Investigation of the effect of pamidronate disodium on the in vitro viability of osteosarcoma cells from dogs

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Objective—To determine the effect of pamidronate disodium on the in vitro viability of osteosarcoma cells and non-neoplastic cells from dogs.

Sample Population—3 osteosarcoma and 1 fibroblast cell line derived from dogs.

Procedure—Cell counts and cell viability assays were performed in cultures of osteosarcoma cells (POS, HMPOS, and COS31 cell lines) and fibroblasts after 24, 48, and 72 hours of incubation with pamidronate at concentrations of 0.001 to 1,000 µM or with no drug (control treatment). Percent cell viability was determined in cell samples for each concentration of pamidronate and each incubation time. A DNA fragmentation analysis was performed to assess bisphosphonate-induced apoptosis.

Results—Osteosarcoma cell viability decreased significantly in a concentration- and time-dependent manner at pamidronate concentrations ranging from 100 to 1,000 µM, most consistently after 48 and 72 hours’ exposure. In treated osteosarcoma cells, the lowest percentage cell viability was 34% (detected after 72 hours’ exposure to 1,000 µM pamidronate). Conversely, 72 hours’ exposure to 1,000 µM pamidronate did not significantly reduce fibroblast viability (the lowest percentage viability was 76%). After 72 hours of exposure, pamidronate did not cause DNA fragmentation in POS or HMPOS cells.

Conclusions and Clinical Relevance—Results indicate that pamidronate may have the potential to inhibit osteosarcoma growth in dogs, possibly through a nonapoptotic mechanism. The clinical relevance of these in vitro findings remains to be determined, but administration of pamidronate may potentially be indicated as an adjuvant treatment in chemotherapeutic protocols used in dogs. (Am J Vet Res 2005;66: 885–891)

Osteosarcoma is the most common primary bone tumor of dogs, accounting for 85% of all malignant skeletal neoplasms in that species. Locally, appendicular osteosarcoma is usually both osteoprodutive and osteolytic. Untreated lesions cause considerable pain and often result in pathological fracture of the affected bone and metastasis to the lungs, bones, or soft tissue sites. Amputation with or without adjuvant chemotherapy is the most common surgical procedure for treatment of osteosarcoma in the appendicular skeleton. Dogs with osteosarcomas and concurrent neoplastic or orthopedic disease or that are extremely large or obese may be poor candidates for amputation. For these animals, present treatment alternatives include limb-sparing surgery, fractionated radiation therapy, and stereotactic radiosurgery. However, limb sparing is only done routinely when the tumor is located in the distal portion of the radius, and limb-sparing surgery and radiation therapy are not always feasible because of the high cost and limited availability. Thus, many owners of dogs with appendicular osteosarcoma opt to treat the associated bone pain via administration of analgesics and have euthanasia performed when the quality of life of the affected dog becomes unacceptably poor.

Bisphosphonates are analogs of endogenous pyrophosphate, a naturally occurring inhibitor of bone metabolism. Bisphosphonates can be categorized into 2 classes. First-generation bisphosphonates are metabolized by intracellular enzymes into nonhydrolyzable analogs of ATP, likely leading to the inhibition of several ATP-dependent intracellular interactions. Second-generation bisphosphonates are nitrogen-containing aminobisphosphonates (eg, pamidronate and alendronate) with the amino group contained in the aliphatic carbon chain. The length and content of the aliphatic carbon chain appear to be important factors in the potency and pharmacologic activity of these agents.

Bisphosphonates have been used in the treatment of a variety of diseases including osteoporosis, human breast carcinoma, multiple myeloma, and Paget disease. Pamidronate disodium is a bisphosphonate that is administered IV and that antagonizes the mevalonate pathway, which is critical for a multitude of cellular functions. Pamidronate binds strongly to bone at sites of active remodeling and inhibits bone resorption for weeks or months without affecting bone growth and mineralization. In humans, it has been used to treat benign conditions, such as osteoporosis, and malignant diseases, such as hypercalcemia of malignancy and bone metastasis. Pamidronate also reduces the skeletal morbidity rate in patients with myeloma and breast cancer.

