[CANCER RESEARCH 63, 287–289, January 15, 2003]

Advances in Brief

Recombinant Osteoprotegerin Decreases Tumor Burden and Increases Survival in a Murine Model of Multiple Myeloma[1]

Karin Vanderkerken,[2] Evy De Leenheer, Claire Shipman, Kewal Asosingh, Angelo Willems, Ben Van Camp, and Peter Croucher

Department of Hematology and Immunology, Free University Brussels, Brussels, Belgium [K. V., E. D. L., K. A., A. W., B. V. C.], and Nuffield Department of Orthopaedic Surgery, University of Oxford, Nuffield Orthopaedic Centre, Oxford, United Kingdom [E. D. L., C. S., P. C.]

Abstract

The aim of the present study was to determine whether modifying the local bone environment with osteoprotegerin (OPG), the soluble decoy receptor for receptor activator of nuclear factor-κB (RANK) ligand, could affect tumor burden and survival in the 5T33MM murine model of multiple myeloma. Treatment of mice, injected with 5T33MM cells, with recombinant OPG (Fc-OPG) caused a significant decrease in serum paraprotein and tumor burden and a significant increase in time to morbidity. This was associated with a decrease in osteoclast number *in vivo* but had no effect on apoptosis and proliferation of 5T33MM cells *in vitro*. These data indicate that targeting the bone microenvironment by inhibiting the interaction between RANK ligand and RANK with Fc-OPG not only inhibits the development of myeloma bone disease but also decreases tumor growth and increases survival.

Introduction

MM[3] is associated with the development of a bone disease characterized by local osteolytic bone lesions, pathological fractures, and hypercalcemia. Recent studies suggest that this may be mediated by abnormal regulation of the RANKL system in the local bone marrow microenvironment. Expression of RANKL has been reported to be increased in stromal cells in the bone marrow of patients with MM (1–3). Furthermore, RANKL was also shown to be expressed directly, both by human and murine MM cells (4–8).

The demonstration that this system is abnormally regulated in MM has raised the possibility that targeting this system may have therapeutic potential. In support of this, we have previously reported that Fc-OPG, the soluble decoy receptor for RANKL, prevents the development of lytic bone disease in the 5T2MM model for myeloma (8). Furthermore, a soluble form of RANK has also been shown to prevent the development of MM bone disease in the severe combined immunodeficient (SCID)/hu model of MM (2, 9).

However, it is unclear whether inhibiting the development of MM bone disease with recombinant Fc-OPG alters the local environment and indirectly affects tumor growth. The aim of the present study was to establish whether Fc-OPG affects the growth of myeloma cells and modulates survival *in vivo* in a murine model of myeloma.

Materials and Methods

The 5T33MM Model. The 5T33MM murine model of MM originated spontaneously in elderly C57BLKaLwRij mice (10, 11). The cells were propagated by i.v. transfer of the diseased bone marrow into young recipients. All of the animals were housed under conventional conditions (license number LA123028). All of the procedures involving mice were approved by the local Ethics Committee. Cell isolation was performed as described previously (12).

Expression of RANKL by 5T33MM Murine Myeloma Cells. The bone marrow from 5T33MM-bearing mice was flushed from the tibiae and femur, and mononuclear cells isolated by density gradient centrifugation (12). Expression of the mRNA for RANKL in purified 5T33MM cells and whole bone marrow was determined by RT-PCR as described previously (8). Expression of RANKL on the surface of 5T33MM cells was determined by flow cytometry. Briefly, mononuclear cells were stained with a goat-anti-RANKL antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by rabbit-antigoat IgG FITC (DAKO Diagnostics, Heverlee, Belgium). 5T33MM cells were identified by cytoplasmic staining for the idiotype protein (13).

Treatment of the Mice with Fc-OPG: Antitumor Effect. Twenty-four C57BL/KaLwRij mice were injected i.v. with 5T33MM cells and the development of MM was monitored by measuring serum paraprotein concentrations (8). Six age-matched mice were included as controls. On the day of injection of 5T33MM cells, mice were treated with Fc-OPG (a gift from Dr. Colin Dunstan, Amgen, Thousand Oaks, CA) at 25 mg/kg i.v. three times a week ($n = 12$) or vehicle ($n = 12$) for 4 weeks. All of the animals that were given injections of 5T33MM cells developed MM, as demonstrated by the presence of a serum paraprotein. Twenty-eight days after injection of the MM cells, mice were sacrificed, bone marrow was isolated from the hind legs, and the proportion of tumor cells determined by staining the cells with an anti-5T33MM idiotype antibody and analysis by flow cytometry.

