Interference with the Microenvironmental Support Impairs the De novo Formation of Bone Metastases In vivo

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Abstract

Interference with the microenvironmental growth support is an attractive therapeutic strategy for repressing metastatic tumor growth. Bone is a highly dynamic tissue that is continuously remodeled by bone resorption and subsequent bone formation. Growth factors supporting bone metastatic growth are released especially during bone resorption. Differently from most other tissues, drugs that can limit local turnover, such as bisphosphonates, are available for bone. In the present study, we tested the hypothesis that inhibition of bone turnover can affect development and growth progression of experimental bone metastasis. Whole-body bioluminescent reporter imaging was used for the detection, monitoring, and quantification in vivo of the growth progression of bone metastases induced by intracardiac or intraosseous injection of luciferase-transfected breast cancer cells (MDA-231-B/luc) to nude mice. Suppression of bone turnover by bisphosphonates, before bone colonization by cancer cells, inhibited by a great extent the number of developing bone metastases. Tumor growth in the few, but still developing, bone metastases was affected only transiently. Reduction of bone turnover had no effect on growth progression of bone metastases, which were already established when bisphosphonate treatment was initiated, despite a substantial reduction in osteolysis. Therefore, cancer cells metastatic to bone, after an initial growth phase that depends on the interaction with the local stroma, become independent of microenvironmental growth factor support and progress autonomously. Inhibition of bone turnover may represent a useful adjuvant therapy especially for cancer patients at risk to develop bone metastasis. (Cancer Res 2005; 65(17): 7682-90)

Introduction

Micrometastases, persisting in various tissues of cancer patients after removal of the primary tumor, represent the pathophysiologic basis for cancer relapse as overt metastases. Preferential colonization of certain tissues by cancer cells and their subsequent growth are determined by interaction with the tissue-specific microenvironment (1, 2). Therefore, pharmacologic interference with the microenvironmental growth support is becoming an attractive therapeutic strategy for repressing metastatic tumor growth (3).

Bone metastases are common in breast and prostate cancer and cause considerable morbidity (4–9). Evidence from animal studies and more recently of human studies supports the concept that the rate of bone remodeling is directly related to the occurrence and progression of bone metastases (10–15).

The skeleton is a highly dynamic tissue that is continuously renewed through the process of bone remodeling. This occurs at multiple sites in the skeleton by temporary structures called basic multicellular units (BMU; refs. 16, 17). The number and the activity of these BMUs determine the rate of bone turnover (18). During bone remodeling, osteoblasts and osteoclasts, the cellular components of the BMU, secrete paracrine factors that induce chemotaxis and cell adhesion, support cell survival and growth, and stimulate angiogenesis (19, 20).

Furthermore, there is experimental evidence showing that growth factors, such as transforming growth factor-β (TGF-β), are released from the bone matrix and stimulate the secretion of bone active cytokines, which not only enhance bone resorption but also stimulate further tumor growth (viscous cycle; refs. 19–25).

Taken together, this evidence suggests that pharmacologic suppression of bone remodeling may not only protect skeletal integrity at the metastatic site and reduce the incidence of skeletal complications, as has been shown with bisphosphonates, but also interfere with the local growth support of cancer cells, thus preventing the development and progression of bone metastases. We tested this hypothesis by bisphosphonate inhibition of bone turnover in animal models of bone metastasis induced by luciferase-expressing human MDA-MB-231 breast cancer cells and monitored by whole-body bioluminescent reporter imaging (BLI) as described earlier (26).

BLI allows spatiotemporal and quantitative analyses of tumor growth and, due to its sensitivity, is ideally suited to evaluate the effectiveness of therapeutic approaches that target both early stages of metastatic development and advanced metastatic disease. BLI gives detailed information on localization and growth of minimal metastatic deposits in the bone marrow of experimental animals at a stage largely preceding tumor detection by other methods. The simultaneous assessment of bone destruction by morphometrical analyses (radiographs and histomorphometry) and of tumor burden (BLI) allows discrimination between bone-sparing effects (inhibition of tumor-induced osteolysis) and interference with tumor progression (viability and growth). Evidence is presented that cancer cells metastatic to bone, after an initial stage that is probably necessary to initiate a self-maintaining autocrine growth, become independent from the microenvironmental growth factor support and thus expand autonomously and independently of pharmacologic reduction of bone turnover.
Materials and Methods