Administration of bisphosphonates is considered to be ideal for use as a palliative treatment of humans with osteolytic diseases because of their availability...
and relatively low cost; furthermore, treatments are associated with a low incidence of adverse effects and complications and with improvement in patients’ quality of life.

Recently, pamidronate has been shown to directly inhibit growth of human and rat osteosarcoma cell lines.23,24 In a study by Sonnemann et al,17 the inhibitory effect on cell proliferation was found to be independent of apoptosis. In a study by El-Abdaimi et al,25 pamidronate was administered SC to nude mice inoculated with a human breast cancer cell line; fewer than 10% of mice injected with high doses of pamidronate developed bone metastases, whereas 85% of mice receiving the vehicle alone developed metastases.

The bisphosphonates alendronate and zoledronate have recently been shown to inhibit canine osteosarcoma cell growth in vitro.25,26 To the authors’ knowledge, there is only 1 report26 of the use of bisphosphonates in dogs with naturally occurring tumors. Alendronate was used in efforts to palliate primary osteosarcoma in 2 dogs; 1 dog had osteosarcoma of the tibia, and the other had osteosarcoma of the maxilla. According to the report,26 both dogs remained comfortable as a result of treatment and survived for 12 and 10 months, respectively. However, because there were only 2 dogs studied, there was no control group involved in the assessment; furthermore, because survival was the only variable measured, it is difficult to conclude what benefit alendronate provided. That case report along with evidence for efficacy in the treatment of various malignant bone diseases in humans has prompted veterinary clinicians to use bisphosphonates in the treatment of some dogs with primary osteosarcoma.

The objective of the study of this report was to determine the effect of pamidronate disodium on the in vitro viability of 3 osteosarcoma cell lines and non-neoplastic cells (fibroblasts) from dogs. A further objective was to assess the potential role of apoptosis in cellular death resulting from treatment with pamidronate disodium. Our hypothesis was that pamidronate disodium would decrease the viability of tumor cells but not affect the viability of canine fibroblasts and that the effect would be independent of apoptosis.

Materials and Methods

Cell lines and culture maintenance—Three canine osteosarcoma cell lines were evaluated: POS,27 HMPOS,28 and COS31.29 The POS (parent osteosarcoma) cell line was originally developed from a primary osteosarcoma of the proximal portion of the left femur of a 1.5-year-old male mongrel dog. The HMPOS (highly metastatic parent osteosarcoma) cell line was a pulmonary metastatic derivative of the POS cell line. The COS31 cell line was established from a dog with a naturally occurring osteosarcoma. The fibroblast cell line was derived from a canine trachea.37 Osteosarcoma cell lines were cultured in RPMI 1640 medium supplemented with UV-treated fetal calf serum, L-glutamine, and antibiotics (penicillin [0.0625 g/L] and streptomycin [0.1 g/L]) at 37°C in 5% carbon dioxide and 95% room air. Fibroblasts were cultured in Dulbecco modified Eagle medium supplemented with the same additives and under the same conditions as used for culture of the osteosarcoma cells. The cells were grown to confluence, detached from the plates with trypsin, and washed with buffered physiologic saline (0.9% NaCl) solution (pH 7.4). Cells were stained with trypan blue3 and counted with a hemacytometer.

Assessment of cell viability—Cell counts and the cell viability assay were performed in triplicate in 96-well flat-bottomed microtiter plates. The MTT assay is a colorimetric test for cell viability. In this assay, viable cells with mitochondrial activity reduce the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to blue formazan crystals, which then can be measured spectrophotometrically. Each assessment was completed concurrently for each plate; the top 7 rows were designated for the MTT assay, and the bottom row was designated for cell counts. The POS, COS31, and fibroblast cells were seeded at 10,000 cells/well, and the HMPOS cells were seeded at 5,000 cells/well because of their high growth rate. Plates were placed in the incubator at 37°C with 5% carbon dioxide and 95% air for 24 hours. At 24 hours, the medium in each plate was removed and replaced with 100 µL of medium containing pamidronate. A stock solution was diluted to obtain pamidronate concentrations of 1 µM, 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 µM, 0.1 µM, 0.01 µM, and 0.001 µM. The microtiter plates were set up such that the number of samples receiving each concentration and the control treatment (no drug) was the same (ie, n = 8).

