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

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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 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 C57B1/KaLwRij 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-antigot 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 C57B1/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 Proliferation in Vitro. 5T33MM (105 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/105 cells; BD PharMingen, Erembodegem, Belgium) and propidium iodide (100 ng/ml/105 cells). 5T33MM cells were also cyt centrifuged 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). [3H]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,
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.
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 \((P < 0.02; \text{Fig. 2A})\) and a small but significant reduction in the proportion of 5T33MM cells in the bone marrow \((P < 0.02, \text{Fc-OPG-treated group versus vehicle-treated group; Fig. 2B})\).

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 \((7.8 \text{ days}, 25\% \text{ increase}, \ P < 0.02, \text{Fc-OPG treated group versus vehicle; Fig. 3})\).

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 \textit{in vitro}, and on osteoclast number \textit{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 analysis demonstrated a significant delay in the onset of morbidity \((0.25 \text{ for vehicle-treated group; Fig. 2A})\) versus \((0.02, \text{Fc-OPG treated group})\) (Fig. 2B).

Staining of osteoclasts for TRAP on sections for long bones demonstrated a reduction in the number of osteoclasts \((7.00, 7.8 \text{ days}, 25\% \text{ increase}, \ P < 0.02, \text{Fc-OPG treated group versus vehicle; Fig. 3})\).

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 \textit{in vitro}, and on osteoclast number \textit{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 analysis demonstrated a significant delay in the onset of morbidity \((0.25 \text{ for vehicle-treated group; Fig. 2A})\) versus \((0.02, \text{Fc-OPG treated group})\) (Fig. 2B).

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