[CANCER RESEARCH 64, 772–775, January 15, 2004]

Letters to the Editor

Do Human Myeloma Cells Directly Produce the Receptor Activator of Nuclear Factor κB Ligand (RANKL) or Induce RANKL in the Bone Marrow Microenvironment?

Farrugia *et al.* (1) in the September 1, 2003 issue of *Cancer Research* have arisen an interesting and controversial question about the direct expression or production of the critical osteoclastogenetic receptor activator of nuclear factor κB ligand (RANKL) by human myeloma cells. Farrugia *et al.* (1) have shown that sorted $CD38^{+++}$ subpopulation expressed RANKL protein by flow cytometry and RANKL mRNA, as well as its receptor, receptor activator of nuclear factor κB, mRNA by reverse transcription-PCR. Moreover, in a coculture system with peripheral blood mononuclear cell they show that sorted $CD38^{+++}$ cells induced osteoclast formation that is abolished by osteoprotegerin (OPG) treatment. On the basis of their data the authors conclude that myeloma cells directly produce RANKL and that RANKL is directly involved in the osteoclast formation induced by myeloma cells. These data are in contrast with the evidence reported by our group (2, 3) and other authors (4–6). In particular we found that purified CD138-positive myeloma cells of 26 patients in different stage of disease were negative for RANKL mRNA expression using a specific primer pair that recognizes both *trans*-membrane and soluble RANKL isoform (2). Moreover we found that several human myeloma cell lines (RPMI-8226, U266, OPM-2, LP-1, XG-1, XG-6, MDN, and JJN3) did not express RANKL and receptor activator of nuclear factor κB mRNA or produced soluble RANKL (2, 3). Similarly, Pearse *et al.* (4) tested ARP-1, U266, RPMI-8226, H929, and EBV-positive ARH-77 cell lines, and failed to find RANKL mRNA or protein. Moreover, RANKL immunostaining, performed by different authors on bone marrow biopsies of multiple myeloma (MM) patients has shown that myeloma cells are negative for RANKL expression (1, 4–6). In line with these observations, Shaughnessy *et al.* (6), using the microarray technology, confirmed recently that RANKL has not been detected in purified CD138-positive MM cells of either 83 osteolytic or 87 nonosteolytic MM patients, as well as in normal bone marrow plasma cells. All of these experimental evidences demonstrate that human myeloma cells do not express or directly produce the critical osteoclastogenetic factor RANKL in contrast with the observations of Farrugia *et al* (1). Why is there this discrepancy? Could it be due to the different type of isolation of plasma cells?

We have some observations and questions about the methodologies and the conclusions presented by the authors.

First they have sorted and tested $CD38^{+++}$ cells instead of CD138-positive cells. CD138 is widely accepted as specific marker for myeloma cells and it is usually used to isolate MM cells (7). Sorting plasma cells by CD38 antigen expression alone the authors cannot exclude the presence of other contaminating cells that are known to express RANKL such as, in particular, activated T lymphocytes (2, 8). Did the authors test RANKL mRNA and protein on CD138-positive cells and on human myeloma cell lines that are a model of high purified MM cells? Moreover, the monoclonal antibody used to detect RANKL by flow cytometry (MAB626; R&D), the same used by Heider *et al.* (9), is not validated for flow cytometry application. So, the authors should prove the specificity of this mAb.

When the authors investigate the RANKL mRNA expression in sorted cells on the basis of CD38 expression they show that both $CD38^{+}$ and $CD38^{+++}$ express RANKL. Thus, how could they explain that $CD38^{+}$ cells do not induce osteoclast formation in their system in contrast to $CD38^{+++}$ cells? Anyway, because both $CD38^{+}$, which are not myeloma cells, and $CD38^{+++}$ cells express RANKL, we could suppose that the direct RANKL production by myeloma is not a critical determinant in myeloma-induced osteoclast formation.

In the article the authors say that they have used a more sensitive PCR technology in comparison with our method (2, 3). But, if they had to increase the sensitivity of PCR to obtain RANKL mRNA signal in plasma cells, they support the hypothesis that RANKL expression in $CD38^{+++}$ cells is very low. Moreover, using a more sensitive method they could have a high risk to amplify cDNA derived from contaminating cells considering that they do not use CD138 antibody to isolate plasma cells.

The last point is about the osteoclastogenetic system. Using all of the peripheral blood mononuclear cells to generate osteoclasts, instead of purified $CD14^{+}$ monocytes (10), the authors are not able to demonstrate that the direct RANKL production by $CD38^{+++}$ is critical in osteoclast formation, because T lymphocytes are present in their system and it has been demonstrated that myeloma cells upregulate RANKL in T lymphocytes.

