KELLI LYNN BOYD
The Comparison of the Effects of Risedronate on Bone Metastasis of Two Malignant Rat Mammary Tumor Cell Lines, MTA and MTB-01
(Under the direction of ELIZABETH WYNNE HOWERTH)

The goals of this study were to 1) demonstrate that the MTA rat mammary adenocarcinoma cell line is nonresponsive to treatment with the bisphosphonate risedronate and 2) compare the in vitro cellular responses of the MTA (nonresponder) and a related rat mammary adenocarcinoma cell line, MTB-01 (responder) to risedronate in order to identify factors responsible for the MTA cell line's response to bisphosphonate treatment.

To establish the MTA cell line as nonresponsive to risedronate treatment, Berlin-Druckery rats received an intracardiac injection of 10^5 MTA cells. Risedronate was administered daily to test animals beginning on the day of tumor cell inoculation. Control animals received MTA cells and daily saline injections. Animals were euthanized on day 14 and metastatic bone lesions were enumerated and area measurements were taken microscopically from femurs and vertebrae. There was no statistically significant difference in the size or number of metastatic foci in treated verses untreated groups.

The second set of experiments focused on identification of cellular characteristics of the MTA cell line that may account for the lack of response to risedronate. To accomplish this objective, the effects of risedronate on MTA cells (nonresponder) and MTB-01 cells (responder) were compared utilizing in vitro assays measuring apoptosis, adhesion, and matrix metalloproteinase (MMP) production. Apoptosis was measured by cell morphology at the light microscopic level, DNA fragmentation, TUNEL staining,

MiCK assay, Annexin-V binding, and transmission electron microscopy. Gelatin zymography was used to identify MMP-2 or MMP-9 protease production.

When compared to MTA cells, MTB-01 cells were susceptible to risedronate-induced apoptosis, had decreased ability to bind to risedronate-treated bone, and did not produce MMP-2 or MMP-9 proteases. MTA cells were less susceptible to risedronate-induced apoptosis and produced MMP-2. Additionally, adhesion of MTA cells to bone matrix was not diminished by risedronate treatment. Our results suggest that the non-responsive nature of the MTA cell line may be due to MMP-2 production (possibly allowing ongoing destruction of risedronate-treated bone), continued adhesion to risedronate-treated bone matrix, and decreased susceptibility to risedronate-induced apoptosis.

INDEX WORDS: Bone metastasis, risedronate, rodent model, apoptosis, matrix metalloproteinase, cell adhesion

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by

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INTRODUCTION

Skeletal metastases frequently complicate some of the most commonly occurring human cancers, including those of the breast, lung, and prostate.[1,2] Bone lesions are commonly the earliest manifestations of distant metastatic disease in breast cancer patients. Morbidity is associated with destruction of the bone matrix. Patients may develop severe bone pain, pathologic fractures, hypercalcemia (possibly life-threatening), nerve impingement, and decreased motility. After initially colonizing marrow spaces, tumor cells orchestrate bone destruction by releasing chemical signals that directly (or perhaps indirectly via osteoblast intermediation) stimulate osteoclastic bone resorption. Osteoclastic osteolysis is recognized as the most important mechanism of bone destruction in metastatic sites,[3] though some bone-metastatic tumor cells are also capable of direct destruction of bone matrix without osteoclast involvement.[4] How significant the contribution of direct tumoral osteolysis may be in certain cases is not known. Therapy aimed at decreasing or preventing bone metastasis with its associated morbidity could greatly improve the quality of life or extend the disease-free interval in some cancer patients.

In addition to causing the destructive skeletal lesions and metabolic imbalances noted above, osteolysis is also important in creating a suitable microenvironment that supports tumor cell colonization and expansion in bone. In the microenvironment of normal remodeling bone, many factors are released from the resorbing matrix. Substances such as transforming growth factor ß and insulin-like growth factor are

thought to be strong stimuli for chemotaxis and/or proliferation of tumor cells and may influence their localization in skeletal sites. In response to TGF-ß and insulin –like growth factor, cultured human breast cancer cells have been shown to release parathyroid-hormone related protein, stimulating further resorption and thus creating a vicious cycle within the bone microenvironment.[4-7]

Osteolysis in metastatic lesions is primarily mediated by osteoclasts responding to signals released by tumor cells. Osteoclast inhibition is a potential mechanism that could interrupt this cycle. Bisphosphonates, a group of drugs derived from bisphosphonic acid, have been shown to repress bone resorption by potent inhibition of osteoclast recruitment and/or function in normal bone and in metastatic bone lesions.[8,9] Several bisphosphonate derivatives have shown beneficial effects on bone metastasis in human[10-14] and animal[15-17] trials. Animal trials have generally shown a decrease in number and size of metastatic lesions, as well as decreased invasion and resorption by contiguous tumors. Several human trials report decreased bone pain, pathologic fractures, increased mobility, correction of hypercalcemia, and decreased formation of lytic lesions as evaluated by radiography and/or scintigraphy during the trial period.[10-14, 18]

Various bisphosphonates, synthetic derivatives of bisphosphonic acid capable of inhibiting osteoclastic bone resorption, have been effective in treating and preventing destructive bone metastatic lesions in animal models[15,19,20] and human clinical trials.[11,14,21,22] Positive responses have included decreased pain and increased mobility, resolution of lytic bone lesions, and decreased occurrence of newly developed lytic bone metastases. Pamidronate is approved for myeloma patients and those with osteolytic breast cancer metastases.[23] Manipulations of chemical structure have resulted in new-

generation bisphosphonates capable of 100 to 10,000-fold increased anti-resorptive activity.[24] Among the most potent are the nitrogen-containing heterocyclic ring derivatives such as risedronate, ibandronate, and zoledronate, compounds currently being evaluated for therapeutic efficacy for osteolytic diseases.

The influence of bisphosphonates on size and destructiveness of lesions was expected, but specific mechanisms responsible for the decreased incidence of bone metastases in treated patients are not known. In only a few cases, bisphosphonates have been shown to be directly cytotoxic to tumor cells.[9] Their most significant influence on lesion incidence may be indirectly related to osteoclast inhibition. Inhibition of osteoclastic resorption halts the release of growth factors and other factors from the bone matrix, potentially depriving tumor cells of important signals needed for colonization of bone. Evaluation of adhesive, chemotactic, invasive, and apoptotic responses of bone-metastatic tumor cells under the influence of bisphosphonate treatment, in this project, may offer some clarity to this issue.

Bisphosphonate therapy shows great promise for patients with metastatic bone disease. However, human and animal trials have identified individuals that have not responded to treatment. In some cases there was no decrease in number or size of lytic lesions while other patients had persistent hypercalcemia.[11,13] Research defining poor response to bisphosphonate therapy is relatively unexplored. Failure of therapy may be attributable to inherent differences in potency of the various bisphosphonate derivatives used in these studies or to differences in tumor phenotypes, such as the ability of some tumors to lyse bone directly without involvement of osteoclasts.[11,22,25] Direct osteolysis by tumor cells independent of osteoclasts has been recognized for years and this capacity

may vary widely between different tumors. Direct tumoral osteolysis, possibly mediated by tumor proteases, may contribute to the poor response of some tumors to bisphosphonate therapy. Tumor cells that respond less favorably to bisphosphonates may possess properties allowing them to directly lyse bone and/or to survive and maintain adhesive, invasive and proliferative responses in the presence of products released from treated bone matrix. Tumor responding more favorably to treatment may be limited in one or more of these characteristics. Knowledge of this process may lead to identification of *s*pecific markers to predict the responsiveness of bone metastatic tumors to antiosteolytic therapy and design of alternative or adjunct treatment strategies that might benefit patients with unresponsive bone-metastatic tumors.

This project seeks to enhance our knowledge concerning the development of metastases in bisphosphonate-treated bone. We have employed a rat model consisting of two related cell lines capable of faithfully reproducing metastasis to bone following injection into the left cardiac ventricle. The bisphosphonate used was the extremely potent third generation derivative, risedronate. One tumor line (MTB-01) has been shown previously to respond well to risedronate therapy. Our pilot data suggested that the response of the second line (MTA) to risedronate is much less favorable. The goal of this study is to establish the MTA cell line as a non-responder to risedronate therapy and compare the responses of the responding line (MTB-01), and the non-responding line (MTA) in various *in vitro* assays. These assays include measuring matrix metalloprotienase production, adhesion, invasion, chemotaxis, and degree of apoptosis after cellular exposure to risedronate. Comparison of the effects of risedronate treatment on these cellular activities will provide a valuable

tool for investigations of specific tumor properties responsible for sub-optimal responses to bisphosphonates. Specific objectives of this work were:

1) to establish the MTA rat mammary adenocarcinoma cell line as a nonresponder to treatment with the bisphosphonate risedronate

2) to compare the in vitro cellular responses of the MTA (non-responder line) and a related rat mammary adenocarcinoma cell line, MTB-01 (responder line) to risedronate in order to identify factors that make the MTA cell a poor responder to bisphosphonate treatment.

References

1. Stoll BA. Bone Metastasis: Monitoring and Treatment. New York: Raven Press; 1983;Natural history, prognosis, and staging of bone metastases. p. 1-20.
2. Guise TA, Mundy GR. Cancer and bone. Endocrinology review 1998;19:18-54.
3. Argeullo F, Baggs RB, Duerst RE, et al. Pathogenesis of vertebral metastasis and epidural spinal cord compression. Cancer Research 1990;42-54.
4. Orr FW, Lee J, Duivenvoorden WCM, et al. Pathophysiologic Interactions in Skeletal Metastasis. Cancer 2000;89:(12)2912-8.
5. Mundy GR. Mechanisms of osteolytic bone destruction. Bone 1991;12 Supplement:S1-6.
6. Orr FW, Varani J, Gondek MD, et al. Chemotactic responses of tumor cells to products of resorbing bone. Science 1979;203:176-9.
7. Orr FW, Varani J, Gondek MD, et al. Partial characterization of a bone-derived chemotactic factor for tumor cells. American Journal of Pathology 1980;99:43-52.

8. Mundy GR, Yoneda T. Bisphosphonates as anticancer drugs. New England Journal of Medicine 1998;339:398-400.
9. Rogers MJ, Gordon RK, Benford HL, et al. Cellular and molecular mechanisms of action of bisphosphonates. Cancer 2000;88:2961-78.
10. Clemmesen B, Ravn P, Zegels B, et al. A 2-year phase II study with 1-year follow-up of risedronate (NE-58095) in postmenopausal osteoporosis. Osteoporosis International 1997;7:488-95.
11. Cleton FJ, van Holten-Verzantvoort AT, Bijvoet OLM. Rec Res cancer Res. 116th ed. New York: Springer-Verlag; 1989;Effects of long-term bisphosphonate treatment on morbidity due to bone metastases in breast cancer patients. p. 73-78
12. Cohen-solal ME, Roux CH, Valentin-opran A, et al. Histomorphometric effect of six month treatment with oral risedronate in patients with multiple myeloma. Bone 1993;14:505-9.
13. Diel IJ, Solomayer E, Costa SD, et al. Reduction of new metastases in breast cancer with adjuvant clondronate treatment. New England Journal of Medicine 1998;339:357-63.
14. Elte JWF, Bijvoet OLM, Cleton FJ, et al. Osteolytic bone metastases in breast carcinoma pathogenesis, morbidity, and bisphosphonate treatment. European Journal of Cancer Clinical Oncology 1986;22:493-500.
15. Guaitani A, Sabatini M, Polentarutti N, et al. Garattini S, editors.Bone Resorption, Metastasisi, and Diphosphonates. New York: Raven Press; 1985;Effects of disodium etidronate on tumor-induced hypercalcemia, bone invasion, and metastasis in rodents. p. 51-8.

16. Hall DG, Stocia G. Effect of the Bisphosphonate Risedronate on Bone Metastases in a Rat Mammary Adenocarcinoma Model System. Journal of Bone and Mineral Research 1994;9:221-30.

17. Sasaki A, Boyce RW, Story B, et al. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res 1995;55:3551-7.

18. Selander KS, Monkkonen J, Karhukorpi EK, et al. Characteristics of clodronate-induced apoptosis inosteoclasts and macrophages. Molecular Pharmacology 1995;50:1127-38.

19. Kelmas PD, Balena R, Confravreux E, et al. Bisphosphonate risedronate prevents bone loss in women in artificial menopause due to chemotherapy of breast: a double blind placebo-controlled study. J Clin Oncol 1997;15: 955-62.

20. Nemoto R, Satou S, Miyagawa I, et al. Inhibition by a new bisphosphonate (AHBuBP) of bone resorption induced by MBT-2 tumor of mice. Cancer 1991;67:643-8.

21. Fittion A, McTavish D. Pamidronate. A review of its pharmacological properties. Drugs 1991;41:289-318.

22. Thiebaud D, Leyvraz S, von Fliedner V, et al. Treatment of bone metastases from breast cancer and myeloma with pamidronate. European Journal of Cancer 1991;27:37-41.

23. Mortensen L, Charles P, Bekker PJ, et al. Risedronate increases bone mass in an early postmenopausal population: two years of treatment plus one year of follow up. Journal of Clinical Endocrinologic Metabolism 1998;83:396-502.

24. Adami S, Zamberlan N. Adverse effects of bisphosphonates: a comparative review. Drug Safety 1996;14:158-70.

25. Roux CH, Ravaud P, Cohen-solal ME, et al. Biologic, histologic, and densitometric effects of oral risedronate on bone in patients with multiple myeloma. Bone 1994;15:41-9.

LITERATURE REVIEW

This review will discuss general and specific mechanisms involved in tumor metastasis. Processes specifically pertinent to tumor cells in the bone microenvironment, including tumor cell proliferation, adhesion, and invasion will be emphasized. Bisphosphonate drugs and their effects on cells within bone will be discussed, followed by a review of the work that has been done in animal metastasis models and in human trials to evaluate these drugs as therapeutic agents for skeletal metastasis. A major point of this dissertation is that while positive responses have been shown for many tumor types and with several bisphosphonate derivatives, a favorable response to therapy is not always obtained and reasons for these treatment failures are currently unknown.

Mechanisms of tumor metastasis

Distant metastasis of solid tumors is a multistep process requiring interaction between tumor cells, normal host cells, and extracellular matrix components. Micrometastatic tumor cells can be detected in 25-75% of patients with common malignancies.[1] All of these lesions will not become clinically significant disease. The presence of tissue specific cytokines and growth factors in the microenvironment of different organs plays a major role in the formation of metastatic lesions. Implantation of tumor cells into a favorable microenvironment results in predictable patterns of metastasis for specific tumor types. This "seed and soil" theory was proposed by Paget over one hundred years ago.[2] Patients with breast, prostate, and lung carcinomas often have a significant portion of the tumor burden in bone at the time of death suggesting that the bone microenvironment is fertile soil for the growth of these types of tumors. This

project focused specifically on bone metastasis of two rat mammary carcinoma cell lines and the effect of treatment with a bisphosphonate, risedronate, on the metastatic pattern and selected steps in the process of metastasis.

Formation of metastatic lesions is dependent on the capability of the tumor cells to successfully complete a multistep process known as the metastatic cascade. This process can be divided into general steps that probably occur: (1) loss of cellular adhesion molecules necessary for detachment from the primary site, (2) production of proteolytic enzymes needed for invasion into the extracellular matrix and blood vessels at the primary site, (3) evasion of the host immune surveillance, (4) attachment and invasion at the metastatic site, and (5) survival and proliferation in the new microenvironment. In addition, bone metastatic neoplasms must possess additional unique properties that allow for their survival and proliferation in the bone microenvironment. Development of strategies to block any one of the steps in this cascade could prove useful in the prevention of metastatic lesions.

Each of these steps is carried out through specific molecular events that occur in both the tumor and host cells.[2, 3] These steps involve the detachment of the tumor cells from adjacent cells or stroma and invasion into the adjacent host organ tissue or directly into tumor capillaries allowing access to the general circulation. Invasion and extravasation of the tumor cells involve similar events. The cells must attach to the basement membrane and secrete proteolytic enzymes to degrade the basement membrane. The cells must then be able to migrate through the degraded membrane to the extravascular space, which in the case of bone metastasis is the marrow cavity.[4, 5]

The loss or expression of cellular adhesion molecules (CAMs) is crucial to the process of metastasis. Molecules such as E-cadherin and laminin play an essential role in tumor cell invasion. These adhesion molecules mediate cell-cell interaction and cell to extracellular matrix attachments. Loss of these molecules results in detachment of tumor cells from the primary mass, initiating invasion into the surrounding tissue and the metastatic process.[6] Loss of these adhesion molecules during invasion and while within the circulation is essential for metastasis to proceed. The re- expression and possibly increased expression of these adhesion molecules may be necessary for adhesion of tumor cells at the metastatic site. Metastatic breast and ovarian carcinomas have been shown to have variable expression of E-cadherin depending on the culture conditions *in vitro* and environmental factors *in vivo*.[7] Therefore, expression of adhesion molecules by cancer cells may be decreased or increased depending on the stage of metastasis and the sites of metastasis.

