inhibition may be true, but the magnitude of change in platelet deposition is actually not impressive compared to what is seen with blockade of both P2Y_{12} and P2Y_{1} receptors concurrently. Data on limited effects of each inhibitor separately in bulk shear stress (viscometry) studies were already presented in our earlier paper.1

To the editor:

**Immunocytochemistry reveals RANKL expression of myeloma cells**

Two highly interesting papers related to osteoclastic activation in multiple myeloma were published in the December 15 issue of *Blood*.1,2 These studies were related to the imbalance of 2 molecules involved in the regulation of bone resorption: receptor activator of NF-kappaB ligand (RANKL) and osteoprotegerin. Croucher and colleagues2 found that mouse myeloma cells expressed RANKL mRNA and that RANKL protein could be demonstrated on the cell membrane. Contrary to these findings, Giuliani and colleagues1 could detect neither RANKL mRNA in human myeloma cells nor RANKL protein using immunohistochemistry of formalin-fixed, decalcified, and paraffin-embedded bone marrow specimens in patients with multiple myeloma. Although these discrepancies might let the reader assume that there are crucial differences between the murine model used2 and human myeloma disease, we wish to make the readers aware of our results, which show a strong RANKL expression of plasma cells in patients with multiple myeloma and osteolytic lesions. Pearse et al3 demonstrated that myeloma stimulates osteoclastogenesis by triggering an increase in RANKL and a decrease in osteoprotegerin expression in stromal cells, but RANKL expression could not be demonstrated in myeloma cells.3 On the other hand, human myeloma cell lines have been shown to express RANKL by other investigators.4 The immunohistochemical evaluation of formalin-fixed, decalcified, and paraffin-embedded tissue sections may be associated with some limitations, since it is known that decalcification of formalin-fixed tissue samples reduces its antigenicity.5 To avoid the potential problems associated with this technique, we studied the RANKL expression in multiple myeloma patients with osteolytic lesions by immunocytochemistry using bone marrow smears and cytopsins. Intracellular staining of bone marrow cells was performed using the same monoclonal mouse antibody against human RANKL as Giuliani and colleagues (clone 70525.11, R&D Systems, Minneapolis, MN).

The antibody was applied for 30 minutes in a 1:10 dilution (antibody-diluent with background reducing components, Dako, Carpinteria, CA) for detection of RANKL-expressing cells. Rabbit antimouse (secondary antibody, Dako, Glostrup, Denmark) was applied for 30 minutes in a 1:50 dilution. Visualization was performed by the alkaline phosphatase/anti–alkaline phosphatase double bridge technique (APAAP; Dako). For intensification, the secondary antibody was used again for another 10 minutes of incubation, followed by another APAAP application for 10 minutes (Fuchsin + Substrate-Chromogen-System, Dako). Counterstaining was done with hematoxylin. Control stainings were performed the same way but without using the primary antibody or using an irrelevant antibody (pancytokeratin). SaOs-2 cells, a human osteosarcoma cell line, which is known to express RANKL, served as a positive control.

We could detect a strong cytoplasmatic expression of RANKL in bone marrow plasma cells in all 6 multiple myeloma patients investigated (Figure 1). Negative controls showed no staining reaction, and SaOs-2 cells were strongly positive for RANKL. Complementary to these results of cytoplasmatic RANKL expression, we performed flow cytometry for the analysis of RANKL expression on the cell surface using the same bone marrow aspirates. Plasma cells were identified as CD38 strongly positive (+ +) and CD138 (B-B4) positive (+) cells as described previously.6 Immunofluorescence staining of RANKL on the cell surface was performed using the same monoclonal mouse antibody against

Figure 1. Immunocytochemistry of bone marrow myeloma cells and controls. (A) RANKL immunostaining shows strong positivity in myeloma cells obtained by bone marrow aspirate from a patient with multiple myeloma and osteolytic lesions. (B) Negative control staining using an irrelevant antibody (pancytokeratin). (C) SaOs-2 cells, a human osteosarcoma cell line, which is known to express RANKL, served as a positive control.

