Multiple Myeloma • Research Paper

The RANK/RANK ligand system is involved in interleukin-6 and interleukin-11 up-regulation by human myeloma cells in the bone marrow microenvironment

[haematologica]
2004;89:1118-1123

NICOLA GIULIANI
SIMONA COLLA
FRANCESCA MORANDI
VITTORIO RIZZOLI

A B S T R A C T

Background and Objectives. The receptor activator of NF-κB ligand (RANKL) has a critical role in osteoclast activation. Recently it has been demonstrated that human multiple myeloma (MM) cells do not express RANKL but up-regulate RANKL in bone marrow stromal cells (BMSC). To further investigate the role of RANKL in the pathophysiology of MM we evaluated the expression of its receptor RANK in MM cells and in the BM environment and the potential role of RANKL in the interaction of myeloma cells with the microenvironment.

Design and Methods. RANK mRNA and protein expression were evaluated by reverse transcription polymerase chain reaction and Western blot analysis in human myeloma cell lines (HMCL), fresh purified MM cells, BMSC and endothelial cells. Moreover the effect and the role of RANKL on cytokine secretion were evaluated in BMSC, in endothelial cells and in co-culture conditions with myeloma cells.

Results. We found that RANK is expressed in BMSC and endothelial cells but not in myeloma cells. Consistently, RANKL did not have a direct effect on myeloma cell survival, but RANKL treatment induced a significant increase of interleukin (IL)-6 and IL-11 secretion by both BMSC and endothelial cells. Moreover, in a co-culture system we found that myeloma cells up-regulated both IL-6 and IL-11 secretion by BMSC and endothelial cells through cell-to-cell contact. The presence of the RANK-Fc that blocks the RANK/RANKL interaction significantly inhibited HMCL-induced secretion of IL-6 and IL-11.

Interpretation and Conclusions. Our data provide new notions on the role of the RANKL system in the pathophysiology of MM.

Key words: multiple myeloma, RANKL, interleukin-6, interleukin-11, microenvironment.

From the Chair of Hematology and BMT Unit, University of Parma, Italy.

Correspondence:
Dr. Nicola Giuliani, MD,
Chair of Hematology and BMT Unit, University of Parma, via Gramsci 14, 43100 Parma, Italy.
E-mail: n_giuliani@yahoo.com
nicola.giuliani@unipr.it

Multiple myeloma (MM) is a plasma cell malignancy characterized by the accumulation of long surviving plasma cells in the bone marrow (BM) and the capacity to induce osteolytic bone lesions.[1-2] The interaction of MM cells with the microenvironment has been postulated to be critical in the pathophysiology of MM[2] through the modulation of cytokines that support myeloma cell proliferation, survival and induce osteoclastic bone resorption.

The receptor activator of NF-κB ligand (RANKL) is a critical osteoclastogenic factor produced by BM stromal cells (BMSC) and osteoblasts which, by interacting with a specific receptor on osteoclastic cells, namely RANK, induces osteoclast formation and survival.[3] Whether human myeloma cells produce RANKL directly is controversial.[4-6] We and other authors demonstrated that neither human myeloma cell lines nor CD138$^+$ MM cells express RANKL mRNA[4-5] and protein whereas other groups showed, by flow cytometry, that plasma cells are positive for RANKL.[6]

However it has been clearly demonstrated that human MM cells up-regulate RANKL and down-regulate its antagonist, osteoprotegerin (OPG), in human BMSC and human osteoblasts leading to osteoclast formation and activation.[5]

Recent data indicate that myeloma cells also induce RANKL production by endothelial cells supporting osteoclast formation.[7]

Higher levels of RANKL have been detected in the serum from MM patients than in serum from normal subjects and the RANKL/OPG ratio in MM patients was found to correlate with IL-6 levels and the patients' survival.[8] Moreover studies performed in MM mouse models have shown that the administration of OPG as well as the RANKL

inhibitor RANK-Fc not only inhibited bone resorption but also led to a reduction in tumor burden.[4,9]