Treatment of the Mice with Fc-OPG: Effect on Survival. To determine the effect of Fc-OPG on survival in the 5T33MM model, a similar experiment (as described above) was performed. Twenty-four mice were given injections of 5T33MM cells; 12 were treated with vehicle and 12 with Fc-OPG from the time of tumor injection. Eight age-matched control animals were included. Treatment continued until each animal showed signs of morbidity, which included hind limb paralysis or cachexia, at which point they were sacrificed.

Assessment of Tumor Cell Apoptosis *in Vitro*. 5T33MM (10^5 cells/ml) cells were incubated with different concentrations of OPG (ranging from 0.1 to 10 μg/ml) for 4–20 h, and the percentage apoptotic cells was determined by flow cytometry after staining with annexin V-FITC (5 μl/10^5 cells; BD PharMingen, Erembodegem, Belgium) and propidium iodide (100 ng/ml/10^5 cells). 5T33MM cells were also cytocentrifuged on glass slides and stained with DAPI (1 μg/ml, 10 min). The proportion of apoptotic cells was then determined by fluorescence microscopy (14).

Assessment of Tumor Cells Proliferation *in Vitro*. 5T33MM cells were cocultured with irradiated bone marrow fibroblasts for 16–48 h (12) in the presence, or absence, of various concentrations of OPG (ranging from 0.1 to 10 μg/ml). [^{3}H]thymidine incorporation was used to evaluate the proliferation.

Assessment of the Effect of OPG on Osteoclast Number *in Vivo*. Femora were removed, fixed in 4% formalin, decalcified in EDTA, and embedded in paraffin; 4-μm sections were cut and stained for the presence of TRAP (Sigma,

Received 10/1/02; accepted 12/2/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (FWO), Onderzoeksraad-VUB, Belgische Federatie tegen Kanker, and the Leukaemia Research Fund, and by a Multiple Myeloma Research Foundation Fellow Award. K. V. and K. A. are postdoctoral research fellows of FWO-Vlaanderen; P. C. is a Leukaemia Research Fund Bennett Senior Fellow.

[2] To whom requests for reprints should be addressed, at Free University Brussels, Department of Hematology and Immunology, Laarbeeklaan 103, B-1090 Brussels, Belgium. Phone: 32-2-477-4573; Fax: 32-2-477-45-68; E-mail: Karin.Vanderkerken@vub.ac.be.

[3] The abbreviations used are: MM, multiple myeloma; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; OPG, osteoprotegerin; Fc-OPG, recombinant OPG; RT-PCR, reverse transcription-PCR; TRAP, tartrate-resistant acid phosphatase; DAPI, 4′,6 diamidino-2-phenylindole.

