Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression

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Communicated by Robert A. Good, University of South Florida, St. Petersburg, FL, July 27, 2001 (received for review January 9, 2001)

Bone destruction, caused by aberrant production and activation of osteoclasts, is a prominent feature of multiple myeloma. We demonstrate that myeloma stimulates osteoclastogenesis by triggering a coordinated increase in the tumor necrosis factor-related activation-induced cytokine (TRANCE) and decrease in its decoy receptor, osteoprotegerin (OPG). Immunohistochemistry and *in situ* hybridization studies of bone marrow specimens indicate that *in vivo*, deregulation of the TRANCE–OPG cytokine axis occurs in myeloma, but not in the limited plasma cell disorder monoclonal gammopathy of unknown significance or in nonmyeloma hematologic malignancies. In coculture, myeloma cell lines stimulate expression of TRANCE and inhibit expression of OPG by stromal cells. Osteoclastogenesis, the functional consequence of increased TRANCE expression, is counteracted by addition of a recombinant TRANCE inhibitor, RANK-Fc, to marrow/myeloma cocultures. Myeloma–stroma interaction also has been postulated to support progression of the malignant clone. In the SCID-hu murine model of human myeloma, administration of RANK-Fc both prevents myeloma-induced bone destruction and interferes with myeloma progression. Our data identify TRANCE and OPG as key cytokines whose deregulation promotes bone destruction and supports myeloma growth.

Multiple myeloma (MM) is a B cell neoplasm characterized by clonal expansion of plasma cells. MM typically involves bone and marrow, suggesting dependence on these microenvironments for survival and proliferation (1–3). In addition, MM stimulates production of activated osteoclasts (OCs) from monocytic precursors, leading to severe osteoporosis and bone destruction in most patients (4–6). A variety of MM-produced cytokines, including IL-1β, IL-6, IL-11, tumor necrosis factor (TNF) α, and lymphotoxin-α, can stimulate osteoclastogenesis *in vitro*. However, production of these cytokines in patients with MM is heterogeneous, thus none has been shown to be the common mediator of MM-induced osteoclastogenesis (6).

Evidence from gene-deleted and transgenic mice indicates that generation of activated OCs from monocytic precursors is controlled by coordinate expression of the TNF-related activation-induced cytokine (TRANCE; OPGL, RANKL, ODF, TNFSF11) and its decoy receptor osteoprotegerin (OPG; OCIF, TNFRSF11b) (7–11). TRANCE is expressed on the surface of activated T cells, marrow stromal cells, and osteoblasts as a 45-kDa transmembrane protein and, in solution, as a 31-kDa product of metalloproteinase cleavage (13–17). TRANCE triggers development and activation of OCs by binding to its functional receptor, RANK (TNFRSF11a), expressed on OCs and their precursors as an integral membrane protein (17). OPG is secreted by stromal cells as a soluble 110-kDa disulfide-linked homodimer (11, 12). Mice that lack either TRANCE or RANK, or that overexpress OPG, develop osteopetrosis because of decreased OC activity (7–11). Conversely, mice that lack OPG exhibit profound osteoporosis as a consequence of unopposed TRANCE activity (9). In addition, mice deficient in either TRANCE or receptor activator of NF-κB (RANK) exhibit defective lymph node organogenesis and early B cell development (7, 10). However, the role of TRANCE and RANK in plasma cell differentiation and survival has not been evaluated.

We present evidence that MM disrupts the balance between TRANCE and its inhibitor, OPG. In addition, we show that TRANCE inhibition prevents MM-induced bone destruction and interferes with MM progression in two murine models of human MM. These findings identify TRANCE and OPG as key factors whose deregulation promotes bone destruction and supports MM growth.

Materials and Methods

Human Samples, Experimental Animals, and Reagents. This study was performed in accordance with federal and institutional guidelines for human subject and animal research. Generation of SCID/ARH-77 and SCID-hu-MM mice has been described (18–21). To generate SCID-hu-MM mice, 5×10^6 mononuclear cells from marrow of patients with MM-associated bone disease were injected into the xenograft. TRANCE-deficient mice were generated as described (8). Human transforming growth factor (TGF) β1 was obtained from R & D Systems. PGE_2 was obtained from Sigma. Human CSF-1 was the kind gift of R. Stanley (Albert Einstein Medical College, Bronx, NY). RANK-Fc, a fusion of murine RANK (amino acids 22–201) with the Fc region of human IgG_1 that can block both murine and human TRANCE activity, was prepared in *Sf9* cells (PharMingen) (15). TRANCE and OPG were prepared in 293T cells (15).