Following 24, 48, and 72 hours of drug exposure, cell counts were performed for the POS, HMPOS, and COS31 cells by use of the trypan blue dye exclusion assay for cell viability. In preparation for counting, cells in the bottom row of the 96-well microtiter plates were washed with saline solution (pH 7.4) and then detached with 10 µL of trypsin. Following the addition of new medium, 10 µL of the cell-rich medium was mixed with 10 µL of trypan blue in another microtiter plate. Ten microliters of the mixed solution was then loaded onto a hemacytometer on an inverted light microscope for cell counting.

Plates containing fibroblast, POS, HMPOS, and COS31 cells were incubated for 24, 48, and 72 hours, after which 10 µL of MTT reagent4 in PBS solution was added to achieve a final concentration of 5.5 mg/mL in each well with the exception of the last row, which was used to obtain cell counts. A 4-hour incubation period followed, after which 100 µL of 50% dimethylformamide in 10% SDS was added to dissolve the insoluble product. The optical density (OD) of the wells was measured at 540 nm by use of an ELISA microplate reader.7 Percentage viability of cells was determined for each concentration and for each incubation time by use of the following equation:

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\%\text{ Viability} = 100 \times \frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated cells}}
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DNA fragmentation analysis for the evaluation of apoptosis—Samples of POS and HMPOS cells that had been treated with pamidronate (ie, 72 hours of incubation with 500 and 1,000 µM pamidronate) or not treated (control samples) were lysed in 5 mL of Tris buffer for 1 hour at 37°C. An additional 1 mL of Tris buffer was added along with 5 µL protease K, and the mixture incubated for 3 hours at 56°C. At the midpoint of the incubation time, an additional 5 µL protease K was added. Samples were then extracted with phenol-chloroform and precipitated with ethanol and 3M sodium acetate. Following centrifugation, ethanol was decanted and the microcentrifuge tube was inverted to dry. The DNA was dissolved in Tris-Cl-EDTA buffer, and the RNA was digested with RNase A (50 µg/mL) at 37°C for 30 minutes. Following digestion, aliquots of 4 µg of each sample underwent electrophoresis for approximately 3 hours at 70 V with molecular weight markers of 123-bp multiples on a 1% agarose gel. The gel was placed in ethidium bromide (1 µg/mL) for 30 minutes and then placed in purified water for 30 minutes; the gel images were captured on a computer.
Data analyses—Statistical analysis to compare cell viability between treated wells and control wells after the same incubation time (ie, 24, 48, and 72 hours) was performed by use of a 1-way ANOVA; values of $P < 0.05$ were considered significant. A pairwise multiple comparison procedure was performed by use of the Dunnett method. Cells were visually inspected for evidence of DNA fragmentation.

Results
For all control (untreated) samples, viable cell number increased in near-linear fashion with increasing incubation time (Figure 1).

Effect of pamidronate on viability of POS cells—Pamidronate decreased the viability of POS cells in a concentration- and time-dependent manner with significant decreases occurring at concentrations of 500 µM (72 hours of incubation) and 1,000 µM (48 and 72 hours of incubation; Figures 1 and 2). Microscopic examination of the cells after exposure to pamidronate revealed loss of attachment to the plate, loss of normal morphologic features, and cellular fragmentation (Figure 3). Subjectively, the percentage of nonviable cells appeared to increase (more cells stained with try-
Pan blue) with increasing pamidronate concentrations. Pamidronate concentrations < 500 µM did not significantly affect viability of POS cells. The lowest percentage cell viability for POS cells was 43%, which was detected after 72 hours of incubation with pamidronate at a concentration of 1,000 µM.

