic activity using mouse bone marrow cultures. The conditioned medium showed strong osteoclastogenic activity (Figure 4). The conditioned medium of 5TGM1 cells cultured on noncoated plates exhibited no activity of osteoclast formation and bone resorption. The rsVCAM-1 itself had no effects on bone resorption (data not shown).

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Production of osteoclastogenic activity in ST2 stromal cells in contact with $\alpha_\beta_1$-integrin

To exclude the possibility that ST2 bone marrow cells produce osteoclastogenic activity when they contact $\alpha_\beta_1$-integrin expressed on 5TGM1 myeloma cells in the co-cultures, ST2 cells were cultured on monolayer of fixed CHO cells expressing $\alpha_\beta_1$-integrin. We observed that attachment of ST2 cells to fixed CHO cells expressing $\alpha_\beta_1$-integrin was increased compared with fixed CHO cells with empty vector or noncoated plates (data not shown). However, none of conditioned media harvested from these cultures showed osteoclastogenic activity in the bone marrow culture assay (Figure 7). These data strongly suggest that 5TGM1 myeloma cells are responsible for the production of osteoclastogenic and bone-resorbing activity in the co-cultures.
factor-α (TNFα), tumor necrosis factor-β (TNFβ), and PTH-P. Bone-resorbing activity of the antibody-treated conditioned media were then determined in the fetal rat long bone assay. In a preliminary experiment using the fetal rat long bone assay, 10^{-7} mol/L IL-1α and IL-1β, 500 ng/ml IL-6 together with 500 ng/ml soluble IL-6 receptor, 10^{-7} mol/L TNFα and TNFβ, and 25 ng/ml PTH-P markedly stimulated bone resorption. We confirmed that each of the antibodies at a concentration used here blocked the bone-resorbing activity of each corresponding cytokine in this assay (data not shown). However, none of these antibodies

Discussion
Progressive osteoclastic bone destruction is one of the most detrimental complications of multiple myeloma. Earlier studies have suggested that production of osteoclast-activating cytokines by myeloma cells is responsible for the aberrant increase in osteoclastic bone destruction in patients. Here, we studied the murine myeloma cell line, 5TGM1, which causes typical myeloma bone diseases with extensive osteoclastic osteolysis in syngeneic mice, for its capacity to produce bone-resorbing cytokines to understand the mechanism responsible for myeloma-induced bone disease. Like many other human and murine myeloma cells, 5TGM1 conditioned medium from 5TGM1 cultures showed marginal bone-resorbing activity. In contrast, our data showed that 5TGM1 cells in direct contact with the ST2 bone marrow stromal cells or primary mouse bone marrow stromal cells in the co-cultures produced increased osteoclastogenic activity. Prevention of contact between STGMI and stromal cells in trans-well cultures resulted in blocked bone-resorbing activity present in the conditioned media (Figure 8).
a marked decrease in the production of this activity. More importantly, the results that 5TGM1 cells cultured in rsVCAM-1–coated plates produced osteoclast-stimulating activity in the absence of bone marrow stromal cells indicate that 5TGM1 myeloma cells are responsible for producing this activity in the co-cultures. These data demonstrate that the cell-cell contact with bone marrow stromal cells is essential for 5TGM1 myeloma cells to cause osteoclast stimulation. It is, therefore, suggested that the absence of stromal cells, a critical cellular component of bone marrow, may explain the failure of myeloma cells that are freshly isolated from patients with extensive osteolytic skeletal lesions or currently available human and murine myeloma cell lines to consistently produce discernible osteoclast-activating cytokines in culture. Figure 6A in which several human myeloma cell lines show the production of osteoclastogenic activity only in the presence of rsVCAM-1 further supports this notion.

We next examined which CAMs were involved in the direct cell-cell interactions between 5TGM1 cells and marrow stromal cells that are necessary for the production of osteoclastogenic cytokines. Our experiments using neutralizing antibodies to VCAM-1 and αβ1-integrin indicate that VCAM-1 and αβ1-integrin are involved in this cell-cell interaction. It has been previously reported that VCAM-1 is constitutively expressed in bone marrow stromal cells and that myeloma cells express αβ1-integrin, which serves as the receptor for VCAM-1.12,16 VCAM-1/αβ1-integrin–mediated cross-talk with marrow stromal cells has been shown to be critical to the growth,9,12 metabolism,12 cellular signaling,13 specific localization,15 and homing1 of myeloma cells in the marrow cavity. Other studies have suggested the importance of the marrow microenvironment on the expression of the malignant phenotype of myeloma cells.7,9 Our results are consistent with these earlier studies. In addition, recent reports have demonstrated that CHO cells overexpressing αβ1-integrin increase osteoclast formation in vitro28 and preferentially develop bone metastases.21 They have also shown that administration of neutralizing antibodies to VCAM-1 or αβ1-integrin selectively decreases bone metastases but not pulmonary metastases.23 These results suggest that VCAM-1/αβ1-integrin interactions also play a critical role in the preferential colonization of solid tumors to bone. Our findings indicate that VCAM-1/αβ1-integrin interactions are, at least in part, responsible for the cell-cell communication between marrow stromal cells and 5TGM1 myeloma cells, which consequently leads to increased production of osteoclast-activating cytokines. In further support of this notion, our data demonstrate that several human myeloma cell lines produce osteoclastogenic activity when they contact rsVCAM-1 in culture.

