Prolonged Antiresorptive Treatment of Lytic Prostate Cancer Xenografts in Mouse Bone Results in Tumor Necrosis


Bone Research Program, ANZAC Research Institute, University of Sydney at Concord, Concord NSW 2139, Australia [J. M. Blair, J. R. K. Modzelewski, M. J. Seibel]; Oncology Research Centre, University of New South Wales Department of Clinical Medicine, Prince of Wales Hospital, Randwick NSW 2031, Australia [L. A. Perryman, P. J. Russell]; Biomedical Engineering, AMME, University of Sydney, Sydney, NSW 2006, Australia [C. R. Dunstan]

AIM: In this study, we examine the time dependence of commencing antiresorptive treatments, using an Fc-fusion osteoprotegerin construct (Fc-OPG), on the growth, viability and lytic effects of PC3 human prostate cancer xenografts in male mouse bone.

METHODS: Male mice were implanted with PC3 cells by intratibial injection. Mice were randomized into three groups (n = 5–7 per group) to receive vehicle or 3 mg/kg Fc-OPG three times weekly from the week before cell injection (week -1; early treatment), week 0 (simultaneous treatment) or week 2 (delayed treatment).

RESULTS: When compared with vehicle-treated controls, Fc-OPG treatments protected bone and reduced bone turnover marker levels. In early and simultaneous Fc-OPG treated mice, necrosis of bone-enclosed tumor and the adjacent endosteal bone was observed. No tissue or bone necrosis was seen in sham-injected or vehicle-treated controls or in the contralateral tibiae of Fc-OPG treated mice.

CONCLUSION: The necrosis of bone-confined tumor in Fc-OPG-treated mice suggests that prolonged blockage of bone erosion during the early development of a tumor mass in bone can limit vascular supply, leading to necrosis. The loss of osteocyte viability was only seen adjacent to necrotic tumor, which suggests that this effect is not due to bone resorption inhibition per se but to interactions between inhibition of bone resorption and tumor effects.


Introduction

The establishment and development of skeletal metastases and their concomitant effects on serum calcium levels, bone stability and pain are known complications of advanced cancer of the prostate (CaP) [1]. The normal bone remodelling cycle of coordinated bone resorption by osteoclasts followed by formation of new bone by osteoblasts is disrupted by metastatic CaP to bone (reviewed in [2]). Although most CaP bone metastases are osteolytic and produce excess bone, osteolytic and mixed lesions have also been detected, even within the same patient [3] and there is a marked concomitant increase in bone resorption in patients with osteolytic lesions, as has been shown by analysis of serum bone turnover markers [4-6].

Bone resorption in healthy adult bone is regulated, at least in part, by RANK (receptor activator of NF-κB), RANK ligand (RANKL) and osteoprotegerin (OPG). RANKL is expressed by bone stromal cells and bone-forming cells and interacts with its cognate receptor, RANK, on osteoclasts and their precursors, initiating and maintaining a proosteoclastogenic stimulus that results in increased numbers and activity of osteoclasts [7,8]. OPG is a decoy receptor for RANKL that binds RANKL and prevents its interaction with RANK [9,10]. The central roles of these molecules were demonstrated by the deletion of RANKL or RANK resulting in osteopetrosis due to impaired osteoclastogenesis [11], or by deletion of OPG [12] leading to osteoporosis.

The OPG-RANKL-RANK axis also mediates the lytic effects of bone metastasis. Tumor cells secrete factors, such as parathyroid-related protein (PThRP), which increase the RANKL:OPG ratio, leading to lytic bone destruction by osteoclasts. Altered expression of OPG and/or RANKL has been demonstrated in bone metastases from cancers of the prostate, breast, lung, kidney, and thyroid gland [13-16]. Therapeutic targeting of OPG/RANKL/RANK has proven successful in mouse xenograft models by preventing or...
treating bone destruction due to multiple myeloma [17,18] and breast cancer [19]. Furthermore, early phase-I studies indicate that treatment with OPG is able to suppress bone resorption in human patients with either multiple myeloma or breast cancer bone metastases [20]. One antiresorptive agent, Fc-OPG, effectively reduced CaP-induced bone destruction for xenograft models in which there is an osteoblastic component [21,22].