E-cadherin is a calcium dependant cell surface glycoprotein responsible for epithelial cell-specific adhesion. E-cadherin causes homotypic cell aggregation which may be important in embryogenesis and morphogenesis.[8] It has been shown that E-cadherin has an important role in cancer invasion and metastasis.[9] Treatment of noninvasive Mardin-Darby canine kidney cells with antibodies to E-cadherin renders them more invasive.[10] In highly invasive cancer cells, over expression of E-cadherin dramatically suppressed their invasiveness. Addition of E-cadherin specific antisense RNA made noninvasive epithelial cells invasive. In the less invasive mammary carcinoma cell line MCF-7, E-cadherin expression is increased, while in, the highly invasive MDA-MD-231 cell line, low levels of E-cadherin are detected.[11] After injection

of MDA-MD-231 cells into the left ventricle of nude mice, osteolytic metastases are detectable by 4 weeks. In contrast, when high E-cadherin expressing MCF-7 cells are injected into the left ventricle, osteolytic lesions are not detectable for 8 weeks. Additionally, transfection of the MDA-MD-231 cell line with E-cadherin cDNA decreases the ability of this cell line to metastasize.[12]

Integrins are some of the most abundant CAMs and are also responsible for cell to cell and cell to matrix interactions. Integrins have also been shown to mediate attachment of tumor cells to vascular endothelium and to matrix proteins such as laminin and fibronectin.[13] Integrins underlie the endothelial basement membrane. Attachment to integrins is a key step in tumor colonization. Human myeloma cells A375 have been shown to express high levels of $\alpha_v\beta_3$ integrin when binding to and invading the basement membrane matrix matrigel.[14] Antagonist to laminin have been shown to decrease the development of osteolytic bone metastasis by A375 cells in nude mice.[15]

The $\alpha3\beta1$integrin is elevated in several types of metastatic tumors. This receptor recognizes a variety of extracellular matrix proteins including laminin-1, laminin-5, fibronectin, collagen, and entactin. Mammary carcinomas frequently show atypical patterns of $\alpha3\beta1$ expression. This integrin promotes the adhesion and spreading of metastatic breast carcinoma cells on lymph node stroma.[16] Recently, MDA-MB-231 cells were shown to possess $\beta1$, $\alpha2$, $\alpha3$, $\alpha5$, and $\alpha6$ subunits along with moderate levels of $\alpha v\beta3$ integrin. In this study, $\alpha3\beta1$ integrin was shown to be critical for the invasion and migration of the MDA-MB-231 cells. Immunohistochemistry for $\alpha3\beta1$ in primary breast carcinomas and their corresponding lymph node metastasis showed a statistically significant increase in $\alpha3\beta1$ expression in the metastatic tumor cells regardless of the

histiogenesis of the primary mass. Additionally, increased α3β1 was identified at non-nodal sites of metastasis including liver, lung, and brain, as well as in local recurrences. Antibodies to the α3 integrin subunit greatly inhibited MDA-MB-231migration toward laminin-1 and correlated with a decrease in matrix-metalloproteinase-9 production, suggesting that α3β1 binding may also play a role in cell signaling and extracellular matrix degradation.[17]

Intimately associated with the process of cellular attachment and detachment is the degradation of the extracellular matrix. Proteolytic degradation of the basement membranes of the blood vessels and the tissue stroma is necessary for cancer cells to exit the circulation. Disruption of the basement membranes involves enzymes such as type IV collagenase and other proteolytic enzymes. Tumor cells move across the basement membrane by directed migrational chemotaxis, [18] but random movement (chemokinesis) may also be involved. Motility factors produced by the tumor cells can enhance movement through the basement membrane defects.[5]

Bone metastasis

Histologic growth pattern, [19] expression of c-erb-B2, [20] parathyroid hormone-related protein (PTHrP) 1-139 isoform mRNA, [21] bone sialoprotein, [22] loss of heterozygosity, [23] and activation, mutation, or inhibition of "metastasis" and "antimetastasis" genes[24] that have been associated with the ability to metastasize to bone. Bone is a unique metastatic target. It is a physically defined microcompartment accessible primarily via the blood stream. In some cases bone metastasis may occur because this is the first compartment reached when a malignant cell leaves the primary tumor. Bone metastasis usually originate within the red marrow, where there are vascular

sinusoids lined by endothelium that lack a basement membrane and have 60-Angstrom fenestrae.[25] Dynamic cell populations in the marrow and bone play a role in the survival of tumor cells in the marrow. In some cases tumor cells may be kept dormant by the actions of CD8+ immune T-cells.[26] The marrow is also a good source of mitogens that can stimulate the growth of prostate[27, 28] or breast carcinoma cells.[29, 30] Growth factors released during homeostatic remodeling can promote the expression of metastatic phenotype of osteotropic cells and provide powerful stimuli for chemotaxis of additional cancer cells to the bone.[31, 32] Also, cancer cells can mediate the skeletal metabolism (osteolysis), creating a vicious cycle that promotes the development of additional metastatic lesions[33,34] **(Figure 1).**

The arrival of tumor cells into the bone microenvironment usually instigates extensive osteolysis of the mineralized bone matrix. Cancer cells can use a variety of mechanisms to induce bone resorption, but stimulation of osteoclast-mediated osteolysis through the production of parathyroid related peptide (PTHrP), [21] TGF-β, Interleukin-1 (IL-1), IL-6, and IL-11, [35] and/or tumor necrosis factor α and β, [36] is thought to be the most important mechanism by which bone resorption occurs in metastatic sites. PTHrP released by some cancer cells has been shown to act on osteoblasts to promote the expression of the receptor-activated nuclear factor (NF)-κB ligand (RANKL). RANKL subsequently binds its receptor RANK in osteoclasts and enhances osteoclast activity.[33]

Additionally, osteolysis can be carried out by tumor-associated macrophages and directly by metastatic cancer cells. In B16F1 melanoma cells, [37] MDA-MB-231,[38] and MTA carcinoma cells,[39] direct contact of the tumor cells with the resorbing bone in the absence of osteoclasts suggests direct bone lysis. *In vitro*, cancer cells have also been

shown to form bone pits and to degrade nondecalcified bone matrix and osteoblastic matrix.[38]

Matrixmetalloproteinases (MMPs) have been implicated as mediators of direct tumor osteolysis. MMPs are zinc and calcium dependant enzymes synthesized as inactive zymogens (proenzymes) in connective tissue. Most are activated by tissue or plasma proteinases, opportunistic bacterial proteinase, or by other MMPs. They constitute the principle matrix degrading proteinases. Some of the normal processes in which MMPs play a role include, embryonic development, organ morphogenesis, ovulation, endometrial cycling, hair follicle cycling, bone remodeling, and angiogenesis and wound healing. MMPs also play a role in pathologic processes such as arthritis, tumor invasion and metastasis, tumor angiogenesis, multiple sclerosis, Alzheimer's disease, atherosclerosis, breakdown of the blood brain barrier in cerebral ischemia, nephritis, and tissue ulceration.[40] Of most significance to this project is the role MMPs play in skeletal metastasis. Recently MMP-9 was shown to be involved in tumor-mediated osteolysis by a metastatic prostate carcinoma cell line, PC-3. These proteinases are likely to mediate direct osteolysis by other cancer cell lines.[41]

MMPs, along with other cysteine proteases, such as, cathepsin K, are mediators of normal bone growth and remodeling. Type I collagen is the most abundant collagen and is the protein most abundantly present in the bone matrix. MMP-1, interstitial collagenase, MMP-13, and collagenase 3, can degrade native Type I collagen.[42] MMP-2, 72kDa Type IV collagenase, (gelatinase A), and MMP-9, 92kDa Type IV collagenase, (gelatinase B), attack degraded type I collagen. MMP-3, stromelysin-1, and membrane MMPs play a role in final activation of MMP-1 and MMP-9.[43]

Cultured human osteoclasts have been shown to produce MMP-1, MMP-2, and MMP-9. MMP-1 has been localized to human osteoblasts where it is present in much higher concentrations compared to the amount produced by osteoclasts. This supports the theory that osteoblasts play a role in removal of surface collagenous osteoid before osteoclast attachment.[44]

Degradation of bone collagen I by cancer cells is associated with the secretion of MMP-2 and MMP-9 and can occur in devitalized bone devoid of osteoclasts.[45] MMPs play a role in the invasion and destruction of bone by multiple myeloma and by giant cell tumors of bone.[46] Inhibition of MMP activity by methods that inhibit MMP release or production or by over expression of tissue inhibitors of MMPs decreases the colonization and destruction of bone marrow in bone metastasis models. Batimastat, a synthetic inhibitor of MMPs, has been shown to block the activity of MMPs produced by MDA-MB-231 cells as measured by *in vitro* zymography. Decreased degradation of osteoblast-like matrix and decreased formation of resorption pits were also observed. Administration of batimastat to nude mice receiving left ventricular injection of these cells reduced osteolysis, tumor growth and replacement of the marrow by tumor.[38] Another cell-derived factor capable of resorbing hydroxyapatite, without osteoclasts or macrophages is neurochondrin. Neurochondrin has been identified in BW 5147 T-lymphoma cells, chondrocytes, osteoblasts and osteocytes.[47]

Cancer cell proliferation in the bone microenvironment

Bone provides a rich source for mitogens and agonists for metastatic cancer cells. Osteoblast-derived growth factors within the bone matrix that mediate differentiation and proliferation of indigenous bone cells can also promote the proliferation of some

established cancer cells lines. *In vitro*, conditioned media from bone resorbing cell cultures will stimulate the growth of metastatic competent cell lines to a degree that parallels the extent of bone resorption.[30] These mitogenic factors include, undefined components of the bone matrix, products of resorbing bone, defined bone-derived growth factors, platelet-derived growth factor, and hematopoietic growth factors. Defined bone-derived growth factors include, basic fibroblastic growth factor, insulin like growth factors, IL-6 and IL-1.[1] Granulocyte-macrophage colony-stimulating factor, IL-3, and transferrin comprise the hematopoietic growth factors. Purified insulin-like growth factor –1 (IGF-1) and IGF-II significantly increased *in vitro* proliferation of prostatic carcinoma cell lines. Some of these factors such as TGF-β, may have an inhibitory or biphasic effect on the tumor cells depending on the histiogenesis, the stage of differentiation, or progression.[48] Bone sialoprotein, a secreted glycoprotein found in bone matrix, elicits a proliferative response in the MDA-MB-231 cell line.[28]

In vivo experiments have shown that factors released from bone can regulate cancer cell proliferation. Mice deficient in hematopoietic stem cell factor have smaller metastatic burdens of B16 melanoma cells compared to normal control mice.[49] In rats with spontaneous metastasis of Walker 256 rat mammary carcinoma, tumor cells adjacent to the endosteal bone surface have a growth rate greater than cells situated >50μm away from the bone surface.[50] Artificial stimulation of bone resorption in this model increased the growth rate of cells in the bone-metastatic site but not in other organs, thus providing additional supporting evidence that bone-derived growth factors stimulate the growth of Walker 256 cells.[29]

Adhesion to bone

Adhesion of tumor cells to bone marrow endothelium, extracellular matrix, and bone marrow stroma is essential for the localization and proliferation in bone-metastatic sites. Selective adhesion of cancer cells is mediated by specific ligands that interact with substrates in bone such as α4 integrin counterreceptors[51] and laminin.[15] These attachments can regulate metastasis by promoting localization, [52] spreading, [53] migration, and proliferation[30] of cancer cells and by inducing the local expression of cytokines such as IL-6 that stimulate osteoclastic bone resorption, thus causing the local release of bone-derived growth factors.

Several *in vitro* experiments have identified specific receptors involved in preferential adhesion to bone marrow stromal components by metastasizing cancer cells. Rat prostate carcinoma cells Dunning Mat-LyLu preferentially adhere to bone marrow endothelial cells and bone marrow stromal cells.[54] Another prostatic carcinoma cell line was shown to have preferential adhesion to cells from the bone marrow stroma and specific adhesion to bone marrow endothelial cells that is mediated by galectin-3.[55] Adhesion of murine myeloma cells (B9/BM) to bone marrow endothelium occurs through LFA-1 and CD-44 attachments.[51] Human myeloma cells expressing α4 integrin (VLA-4) adhere to the marrow endothelium, which results in subsequent IL-6 secretion and osteoclastic resorption.[56]

In vivo, manipulations of α4 integrin in experimental models of cancer have had similar results. Chinese hamster ovary cells injected into nude mice result in pulmonary metastasis. Transfecting CHO cells with α4 integrin results in bone metastatic lesions in nude mice. Injection of transfected cells and antibodies to α4 integrin inhibits formation

of bone metastasis but not pulmonary lesions. Human leukemia cells, K562 erythroleukemia cells, were also transfected with α4 integrin, which conferred bone-metastatic potential to these cells in SCID mice.[52] Blocking adhesion of human melanoma (A275) cells to laminin through injection of a synthetic agonist inhibited the formation of bone metastasis.[15]

Human myeloma cells,[57] PC-3 prostate carcinoma cells,[53] and 1˚ prostate epithelial and carcinoma cells[58] bind collagen I through α2β1 integrin and syndecan, which results in cell adhesion and spreading. Interaction of metastatic cells with TGF-β induces further expression of α2β1 integrin. Human prostate carcinoma cells (LNCaP and DU145) bind bone extract and fibronectin through αvβ3 and α2β3integrin, respectively, resulting in cell adhesion and spreading.[27] Binding of vitronectin by αvβ3 on PC-3 carcinoma cells promotes cell migration. Cell migration and proliferation are stimulated through the binding of bone sialoprotein and αvβ5 integrin on MDA-MB-231 breast carcinoma cells. Inhibition of α3β1 integrin in MDA-MB-231inhibited migration and invasion of these cells and was also correlated with a significant decrease in MMP-9 production.[30]

Chemotaxis and migration

Chemotaxis and migration of cancer cells is essential for invasion and extravasation at metastatic sites. Ameboid-like properties of cells allow for movement through pseudopodial extensions. Highly motile cells are capable of producing osteolytic metastasis compared to cells rendered less motile due to over expression of heat shock protein 27.[59] Growth factors released during homeostatic remodeling, as discussed previously, can be potent mitogens for cancer cells. These factors also play a role in

recruitment of cancer cells to bone (chemotaxis and migration). In early experiments investigating mechanisms of bone metastasis, bone-conditioned media were shown to initiate directed migration (chemotaxis) of Walker 256 cells.[50] These experiments suggested the existance of a feedback loop between the cancer cells and the resorbing bone. Soluble factors released by the Walker 256 cell line enhanced bone resorption, increasing the release of chemattractants from the bone.[32, 60] Later work has identified specific products that promote chemotaxis, adhesion, and invasion, including Type I collagen and Type I collagen fragments,[61] α_2 HS glycoprotein, osteoclacin, peptides containing amino acids found in the collagen helix,[62] and TGF-β.[63] Osteonectin promotes MMP production in prostate and breast cancer cells and plays a role in bone metastasis by promoting migration, protease activity, and invasion.[64] Conditioned media harvested from osteoblast cultures stimulated chemotaxis and invasion of artificial basement membrane (Matrigel) by prostate carcinoma cells. This media stimulated production of urokinase (uPA) and MMP-9 by the cancer cells. Antibodies or inhibitors of uPA and MMP-9 reduced Matrigel invasion.[65] In MDA-MB-231 cells, TGF-β receptor-mediated pathways initiate the secretion of PTHrP, resulting in osteolysis and increased metastasis *in vivo*.[66] TGF-β also induces the production or MMP-1 and MMP-9 in these cells, contributing to osteolysis. In three breast carcinoma cell lines, MDA-MB-231, MCF-7, and T-47D exposure to substances such as IL-6, IL-11 induced significant migration while laminin inhibited migration. However, the potency of chemotaxis or chemoinvasion can vary with the specific cell line, the cytokines, and the extracellular matrices.[67]

Ongoing investigations into these specific mechanisms involved in cancer cell metastasis to bone provide useful targets for therapies designed specifically for the bone microenvironment. Work of this nature has the potential to uncover therapeutic modalities that may prevent bone-metastasis.

Bisphosphonates

The study of bisphosphonates began over 30 years ago when they were shown to precipitate calcium *in vitro* and *in vivo*. Soon after, they were also proven to inhibit bone resorption. In the past decade the use of this class of drugs in clinical settings has increased tremendously and various derivatives have proven to be clinically effective treating and preventing metastatic lesions in animal models and human clinical trials.[68-70]

Bisphosphonates (BPs) are analogues of pyrophosphate, an endogenous regulator of bone mineralization (**Figure 2 a&b).** These chemicals are capable of binding divalent metal ions such as Ca^{2+}, Mg^{2+}, and Fe^{2+}. The high affinity of bisphosphonates for Ca^{2+}, ions is the basis for their use in treating bone diseases.[71] The resorption of bone delivers bisphosphonates to the sites of active remodeling where they are potent inhibitors of osteoclast function and therefore, bone resorption. This property led to the use of these drugs in Paget's disease, osteoporosis, and osteolytic bone metastasis. Manipulation of the chemical structure has provided various derivatives with decreased inhibitory effects on bone mineralization and increased inhibition of bone resorption, resulting in 100 to 10,000-fold increases in potency of some compounds. Increased potency has been achieved by addition of a nitrogen atom at critical side chain positions or heterocyclic ring groups containing nitrogen. Risedronate, ibandronate, and zoledronate are among

the potent nitrogen-containing heterocyclic ring bisphosphonates[72] (**Figure 3).** The cellular mechanisms of action of these drugs are just beginning to be understood.

BPs have a high affinity for bone mineral and are quickly cleared from the circulation and localized to hydroxyapatite bone surfaces. BPs are found in highest concentrations in areas of bone resorption rather that bone formation.[73, 74] The tissue targeting of BP to bone especially sites of resorption suggests that BP have direct effects on osteoclasts or other bone cells in the immediate vicinity. The ability of these drugs to chelate Ca^{2+} is reduced in an acidic environment. Therefore, in the highly acidic environment of the osteoclast resorption chamber, BP's are released from the bone matrix and can achieve high concentrations in solution.[74]

Working osteoclasts are actively endocytotic, therefore, BPs released in to the resorption pits are likely to be taken up by osteoclasts. In rats treated with radiolabeled BP, 50% of the osteoclasts on trabecular bone surfaces were shown to contain BP after only 12 hours.[75] The theory that bone resorption must occur for the BP to be released is supported by the finding that mouse osteoclasts in oc/oc mice that are incapable of forming ruffled borders and bone resorption, are not affected when cultured on BP-treated bone.[76] Inhibition of osteoclastic resorption by calcitonin also protects osteoclasts from the affects of BP treated bone.[77] The affect of BPs on osteoclasts appears to occur by intracellular mechanisms. However, BPs are negatively charged and not membrane permeable and the ways these drugs enter the cell have not been clearly defined.