Histology. Bone marrow from 14 MM patients with bone disease and 13 non-MM patients [five normal, two non-Hodgkin's lymphoma (NHL), one Hodgkin's disease, one chronic lymphocytic leukemia, one chronic myelogenous leukemia, and three monoclonal gammopathy of unknown significance (MGUS) without evidence of progression to MM during 18–48 months follow-up] was evaluated for TRANCE and OPG expression. Concurrent samples from two biopsy sites were available for three MM and three non-MM patients (one NHL, one Hodgkin's disease, one normal). The MM and non-MM groups

Abbreviations: Dpd, deoxypyridinoline crosslinks; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; OAF, osteoclast activating factor; OC, osteoclast; OPG, osteoprotegerin; SCID, severe combined immunodeficiency; TRANCE, TNF-related activation-induced cytokine; TRAP, tartrate-resistant acid phosphatase; RANK, receptor activator of NF-κB; TNF, tumor necrosis factor; TGF, transforming growth factor; NHL, non-Hodgkin's lymphoma; RT, reverse transcription.

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did not differ significantly in distribution of age or sex. TRANCE expression was also evaluated in five plasmacytomas arising from bone: four from patients with concurrent MM and one from a patient with a solitary plasmacytoma.

Immunohistochemistry. Four-micrometer sections of formalin-fixed, decalcified, bone marrow or formalin-fixed plasmacytoma were heated in an 80°C oven for 60 min, deparaffinized, rehydrated, and treated with 1.5% hydrogen peroxide for 10 min. Antigen retrieval was accomplished by pretreatment for 10 min with either microwave (OPG) or 0.5% pepsin (TRANCE). Three anti-TRANCE antibodies, MAB626 (R & D Systems), IMG-133 (Imgenex), and sc-7627 (Santa Cruz Biotechnology), gave similar staining patterns at 1:100, although the goat polyclonal antibody (sc-7627) produced background staining that was not seen with either monoclonal antibody. Two anti-OPG antibodies, IMG-103 (Imgenex, San Diego, 1:300 dilution) and sc-8468 (Santa Cruz Biotechnology, 1:1,000), were used with similar staining. Staining for either TRANCE or OPG could be blocked by incubation with specific peptide. Sections incubated with rabbit or murine primary antibodies were blocked with ChemMate blocking antibodies (Ventana Medical Systems, Tuscon, AZ) and stained by using the ChemMate secondary detection kit-peroxidase/diaminobenzidine. Sections incubated with goat primary antibodies were blocked with normal goat serum (Santa Cruz Biotechnology) and stained by using the goat ABC staining system (Santa Cruz Biotechnology). Consistent results were obtained for slides from each individual stained on different days and also for marrow samples taken from a second site. Normal tonsil served as control for TRANCE staining. Vascular staining, which was consistent among all samples, served as control for OPG staining.

***In Situ* Hybridization.** Bone marrow from nine MM and five non-MM (one MGUS, two NHL, two normal) patients was processed with [α-^{33}P]UTP-labeled sense and antisense riboprobes as described (22). The *Bam*HI/*Eco*RI fragment of human TRANCE cDNA (nucleotides 350–930) was used as probe. Sense riboprobes gave no signal above background.

Tartrate-Resistant Acid Phosphatase (TRAP) Cytochemistry. Xenografts were harvested from SCID-hu mice, fixed in formalin, decalcified with EDTA, and embedded in paraffin. Five micrometer deparaffinized sections were immersed in acetone and stained for TRAP according to the manufacturer's instructions (Sigma). TRAP-positive multinucleated giant cells (OCs) in four nonoverlapping mm^2 areas were counted.

Cell Culture. MG63, U2OS, ARH-77, U266, H929, and RPMI 8226 cells were obtained from American Type Culture Collection. ARP-1 cells were the generous gift of J. Hardin (University of Arkansas for Medical Sciences). Primary murine stromal cells were isolated from the calvaria of newborn 129 SV mice as described (23). Conditioned media, generated over 4 days by coculture of MM cell lines with primary murine stromal cells, was concentrated 4-fold by using Centriprep 10 columns (Millipore) and used at a dilution of 1:4. *In vitro* osteoclastogenesis was performed as described (24). Briefly, murine marrow was cultured with CSF-1 (50 ng/ml), PGE_2 (1 μM), and TRANCE (1 μg/ml). In some experiments, TRANCE was replaced by primary murine stromal cells isolated from wild-type or TRANCE-deficient mice cocultured with one of three human MM cell lines. TRAP activity was analyzed according to the manufacturer's instructions (Sigma).

