INTRODUCTION:
Osteoclastogenesis and osteolysis involve adhesion and degradation of matrix. Osteoclast-like multinucleated giant cells are commonly seen in destructive bone tumors and have capacity for bone destruction. Osteoclasts are known to express several adhesion molecules and degrading enzymes which can be blocked to inhibit pathologic osteolysis. A dominant role of osteoclasts in tumor-induced osteolysis has been confirmed by several investigators using bone-resorbing cell lines. Bisphosphonates have been used for the treatment of osteoporosis and metastatic bone cancers. The known mechanisms of bisphosphonates include blockage of mevalonate pathways with resulting apoptosis of osteoclasts and precursor cells. In our GCT culture, a naturally occurring co-culture of neoplastic stromal cells, monocytes and multinucleated osteoclast-like giant cells, geranylgeraniol, a metabolic intermediate of mevalonate pathway, did not block the effect of bisphosphonates, suggesting the possibility of multiple targets. For these reasons, we investigated other therapeutic targets of bisphosphonates in GCT as well as apoptotic pathways.

MATERIALS AND METHODS:
(1) Characterization of GCT Culture: We established 3 cell cultures from GCT tissues after IRB approval. GCT culture consisted of stromal cells, round mononuclear cells and multinucleated osteoclast-like cells. These cultures showed tartrate-resistant acid phosphatase (TRAP) positive cells without exogenous osteoclastogenic factors. The cells with treated with various doses of Pamidronate and Zolendronate ranging from 10 µM to 1,000 µM.

(2) Cell Death Assay: After 3 days, cells were pelleted by centrifugation and resuspended in 5 ml PBS. Cell concentration was measured and the cells were then pelleted again and resuspended in binding buffer (A.G. Scientific) to the density of 5 x 10^5 cells/ml. Five phosphates have V-FITC (A.G. Scientific) was added to 195 µl of cell suspension and incubated in the dark for 10 minutes. The cells were pelleted and washed with PBS and resuspended again in 190 µl of binding buffer. Ten µl of 20 µg/ml propidium iodide (A.G. Scientific) was added to the cell suspension and flow cytometry was performed within 30 minutes using a FACS Calibur (Becton Dickson). Monoparametric cytograms of annexin V-FITC fluorescence (FL1) versus number of events were created using the CellQuest program gating for living cells and excluding dead cells on the basis of their propidium iodide uptake.

(3) Caspase-3 and PARP Western Blotting: Caspase-3 is a downstream effector of apoptosis and is present as an inactive, uncleaved form in the cytoplasm. The active, cleaved form can be detected using western blotting. In order to confirm the functionality of caspase-3, we assessed the form of caspase-3 from day 1 to day 3 while there was a decrease in PARP from day 1 to 3 (Figure 2). These findings suggest that caspase-3 is activated by Pamidronate and one of the substrates of caspase-3, PARP, is degraded by an activated caspase-3.

(3) Altered Gene Expression
(4) cDNA Miinarray for Adhesion Molecules and Cytokines: We extracted RNA and prepared cDNA for mini-array containing 400 genes related to adhesion, cytokines, signal transduction and osteogenesis. Biotin-labeled cDNA probes were made using RT PCR, and hybridized overnight in vitro following protocol via SuperArray GEArray™ S and Q Series Kits (for chemiluminescent detection). The computer program ScanAlyzer allowed us to obtain data from our arrays and GEArray Analyzer 1.3 allowed for analysis. Due to their importance in cellular adhesion and osteoclastogenesis, our analysis focused on integrin α5, integrin αV, integrin β1, collagen binding protein-1 (CBP-1), thrombospondin-1 (TSP-1), osteonectin (SPARC), cathepsin K, matrix metalloproteinase-2 (MMP-2), and fibronectin-1 (FN-1).

RESULTS:
Pamidronate and Zolendronate decreased cell proliferation and induced cell death in cultured GCT stromal cells.

(1) Cell Death Assay: Exposure to different concentrations of bisphosphonate produced morphological changes and a decrease in the number of adherent cells after only 24 hours of treatment compared to controls in a dose-dependent manner. Characteristics of apoptosis such as condensed nuclei and cell shrinkage were visible in most of the adherent cells after 48 hours (Figure 1).

(2) Activation of Apoptosis via Caspase-3: Western blotting after treatment of GCT cells with 100 µM of Pamidronate demonstrated an increasing amount of the active form of caspase-3 from day 1 to day 3 while there was a decrease in PARP from day 1 to 3 (Figure 2). These findings suggest that caspase-3 is activated by Pamidronate and one of the substrates of caspase-3, PARP, is degraded by an activated caspase-3.

DISCUSSION:
Our findings suggest that bisphosphonates can inhibit osteoclastic function during tumor-induced osteolysis by downregulating matrix degrading enzymes and adhesion molecules in addition to pro-apoptotic effects via specific pharmacologic action sites. Although we successfully demonstrated activation of caspase-3 by bisphosphonates, it remains unclear as to how osteonectin, MMP-2 and cathepsin K are regulated by bisphosphonates.