Pathogenesis and treatment of skeletal metastasis : studies in animal models

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Chapter 5

Inhibition of Bone Resorption and Growth of Breast Cancer in the Bone Microenvironment

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Abstract

Breast cancer frequently metastasizes to bone, where tumor cells induce osteoclasts to locally destroy bone. During bone resorption, growth factors are locally released that may support bone metastatic growth. Differently from most other tissues, drugs that can limit local turnover are available for bone.

We examined the hypothesis that inhibition of bone resorption by two different mechanisms may also affect the growth of cancer cells in bone. For this, we have tested the effects of high doses of osteoprotegerin (OPG) and zoledronic acid (ZOL), the most potent bisphosphonate available, on progression of breast cancer cells in the bone microenvironment using whole body bioluminescent imaging (BLI).

Both treatments significantly inhibited the development of radiographically detectable osteolytic lesions. Histological examination corroborated the radiographic findings, showing that both treatments preserved the integrity of bone trabeculae and prevented bone destruction, (significantly higher trabecular bone volumes vs. vehicle). However, whereas practically no TRAcP-positive osteoclasts were observed in tibiae preparations of animals treated with Fc-OPG, TRAcP-positive osteoclasts were still present in the animals treated with ZOL. Intra-bone tumor burden was reduced upon ZOL and Fc-OPG treatment. Although there appeared to be a trend for less overall total tumor burden upon treatment with both compounds, this was not significant as assessed by BLI and histomorphometrical analysis due to the extramedullary growth of cancer cells which was not affected by these treatments.

Collectively, anti-resorptive agents with different mechanisms of actions — ZOL and FcOPG — significantly reduced cancer-induced osteolysis and intraosseous tumor burden, but failed to restrain local tumor growth. However, interference with the bone microenvironmental growth support could still be of therapeutic relevance when given to patients early in the course of bone metastatic disease.

Introduction

Micrometastases persisting in various tissues of cancer patients after removal of the primary tumor represent the pathophysiological basis for cancer relapse as overt metastases. The survival of these cells and the development of metastases depend on the growth support provided by the microenvironment and the ability of cancer cells to adapt to this environment [1-3]. Bone provides a favorable microenvironment for the survival and growth of certain common cancers, including breast cancer which metastasizes frequently to the skeleton and causes significant morbidity and deterioration of life [4-6].

Studies in animal models of bone metastases strongly suggest that the rate of bone turnover enhances the occurrence and progression of metastases in the bone-bone marrow microenvironment [7-9]. These findings are supported by clinical studies which showed a significant association between the rate of bone resorption, assessed by biochemical markers of bone remodeling, and disease progression in the skeleton [10,11]. It was, therefore, hypothesized that suppression of bone resorption by agents that inhibit the formation and/or activity of the osteoclasts may not only protect skeletal integrity in metastatic disease but may also affect local tumor growth.

Previous studies of animal models with bone metastasis from breast cancer treated with bisphosphonates showed that reduction of bone turnover prior to bone colonization by cancer cells, could significantly reduce the number and progression of bone metastases [12-15]. However, when bisphosphonates were given to animals with established bone metastases they did not consistently affect the growth potential of the breast cancer cells in the bone microenvironment, despite a substantial anti-osteolytic effect [12,13]. There are several potential explanations for these findings: tumor growth response is related to the magnitude of suppression of bone resorption and, hence, the potency, and/or the dose (dosing regimen) of the bisphosphonates given; the fundamental mechanism of inhibition of bone resorption by the bisphosphonates; the initial growth phase of cancer cells in bone is dependent upon the interaction with the bone microenvironment, but eventually becomes independent of the bone microenvironent and can progress autonomously [13,50]. To address these questions we assessed the effects of very high doses of the most potent bisphosphonate currently available, zoledronic acid (ZOL), and we compared these to the effects of osteoprotegerin (Fc-OPG), that inhibits bone resorption by a different mechanism of action, in an experimental model of intra-tibial injection of human breast cancer cells.

MATERIALS & METHODS

Cell lines and culture conditions

The luciferase expressing bone-seeking clone MDA-MB-231 (MDA-231)-B/Luc+ was cultured as previously described [16,17]. Cell suspensions of MDA-231-B/Luc+(1.0 x 10^5 cells /10 µl PBS) were prepared for intraosseous injection as described previously [13,17].