All these pieces of evidence suggest that RANKL could be critical in the pathophysiology of MM, not only for the development of bone lesions. To investigate the role of RANKL in MM further, we evaluated the expression of this ligand's receptor, RANK, in MM cells and in the BM environment and the potential relationship between RANKL and IL-6 and IL-11, which are cytokines up-regulated by MM in the BM environment.[10,11]

Design and Methods

Cells and cell culture conditions

IL-6-dependent human myeloma cell lines (HMCL) XG-6 and XG-1 were established in Dr. Bataille's laboratory. U266 and MG-63 were obtained from the American Type Culture Collection (Rockville, MD, USA); RPMI-8226 and OPM-2 were purchased from DSM (Brunswick, Germany). Human BM endothelial cell line (BMEC-1) and the microvascular cell line HMEC were kind gifts from Dr. Kenneth Pienta and Dr. Paola Romagnani, respectively and were cultured as described elsewhere.[12,13]

Primary human osteoblasts, transfected with SV40, (hOB) were obtained from Dr. Riggs (Mayo Clinic, Rochester, MO, USA). Fresh MM cells from 15 newly diagnosed MM patients (stage I-III) were purified using an immunomagnetic method with $CD138^+$ coated antibody (MACS Miltenyi). BMSC were isolated and cultured as previously described[5] from either normal subjects or from BM mononuclear cells of MM patients after depletion of $CD138^+$ cells by the immunomagnetic method.

Confluent BMSC or endothelial cells (2×10^6) were incubated in the presence or absence of RANKL (100 ng/mL) (R&D System, Minneapolis, MN, USA) or RANK-Fc (20-100 ng/mL) (R&D) for 48 hours; RANK-Fc is a decoy receptor for RANKL that prevents this ligand's interaction with RANK. In co-culture conditions, HMCL (10×10^6 cells) were added to confluent BMSC (2×10^6) directly or placed into a transwell insert (0.45 μM pore size) for 48h, in 5 mL of RPMI-1640 medium with 2% fetal calf serum and incubated in the presence or absence of RANK-Fc (20-100 ng/mL) or anti vascular endothelium growth factor (VEGF) monoclonal antibody (2 μg/mL) (R&D) or anti IgG control. In one series of experiments HMCL were incubated in the presence or absence of RANK-Fc (100 ng/mL) and cell survival was evaluated by flow cytometry using annexin V (Becton Dickinson, USA).

RNA isolation and reverse-transcriptase polymerase chain reaction amplification

RANK and RANKL mRNA expression were evaluated by RT-PCR. Total RNA (1 μg) was reverse-transcribed and cDNA was amplified by PCR with specific primers for RANK: sense: 5'GGGAAAGCACTCACAGCTAATTTG-3', antisense: 5'-GCACTGGCTTAAACTGCTATTCTCC-3'; RANKL: sense: 5'-TGGATCACAGCACATCAGAGCAGAG-3', antisense: 5'-ATACTCTGTAGCTAGGTCTCCTGAAG-3'. PCR reactions were performed in a thermal cycler (MiniCycler™ MyResearch, Watertown, USA) for 30 cycles (annealing temperature: 60°C).

Western blot analysis

RANK protein expression was evaluated by Wester blot analysis on cell lysates according to a previously described procedure.[5] Briefly, cells were resuspended in 100 μL of lysis buffer [10 mM Tris-HCL, pH 7.6, 150 mM NaCl, 5 mM EDTA, 2 mM PMSF, 2 mg/mL aprotinin (Sigma, St Louis, MO, USA) and 1% Triton X-100]. Protein levels were determined using a standard procedure (Uptima, Interchim, France). After 40 min on ice, lysates were cleared by centrifugation at 12,000 g for 30 min at 4°C. Proteins (70 μg) were separated by SDS-PAGE using 10% polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane. After blocking, membranes were incubated overnight with a monoclonal anti-RANK antibody (R&D Systems, Minneapolis, MN, USA). After washing, membranes were incubated with an horseradish peroxidase-conjugated goat anti-mouse antibody (1:10.000) (Becton Dickinson) at room temperature for 30 min. Blots were developed using the Supersignal West Dura Extended Duration Substrate detection system (Pierce, Rockford, IL, USA).