Cell line and culture conditions. A subclone from human MDA-MB-231 breast cancer cells inducing invariably bone metastases after intracardiac inoculation (bone-seeking clone MDA-231-B) has been established by four consecutive sequential cycles of intracardiac inoculation of MDA-MB-231 in vivo and subsequent expansion in vitro of the cell population recovered from the resulting bone metastases as described earlier (26). MDA-231-B cells were stably transfected with a cytomegalovirus (CMV) promoter-driven mammalian expression vector for luciferase, CMV-luc, and one clone with the highest expression of luciferase expression (MDA-231-B/luc+) was successfully used for in vivo optical imaging as described previously (26). MDA-231-B/luc+ cells were cultured in DMEM (Life Technologies, Breda, the Netherlands) containing 4.5 g/L glucose and supplemented with 10% FCS (Life Technologies) and 800 μg/mL gentamicin/G418 (Life Technologies). Cells were regularly certified free of Mycoplasma contamination.

All MDA-231-B/luc+ cell cultures were harvested at subconfluence after being re-fed with fresh medium 24 hours before inoculation preparation. Cell suspensions of MDA-231-B/luc+ (1 × 10^6 cells/100 μL PBS for intracardiac inoculation or 1 × 10^5 cells/10 μL PBS for intraskeletal inoculation) were prepared as described previously (26–28).

Animals. Female nude (BALB/c nu/nu) mice were purchased from Charles River (L’Arbresle, France). They were housed in individual ventilated cages under sterile condition according to the Dutch Guidelines for the Care and Use of Laboratory Animals. Sterile food and water were provided ad libitum. Mice were 6 weeks old when used for the intracardiac or intraskeletal inoculation of cancer cells.

For surgical and analytic procedures (intraskeletal inoculation, whole-body imaging, and skeletal radiography), mice were anesthetized by i.p. injection of 50 μL of 1:1:1 mixture: ketamine HCl (stock solution of 100 mg/ml, Nutemate; 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 done under isoflurane anesthesia (0.8 L/min, isoflurane, Air Products, Waddinxveen, the Netherlands) using the Vanox system (VetTech Solutions Ltd., United Kingdom). At the end of the experimental period, animals were sacrificed by cervical dislocation.

Induction of systemic metastases by intracardiac injection of MDA-231-B/luc+ cells. A single-cell suspension of 1 × 10^6 MDA-231-B/luc+ cells per 100 μL PBS was injected into the left cardiac ventricle as described previously (29, 30). The progression of the cancer cell growth was monitored weekly by optical imaging (26). At the same time points, osteoblastic bone metastases were monitored by radiographs and analyzed (26–28).

After the experimental period, the animals were sacrificed, and selected long bones site of cancer metastasis were dissected and processed for histomorphometric and immunohistochemical analysis (see below).

Intraskeletal inoculation of MDA-231-B/luc+ cells. A single-cell suspension of MDA-231-B/luc+ cells was injected into the right tibiae as described previously (26–28). In brief, two holes, 4 mm to 5 mm apart with a diameter of ~0.35 mm each, were drilled through the bone cortex of the upper right tibia with the aid of a dental drill. Space in the bone marrow was created by flushing out the bone marrow from the proximal end of the shaft. The upper hole was sealed by surgical wax and 1 × 10^6 MDA-231-B/luc+ cells per 10 μL PBS were slowly inoculated via a 30-gauge needle through the lower hole. Finally, the lower hole was sealed with surgical wax and the cutaneous wound was sutured. The progression of cancer cell growth was monitored weekly by optical imaging and osteoblastic bone metastases were monitored by radiography (26–28). After the experimental period, the animals were sacrificed, the tibia site of intraskeletal inoculation was dissected and processed for histomorphometric and immunohistochemical analysis (see below).