Animals

Female nude (BALB/c *nu/nu*) mice were purchased from Charles River (L'Arbresle, France) and were used for the studies with human MDA-231-B/Luc+breast cancer cells.

Mice were housed in individual ventilated cages under sterile condition. Sterile food and water were provided *ad libitum*. Mice were 6 weeks old when used for intraosseous inoculation of cancer cells. Animal experiments were approved by the local committee for animal health, ethics and research of Leiden University and carried out in accordance with European Communities Council Directive 86/609/EEC.

For surgical and analytical procedures (intraosseous inoculation, BLI, radiography) mice were anesthetized by intraperitoneal injection of a 50 µl 1:1:1 mixture; Ketamine HCl (Stock solution of 100 mg/ml Nimatek; Vetimex Animal Health B.V., Bladel, The Netherlands) + Xylazine (2 % Rompun, Bayer AG, Leverkusen, Germany) + PBS (pH 6.8). Intracardiac inoculation of cancer cells was performed under Isofluorane anesthesia (0.8 L/min, Isofluorane, Air Products, Waddinxveen, The Netherlands) using the Vapex3 system (VetTech Solutions Ltd, United Kingdom). At the end of the experimental period animals were sacrificed by cervical dislocation.

Histomorphometry, Histochemistry and Immunohistochemistry

Dissected long bones were fixed in 4 % paraformaldehyde (pH 6.8), decalcified as described previously and processed for paraffin embedding [16,18]. Five micrometer longitudinal sections were cut through the sagittal plane of the tibiae containing tumors induced by the intra-bone inoculation of MDA-231-B/Luc+cells. Sections were either submitted to Goldner staining or histochemical staining for Tartrate Resistant Acid Phosphatase (TRAcP) as described previously [13].

Histomorphometric analysis of tumor burden was performed on central sections through the tumor (largest tumor area) as described previously [13]. Subsequently, a distinction was made between intraosseous and extramedullary tumor burden. For this, the digital image of the total tumor area was subdivided into an area delimited by the bone cortex or, were this has been partially resorbed as a result of the tumor-induced osteolysis, by a virtual line joining the remnants of the bone cortex, to define 'intraosseous' tumor growth. Evidently, the intraosseous, or intra-bone, tumor burden was critically selected between the bone trabeculae. Accordingly, the extramedullary tumor growth was defined as tumor cells growing surrounding the bone cortex or its remnants.

Histomorphometric measurements of trabecular bone volumes were performed after Goldner staining on the same central sections as used for tumor burden measurements. Trabecular bone volume (TBV) was estimated in the proximal tibia by measuring the total area of trabecular structures in an area 0-2 mm distal to the capillary invasion front of the growth plate. TBV is expressed as percentage of the total area that was covered by trabeculae.

Statistical Analyses

All data are represented as means ± SE. Statistical evaluation was carried out by ANOVA using post-hoc of LSD.

RESULTS

Effect of ZOL and Fc-OPG on bone destruction

Compared to vehicle treatment, both treatments inhibited significantly the development of radiographically detectable osteolytic lesions (Fig. 1). There was no significant difference between the two treatment groups, but Fc-OPG prevented completely the development of radiographically evident osteolytic lesions.

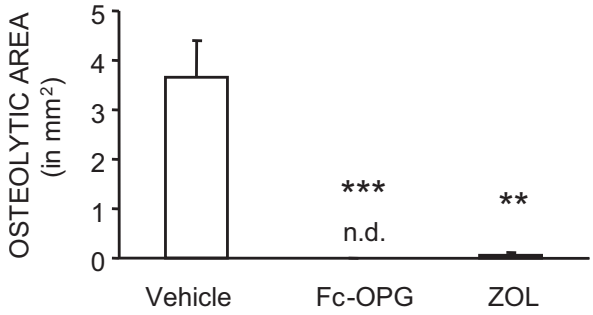

Figure 1 **Radiographically evident osteolytic lesions after 42 days of experimental treatment with Fc-OPG or ZOL.** n.d. = not detected; ** p <0.01, *** p < 0.001 compared to vehicle.

Histological examination of tibiae inoculated with tumor cells corroborated the radiographic findings (Fig. 2*A*). In vehicle-treated animals trabecular bone was destroyed and was replaced by tumor. In contrast, treatment with either ZOL or Fc-OPG preserved the integrity of bone trabeculae and prevented bone destruction (Fig. 2). In addition, trabecular bone volume (TBV) was similar between the two actively treated groups and significantly higher than in animals treated with vehicle alone (Fig. 2*B*).