Our current study sees to evaluate whether treatment (early treatment) with Fc-OPG can enhance its effects on tumor establishment and bone protection in osteolytic CaP tumors in mouse bone, relative to simultaneous or delayed Fc-OPG treatment.

Materials and Methods

Cell culture

All cell culture reagents were supplied by Invitrogen (Mount Waverley, Victoria, Australia) unless otherwise stated. The PC-3 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was maintained under standard tissue culture conditions in RPMI medium supplemented with 10% FBS. The cells were transfected with the phHygEGFP vector (Clontech, Mountain View, CA, USA) to express enhanced green fluorescent protein (EGFP) and were selected for over eight weeks in hygromycin B; however, the level of green fluorescent protein expression was not sufficiently strong to permit in vivo detection of fluorescence. Fc-OPG was supplied as a generous gift by Amgen, Inc. (Thousand Oaks, CA, USA).

In vitro analyses

Five thousand PC-3-EGFP cells per well were plated in triplicate into 96-well plates and were allowed to adhere overnight. The plating media were replaced with RPMI and 0.1% FBS including vehicle or 5-80 µg/ml of Fc-OPG. Cells were tested for viability after 6-day incubation using the WST-1 mitochondrial activity assay (Takara Bio Inc., Shiga, Japan) and the Cytotox96 assay (Promega Co., Annandale, NSW, Australia). All experiments were performed three times independently.

In vivo studies

All mouse experiments were approved by the University of New South Wales institutional Animal Care and Ethics Committee. Five to seven week-old male non-obese diabetic severe combined immunodeficient (NOD-SCID) mice were housed under sterile specific pathogen-free conditions and were fed mouse chow and water ad libitum.

Following a pilot study to determine the time course of tumor development, in which injection of $4 \times 10^6$ PC-3-EGFP cells in 20 µl PBS into the tibia produced detectable lytic tumors in 100% of the mice within 6 weeks of cell injection (data not shown), mice were randomised into three groups (n = 5–7 per group) to receive vehicle or 3 mg/kg Fc-OPG three times weekly from the week before cell injection (week -1; early treatment), week 0 (simultaneous treatment) or week 2 (delayed treatment). The dose of Fc-OPG was previously shown to be effective in preventing bone destruction in a highly lytic breast cancer xenograft model [19]. Radiographic images were obtained using a digital MX-20 specimen radiography system (Faxitron X-ray Co., Wheeling, IL, USA). Radiographic images of some week 6 mice were previously published to illustrate lytic lesions [16]. Age-matched un.injected and sham-injected mice (injected with PBS into the left tibia, n = 4 per group) were included as controls. All mice were euthanased by over-inhalation of anaesthetic in week 6.

Serum analysis

Serum was prepared from the blood samples obtained at weeks 0, 2 and 6, and was frozen in aliquots at -80°C, pending analysis of bone turnover markers. Levels of mouse tartrate-resistant acid phosphatase type 5b (TRAP5b), a marker of bone resorption, were analysed by ELISA (Suomen Bioanalytikka, Finland). The sensitivity was 10^4 U/ml and intra-assay and inter-assay variations were 4.2% and 5.5%, respectively. Those of mouse osteocalcin, a marker of bone formation, were analysed by immunoradiometric assay (Immunotopics International, LLC, San Clemente, CA, USA) with a sensitivity of 0.1 ng/ml and an intra-assay variation of 3.1%. Both assays were performed according to the manufacturers’ instructions.

Microtomography (µCT) analysis

At sacrifice at week 6, the tumor-bearing and contralateral control tibias were harvested and fixed at 4°C in 70% ethanol prior to analysis using a SkyScan 1072 desktop X-ray microtomograph (SkyScan N.V., Aartselaar, Belgium) set at an X-ray tube voltage of 80 kV and a current of 100 µA, and using a 1-mm aluminium filter. Specimens were rotated through 180° in 0.5° increments. Three hundred and fifty coronal slices were obtained for each tibia and 178 of these slices were used in the analysis. The bone region of interest was manually identified on each slice, excluding 2 mm immediately distal to the growth plate and extending 4 mm distally into the tibial shaft. Bone volume (BV) was