Bisphosphonate reporter imaging and quantification of the bioluminescent signal. Whole-body optical imaging of tumors induced by the luciferase-expressing MDA-MB-231 cell line was done as described earlier (26). After i.p. administration of 2 mg β-luciferin (Perbio Science Nederland B.V., Etten-Leur, the Netherlands), the animals were immediately transferred to a light-tight chamber, and reference gray-scale body-surface images were taken using a intensified charge-coupled device camera (C2400-77A-H1, Hamamatsu Photonics K.K., Hamamatsu, Japan) fitted with a 25 mm/0.95 f (optical aperture) objective (Schneider Optik, Kreuznach, Germany). Five minutes after administration of β-luciferin, photon emission was integrated for a period of 3 minutes and processed using an Argo 20 image processor (M4314 image intensifier (Hamamatsu Photonics). Gray-scale images and bioluminescent images were superimposed using OpenLAB software (Improvision, Coventry, United Kingdom). The relative light intensity was visualized by pseudocolors. Analyses for each metastatic site were done after definition of the region of interest (ROI; OpenLAB software) and quantified as described previously (26). Values are expressed as relative light units (RLU).

Radio graphical analyses. The formation of osteolytic lesions was assessed by radiography (Kodak X-OMAT TL film, Eastman Kodak Co., Rochester NY) using a Hewlett Packard X-ray system Faxitron 43805 and osteolytic areas were measured using NIH-Image 1.62b7 software as described earlier (27, 28).

Histomorphometry, histochemistry, and immunohistochemistry. Dissected long bones were fixed in 4% paraformaldehyde (pH 6.8), decalcified, and processed for parafin embedding (27, 28). Longitudinal sections (5 μm) were cut through the sagittal plane of the tibia containing tumors induced by the intraskeletal inoculation of MDA-231-B/luc+ cells. Sections were either submitted to Goldner staining or histochemical staining for tartrate-resistant acid phosphatase (TRACP; ref. 27, 28).

Histomorphometric measurements of tumor burden were done on central sections through the tumor (largest tumor area). Tumor growth in bone could be readily identified by cytokeratin staining alone or in combination with H&E staining. Total tumor areas, as an estimate of total tumor burden, was measured by image analysis using NIH-Image 1.62b7 image analyses software as described previously (27, 28). Subsequently, a distinction was made between the total tumor burden and the intraskeletal and extraosseous tumor burden. For this, the digital image of the total tumor area was subdivided into an area delimited by the bone cortex or, where 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 intraskeletal tumor growth. In addition, extraosseous tumor growth was defined as the extramedullary growth of cancer cells.

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

Bisphosphonate treatment. Treatment protocols to establish the effects of pamidronate and olpadronate on the generation and progression of systemic bone metastases induced by intracardiac injection of MDA-231-B/luc+ cells were done according to the following two schemes (Fig. 1). (a) Preventive protocol: effect of long-term bisphosphonate treatment on de novo generation and growth of systemic bone metastases (Fig. 1A). Beginning from 2 days before inoculation of MDA-231-B/luc+ cells, the mice were treated by daily s.c. injections with 1.6 μmol/kg/d olpadronate for the whole duration of the in vivo study (48 days). The generation and progression of bone metastases were monitored by BLI and radiography at days 7, 14, 21, 28, 33, 39, and 46. After 48 days of bisphosphonate treatment (day 46 from intracardiac injection of cancer cells), the animals were sacrificed.

(b) Curative protocol: effect of short-term bisphosphonate treatment on growth of established bone metastases (Fig. 1B). Twenty-eight days after intracardiac injection of MDA-231-B/luc+ cells, the animals were equally distributed into two experimental groups based on a comparable bone metastatic tumor burden/mouse as detected by BLI. Subsequently, one experimental group received 1.6 μmol/kg/d olpadronate, whereas the other received vehicle (0.9% NaCl) for the following 18 days. The progression of bone metastasis was monitored by BLI and radiography at days 5, 11, and 18. After 18 days of bisphosphonate treatment (day 46 from intraskeletal inoculation of cancer cells), the animals were sacrificed.
resorption doses were shown previously to completely inhibit osteoclastic bone resorption. Alternatively, to achieve constantly elevated blood levels of resorption-inhibitory doses of olpadronate dissolved in 0.9% NaCl, a dose 10-fold higher than the maximal bisphosphonate dissolved in 0.9% NaCl (50 animals were treated for a period of 40 days by daily s.c. injections of the continuous presence of bisphosphonates for up to 48 hours. Continuous treatment of MDA-231-B/luc+ cells with a dose range of bisphosphonates (10^{-4}-10^{-8} mol/L for 24 and 48 hours) did not affect functional expression of the luciferase gene in MDA-231-B/luc+ cells in vitro as measured in luciferase assays and semiquantitative PCR (data not shown).