The effect of both ZOL and Fc-OPG on bone destruction was due to their action on bone resorption as evidenced by the significant decrease in TRAcP-positive (TRAcP+) osteoclasts (Fig. 3). However, whereas practically no TRAcP+ cells were observed in tibiae preparations of animals treated with Fc-OPG, such cells were still present in the animals treated with ZOL (Fig. 3*B*). Though, in the ZOL-treated animals, many of the TRAcP+ osteoclasts that are still present are not tightly attached to the bone surface and represent non-active osteoclasts not capable of resorbing bone (Fig. 3*A*). This discrepancy between the effects on osteoclasts of the two agents is probably due to their different mechanisms of actions. Fc-OPG inhibits the formation and activity of osteoclasts whereas ZOL acts primarily on mature osteoclasts and

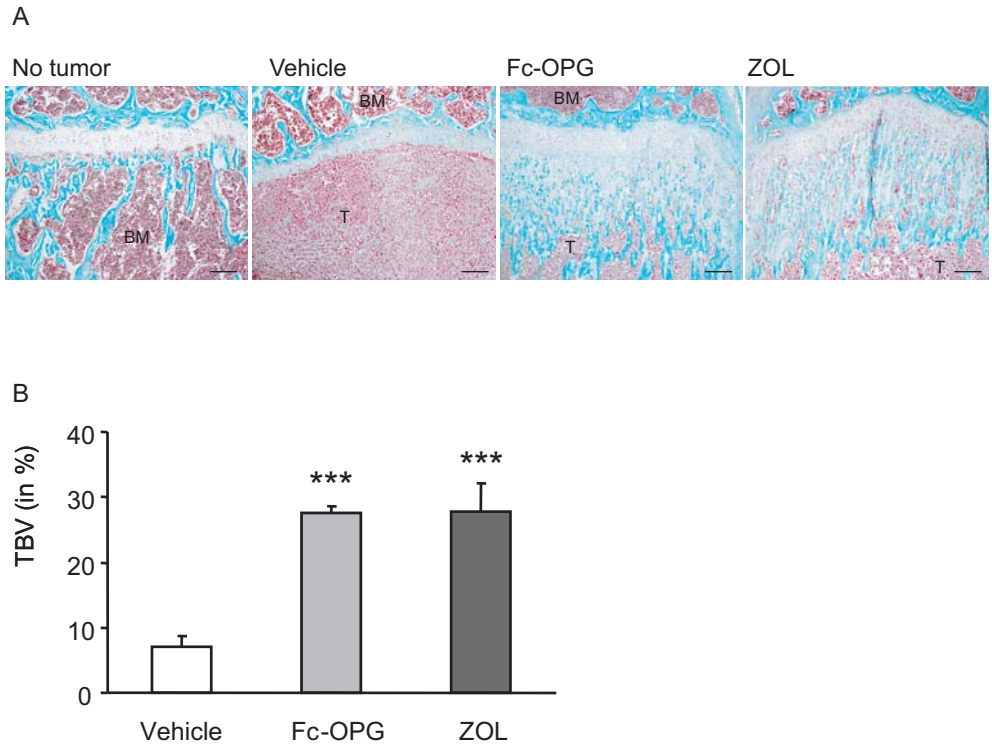

Figure 2 **Effects of Fc-OPG and ZOL treatment on bone trabeculae as shown by Goldner staining.** *A,* Fc-OPG and ZOL treatment inhibited cancer-induced bone destruction leading to a significant increase in trabecular bone area when compared with vehicle treated animals. Scalebars = 100µm; BM = bone marrow; T = tumor. *B,* trabecular bone volume (TBV) was significantly increased by Fc-OPG and ZOL treatment to a comparable extent when compared with vehicle treatment as determined by histomorphometric analysis. *** p <0.001 compared to vehicle.

increases their rate of apoptosis. Therefore, the TRAcP+ cells persisting in the ZOL treatment group are probably non-functioning osteoclasts, as shown previously [20-22].

Effect of ZOL and Fc-OPG on tumor growth

A. ZOL and Fc-OPG did not decrease the total tumor growth in the hindlimb assessed by BLI. Representative examples of animals treated with vehicle, ZOL or Fc-OPG are shown in figure 4*A* and the overall results of treatment are depicted in figure 4*B*. Although there appeared to be a trend for less tumor burden with both active treatments at the end of the experiment, this was not significantly different from vehicle-treated animals.