Reverse Transcription (RT)-PCR. mRNA was prepared by using Trizol (GIBCO) and OLIGOTEX (Qiagen, Chatsworth, CA). cDNA was generated by using Moloney murine leukemia virus RT and oligo(dT) (Amersham Pharmacia). PCR was performed for 40 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) by using the following primer pairs to detect murine TRANCE and actin, respectively: 5′-ATCAGAAGACAGCACTCAC-3′/5′-TTCGTGCTCCCTC-CTTTCAT-3′ and 5′-GTGACGAGGCCCAGAGCAAGAG-3′/5′-AGGGGCCGGACTCATCGTACTC-3′.

PCR was performed for 35 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) by using the following primer pairs to detect human OPG and actin, respectively: 5′-GTGGTGCAAGCTG-GAACCCCAG-3′/5′-AGGCCCTTCAAGGTGTCTTG-GTC-3′ and 5′-CCTTCCTGGGCATGGAGTCCT-3′/5′-GGAGCAATGATCTTGATCTTC-3′.

Northern Analysis. RNA was prepared by using Trizol, separated by agarose gel electrophoresis in formaldehyde (20 μg total RNA/lane) and blotted to Hybond N+ (Amersham Pharmacia). Hybridization was performed by using an [α-^{32}P]UTP-labeled antisense riboprobe generated by using T7 polymerase (Ambion) and a PCR fragment of human OPG (nucleotides 478–1,124) linked to the T7 promoter.

ELISA. Titers of MM paraprotein were determined as described (21) by using Immulon 2HB microtiter plates (Dynex Technologies, Chantilly, VA) and antibodies purchased from Southern Biotechnology Associates. Urinary crosslinked deoxypyridinoline (Dpd) was assayed according to the manufacturer's instructions (Metra Biosystems, Mountain View, CA). To compensate for diurnal variation in Dpd excretion, urine was collected at the same time on consecutive days and assayed separately for Dpd and creatinine (Sigma); the measured Dpd (nmol)/creatinine (mmol) was then averaged (25).

Results

Deregulation of TRANCE and OPG in Marrow of Patients with MM. Bone marrow biopsies from MM and non-MM patients were evaluated for TRANCE and OPG expression by using riboprobes specific for TRANCE and antibodies specific for TRANCE and OPG (Fig. 1*A*). Both immunohistochemistry and *in situ* hybridization reveal foci of increased TRANCE expression in MM marrow samples but little TRANCE expression in most non-MM samples. Within MM-infiltrated marrows, TRANCE expression is increased in areas that also possess normal marrow elements. In areas of marrow completely replaced by MM, almost all cells express light chain, but TRANCE-positive cells are extremely rare. Similarly, TRANCE was not expressed by Ig-positive cells in any of the five plasmacytomas of bone evaluated, although TRANCE was expressed by rare cells within the plasmacytoma and by lining cells found at the periphery of the tumor in several specimens. Comparison with sections of bone marrow stained for other markers suggests that CD3+, CD30+ activated T cells are the major TRANCE-positive cells in non-MM bone marrow and are a subset of the TRANCE-positive cells in MM bone marrow. In MM, other stromal elements, but not CD34+ endothelial cells, also express TRANCE. Consistent with these observations, we did not find TRANCE expression by the MM cell lines ARP-1, U266, RPMI 8226, H929, and ARH-77, as assessed by RANK-Fc binding or by RT-PCR (data not shown). Together, these results indicate that TRANCE expression is increased in MM-infiltrated marrow by the interaction of malignant plasma cells with activated T cells and stromal cells.

In addition to increased expression of TRANCE, MM-infiltrated marrows exhibit decreased expression of the TRANCE inhibitor, OPG. In normal marrow, OPG-specific antibodies show intense staining of megakaryocytes, stromal cells, and vessels. In marrow infiltrated by MM, vascular staining is evident, but staining of megakaryocytes and stromal cells is markedly decreased. The pattern of increased TRANCE expres-