ELISA assays

RANKL, IL-6, IL-11, IL-1 and tumor necrosis factor α (TNF-α) levels were measured in the conditioned media of cell cultures using ELISA assays (IL-6 and IL-11: R&D; IL-1 and TNF-α: Endogen Woburn MA, USA). The ELISA assay for RANKL, purchased from Biomedica (Vienna, Austria) is an enzyme immunoassay designed to determine the soluble un-complexed human RANKL.

Soluble RANKL binds to the pre-coated recombinant OPG and forms a sandwich with the detection antibody. The detection antibody is a rabbit anti-human soluble RANKL that is specific for RANKL with negligible cross-reactivity (<1%) to human TRAIL, as certified by the producer.

Results

RANK expression in myeloma cells and in the microenvironment

The human myeloma cell lines RPMI-8226, U266, XG-1 and XG-6 but not OPM-2 were negative for RANK mRNA expression. Similarly, fresh purified $CD138^+$ cells of all MM patients tested did not express RANK mRNA as shown for two representative patients in Figure 1A.

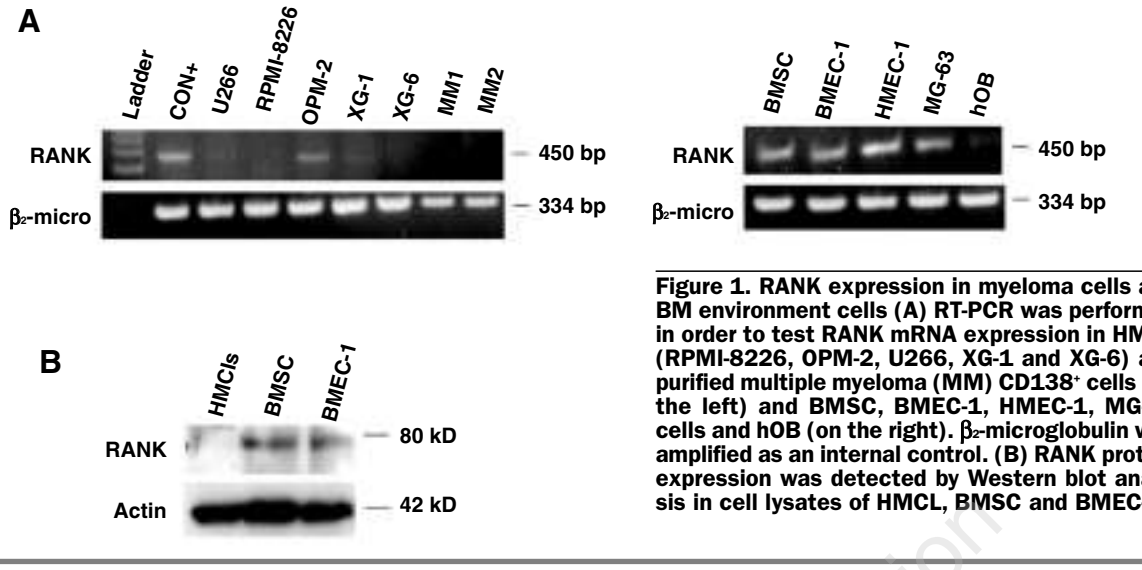

Figure 1. RANK expression in myeloma cells and BM environment cells (A) RT-PCR was performed in order to test RANK mRNA expression in HMCL (RPMI-8226, OPM-2, U266, XG-1 and XG-6) and purified multiple myeloma (MM) CD138⁺ cells (on the left) and BMSC, BMEC-1, HMEC-1, MG-63 cells and hOB (on the right). β_2-microglobulin was amplified as an internal control. (B) RANK protein expression was detected by Western blot analysis in cell lysates of HMCL, BMSC and BMEC-1.