Figure 1: Effects of Fc-OPG treatment on the proliferation and viability of PC-3-EGFP cells in vitro. Following exposure to 5-80 µg/ml Fc-OPG or vehicle control for one week, PC-3-EGFP proliferation and viability were measured using WST-1 (A) and CytoTox96 (B), respectively. Data are expressed as ratios to vehicle-treated control and were analyzed by parametric one-way ANOVA followed by Bonferroni’s post-tests for multiple comparisons. Data are presented as the mean ± SD of three independent experiments.
calculated using the marching cubes volume model. Tissue volume was not analysed as tumor had compromised the cortex in the vehicle control tibias. All data were expressed as percentages relative to the contralateral control leg from the same mouse.

**Tissue analysis**

After μCT analysis, bone tissues were decalcified in 10% EDTA, pH 7.0 for one week and were then processed and embedded in paraffin and sectioned for standard histological analysis. Three 5-μm sections at 250-500 μm separation were obtained in the sagittal plane of the paraffin-embedded, decalcified bone specimens and were analysed in a blinded manner under 200× magnification for total intramedullary tumor area, intramedullary necrotic tumor area, total cortical bone, osteoclast surface, and necrotic bone area. Due to the extensive bone destruction, particularly in mice treated with vehicle, it was frequently not possible to differentiate intramedullary versus extraskeletal tumor masses. Thus, for all mice, tumor area and tumor necrosis area were measured over the whole section, including tumor both associated with the bone and tumor expanded into the surrounding soft tissues. Data were averaged across the three sections and were expressed as mean values.

**Statistical analyses**

Data were analysed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Normally distributed data were analysed by one-way ANOVA and two-way ANOVA (general linear model, GLM) followed by Tukey’s or Bonferroni post-tests as indicated. *In vivo* experiments produced data for some parameters that were not normally distributed; therefore, for these data, non-parametric one-way ANOVAs (Kruskal-Wallis H tests) and non-parametric GLM tests followed by Bonferroni post-tests were used. Significance was accepted where *p* < 0.05. Data were found to be statistically homogeneous for all measured variables for the tumor-inoculated control mice commencing vehicle treatment at the different times.

**Results**

**In vitro study**

Fc-OPG treatment at concentrations up to 80 μg/ml did not alter PC-3-EGFP cell proliferation and marginally decreased an index of endogenous cytotoxicity, and so was unlikely to impair tumor growth directly (Figure 1).

**Table 1: Measurement of bone volumes (BV) by μCT in the tibial metaphysis**

<table>
<thead>
<tr>
<th></th>
<th>BV (% of contralateral limb)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham controls</td>
<td>85.2 ± 15.4</td>
</tr>
<tr>
<td>Early vehicle</td>
<td>65.0 ± 15.1</td>
</tr>
<tr>
<td>Early Fc-OPG</td>
<td>113.2 ± 12.5</td>
</tr>
<tr>
<td>Simultaneous</td>
<td>44.6 ± 37.0</td>
</tr>
<tr>
<td>Simultaneous Fc-OPG</td>
<td>136.5 ± 35.9*</td>
</tr>
<tr>
<td>Delayed vehicle</td>
<td>54.7 ± 30.3</td>
</tr>
<tr>
<td>Delayed Fc-OPG</td>
<td>130.6 ± 60.2*</td>
</tr>
</tbody>
</table>

*Results were expressed as percentages relative to contralateral controls; for each treatment group, data are shown as mean ± SD. n = 4-7 mice per group.

*P < 0.05 vs. vehicle, **P < 0.001 vs. vehicle.

**Effects of Fc-OPG treatment on serum markers of bone turnover**

Systemic levels of bone resorption, as indicated by serum mouse TRAP5b levels, were decreased profoundly to a similar extent in all groups following commencement of Fc-OPG treatment. This suppression was maintained until the end of the study (Figure 2A). By week 6, serum osteocalcin was also significantly reduced in the Fc-OPG-treated groups when compared with their respective vehicle-treated controls (Figure 2B). Inoculation with tumor cells did not change serum osteocalcin or mouse TRAP5b levels in vehicle-treated mice compared to sham-injected controls.

