

Bisphosphonate Risedronate Reduces Metastatic Human Breast Cancer Burden in Bone in Nude Mice¹

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ABSTRACT

Human breast cancer frequently metastasizes to the skeleton to cause osteolysis and subsequent pain, pathological fracture, and hypercalcemia. Because bone continuously releases growth factors stored in bone matrix by bone resorption during physiological remodeling and, thus, possibly provides a favorable microenvironment for metastatic breast cancer cells to proliferate, inhibitors of bone resorption used either prophylactically or in patients with established disease, therefore, would seem likely to be useful adjuvant therapy in patients with breast cancer. However, the parameters for monitoring progressive osteolytic bone disease in humans are imprecise. We examined the effects of the third generation bisphosphonate, risedronate, which is a specific inhibitor of osteoclastic bone resorption, in a bone metastasis model in nude mice in which intracardiac injection of the human breast cancer cell line MDA-231 leads to osteolytic bone metastases. Risedronate (4 µg/animal/day) was given s.c. to animals (a) after radiologically small but defined osteolytic metastases were observed; (b) simultaneously with MDA-231 cell inoculation through the entire experimental period; or (c) by short-term prophylactic administration before inoculation of MDA-231 cells. In all experiments, risedronate either slowed progression or inhibited the development of bone metastases assessed radiographically. Furthermore, mice treated continuously with risedronate showed significantly longer survival than did control mice. Histomorphometrical analysis revealed that osteoclast numbers were diminished at metastatic tumor sites. Unexpectedly, there was also a marked decrease in tumor burden in bone in risedronate-treated animals. In contrast, the growth of metastatic breast cancer in soft tissues surrounding bones was not affected by risedronate. Moreover, risedronate had no effects on the local growth of s.c. implanted MDA-231 breast cancers in nude mice or on MDA-231 cell proliferation in culture. These data demonstrate that risedronate decreases metastatic MDA-231 breast cancer burden selectively in bone, as well as suppresses progression of established osteolytic lesions and prevents the development of new osteolytic lesions; thus, the data suggest that inhibition of osteoclastic bone resorption may be a useful adjunctive therapy for the treatment of cancers that have colonized in bone.

INTRODUCTION

The majority of patients with advanced breast cancer manifest osteolytic bone metastases (1-3). The occurrence of an osteolytic metastasis is a catastrophic complication for patients with breast cancer. Not only do these metastases frequently cause intractable bone pain, but they also lead to increased susceptibility to fractures, hypercalcemia, and nerve compression syndromes. Most importantly, once cancer cells become housed in the skeleton, the cancer is essentially noncurable with current treatment regimens. Any agent that could prevent the development of bone metastases or treat bone metastases

once they become established would, therefore, be a major therapeutic advance in the treatment of breast cancer patients.

Despite the importance of this clinical problem, there are few currently available treatments which are satisfactory for either the prevention or treatment of breast cancer metastasis to bone. The bisphosphonates are effective inhibitors of osteoclastic bone resorption, which are predominantly being used in the treatment of cancer-associated hypercalcemia and other states of increased bone resorption, such as Paget's disease of bone (4). Because our earlier study indicates that the primary cellular mechanism of osteolysis associated with metastatic cancer is osteoclast mediated (5), it is likely that bisphosphonates should also be efficacious in this situation. However, definitive information is scarce due to the difficulties of assessing beneficial effects in bone in patients with advanced cancer who are usually receiving many therapies and to the lack of useful animal models.

Recently, Arguello *et al.* (6) established a bone metastasis model in which intracardiac injection of cancer cells causes the development of osteolytic lesions. We have used this approach by injecting human cancer cells into the left cardiac ventricle of nude mice, and we have found that some human cancer cells (melanoma and breast cancer cells) can form osteolytic lesions characteristic of those of osteolytic metastases observed in patients (7).

In the present study, using this nude mouse model, we examined the effects of a potent bisphosphonate on established osteolytic bone metastases and on the development of new bone metastases. We have found that risedronate, a third generation bisphosphonate (4), not only inhibits bone resorption by osteoclasts in metastatic lesions, but markedly suppresses the progression of established osteolytic lesions and prevents the development of new osteolytic bone metastases. Furthermore, stereological and histomorphometrical studies have demonstrated that the volume of metastatic breast cancer in bone is significantly and selectively decreased, and that the survival time of the treated mice was increased. These results suggest that inhibition of bone resorption by risedronate inhibits growth of breast cancer cells that have metastasized to bone, and that the bisphosphonates may be useful adjunctive agents for the treatment of metastatic breast cancer in bone.

MATERIALS AND METHODS

Breast Cancer Cells

A human breast cancer cell line, MDA-231 (kindly provided by Dr. C. Kent Osborne, University of Texas Health Science Center, San Antonio, TX), was cultured in DMEM (Hazleton Biologics, Inc., Lenexa, KS) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin solution (GIBCO-BRL Laboratory, Grand Island, NY) in a humidified atmosphere of 5% CO₂ in air. MDA-231 cells were isolated from pleural effusion of a 51-year-old white woman with poorly differentiated adenocarcinoma in 1973 (8). The cells show 67 chromosomes and karyotypes. They were found to be estrogen receptor negative (8).

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Intracardiac Injection of MDA-231 Human Breast Cancer Cells in Nude Mice

All cultures used for intracardiac injections were at subconfluency and were refed with fresh medium 24 h before inoculation into nude mice. Cells (1×10^5) were suspended in 0.1 ml of PBS and then injected into the left heart ventricle of female BALB/c-*nu/nu* mice (Harlan Industries, Houston, TX) with the use of a 27-gauge needle under the anesthesia with pentobarbital (0.05 mg/g) according to the modification of the method described by Arguello *et al.* (9).