Experiments with rodent, avian, and human osteoclast-like cells provide strong evidence that BPs act directly on osteoclasts.[78, 79] Several bisphosphonates, including clodronate, etidronate, and pamidronate, were observed to cause degenerative toxic

changes in rat and mouse osteoclasts *in vitro* and *in vivo*.[80, 81] More recent work has demonstrated that BPs, including etidronate, clodronate, pamidronate, and risedronate, at concentrations of 10^{-7} induce apoptosis in mouse, rat, and rabbit osteoclasts *in vivo* and *in vitro*.[82, 83] Osteoclasts undergoing apoptosis often become nonadherent, which in itself has been shown to induce apoptosis in osteoclasts. Some studies have determined that BPs do not inhibit osteoclast binding to bone,[73, 84] while others have shown that clondronate may interfere with attachment of osteoclasts to certain bone matrix proteins via surface integrins. Additionally, BPs can decrease the attachment of tumor cells to bone.[85, 86]

Apoptosis of osteoclasts would inhibit bone resorption, however in studies with aledronate, pamidronate, and etidronate, decreased bone resorption was not associated with osteoclast toxicity or with a decrease in osteoclast number except at high doses.[87] BP can, however, cause more subtle changes in osteoclasts that decrease their ability to resorb bone. Osteoclasts treated with bisphosphonate *in vitro* and *in vivo* lose their ruffled border, the convoluted region of the plasma membrane adjacent to the bone surface that is necessary for the resorption process.[76, 81,87] Another consistent feature is the disruption of the osteoclast cytoskeleton with the loss of actin rings.[73, 76] Actin rings are unique to osteoclasts and are composed of F-actin, vinculin, and other cytoskeletal proteins.[78] Concentrations of BP that disrupt the ruffled border and actin rings will prevent bone resorption, but do not cause osteoclast toxicity. This change may even be reversible upon the cessation of BP treatment.

Along with the affects on mature osteoclasts, several studies have suggested that BPs influence osteoclast precursors. Low concentrations of pamidronate prevented the

recruitment and differentiation of osteoclasts in 17-day-old fetal mouse metacarpals, which lack mature osteoclasts. Clondronate and etidronate had no effect.[88] Direct treatment of osteoclast precursors with pamidronate and nitrogen-containing BPs did not inhibit osteoclast development and did not prevent migration or proliferation at the bone surface; this inhibition of osteoclast precursors appeared to be dependant on mineral-bound bisphosphonate. Treatment of bone marrow cultures prevented osteoclast formation and it was shown that the potency for decreasing osteoclast formation correlated with the antiresorptive potency (risedronate>pamidronate>neridronate> clodronate>etidronate). [89] The importance of the mineral-bound BP was emphasized when it was shown that bone marrow isolated from mice treated with aledronate could form osteoclasts when cultured concurrently with osteoclast-free bone explants ex vivo.[90] However, several studies have determined that BPs have no effect on osteoclast formation *in vitro*.[91] Furthermore, osteoclast resorption with low doses of aminobisphosphonates, pamidronate and opadronate, can actually be enhanced.[87, 92] A transient increase in the number of osteoclasts has also been observed immediately after BP treatment.[93] Given these conflicting results, the influence of BPs on formation of osteoclasts is uncertain.

The effects of BP on osteoblasts appear to have a significant effect on the prevention of bone resorption. Treatment of osteoblast-like cells with ibandronate or clodronate inhibited bone resorption when these cells were subsequently cultured for 24 hours with osteoclasts.[94] Similar responses were observed when osteoclasts were cultured with conditioned media from osteoblast-like cell cultures. Subsequently, osteoblast-like cells were shown to release a soluble factor of low molecular weight that acted on

osteoclast precursors and prevented osteoclast formation. This factor was shown to be released when cells were treated for a little as five minutes and with concentrations of BP as low as 10^{-11}. [95] *In vitro*, UMR106 osteoblast-like cells and calvarial osteoblasts, were shown to release factors that prevent bone resorption.[96] The importance of these *in vitro* findings on *in vivo* bone resorption is unclear at this time.

Determining the exact mechanism of action of BPs on osteoclasts has been a difficult task complicated by the fact that isolating and culturing large numbers of pure osteoclasts is difficult. Most of the early work has been performed in J477 macrophages. This cell line has similarities to osteoclast in that, it is highly endocytotic, has the capacity to demineralize bone matrix, and comes from the same hematopoietic colony-forming unit-granulocyte macrophage lineage. Treatment of these cells with BP results in prevention in development of macrophage lineage, inhibition of the break down of bone, and apoptosis.[97-99]

Nitrogen-containing BPs cause apoptosis via inhibition of the mevelonate pathway, [100] which is responsible for the production of cholesterol and isoprenoid lipids such as isopentyl diphosphate (IPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). Loss of these lipids prevents prenylation of small GTPases such as Ras, Rho, and Rac. These are important signaling proteins that regulate a variety of cell processes important for osteoclast function, including cell morphology, integrin signaling, membrane ruffling, trafficking of endosomes, and apoptosis. Therefore, blocking the mevelonate pathway could account for the effects of nitrogen-containing BPs on osteoclast function.[101] The exact enzymes of the mevelonate pathway that are inhibited by BP are being identified.

In contrast, non-nitrogen containing BPs do not inhibit the mevelonate pathway or protein prenylation in macrophages or osteoclasts.[100] These bisphosphonates closely resemble PPi in that they can be incorporated into nonhydrolyzable analogues of ATP. Inhibition of osteoclast function and apoptosis is most likely due to the intracellular accumulation of these metabolites.

Many questions into the mechanism of actions of BPs remain unanswered. An area of interest in recent research is the potential of these substances to exert anticancer effects. Exposure to bisphosphonate has been shown to alter adhesive[85, 102,103] and invasive[85, 68] properties in some tumor cell lines. In addition to initiating apoptosis in osteoclasts, BPs have also been shown to induce apoptosis in certain carcinoma cell lines [104,106]and myeloma cells [100,106] *in vitro* and *in vivo*. The *in vitro* concentrations required to cause apoptosis in cancer cells are much higher than the concentrations that inhibit bone resorption and induce osteoclast apoptosis.

Bisphosphonates and animal models

A significant body of work with animal models of bone metastasis and BP treatment has been completed. The first evidence of a reduction in osteolysis was observed with etidronate in murine models. Inhibition of osteolysis was identified and one study showed increased survival times. However, the weak effects and disturbances of etidronate were noted at this time. **Table 1** summarizes the more recent work that has been done with rodent models of neoplasia and bisphosphonate therapy.

Work with these animal models has shown that bisphosphonates can inhibit the formation and progression of bone metastasis.[108, 111-115] Experiments by Yoneda *et al.* have shown that treatment with bisphosphonates before bone metastases are detectable

may be valuable in the prevention of metastatic lesions.[114, 115] An isolated report of increased skeletal tumor mass in Fischer rats receiving daily pamidronate after inoculation with Walker 256 carcinoma cells[109] is contradictory to what was observed by Krempien *et al.* in two similar independent experiments,[107,108] and has not been reported again.

Bisphosphonates and clinical trials

Currently seven BPs are approved for clinical use for various conditions. Two of these drugs, clondronate and pamidronate, are approved for the treatment of metastatic bone disease.[116] The first clinical trial with clondronate enlisted women with local or distant progression of breast cancer but without skeletal metastasis. At the end of the study the number of patients with bone metastasis and the overall number of metastasis were decreased in the BP treated group. Also, skeletal complications were reduced in the group receiving clondronate. Three additional clinical trials with daily oral administration were performed.[117] Two of the studies reported decrease in metastatic lesions. Of these two studies, Diel *et al.* observed decreased visceral metastasis and overall survival time was increased.[118] In the third of these studies, skeletal metastasis was unchanged, visceral metastasis was increased and overall survival time was decreased.[117] The results of this study are contradictory to the results seen in other human and animal trials and may be due to a small sample size which can lead to random results.

Trials with pamidronate by Conte *et al.* revealed an increase in time to bone progression and complication free interval. A reduction in bone metastasis was not observed.[119] Additional studies have confirmed the lack of inhibition of metastatic

lesions. Pamidronate should not be considered ineffective because in these latter trials, the drug was given orally. Pamidronate is poorly absorbed from the gastrointestinal tract and causes esophagitis and gastritis with ulceration at doses that will inhibit osteolysis.

Not all patients will respond to bisphosphonates. Selected patients will not have resolution of hypercalcemia or decreases in skeletal complications. New ways of using bisphosphonates are being employed. The first method is to increase the dose and frequency of the BP infusions. Data regarding the efficacy of this treatment is not yet available.[120]

Another approach is to utilize the more potent third generation heterocyclic ring-containing BPs. Recently a Phase II trial with Zoledronate, which is 500-1000 times more potent than pamidronate, was completed in patients with multiple myeloma and metastatic breast cancer. Data to date shows that a five-minute infusion of zoledronate is at least as effective as a two-hour infusion of pamidronate.[120]

In this study we are utilizing the third generation bisphosphonate risedronate. Risedronate has been evaluated for ability to maintain bone mass in rats, dogs, and postmenopausal women.[121-123] Two recent studies examining the influence of oral risedronate on bone resorption and formation in patients with multiple myeloma demonstrated reduced osteoclast number, decreased osteoclast activity, and maintenance of osteoblast activity (neither trial was designed to evaluate the treatment effect on the neoplastic bone lesions). [124,125] In a small trial involving patients with Paget's disease, oral risedronate increased bone mineral density in both pagetic and non-pagetic bone.[126] In these osteolytic human diseases, risedronate administered orally was beneficial for decreasing bone resorption and maintaining or increasing bone mass. Risedronate is

approved for the treatment of osteoporosis. It is manufactured by Proctor and Gamble incorporated and the trade name is Actonel®. Risedronate has not yet been thoroughly evaluated for efficacy in treatment of human bone metastasis.

Conclusion

Bisphosphonates have shown promise in the treatment of osteolytic bone metastasis in human trials and animal models. The approval for the clinical use of more potent third generation BPs in skeletal metastasis is promising. Even so, some patients have incomplete response or no response to BP therapy. Animal models provide an excellent tool for identifying the mechanisms of action of BPs. In addition, manipulation of cellular properties and experimenting with various drug combinations may help identify therapeutic protocols that could be useful in human patients.

Little work addressing patients with incomplete or no responses to BPs has been pursued. The focus of this work has been to characterize an ENU induced rat mammary carcinoma cell line, MTA, as a nonresponder to risedronate therapy. Establishing this cell line as a nonresponder to BP will provide a useful tool for the investigation into BP mechanisms of action and ways some cells might evade the effects of BPs.[39] The availability of predictable animal models of both BP-responsive and BP-unresponsive bone-metastatic tumors will provide useful systems for identifying new therapeutic targets or designing multiple treatment modalities for patients with cancers less responsive to BP treatment.

References

1. Orr FW, Lee J, Duivenvoorden WCM, *et al.* Pathophysiologic Interactions in Skeletal Metastasis. Cancer 2000;89:(12)2912-8.

2. Paget S. The distribution of secondary growths in cancer of the breast. Lancet 1889;1:571-3.
3. Mundy GR. Mechanisms of osteolytic bone destruction. Bone 1991;12 Supplement:S1-6.
4. Liotta LA, Tryggvason K, Garbisa S, *et al.* Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980;284:67-8.
5. Liotta LA, Mandler R, Murano G, *et al.* Tumor cell autocrine motility factor. Proceedings of the National academy of Science USA 1986;83:3302-6.
6. Beavon IR. The E-cadherin-catenin complex in tumor metastasis: structure, function, and regulation. European Journal of Cancer 2000;36:1607-20.
7. Marcel MM, Behrens J, Birchmeirer W, DeBrune GK, Vleminckx K, Hoogewijs A. Down regulation of E-cadherin expression in Madin-Darby kidney cells inside tumors of nude mice. Int J Cancer 1991; 47:922-8.
8. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 1991;251:1451-5.
9. Yoshida R, Kimura N, Harada Y, *et al.* The loss of E-cadherin, alpha-and beta-catenin expression is associated with metastasis and poor prognosis in invasive breast cancer. International Journal of Oncology 2001;18:(3)513-20.
10. Behrens J, Mareel MM, Van Roy FM, *et al.* Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. Journal of Cell Biology 1989;108:(2435)2347.

11. Sommers CL, Thompson EW, Torri JA, *et al.* Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. Cell Growth and Differentiation 1991;2:365-72.
12. Mbalaviele G, Dunstan CR, Sasaki A, *et al.* E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in experimental metastasis model. Cancer Research 1996;56:4063-70.
13. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11-25.
14. Seftor REB, Seftor EA, Gehlsen KR, *et al.* Role of the $alpha_v$ beta3 integrin in human melanoma cell invasion. Proc Natl Acad Sci USA 1992;89:1557-61.
15. Nakai M, Mundy GR, Williams PJ, *et al.* A synthetic antagonist to laminin inhibits the formation of osteolytic metastases by human melanoma cells in nude mice. Cancer Res 1992;52:5395-9.
16. Tawil NJ, Gowri V, Djoneidi M, *et al.* Integrin alpha3beta1 can promote adhesion and spreading of metastatic breast carcinoma cells on the lymph node stroma. International Journal of Cancer 1996;66:703-10.
17. Morini M, Mottloese M, Ferriari N, *et al.* The alpha3beta1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. International Journal of Cancer 2000;87:336-42.
18. Muller A, Homey B, Soto H, *et al.* Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50-6.

19. Koyama T, Hasebe T, Tsuda H, *et al.* Histological factors associated with initial bone metastasis of invasive ductal carcinoma of the breast. Japan Journal of Cancer Research 1999;90:294-300.
20. Engebraaten O, Fodstad O. Site specific experimental metastasis patterns of two human breast cancer cell lines in nude rats. International Journal of Cancer 1999;82:219-25.
21. Guise TA, Mundy GR. Cancer and bone. Endocrinology review 1998;19:18-54.
22. Kim RH, Sodek J. Transcription of bone sialoprotein gene is stimulated by v-Src acting through an inverted CCAAT box. Cancer Research 1999;59:565-71.
23. Hampl M, Hampl JA, Reiss G, *et al.* Loss of heterozygosity accumulation in primary breast carcinomas and additionally in corresponding distant metastases is associated with poor outcome. Clinical Cancer Research 1999;5:1417-25.
24. Hildebrant T, Preiherr J, Klostermann S, *et al.* Identification of URIM, a novel gene up-regulated in metastasis. Anticancer Research 1999;19:525-30.
25. Argeullo F, Baggs RB, Duerst RE, *et al.* Pathogenesis of vertebral metastasis and epidural spinal cord compression. Cancer Research 1990;42:54
26. Muller M, Gounari F, Prifti S, *et al.* EblacZ tumor dormancy in bone marrow and lymph nodes: active control of proliferating tumor cells by CD8+ immune T cells. Cancer Research 1998;58:5439-46.
27. Hullinger TG, McCauley LK, Dejoode ML, *et al.* Effect of bone proteins on human prostate cancer cell lines *in vitro*. Prostate 1998;36:14-22.
28. Ritchie CK, Andrews LR, Thomas KG, *et al.* The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis:

implications for the development of metastatic disease. Endocrinology 1997;138:1145-50.

29. Kostenuik P, Singh G, Suyama KL, *et al.* Stimulation of bone resorption results in a selective increase in the growth rate of spontaneously metastatic Walker 256 cancer cells in bone. Clinical and Experimental Metastasis 1992;10:411-8.
30. Sung V, Stubbs JT, Fisher L, *et al.* Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta3 and alpha(v)beta5 integrins. Journal of Cell Physiology 1998;176:482-94.
31. Mundy GR, Poser JW. Chemotactic activity of the gammacarboxyglutamic acid containing protein in bone. Calcified Tissue International 1983;35:164-8.
32. Orr FW, Varani J, Gondek MD, *et al.* Chemotactic responses of tumor cells to products of resorbing bone. Science 1979;203:176-9.
33. Thomas RG, Guise TA, Yin JJ, *et al.* Breast cancer cells interact with osteoblasts to support osteoclasts formation. Endocrinology 1999;140:4451-8.
34. Winding B, Misander H, Sveigaard C, *et al.* Human breast cancer cells induced angiogenesis, recruitment and activation of osteoclasts in osteolytic metastasis. J Cancer Res Clin Oncol 2000;126:631-40
35. Lacroix M, Siwek B, Marie PJ, *et al.* Production of interleukin-11 by breast cancer cells. Cancer Lett 1998; 127:29-35.
36. Garret IR. Bone destruction in cancer. Seminars in Oncology 1993;20:4-9.

37. Sanchez-Sweatman OH, Lee J, Orr FW, *et al.* Direct osteolysis induced by metastatic murine melanoma cells: role of Matrix metalloproteinases. European Journal of Cancer 1997;33:918-25.

38. Lee J, Bone EA, Watson PH, *et al.* The effects of matrix metalloproteinase (MMP) inhibitor Bamistat on bone metastasis. Proceedings American Cancer Research 1998;39:299

39. Boyd K, Neuwirth L, Hall DG. Characterization of a risedronate-nonresponsive bone-metastatic rat mammary tumor cell line. Submitted 2001;

40. Delaisse J, Engsig MT, Everts V, *et al.* Proteinases in bone resorption: obvious and less obvious roles. Clinica Chimica Acta 2000;291:223-34.

41. Sanchez-Sweatman OH, Orr FW, Singh G: Human Metastatic Prostate PC3 Cell lines Degrade Bone Using Matrix Metalloproteinases. Invasion and Metastasis 2000;18: 297-305.

42. Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. Cancer Biology 2000;10:415-33.

43. Kusano K, Miyaura C, Inada M, *et al.* Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 1998;139:1339-45.

44. Bord S, Horner A, Hembry RM, Reynolds JJ, *et al.* Production of Collagenase by human osteoblasts and osteoclasts in vivo. Bone 1995;17:255-60.

45. Stearns ME, Wang M. Aledronate blocks metalloproteinase secretion and bone collagen I release by PC-3 ML cells in SCID mice. Clinical and Experimental Metastasis 1998;16:693-702.

46. Ueda Y, Imai K, Tsuchiya H, *et al.* Matrix metalloproteinase 9 (gelatinase B) is expressed in multinucleated giant cells of human giant cell tumor ob bone and is associated with vascular invasion. American Journal of Pathology 1996;148:611-22.

47. Ishiduka Y, Mochizuki R, Yanai K, Takatsuka M, *et al.* Induction of hydroxyapatite resorptive activity in bone marrow cell populations resistant to bafilomycin A1 by a factor with restricted expression to bone and brain, neurochondrin. *Biochim Biophys Acta* 1450:92-98, 1999.

48. Schwarz LC, Gingras M, Goldberg G, *et al.* Loss of growth factor dependence and conversion of transforming growth factors-1 inhibition to stimulation in metastatic H-ras-transformed murine fibroblasts. Cancer Research 1988;48:6999-7003.

49. Argeullo F, Furlanetto FW, Baggs RB, *et al.* Incidence an distribution of experimental metastases in mutant mice with defective organ microenvironments (genotypes S1/S1d and W/Wv. Cancer Research 1992;52:2304-9.

50. Jung A, Bornand J, Mermillod B, *et al.* Inhibition by diphosphonates of bone resorption induced by the Walker tumor of the rat. Cancer Research 1984;44:3007-11.