**Effects of Fc-OPG treatment on bone radiographic changes**

All vehicle-treated mice bearing intratibial tumors exhibited profound bone destruction by both standard radiographic (Figure 3A-3E) and μCT analyses (Figure 3F-3J), including loss of trabecular bone and destruction of the cortical bone. Although variation was found in vehicle-treated mice, BV was not significantly different between groups commencing vehicle treatment at different times. Treatment with Fc-OPG effectively inhibited bone destruction with all treatment regimens (Figure 3C-3E and 3H-3J, Table 1) though small perforations of the cortex were seen in some mice on delayed treatment (Figure 3J). In the early prophylaxis arm of the study, the Fc-OPG-treated mice displayed accumulation of mineralized cartilage and bone adjacent to the growth plate in both the control and tumor-bearing tibias (Figure 3C). This is a known effect of antiresorptive treatments of young growing animals with either bisphosphonates or OPG [9,23]. This effect was diminished in the simultaneous and delayed
treatment arms, consistent with the shorter duration of treatment and the reduced growth rate of mice at the ages when treatment was commenced.

Effects of Fc-OPG treatment on bone and tumour: histological and histomorphometric assessment

Tumor deposits were consistently seen in the medullary cavity of the tibiae in all groups (one representative example from each group is shown in Figure 4 under 4× (upper panel) and 30× (lower panel) magnification. Tumor area ranged considerably in this study (Figure 5A); in addition, due to the aggressive growth of this tumor and the relatively long duration of the study, large tumor masses were also frequently seen in the surrounding soft tissues in all treatment groups, presumably as a result of cortical bone destruction or by migration through spaces in the cortical bone around vessels. Two-way analysis of variance indicated a significant but modest inhibitory effect of Fc-OPG treatment overall on tumor area (Figure 5A, P = 0.038), though significance was not reached for any individual treatment group. Tumor area was quite variable largely due to the different size of extraskelatal tumor masses, which were carefully retained during specimen preparation.

Tumor necrosis was found in the tibiae of a majority of mice in the early (5/6) and simultaneous (4/7) treatment groups (Figures 4 and 5B). Necrosis extended over a large area of the medullary cavity and appeared to affect tumor and host cells, including endothelial cells and osteocytes in adjacent bone. Nonviable bone was observed adjacent to necrotic tissue in the medullary cavity and was restricted to a region that averaged 90 ± 33 μm in width adjacent to the endosteal surface of the bone (indicated by arrows in Figure 4B). Significant tumor necrosis was not seen in extraskelatal tumor masses, even when several millimeters in diameter. In some mice treated with Fc-OPG (6/15), necrotic areas in the tibiae showed evidence of recovery with invasion of fibroblast-like cells and blood vessels. While small foci of tumor necrosis were occasionally seen in vehicle-treated mice (3/15), extensive tumor necrosis or the presence of nonviable bone was not seen in tumor-bearing, vehicle-treated mice, and nonviable bone was not seen in the non-tumor-bearing bones of Fc-OPG-treated mice.

Histologic evaluation confirmed that bone destruction was inhibited by Fc-OPG treatment. Destruction of cortical bone, with perforation and/or erosion of endosteal surfaces, and loss of trabecular bone was clearly most pronounced in vehicle-treated mice, though it was also apparent with delayed treatment (Figure 5C). Early treatment with Fc-OPG increased BV significantly, in part due to the growth-related accumulation of bone adjacent to the growth plate (Figure 4). This effect was diminished with later treatment groups. Fc-OPG treatment reduced osteoclast numbers similarly

Figure 3: Effects of PC-3-EGFP tumor growth and Fc-OPG on the radiographic (A-E) and μCT (F-J) appearance of the injected tibiae. Normal bone morphology is shown in sham-injected mice (A, F) while profound bone destruction is evident in vehicle-treated mice (B, G). Mice treated with Fc-OPG from early (C, H), simultaneous (D, I) and late (E, J) time points show preservation of gross bone structure.

Figure 4: Haematoxylin and Eosin stained sections of the proximal region of tibiae at low magnification (upper panel, 4× objective) or high magnification (lower panel, 30× objective). Early and simultaneous Fc-OPG treatment showed preservation of cortical bone and induction of tumor necrosis (N). Tumor necrosis was associated with necrosis of osteocytes in adjacent bone. The boundary of necrotic bone is indicated by arrows.
regardless of time of commencing treatment (Figure 5D and Table 2) consistent with changes seen in TRAP5b levels.