Bisphosphonate

A potent third generation bisphosphonate risedronate (4) was kindly provided by Rhône-Poulenc-Rorer (Collegeville, PA; Fig. 1). Stock solution of risedronate was made in PBS.

Experimental Protocols of Administration of Risedronate

The summary of the experiments is depicted in Fig. 2. In all experiments, 4-week-old female nude mice received MDA-231 cells (day 0) and subsequently were kept in our animal facilities as described (10). The number of nude mice studied in each experiment is described in each figure. Survival of mice was determined only in Protocol 2.

Protocol 1: Effects of a short period of treatment with risedronate on established bone metastases.

Animals were inoculated with MDA-231 breast cancer cells in the left heart ventricle (day 0) and examined for the development of osteolytic lesions by radiography at day 17. Animals that showed distinct osteolytic metastases on radiographs were divided into two groups. One group of mice received PBS and another group of mice received risedronate (4 $\mu\text{g}/\text{mouse}/\text{day}$) s.c. once a day from day 17 to 28. At the end of the experiments, nude mice were examined again by radiography for osteolytic bone metastases. Changes in numbers of osteolytic lesions were assessed by comparing the radiological films of each individual mouse taken at day 17 with those taken at day 28. Data are shown as:

% of increase

$$= \frac{\text{Osteolytic metastasis number at day 28} - \text{osteolytic metastasis number at day 17}}{\text{Osteolytic metastasis number at day 17}} \times 100$$

Protocol 2: Effects of continuous treatment with risedronate on the development of new bone metastases.

From the same day as the inoculation of MDA-231 breast cancer cells (day 0), risedronate (0.4, 4, and 40 $\mu\text{g}/\text{mouse}/\text{day}$) was s.c. injected once a day for 28 days. The control group received PBS. Radiographs were taken at day 28 to assess the presence of osteolytic bone metastases. Mice were then kept untreated until they died. Survival of each animal was determined by the duration between day of cell inoculation and death of mice.

Protocol 3: Effects of a short period of prophylactic treatment with risedronate on the development of new bone metastases.

Risedronate was administered s.c. into female nude mice (3 weeks old) once a day for 7 days before cell inoculation (day -7). Seven days later, the administration of risedronate was discontinued, and nude mice were then inoculated with MDA-231 breast cancer cells into their left ventricle (day 0).

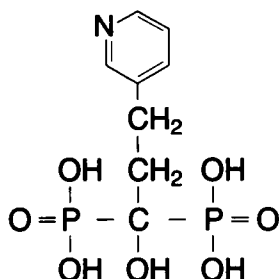


Fig. 1. Chemical structure of risedronate; 2-(3-pyridinyl)-1-hydroxyethylidene-bisphosphonic acid.

Experimental Protocol

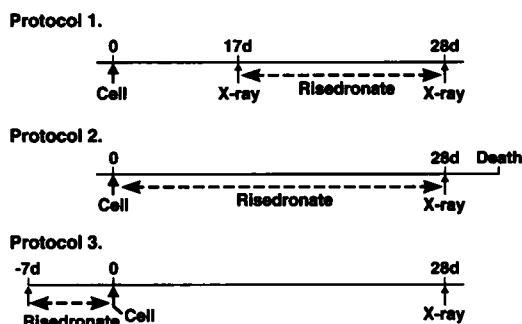


Fig. 2. Summary of experimental protocol. MDA-231 cells (*cell*) were inoculated into the left cardiac ventricle of 4-week-old female nude mice [day (*d*) 0]. Risedronate (4 $\mu\text{g}/\text{mice}$) was given daily s.c. All mice were sacrificed at day 28 except for in Protocol 2 in which the survival of mice was assessed.

Radiographs of these animals were taken, and the mice were sacrificed for histological examination at day 28. Control mice received PBS.

Scoring of Bone Metastasis Number

The number of osteolytic bone metastases was enumerated on radiographs as described (7). Animals were anesthetized deeply with the use of pentobarbital (0.05 mg/g body weight), laid down in the prone and lateral position against the films (22 x 27 cm; X-OMAT AR; Eastman Kodak Co., Rochester, NY), and exposed to X-rays at 35 kVp for 6 s with the use of a Faxtron radiographic inspection unit (Field Emission Corporation, Inc., McMinnville, OR). Films were developed with the use of a RP X-OMAT processor (Kodak). All of the radiographs of bones in nude mice were evaluated extensively and carefully by 3 different individuals, all of whom were without knowledge of the experimental protocols. Osteolytic metastatic foci as small as 0.5 mm in longer diameter, which were recognized as demarcated radiolucent lesions in the bone, were projected and amplified on the monitor and enumerated manually.

Histological, Histomorphometrical, and Stereological Examinations

Forelimbs and hindlimbs from animals in each treatment group were fixed with 10% formalin in PBS (pH 7.2) and decalcified in 14% EDTA solution for 2-3 weeks. Paraffin sections were made after conventional methods and stained with hematoxylin and eosin.

Histomorphometrical analysis of bone volume was assessed at sites of metastases in proximal tibial metaphyses on hematoxylin and eosin-stained sections at a standard location in each bone just distal to the epiphyseal plates with the use of a Bioquant image analysis system (R & M Biometrics, Nashville, TN) and an Olympus BH-2 microscope as described (11). Osteoclasts were identified at the interface between tumor and bone in the proximal tibial metaphyses and expressed per mm of this interface distance.