**Results**

Effect of bisphosphonate treatment on the de novo generation and progression of systemic bone metastases (preventive protocol). In this experiment, we investigated whether pharmacologic inhibition of bone turnover before inoculation of cancer cells, and continued for the duration of the experiment, could prevent the initiation and maintenance of a positive interaction between the bone microenvironment and cancer cells (Fig. 1).

Consistent with our previous findings (26), BLI showed that MDA-231-B/luc+ cells metastasize exclusively to the skeleton. Olpadronate treatment decreased significantly the number of bone metastases per animal by ~70% (Fig. 2A). Radiographical analysis in the same animals showed, from day 40 onwards, almost no osteolytic bone lesions in bisphosphonate-treated animals compared with control (Fig. 2A).

During the early experimental period, olpadronate treatment significantly delayed the progression of the bone metastases, detected by BLI as total tumor burden per animal (Fig. 2C, days 26-33), and tumor burden for each bone metastatic site (Fig. 2D, days 26-40). However, this effect was only transient, and at later stages, both the total burden per mouse (Fig. 2C, days 40-47) and the tumor burden per bone metastatic site (Fig. 2D, day 47) in the olpadronate-treated animals became equivalent to those of the vehicle-treated animals.

Effect of bisphosphonate treatment on growth of established bone metastases (curative protocol). In this experiment, we investigated the effect of pharmacologic inhibition of bone turnover on the bone microenvironmental growth support for cancer metastases that are already in an advanced stage of progression (Fig. 1B).

Olpadronate treatment did not affect the number of bone metastases detected by BLI (Fig. 3A) but significantly reduced the number of osteolytic lesions per mouse as assessed by radiography at the end of treatment period (Fig. 3B, day 18). A discrepancy between BLI and radiography in the number of bone metastases detected in control and olpadronate-treated animals can be noticed (Fig. 3A and B).

Olpadronate treatment transiently, but not significantly, affected total tumor burden per animal (Fig. 3C, day 5). However, at later time points, tumor growth was not further affected by olpadronate (Fig. 3C), and at the end of the experiment, it even enhanced significantly tumor burden per bone metastatic lesion (Fig. 3C and D).

As shown in a representative radiograph of an olpadronate-treated animal (Fig. 4), there is minimal osteolysis in the left proximal tibia and proximal humerus. However, the photon emission detected by BLI of the same bone areas (Fig. 4) indicates that these bones are sites of metastatic growth of MDA-231-B/luc+ cells. Due to the limited intraosseous space, most of this growth seems to have taken place in the soft tissues surrounding bone as revealed on the radiograph by the tumor shadow present especially around the left humerus (Fig. 4).

Effect of bisphosphonate treatment on intraosseous growth of breast cancer cells. To investigate in more detail the cellular

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**Figure 1.** Preventive and curative protocols for bisphosphonate treatment in a heart injection model of bone metastasis. A, in the preventive protocol, 4-week-old female BALB/c nu/nu mice were treated with 1.6 μmol/kg/d olpadronate for 2 days before inoculation of cancer cells. Subsequently, 10^6 MDA-231-B/luc+ cells were inoculated into the left cardiac ventricle and the animals were treated by daily s.c. injections with 1.6 μmol/kg/d olpadronate for the duration of the experiment. The formation of bone metastases (and quantification of individual metastasis) was monitored and quantified weekly by whole-body BLI. In the same animals, the formation of osteolytic lesions was followed by radiography. B, in the curative protocol, 10^6 MDA-231-B/luc+ cells were inoculated into the left cardiac ventricle of 4-week-old female BALB/c nu/nu mice. Animals were monitored weekly to assess the formation of distant metastases and osteolysis (BLI and radiography). Starting from 28 days after cancer cell inoculation, the animals were equally distributed into two experimental groups and were given either 1.6 μmol/kg/d olpadronate or vehicle. Subsequently, the progression of bone metastasis was monitored by BLI and radiography at days 5, 11, and 18. After 18 days of bisphosphonate treatment (day 46 after intraosseous inoculation of cancer cells), the animals were sacrificed.