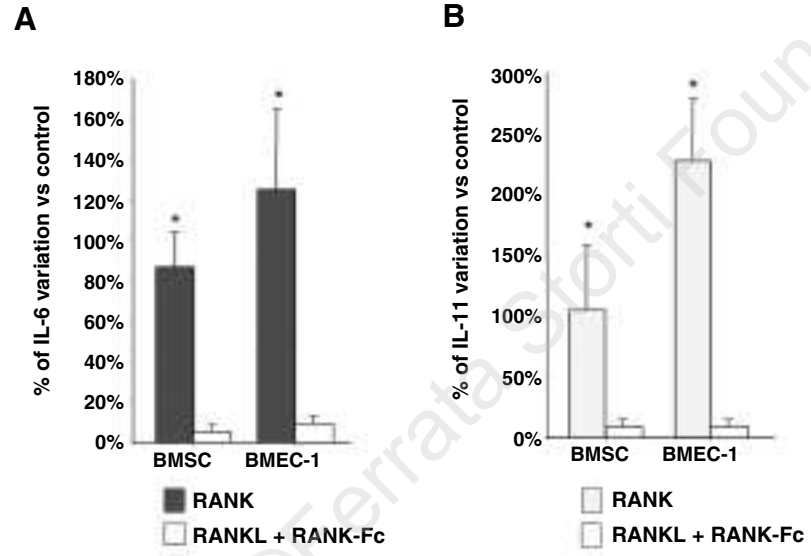

Figure 2. RANKL effect on IL-6 and IL-11 secretion by the microenvironment. Effect of RANKL treatment on IL-6 (A) and IL-11 (B) secretion by BMSC and BMEC-1. IL-6 and IL-11 levels were detected in the conditioned media of cells after 48 hours of incubation in the presence of RANKL (100 ng/mL) with or without RANK-Fc (100 ng/mL). The graphics represent the% mean variation versus control ± standard deviation of three independent experiments. *=p<0.01.

The lack of RANK expression in HMCL and fresh purified MM cells was confirmed by Western blot analysis (Figure 1B). Moreover, treatment with RANKL had no observed direct effect on HMCL proliferation, survival or cytokine production. On the other hand, we found that RANK is expressed at both mRNA and protein levels in BMSC, in both microvascular endothelial cells, HMEC-1 and BMEC-1, in the osteoblast-like cell line, MG-63, but not in hOB (Figures 1A and 1B).

Effect of RANK ligand on cytokine secretion by the microenvironment

RANKL treatment induced a statistically significant, dose-dependent increase of IL-6 secretion by both BMSC and endothelial cells after 48 hours (mean±SD% of increase vs. control: +87±17% and +125±40%, respectively; p<0.01) (Figure 2A). IL-11 secretion was up-regulated in BMSC and BM endothelial cells by RANKL (+105±52% and +228±50%, respectively; p<0.01) (Figure 2B). In contrast, we found that RANKL did not induce IL-1 or TNF secretion by BMSC or endothelial cells (*data not shown*).