51. Okado T, Hawley RG. Adhesion molecules involved in the binding of murine myeloma cells to bone marrow stromal elements. International Journal of Cancer 1995;63:823-30.

52. Matsuura N, Puzon-McLaughlin W, Irie A, *et al.* Induction of experimental bone metastasis in mice by transfection of integrin alpha 4 beta1 into tumor cells. American Journal of Pathology 1996;148:55-61.

53. Kostenuik P, Sanchez-Sweatman OH, Orr FW, *et al.* Bone cell matrix promotes the adhesion of human prostatic carcinoma cells via the alpha 2 beta 1 integrin. Clinical and Experimental Metastasis 1996;14:19-26.

54. Haq M, Goltzman D, Tremblay G, *et al.* Rat prostate adenocarcinoma cells disseminate to bone and adhere preferentially to bone marrow-derived endothelial cells. Cancer Research 1992;52:4613-9.

55. Lehr JE, Pienta KJ. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. Journal National Cancer Institute 1998;90:118-23.

56. Uchiyama H, Barut BA, Mohrbacher AF, *et al.* Adhesion of Human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood 1993;82:3712-20.

57. Ridley RC, Xaio H, Woodliff J, *et al.* Expression of syndecan regulates human myeloma plasma cell adhesion to type I collagen. Blood 1993;81:767-74.

58. Lang SH, Clarke NW, George NJR, *et al.* Primary prostatic epithelial cell binding to human bone marrow stroma and the role of alpha 2 beta 1 integrin. Clinical and Experimental Metastasis 1997;15:18-27.

59. Lemieux P, Harvey, Guise TA, *et al.* Low cell motility induced by hsp27 over expression decreases osteolytic bone metastases of human breast cancer cells *in vivo*. Journal of Bone and Mineral Research 1999;14:1570-5.

60. Orr FW, Varani J, Gondek MD, *et al.* Partial characterization of a bone-derived chemotactic factor for tumor cells. American Journal of Pathology 1980;99:43-52.

61. Wass JA, Varani J, Pointek GE, *et al.* Responses of normal and malignant cells to collagen, collagen-derived peptides and the C5-related tumor cell chemotactic peptide. Cell Differ 1981;10:329-32.

62. Mundy GR, Poser JW. Chemotactic activity of the gammacarboxyglutamic acid containing protein in bone. Calcified Tissue International 1983;35:164-8.

63. Festuccia C, Bologna M, Gravina GL, *et al.* Osteoblast conditioned media contains TGF-beta-1 and modulates the migration of prostate tumor cells and their interactions with extracellular matrix components. International Journal of Cancer 1999;81:395-403.

64. Jacob K, Webber M, Benayahy D, *et al.* Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. Cancer Research 1999;59:4453-7.

65. Festuccia C, Giunciuglio D, Guerra F, *et al.* Osteoblasts modulate secretion of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) in human prostate cancer cells promoting migration and matrigel invasion. Oncology Research 1999;11:17-31.

66. Yin J, Selander KS, Chirgwin JM, *et al.* TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. Journal of Clinical Investigation 1999;103:197-206.

67. Arihiro K, Oda H, Kaneko M, *et al.* Cytokines facilitate chemotactic motility of breast carcinoma cells. Breast Cancer 2000;7:221-30.

68. Guaitani A, Sabatini M, Polentarutti N, *et al.* Effects of disodium etidronate on tumor-induced hypercalcemia, bone invasion, and metastasis in rodents. *In*: S. Garattini (ed.), Bone Resorption, Metastasis, and Diphosphonates, pp. 51-58. New York: Raven Press, 1985.

69. Jung A, Bornand J, Mermillod B, *et al.* Inhibition by diphosphonates of bone resorption induced by the Walker tumor of the rat. Cancer Research 1984;44:3007-11.

70. Nemoto R, Satou S, Miyagawa I, *et al.* Inhibition by a new bisphosphonate (AHBuBP) of bone resorption induced by MBT-2 tumor of mice. Cancer 1991;67:643-8.

71. Rogers MJ, Gordon RK, Benford HL, *et al.* Cellular and molecular mechanisms of action of bisphosphonates. Cancer 2000;88:2961-78.

72. Gatti D, Adami S. New bisphosphonates in the treatment of bone disease. Drugs Aging 1999;15:(4)285-96.

73. Sato M, Grasser W, Endo N, *et al.* Bisphosphonate action. Aledronate localization in rat bone and effects on osteoclast ultrastructure. Journal of Clinical Investigation 1991;88:2091-105.

74. Ebetino F, Francis MD, Rogers MJ, *et al.* Mechanisms of actions of etidronate and other bisphosphonates. Rev Contemp Pharmacother 1998;9:233-43.

75. Masarachia PJ, Weinreb M, Balena R, *et al.* Comparison of the distribution of 3H-aledronate and 3H-etidronate in rat and mouse bones. Bone 1996;19:281-90.

76. Murakami H, Takahashi N, Sasaki T, *et al.* A possible mechanism of the specific action of bisphosphonates on osteoclasts: tiludronate preferentially affects polarized osteoclasts having ruffled borders. Bone 1995;17:137-44.

77. Plotkin LI, Weinstein RS, Parfitt AM, *et al.* Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcition. Journal of Clinical Investigation 2000;104:(10)1363-74.

78. Selander KS, Lehenkari P, Vaananen HK. The effects of bisphosphonates on the resorption cycle of isolated osteoclasts. Calcified Tissue International 1994;55:368-75.

79. Muller A, Homey B, Soto H, *et al.* Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50-6.

80. Rowe DJ, Hausmann E. The alteration of osteoclast morphology by diphosphonates in bone organ culture. Calcified Tissue Research 1976;20:53-60.

81. Schenk KS, Merz WA, Muhlbauer R, *et al.* Effect of ethane-1-hydroxy-1, 1-disphosphonate(EHDP) and dichloromethylene diphosphonate (Cl_2MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. Calcified Tissue Research 1973;11:196-214.

82. Hughes DE, Wright KR, Uy HL, *et al.* Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. Journal of Bone and Mineral Research 1995;10:1478-87.

83. Hiroi-Furuya E, Kameda T, Hiura K, *et al.* Etidronate (EHDP) inhibits osteoclastic -bone resorption, promotes apoptosis, and disrupts actin rings in isolate-mature osteoclasts. Calcified Tissue International 1999;64:219-23.

84. Azuma Y, Sato H, Oue Y, *et al.* Alendronate distrubuted on bone surfaces inhibits osteoclastic bone resorption *in vitro* and in experimental hypercalcemia. Bone 1996;19:235-45.

85. Bossier S, Ferreras M, Pevruchaud O, *et al.* Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Research 2000;60:(11)2949-54.

86. Van der Pluijm G, Vloedgraven H, Papapoulos S, *et al.* Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components. Laboratory Investigation 1997;77:665-75.

87. Sato M, Grasser W. Effects of bisphosphonate on isolated rat osteoclasts as examined by reflected light microscopy. Journal of Bone and Mineral Research 1990;5:31-40.

88. Boonekamp PM, van der Wee-Pals LJA, van Wijk-van-Lennep MLL, *et al.* Two modes of action of bisphosphonates on osteoclastic resorption of mineralized matrix. Bone Miner 1986;1:27-39.

89. Hughes DE, MacDonald BR, Russell RGG, *et al.* Inhibition of osteoclast-like cell formation by bisphosphonates in long term cultures of human bone marrow. Journal of Clinical Investigation 1989;83:1930-5.

90. van Beek E, Lowik C, Papapoulos S. Effect of alendronate treatment on the osteoclastogenic potential of bone marrow cells in mice. Bone 1997;20:335-40.

91. Flanagan AM, Chambers TJ. Inhibition of bone resorption by bisphosphonates: interactions between bisphosphonates, osteoclasts and bone. Calcified Tissue Internation 1991;49:407-15.

92. Van der Pluijm G, Lowik C, de Groot H, *et al.* Modulation of PTH-stimulated osteoclastic resorption by bisphosphonates in fetal mouse bone explants. Journal of Bone and Mineral Research 1991;6:1203-10.

93. Holt I, Marshall MJ, Davie MW. Pamidronate stimulates recruitment and decreases longevity of osteoclast nuclei in mice. Semin Arthitis Rheum 1994;23:263-4.

94. Sahni M, Guenther HL, Fleisch H, *et al.* Bisphosphonates act on rat bone resorption through mediation of osteoblasts. Journal of Clinical Investigations 1993;91:2004-10.

95. Vitte C, Fleisch H, Guenther HL. Bisphosphonates induce osteoblasts to secrete and inhibitor of osteoclast-mediated resorption. Endocrinology 1996;18:(9)14.

96. Yu X, Scholler J, Foged NT. Interaction between effects of parathyroid hormone and bisphosphonate on regulation of osteoclast activity by the osteoblast-like cell line UMR-106. Bone 1996;19:339-45.

97. Chambers TJ. Diphosphonates inhibit bone resorption by macrophages *in vitro*. Journal of Pathology 1980;132:255-62.

98. Coxon FP, Benford HL, Russell RGG, *et al.* Protein synthesis is required for caspase activation and induction of apoptosis by bisphosphonate drugs. Mol Pharmacol 1998;54:(4)631-8.

99. Luckman SP, Coxon FP, Ebetino F, *et al.* Heterocyclic-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure-activity relationships in J774 macrophages. Journal of Bone and Mineral Research 1998;13:(11)1668-78.

100. Shipman CM, Croucher PI, Russell RGG, *et al.* The bisphosphonate icandronate (YM175) causes apoptosis of human myeloma cells *in vitro* by inhibiting the mevalonate pathway. Cancer Research 1998;58:(23)5294-7.

101. Rogers MJ, Gordon S, Benford HL, *et al.*Cellular and molecular mechanisms of action of bisphosphonates. Cancer. 2000;88:2961-78.

102. Magnetto S, Bossier S, Delmas PD, *et al.* Additive antitumor activities of taxoids in combination with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. International Journal of Cancer 1999;83:(2)263-9.

103. Van der Pluijm G, Vloedgraven H, van Beek E, *et al.* Bisphosphonates inhibit the adhesion of breast cancer cells to bone matrices *in vitro*. Journal of Clinical Investigation 1996;98:698-705.

104. Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. British Journal of Cancer 2000;82(8):1459-68.

105. Ito M, Amizuka N, Nakajima T, *et al.* Ultrastructural and cytochemical studies on cell death of osteoclasts induced by bisphosphonate treatment. Bone 1999;25:(4)447-52.

106. Shipman CM, Rogers MJ, Apperley JF, *et al.* Anti-tumor activity of bisphosphonates in human myeloma cells. Leuk Lymphoma 1998;32:(1-2)129-38.

107. Krempien B, Wingen F, Eichmann T, *et al.* Protective effect of a prophylactic treatment with the bisphosphonate 3-amino-1hydroxypropane-1,1bisphonic acid on the development of tumor osteopathies in rat: experimental studies with the Walker Carcinosarcoma 256. Oncology 1988;45:41-6.

108. Krempien B, Manegold C. Prophylactic treatment of skeletal metastases, tumor-induced osteolysis and hypercalcemia in rats with the bisphosphonate CL2MBP. Cancer 1993;72:91-8.

109. Kostenuik P, Orr FW, Suyama KL, *et al.* Increased growth rate and tumor burden of spontaneously metastatic Walker 256 cancer cells in the skeleton of bisphosphonate treated rats. Cancer Research 1993;5472-7.

110. Muller M, Green JR, Fabbro D. The bisphosphonate pamidronate inhibits the growth of a murine myeloma cell line in syngeneic mice. Blood 1996;88:2333.

111. Sasaki A, Boyce RW, Story B, *et al.* Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res 1995;55:3551-7.

112. Hall DG, Stocia G. Effect of the Bisphosphonate Risedronate on Bone Metastases in a Rat Mammary Adenocarcinoma Model System. Journal of Bone and Mineral Research 1994;9:(2)221-30.

113. Sasaki A, Kitamura K, Alcalde RE, *et al.* Effect of a newly developed bisphosphonate, YH529, on osteolytic bone metastases in nude mice. International Journal of Cancer 1998;77:279-85.

114. Yoneda T, Sasaki A, Dunstan CR, *et al.* Inhibition of osteolytic bone metastasis of breast cancer by combined treatment with the bisphosphonate Ibandronate and tissue inhibitor of the matrix metalloproteinase-2. Journal of Clincal Investigation 1997;99:2509-17.

115. Yoneda T, Michigami T, Yi B, Williams PJ, Niewolna M, Hiraga T. Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. Cancer 2000;88(12 Suppl):2979-788.

116. Ma YF, Pan Z, Jee WSS, *et al.* Intermittent on/off prostaglandin E_2 and risedronate are equally anabolic as daily PGE_2 alone treatment in cortical bone of ovariectomized rats. Journal of Bone and Mineral Research 1997;12:2108-12.

117. Diel IJ, Solomayer EF, Bastert G. Bisphosphonates and the prevention of metastasis: first evidences from preclinical and clinical studies. Cancer. 2000;88:3080-8.

118. Diel IJ, Solomayer E, Costa SD, *et al.* Reduction of new metastases in breast cancer with adjuvant clondronate treatment. new England Journal of Medicine 1998;339:357-63.

119. Conte PF, Latreille J, Mauriac L, *et al.* Delay in progression of bone metastases in breast cancer patients treated with intravenous pamidronate: results from a multinational randomized controlled trial. Journal of Clinical oncology 1996;14:2552-9.

120. Lipton A. Bisphosphonates and breast carcinoma. Cancer 2000;88 Supplement:(12)3033-7.

121. Boyce RW, Paddock CL, Franks AF, *et al.* The effects of intermittent hPTH(1-34) alone and in combination with $1,25(OH)_2D_3$ or risedronate on endosteal bone remodeling in canine cancellous and cortical bone. Journal of Bone and Mineral Research 1996;11:600-13.

122. Li QN, Jee WSS, Ma YF, *et al.* Risedronate pretreatment does not hamper the anabolic effects of prostaglandin E_2 in OVX rats. Bone 1995;17 Supplement:(4)261S-6S.

123. Clemmesen B, Ravn P, Zegels B, *et al.* A 2-year phase II study with 1-year follow-up of risedronate (NE-58095) in postmenopausal osteoporosis. Osteoporosis International 1997;7:488-95.

124. Cohen-solal ME, Roux CH, Valentin-opran A, *et al.* Histomorphometric effect of six month treatment with oral risedronate in patients with multiple myeloma. Bone 1993;14:505-9.

125. Roux C, Ravaud P, Cohen-Solal M, *et al.* Biologic, histologic and densitometric effects of oral risedronate on bone in patients with multiple myeloma. *Bone* 1994; 15:41-9.

126. Patel S, Pearson D, Bhallah A, *et al.* Changes in bone mineral density in patients with Paget's disease treated with risedronate. *Ann Rheum Dis* 1997; 56:405-9.

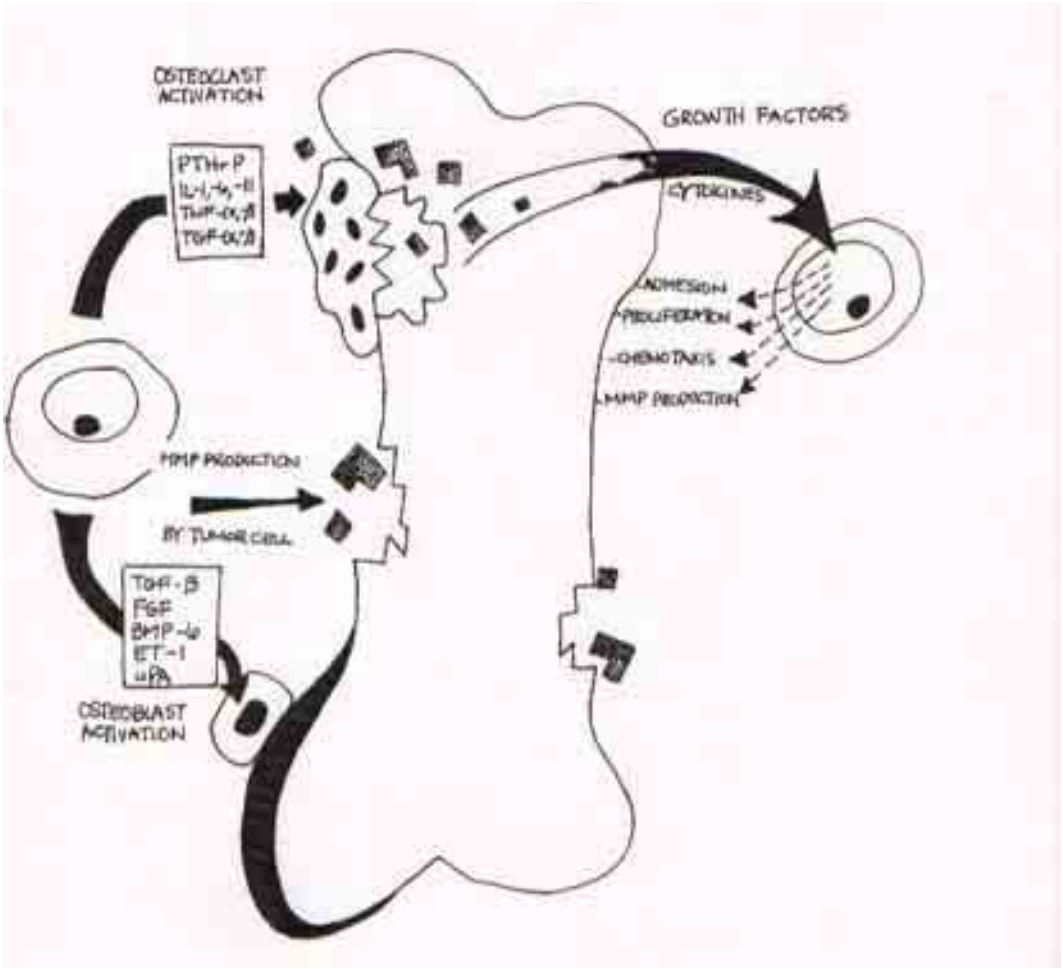


Figure1: Schematic depicting the vicious cycle set into motion when tumor cells metastasize to bone.