B. Histologically, the intra-bone tumor burden was significantly decreased with both treatments (Fig. 5*B*), but cancer cells were still present in bone marrow spaces (Figs. 2*A*, 3*A* and 5*A*). In contrast, extramedullary growth of cancer cells was not affected (Fig. 5*B*).

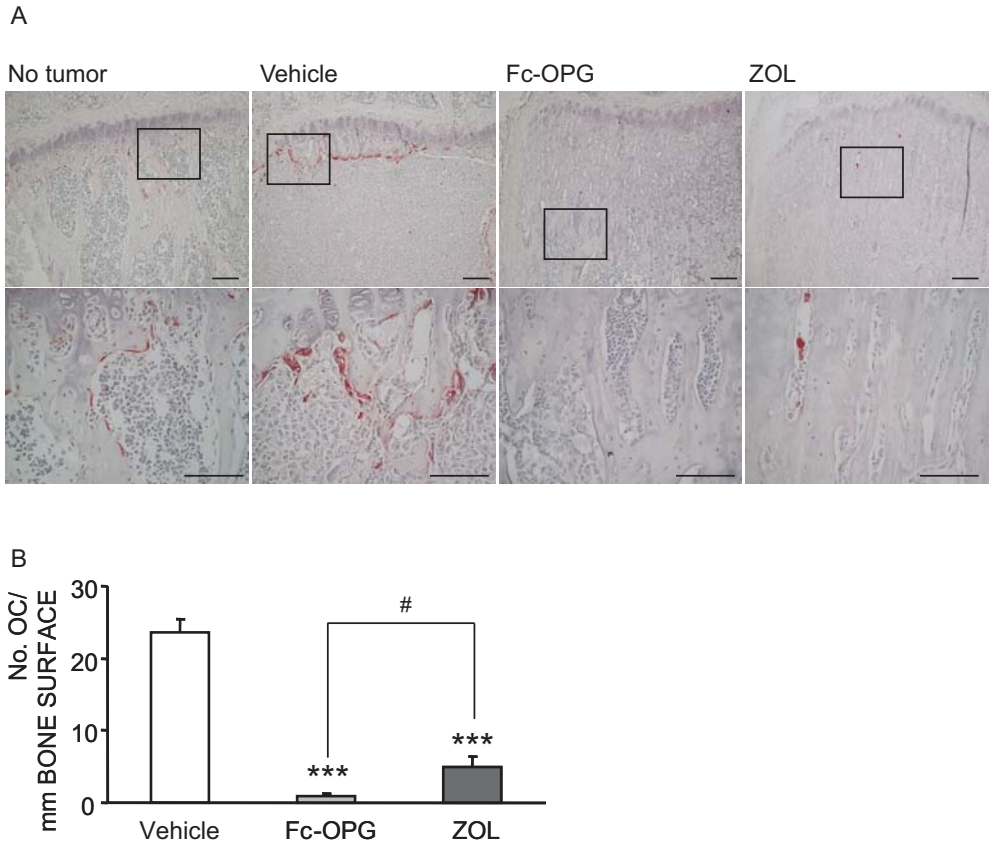

Figure 3 **TRAcP staining to examine osteoclast numbers.** Osteoclasts were stained red with TRAcP staining. *A,* in the ZOL treated animals, many of the TRAcP+ osteoclasts that are still present are not tightly attached to the bone surface and represent non-active osteoclasts not capable of resorbing bone. In the Fc-OPG group the osteoclasts were almost totally eradicated. Selected areas in the figures at the top are enlarged in the figures at the bottom. Scalebars are 50μm (figures at the top) and 80μm (figures at the bottom). *B,* both Fc-OPG and ZOL treatment significantly reduced the number of osteoclasts compared to vehicle treatment. In addition, Fc-OPG treatment resulted in a superior deletion of osteoclasts when compared to ZOL treatment. *** $p < 0.001$ compared to vehicle; # $p < 0.05$.

The overall tumor burden (combined intra-bone and extramedullary growth) decreased, but not significantly so, which is full agreement with the BLI data.