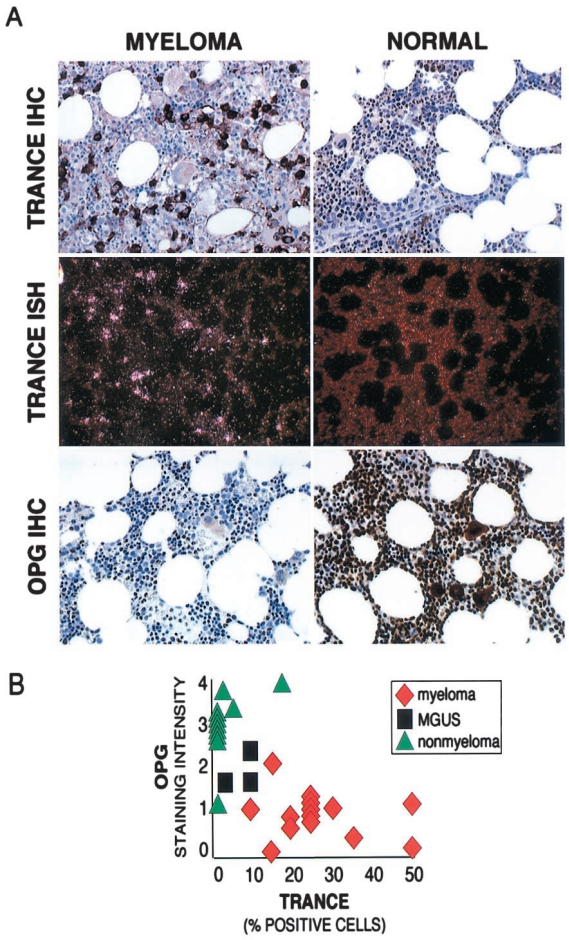

Fig. 1. Marrow infiltration by MM is associated with increased TRANCE and decreased OPG expression. (*A*) Representative images of MM and normal bone marrow following immunohistochemistry (IHC, light field ×600) and *in situ* hybridization (ISH, dark field ×200). Intense staining by a TRANCE-specific antibody and increased hybridization by a TRANCE-specific riboprobe are seen in MM, but not normal marrow. In contrast, staining by OPG-specific antibody is dramatically decreased in MM compared with normal marrow. (*B*) Graphic display of TRANCE and OPG protein expression. Bone marrow biopsies from 14 MM and 13 non-MM patients (three MGUS, two NHL, one chronic lymphocytic leukemia, one chronic myelogenous leukemia, one Hodgkin's disease, and five normal) were evaluated for expression of TRANCE and OPG by IHC and independently graded by two investigators without knowledge of the diagnosis. TRANCE staining was scored on a scale from 0 to 100 based on the percentage of positive cells in foci of increased staining. The mean scores for TRANCE expression in MM and non-MM samples were 26 and 4.5, respectively ($P < 0.001$, Mann–Whitney test). OPG expression was graded on a 0 (none) to 4 (heavy) scale based on the number and intensity of cells stained. The mean scores for OPG expression in MM and non-MM samples were 1.0 and 2.6, respectively ($P < 0.001$, Mann–Whitney test).

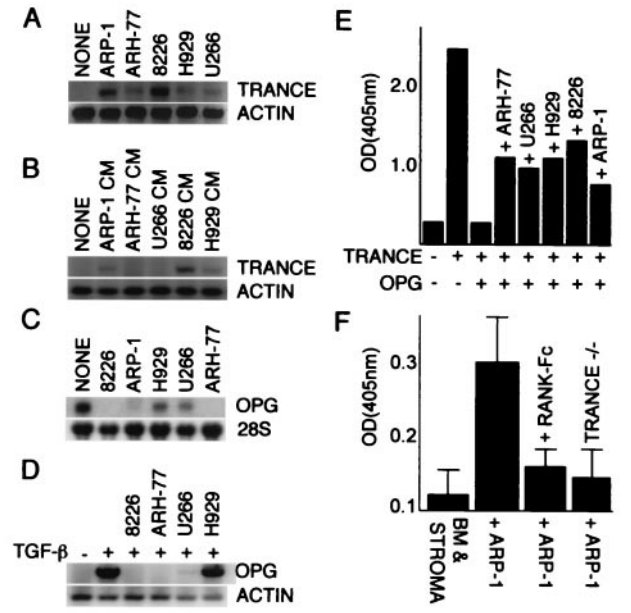

Fig. 2. MM triggers OC development through deregulation of the TRANCE–OPG cytokine axis. (*A*) MM stimulates stromal expression of TRANCE. Primary murine stromal cells were cultured for 4 days without (lane 1) or with (lanes 2–6) each of five MM cell lines (10^5 cells/ml). Stromal cell mRNA was isolated and TRANCE expression was determined by RT-PCR by using primers specific for murine TRANCE and β-actin. (*B*) Media conditioned by MM–stroma coculture stimulates stromal expression of TRANCE. Primary murine stromal cells were cultured for 4 days without (lane 1) or with (lanes 2–6) media condition by the coculture of stroma with each of five MM cell lines. Stromal TRANCE mRNA expression was determined as above. (*C*) MM inhibits stromal expression of OPG. MG63 cells were cultured without (lane 1) or with (lanes 2–6) five MM cell lines (10^5/ml). After 4 days, stromal RNA was isolated and subjected to Northern analysis by using ribosomal and OPG-specific riboprobes. (*D*) MM inhibits TGF-β-induced expression of OPG. U2OS cells were stimulated with TGF-β1 (200 nM) in the absence (lane 2) or presence (lanes 3–6) of four MM cell lines (10^5/ml). After 4 days, stromal mRNA was isolated and OPG expression determined by RT-PCR by using primers specific for OPG and β-actin. (*E*) MM subverts OPG function *in vitro*. TRANCE (1 μg/ml) triggers the development of OCs from precursors present in CSF-1-treated murine marrow (column 2). OPG (1 μg/ml) inhibits TRANCE-induced osteoclastogenesis (column 3). Human MM cell lines ARH-77, U266, H929, RPMI 8226, and ARP-1 partially overcome the suppressive effect of OPG (columns 4–8). Osteoclastogenesis is assessed semiquantitatively by using a colorimetric assay for TRAP with results expressed as $OD_{405\ nm}$. (*F*) MM-induced osteoclastogenesis is TRANCE dependent. Coculture of primary stroma with MM (ARP-1) triggers the generation of OCs from CSF-1-treated murine marrow (BM) (column 2). OCs fail to develop in the absence of MM (column 1), in the presence of 1 μg/ml RANK-Fc (column 3), or if TRANCE-deficient mice (TRANCE −/−, column 4), rather than wild-type littermates, are used as the source of stromal cells. OCs do not develop in the absence of marrow or stroma (not shown).