RANKL is involved in IL-6 and IL-11 up-regulation in the microenvironment by myeloma cells

First, we found that HMCL and fresh purified MM cells did not express RANKL mRNA (Figure 3) or produce RANKL, as shown for the XG-1 cell line in Figure 4, then we found that HMCL up-regulated RANKL secretion in

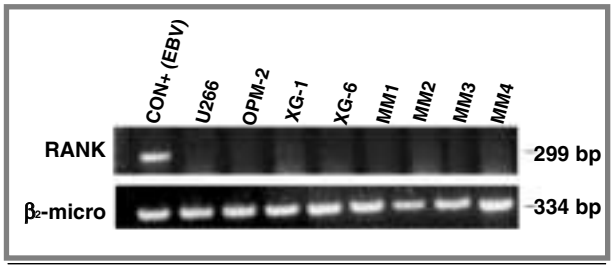

Figure 3. Lack of RANKL mRNA expression by human myeloma cells. RT-PCR was performed on HMCL and fresh purifed CD138+ MM cell mRNA using specific primers for RANKL as described in the *Methods* section. EBV+ cells were used as a positive control for RANKL and β2-microglobulin as an internal control of PCR amplification.

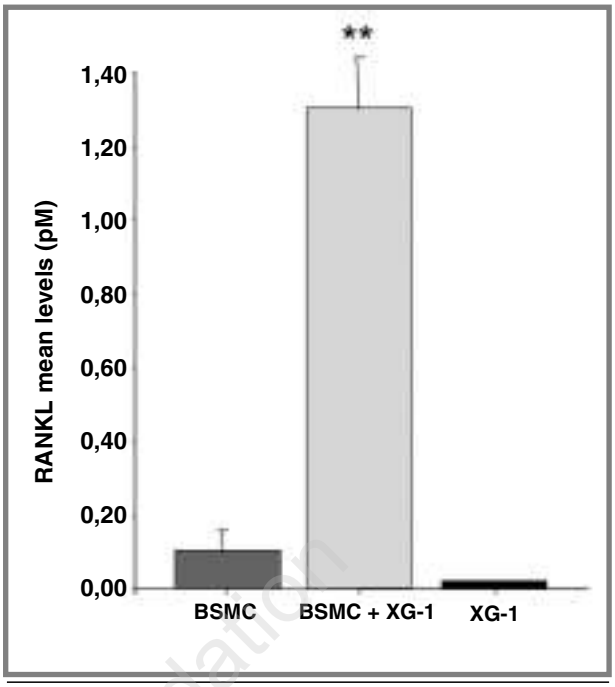

Figure 4. RANKL levels in a co-culture system with BMSC and XG-1. Using an ELISA, RANKL levels were assayed in the conditioned media of BMSC, BMSC co-cultured with XG-1 and XG-1 alone. The graph shows the mean concentration of RANKL, ± standard deviation of three independent experiments. **= $p<0.01$.

BMSC in a cell-to-cell contact co-culture system as shown for XG-1 (Figure 4). Moreover, in the co-culture system we found that HMCL up-regulated IL-6 and IL-11 secretion by BMSC through the cell-to-cell contact. The presence of RANK-Fc in this system significantly inhibited XG-1 induced IL-6 (-67±12%; $p<0.01$) (Figure 5A) and IL-11 (-55±1.4%; $p<0.05$) (Figure 5B) secretion by BMSC, whereas RANK-Fc did not affect IL-6 or IL-11 secretion by BMSC in the absence of HMCL in accordance with the low RANKL levels detected in the supernatant of the BMSC. Similar results were obtained using other HMCL or fresh purified MM cells in co-culture. RANK-Fc was able to blunt the IL-6 up-regulation induced in BMSC by U266 and RPMI-8266 with a lower effect as compared to XG-1 and by CD138+ purified MM cells (Figure 5C). The IL-6 levels secreted by normal and MM BMSC did not differ significantly in control and co-culture conditions, as also reported by others.[14] A similar inhibitory effect of RANK-Fc was observed on IL-6 up-regulation in the co-culture condition (Figure 5D).

Discussion

In this study, first, we show that the osteoclast receptor RANK is also present on BMSC and endothelial cells but not on myeloma cells or primary osteoblastic cells. The lack of RANK expression on HMCL and MM cells is in line with previously reported results on BM plasma cells from MM patients, studied using immunohistochemical techniques.[15] However, using a more sensitive PCR technology, Farrugia *et al.* found that myeloma cells express RANK mRNA.[6] Nevertheless, we found that treating myeloma cells with RANKL had no effect on these cells' survival or cytokine production, indicating the lack of a functional RANK receptor on myeloma cells.