Each experimental group for both protocols consists of eight animals and experiments were repeated at least twice.

To investigate the effect of bisphosphonates on tumor development and osteolysis after intraosseous inoculation of MDA-231-B/luc+ cells, beginning from day 3 after inoculation (day of inoculation = day 0), the animals were treated for a period of 40 days by daily s.c. injections of bisphosphonate dissolved in 0.9% NaCl (50 μL volume) at the following doses: 16 μmol/kg/d pamidronate and 1.6 μmol/L/kg/d olpadronate. These doses were shown previously to completely inhibit osteoclastic bone resorption in vivo (31, 32). After this treatment period, the animals were sacrificed. Alternatively, to achieve constantly elevated blood levels of bisphosphonate during the entire period of treatment, 16 μmol/kg/d olpadronate dissolved in 0.9% NaCl, a dose 10-fold higher than the maximal resorption-inhibitory dose in vivo was continuously infused by osmotic mini-pumps (200 μL, 0.25 μL/h; Alzet Scientific products, Alza Corp., Mountain View, CA) over a period of 28 days, beginning from day 0 after intraosseous inoculation of MDA-231-B/luc+ cells. Osmotic mini-pumps filled with vehicle solution (0.9% NaCl in water) were implanted in a control group. The animals were sacrificed at the end of the treatment period. Experimental groups consist of five animals and experiments were repeated at least twice.

**Bisphosphonate effect on the expression of luciferase in MDA-231-B/luc+ cells.** To exclude putative bisphosphonate effects on luciferase expression by MDA-231-B/luc+ cells, the breast cancer cells were cultured in the continuous presence of bisphosphonates for up to 48 hours. Continuous treatment of MDA-231-B/luc+ cells with a dose range of bisphosphonates (10^{-4}-10^{-8} mol/L for 24 and 48 hours) did not affect functional expression of the luciferase gene in MDA-231-B/luc+ cells in vitro as measured in luciferase assays and semiquantitative PCR (data not shown).

**Statistical analysis.** All data are represented as means ± SE for animal studies and mean ± SD for histomorphometric analyses. Statistical evaluation was carried out by ANOVA or two-tailed Student’s t test.
events during inhibition of bone turnover, progression and growth of cancer cells was analyzed in a model of tumor growth in the bone marrow. For this purpose, MDA-231-B/luc⁺ cells were inoculated directly into the tibiae of nude mice (26–28), which were then treated by daily s.c. injections with olpadronate or pamidronate.

Continuous bisphosphonate treatment did not significantly affect growth of the cancer cells as determined by BLI (Fig. 5A and C).

Figure 2. Prevention of bone metastasis formation by the bisphosphonate olpadronate after inoculation of MDA-231-B/luc⁺ into the left cardiac ventricle of nude mice (preventive protocol). Olpadronate (or vehicle) was given 2 days before inoculation of MDA-231-B/luc⁺ cells into the left cardiac ventricle and continued for the duration of the experiment (1.6 μmol/kg/d for 48 days). Distribution and growth of MDA-231-B/luc⁺ cells was monitored weekly by BLI and radiography. Under both experimental conditions, MDA-231-B/luc⁺ cells metastasize exclusively to the skeleton. A, number of bone metastases per mouse determined by BLI. B, number of bone metastases per mouse determined by radiographical analysis. C, total tumor burden per mouse (BLI). D, tumor burden per bone metastatic lesion (BLI). BLI measurements (C and D) are expressed as RLU. Open columns, vehicle-treated animals; filled columns, olpadronate-treated animals. Columns, mean (n = 8); bars, SE. *, P < 0.05; **, P < 0.001.