Moreover, the osteoclastogenetic effect observed by the authors using the $CD38^{+++}$ cells is little as compared with what is observed by myeloma cells in the presence of stromal cells, suggesting that stromal cells are critical in the osteoclastogenetic effect of myeloma cells (1, 11).

Several evidence have demonstrated that RANKL/OPG expression and ratio in stromal/osteoblastic cells is the critical point in the regulation of osteoclast formation both in physiological and pathophysiological conditions (12). Moreover a role of T lymphocytes has been postulated recently in the regulation of bone resorption through RANKL expression (8). In line with these observations, different groups have shown that myeloma cells induce RANKL and inhibit its soluble antagonist OPG in bone marrow stromal/osteoblastic and T cells both in a coculture system and *in vivo* in MM patients. Moreover, immunohistochemistry, performed on bone marrow biopsies by several authors, has also confirmed the presence of an imbalance in RANKL/OPG expression in the bone marrow environment. All of these evidence strongly suggest that RANKL/OPG system is mainly involved in the activation of osteoclastic cells by myeloma cells indirectly through the bone marrow environment.

Nicola Giuliani
Simona Colla
Vittorio Rizzoli
Chair of Hematology, BMT Unit
University of Parma
Parma 43100, Italy

Sophie Barillé-Nion
Régis Bataille
Institut National de la Santé et de la Recherche Médicale U463
Nantes 44093, France

Received 9/30/03; accepted 10/2/03.
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Requests for reprints: Nicola Giuliani, Chair of Hematology and BMT Unit, University of Parma, via Gramsci 14, 43100 Parma, Italy. Phone: 00390 521290787; Fax: 00390 521292765; Email: N_Giuliani@yahoo.com or Nicola.Giuliani@unipr.it.

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Identification and Clinical Relevance of Receptor Activator of Nuclear Factor κB Ligand Expression of Myeloma Cells

Letter

We read the publication by Farrugia *et al.* (1) with great interest. A novel system of cytokines was identified as key mediators in osteoclastogenesis, including the receptor activator of nuclear factor κB ligand (RANKL), receptor activator of nuclear factor κB, and osteoprotegerin (2). RANKL binds to its specific receptor, receptor activator of nuclear factor κB, which is located on osteoclastic precursors and induces osteoclastogenesis. Osteoprotegerin acts as a decoy receptor for RANKL and neutralizes its biological effects. Farrugia *et al.* (1) reported that RANKL is expressed by human myeloma cells, and this expression correlates with bone destruction. This publication is important because there has been a controversy whether myeloma cells express RANKL (3). Whereas several authors reported RANKL expression by myeloma cells (4–6), no expression could be detected by others using immunohistochemistry (7, 8). We have used immunocytochemistry rather than immunohistochemistry to show the RANKL expression of human myeloma cells in bone marrow aspirates (9). The reason was that the immunohistochemical evaluation of formalin-fixed, decalcified, and paraffin-embedded tissue sections may be associated with limitations because it is known that decalcification of formalin-fixed tissue samples reduces its antigenicity. Bypassing the need for formalin fixation and decalcification, we could detect a strong cytoplasmatic expression of RANKL in bone marrow plasma cells in all of the investigated multiple myeloma patients with lytic bone disease (9). These results are in line with the observation by Farrugia *et al.* (1) that the epitope identified by the monoclonal RANKL antibody was sensitive to paraformaldehyde-fixation.

Farrugia *et al.* (1) found a significantly higher RANKL expression on plasma cells from patients with multiple myeloma and lytic bone disease in comparison to patients without osteolysis. Plasma cells were defined as CD38+++ cells using two-color flow cytometry. We had used three-color flow cytometry in bone marrow aspirates from 50 patients with multiple myeloma with CD38 PC5, CD138 FITC, and a monoclonal antihuman RANKL antibody conjugated to PE (10). Plasma cells were identified as cells strongly positive for CD38 and coexpressing CD138. The bone marrow plasma cells from controls showed no or only a weak surface expression of RANKL, and the median mean fluorescence index was 6. The expression of RANKL on bone marrow plasma cells from myeloma patients was significantly correlated with the presence (median mean fluorescence index = 60) or absence (median mean fluorescence index = 16) of bone lesions ($P < 0.0005$; Ref. 10). Additionally we isolated primary bone marrow plasma cells using another method than Farrugia *et al.* (1), namely immunomagnetic cell sorting using an antihuman CD138 antibody conjugated to magnetic beads (CD138 MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany). RANKL mRNA could be detected in freshly isolated bone marrow plasma cells from myeloma patients with osteolytic myeloma bone disease. These data show that human bone marrow myeloma cells directly produce RANKL on mRNA and protein level, and the level of RANKL protein expression on myeloma cells correlates with lytic bone disease in patients with multiple myeloma.