On the other hand we showed that the osteoclast receptor RANK[3] can be present on BMSC and endothelial cells but not on primary osteoblastic cells, as also recently reported.[16] Further, we demonstrated that RANKL treatment induces IL-6 and IL-11 secretion by both BMSC and endothelial cells and that when the RANKL/RANK interaction was blocked, myeloma-induced IL-6 and IL-11 secretion was blunted. This finding indicates that RANKL, induced by myeloma cells in the BMSC, in turn supports IL-6 and IL-11 production in the BM environment. Of course, other cytokines beside RANKL, such as vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF) produced directly by myeloma cells or by BMSC, could be involved in IL-6 and IL-11 up-regulation.[10,17] In line with these observations we found that blocking VEGF with a monoclonal antibody was able to inhibit IL-6 secretion in our co-culture system (*data not shown*). The capacity of RANK-Fc to blunt IL-6 upregulation in the co-cultures was observed for several HMC; the effect was more pronounced for XG-1 than for U266 or RPMI-8266. This could be explained, at least in part, by the different sensitivity of these cell lines to IL-6, XG-1 being IL-6-dependent cells while U266 and RPMI-8266 are not.

On the basis of our evidence we can hypothesize that RANKL does not have a direct effect on myeloma cell proliferation and survival because of the lack of RANK on myeloma cells but that the ligand may contribute to stimulating myeloma cell proliferation and survival indi-