Figure 3. Effects of olpadronate treatment on existing bone metastases induced by inoculation of MDA-231-B/luc⁺ into the left cardiac ventricle of nude mice according to the curative protocol (see Fig. 1B). Daily s.c. administration of olpadronate (1.6 μmol/kg/d for 18 days) was initiated when bone metastases had already developed (day 28) following intracardiac injection of MDA-231-B/luc⁺ cells. The fate and growth of MDA-231-B/luc⁺ cells were monitored weekly by BLI and radiography. Under both experimental conditions, MDA-231-B/luc⁺ cells metastasize exclusively to the skeleton. A, number of bone metastases per mouse determined by BLI. B, number of bone metastases per mouse determined by radiographical analysis. C, total tumor burden per mouse (BLI). D, tumor burden per bone metastatic lesion (BLI). BLI measurements (C and D) are expressed as RLU. Open columns, vehicle-treated animals; filled columns, olpadronate-treated animals. Columns, mean (n = 8); bars, SE. *, P < 0.05.
As expected, both bisphosphonates strongly and significantly inhibited the development of osteolytic lesions (Fig. 5B and C). Histomorphometric analysis (Fig. 5D) confirmed that bisphosphonate inhibited cancer-induced bone destruction leading to a significant increase in trabecular bone area associated with a significant decrease in TRAcP* resorbing osteoclasts at the bone surface (Table 1). As a consequence of this bone-sparing effect of bisphosphonate treatment, the intrabone tumor burden was significantly decreased, although it was clear that the cancer cells were filling completely the bone marrow spaces (Fig. 5D). In contrast to the intraosseous tumor burden, no significant inhibition of the overall tumor burden (intraosseous and in surrounding soft tissues) was observed (Fig. 5D), which is in line with the optical imaging data presented in Fig. 5A and C.

Previously, it was found that the intensity of the signal detected by BLI is a function of the tissue composition and depth of the bioluminescent source (33, 34). There is the possibility that changes in the tissue type invaded by the tumor growth and/or representation of bone tissue, with consequent differences in light penetration and light scattering between the experimental groups (vehicle versus bisphosphonate), may have generated artifactual results in determining tumor burden by BLI. To exclude this, the treatment/control ratios of the tumor burden values obtained by BLI and histomorphometric analyses were compared. No differences in ratios between these two measurement methods were found (0.98 ± 0.16 and 1.17 ± 0.22 treatment/control ratios for pamidronate and olpadronate, respectively). Thus, potential differences in quenching and/or light scattering by bone cannot account for the observed lack of in vivo growth-inhibitory effects during bisphosphonate treatment and confirm the reliability of BLI as method to quantify tumor burden.

Discussion

Interference with the microenvironmental growth support is currently being evaluated as a therapeutic strategy for the treatment of metastatic disease. Bone metastasis is a paradigm of the interactions that take place at the tumor-stroma interface (1, 2) and evidence from animal and clinical studies support the notion that bone turnover, particularly bone resorption, contributes substantially to initiation and maintenance of local tumor growth through release of growth factors and bone resorbing cytokines (vicious cycle). Differently from other tissues, bone...
turnover can be reduced by pharmacologic means; thus, animal models of bone metastasis offer the unique opportunity to test in vivo the therapeutic efficacy of the interference with the tumor-stroma interface.

In this study, we investigated whether inhibition of bone turnover by bisphosphonate administration could prevent the development of bone metastases and/or repress tumor growth in already developed bone metastases. Our results show that...
inhibition of bone turnover by bisphosphonates, before the establishment of a micrometastatic spreading induced by intracardiac inoculation of breast cancer cells (preventive protocol), reduces by a great extent, although not completely, the development of bone metastases as detected by BLI. Although not significant, a tendency toward this effect is also present when bisphosphonates are given later, after the establishment of a micrometastatic spreading (curative protocol), when some bone metastases had already been generated, but a residual number had still to develop. The limited residual number and thus the low statistical power may explain the lack of significance.

The dose of olpadronate given (1.6 μmol/kg body weight/d) has been shown previously to efficiently inhibit bone resorption in vivo (32), but in the present study, it did not prevent the formation of bone metastases by >70%. This level of inhibition is consistent with the observation that in the experimental animal not more than 70% of the bone resorption can be inhibited by high dose of bisphosphonates (35). Possibly, this persistently low level of bone resorption is sufficient to provide growth support to a minimal number of micrometastatic foci.

Although preventive bisphosphonate inhibition of bone turnover precludes to a great extent the generation of bone metastases, the progression of tumor growth in the small number of still developing metastases, as detected by BLI, is significantly reduced only during early stages. A similar trend is also seen when bisphosphonate are given to mice with already developed bone metastases. However, at later stages, bone metastatic growth is even accelerated, leading to a significant increase in tumor burden per metastatic lesion. This result is in line with earlier observations demonstrating that bisphosphonate treatment, despite preserving trabecular bone, does not inhibit bone metastatic growth and may even increase skeletal tumor burden (36). This suggests that bone metastatic growth, once it is initiated and reaches a critical mass, proceeds independently of the bone microenvironmental growth support and is self-maintained by autocrine mechanisms.