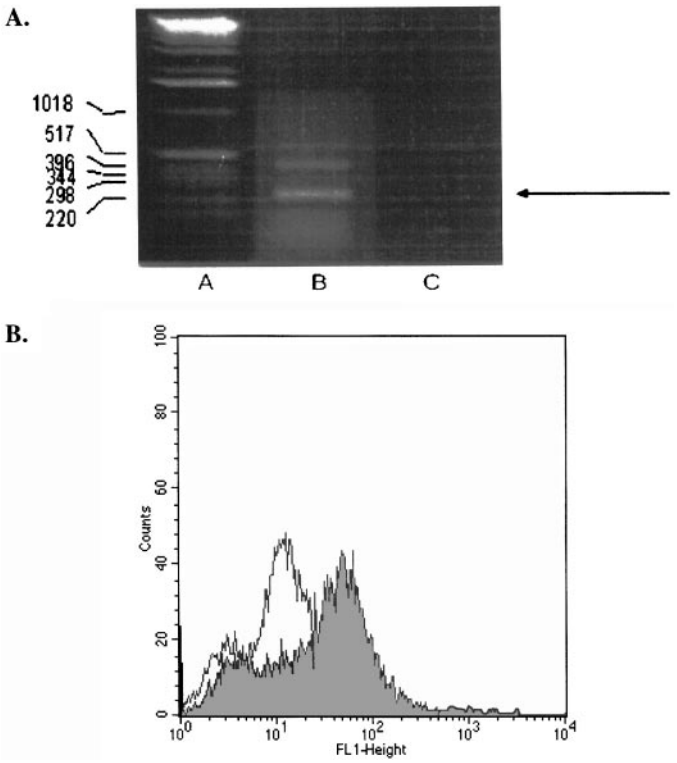

Fig. 1. *A*, RT-PCR demonstrating the expression for RANKL; *A*, 1-kb DNA ladder (Life Technologies, Inc.); *B*, 5T33MMvivo cells; *C*, negative control]. The band (*arrow*) was sequenced and demonstrated to be identical to murine RANKL. *B*, fluorescence-activated cell sorting (FACS) analysis demonstrating the membranic expression of RANKL (*dark open profile*) of 5T33MM cells, when compared with isotype-matched controls (*light open profile*).

Poole, United Kingdom). The number of TRAP-positive osteoclasts lining cancellous bone surfaces was determined as described previously (8).

Statistics. The unpaired Student *t* test was used to assess differences in tumor burden between OPG-treated and vehicle-treated control groups. The effect of Fc-OPG on survival was determined using a Kaplan-Meier analysis and a log-rank (Mantel-Cox) test.

Results and Discussion

MM is associated with a characteristic bone disease. Interactions between MM cells, soluble factors derived from MM cells, and the bone marrow microenvironment are critical for the recruitment of osteoclasts and for initiating and promoting the formation of lytic bone lesions. The RANKL/RANK system is believed to play a role in this process of osteoclast formation and the development of myeloma bone disease (1, 2, 8, 9). However, it is less clear whether inhibiting bone resorption with specific antagonists to this system has an effect on tumor growth and survival. In previous studies (9), treating 5T2MM-bearing mice for 4 weeks with Fc-OPG, demonstrated a 25% reduction in serum paraprotein. However, to evaluate any potential antitumor effect of altering the bone microenvironment by Fc-OPG a treatment from the time of inoculation of the tumor cells is important. Because a long-term treatment in the 5T2MM model (with a mean take-time of 12 weeks) caused the formation of antibodies against human Fc-OPG, which compromised the anti-osteoclast activity of the Fc-OPG, an alternative model was required. The 5T33MM model was chosen. This model has, compared with the 5T2MM model, a more aggressive growth, with a mean take-time of 4 weeks. Long-term treatment during the 4 weeks of the experiment did not generate detectable levels of antibodies against Fc-OPG in this model and, thereby, also excluded potential direct humoral antitumor effects. We first examined the expression of RANKL by 5T33MM cells. RT-PCR demonstrated expression of mRNA for RANKL (Fig. 1). Flow cytometric analysis confirmed expression of RANK on the surface of 5T33MM cells (Fig. 1). These data support previous reports demonstrating the expression of RANKL by 5T2MM cells and human MM cells (4–8) and suggesting that tumor cells may be able to interact directly with RANK-expressing cells such as osteoclasts and their precursors.

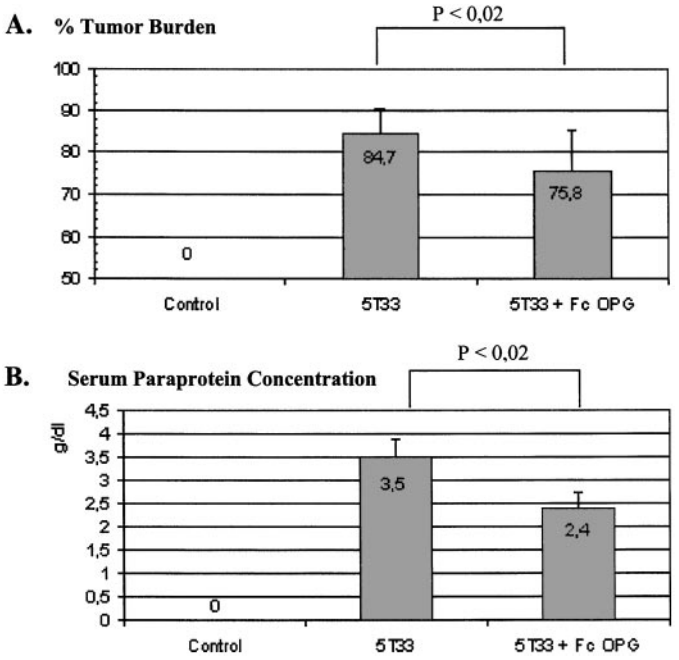

Fig. 2. The effect of treatment with Fc-OPG on 5T33MM-bearing mice. *A*, serum paraprotein concentration as determined by serum electrophoresis. *B*, tumor burden as determined by fluorescence-activated cell sorting (FACS) analysis. Data are expressed as percentage 5T33MM cells of total nuclear cell number.