sion in association with decreased OPG expression is not found in marrow specimens from normal individuals or patients with non-MM B cell malignancies (Fig. 1*B*). Specimens from three patients with MGUS demonstrated intermediate changes in TRANCE and OPG expression.

Deregulation of TRANCE and OPG by MM *in Vitro*. MM induces stromal TRANCE expression *in vitro*. Primary murine stromal cells express TRANCE when cocultured with human MM cell lines (Fig. 2*A*) or when cultured in the presence of media conditioned by stroma–MM coculture (Fig. 2*B*), but not when cultured with media conditioned by MM alone (data not shown). These results suggest that both direct MM–stroma cell contact and soluble factors contribute to TRANCE induction by MM. The suppression of OPG observed in marrow from MM patients also seems to be a direct effect of MM. In coculture, MM cell lines inhibit constitutive OPG mRNA expression by the human osteosarcoma cell line MG63 (Fig. 2*C*). In addition, the induction by TGF-β1 of OPG mRNA expression in the human osteosarcoma cell line U2OS (Fig. 2*D*) is prevented by coculture with MM cell lines. MM can also subvert the function of OPG as a TRANCE antagonist (Fig. 2*E*). Addition of TRANCE to CSF-1-treated murine marrow triggers the development of OCs (24). Although OPG inhibits TRANCE-induced osteoclastogenesis, this inhibition is partially overcome by coculture with human MM cell lines.

MM-Triggered Osteoclastogenesis Depends on TRANCE. The functional consequence of MM-induced TRANCE expression is

demonstrated by the ability of MM–stroma cell cocultures to trigger the *in vitro* generation of OCs from murine marrow. Coculture of murine marrow with primary murine stromal cells and the human MM cell lines H929, RPMI 8226, or ARP-1 results in the development of OCs, evident as multinucleated giant cells expressing TRAP. OCs do not develop if RANK-Fc, a synthetic TRANCE antagonist, is added to the culture, or if TRANCE-deficient mice are used as the source of stromal cells. Results of ARP-1-triggered osteoclastogenesis are presented (Fig. 2*F*).

MM-Induced Bone Destruction Requires TRANCE. In mice with severe combined immunodeficiency (SCID) injected intravenously with the human MM cell line ARH-77, osteolytic lesions develop in 100%, and hind limb paralysis develops in 80% (18, 19). We injected SCID/ARH-77 mice with either the synthetic TRANCE antagonist, RANK-Fc, or $hIgG_1(\lambda)$ (200 μg three times weekly) starting the day after injection of ARH-77. At 6 weeks, RANK-Fc-treated mice exhibited significantly less bone turnover, as measured by urinary Dpd excretion ($P < 0.01$; Fig. 3*A*). Animals treated with $hIgG_1$ also developed obvious radiographic evidence of skeletal destruction, whereas RANK-Fc-treated animals did not (Fig. 3*B*). Over a 7-week period, four of the five mice that received $hIgG_1$ became unable to move their hind limbs, whereas none of the four mice treated with RANK-Fc developed paralysis ($P < 0.01$; Fig. 3*C*). At autopsy, many of the vertebral bodies of the $hIgG_1$-treated mice were infiltrated by MM, with evidence of bony destruction and enlargement because of tumor growth. In contrast, vertebral bodies from RANK-Fc-treated animals were grossly intact. Despite this difference in bone destruction, both groups had comparable titers of $hIgG_1(\kappa)$, the antibody produced by the ARH-77 cell line (Fig. 4*A*). ARH-77 is an Epstein–Barr virus-transformed cell line derived from a patient with plasma cell leukemia (26). Unlike primary MM, growth of ARH-77 in SCID mice is not limited to bone; extraosseous growth of ARH-77 cells was similar in both RANK-Fc and $hIgG_1$-treated animals.