Assessment of tumor volume by stereological technique was conducted as described previously (12). Systematic random sections were prepared and sampled every 300 or 900 μm through the full thickness of the blocks. These sections were stained with a Masson's trichrome stain and projected onto a tabletop with Olympus BH-2 microscope (Olympus, Inc., Tokyo, Japan) equipped with a vertical projector at a final magnification of $\times 30$. Tumor volumes were estimated by overlaying a point grid with a point area of 3.33 mm^2 , correcting for magnification, and counting the points overlying tumor tissue. Total numbers of points associated with the tumor metastases were recorded on each section for each hindlimb. The total tumor volume was estimated by summing the counts, multiplying by area associated with the point (3.33 mm^2) and the distance between the sections (300 or 900 μm).

Statistical Analysis

Statistical significance of the difference in numbers of osteolytic metastases and tumor volume between risedronate-treated groups and untreated groups was determined by the Mann-Whitney test for nonparametric samples. Statistical differences of survival rate of the animals were analyzed by a generalized

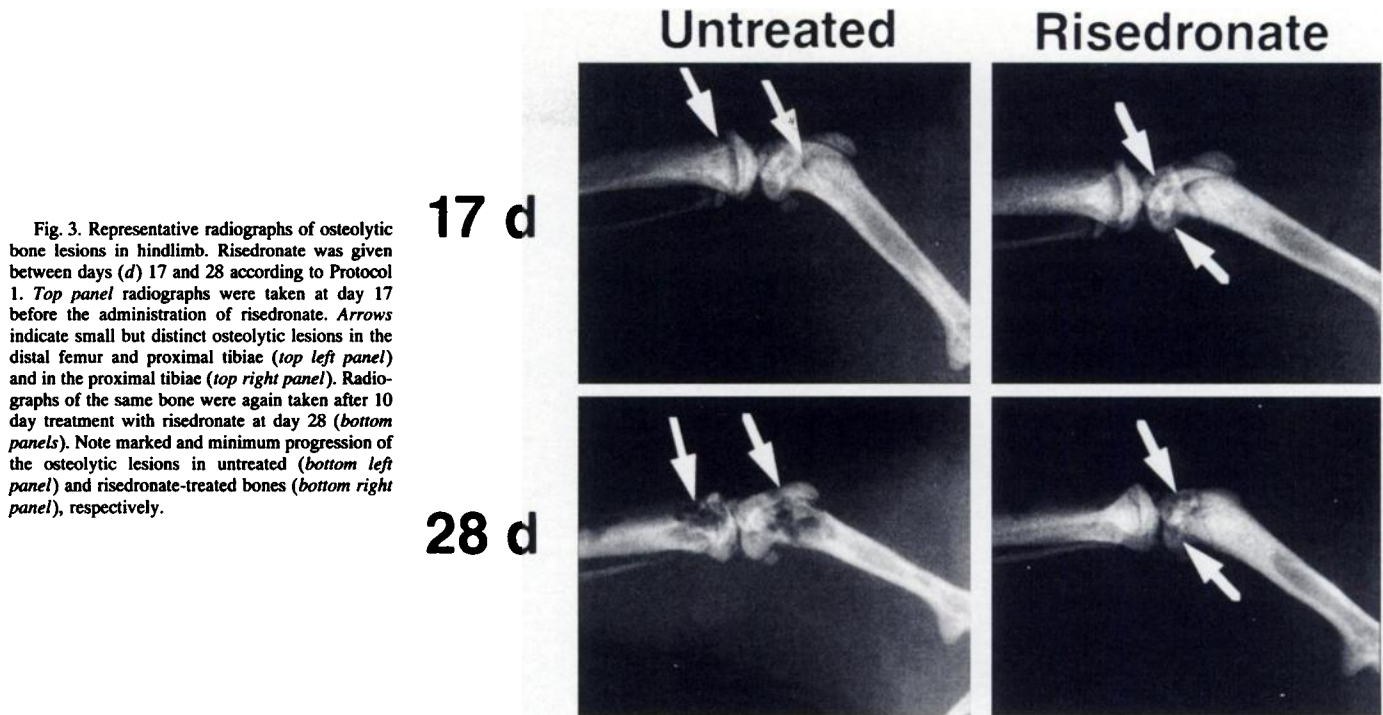


Fig. 3. Representative radiographs of osteolytic bone lesions in hindlimb. Risedronate was given between days (*d*) 17 and 28 according to Protocol 1. Top panel radiographs were taken at day 17 before the administration of risedronate. Arrows indicate small but distinct osteolytic lesions in the distal femur and proximal tibiae (top left panel) and in the proximal tibiae (top right panel). Radiographs of the same bone were again taken after 10 day treatment with risedronate at day 28 (bottom panels). Note marked and minimum progression of the osteolytic lesions in untreated (bottom left panel) and risedronate-treated bones (bottom right panel), respectively.

Wilcoxon test (13). Statistical analysis of bone volumes and osteoclast numbers in the treatment and control groups was assessed with the use of ANOVA. All data were presented as the mean \pm SE.