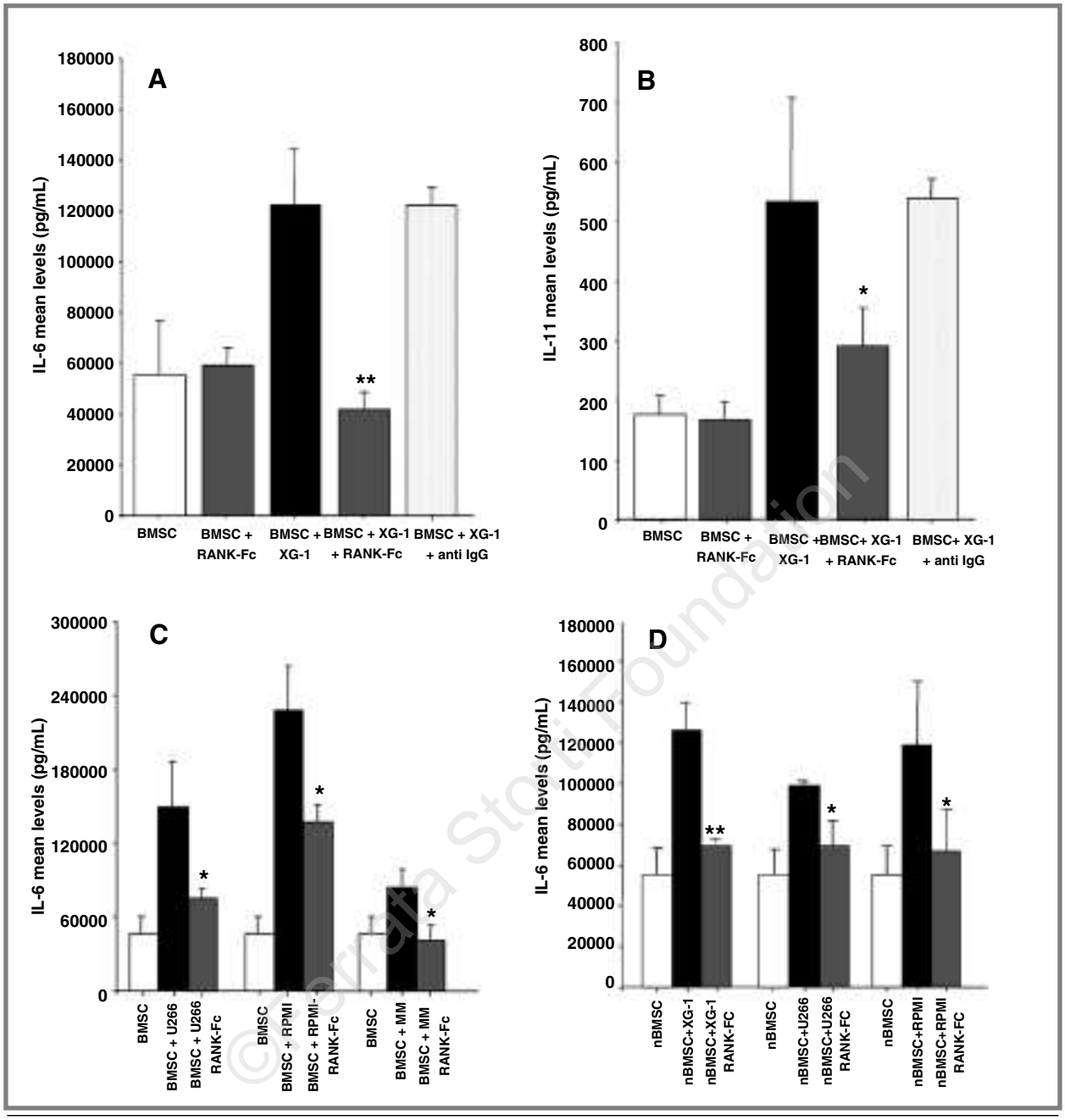

Figure 5. RANKL involvement in IL-6 and IL-11 up-regulation by myeloma cells. BMSC obtained from MM patients were co-cultured with the HMCL XG-1 (A and B) or U266, RPMI and fresh purified MM cells (C) in the presence or absence of RANK-Fc or anti-IgG control for 48 hours. After the culture period supernatants were collected and tested for IL-6 (A and C) and IL-11 (B) levels by ELISA. In parallel experimental conditions, BMSC obtained from healthy subjects, (nBMSC) were incubated with or without the HMCL XG-1 or U266 or RPMI-8226 in the presence or absence of RANK-Fc and IL-6 levels were evaluated in the conditioned media after 48 hours by ELISA (D). The graphs show the mean of IL-6 or IL-11 levels ± standard deviation of three independent experiments. *= $p<0.05$; **= $p<0.01$.

rectly through the induction of IL-6 in the BM environment. This hypothesis is in line with a recently observed relationship between IL-6 levels and RANKL/OPG ratio in MM patients in whom RANKL levels were correlated with prognosis.[8] Moreover, the induction of IL-6 and IL-11 in BMSC by RANKL could support and increase the osteoclastogenetic effect of RANKL given that IL-6 and IL-11 are known to stimulate osteoclastogenesis.[18]

The role of endothelial cells in supporting myeloma cell growth was previously postulated[19] and confirmed by the observation of increased BM angiogenesis in MM patients with active and progressive disease.[20] Recently, it has also been suggested that endothelial cells may support osteoclast formation induced by myeloma cells

through RANKL production[7] and that RANKL in turn can stimulate angiogenesis.[21] Our data show that BM endothelial cells express RANK and produce IL-6 and IL-11 in response to RANKL, suggesting that they could be involved in myeloma induced IL-6 and IL-11 secretion in the BM environment.

In conclusion, our data indicate that RANK is expressed in the BM environment and that RANKL affects IL-6 and IL-11 levels in the BM microenvironment being involved in the up-regulation of these cytokines by human MM cells. This body of evidence provides new notions on the role of the RANKL system in the pathophysiology of MM.