The lack of effect by interference with the microenvironmental support on growth progression of already developed bone metastases was corroborated in a further experiment in which tumors were induced by intraosseous inoculation of breast cancer cells. Also in this case, continuous inhibition of bone resorption, achieved by bisphosphonates given either s.c. or by sustained high-dose release, did not affect tumor growth despite a clear bone-sparing effect shown radiographically and histomorphometrically. Although the tumor mass confined within the bone marrow cavity was decreased as a result of the reduced intramedullary space made available by persisting bone trabeculae, the total tumor mass was compensated by growth of cancer cells forced to invade the soft tissues (muscle, tendon, and connective tissue) surrounding the bone cortex. Bone tissue may partially segregate the cancer cells within the medullary cavity and prevent them from invading the surrounding soft tissues. Instead, on inhibition of bone resorption, the invasive growth is most likely forced to modify its direction from intramedullary to extramedullary. It is important to note that MDA-MB-231 breast cancer cells can migrate from bone marrow to periosteal surfaces (26) probably through vascular channels.

Previous studies of the effects of bisphosphonates on bone metastatic growth in comparable animal models have shown contradictory results (30, 36–40). Bisphosphonates were reported to reduce the tumor burden in established bone metastases but not in soft tissue and visceral metastases. This effect seemed to be mediated by induction of apoptosis of the cancer cells (30, 37). Treatment with bisphosphonate, given before the development of evident metastasis, resulted in a marked reduction of the number of bone metastases (30, 38–40). However, another preventive study showed that bisphosphonate treatment resulted in an increased tumor burden in the developing bone metastases but not in visceral metastases (38). In most of these investigations, tumor burden has been quantified indirectly by measuring the osteolytic area on bone radiographs.

The difference between BLI and radiography in the number of bone metastatic foci detected in either bisphosphonate- or vehicle-treated animals is consistent with our previous report (26) and corroborates further the higher sensitivity, reliability, and statistical power of the BLI method. BLI provides a direct indication of tumor viability and growth, whereas radiography does not detect actual tumor burden but reveals only osteolysis, which is an indirect, late, and inconsistent sign of the presence of a tumor (26). In fact, determination of the osteolytic area depends not only on tumor cell mass but also on the ability of the cancer cells to secrete bone-resorbing cytokines. Furthermore, the determination of the osteolytic area does not take in account extension of the tumor burden in the surrounding soft tissues.

### Table 1. Effects of bisphosphonate treatment on osteoclast numbers at the bone surface of MDA-231-B/luc breast cancer cells growing in tibiae of 6-week-old female nude mice

<table>
<thead>
<tr>
<th></th>
<th>No. osteoclast/mm bone surface, mean (SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>22 (4)</td>
<td>—</td>
</tr>
<tr>
<td>Pamidronate 16 μmol/kg/d</td>
<td>5 (1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Olpadronate 1.6 μmol/kg/d</td>
<td>4 (2)</td>
<td>&lt;0.01</td>
</tr>
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**NOTE:** n = 5 per experimental group. P represents bisphosphonate-treated versus vehicle-treated animals.

### Table 2. Histomorphometric analysis of TBV (%), intrabone tumor burden (mm³), and total tumor volume (mm³) of MDA-231-B/luc breast cancer cells growing in tibiae of 6-week-old female nude mice

<table>
<thead>
<tr>
<th></th>
<th>Vehicle, mean (SD)</th>
<th>Olpadronate, mean (SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBV</td>
<td>3 (2)</td>
<td>13 (3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Intrabone tumor burden</td>
<td>67 (18)</td>
<td>36 (5)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total tumor volume</td>
<td>167 (24)</td>
<td>107 (24)</td>
<td>NS</td>
</tr>
</tbody>
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**NOTE:** Olpadronate (or vehicle) was released at a high dose by osmotic mini-pumps (16 μmol/kg/d, 24 hours/d) for 28 days. Mean (SD), n = 5. NS, not significant.
Therefore, determination of only the osteolytic area is invalid as a measurement of bone tumor mass. Parallel histomorphometric evaluation can avoid this pitfall. In this case, however, histomorphometric analysis should not be restricted to evaluation of intraosseous tumor burden. Only one study assessed this in neighboring tissues and showed that inhibition of bone resorption by bisphosphonates restrained intraosseous tumor growth but increased tumor burden in the soft tissues (30). This effect is perfect in line with the results presented here with both histomorphometry and BILI.