In a second model of MM-associated bone disease, we used SCID-hu mice injected with marrow from three MM patients. In this SCID-hu–MM model, the human bone xenograft becomes infiltrated by MM and eroded by OCs, demonstrating pathology similar to that seen in MM patients (20, 21). Three pairs of SCID-hu mice were generated, with each pair receiving aliquots of marrow from a single individual. Injection of either RANK-Fc or $hIgG_1$ (200 μg three times weekly) was started when titers of MM paraprotein were detected in both mice of a pair. In each case, the mouse with the higher paraprotein titer received RANK-Fc. After 8 weeks, resorption of the xenograft was evident radiographically in all three mice treated with $hIgG_1$ but in none of the mice treated with RANK-Fc (Fig. 3*D*). Consistent with this result, xenografts from mice treated with RANK-Fc contained significantly fewer OCs than did xenografts taken from mice that received IgG_1 ($P < 0.001$; Fig. 3*E*).

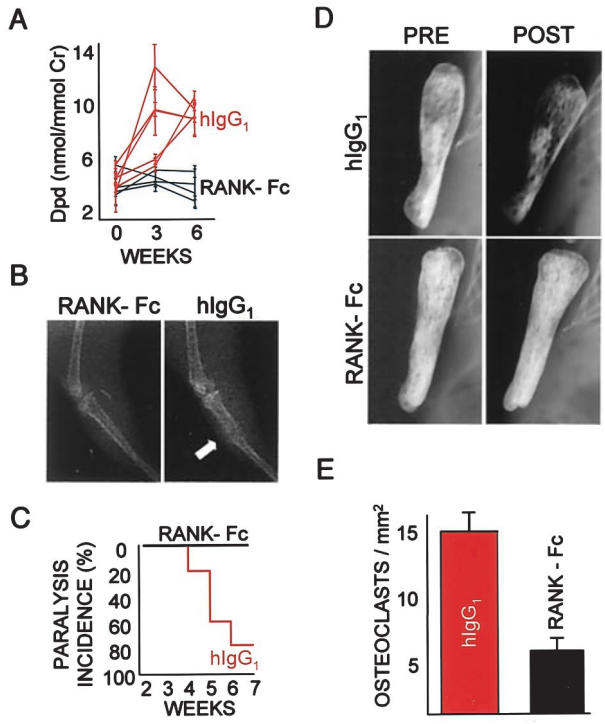

Fig. 3. TRANCE inhibition by RANK-Fc blocks MM-induced bone destruction in two murine models. Irradiated (200 cGy) SCID mice were injected intravenously with 10^6 cells of the human MM line ARH-77. Intravenous administration of either RANK-Fc or $hIgG_1(\lambda)$, both at 200 μg three times weekly, began the following day. At 3 and 6 weeks, both groups had comparable titers of $hIgG_1(\kappa)$, the antibody produced by the ARH-77 cell line. (*A*) Urinary excretion of crosslinked Dpd in SCID/ARH-77 mice. Bone turnover was assessed at 0, 3, and 6 weeks by measuring urinary excretion of Dpd. At 6 weeks, mice treated with RANK-Fc exhibited significantly less bone turnover ($P < 0.01$, Student's *t* test). (*B*) Osteolysis in SCID/ARH-77 mice. Osteolysis was evident in $hIgG_1$ but not RANK-Fc-treated animals. Representative radiographs taken following 6 weeks of therapy are shown. (*C*) Incidence of hind limb paralysis in SCID/ARH-77 mice. Over 7 weeks, four of the five mice that received $hIgG_1$ developed hind limb paralysis as a result of vertebral bone destruction, whereas none of the mice treated with RANK-Fc developed paralysis ($P < 0.01$, Student's *t* test). (*D*) Xenograft osteolysis in SCID-hu–MM mice. SCID-hu mice inoculated with primary MM were treated with either RANK-Fc or $hIgG_1$ (200 μg three times weekly). Injections began when titers of MM paraprotein were detected in both mice of a pair, the mouse with the higher titer receiving RANK-Fc. Osteolysis of the xenograft was evident in $hIgG_1$ but not RANK-Fc-treated animals. Radiographs taken before (PRE) and following (POST) 8 weeks of therapy are shown for one pair of mice. (*E*) Osteoclastogenesis in the xenografts of SCID-hu–MM mice. Xenografts from SCID-hu–MM mice were removed after treatment with either $hIgG_1$ or RANK-Fc and stained for TRAP. Xenografts taken from RANK-Fc recipients had significantly fewer TRAP+ multinucleated giant cells (OCs) per mm^2 than did xenografts taken from $hIgG_1$ recipients ($P < 0.001$, Student's *t* test).