DISCUSSION

In this study we tested the hypothesis that suppression of bone resorption could reduce the growth and progression of breast cancer cells in the bone microenvironment. For this we have used a mouse model of intra-tibial injection of cancer cells as a model for late events

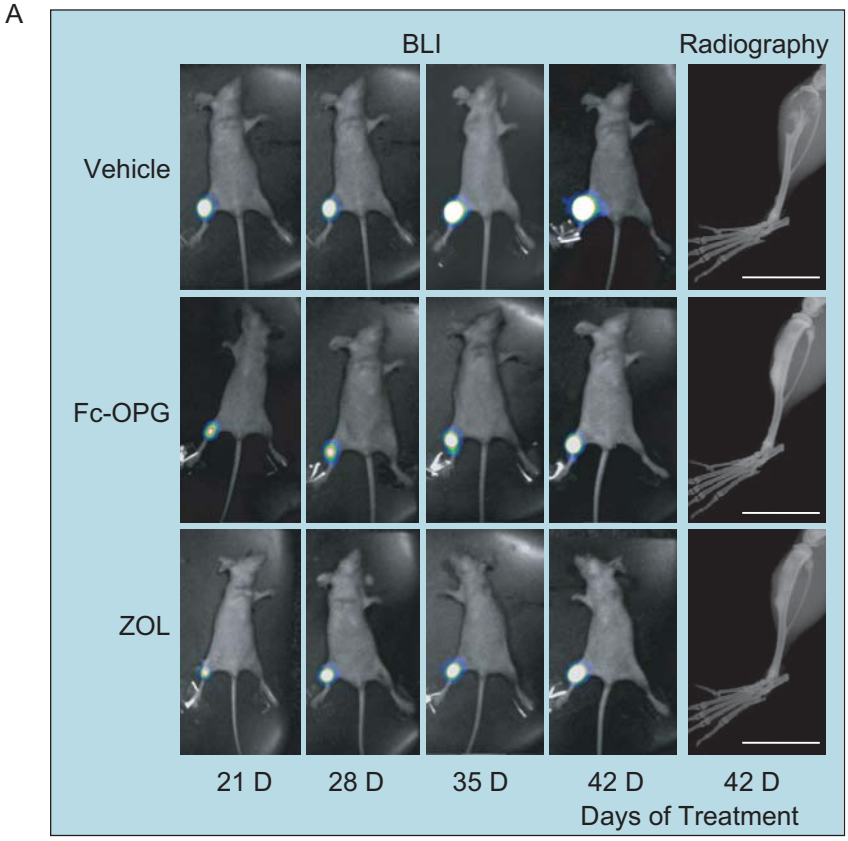

Figure 4 **Tumor growth as detected by bioluminescent imaging.** *A*, representative examples of Fc-OPG and ZOL treated mice with intraosseous tumor growth and tumor-induced bone destruction as detected by bioluminescent imaging (BLI) and radiography. Scale bars in X-rays: 10 mm. *B*, tumor burden as measured by BLI and quantified in 10^5 relative light units (RLU).