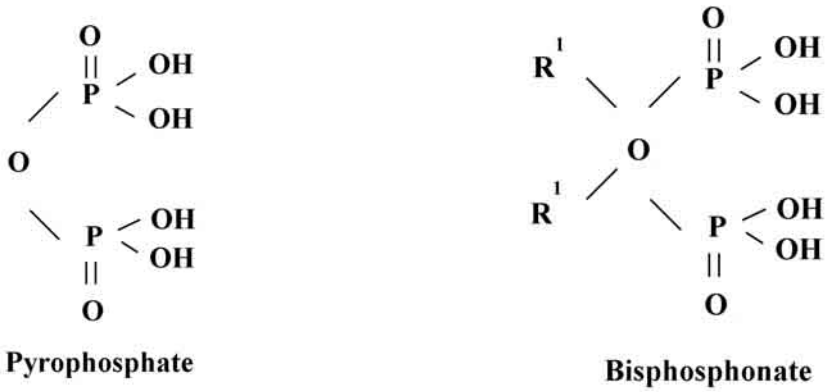
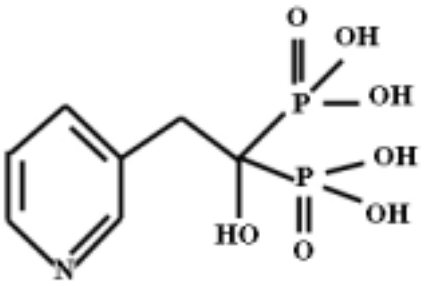


Figure 2 a: Chemical structure of Pyrophosphate b. Chemical structure of first generation bisphosphonates (non-nitrogen containing)



Risedronate

Figure 3: Chemical structure of risedronate a third generation bisphosphonate (contains nitrogen in a heterocyclic ring)

CHAPTER 1.

CHARACTERIZATION OF A RISEDRONATE-NONRESPONSIVE BONE-METASTATIC RAT MAMMARY TUMOR CELL LINE[1]

[1] Boyd, Kelli, Lisa Neuwirth, and D. Greg Hall. Submitted to Clinical and Experimental Metastasis 5/1/01.

Abstract. Bisphosphonates, potent osteoclast inhibitors, have shown promise in reducing the destructiveness and incidence of bone-metastatic lesions. Positive responses to bisphosphonate therapy include decreased number and size of bone-metastatic lesions and normalization of hypercalcemia. However, human and animal trials have identified individuals whose bone metastatic cancers have failed to respond to bisphosphonate treatment. Specific properties that render bone-metastatic tumor cells responsive or nonresponsive to bisphosphonates are not known. Animal models could provide a useful tool for investigating mechanisms responsible for these treatment failures. In this study, we identify the MTA rat mammary tumor cell line as nonresponsive to bisphosphonate, specifically risedronate, therapy. In an *in vivo* bone-metastasis assay, a therapeutic trial showed that the incidence and size of MTA bone-metastatic lesions were not influenced by risedronate treatment when compared with lesions in untreated animals. In contrast, the related bone-metastatic rat mammary carcinoma cell line (MTBo2) was previously shown to respond favorably to this risedronate treatment protocol [1]. The MTA line may provide a useful model for studying the properties of bisphosphonate nonresponsive bone-metastatic cells.

INDEX WORDS**:** animal model, bisphosphonate, bone metastasis, rat, risedronate

Introduction

Bone metastasis is an important complication of some of the most commonly occurring human cancers. Morbidity is associated with massive bone lysis that may cause severe bone pain, fractures, and life-threatening hypercalcemia [2,3]. Localization of tumor cells to bone is augmented by growth factors released from the bone matrix during normal skeletal remodeling. In the bone microenvironment, osseous resorption is carried out by osteoclasts activated directly or indirectly by chemical mediators. Bone lysis releases growth factors from the bone matrix, which serve as potent chemotactic factors for tumor cells, enhancing tumor colonization of bone and additional bone destruction [3,4].

Advancements in palliative therapy for patients with skeletal metastasis have included the use of bisphosphonate drugs, which in many patients have been shown to decrease bone pain, reduce fracture incidence, and decrease hypercalcemia. Bisphosphonates are synthetic derivatives of bisphosphonic acid that inhibit bone resorption by inhibiting osteoclast development and activity. These drugs concentrate within the bone matrix and are released into the acidic microenvironment of the osteoclast resorption chambers, exposing osteoclasts to high concentrations of drug. Addition of a nitrogen-containing ring to the early generation bisphosphonates resulted in a tremendous increase in antiresorptive activity. These potent aminobisphosphonates have been shown to inhibit the mevalonate/isoprenoid pathway, resulting in osteoclast apoptosis and loss of activity. Bisphosphonate-induced apoptosis has also been demonstrated in macrophages [5,6], osteoclasts [7-10] certain carcinoma cell lines [11,12], and myeloma cells [13,14].

Although patient responses to bisphosphonates have been encouraging, some patients do not respond to bisphosphonate therapy [13,15,16]. To our knowledge, there is no current research addressing this population of nonresponsive patients. Identification of bisphosphonate nonresponsive bone-metastatic tumor cell lines in animal models could provide a useful system for investigations into factors responsible for treatment failures.

The MTA cell line was cloned from an N-Ethyl-N-Nitrosourea (ENU)-induced rat mammary tumor [17] and reliably reproduces bone-metastatic lesions when injected into the left cardiac ventricle of syngeneic rat hosts. MTA is related to the MTBo2 line, which has been shown previously to be risedronate-responsive [1]. In this study, we evaluated the effect of risedronate therapy on the size and incidence of MTA bone-metastatic lesions and demonstrated a lack of treatment response in comparison to untreated control animals.

Materials and methods

Chemicals

Risedronate (Proctor and Gamble, Inc., Cincinnati, OH) was obtained from Dr. George Stoica (Texas A&M University). Test animals received 0.2 mg/kg/day of risedronate administered subcutaneously at one site between the scapulas.

Tumor cell line

The MTA tumor cell line originated from an N-ethyl-N-nitrosourea-induced mammary adenocarcinoma in a female Berlin-Druckrey IV (BDIV) rat. Conditions and procedures for cell culture and harvesting for experiments were as previously described

[8, 17]. Widespread metastasis including colonization of bone occurs by day 14 following injection of $1X10^5$ monodispersed cultured tumor cells into the left cardiac ventricle of syngeneic rats.

Animal inoculations and treatment groups

The 30 to 45-day-old BDIV rats utilized in the experiment were maintained at the University of Georgia in accordance with institutional animal care guidelines. For tumor cell inoculations, animals received an intramuscular injection of anesthetic cocktail, 50 mg ketamine and 15 mg xylazine per 150 grams of body weight. Each anesthetized rat received $1 X 10^5$ cells in a volume of 0.1 ml Hank's Balanced Salt Solution, utilizing ultrasound to visualize the cardiac left ventricular chamber. Animals were divided into two groups. Animals in the treatment group (n=15) received 0.2 mg/kg risedronate daily beginning on the day of tumor cell inoculation and continuing until the day of sacrifice. Untreated animals (n=14) received daily subcutaneous injections of sterile physiologic saline.

Pathology

Animals were euthanized by halothane overdose on day 14 post tumor cell inoculation. At necropsy, both femurs and the cervical, thoracic, and lumbar vertebra were fixed by immersion in 10% neutral buffered formalin. After fixation, the bones were decalcified in 12.5% EDTA solution, and sagittal hemisections were processed routinely for paraffin embedding. Three-micron-thick histologic sections were stained with hematoxylin and eosin.

Sagittal sections of both distal femoral metaphyses and all cervical, thoracic, and lumbar vertebral bodies were used for data collection. Lesions were counted if all or part of the lesion was within the preexisting limits of the examined skeletal structures. Not included in lesion counts were, metastatic foci limited entirely to hematopoietic marrow where there would have been no interaction with cortical or trabecular bone while tumors were still relatively small.

Area measurements of bone metastatic lesions were obtained from the histologic samples described above. Measurements were obtained from digital images captured at 40X and 100X magnifications using a Polaroid Digital Microscope Camera with Polaroid volume 2.0 DMC software and analyzed using Image-Pro Plus software version 3.0 (©Media Cybernetics, Silver Spring, MD). Tissue components such as fibroblasts and osteoclasts on the tumor front were included in measurements of the tumor area, because these components are known to have an important role in altering the bone and bone marrow microenvironment to the advancement of the metastatic lesions. Any portion of the tumor mass that was determined to extend beyond the preexisting limits of the bone (i.e., beyond the cortex) or proximal to the preexisting limits of femoral metaphyseal trabecular bone was not included in area measurements. These portions of lesions were excluded because tumor cell-bone matrix interactions were presumably not involved in lesion expansion in these areas.

Statistics

Comparison of tumor areas in vertebral bodies and femurs were evaluated using a Kolmogorov-Smirnov test for normality and Student's t-test.

Results

Descriptive comparison of bone metastasis in treated and untreated animals

Areas of bone altered morphologically by risedronate treatment in the immature skeletons of these animals were not spared of destructive metastases. Multiple large destructive bone metastases were present in both treated and untreated animals (Figures 1.1 and 1.2). Subjectively, there was no detectable difference in the number or size of metastatic foci in the two groups.

In untreated animals, metastases consisted of sheets of neoplastic cells effacing and replacing the preexisting marrow, marrow stroma, and trabecular or cortical bone. A resorption front of osteoclasts overlying resorption pits along trabecular and cortical bone surfaces was observed on the periphery of the metastatic foci (Figure 1.3a&b). Cortical bone destruction ranged from mild thinning to complete obliteration in the examined plane of the tissue section.

As has been previously reported [1] , expansion of epiphyseal and metaphyseal bone demonstrated that the risedronate had effectively inhibited remodeling in treated animals. There was an apparent decrease in numbers of osteoclasts lining bone surfaces associated with metastatic lesions. More commonly, neoplastic cells were adjacent to uneven trabecular and cortical bone surfaces, giving the impression that bone destruction was occurring via direct osteolysis by tumor cells (Figure 1.4a&b). In areas of massive bone destruction, tumor cells completely surrounded fragments of necrotic bone. Where cortical thinning was identified, tumor cells lined irregular endosteal surfaces, typically in the absence of osteoclasts.

Enumeration of bone-metastatic lesions

Large destructive metastatic lesions were observed in distal femoral metaphyses and in multiple vertebral bodies of both treated and untreated animals. The total numbers of metastases in distal femoral metaphyses and in cervical, thoracic, and lumbar vertebral bodies were determined for each animal. In femoral metaphyses, lesions in both treated and untreated animals often appeared to coalesce such that distinction and enumeration of individual lesions became difficult. Large lesions that counted as a single focus may have actually originated as independent lesions, resulting in lesion undercounts. However, this circumstance occurred as often in treated as in untreated animals. This problem was not encountered in counting lesions in vertebral bodies. The data were normally distributed, and there was no significant difference in mean number of lesions in treated versus untreated groups for either femurs (Figure 1.5, $t=0.19$) or vertebral bodies (Figure 1.6, $t=0.43$).

Histomorphometry of lesion area

Area of bone metastatic lesions was measured in sagittal sections of femur metaphyses and in vertebral bodies. Mean tumor areas in femoral metaphyses (Figure 1.7) and in vertebral bodies (Figure 1.8) were not significantly different between the treated and untreated animals ($t = 0.20$ for femurs, $t = 0.44$ for vertebral bodies).

Discussion

These experiments demonstrate that daily risedronate treatment at the indicated dose had no effect on the incidence or size of bone metastatic lesions produced by MTA cells in syngeneic hosts. This is in contrast to the positive treatment response previously

demonstrated for the related cell line MTBo2 [1]. This animal model may provide a unique system with which to investigate factors that influence positive and negative outcomes of bisphosphonate treatment for bone metastasis.

Morbidity associated with bone metastasis is related primarily to destruction of bone matrix, which in most situations is mediated primarily by osteoclasts and influenced by substances produced by the neoplastic cells. Substances known or thought to exert such an influence on bone lysis include parathyroid hormone-related protein (PTH-rP) [18], transforming growth factor β [4]; interleukins 1, 6 and 11 [4]; leukemia inhibitory factor (LIF) [4]; tumor necrosis factors β and α [4]; and insulin-like growth factor-II [4,18]. Inhibition of osteoclastic bone resorption by therapeutic bisphosphonates has decreased morbidity from bone metastasis in many patients, both by decreasing the destructiveness of already established lesions and preventing development of new bone lesions.

Positive responses to bisphosphonate therapy have not been universal, with treatment failures reported in a subset of human patients and in some animal models [15,19,20]. Reasons for these occasional unfavorable treatment outcomes are not currently known but presumably are caused in part by phenotypic differences that allow some bone-metastatic cells to successfully colonize and grow in bone in spite of decreased osteoclast involvement. One possibility would be that some tumor cells are more capable than others of directly lysing bone without osteoclast assistance. Direct tumoral osteolysis has been demonstrated in some tumor cell lines [21-23]. Recently, a prostate carcinoma cell line has been shown to directly lyse bone via the release of matrix metalloproteinase-9 (mmp-9) [24]. Although capacity for direct osteolysis remains

unproven for MTA cells, the histologic appearance of bone-metastatic lesions in treated animals described in this paper suggests such a capability in that there often appeared to be direct contiguity between resorbing bone and neoplastic cells without obvious osteoclast involvement.

Differences in dependency of tumor cells on bone matrix-derived growth factors for stimulation of functions such as chemotaxis and proliferation may also contribute to variable responses to bisphosphonate treatments. It is also possible that properties important for bone colonization (adhesion, chemotaxis, protease secretion, proliferation, cell survival) in responsive tumor cells are adversely affected by high concentrations of bisphosphonate released from bone matrix. Whereas nonresponsive tumor cells, such as MTA cells, are refractory to these inhibitory influences. Exposure to bisphosphonate has been shown to alter adhesive [25-27] and invasive [28-29] properties and to induce apoptosis [11-14] in some tumor cell lines. Whether such properties are affected to a lesser degree in bisphosphonate-nonresponsive tumor cells remains a topic for investigation.

The first step toward answering these questions is the identification and characterization of bisphosphonate-nonresponsive tumor cell lines. In this paper, we describe lack of response of MTA rat mammary tumor cell bone metastatic lesions to the same risedronate treatment protocol that was effective in decreasing the incidence and size of bone metastases caused by the related cell line, MTBo2 [1]. An important initial study will be to determine whether the resistance of MTA (and susceptibility of MTBo2) extends to other bisphosphonate derivatives in addition to risedronate. These related cell lines, cloned separately from the same ENU-induced mammary tumor provide a system

for investigating mechanisms responsible for differences in responsiveness of bone-metastatic cells to bisphosphonate treatment.

Acknowledgement

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References:

1. Hall DG and Stoica G Effect of the bisphosphonate risedronate on bone metastases in a rat mammary adenocarcinoma model system. J Bone and Miner Res 1994;9:221-230.
2. Galasko CSB Development of skeletal metastases, *In*: CSB Galasko (ed.), Skeletal Metastases, Boston: Butterworth and Co. (Publishers) Ltd 1986; pp. 22-51.
3. Mundy GR, Yoneda T Mechanism of osteolytic bone resorption. N Engl J Med 1998; 339:398-400.
4. Orr FW, Lee J, Duivenvoorden WCM, Singh G: Pathophysiologic interactions in skeletal metastasis. Cancer suppl. 2000; 88:2912-2918.
5. Rogers MJ, Chilton KM, Coxon FP, et al. Bisphosphonates induce apoptosis in mouse macrophages like cells by a nitric-oxide-independant mechanism. Journal of Bone and Mineral Research 1996;11:1482-91.
6. Selander KS, Monkkonen J, Karhukorpi EK, et al. Characteristics of clodronate-induced apoptosis inosteoclasts and macrophages. Molecular Pharmacology 1995;50:1127-38.

7. Coxon FP,. Helfrich MH, Van'tHof R, Sebti S, Ralston SH, Hamilton A, and Rogers M.J. Protein geranylgeranylation is required for osteoclast formation function, and survival: inhibition by bisphosphonates and GGTI-298. J Bone Minerl Res2000; 15:1467-1476.
8. Hughes DE, Wright KR, Uy HL, et al. Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. Journal of Bone and Mineral Research 1995;10:1478-87.
9. Ito M, Amizuka N, Nakajima T, et al. Ultrastructural and cytochemical studies on cell death of osteoclasts induced by bisphosphonate treatment. Bone 1999;25:447-52.
10. Reszka AA, Halasy-Nagy JM, Masarachia PJ, et al. Bisphosphonates act directly on the osteoclast to induce caspase cleavage of kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase. Journal of Biol Chem 1999;274:34967-73.
11. Senaratne SG, Piranov G, Mansi JL, Arnett TR, and Colston KW Bisphosphonates induce apoptosis in human breast cancer cell lines Br J Cancer 2000; 82:1459-1468.
12. Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. British Journal of Cancer 2000;82:1459-68.
13. Shipman CM, Croucher PI, Russell RG, Helfrich MH, and Rogers MJ The bisphosphonate icandronate (YM175) causes apoptosis of human myeloma cells

in vitro by inhibiting the mevalonate pathway. Cancer Research 1998; 58:5294-5297.

14. Shipman CM, Rogers MJ, Apperley JF, et al. Bisphosphonates induce apoptosis in human myeloma cells; a novel antitumor activity. British Journal of Haematology 1997;98:665-72.
15. Roux C, Ravaud P, Cohen-Solal M, de Vernejoul MC, Guillemant S, Cherruau B, Delmas P, Douados M, Amor B Biologic, histologic and densitometric effects of oral risedronate on bone in patients with multiple myeloma. Bone 1994;15:41-9.
16. Smith JA Palliation of painful bone metastases from prostate cancer using sodium etidronate: results of a randomized, prospective, double-blind, placebo-controlled study. J Urology 1989; 141: 85-87.
17. Hall DG, Stoica G: Characterization of brain and bone-metastasizing clones selected from an ethylnitrosourea-induced rat mammary carcinoma. Clin Exp Metastasis 1994; 12:283-295.
18. Tumber A, Morgan HM, Meikle MC, Hill PA: Human breast cancer cells stimulate the fusion, migration and resorptive activity of osteoclasts in bone explants. Int J Cancer 2001; 91:665-672.
19. Cleton FJ, van Holten-Verzantvoort AT, and Bijvoet OLM Effect of long-term bisphosphonate treatment on morbidity due to bone metastases in breast cancer patients. *In:* KW Brunner, H Fleisch, and H-J Senn (eds.). *Rec Res Cancer Res* 1989;116:73-78.