Ulrike Heider
Ivana Zavrski
Christian Jakob
Claudia Fleissner
Orhan Sezer
Universitätsklinikum Charité
Oncology and Hematology
Berlin, 10117 Germany

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Received 10/23/03; accepted 10/27/03.

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Reply

We appreciate the careful and critical reading of our article by Drs. Giuliani and Sezer and the opportunity to respond to their comments. As highlighted in the letter from Dr. Sezer, the topic of our article (1) has been the subject of much controversy in the field of myeloma biology (2–6) and centers on whether myeloma plasma cells express the potent pro-osteoclastogenic molecule receptor activator of nuclear factor κB ligand (RANKL) or secrete factor(s) that cause an increase in RANKL expression by surrounding stromal cells/T cells in the bone marrow (2–6). Whereas our article in no way precludes the latter scenario, it does show by a variety of methodologies that myeloma plasma cells express RANKL mRNA and functional protein. Moreover, it shows that myeloma plasma cells also express the soluble isoform of RANKL, shown previously to be expressed by activated T cells in inflammatory conditions such as rheumatoid arthritis (7, 8). Consistent with work of Heider *et al.* (9), our studies show that RANKL protein expression by plasma cells is associated significantly with the presence of osteolytic lesions in patients, highlighting the potential prognostic and therapeutic value of our findings.

We acknowledge the concerns raised by Giuliani *et al.*, but feel that a number of their comments are inconsistent with recent published findings (6, 9). Firstly, Giuliani *et al.* quoted data from their laboratory (2, 3) and others (4, 10) who show that patient-derived CD138-positive plasma cells and human myeloma cell lines do not express RANKL mRNA and protein. In contrast, our studies unequivocally show that the human myeloma cell lines RPMI-8226, U266, WL-2, and the EBV-positive ARH-77[1] as well as patient-derived myeloma cells all express RANKL mRNA and protein (1). Whereas initially reporting that plasma cells in bone marrow trephines lacked RANKL expression (4), Yaccoby *et al.* (11) have reported recently that CD138-isolated human plasma cells express membrane-associated RANKL. Furthermore, studies by Croucher *et al.* (12) show that the widely used murine myeloma cell line model, 5TMM, expresses surface RANKL. Whereas we are unable to explain the microarray studies published by Zhan *et al.* (13), we postulate that this may relate to the relatively low level of RANKL transcript within these cells and the relative instability of this transcript. In considering the biological significance of this low level of RANKL expression, it must be remembered that it is the relative expression of RANKL to its inhibitor osteoprotegerin, which is critical in determining the osteoclastogenic effect. To this end, our studies also clearly show that $CD38^{+++}$ plasma cells do not express detectable levels of osteoprotegerin mRNA (1). Therefore, in situations of significant plasma cell burden within the bone marrow microenvironment, this would result in a high ratio of RANKL to osteoprotegerin expression and a resultant pro-osteoclastogenic environment.

Secondly, Giuliani *et al.* question our use of CD38 expression to isolate myeloma plasma cells instead of the "widely accepted specific marker of myeloma cells," CD138. Our experience, as with a number of other groups, shows that CD138 is variably expressed and sometimes lacking in patients with significant plasma cell burden (14, 15). On this basis, we chose to use high expression of CD38 to isolate bone marrow-derived plasma cells. We strongly reject the suggestion that our results are due to "other contaminating cells that are known to express RANKL such as activated T lymphocytes," because the purity of all of our sorted populations was routinely assessed and found to be >99%. Whereas we recognize that activated T lymphocytes are capable of expressing the CD38 antigen (16), we as others have reported (14), find no evidence of contaminating $CD3^+$ T lymphocytes in the $CD38^{+++}$ population. Although we have not examined RANKL mRNA expression in sorted CD138 plasma cells, studies by Sezer *et al.* suggest that magnetic activated cell sorting purified $CD138^+$ cells do indeed express RANKL mRNA (9). Our two-color flow cytometric analysis on bone marrow derived from several myeloma patients show that $CD138^+$ cells react with two anti-RANKL antibodies, MAB626 (R& D Systems) and polyclonal anti-RANKL, Sc-9073 (Santa Cruz Biotechnology). Once again, these findings are consistent with findings from the laboratory of Dr. Sezer as outlined in his letter. Furthermore, using flow cytometry, recent studies from our laboratory show that sf21-derived huRANKL (the immunogen used to raise MAB626) can block the binding of MAB626 to the human myeloma cell lines 8226, confirming the specificity of this monoclonal antibody (Fig. 1).