RESULTS

Osteolytic Bone Metastases and Cachexia Caused by MDA-231 Breast Cancer Cells in Nude Mice. Nude mice given injections with MDA-231 breast cancer cells into the left ventricle of the heart showed osteolytic lesions in limbs, vertebrae, pelvis, and maxilla between 3 and 5 weeks after tumor inoculation. Some mice developed hindlimb paralysis, probably due to spinal cord compression. Furthermore, they also manifested cachexia with a marked loss of muscle, adipose tissue, and body weight (data not shown). These results are consistent with the findings that are described in our previous report in which a human melanoma A375 was studied (7).

Effects of a Short Period of Treatment with Risedronate on Established Bone Metastases (Protocol 1). Radiographs taken at day 17 demonstrated a few small but distinctive osteolytic lesions (Fig. 3, top panel, arrows). The animals were then treated with or without risedronate (4 μ g/mouse/day) for the subsequent 10 days. At

day 28, the same bones were again examined radiologically for changes in the number of osteolytic lesions and in the size of those detected previously. The size of established osteolytic lesions increased progressively, and the total number of osteolytic metastases was markedly increased in the untreated group (Fig. 3, left panel). In sharp contrast, there were few changes in the size and number of the osteolytic lesions in the risedronate-treated group (Fig. 3, right panel).

Quantitative assessment of changes in the number of osteolytic bone metastases on radiographs demonstrated that the administration of risedronate significantly suppressed this increase in the number of osteolytic bone metastases that occurred between days 17 and 28 in the control groups (Fig. 4). We did not quantitatively assess for changes in size in this experiment.

Histological examination of bones from the untreated control group revealed that most of the cancellous bone in the proximal tibiae, distal femora, and proximal humeri had been replaced by the metastatic MDA-231 breast cancer, which in most mice extended through defects in the cortical bone into the adjacent soft tissue (Fig. 5A). Numerous active osteoclasts were present along the trabecular bone surface surrounded by the metastatic MDA-231 breast cancer cells invading aggressively in the bone marrow (Fig. 5E). In contrast, in the bones from risedronate-treated mice, there were much fewer osteoclasts (Fig. 5F), and invasion of the metastatic MDA-231 breast cancer cells within the bones was confined to the proximal parts of the metaphyses where lytic lesions had been identified radiologically before risedronate administration (Fig. 5B).

Histomorphometric analysis of tibial metaphyses showed that treatment with risedronate significantly decreased tumor volume (Fig. 6, left panel, B) and reduced osteoclast numbers along the tumor/bone interface (Fig. 6, right panel, B). Histomorphometric analysis of the changes in bones was confined to sections of the proximal tibial metaphyses because these were involved in all of the animals, and the appearances at these sites were very similar to those seen in sections of femoral and humeral metaphyses.

Tumor burden in bone and in the surrounding soft tissue adjacent to



Fig. 4. Effect of risedronate on a change in numbers of established osteolytic bone metastases. Risedronate was given between days 17 and 28 according to Protocol 1. Numbers of osteolytic lesions in the same bones were counted at days 17 and 28, and changes in the number of osteolytic lesions were determined as described in "Materials and Methods." Columns, mean; bars, SE. Numbers in parentheses, numbers of animals studied. *, significantly different from untreated group ($P < 0.01$).