20. Shevrin DH, Gorny KI, Rosol TJ, and Kukreja SC. Effect of etidronate disodium on the development of bone lesions in an animal model of bone metastasis using the human prostate cancer cell line PC-3. *Prostate* 1991; 19: 149-154
21. Eilon G, Mundy GR: Direct resorption of bone by human Breast cancer cells in vitro. *Nature* 1978; 276:726-728.
22. Shivas AA, Black JW, Finlayson ND: The growth of Brown-Pearce carcinoma in the medullary cavity of the rabbit femur. Br J Cancer 1963;17:711-714.
23. Eilon G, Mundy GR: Association of increased cyclic adenosine 3':5'-monophosphate content in cultured human breast cancer cells and release of hydrolytic enzymes and bone-resorbing activity. Cancer Res 1983;43:5792-5794.
24. Sanchez-Sweatman OH, Orr FW, Singh G: Human Metastatic Prostate PC3 Cell lines Degrade Bone Using Matrix Metalloproteinases. Invasion and Metastasis 2000;18: 297-305.
25. Magnetto S, Bossier S, Delmas PD, et al. Additive antitumor activities of taxoids in combination with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. International Journal of Cancer 1999;83:263-9.
26. Bossier S, Magnetto S, Frappart L, et al. Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. Cancer Research 1997;3890-4.
27. Van der Pluijm G, Vloedgraven H, van Beek E, et al. Bisphosphonates inhibit the adhesion of breast cancer cells to bone matrices *in vitro*. Journal of Clinical Investigation 1996;98:698-705.

28. Bossier S, Ferreras M, Pevruchaud O, et al. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Research 2000;60:2949-54.
29. Guaitani A, Sabatini M, Polentarutti N, et al. Garattini S, editors.Bone Resorption, Metastasisi, and Diphosphonates. New York: Raven Press; 1985;Effects of disodium etidronate on tumor-induced hypercalcemia, bone invasion, and metastasis in rodents. p. 51-8.

Figure 1.1: Histologic appearance of a bone metastasis in a vertebral body from an untreated animal. A large tumor mass effaces the medullary space and has destroyed the preexisting trabecular bone (H&E. Bar = 100μm).

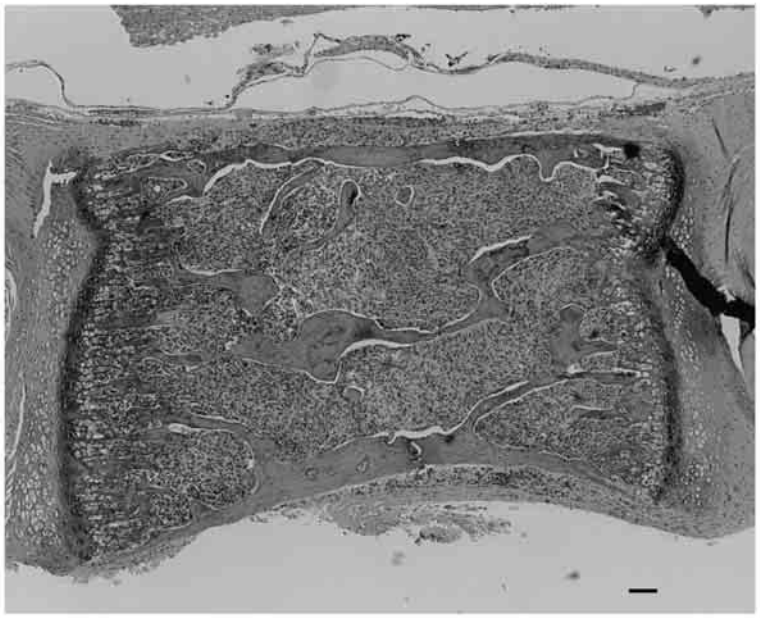


Figure 1.2: A bone metastasis in a vertebral body from a risedronate-treated animal. The skeletal architecture has been altered to the same extent as lesions in untreated animals (H&E. Bar = 100μm).

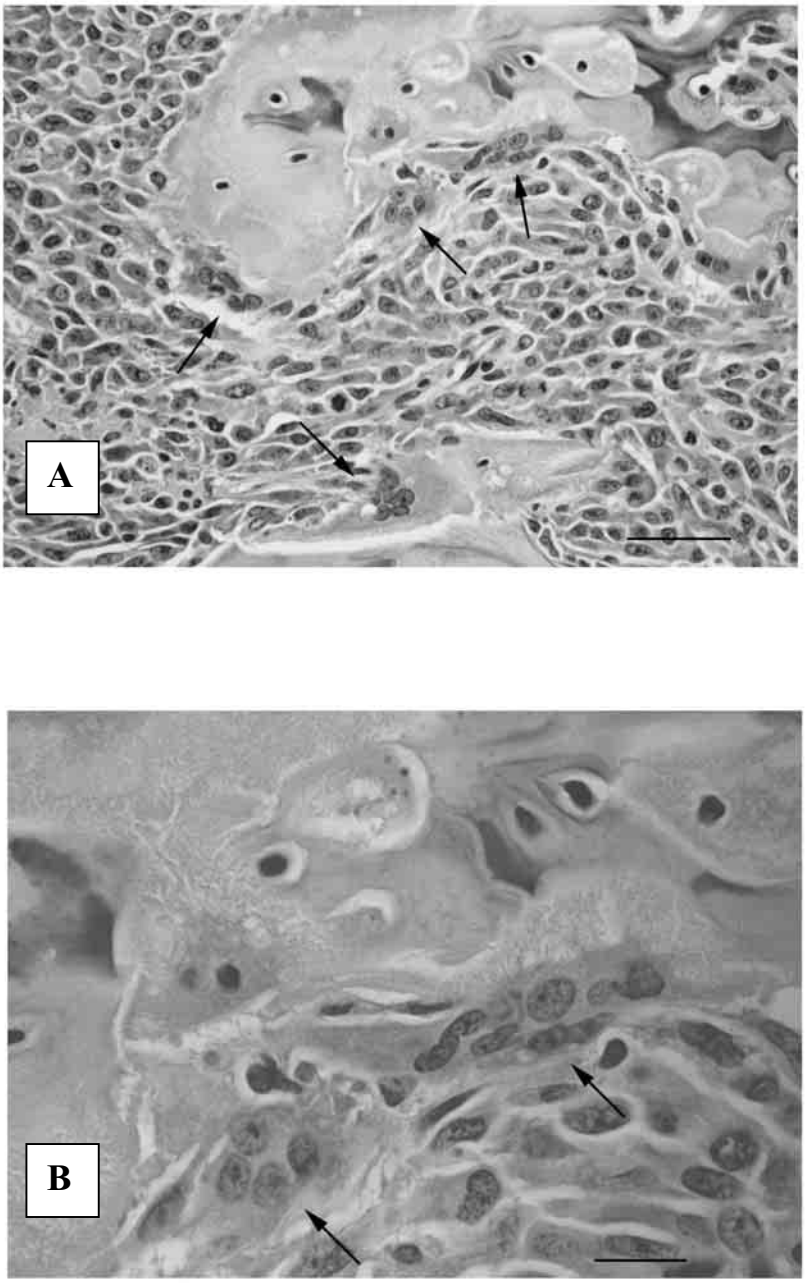


Figure 1.3: Bone metastasis in an untreated animal. **a.** Osteoclasts (arrow) within resorption lacunae are prominent along the tumor front (H&E. Bar = 40μm). **b.** Two osteoclasts (arrows), separated from the tumor by a delicate fibrovascular stroma, and actively resorbing the bone matrix (H&E. Bar = 30μm).

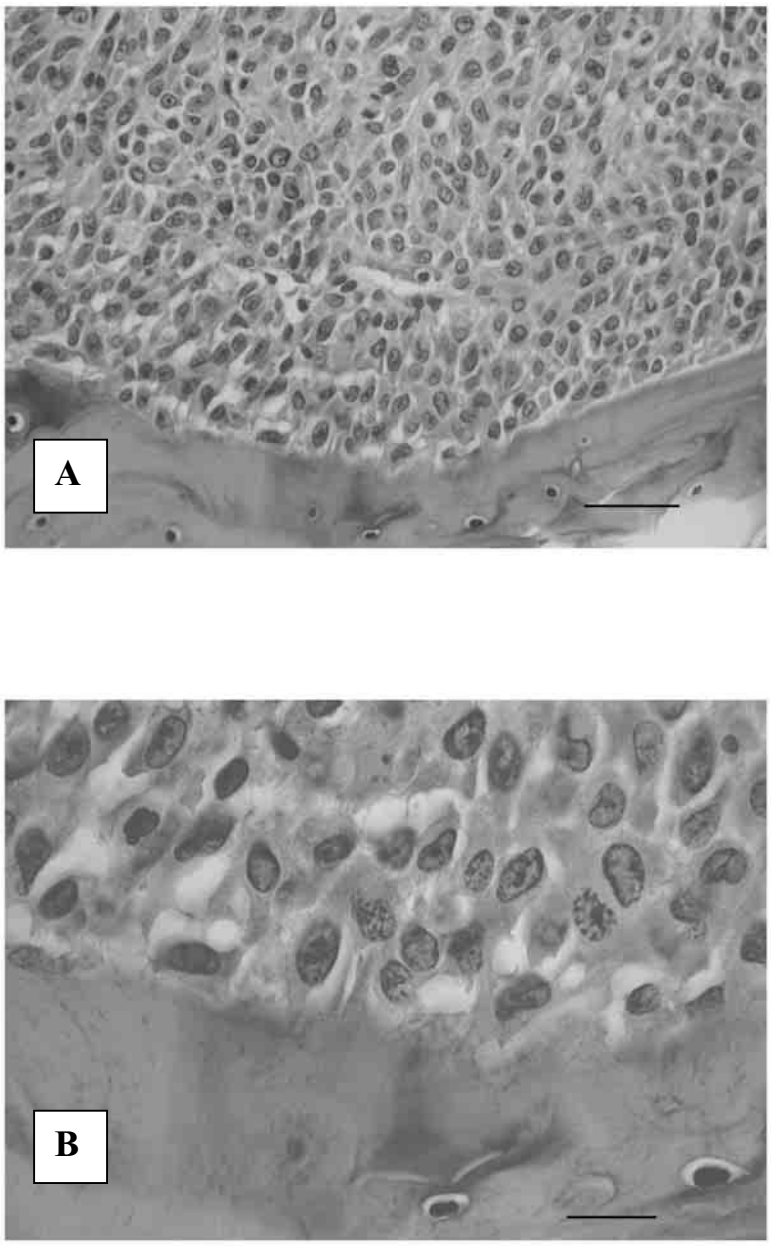


Figure 1.4: Bone metastasis in a risedronate-treated animal. **a.** Sheets of tumor cells in the medullary space and along bone surfaces. Osteoclasts are absent (H&E. Bar = 40µm). **b.** Tumor cells along an uneven bone surface give the impression of direct osteolysis by neoplastic cells (H&E. Bar = 30µm).

Figure 1.5: Total numbers of bone-metastatic lesions in distal femoral metaphyses of risedronate-treated and untreated animals. Horizontal lines represent the mean number of metastases for the group (t=0.19).

Figure 1.6: Total numbers of bone metastases in vertebral bodies of risedronate-treated and untreated animals. Horizontal lines represent mean number of metastases for the group (t=0.43).

Figure 1.7: Mean tumor areas in distal femoral metaphyses were not significantly different in risedronate-treated animals vs. untreated control animals. Horizontal lines represent mean lesion area for the group (t=0.20).

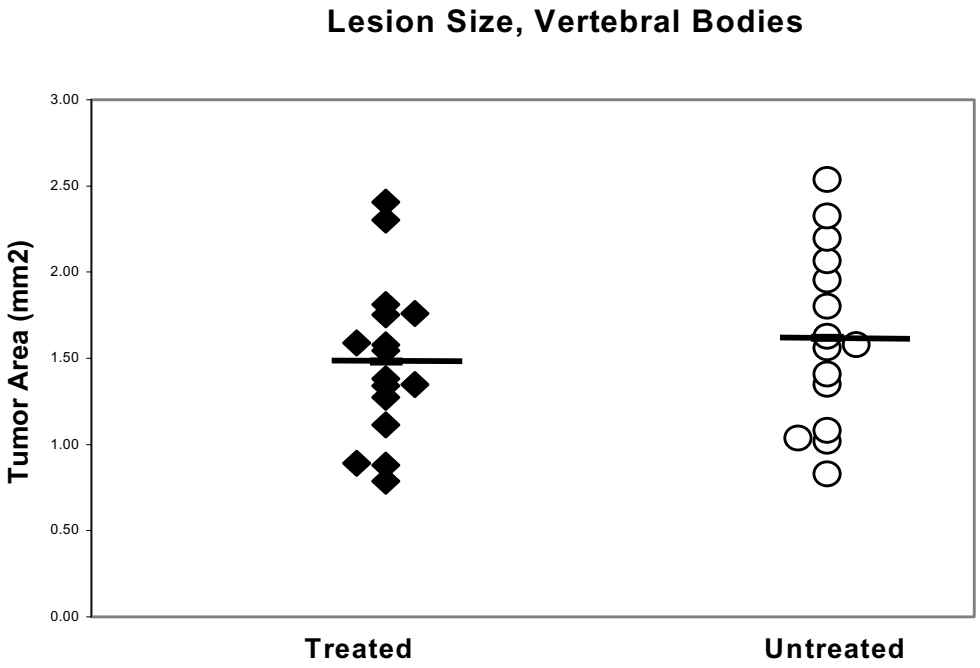


Figure 1.8: Mean tumor areas in vertebral bodies were not significantly different in risedronate-treated animals vs. untreated control animals. Horizontal lines represent mean lesion area for the group (t=0.44).

CHAPTER 2.

COMPARISON AND CHARACTERIZATION OF THE IN VITRO RESPONSES OF MTA (NONRESPONDER) AND MTB-01 (RESPONDER) MAMMARY CARCINOMA CELL LINES TO THE BISPHOSPHONATE, RISEDRONATE[1]

[1]Boyd, K. B., Cornell, K. K., and Howerth, E.W. To be submitted to Clinical and Experimental Metastasis

Abstract. Bisphosphonates are potent inhibitors of bone resorption and have proven useful in the treatment of diseases that result in osteolysis including palliative therapy in cancers that metastasize to bone such as mammary cancer. Although, many patients have favorable responses to bisphosphonate therapy, patients with poorly responding neoplasms have been identified. There has been little investigation into mechanisms by which this may occur. To identify factors that may account for the lack of response to risedronate we compared two rat mammary carcinoma cell lines, the MTA cell line which has previously been shown to be a nonresponder to risedronate and the MTB-01, which has been shown to have favorable responses to risedronate therapy. To accomplish this objective, we utilized in vitro assays measuring apoptosis, adhesion, and matrix metalloproteinase (MMP) production. Apoptosis was measured by cell morphology at the light microscopic level, DNA fragmentation, TUNEL staining, MiCK assay, Annexin-V binding, and transmission electron microscopy. Gelatin zymography was used to identify MMP-2 or MMP-9 protease production. When compared to MTA cells, MTB-01 cells were susceptible to risedronate-induced apoptosis, had decreased ability to bind to risedronate-treated bone, and did not produce MMP-2 or MMP-9 proteases. MTA cells were less susceptible to risedronate-induced apoptosis and produced MMP-2. Additionally, adhesion of MTA cells to bone matrix was not diminished by risedronate treatment. Our results suggest that the non-responsive nature of the MTA cell line may be due to MMP-2 production (possibly allowing ongoing destruction of risedronate-treated bone), continued adhesion to risedronate-treated bone matrix, and decreased susceptibility to risedronate-induced apoptosis.

INDEX WORDS: Bone metastasis, mammary carcinoma, risedronate, rodent model, apoptosis, matrix metalloproteinase, cell adhesion

Introduction

Metastasis of cancer cells to bone is a common occurrence in mammary and prostate cancers.[1] Osteolysis associated with the spread of these tumors to bone can result in severe bone pain, fractures, spinal cord impingement, and life threatening hypercalcemia. Palliative treatment of bone metastasis has included the use of bisphosphonate drugs.[2-5] Bisphosphonates are pyrophosphate analogs that concentrate in the bone matrix. Three generations of bisphosphonates have been produced with the third generation of drugs, those with a nitrogen containing heterocyclic ring, being the most potent. [6] Although bisphosphonates inhibit osteoclast mediated bone resorption,[6-8] induce osteoclast apoptosis,[9-11] and decrease the recruitment and proliferation of osteoclast progenitors,[12,13] the mechanisms of action of these drugs are still not completely understood. Bisphosphonates have been shown to decrease migration, invasion,[14] and adhesion to bone matrix[15,16] of prostate and mammary carcinoma cells, and to induce apoptosis in myeloma cells,[17-19]associated macrophages,[20] and in mammary carcinoma cell lines.[21] These findings suggest that in addition to osteoclast inhibition, bisphosphonates in the bone microenvironment may posses some anti-tumor activity.

Bisphosphonate therapy in patients with bone metastasis can result in resolution of hypercalcemia, decreased bone pain, decreased fracture incidence, and a decrease in new metastatic lesions.[22] In animal models of bone metastasizing mammary carcinoma, bisphosphonate therapy decreased size and incidence of metastatic lesions and has shown some promise in prophylactic treatment.[23-26] As with most drug therapies, however, positive responses to bisphosphonates are not universal.[21] The

mechanisms by which some tumor cells are able to successfully metastasize and proliferate in the bone microenvironment in the presence of bisphosphonate are poorly studied. The way in which the incorporation of these drugs into the bone matrix alters the matrix and directly affects the final steps of the metastatic cascade also is not clear.

The ability of tumor cells to invade extracellular matrices and basement membranes and the ability to directly lyse bone have been associated with the production of matrix metalloproteinases (MMPs).[27-30] Direct bone resorption by tumor cells mediated by MMPs, releasing potent mitogens and tumor agonists such as basic fibroblastic growth factor, transforming growth factor beta, and insulin like growth factors may represent a way that tumor cells can survive in the presence of high concentrations of bisphosphonate in the bone microenvironment. In addition to promoting tumor growth, substances released from bone serve as potent chemoattractants for tumor cells thereby potentiating bone metastasis and osteolysis.[1]

Some potential traits of tumor cells able to escape the effects of high concentrations of bisphosphonates in the bone microenvironment may include resistance to bisphosphonate induced apoptosis, the ability to adhere to the bisphosphonate treated bone surface, retention of invasive properties, and the ability to directly degrade the treated bone matrix. We have characterized a rat mammary carcinoma cell line, designated MTA, as a nonresponder to risedronate, a potent third generation bisphosphonate.[31] This cell line is capable of producing extensive osteolytic metastasis in the face of risedronate treatment, whereas, in other rodent models of mammary cancer,[23,24] risedronate has been shown to decrease the number and size of bone

metastatic lesions. Thus, the MTA cell line might be useful for studying factors that make certain tumors refractory to risedronate therapy.