Effect of pamidronate on viability of HMPOS cells—Pamidronate decreased the viability of HMPOS cells in a concentration-dependent manner with significant decreases occurring at concentrations of 500 µM (48 and 72 hours of incubation) and 1,000 µM (24, 48, and 72 hours of incubation; Figures 1 and 2). Pamidronate concentrations < 500 µM did not significantly affect viability of HMPOS cells. The lowest percentage cell viability for HMPOS cells was 33.4%, which was detected after 72 hours of incubation with pamidronate at a concentration of 1,000 µM.

Effect of pamidronate on viability of COS31 cells—Pamidronate decreased the viability of COS31 cells in a concentration- and time-dependent manner with significant decreases occurring at concentrations of 100 µM (72 hours of incubation), 500 µM (72 hours of incubation), and 1,000 µM (48 and 72 hours of incubation).
Fragmentation in either of the 2 osteosarcoma cell lines was not significantly different from that of the control concentration of 1,000 µM. The lowest percentage cell viability for canine fibroblasts was 76% of control value, which occurred 21% reduction in cell viability after 72 hours of incubation. In the present study, the 3 osteosarcoma cell lines had similar sensitivities to pamidronate, and significant decreases in cell viability occurred after 72 hours of incubation at pamidronate concentrations of 100, 500, and 1,000 µM. The effect of pamidronate on canine fibroblasts was also similar to that observed for human fibroblasts in that there was a decrease in cell viability of only 24% at maximal drug concentration (500 µM). There is no evidence of apoptosis (ie, no DNA laddering) in either tested cell line.

Effect of pamidronate on viability of fibroblast cells.—Treatment of canine fibroblast samples with pamidronate at any tested concentration and at any exposure time did not cause a significant decrease in cell viability, compared with viability of control samples (Figure 2). The lowest percentage cell viability for fibroblasts was 76% of control value, which occurred after 72 hours of incubation with pamidronate at a concentration of 1,000 µM; however, this decrease was not significantly different from that of the control value.

Assessment of apoptosis in pamidronate-treated cells.—After 72 hours’ exposure, pamidronate at concentrations of 500 and 1,000 µM did not cause DNA fragmentation in either of the 2 osteosarcoma cell lines evaluated (Figure 4). Thus, the cytotoxic effect of pamidronate did not appear to be a result of apoptosis induction.

Discussion
Bisphosphonates were originally used in the treatment of osteoporosis because of their ability to inhibit bone resorption. They are used clinically in the treatment and prevention of osteolytic diseases, such as metastasis to bones, and are gaining popularity in the oncology field of medicine. Recently, pamidronate was shown to directly inhibit the viability of osteosarcoma cells from humans. Reported effects of bisphosphonates include inhibition of tumor cell proliferation, induction of tumor cell apoptosis, inhibition of tumor cell adhesion and invasion, inhibition of tumor angiogenesis, and inhibition of bone metastasis in vivo. To the authors’ knowledge, this is the first published report on the effect of pamidronate on the viability of osteosarcoma cells from dogs. Our data obtained by use of canine cell lines have indicated that pamidronate has concentration- and time-dependent inhibitory effects on osteosarcoma cell viability in vitro, whereas it has no significant inhibitory effect on non-neoplastic canine mesenchymal cells (ie, fibroblasts).

Previous studies have revealed that bisphosphonates inhibit tumor cell growth in a time- and dose-dependent manner. In a study by Sonnemann et al., a significant decrease in the viability of osteosarcoma cells from humans was detected at pamidronate concentrations ≥ 50 µM and the effect was greatest after 72 hours of incubation. In the same study, pamidronate had only minimal effects on human fibroblasts, with a 21% reduction in cell viability after 72 hours of incubation. In the present study, the 3 osteosarcoma cell lines had similar sensitivities to pamidronate, and significant decreases in cell viability occurred after 72 hours of incubation at pamidronate concentrations of 100, 500, and 1,000 µM. The effect of pamidronate on canine fibroblasts was also similar to that observed for human fibroblasts in that there was a decrease in cell viability of only 24% at maximal drug concentration and exposure time. Other investigators have also documented time- and concentration-dependent inhibitory effects of pamidronate on proliferation of a variety of human and murine tumor cell lines at pamidronate concentrations ranging from 1 to 500 µM.