**Discussion**

The treatment regimens selected in our study were designed to examine effects of the antiresorptive treatment upon establishment versus expansion of the intratibial tumors. All regimens of Fc-OPG treatment effectively inhibited bone destruction mediated by the PC-3-derived cells in vivo as determined using radiography, microcomputed tomography, serology, and tissue analyses. The inhibition of bone resorption was associated overall with a modest reduction in tumor area, though this did not reach significance for individual treatment groups. Inhibition of tumor growth in this and other cell lines by Fc-OPG or bisphosphonates has been reported previously (reviewed in [16]). By radiography, both the incidence and size of lytic lesions were decreased by Fc-OPG treatment, and Fc-OPG treatment was effective when commenced before or after tumor establishment but gave best bone protection when provided from early time points. However, histological analysis showed that tumor was readily detectable in all PC-3-EGFP-injected mice, indicating that presence of normal bone resorption was not a requirement for tumor establishment in this model. This is supported by the findings of Kiefer et al. [22] in their study of the effects of Fc-OPG on the establishment and growth of the osteoblastic LuCaP 23.1 prostate cancer xenograft, but is in contrast with the findings of others that the establishment of a mixed prostate cancer xenograft [21] and lytic myeloma [23] in bone was inhibited with Fc-OPG treatment. These differences could be due to how the various cancer cell lines interact with bone, as well as dosage and timing differences. The study with LuCaP 23.1 cells used a higher dose (6 mg/kg, three times
weekly) though effects on bone resorption and tumor-associated bone destruction were similar. Analysis of the mouse TRAP5b serum marker of bone resorption and histological assessments showed that Fc-OPG profoundly reduced but did not eliminate bone resorption from the time of treatment initiation; it is therefore possible that these low bone resorption levels are sufficient for the PC-3-EGFP cell line to establish in bone, although PC-3-EGFP cells do not require the bone environment for growth, as they can grow robustly either in vitro or at subcutaneous sites (data not shown). The lack of increased TRAP5b in the tumor-bearing mice not treated with Fc-OPG, is surprising, reflecting that even though locally lytic, the amount of bone resorption induced by the tumor was too small relative to the total skeletal levels of bone resorption to significantly elevate levels of this resorption marker. In addition to its effects upon bone resorption, Fc-OPG treatment inhibited bone formation, as determined by significant decreases in mouse serum osteocalcin levels, and a limited localized periosteal bone formation response was observed in mice wings tumor had perforated the cortical bone, indicating that some repair responses were preserved. Fc-OPG-treated mice exhibited viable bone and tumor in these extraosseous sites.

We found that pretreatment (early treatment) of mice with Fc-OPG significantly affected the viability of bone-enclosed tumor in mice receiving PC-3-EGFP cells by intratibial injection, leading to substantial areas of tumor necrosis. This effect was also seen, though to a lesser extent, when treatment commenced at the time of tumor inoculation (simultaneous treatment). However, tumor that had escaped the bone environment to grow in the soft tissues surrounding the bone did not show similar extensive necrosis in any group, suggesting that Fc-OPG treatment had no direct effect upon tumor; this is supported by our in vitro observations that Fc-OPG treatment did not increase cytotoxicity and had no effect upon tumor cell viability. RANKL can both induce angiogenesis [24] and endothelial cell survival [25]; therefore, Fc-OPG treatment could inhibit these processes and compromise vasculature. This would be expected to result in hypoxia and a microenvironment poor in nutrients, growth factors and survival factors. However, no vascular effects were seen in non-tumor-bearing limbs and have not been reported in RANK- or RANKL-deficient mice or in transgenic mice overexpressing OPG [9], suggesting that the RANKL pathway is not critical for maintaining angiogenesis. It is thus likely that tumor necrosis was produced as a result of indirect effects of Fc-OPG treatment on the bone. Bone serves as a physical barrier to tumor growth: as a tumor grows, it increases the medullary pressure on the vascular supply and Fc-OPG, by its ability to block osteoclastogenesis and bone resorption, maintains the integrity of the bone structure, which could result in mechanical ischaemia, as has been suggested previously (reviewed in [26]). The antiresorptive functions of Fc-OPG would also be expected to have growth-supporting effects from the extracellular matrix [27] and decrease the availability of tumor cell adhesion sites that would otherwise be available due to exposure of integrins and other adhesion molecules [28].