The objectives of this study were to compare *in vitro* properties of MTA cells (nonresponding cell line) to those of a related rat mammary carcinoma cell line, MTB-01, [23] (responder cell line) to identify potential factors which may contribute to the resistance of MTA cells to risedronate therapy. We evaluated the effects of risedronate on apoptosis, adhesion to bone, and matrix metalloproteinase production of both cell lines. Our results suggest that MMP-2 production, adhesion to risedronate treated bone matrix, and decreased susceptibility to risedronate induced apoptosis may contribute to the nonresponsive nature of MTA cells.

Materials and Methods

Cell lines

Rat mammary cancer cell lines MTA and MTB-01 were utilized in this study. These cell lines were selected from a mammary tumor that developed in a Berlin-Druckrey IV female rat treated with a single injection of N-ethyl nitrosurea. Cells were cultured in Dulbecco's Modified Eagle Medium,(DMEM),(Life Technologies, Grand Island, NY), 5% fetal calf serum (FCS), (Atlanta Biologicals, Norcross, GA) with 1mM L-glutamine (Sigma, St. Louis, MO), and 1% penicillin/streptomycin (Sigma, St. Louis, MO), at 37ºC in a humidified atmosphere containing 5% CO_2 in air. MTB-01 is an adherent cell line. MTA cells grow with a portion of the cells adhering and the remaining population in suspension.

Bisphosphonate

Risedronate (Proctor and Gamble, Inc., Cincinnati, OH) was obtained from Dr. George Stoica (Texas A&M University). A stock solution of (10^{-4}M) was prepared in phosphate-buffered saline (PBS). For addition to cell cultures, serial dilutions were prepared in serum free DMEM.

Apoptosis

Cells were cultured in the presence of various concentrations of risedronate ranging from 10^{-4}M – 10^{-8}M in DMEM supplemented with 5% FCS, 1M L-glutamine and 1% antibiotic. Control cells were cultured in similar media without risedronate. Cells were collected at multiple time points and evaluated for apoptosis using cellular morphology by light microscopy, DNA fragmentation, modified TdT-mediated dUTP Nick End Labeling (TUNEL), Annexin-V binding, microculture kinetics (MiCK) assay and transmission electron microscopy. Cellular morphology was evaluated on cytocentrifuge preparations stained with Diff-Quik (Jorgensen Laboratories, Loveland, CA).

DNA Fragmentation

Cells were cultured in 25 mm^2 flasks for 48 and 72 hrs in the presence of 10^{-4} and 10^{-5} M risedronate. As a negative control, both cell lines were cultured in media without risedronate and collected at each time point. Cells were harvested by scraping and DNA was isolated using the Puregene® DNA isolation kit (Gentra, Minneapolis, MN). The DNA content of each sample was determined using spectrophotometry and 1μg of

purified DNA was loaded into a 2.5% agarose gel. The samples were electrophoresed at 25 volts for 5 hrs, stained with ethidium bromide (1µg/ml), and examined for laddering.

Modified TUNEL

DeadEnd colorimetric apoptosis detection system (Promega, Madison, WI) was used. Cells were cultured in previously described media in 96- well plates in the presence of 10^{-4} and 10^{-5} M risedronate. Negative control cells from both cell lines were cultured in supplemented media without risedronate and collected at corresponding time points. Adherent cells were scraped from 2 mm^2 wells, and cytocentrifuge preparations were prepared. MTB-01 cells were collected at 24, 48, 65, and 72 hrs. MTA cells were collected at these times and also at 96 and 120 hrs. Cell preparations were immersed in 10% neutral buffered formalin for 25 minutes, then washed in PBS for five minutes. Slides were loaded with TdT reaction mixture (biotinylated nucleotide mix and TdT enzyme) and incubated at 37° C for 1 hr. The reaction was stopped by immersing the slides in 2X SSC. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide. Slides were then incubated with horseradish-peroxidase-labeled streptavidin solution then combined with diaminobenzidine (DAB) substrate for color development. Initially, the number of TUNEL positive cells per 100 cells were going to be counted. However, in the samples where TUNEL-postive cells were identified, low total cell numbers precluded counting 100 cells.

Annexin-V

Externalization of phosphatidyl serine by apoptotic cells was detected with the Annexin-V-BIOTIN apoptosis detection kit (Oncogene Research Products, San Diego,

CA). MTA and MTB-01 cells were cultured for up to 72 hours in the presence of 10^0 (without risedronate), 10^{-4}, or 10^{-5} M risedronate. The MTB-01 cells were evaluated at 40, 48, and 72 hrs. Based on the results of previous experiments with MTA cells , a single time point of 72 hrs was chosen for evaluation by flow cytometry. At each time point, $5x10^5$ cells were collected, centrifuged at 1000g for 5 min, resuspended in PBS, then incubated for 15 minutes at room temperature in the dark with Annexin-V-BIOTIN (An). After incubation cells were centrifuged and resuspended in binding buffer with propidium iodide (PI) and streptavidin fluorescein isothiocyanate (FITC) conjugate (Oncogene Research Products, San Diego, CA). Samples were placed in the dark on ice until fluorescent microscopy or flow cytometry was performed. FITC fluorescence was measured at 515-545 nm and fluorescence of DNA-PI complexes at 550-606 nm. Cell debris was excluded from analysis by appropriate forward light scatter threshold and compensation was used when necessary. Twenty thousand cells were measured for each treatment group. Four quadrants of the cytograms were set using negative controls. Proportions of cells in each quadrant were expressed as percentage of the total population. The lower left quadrant represented viable An- PI- cells. The lower right quadrant showed early apoptosis with preserved plasma membrane integrity (An+ PI –). The upper right quadrant showed cells that have lost membrane integrity and are An+ PI+. The percentage of cells in each quadrant was compared between treated and untreated controls and between both cell lines.

Microculture Kinetics Assay (MiCK)

Microculture kinetic plate assay was performed as previously described with minimal modification.[32,33] Briefly, MTB-01 and MTA cells cultured in DMEM (without phenol red) supplemented with 5% FCS cells were seeded into 96 well plates at 10^5 cells per well. Cells were allowed 6 to 8 hours to adhere. Risedronate was then added to the wells at various concentrations (10^0, 10^{-4},10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}M). Cells were placed in the incubator for 30 minutes to allow for equilibration with the $C0_2$. The wells were covered with 50 ul sterile mineral oil and the plate was placed in a spectophotometer at 37°C. Optical density (OD) was measured at 590 nm each hour for 24 or 72 hours. The reader was calibrated to zero absorbance using wells containing only complete medium without cells. OD readings were plotted against time to provide a kinetic representation of the drug responses. Apoptosis is indicated graphically by an "apoptotic curve" with a characteristic region showing a steep increase in the OD .

Transmission electron microscopy

MTB-01 cells were cultured in the presence of 10^{-4} risedronate for 72 hours. Cells were collected and fixed in 2% glutaraldehyde-2% paraformaldehyde-0.2% picric acid in 0.1M Cacodylate-HCl buffer. Cells were pelleted and resuspended in 50ºC Molten Agar (Difco Laboratories, Detroit, MI) then pelleted and placed at 4º C until the pellet hardened. Sections of the agar pellet 1 mm thick were post fixed with 1% osmium tetroxide in 0.1M phosphate then transferred to 0.5% uranyl acetate (aqueous) for enbloc staining. Samples were dehydrated through a series of ethanol rinses, then embedded in Epon-Araldite resin. Ultrathin sections were mounted on 200 mesh high transmission

nickel grids subbed with 0.3% formavar and post stained with 5% methanolic uranyl acetate and Reynold's lead citrate. Grids were viewed with a JEM-1210 Transmission Electron Microscope.

Adhesion to treated and untreated bone

To obtain risedronate treated bone, an adult male rat received daily subcutaneous injections of 0.2 mg/kg risedronate for seven days. Treated and untreated rats were euthanized and right and left femurs were collected. The bone was taken through three freeze thaw cycles to devitalize resident bone cells and then sonicated to remove any adhering cells or debris. Cross sections of the femoral diaphysis 5μm in width were collected and frozen at -20°C until use.

Suspensions of MTA and MTB-01 cells at a concentration of 10^5cells in 100 ul of medium (DMEM 5% FCS) were seeded into a 48 well plate containing a single cross section of either treated or untreated femur. Cells were allowed 1 hour for adhesion. The bone fragments were fixed in 10% neutral buffered formalin and stained with toluidine blue. Images were captured using a Polaroid Digital Microscope Camera with Polaroid volume 2.0 DMC software and analyzed using Image-Pro Plus software version 3.0 (©Media Cybernetics, Silver Spring, MD). Total number of cells per mm^2 were measured and averaged from two independent experiments. Results were analyzed by a Student's t-test.

Zymography

MTA and MTB-01 cells were seeded in 75 cm^2 flasks in DMEM supplemented with 5% FCS. When the cells reached 80-90% confluency, the media was removed and cells were rinsed with Hank's balanced salt solution (HBSS) (Sigma, St. Louis, MO). Cells were then cultured for 24 hr in serum free DMEM containing 10^0, 10^{-4}, or 10^{-5} M risedronate. The media were collected and concentrated using YM-3 Centriprep Centrifugal Filter Devices (Millipore Corp.) Protein concentration of the media was measured at 590 nm on a spectrophotometer, using Bio-rad protein assay (Bio-Rad, Hercules, CA) . MMP activity was assessed by gelatin zymography as previously described.[34] Briefly, 5-10 μg of protein from each sample in zymography sample buffer(Bio-Rad, Hercules, CA) was loaded into wells of a 10% Tris-glycine acrylamide gel, with 0.1% gelatin (Bio-Rad). Electrophoresis was performed in Tris buffer (Bio-Rad) at 100V for 120 min. The gel was washed and agitated in renaturing buffer Triton X-100 (Bio-Rad) for 30 minutes at RT followed by overnight incubation in 50mM Tris, 200mM NaCl, 10mM $CaCl_2$, 0.02% Brij pH 7.6 at 37°C with agitation. Gels were stained for 60 minutes in Coomassie Blue dye, then destained in 40% methanol/10%acetic acid in water. Gelatinase activity was indicated by clear bands on the gel. Purified human MMP-2 and MMP-9 (Chemicon International, Temecula, CA) were run on each gel as positive controls. Digital images of the cells were captured with a Canoscan D660U (Canon, U.S.A.) The areas of the gelatinolytic bands were measured using Image-Pro Plus software version 3.0 (©Media Cybernetics, Silver Spring, MD) and compared between the two cell lines and between the treatment groups of individual cell lines. Area measurements were analyzed by Student's t-test.

Results

Apoptosis

In the MiCK assay, a steep increase in the slope of the OD reading indicative of membrane blebbing or zeiosis characteristic of early apoptosis[32] was detectable in the MTB-01 cells from 20 – 40 hours in cells exposed to 10^{-4}M risedronate **(Figure 2.1).** A steep slope in the OD curve did not occur in cultures of MTA cells exposed to risedronate for up to 72 hrs. Instead, a linear increase in the OD reading indicative of cell proliferation was observed **(Figure 2.2).**

In the MTB-01 cell line, DNA fragmentation, shown as laddering on agarose gels **(Figure 2.3 a&b)**, and positive TUNEL staining were observed at 72 hours of exposure to 10^{-4}M risedronate **(Figure 2.4),** but not at 48hrs. In contrast, DNA fragmentation **(Figure 2.3 a&b)** and TUNEL **(Figure 2.5b)** staining were not observed in the MTA cell line in any of the experimental groups.

Morphologic changes characteristic of apoptosis were identifiable in MTB-01 cell lines on Diff Quik stained cytocentrifuge preparations at 48 hours. By 72 hours **(Figure 2.6),** approximately 30% of the cells had changes consistent with apoptotic cell death. Cells were condensed with fragmented nuclei, membrane blebs, and membrane bound fragments of nuclear and cellular material (apoptotic bodies). A decrease in the total number of adhering cells in the MTB-01 cell cultures treated with 10^{-4}M risedronate was observed by phase contrast microscopy at 48 hours, becoming most prominent at 72 hours compared with untreated controls and with cells treated with 10^{-5} M risedronate.

(Figure 2.7) In the MTA cell line, morphologic changes consistent with cell death were not observed after 72 hrs of culture in the presence of 10^{-4}M risedronate. **(Figure 2.5 a)**

MTB-01 cells cultured in the presence of 10^{-4} and 10^{-5}M risedronate were evaluated for externalization of phosphatidyl serine at 40, 48, and 72 hours by Annexin-V binding and flow cytometry. An+PI- staining, indicating apoptosis, was highest at 40 hours in the group treated with 10^{-4}M risedronate. In the later time points, 48 and 72 hours, An+PI+ cells increased and cells An+PI- decreased, indicating a shift toward necrosis as time progressed. **(Figure 2.8)** At all time points, MTB-01 cells treated with 10^{-4} or 10^{-5} M risedronate, consistently had a higher percentage of the cell population that were An+ PI- than the untreated control cells and the percentage of necrotic cells in the treated groups was consistently lower than the untreated control cells**(Table 2.1)**. Because there was no evidence of apoptosis in the MTA cell line in the other assays, this cell line was analyzed for Annexin-V binding at a single time point of 72 hrs after culture with 10^{-4} risedronate. After 72 hrs, a small percentage, 22.4%, of the MTA cells cultured in the presence of risedronate became An+PI- **(Figure 2.8)** compared with only 4.8% of the untreated MTA cells, suggesting that apoptosis occurs after long term exposure to risedronate. **(Table 2.1)**

Transmission electron microscopy was performed on the MTB-01 cells after 72 hours of culture in the presence of 10^{-4}M risedronate. Although classical cellular changes consistent with apoptosis (cellular and nuclear condensation, nuclear fragmentation, and membrane blebbing) was most common, cells with nuclear, organellar, and cellular swelling more characteristic of necrosis were also identified. (**Figure 9a-c).**

Adhesion to the bone matrix

The difference of the average number of MTA cells adhering to the treated bone versus the untreated bone was not statistically significant (266 vs 249 cells/mm^2 respectively p= 0.30). In the MTB-01 line however, a significant decrease in the number of cells adhering to the risedronate treated bone matrix (201 cells/mm^2) compared to the number of MTB-01 cells adhering to the untreated bone matrix (274 cells/mm^2) was observed ($p<0.05$). The number of MTB-01 cells adhering to treated bone was also significantly lower than the number of MTA cells adhering to the treated and untreated bone matrix ($p<0.05$) **(Figure 2.10)**. There was no significant difference between the number of MTB-01 cells adhering to the untreated bone matrix compare to the MTA cellular adhesion to the untreated matrix (p=0.29). Additionally, the average number of MTA cells adhering to the risedronate treated bone matrix was not significantly different than the average number of MTA or MTB-01 cells adhering to the untreated matrix.

Zymography

Samples from cell cultures for zymography were collected on three separate occasions. Initially, gels were loaded with 5 µg of sample from each cell line. Media collected from the MTA cell line had clear bands present at 72 kD that co-migrated with the MMP-2 standard. Clear bands were not observed in the 92 kD area, indicating that MMP-9 production was not detectable by zymography **(Figure 2.11).** There was no detectable difference between the negative control cells and the cells exposed to 10^{-4} or 10^{-5} M risedronate for 24 hours in the area measurements of the clear bands associated with MMP-2 production, indicating that risedronate exposure had no effect on MMP-2 production.

No clear bands were present in samples from the MTB-01 cell line **(Figure 2.12).** The protein concentration was increased from 5µg to 10µg of protein per well for electrophoresis of samples from both cell lines. The 72 kDa bands were evident in the MTA samples but not in the MTB-01 samples, even at these higher substrate concentrations.

Discussion

This study compared the in vitro cellular responses of MTA (nonresponder) and MTB-01 (responder) cell lines to the bisphosphonate, risedronate. The observed differences may provide clues into mechanisms responsible for the differing in vivo responses. For the past decade the usefulness of bisphosphonates in the prevention of osteolysis in bone-metastatic cancer and osteoporosis has been known. It is only recently that the direct antitumor effects of these drugs have been investigated. Concentrations of 10^{-4} M are considered to be slightly higher than what is expected in the bone microenvironment. It is possible, however, this concentration could be found in osteoclast resorption chambers and therefore also possibly achievable where direct osteolysis by tumor cells is occurring.[21] These two cell lines, MTA and MTB-01, were chosen because of their very different in vivo responses to risedronate, thus providing a system for in vitro comparison of the different cellular properties and responses to risedronate.

In this study we specifically evaluated apoptosis, MMP production, and tumor cell adhesion to bone in both cell lines. We found that the nonresponder, MTA, is less susceptible to risedronate induced apoptosis, produces MMP-2 that is unaffected by

risedronate exposure, and adheres to bone by mechanisms that are unaffected by risedronate treatment. Conversely, the responder, MTB-01, was susceptible to risedronate induced apoptosis, did not produce MMP-2 or MMP-9, and had decreased affinity to adhere to risedronate treated bone.

We found evidence of risedronate-induced apoptosis in the responding cell line, MTB-01, but very little apoptosis in the nonresponding cell line, MTA. The MiCK assay detects membrane blebbing one of the first changes seen in apoptosis. [33] Utilizing the MiCK assay, evidence of early apoptosis was detected in the MTB-01 cell line after 28 hrs exposure to risedronate but not the MTA cell line for up to 72 hrs exposure to risedronate.