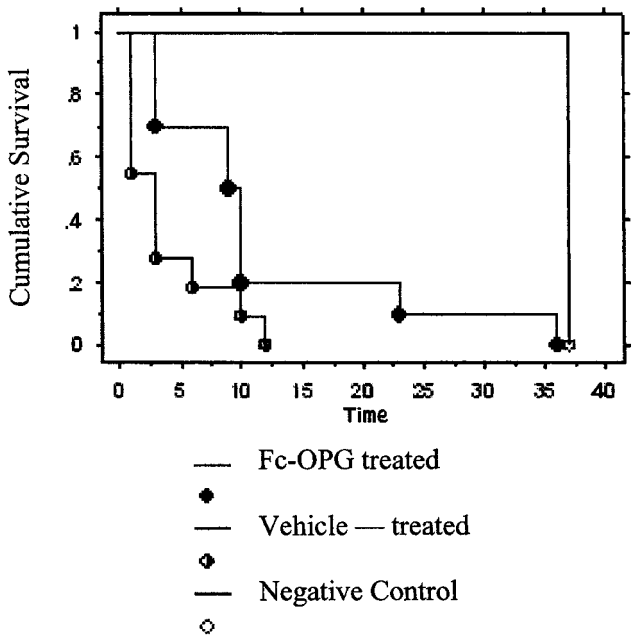

Fig. 3. Kaplan-Meier analysis showing the onset of morbidity. *Day 1*, the first day of onset of morbidity (4 weeks after initiation of the experiment). The onset of morbidity was delayed by a mean of 7.8 days (25%), Fc-OPG treatment when compared with vehicle treatment.

To evaluate the potential antitumor effect of Fc-OPG, mice were given injections of 5T33MM cells and treated with Fc-OPG. Treatment resulted in a 27% reduction in serum paraprotein concentration ($P < 0.02$; Fig. 2*A*) and a small but significant reduction in the proportion of 5T33MM cells in the bone marrow ($P < 0.02$, Fc-OPG-treated group *versus* vehicle-treated group; Fig. 2*B*).

To evaluate the effect of reduced tumor burden on survival, 5T33MM-bearing mice were treated with Fc-OPG in separate experiments and the time to onset of morbidity assessed. Kaplan-Meier analysis demonstrated a significant delay in the onset of morbidity (7.8 days, 25% increase, $P < 0.02$, Fc-OPG treated group *versus* vehicle; Fig. 3).

To determine whether these effects could be mediated by a direct effect on tumor cells or indirectly, by an effect on bone resorption, the effect of Fc-OPG on the apoptosis and proliferation of 5T33MM cells *in vitro*, and on osteoclast number *in vivo*, was determined. Annexin V/propidium iodide staining and DAPI staining of 5T33MM cells, followed by flow cytometry or examination of nuclear morphology, did not reveal any effect of Fc-OPG on the apoptosis of the 5T33MM cells (results not illustrated). Similarly, [^{3}H]thymidine incorporation showed no effect on proliferation.

Staining of osteoclasts for TRAP on sections for long bones demonstrated a reduction in the number of osteoclasts (7.00 +/− 0.25 for the 5T33MM bearing animals, 0 +/− 0 for the 5T33MM bearing animals treated with Fc-OPG). This is consistent with data reported in the 5T2MM model (8) and demonstrate that Fc-OPG is able to block osteoclast formation and may therefore alter the local bone microenvironment.

Taken together, these data strongly suggest that targeting the bone marrow microenvironment, by blocking interactions between RANKL and RANK with Fc-OPG not only inhibits the development of bone disease (8) but also results in a decrease in MM cell growth and an increase in survival. Because Fc-OPG had no direct effect on tumor cells but was able to inhibit osteoclast formation it is likely that this is an indirect effect via a change in the local microenvironment, rather than a direct antitumor effect.