However, it should be noted that solid tumors may behave differently from myeloma in this regard. For example, others have shown that stable transfection of αβ1-integrin cDNA into B10 melanoma cells decreases the capacity of these cells to invade and metastasize.29 In addition, several other studies also reported that αβ1-integrin inhibited metastasis.30 The reasons for these differences are unknown. It is speculated that solid tumor cells, which do not necessarily have predilection for homing to the bone marrow, might elicit different cytoplasmic or nuclear events as a consequence of these cell-cell interactions with host cells compared with myeloma cells that exclusively colonize in the bone marrow.

Myeloma cells express not only αβ1-integrin but also a variety of other integrins.12,16 To determine the involvement of other integrins in the interactions between 5TGM1 cells and stromal cells, we examined αβ1-integrin, which has been shown to be expressed in myeloma cells12,16 and is also a receptor for VCAM-1. However, the β1-integrin was not detected in 5TGM1 cells by RT-PCR. Fibronectin, which is also a ligand for αβ1-integrin and expressed in marrow stromal cells,31 may also be involved in mediating the interactions between 5TGM1 cells and bone marrow stromal cells. In a preliminary experiment, we observed that 5TGM1 cells attached onto fibronectin-coated plates (data not shown), suggesting that fibronectin may also play a role in cell-cell contact between 5TGM1 cells and bone marrow stromal cells. Further studies are required to clarify this point. Nevertheless, our results strongly suggest that αβ1-integrin constitutively expressed in myeloma cells and VCAM-1 constitutively expressed in the marrow stromal cells are critical to establish the partnership between these 2 types of cells in the bone marrow cavity during the progression of massive bone destruction in myeloma.

Different osteoclast-activating cytokines such as IL-1,32 IL-6,33,34 TNF-β,35 PTH-rP36 and hepatocyte growth factor37 have been implicated in the pathogenesis of myeloma-induced osteolyis. We have not as yet identified the osteoclast-stimulating activity produced in the co-cultures, and this is likely to be an extensive undertaking. Neutralizing antibodies to several known osteoclast-activating cytokines including IL-1α and β, IL-6, TNF-α and -β, and PTH-rP failed to block the osteoclast-stimulating activity in the conditioned medium of the co-cultures. These preliminary observations raise the possibility that a novel cytokine may be responsible. Recent studies reported independently from 2 groups suggest that the transmembranous receptor activated nuclear factor (NF)-κB ligand (RANKL)/osteoclast differentiation factor (ODF)/osteoprotegerin ligand (OPGL) expressed in stromal cell/osteoblasts is the final common mediator of osteoclast-activating factors such as 1,25-D3, parathyroid hormone (PTH), prostaglandin E2, and IL-11.38,39 Of interest, our preliminary experiments have shown that both 5TGM1 and ST2 cells express RANKL/ODF/OPGL and that the expression is up-regulated in the co-cultures.30 These results suggest that not only myeloma cells but also bone marrow stromal cells directly enhance osteoclastogenesis and bone resorption. It should be noted, however, that RANKL/ODF/OPGL is a membrane-bound protein and thus not soluble. Whether RANKL/ODF/OPGL also plays a role in the pathogenesis of bone destruction in myeloma is currently unknown but is obviously an important issue to be explored to further understand the molecular mechanism of this devastating complication of myeloma.

In conclusion, our data suggest that the direct cell-cell contact between myeloma cells and marrow stromal cells via αβ1-integrin/VCAM-1 is critical to the production of a yet-unidentified soluble factor(s) that stimulates osteoclastogenesis and bone resorption. We believe this is one of the mechanisms by which myeloma cells exclusively home in the bone marrow and subsequently cause profound bone destruction. If this is proved to be the case universally in myeloma, then disruption of this interaction is a potential new therapeutic intervention for the treatment of myeloma-induced bone disease.

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References


Cell–cell contact between marrow stromal cells and myeloma cells via VCAM-1 and \( \alpha_4\beta_1 \)-integrin enhances production of osteoclast-stimulating activity

Toshimi Michigami, Nobuaki Shimizu, Paul J. Williams, Maria Niewolna, Sarah L. Dallas, Gregory R. Mundy and Toshiyuki Yoneda