Of note was the finding that the endosteal bone was also affected, as evidenced by a band of necrotic osteocytes approximately 80 μm in width in areas adjacent to necrotic tumor. Such loss of osteocyte viability was only seen adjacent to necrotic tumor and was not seen in periosteal regions of the cortical bone or in the contralateral bone of Fc-OPG-treated animals, which suggests that this effect is not due to osteoclast (bone resorption) inhibition per se. Similar observations of increased avascular bone marrow and trabecular bone necrosis have been observed in patients with extremely large skeletal tumors and this phenomenon likely occurs as a result of the expansion of the tumor within the medullary cavity of the bone leading to a loss of vascular supply [29,30]. Whilst large bands of bone necrosis were not observed in the tumor-bearing vehicle-treated mice, we did find infrequent small foci of nonviable osteocytes. One would expect fewer nonviable osteocytes because increased osteoclastic bone resorption would cause extensive bone remodeling, including any sites of pre-existing endosteal necrosis, and because breakthrough of the cortical bone would allow significant vascular supply to both the tumor and endosteum, reducing necrosis.

Osteonecrosis of the jaw (ONJ) is a known clinical side-effect of prolonged antiresorptive treatment using bisphosphonates, particularly in patients receiving high doses for treatment of metastatic bone disease or multiple myeloma. Unlike the avascular necrosis in tumor-bearing bones observed in the current study, ONJ in patients treated with bisphosphonates is usually not associated with metastatic foci in the jaw but is coincident with dental trauma, such as surgery or tooth extraction, or periodontal disease, although spontaneous ONJ has also been reported. ONJ has been reported in patients with bone-metastatic breast and prostate cancer whom have been treated with bisphosphonates [31,32]. Mechanisms involved in bisphosphate-associated ONJ are unclear but are likely to include the possibility that a combination of strong inhibition of bone resorption by bisphosphonate and concurrent dental pathology might impair bone vascular supply and, thus, tissue viability. Alternative pharmacologic actions of bisphosphonates might also be responsible for the induction of ONJ: for example, nitrogen-containing bisphosphonates can induce apoptosis if sufficient levels of the drug are internalized by cells [33], which could affect wound repair processes. Bisphosphonates have also been found to have direct antiangiogenic effects [34], which, if demonstrated in jaw tissues, would result in a direct induction of avascular necrosis or impairment of tissue repair. It is also possible that bisphosphonates may directly modulate inflammation or healing of mucosa, perhaps by inhibition of macrophage activity, as demonstrated by in vitro studies [35,36].

In summary, we found that Fc-OPG had efficacy in treating lytic prostate cancer xenografts in mouse bone by preserving gross bone architecture and by producing intrabone tumor necrosis. To our knowledge, this necrotic action of antiresorptive treatments has not been previously reported. We have not yet determined the mechanism of Fc-OPG action in prostate cancer tumor growth in bone; however, since focal osteonecrosis was found in the endosteal bones adjacent to the intrabone tumors, these data suggest that use of antiresorptive strategies as part of a combination therapeutic approach, with consideration of the long-term implications for bone health, are warranted in patients with profoundly lytic prostate cancer bone metastases.

Acknowledgments

Funding was provided by Amgen Inc., Cure Cancer Australia Foundation and the Clive and Vera Deyl Foundation. The authors wish to thank Kevin McNamara (ARI), Mila Sajinovic, Jayne Lelliott, Hnin Pwint and Kylie Deguara (ORC) for their assistance with histology and in vivo studies, Ms. Katie Levick and Ms. Jenny Norman of the Electron Microscope Unit, University of New South Wales, for assistance with μCT analysis, and Dr. Bill Dougall, Amgen Inc., for helpful discussions.

Disclosure Statement: Amgen Inc. provided part of the funding for this study. Dr. Colin R. Dunstan is a stock-holder of Amgen, Inc.
References