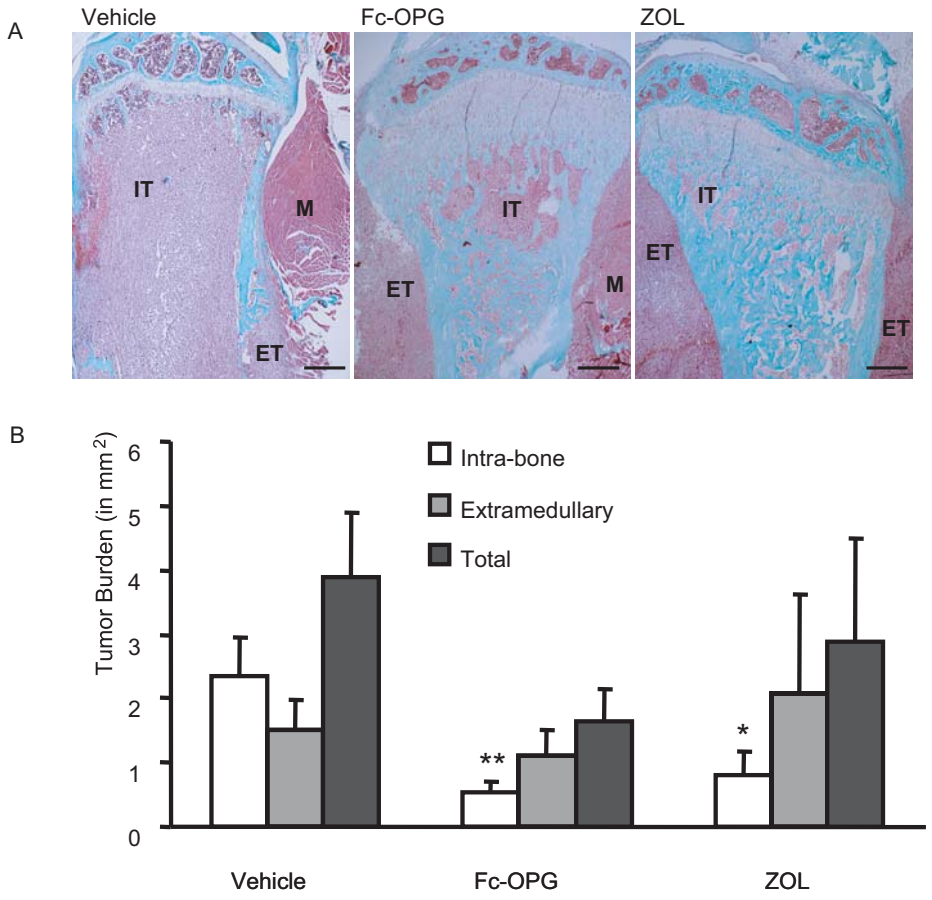

Figure 5 **Effects of Fc-OPG and ZOL treatment on intra-bone and extramedullary tumor growth in tibiae as assessed by histomorhphogical analysis.** *A,* goldner staining showing intra-bone tumor growth (IT), extramedullary tumor growth (ET), and muscle tissue (M). Scale bars: 200 µm. *B,* ** p <0.01 and * p <0.05 compared to vehicle.

in the bone metastatic process. This *in vivo* model, therefore, does not represent earlier stages of bone metastasis (e.g., bone homing), but is ideally suited to monitor the effects of bone resorption inhibitors on the growth of cancer cells in the bone microenvironment. Our results show that the two very potent agents used, ZOL and Fc-OPG, significantly reduce intra-bone tumor burden (as measured by histology). Despite a marked effect on tumor-induced bone resorption and intra-bone tumor burden, ZOL and Fc-OPG failed to reduce the total tumor burden which included extramedullary growth.

Interference with the microenvironmental growth support has been proposed as an attractive strategy for decreasing metastatic tumor growth [23]. Bone is a dynamic tissue that is continuously remodeled by bone resorption and subsequent bone formation [24]. Animal

and human studies have shown that high bone remodeling promotes the formation of bone metastases [8-10,13] through the release of factors from bone matrix that support metastatic growth in the bone marrow [1,25].

Differently from other tissues, agents that specifically decrease bone resorption and remodeling are available and are used clinically or experimentally for the protection of bone destruction by metastases from different primary tumors [26-30]. The question is whether local growth and spread of metastases in bone can also be decreased during this process. To provide an answer to this question, in the present proof-of-concept study we aimed to suppress bone resorption maximally with doses of agents that specifically and effectively inhibit and osteoclast-induced bone resorption.

Bisphosphonates are used extensively in the management of patients with bone metastases [27,29-31]. They suppress bone resorption and reduce significantly the frequency of skeletal-related events [31-34]. Of the currently available bisphosphonates, ZOL is the most potent inhibitor of bone resorption *in vitro* and *in vivo* [21,34] and it has also been reported to induce apoptosis of tumor cells *in vitro* [35-37]. We, therefore, used high doses of ZOL to examine the relation between suppression of bone resorption and local progression of breast cancer cell growth. As expected and previously reported [22,38], ZOL reduced markedly tumor-induced osteolysis, as shown radiographically and histologically, through its effects on osteoclasts. TRAcP+ cells were not totally eradicated but those still persisting were probably inactive apoptotic osteoclasts, as has been previously shown in studies with different bisphosphonates [20,22]. Intraosseous tumor burden was reduced, however, the total tumor burden (intrabone and extramedullary) still progressed and although at the end of the experiment this appeared less compared to controls, the difference was not significant. It should be noted that the dosing regimen we used seems unlikely to be responsible for the observed effects as daily and and once-weekly administration of ZOL has been reported to have similar effects on tumor growth in a different model of bone metastasis [50].