References

1. Giuliani, N., Bataille, R., Mancini, C., Lazzarretti, M., and Barillé, S. Myeloma cells induce imbalance in the osteopotegerin/osteoprotegerin ligand system in the human bone marrow microenvironment. Blood, *98:* 3527–3533, 2001.
2. Pearse, R. N., Sordillo, E. M., Yaccoby, S., Wong, B. R., Liau, D. F., Colman, N., Michaeli, J., Epstein, J., and Choi, Y. Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. Proc. Natl. Acad. Sci. USA, *98:* 11581–11586, 2001.
3. Roux, S., Meignin, V., Quillard, J., Meduri, G., Guiochon-Mantel, A., Fermand, J. P., Milgrom, E., and Mariette, X. RANK (receptor activator of nuclear factor-κB) and RANKL expression in multiple myeloma. Br. J. Haematol., *117:* 86–92, 2002.
4. Oyajobi, B. O., Traianedes, K., Yoneda, T., and Mundy, G. R. Expression of Rank ligand (RankL) by myeloma cells requires binding to the bone marrow stromal cells via an α4β1-VCAM-1 interaction [abstract]. Bone, *5:* S180, 1998.
5. Shipman, C. M., Holen, I., Lippitt, J. M., Vandenberghe, E., and Croucher, P. I. Tumour cells isolated from patients with multiple myeloma express the critical osteoclastogenic factor, RANKL. Blood, *96:* 360a, 2000.
6. Sezer, O., Heider, U., Jakob, C., Zavrski, I., Eucker, J., Possinger, K., Sers, C., and Krenn, V. Immunocytochemistry reveals RANKL expression of myeloma cells. Blood, *99:* 4646–4647, 2002.
7. Altamirano, C. B., Neeser, J. A., Manyak, S., Tang, Y. G., Sjak-Shie, N., Okopnik, J., Ma, M. H., Parker, K. M., Vescio, R., and Berenson, J. R. Malignant multiple myeloma cells expressing RANKL induce the formation of TRAP positive multinucleated cells [abstract]. Blood, *98:* 637a, 2001.
8. Croucher, P. I., Shipman, C., Lippitt, J., Perry, M., Asosingh, K., Hijzen, A., Crabbs, A. C., van Beek, E. J. R., Holen, I., Skerry, T., Dunstan, C., Russell, G. R., Van Camp, B., and Vanderkerken, K. Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma. Blood, *98:* 3534–3540, 2001.
9. Yaccoby, S., Pearse, R. N., Johnson, C. L., Barlogie, B., Choi, Y., and Epstein, J. Myeloma interacts with the bone marrow microenvironment to induce osteoclastogenesis and is dependent on osteoclast activity. Br. J. Haematol., *116:* 278–290, 2002.
10. Radl, J., De Glopper, E., Schuit, H. R., and Zurcher, C. Transplantation of the paraprotein-producing clone from old to young C57Bl/LaLwRij mice. J. Immunol., *122:* 609–613, 1979.
11. Asosingh, K., Radl, J., Van Riet, I., Van Camp, B., and Vanderkerken, K. The 5TMM series: a useful *in vivo* mouse model of human multiple myeloma. Hematol. J., *1:* 351–356, 2000.
12. Vanderkerken, K., Asosingh, K., Braet, F., Van Riet, I., and Van Camp, B. Insulin like growth factor-I acts as a chemoattractant factor for 5T2 multiple myeloma cells. Blood, *93:* 235–241, 1999.
13. Vanderkerken, K., De Raeve, H., Goes, E., Van Meirvenne, Radl, J., Van Riet, I., Thielemans, K., and Van Camp, B. Organ involvement and phenotypic adhesion profile of 5T2 and 5T33 myeloma cells in the C57BL/KaLwRij mouse. Br. J. Cancer, *76:* 451–460, 1997.
14. Shipman, C. M., Rogers, M. J., Apperley, J. F., Russell, R. G., and Croucher, P. I. Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti tumour activity. Br. J. Haematol., *98:* 665–672, 1997.

Recombinant Osteoprotegerin Decreases Tumor Burden and Increases Survival in a Murine Model of Multiple Myeloma

Karin Vanderkerken, Evy De Leenheer, Claire Shipman, et al.

Cancer Res 2003;63:287-289.