With regard to the ability of myeloma plasma cells to support osteoclast differentiation and activation, it seems that Giuliani *et al.* may have misinterpreted the data presented. The $CD38^+$ cells, which also express RANKL, are less capable of supporting TRAP+ osteoclast development, when compared with the $CD38^{+++}$ plasma cell

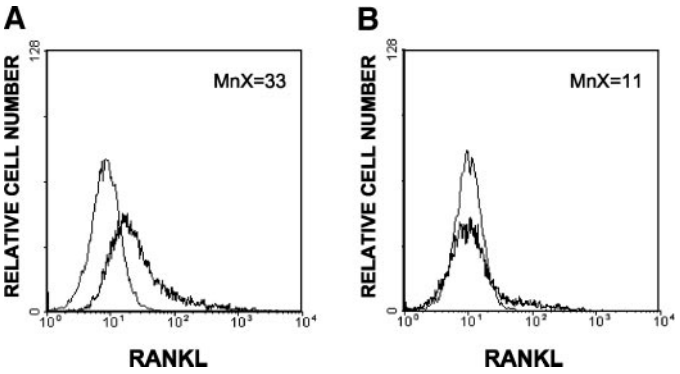

Fig. 1. The expression of membrane-associated receptor activator of nuclear factor κB ligand (RANKL) protein by the HMCL 8226 was measured using indirect immunofluorescence and flow cytometry. Two hundred thousand 8226 cells were stained with a 100 μl solution of MAB626 at a concentration of 10 μg/ml (anti-RANKL; R&D Systems). Specific binding was revealed by incubation with saturating concentration of goat-antimouse IgG-FITC. The fluorescence histograms in *A* show the expression of RANKL protein (*black histogram*) compared with isotype-matched, nonbinding control used under identical conditions (1A6.11, IgG2b; *gray histogram*). In *B*, 100 μl solutions of MAB626 and 1D4.5, both at a concentration of 10 μg/ml, were incubated with 300 ng/ml SF-21-derived RANKL protein (R&D Systems) for 1 h at 37°C for 60 min before staining the HMCL 8226 as in *A*. The reactivity of MAB626 is almost completely inhibited by prior incubation with recombinant RANKL as evidenced by the reduction in transmembrane RANKL expression (*A*; MnX = 33) compared with untreated cells (*B*; MnX = 11).

Received 10/22/03; revised 11/5/03; accepted 11/7/03.
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Requests for reprints: Andrew C. W. Zannettino, Myeloma and Mesenchymal Research Group, Matthew Roberts Foundation Laboratory, Division of Haematology, Institute of Medical and Veterinary Science and The Hanson Institute, Frome Road, Adelaide, South Australia 5000, Australia. Phone: 61-8-8222-3455; Fax: 61-8-8222-3139; E-mail: andrew.zannettino@imvs.sa.gov.au.

[1] A. N. Farrugia and A. C. W. Zannettino, unpublished observations.

population. This, in our opinion, reflects the fact that the $CD38^{+}$ population also expresses mRNA encoding osteoprotegerin. Whereas our laboratory routinely performs osteoclast-forming assays with peripheral blood derived magnetic activated cell sorting separated $CD14^{+}$ monocytes, we find little difference in the phenotype and functional capacity of magnetic activated cell sorting selected $CD14^{+}$ osteoclast precursors and those obtained by adherence as described in the article (1). Moreover, unlike what is suggested by Giuliani *et al.*, we find no evidence of contaminating $CD3^{+}$ T lymphocytes in our osteoclast precursor population. While we recognize that when compared with stromal cells or, as was the case in our study, compared with osteoclasts generated by continuous exposure to recombinant RANKL, the "osteoclastogenic effect" observed "is little" when osteoclast precursors are cultured with the $CD38^{+++}$ cells, it nevertheless demonstrates that myeloma plasma cells are capable of supporting osteoclast formation and activation in a RANKL-dependent manner. Indeed, we believe that the more modest osteoclast formation observed when $CD38^{+++}$ cells were used as the stromal support may relate in part to the poor *in vitro* survival capacity of the primary plasma cells used (1).

We feel that our results reflect the careful nature in which our study was performed and as highlighted in the letter by Sezer, represents one of a number of recent studies that will recognize the importance and the role of plasma cell-derived RANKL in myeloma biology.

Andrew C. W. Zannettino
Beiqing Pan
Amanda N. Farrugia
Gerald J. Atkins
L. Bik To
Myeloma and Mesenchymal Research Group
Matthew Roberts Foundation Laboratory
Division of Haematology
Institute of Medical and Veterinary Science
and The Hanson Institute
Adelaide, South Australia 5000, Australia

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