MM Tumor Progression Depends on TRANCE. RANK-Fc treatment caused a marked reduction in serum paraprotein in the SCID-hu–MM mice (Fig. 4*A*), accompanied by a reduction in tumor burden assessed histologically. These findings were not observed in RANK-Fc-treated SCID/ARH-77 mice, consistent with the observed dependence of primary MM, but not plasma cell leukemia, on bone and marrow microenvironments for growth and survival. RANK-Fc does not seem to decrease MM tumor burden through a direct effect of TRANCE or RANK-Fc on plasma cell survival. *In vitro*, addition of TRANCE or RANK-Fc to cultures of primary MM or MM cell lines has no effect on cell growth or survival after exposure to cytotoxic agents (data not shown). The reduced tumor burden in RANK-Fc-treated SCID-hu–MM mice was accompanied by restoration of OPG and reduction of TRANCE expression within the xenograft, further supporting the causal association of MM with altered expression of TRANCE and OPG (Fig. 4*B*).

Discussion

The interaction of MM with marrow stroma is thought to influence both osteoclastogenesis and survival of the malignant clone (1–5). We present evidence that the interaction of MM with stroma results in deregulation of the TRANCE–OPG cytokine axis and that this deregulation is necessary for both MM-associated bone destruction and tumor progression.

Severe bone destruction is a prominent feature of MM but is infrequently associated with other hematologic malignancies. Consistent with this observation, we find marked alteration in

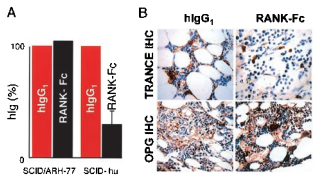

Fig. 4. Effect of RANK-Fc on primary MM in SCID-hu mice. (*A*) Reduction in MM paraprotein. SCID-hu–MM mice demonstrate significantly less MM paraprotein when treated with RANK-Fc ($P < 0.01$, Student's *t* test), whereas paraprotein levels in SCID/ARH-77 mice are not affected by RANK-Fc treatment. For comparison among animals inoculated with MM from different sources, paraprotein levels for RANK-Fc-treated animals are presented as the percentage (mean ± SE) of the paraprotein level measured in the $hIgG_1$-treated animals. (*B*) OPG and TRANCE expression in xenografts from SCID-hu–MM mice (IHC, ×600). Immunohistochemistry performed on xenografts harvested from SCID-hu–MM mice after 8 weeks of treatment demonstrates normalization of TRANCE and OPG expression in the xenografts taken from RANK-Fc-treated animals.

expression of both TRANCE and OPG only in specimens from MM patients. In marrow infiltrated by MM, TRANCE is expressed by stromal and activated T cells at the interface of MM with normal marrow elements. The absence of TRANCE staining in light chain-expressing cells in specimens of plasmacytoma and MM-replaced bone marrow suggests that malignant plasma cells do not themselves produce TRANCE, a conclusion supported by our inability to document TRANCE mRNA or protein in MM cell lines. Our coculture experiments also indicate a stromal, rather than plasma cell, origin for TRANCE; MM can induce development of OCs from marrow precursors in the presence of wild-type, but not TRANCE-deficient, stroma.

MM must interact directly with stroma to initiate deregulation of TRANCE expression. *In situ*, TRANCE is expressed at the interface of MM with normal marrow elements. *In vitro*, TRANCE expression by stroma is induced by coculture with MM but not by addition of MM-conditioned media. This requirement for direct cellular contact is consistent with the study of Michigami *et al.*, who report enhanced OC-stimulating activity by the ST2 stromal cell line after direct contact with 5TGM1 murine myeloma cells (27). *In vitro*, TRANCE expression can also be induced by media conditioned by MM–stroma coculture. Thus, MM–stroma contact may trigger the production of TRANCE-inducing cytokines. Several MM-associated OC-activating factors (OAFs) including IL-1β, produced by malignant plasma cells following interaction with marrow stroma, and IL-6 and IL-11, produced by stroma in response to MM, have been shown *in vitro* to induce stromal expression of TRANCE (28). Other OAFs, such as IL-17 and hepatocyte growth factor, induce stromal expression of IL-11 and may act indirectly to increase TRANCE (29).

The Significance of TRANCE Deregulation by MM Can Be Demonstrated Both *in Vitro* and *in Vivo*. In coculture, OC development in response to MM requires TRANCE expression by stromal cells and is blocked by addition of the TRANCE antagonist RANK-Fc. *In vivo*, administration of RANK-Fc abrogates the development of bony disease in both SCID mice inoculated with the IL-1β-producing ARH-77 cell line and in SCID-hu mice inoculated with primary MM. Taken together, these results indicate that TRANCE is essential for MM-triggered osteoclastogenesis and that antagonism of TRANCE activity can block MM-associated bone destruction.