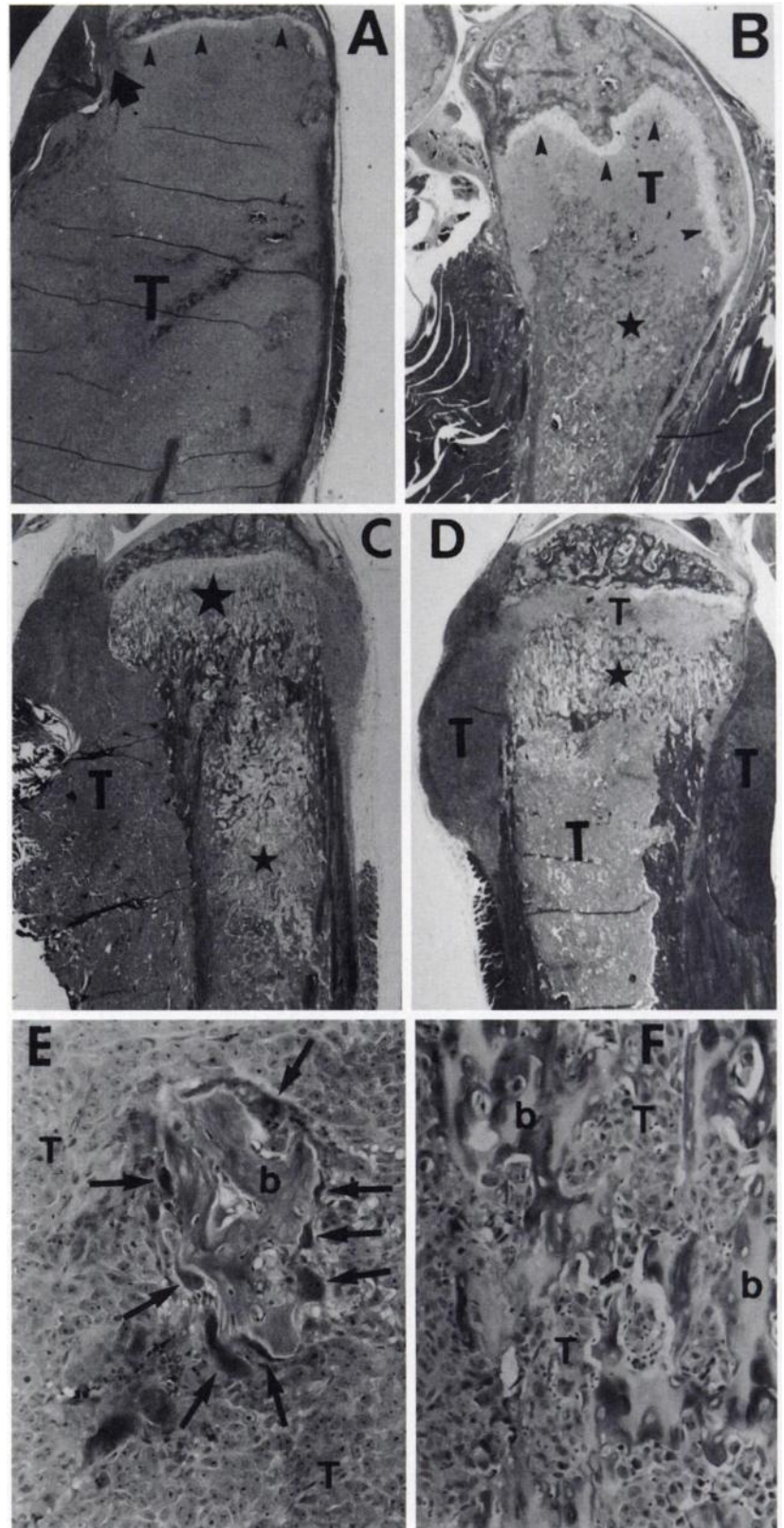


Fig. 5. Histology of bones of nude mice treated without (A) or with risedronate according to Protocol 1 (B), Protocol 2 (C), and Protocol 3 (D); (E) and (F), higher magnification of (A) and (C), respectively. Bones subjected to histological examination here are all proximal tibiae. A, osteolytic lesions in bones of control nude mice. Almost all of the primary and secondary spongiosa bone has been replaced by metastatic breast cancer (T) in control mice. The metastatic breast cancer cells extend from the epiphyseal plate (arrowheads) toward the lower end of the medullary cavity and into the surrounding soft tissue through a large defect in the cortical bone, the edges of which are delineated by large arrows. A previous report has also shown the outgrowth of tumor from the bone to the surrounding soft tissue through the similar defect in cortical bone which is defined as bone foramina of the external venous plexus (24). Hematoxylin and eosin, $\times 25$. B, effect of risedronate on the progression of osteolytic metastases of MDA-231 breast cancer cells. In a nude mouse given risedronate from day 17 to 28 after osteolytic metastases had been detected radiologically in the proximal tibia according to Protocol 1, metastatic breast cancer cells are present at the same sites (T). A few trabeculae remain attached to the epiphyseal plate (arrowheads), and, although some metastatic breast cancer cells are present between trabeculae in the diaphysis (star), most of the bone in the medullary cavity has not been resorbed. Hematoxylin and eosin, $\times 25$. C, effect of risedronate on development of new osteolytic metastases of MDA-231 breast cancer cells. In a nude mouse given risedronate continuously for 28 days according to Protocol 2, islands of metastatic breast cancer cells are present between trabeculae in the medullary cavity (small star), and a large mass of tumor cells is also present in the surrounding soft tissue (T). Although metastatic breast cancer cells are present between trabeculae distal to the epiphyseal plate, the bone trabeculae in the primary spongiosa remain intact (large star). Hematoxylin and eosin, $\times 25$. D, in a nude mouse given risedronate for 7 days before intracardiac injection of MDA-231 breast cancer cells according to Protocol 3, metastatic breast cancer (T) has replaced the primary spongiosa bone beneath the epiphyseal plate and in the diaphysis and has extended into the surrounding soft tissue. However, the metastatic breast cancer has not replaced a band of bone in the metaphysis (star). There are small islands of tumor cells between surviving trabeculae. Hematoxylin and eosin, $\times 25$. E, higher magnification of osteoclastic bone resorption in an untreated mouse. Numerous osteoclasts (arrows) are present between the metastatic breast cancer cells (T) and a surviving island of bone (B). Hematoxylin and eosin, $\times 470$. F, higher magnification of inhibitory effect of risedronate on osteoclastic bone resorption. Metastatic breast cancer cells (T) are present between bone trabeculae (B) in the proximal tibia of a nude mouse given risedronate continuously for 28 days according to Protocol 2. There are no osteoclasts between the metastatic breast cancer cells and the bone. Hematoxylin and eosin, $\times 470$.

bone was compared with the use of stereological analysis to assess for the tissue specificity of the effects of risedronate. Metastatic tumor volume in bone was significantly decreased in the risedronate-treated group, whereas risedronate had no effects on metastatic tumor burden in soft tissue (Fig. 7). The results indicate that the inhibitory effect of risedronate on metastatic breast cancer is restricted to bone. Although

not statistically significant, there was a trend that tumor volume in soft tissue was larger in risedronate-treated animals than it was in untreated animals (Fig. 7). The metastatic deposits consisted largely of solid masses of tumor cells with some areas of central necrosis and little stromal reaction. The solid and necrotic areas of tumor were both included in the measurement of tumor volume.