In vitro, bisphosphonates have been reported to exert direct antiproliferative and proapoptotic effects on cancer cells (41–43), to interfere with cancer cell adhesion to bone matrix proteins (44, 45), and to inhibit matrix metalloproteinases (46, 47) and cancer cell migration and invasion (48, 49). Our results indicate that bisphosphonates have no direct cytostatic effect in vivo.

Collectively, our obtained results substantiate the concept that interference with the bone microenvironmental growth support, through suppression of bone resorption, can affect early developmental steps of bone metastasis. However, the same strategy could not repress progression of overt bone metastases; in addition, bisphosphonates do not seem to exert an effect on cancer cell growth either directly by induction of apoptosis or indirectly by inhibition of angiogenesis.

How can the therapeutic effect of inhibition of bone resorption/turnover on early stages of bone metastasis development be explained? Bone remodeling does not occur throughout the entire bone surface but takes place at microscopic patches of bone resorption and subsequent bone formation. The number and activity of BMUs determine the rate of bone turnover. Osteoblasts and osteoclasts secrete and/or release from bone matrix cytokines, like TGF-β and insulin-like growth factor-I, which can act as paracrine growth factors for neighboring cancer cells that may have colonized bone marrow (19, 22–25, 28, 50–55). Consequently, local growth factor release by an active BMU in close proximity of a bone marrow micrometastasis may evoke the growth and invasive potential of the cancer cells, ultimately leading to the development of a macrometastasis (Fig. 6). We hypothesize that the probability for a bone marrow micrometastasis to become a clinically evident bone metastasis should be related to the chance that micrometastatic cancer cells extravasate in the proximity of an active BMU. In contrast, cancer cells extravasating near to a non-remodeling, "resting" bone surface will persist as "dormant" and eventually become apoptotic. Accordingly, a higher number and density of active BMUs (high bone turnover rate) should favor the probability that bone marrow micrometastases "home" near active BMUs. This may explain the clinical and experimental evidence showing an association between high bone turnover rate and the preferential development of hematogenous metastases at sites of active bone remodeling (14, 15). Conversely, inhibition of bone turnover before the generation of a self-maintaining, autocrine cancer cell growth may arrest the vicious cycle underlying the initial growth support by the BMU and ultimately prevent the development of clinically evident micrometastases.

Figure 6. Hypothesis regarding the importance of bone turnover rate and the therapeutic relevance for inhibiting early developmental steps of bone metastasis. Bone turnover takes place at microscopic patches of bone resorption and subsequent bone formation (BMU). The number and the activity of these BMUs determine the rate of bone turnover. Osteoblasts and osteoclasts, the cellular components of an active BMU, secrete cytokines from bone matrix, which can act as paracrine growth factors for neighboring cancer cells that may have colonized bone marrow (micrometastatic deposits). Consequently, local growth factor release by an active BMU in close proximity of a bone marrow micrometastasis may evoke the growth and invasive potential of the cancer cells, ultimately leading to the development of an overt bone metastasis. Interference with the bone microenvironmental growth support through inhibition of bone resorption/turnover (for instance, by bisphosphonate) may be of therapeutic relevance for inhibiting early developmental steps of bone metastasis. The probability of a bone marrow micrometastasis to become clinically evident bone metastasis may be related to the chance that micrometastatic cancer cells extravasate in the proximity of an active BMU and vice versa.
Assessment of the bone turnover status may lead to a better identification and stratification of the cancer patients at risk to develop bone metastases. Cancer patients, probably harboring micrometastases in the bone marrow at the moment of diagnosis and/or surgery but with high bone turnover rate, either basal (i.e., induced by menopause) or after surgical or chemical gender steroid deprivation, may benefit from a prophylactic reduction of the bone turnover rate by bisphosphonates.

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