The ability of RANK-Fc to block osteoclastogenesis both *in vitro* and *in vivo* emphasizes the importance of the concurrent decrease in OPG expression we observe in MM marrow specimens. OPG, the natural TRANCE decoy receptor, is central to the maintenance of bone homeostasis (9, 11) and can inhibit osteoclastogenesis and bone loss after administration of osteotrophic agents (30–32). The high level of OPG expression we observe in normal marrow would be expected to counteract moderate increases in TRANCE. Therefore, the ability of MM to concurrently down-regulate expression of this natural buffer may be critical to the development of bone disease. *In vitro*, we find that constitutive stromal expression of OPG is reduced by coculture with MM, which may reflect the ability of OAFs, including IL-1β, IL-6, and IL-11, to inhibit transcription of OPG (28). We also find that MM interferes with up-regulation of OPG by TGF-β, which may reflect the ability of OAFs, including hepatocyte growth factor, IL-1β, and TNF-α, to inhibit TGF-β signaling through activation of either mitogen-activated protein kinase or the inhibitory Smad7 (33). TGF-β, which is released from bone matrix during OC resorption, is thought to act as a feedback regulator to control osteoclastogenesis by stimulating stromal expression of OPG and inhibiting stromal expression of TRANCE (34–36). However, TGF-β can also stimulate the growth and maturation of OCs already primed by TRANCE (37). Our finding that MM directly inhibits TGF-β-induced expression of OPG is of particular importance in light of reports that MM cells can produce TGF-β (38) and suggests that MM might simultaneously use TGF-β to enhance osteoclastogenesis, yet block modulation by TGF-β of stromal TRANCE and OPG expression. Our coculture experiments also suggest subversion of OPG function by MM because addition of MM cell lines partially overcomes TRANCE inhibition by OPG. The mechanisms responsible for this activity are unknown, but they may involve the ability of syndecan-1, expressed at high level on the surface of malignant and nonmalignant plasma cells, to bind the heparin-binding domain of OPG (39). It is of particular interest that although all of the MM cell lines we tested increase TRANCE expression, not all interfere with OPG expression by stroma, suggesting that some factors controlling OPG expression are independent of controls on TRANCE.

Our data suggest that the ability to disrupt the TRANCE–OPG cytokine axis, an ability shared by many MM-associated OAFs, is critical to the development of MM-associated bone destruction. Other OAFs, notably VEGF, which activates the CSF-1 receptor c-fms (40), a necessary step in OC development, MIP-1α, which recruits OC precursors (41), and TNF-α, which augments the intracellular signals initiated by TRANCE–RANK interaction (42), seem to act in synergy with TRANCE to stimulate osteoclastogenesis.

Disruption of the TRANCE–OPG axis by MM may also be necessary for tumor progression. Administration of RANK-Fc results in decreased tumor burden in the SCID-hu model of human MM, suggesting a role for TRANCE in MM cell growth. However, unlike other stromally expressed growth factors induced by hematologic malignancies, such as granulocyte colony-stimulating factor, induced by myeloid leukemias, or IL-6, induced by B cell malignancies, TRANCE does not act as a direct survival factor for plasma cells. Rather, TRANCE seems to act indirectly to support MM as part of a paracrine loop involving stroma, OCs, and bone. Inhibition of TRANCE-induced osteoclastogenesis may decrease OC production of cytokines such as IL-6 that are known MM survival factors, it may limit the release during bone resorption of MM growth factors such as IGF-1 (43), or it may simply limit the niche for MM growth. Similar mechanisms have been described for bisphosphonates (44). A reciprocal relationship between osteoclastogenesis and early B lymphopoiesis has been suggested (45–47); our data indicate that osteoclastogenesis and survival of MM cells are also interdependent.

In conclusion, we find that MM deregulates stromal TRANCE and OPG expression to trigger bone destruction and promote tumor growth. The multiple mechanisms used by MM to control

expression of TRANCE and OPG underscore the critical role of this axis in MM biology.

This manuscript is dedicated to the memory of Dr. Joseph Michaeli. We thank J. V. Ravetch for his generous support, D. Louie for assistance in procuring specimens, and K. Witty-Blease, K. Manova, A. Santana, M. Vologodskaia, and L. Lai for technical assistance. This work was supported by grants from the International Myeloma Foundation (to R.N.P.), the Leukemia–Lymphoma Society of America (to R.N.P.), the Charles A. Mastronardi Foundation (to E.M.S.), the Multiple Myeloma Research Foundation (to S.Y.), the Howard Hughes Medical Institute (to Y.C.), and the National Institutes of Health (to J.E., Y.C., and B.R.W.).

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