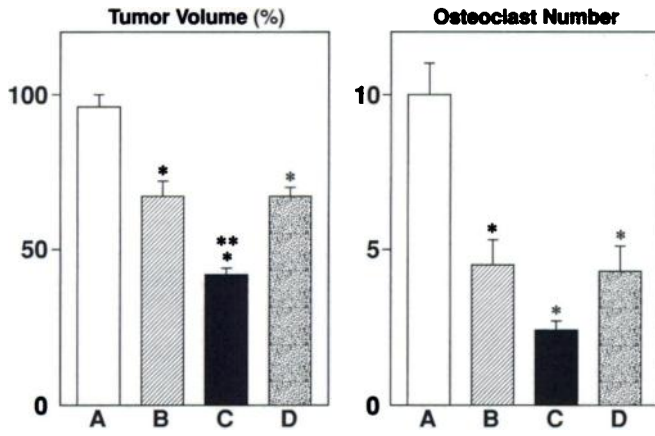


Fig. 6. Histomorphometrical analysis of tumor volume (left) and osteoclast number (right) in bones of mice untreated (A) and treated with risedronate according to Protocol 1 (B), Protocol 2 (C), and Protocol 3 (D). Tumor volume is expressed as percentage of the cancellous space in the tibial metaphysis. Osteoclast number is expressed per mm of the tumor/bone interface in the proximal tibia. Columns, mean; bars, SE ($n = 4$). *, significantly different from the untreated group ($P < 0.01$). **, significantly different from B and D ($P < 0.05$).

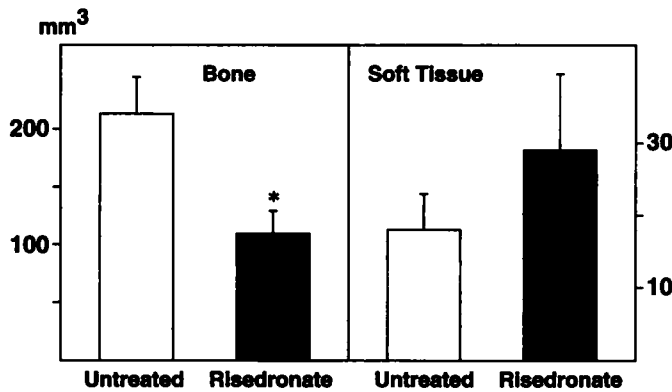


Fig. 7. Comparison of the effects of risedronate on tumor bulk in bone (left) and soft tissue surrounding bone (right) by stereological assessment. Right and left legs of untreated and risedronate-treated (between days 17 and 28 according to Protocol 1) mice were analyzed as described in "Materials and Methods." Columns, mean; bars, SE ($n = 10$ animals for each group). *, significantly different from untreated group ($P < 0.05$).

To determine whether risedronate has a direct growth-inhibitory effect on MDA-231 breast cancer cells, the effect of risedronate on s.c. implanted MDA-231 breast cancer in nude mice was examined. Risedronate showed no effects on s.c. growing MDA-231 breast cancer (Fig. 8). Furthermore, anchorage-independent or anchorage-dependent growth of MDA-231 breast cancer cells in soft agar and monolayer, respectively, was not decreased by risedronate at concentrations of 10^{-6} and 10^{-5} M (data not shown), concentrations at which risedronate markedly inhibits osteoclastic bone resorption in several *in vitro* assays (data not shown).

Effects of Continuous Treatment with Risedronate on the Prevention of New Osteolytic Bone Metastases (Protocol 2). Continuous administration of risedronate ($4 \mu\text{g}/\text{mouse}/\text{day}$) from the time of MDA-231 breast cancer cell inoculation for 28 days resulted in the development of very few radiographically detectable osteolytic bone metastases (data not shown). The number of osteolytic metastases in the risedronate-treated group was markedly lower than that in the untreated group (Fig. 9). A lower dose ($0.4 \mu\text{g}/\text{mouse}/\text{day}$) of risedronate showed less but significant inhibition of osteolytic bone metastases, and a higher dose ($40 \mu\text{g}/\text{mouse}/\text{day}$) had an effect comparable with that seen with the drug at a dose of $4 \mu\text{g}/\text{mouse}/\text{day}$ (data not shown).

Histological examination exhibited that metastatic MDA-231 breast cancer cells were present in the narrow bone marrow spaces between trabeculae, but many of these trabeculae and some of the osteocytes within the bone trabeculae were necrotic, presumably due to pressure effects of metastatic breast cancer cells (Fig. 5C). In comparison with the bones from the control mice, few osteoclasts were present on the bone surfaces adjacent to metastatic breast cancer cells (Fig. 5F).

Histomorphometric analysis of tibial bones from mice in this experiment showed that risedronate significantly decreased metastatic MDA-231 breast cancer burden within the bones and tumor-induced bone loss (Fig. 6, left panel, C) and reduced osteoclast numbers along the tumor/bone interface (Fig. 6, right panel, C).

In the same experiments, we found that risedronate significantly increased the survival rate of cancer-bearing animals (Fig. 10). However, animals treated with risedronate also died 10–14 days after untreated animals died despite the fact that bone metastases were significantly decreased. At necropsy, although they did not show macroscopically visible metastases in organs including lung, liver, kidney, and spleen, large tumor deposits were present in the soft tissues around affected bones.

We did not observe any serious adverse effects of risedronate in mice during daily administration for 28 days except for skin rash at the sites where risedronate was repeatedly injected.