NG was the main investigator, contributing to the performance of experiments, to the design and the concept of the whole study, interpretation of results, drafting and editing the manuscript; SC performed the molecular biology experiments and contributed to the interpretation of the data, the critical revision of the manuscript and editing the manuscript; FM performed cell culture experiments and ELISA assays; VR was involved in the review of the article and gave the final approval version to be published.

The authors reported no potential conflicts of nterest.

Manuscript received June 28, 2004. Accepted July 7, 2004.

References

1. Seidl S, Kaufmann H, Drach J. New insights into the pathophysiology of multiple myeloma. Lancet Oncol 2003; 4: 557-64.
2. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. Nat Rev Cancer 2002;2:927-37.
3. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003;423:337-42.
4. Pearse RN, Sordillo EM, Yaccoby S, Wong BR, Liau DF, Colman N, et al. Multiple myeloma disrupts the TRANCE/ osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. Proc Natl Acad Sci USA 2001; 98: 11581-6.
5. Giuliani N, Bataille R, Mancini C, Lazzaretti M, Barille S. Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment. Blood 2001; 98:3527-33.
6. Farrugia AN, Atkins GJ, To LB, Pan B, Horvath N, Kostakis P, et al. Receptor activator of nuclear factor-κB ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo. Cancer Res 2003;63:5438-45.
7. Okada T, Akikusa S, Okuno H, Kodaka M. Bone marrow metastatic myeloma cells promote osteoclastogenesis through RANKL on endothelial cells. Clin Exp Metastasis 2003;20:639-46.
8. Terpos E, Szydlo R, Apperley JF, Hatjiharissi E, Politou M, Meletis J, et al. Soluble receptor activator of nuclear factor {κ}B ligand-osteoprotegerin ratio predicts survival in multiple myeloma: proposal for a novel prognostic index. Blood 2003;102: 1064-9.
9. Vanderkerken K, De Leenheer E, Shipman C, Asosingh K, Willems A, Van Camp B, et al. Recombinant osteoprotegerin decreases tumor burden and increases survival in a murine model of multiple myeloma. Cancer Res 2003;63:287-9.
10. Uciyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood 1993; 82: 3712-20.
11. Hjertner O, Torgersen ML, Seidel C, Hjorth-Hansen H, Waage A, Borset M, et al. Hepatocyte growth factor (HGF) induces interleukin-11 secretion from osteoblasts: a possible role for HGF in myeloma-associated osteolytic bone disease. Blood 1999;94:3883-8.
12. Lehr J-E, Pienta K-J. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. J Natl Cancer Inst 1998;90:118-23.
13. Lasagni L, Francalanci M, Annunziato F, Lazzeri E, Giannini S, Cosmi L, et al. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. J Exp Med 2003; 197: 1537-49.
14. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood 1993; 82: 3712-20.
15. Roux S, Meignin V, Quillard J, Meduri G, Guiochon-Mantel A, Fermand JP, et al. RANK (receptor activator of nuclear factor-κB) and RANKL expression in multiple myeloma. Br J Haematol 2002; 117: 86-92.
16. Bu R, Borysenko CW, Li Y, Cao L, Sabokbar A, Blair HC. Expression and function of TNF-family proteins and receptors in human osteoblasts. Bone 2003;33:760-70.
17. Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM, et al. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood 2000;95:2630-6.
18. Manolagas SC. Role of cytokines in bone resorption. Bone 1995;17:63S-7S.
19. Astaldi GC, Janssen MC, Lansdorp PM, Zeijlemaker WP, Willems C. Human endothelial culture supernatant (HECS): evidence for a growth-promoting factor binding to hybridoma and myeloma cells. J Immunol 1981;126:1170-3.
20. Vacca A, Ribatti D, Presta M, Minischetti M, Iurlaro M, Ria R, et al. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. Blood 1999; 93:3064-73.
21. Kim HH, Shin HS, Kwak HJ, Ahn KY, Kim JH, Lee HJ, et al. RANKL regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. FASEB J 2003; 17:2163-5.