Figure 2. Flow cytometry of bone marrow myeloma cells. In the same patient as in Figure 1, flow cytometry reveals RANKL expression on the surface of bone marrow plasma cells. (A) Identification of plasma cells according to their CD38 + /B-B4 expression. (B) RANKL expression (solid line) of the gated plasma cells compared with isotype control (dotted line).
human RANKL we used for immunocytochemistry and its isotype control (mouse IgG2b), and fluorochrome conjugated F(ab')2 fragment goat anti–mouse IgG. Using flow cytometry, we could detect a strong expression of RANKL on bone marrow plasma cells in all multiple myeloma patients investigated (Figure 2). Furthermore, we performed Western blot analysis in 6 human myeloma cell lines (IM-9, NCI-H929, LP-1, OPM-2, RPMI 8226, U266) and found RANKL protein expression in all of them.

These results extend our recent findings using flow cytometry alone in 10 previous patients,6 underline that RANKL is directly expressed by plasma cells in the bone marrow of multiple myeloma patients with osteolytic bone lesions, and suggest a direct role of this ligand in the osteoclast activation in multiple myeloma.

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Response:

OPGL/RANKL expression is not detectable in human myeloma cells

The data reported by Sezer et al show the expression of receptor activator of NF-kappaB ligand (RANKL) or osteoprotegerin ligand (OPGL) by plasma cells in human myeloma cells by immunocytochemistry and flow cytometry analysis. In contrast, we previously found no expression of RANKL on myeloma cells checked at the protein level by Western blot and immunohistochemistry and also at the RNA level by reverse transcription–polymerase chain reaction.1 Our data, including positive controls and resulting from the analysis of 10 human myeloma cell lines, 26 samples of primary myeloma cells, and 15 bone marrow specimen from patients with or without osteolytic lesions, are consistent with those reported by Pearse et al2 but contrast with the mouse model described by Croucher et al in which RANKL expression has been observed in the mouse myeloma cell line ST2MM.3 Furthermore, we have demonstrated by in vitro experiments and bone marrow biopsies analyses that human myeloma cells induced the overexpression of RANKL in the human bone marrow environment especially in stromal cells, as observed by Pearse et al. In conclusion, most of the published data indicate that myeloma bone disease is associated with the increase of RANKL expression in bone environment and that, at least in the human disease, the overexpression of RANKL results from the up-regulation of RANKL expression in bone environment rather than the expression of RANKL by myeloma cells themselves.

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References

To the editor:

Recurrent involvement of the MLL gene in adult T-lineage acute lymphoblastic leukemia

MLL (also known as ALL1, HTRX, or HRX) gene translocations are among the most common chromosomal abnormalities in both B-lineage acute lymphoblastic leukemia (B-ALL) and acute myeloid leukemia (AML). This MLL involvement means that these leukemias constitute a distinct disease1 with a particularly poor prognosis, which needs to be more easily identified and better characterized if therapeutic regimens are to be improved. MLL gene rearrangements have been sporadically reported in acute lymphoblastic T-cell leukemia (T-ALL),2,3 but the incidence of MLL involvement in T-ALL could be consistently underestimated because MLL rearrangements would seem to be due to translocations that are difficult to detect by classic cytogenetic methods.

Studying the MLL gene could lead to the identification of a new group of T-ALL, especially if this group also turned out to have a poor clinical outcome. Here we present evidence that the MLL gene could be in fact involved in more than 8% of adult T-ALL.

We looked for MLL abnormalities in bone marrow samples from 47 adults and 34 children with unselected T-ALL. Immunophenotyping and the assignment of the leukemias to the T lineage were based on the classification of the European Group for the Immunological characterization of Leukemias. No correlation was observed between the maturation of T-ALL and the involvement of the MLL gene. All the patients were screened by Southern blot analysis with various probes encompassing exon 3 to exon 22 of
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