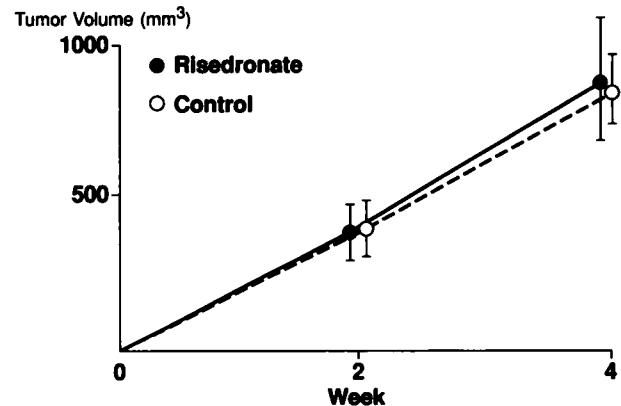


Fig. 8. Effect of risedronate on s.c. growing MDA-231 breast cancer. MDA-231 breast cancer cells ($2 \times 10^6/0.1$ ml PBS) were inoculated s.c. into nude mice. Risedronate ($4 \mu\text{g}/\text{mouse}/\text{day}$) was administered s.c. once a day continuously for 28 days in the same manner as Protocol 2. Tumor size was determined according to the methods described previously (10). Points, mean; bars, SE ($n = 5$ animals).

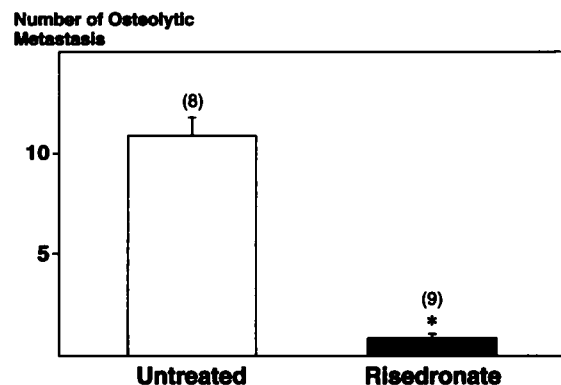


Fig. 9. Effect of risedronate on development of new osteolytic bone metastases. Risedronate was given continuously for 28 days according to Protocol 2. The number of osteolytic lesions was counted on the radiographs at day 28. Columns, mean; bars, SE. Numbers in parentheses, number of animals studied. *, significantly different from untreated group ($P < 0.01$).

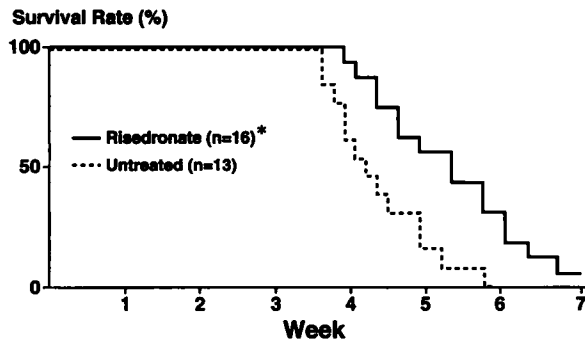


Fig. 10. Effect of risedronate on survival rate of nude mice that received intracardiac inoculation of MDA-231 breast cancer cells. Risedronate was given continuously for 28 days according to Protocol 2. Survival of each individual mouse was determined by the duration between day of cell inoculation and death. Values, mean \pm SE. Numbers in parentheses, number of animals studied. *, significantly different from untreated group ($P < 0.01$).

Effects of Short Period of Prophylactic Administration of Risedronate on the Development of New Osteolytic Bone Metastases (Protocol 3). The radiographs of bones of nude mice that were treated with risedronate for 7 days before MDA-231 breast cancer cell inoculation showed ill defined osteolytic lesions with increased radiopacity at day 28 (Fig. 11, right panel). Because of this ill-defined radiographic appearance of the lesions, it was not possible to count their numbers and to quantitatively compare them with bones of untreated animals. However, the histological pattern of risedronate pretreated bones showed lower numbers of osteoclasts than in control and that metastatic breast cancer invasion was confined to the area immediately beneath the epiphyseal plate where bone would have been formed after cessation of risedronate therapy with few cells present in bone distal to this site (Fig. 5D). This relatively unaffected bone would likely have been formed during the 2 weeks of risedronate therapy. It appeared that metastatic breast cancer invasion into soft tissue surrounding bone was more extensive in risedronate-treated animals than in untreated animals (Fig. 5D).

DISCUSSION

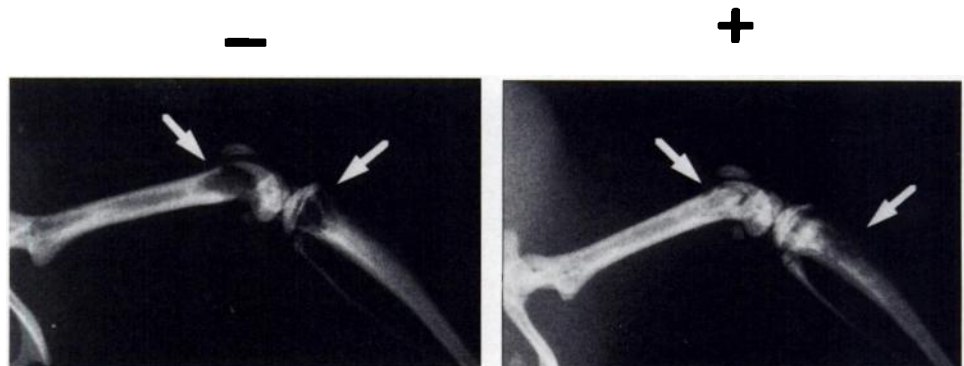
We have used an *in vivo* model in which human breast cancer cells spread to bone and formed osteolytic lesions to study the effects of the bisphosphonate (risedronate), which is a powerful inhibitor of osteoclastic bone resorption, on both the development and the progression of these osteolytic lesions. We have found that risedronate not only suppressed the development of new metastases, but it also inhibited the progression of already-established osteolytic lesions. Moreover, our results show that risedronate diminished metastatic breast cancer burden in bone, probably by indirect mechanisms.