ZOL, as all potent nitrogen-containing bisphosphonates, acts primarily on mature osteoclasts and decreases their activity and life-span [21,39]. To exclude the possibility that for an effect on tumor growth all stages of osteoclastogenesis leading to increased bone resorption need to be optimally suppressed, we also treated animals with Fc-OPG. OPG is the natural inhibitor of RANK ligand (RANKL) [40-42], an essential factor for the formation and activity of osteoclasts [43,44]. Fc-OPG has been shown in animal models to strongly inhibit cancer-induced osteolysis [45-47] and to suppress bone resorption in patients with bone metastases from breast cancer [28]. In our study, Fc-OPG suppressed markedly bone resorption, protected the integrity of bone trabeculae and prevented the development of osteolytic lesions. These effects were due to its actions on osteoclast formation and activity as evidenced by the nearly complete eradication of TRAcP+ cells in bone from treated animals. Furthermore, as was also the case with ZOL, intraosseous tumor burden was significantly reduced. Since there was not an effect of Fc-OPG on extramedullary tumor burden, the total tumor

burden (sum of intraosseous and extramedullary) still progressed, and although throughout the entire experiment this appeared less compared to controls, the difference was not significant.

It should be noted that with both ZOL and Fc-OPG the intra-bone tumor burden diminished considerably as shown in histological sections of the tibiae. This is, at least in part, due to the increase in trabecular bone volume and concomitant decrease in bone marrow volume where tumor cells could reside. In other words, even if tumor cells completely fill bone marrow spaces, the relative intra-bone tumor volume would be less in ZOL and Fc-OPG-treated animals compared to vehicle-treated animals. In fact, space limitations within the bone marrow may direct invasive growth towards the extramedullary.

If we had, thus, confined our assessment of tumor growth strictly within the bone we could have arrived to the wrong conclusion about an effect on tumor growth. This underlines the importance of careful dissection of the tumor-containing bones in such a way that the invaded surrounding soft tissue is not removed. It is important to note that the growth of MDA-231 breast cancer cell outside the bone collar in the surrounding soft tissue was also observed in our previous studies with olpadronate [13] and those of Sasaki and co-workers with risedronate and other BPs [12,15]. In these studies, a bone metastasis model of injection of tumor cells into the left heart ventricle was used, rather than direct injection into bone. Therefore, tumor cells invading the surrounding soft tissue can not fully be explained by a potential artefact of the model of direct injection into bone. In this respect, it is also important to note that MDA-231 cancer cells can migrate from the bone marrow to periosteal surfaces through vascular channels [13].

Zheng and co-workers showed that curative OPG treatment significantly inhibited tumor growth in the bone environment, as assessed by histology [48]. In fact, tumor areas for OPG and vehicle treated mice were comparable to our data. Furthermore, OPG treatment inhibited proliferation and stimulated apoptosis of cancer cells in the bones. Another study, in which tumor cells were injected into the left heart ventricle, showed that curative low and high dose OPG treatment inhibited tumor growth in bone, and that only high dose OPG increased tumor cell apoptosis [49].

So, bone resorption inhibitors could affect tumor progression in bone by decreasing the bone marrow volume where tumor cells could reside, and by stimulating apoptosis and inhibiting proliferation of cancer cells. However, our data demonstrate that tumor progression into extramedullary spaces is not affected by those treatments. In addition, ZOL and Fc-OPG do not have a major impact on overall tumor burden under the experimental conditions described here. Therefore the results of our experiments do not provide support for the notion that suppression of bone resorption in already established metastatic disease in bone arrest tumor growth and progression, despite its inhibitory effects on intra-bone tumor volume. These data favor the hypothesis that metastatic cancer cells in bone after an initial growth phase that depends on their interaction with the bone marrow stroma

and extracellular bone matrix, become increasingly independent of microenvironmental growth factor support and progress autonomously [13]. At this stage, for skeletal protection and arrest of further tumor growth, anti-resorptive agents should probably be combined with compounds with different mechanism of action on the metastatic cascade such as, for example, cytostatics or anti-angiogenic factors.

The important, and clinically relevant question, that still needs to be addressed is whether suppression of bone resorption early in the course of metastatic disease and while cancer cells are still dormant may prevent the development of macrometastases. Animal studies with bone resorption inhibitors given before the colonization of bone by cancer cells strongly suggest that this can be feasible [13-15]. Therefore, this issue needs to be addressed in properly designed clinical studies.

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