In our study, a significant decrease in cell viability in all 3 osteosarcoma cell lines was detected after 48 and 72 hours of incubation at pamidronate concentrations of 1,000 µM. The lowest concentration that resulted in significant inhibition of osteosarcoma cells was 100 µM, but this effect was only detected in the COS31 cell line. In most in vitro studies, including the present one, inhibitory effects of pamidronate have been detected at concentrations approximately 10-fold greater than sera concentrations reported in human patients. Although peak serum concentrations of pamidronate are transient, its strong affinity for bone mineral leads to accumulation at high concentrations over time. Because bone concentration of bisphosphonates increases with time, the longer incubation period used in the present study is probably more clin-
ically relevant. Intravenous administration of pamidronate in humans has been reported to result in serum concentrations that range from 0.5 to 10µM, depending on duration and dosage of IV infusion. Concentrations of alendronate at active osteoclast resorption sites have been reported to range from 100 to 1,000µM. Presently, the concentration of pamidronate to which bone tumor cells are exposed is unknown. Thus, we do not know whether intratumoral concentrations of pamidronate of 100µM or higher are achievable via standard dosing protocols used in humans (eg, 90 mg of pamidronate administered during a 1- to 2-hour IV infusion every 3 to 4 weeks).

The results of the DNA fragmentation analysis performed in the present study indicated that the inhibitory effect of pamidronate at concentrations of 500 and 1,000µM appears to be independent of apoptosis. This is contradictory to findings of a study by Foerter et al, which indicated that there was a proapoptotic effect of the bisphosphonates alendronate and zoledronate on osteosarcoma cells; however, the extent of the apoptotic effect was both drug and cell-line dependent. In our study, osteosarcoma cell death occurred after 72 hours’ exposure to pamidronate at concentrations of 500 and 1,000µM and was not a result of an apoptotic effect. It is possible that this may reflect the lower sensitivity of DNA fragmentation analysis in detecting evidence of apoptosis. Our results are consistent with those of Sonnemann et al; in their study, pamidronate decreased the viability of osteosarcoma cells from humans through a nonapoptotic mechanism as determined via DNA fragmentation analysis and fluorometric analysis. However, like the procedure used by Sonnemann et al, exposure of cells to pamidronate in the present study was carried out in the presence of serum, which is a survival factor for osteosarcoma cells. Further evaluation should be performed to fully characterize the mechanism of the cytotoxic action of pamidronate on osteosarcoma cells.

In our study, pamidronate at a concentration of 0.001µM produced a small increase in viabilities of POS and COS31 cells, compared with the viability of control cells, after 24 hours of incubation. Mathov et al reported a stimulatory effect of pamidronate on proliferation of human osteoblastic cells after short-term drug exposure to concentrations of 0.01 to 1µM. Bisphosphonates have also been shown to increase mineralization and alkaline phosphatase activity in chick periosteal osteoblasts and stimulate osteoblast precursors. In the present study, the cell viabilities of POS and COS31 cells were not significantly greater than the viability of the control cells and were most likely clinically irrelevant because bone concentration of bisphosphonates increases with time. Furthermore, in the study by Sonnemann et al, pamidronate did not have a stimulatory effect on osteosarcoma cells from humans.

Bisphosphonates have the ability to inhibit the osteolytic process and reduce pain associated with bone lesions. Because of the low cost, ease of administration, and availability of pamidronate and the low rate of complications associated with treatment, the potential use of this agent in dogs in which primary tumors are not removed surgically warrants further investigation. Our data have indicated that pamidronate may have the potential to inhibit osteosarcoma growth in dogs. It will be important to determine the clinical relevance of our in vitro findings. If in vivo studies provide similar findings, the administration of pamidronate may also be used as an adjuvant treatment in existing chemotherapeutic protocols.

References