Histomorphometric and stereological analyses demonstrated that progression of metastatic breast cancer in bone was profoundly and selectively decreased in risedronate-treated animals. Although inhibition by risedronate of osteoclastic bone resorption at metastatic sites with the use of a similar model in rat to that described here has been reported recently (14), quantitative data that clearly show a significant and selective inhibition of metastatic breast cancer progression in bone by treatment with bisphosphonate have not been reported previously. However, it is most unlikely that risedronate has direct anticancer effects. The volume of MDA-231 breast cancer that had metastasized to soft tissues surrounding bone was not diminished in risedronate-treated animals. Moreover, risedronate did not suppress enlargement of MDA-231 breast cancer implanted s.c. and failed to inhibit the proliferation of MDA-231 breast cancer cells in culture at concentrations that effectively inhibit osteoclastic bone resorption in organ culture assays. Because the only currently known specific action of bisphosphonates is osteoclast inhibition, we speculate that the decrease in the progression of metastatic MDA-231 breast cancer in bone in risedronate-treated animals was secondary to the inhibition of osteoclastic bone resorption. The results suggest that the rate of bone turnover, which is inhibited by bisphosphonates (15), influences metastatic breast cancer progression in bone.

Bone is a storehouse for a variety of growth factors (16) that are released into the bone microenvironment as a consequence of osteoclastic bone resorption (17). Inhibition of osteoclastic bone resorption by risedronate may limit the supply of growth factors that facilitate metastatic breast cancer cells to proliferate in the bone microenvironment. Thus, as proposed by Paget (18) more than a century ago, bone (soil) may provide a favorable environment for the growth of metastatic breast cancer cells (seed; Refs. 3, 19). Inhibition of osteoclastic bone resorption by risedronate may alter this environment and render it less favorable for metastatic breast cancer progression. In addition, hard calcified bone remaining due to inhibition of osteoclastic bone resorption may limit space availability for breast cancer cells to spread in bone. Our results together with a previous result (14), therefore, have shown an additional potential action of the bisphosphonates on cancer cells in bone that has not been appreciated previously. This may provide a rationale for the use of this drug as an adjuvant chemotherapeutic agent in combination with conventional antitumor therapeutic strategies in the management of cancer-associated bone metastasis.

In the present study, the effects of risedronate were examined in three different protocols. Protocol 1 is relevant to clinical situations in which administration of the drug is initiated subsequent to clinical detection of bone metastases. Protocols 2 and 3 were conducted to examine whether continuous and short-term prophylactic treatment with risedronate, respectively, prevented the development of new bone metastases. These studies are difficult to perform in breast

Fig. 11. Radiographs of osteolytic lesions of nude mice untreated (–) and treated with risedronate (+) for 7 days before cancer cell inoculation according to Protocol 3. Radiographs were taken at day 28.



cancer patients who manifest no clinical evidence of bone metastases, and very little has been explored and is known about the preventive effects of the bisphosphonate on breast cancer metastasis to bone. In all three protocols, we found radiological, histological, and histomorphometrical evidence that osteolytic bone metastasis caused by metastatic breast cancer was markedly suppressed by the treatment of animals with risedronate. The findings obtained in Protocols 2 and 3 raise the possibility that the bisphosphonates might have potential for the prophylactic treatment of patients with cancer that is known or predicted to preferentially metastasize to bone. Furthermore, the result that the risedronate showed equivalent suppressive effects on osteolytic metastases in Protocol 2 (continuous long-term administration) and Protocol 3 (short periods of pretreatment) suggests the feasibility of short-term intermittent administration of the drug, which would decrease the occurrence of undesirable adverse effects.

In contrast to the prolonged survival of animals which were treated continuously with risedronate, most of the clinical studies to date have reported that the bisphosphonates do not increase the survival of patients with cancer and bone metastases (20–22). In these studies, however, administration of bisphosphonates was initiated after patients manifested clinical evidence of bone metastases. In contrast, in experimental studies using animal models, such as ours and others (23) in which bisphosphonates cause an increase in survival rate, the drug is given either from the beginning of cancer cell inoculation or in a prophylactic manner. It is, therefore, possible that prophylactic administration of the bisphosphonates to cancer patients with high risk of bone metastasis might not only be preventive but prolong survival.

It should be noted that our data, although statistically not significant, also suggest that tumor volume in nonbone sites may be increased by risedronate treatment. A previous study using the same bone metastasis model has described the outgrowth of cancer cells from bone into surrounding soft tissue through bone foramina of the external venous plexus (24). It is possible that inhibition of progression in bone might in turn lead metastatic cancer cells to extend via this route to surrounding soft tissue where osteoclastic bone resorption is unnecessary for their invasion presumably. Thus, the bisphosphonate may also affect the invasive behavior of metastatic cancer in bone. This is a very important issue that will need to be studied carefully because if bisphosphonates inhibit cancer progression in bone but, on the other hand, stimulate their invasion into nonbone tissue, their therapeutic usefulness for the treatment of cancer patients with bone metastases will be limited. In addition, a previous study using rat Walker 256 cancer has shown that 3-amino-1-hydroxypropylidene-1,1-bisphosphonate, a second generation of bisphosphonate, increases tumor burden in the skeleton (25). Additional extensive studies are needed to establish the usefulness of the bisphosphonates for the treatment of metastatic cancer in bone.

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