Annexin-V binding detects externalization of phosphatidyl serine (PS) which is another early change seen in apoptosis and occurs shortly after membrane blebbing.[32] In the MTB-01 cell line, Annexin-V staining was observed in all cell samples including the negative control indicating that culture conditions and handling procedures probably contributed to cell death. In cells cultured in the presence of risedronate, regardless of the concentration, the percentage of apoptotic cells (An+PI-) was greater than the negative control indicating that the presence of risedronate in the media enhanced apoptosis. Additionally, the percentage of necrotic cells in cells exposed to risedronate regardless of time or concentration was never as high as the percenage of necrotic cells in the negative control suggesting that the presence of risedronate may influence the type of cell death that occurs. In the treated cells, the highest percentage of apoptotic cells (An+PI-) were observed at the earliest time point (40hrs), but by 72 hrs there was

essentially no difference between the percentage of apoptotic cells (An+PI-) necrotic cells (An+PI+), suggesting as time progresses there is a shift toward necrosis.

Additional confirmation of apoptosis in the MTB-01 cell line included positive TUNEL staining, identification of DNA fragmentation, and cellular changes identified by light microscopy and transmission electron microscopy. All of these detect late changes in apoptosis and, as expected, changes with these assays occurred later that the MiCk and the Annexin-V binding assay Both apoptotic and necrotic cells were identified by transmission electron microscopy, supporting the previous findings that drug induced apoptosis occurs in the MTB-01 cell line, but necrosis also takes place after long term exposure to risedronate.

In the MTA cell line, a small portion of the cell population became An+PI- after 72 hrs in the presence of risedronate indicating early apoptosis. This suggests that in contrast to the MTB-01 cell line, MTA cells may begin to undergo apoptosis only after long term exposure to of risedronate. Apoptosis was not confirmed by TUNEL, DNA fragmentation, and light microscopy in this cell line; however, these tests detect changes that occur late in apoptosis and changes might have been detected if the MTA cell line had been analyzed at later time points.

The results of the apoptosis investigations provide evidence that the MTB-01 cell line is more sensitive to bisphosphonate induced apoptosis in vitro compared to the MTA cell line. Therefore, MTB-01 cells may be less likely to survive in the microenvironment of risedronate-treated bone, which may correlate to fewer or smaller metastatic lesions in vivo following risedronate treatment.

In the next set of experiments investigating the effects of risedronate on tumor cell adhesion to the bone matrix, significant differences were observed between the two cell lines in their ability to adhere to treated bone matrix. While adhesion of the MTB-01 cell line to risedronate treated bone matrix was diminished, adhesion of the MTA cell line to treated bone was unaffected. These findings show another difference between the nonresponding MTA cells and the responding MTB-01 cells that possibly contributes to the decreased ability of the MTB-01 cell line to colonize bone in the face of risedronate treatment.

In the MMP assays, the MTB-01 cell line did not produce MMP-2 or MMP-9. The MTA cell line produced MMP-2 but not MMP-9 and after 24 hours of culture in the presence of risedronate, MMP-2 production was unaffected. MMP-2 is important in the destruction of type-1 collagen. This is the most abundant form of collagen in bone matrix. In histologic sections of MTA bone metastasis, tumor cells are commonly observed along irregular trabecular bone surfaces, suggesting that they are engaged in direct osteolysis. Matrix metalloproteinase-2 production by these cells may allow direct osteolysis after the removal of surface collagenase-1, without osteoclast involvement. As mentioned previously, the ability of tumor cells to directly resorb bone, releasing beneficial growth factors, can potentiate bone metastasis. In the case of the risedronate treated animal, the ability of the tumor cells to lyse bone in the presence of osteoclast inhibition is a potential cause for a poor response to bisphosphonate treatment.

These experiments have identified three specific properties by which the nonresponding line, MTA, and the responding cell line, MTB-01, differ in response to direct and indirect exposure to risedronate. We have identified differences in the ability

to survive in the risedronate-treated bone microenvironment, in the ability to adhere to risedronate treated bone, and in the production of proteolytic enzymes capable of lysing bone. In the MTB-01 cells exposure to risedronate induced anti-tumor effects similar to what has been described in other breast cancer and prostatic carcinoma cell lines such as, decreased adhesion to the bone matrix and the induction of apoptosis. In the nonresponder cell line, MTA, we have identified traits that may contribute to the lack of response to bisphosphonate therapy. Identification of specific properties of tumor cells, such as the ones described in this study, provide targets that may be useful in the development of treatment modalities for human patients with bone-metastatic cancers unresponsive to bisphosphonate treatment.

References:

1. Orr FW, Lee J, Duivenvoorden WCM, et al. Pathophysiologic Interactions in Skeletal Metastasis. Cancer 2000;89:(12)2912-8.
2. Kanis JA, Powles TJ, Paterson AHG, et al. Clondronate decreases the frequency of skeletal metastases in women with breast cancer. Bone 1996;19:663-7.
3. Mundy GR, Yoneda T. Bisphosphonates as anticancer drugs. New England Journal of Medicine 1998;339:398-400.
4. Thiebaud D, Leyvraz S, von Fliedner V, et al. Treatment of bone metastases from breast cancer and myeloma with pamidronate. European Journal of Cancer 1991;27:37-41.
5. Major PP, Lipton A, Berenson J, et al. Oral Bisphosphonates: A review of clinical use in patients with bone metastasis. Cancer 2000;88:(1)6-14.

6. Rogers MJ, Watts DJ, Russell RGG. Overview of bisphosphonates. Cancer 1997;80 supplement:1652-60.
7. Carano A, Teitelbaum SA, Konsek JD, et al. Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts *in vitro*. Journal of Clinical Investigation 1990;85:456-61.
8. Chambers TJ. Diphosphonates inhibit bone resorption by macrophages *in vitro*. Journal of Pathology 1980;132:255-62.
9. Ito M, Amizuka N, Nakajima T, et al. Ultrastructural and cytochemical studies on cell death of osteoclasts induced by bisphosphonate treatment. Bone 1999;25:(4)447-52.
10. Hughes DE, Wright KR, Uy HL, et al. Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. Journal of Bone and Mineral Research 1995;10:1478-87.
11. Coxon FP, Helfrich MH, Van't Hof R, et al. Protein geranygeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. Journal of Bone and Mineral Research 2000;15:(8)1467-76.
12. Hughes DE, MacDonald BR, Russell RGG, et al. Inhibition of osteoclast-like cell formationby bisphosphonates in long term cultures of human bone marrow. Journal of Clinical Investigation 1989;83:1930-5.
13. Sato M, Grasser W. Effects of bisphosphonate on isolated rat osteoclasts as examined by reflected light microscopy. Journal of Bone and Mineral Research 1990;5:31-40

14. Bossier S, Ferreras M, Pevruchaud O, et al. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Research 2000;60:(11)2949-54.
15. Bossier S, Magnetto S, Frappart L, et al. Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. Cancer Research 1997;(57)3890-4.
16. Van der Pluijm G, Vloedgraven H, van Beek E, et al. Bisphosphonates inhibit the adhesion of breast cancer cells to bone matrices *in vitro*. Journal of Clinical Investigation 1996;98:698-705.
17. Shipman CM, Rogers MJ, Apperley JF, et al. Anti-tumor activity of bisphosphonates in human myeloma cells. Leuk Lymphoma 1998;32:(1-2)129-38.
18. Shipman CM, Croucher PI, Russell RGG, et al. The bisphosphonate icandronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. Cancer Research 1998;58:(23)5294-7.
19. Shipman CM, Rogers MJ, Apperley JF, et al. Bisphosphonates induce apoptosis in human myeloma cells; a novel antitumor activity. British Journal of Haematology 1997;98:665-72.
20. Rogers MJ, Chilton KM, Coxon FP, et al. Bisphosphonates induce apoptosis in mouse macrophages like cells by a nitric-oxide-independant mechanism. Journal of Bone and Mineral Research 1996;11:1482-91.

21. Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. British Journal of Cancer 2000;82(8):1459-68.

22. Russell RGG, Rogers MJ. Bisphosphonates: from the laboratory to the clinic and back again. Bone 1999;25:(1)97-106.

23. Hall DG, Stocia G. Effect of the Bisphosphonate Risedronate on Bone Metastases in a Rat Mammary Adenocarcinoma Model System. Journal of Bone and Mineral Research 1994;9:(2)221-30.

24. Sasaki A, Boyce RW, Story B, et al. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res 1995;55:3551-7.

25. Sasaki A, Kitamura K, Alcalde RE, et al. Effect of a newly developed bisphosphonate, YH529, on osteolytic bone metastases in nude mice. International Journal of Cancer 1998;77:279-85.

26. Krempien B, Manegold C. Prophylactic treatment of skeletal metastases, tumor-induced osteolysis and hypercalcemia in rats with the bisphosphonate CL2MBP. Cancer 1993;72:91-8.

27. Sanchez-Sweatman OH, Lee J, Orr FW, et al. Direct osteolysis induced by metastatic murine melanoma cells: role of Matrix metalloproteinases. European Journal of Cancer 1997;33:918-25.

28. Barille S, Akhoundi C, Collette M, et al. Metalloproteinases in multiple myeloma: production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. Blood 1997;90:1649-55.

29. Yoneda T, Sasaki A, Dunstan CR, et al. Inhibition of osteolytic bone metastasis of breast cancer by combined treatment with the bisphosphonate Ibandronate and tissue inhibitor of the matrix metalloproteinase-2. Journal of Clincal Investigation 1997;99:2509-17.

30. Sanchez-Sweatman OH, Orr FW, Singh G: Human Metastatic Prostate PC3 Cell lines Degrade Bone Using Matrix Metalloproteinases. Invasion and Metastasis 2000;18: 297-305.

31. Boyd K, Neuwirth L, Hall DG: Characterization of a Risedronate-Nonresponsive Bone-Metastatic Rat Mammary Tumor Cell Line. Submitted to Clinical and Experimental Metastasis, March 1, 2001.

32. Kravtsov V, Fabian I. Automated monitoring of apoptosis in suspension cell cultures. Lab Invest 1996; 74:557-63.

33. Kravtsov VD, Greer JP, Whitlock JA, et al. Use of the microculture kinetic assay of apoptosis to determine chemosensitivities of leukemias. Blood 1998; 92:968-80.

34. Huessen C, Dowdle EB. Electrophoretic analysis of plasminogen activators I polyacrylamide gels containing sodium dodecylsulfate and copolymerized substrates. Anal Biochem 1980; 102:196-202.

Table 2.1. MTA and MTB-01 cell staining with Annexin-V and PI evaluated by flow cytometry. Results are expressed as relative percentages for each category. After 72 hrs exposure to risedronate MTA cells are beginning to display apoptosis. In the MTB-01 cell line the control cells have the highest population of necrotic cells. The highest percentage of apoptotic cells is observed after 40 hrs exposure to 10^{-4} risedronate which correlates with the findings in the MiCK assay.

Cell line	M[1] risedronate	Time in hours	(An-PI-)[2] % viable	(An+PI-)[3] % apoptotic	(An+PI+)[4] % necrotic
MTA	10^{0}	72	92.2	4.8	2.6
MTA	10^{-4}	72	68.5	22.4	8.7
MTB-01	10^{0}	72	28.8	20.6	41.2
MTB-01	10^{-4}	40	19.7	52.2	25.9
MTB-01	10^{-5}	40	24.0	35.2	37.2
MTB-01	10^{-4}	48	21.9	37.2	37.7
MTB-01	10^{-5}	48	23.8	31.8	39.2
MTB-01	10^{-4}	72	24.7	37.8	35.5
MTB-01	10^{-5}	72	30.8	38.9	28

1- M= Molar cocentration
2- An-PI-= Annexin-V negative/Propidium Iodide negative
3- An+PI-= Annexin-V positive/Propidium Iodide negative
4- An+PI+= Annexin-V positive/Propidium Iodide positive

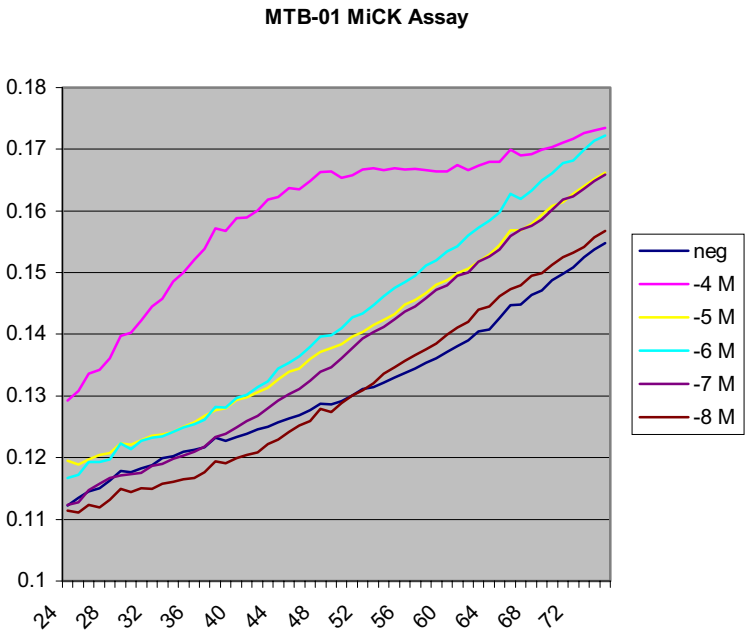


Figure 2.1: OD kinetics readings of the MTB-01 cell line 24-72 hrs culture with 10^{-0}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, or 10^{-8}M risedronate. There was a constant linear increase in the OD reading over the 72 hr period consistent with cell proliferation in control cells and in cells exposed to risedronate at all concentrations except 10^{-4}M. Note in the cells exposed to 10^{-4}M risedronate there is a typical apoptotic curve (sharp incline in the OD measurement), indicative of membrane blebbing, present from 24-40hrs.

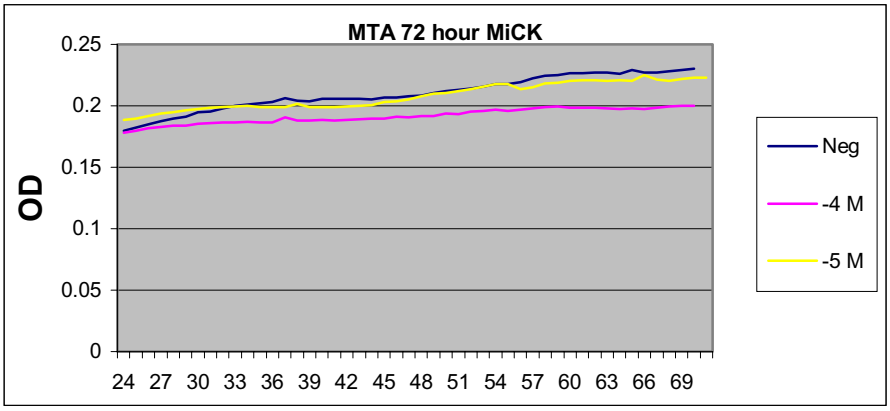


Figure 2.2: OD kinetics readings of the MTA cell line from 24-72 hrs culture with 10^{-0}, 10^{-4}, or 10^{-5}M risedronate. There is a constant linear increase in the OD reading over the 72 hr period consistent with cell proliferation. There is no apoptotic curve (sharp incline in the OD measurement) present even after exposure to the highest concentration of risedronate.

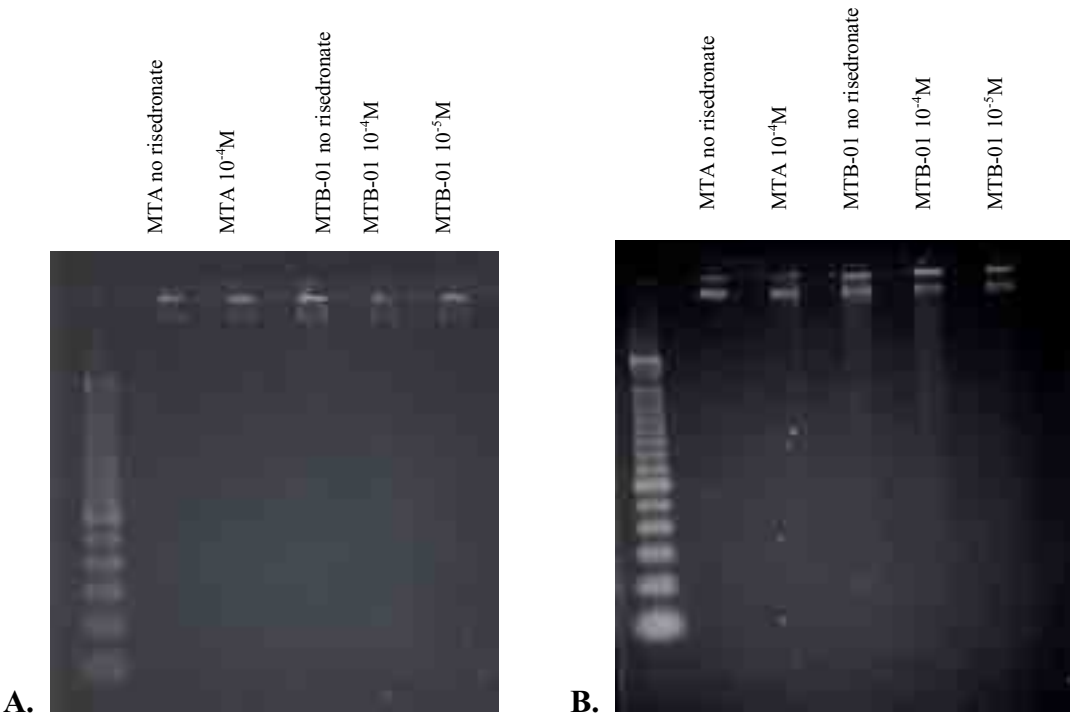


Figure 2.3: Gel electrophoreses of DNA isolated from MTA and MTB-01 cells after 48 and 72 hours in culture with or without risedronate. a) 48 hr sample, Lane 1: DNA Ladder , Lane 2: MTA cells without risedronate, Lane 3: MTA cells exposed to 10^{-4}M risedronate, Lane 4: MTB-01 cells without risedronate, Lane 5: MTB-01 cells exposed to 10^{-4}M risedronate, Lane 6: MTB-01 cells exposed to 10^{-5}M risedronate;
b) 72 hr sample, Lane 1: DNA Ladder , Lane 2 MTA cells without risedronate, Lane 3: MTA cells exposed to 10^{-4}M risedronate, Lane 4: MTB-01 cells without risedronate, Lane 5: MTB-01 cells exposed to 10^{-4}M risedronate, Lane 6: MTB-01 cells exposed to 10^{-5}M risedronate. Laddering is not present at 48 or 72hrs in the MTA cell line exposed to risedronate. Typical laddering is observed in the MTB-01 cell line at 72 hours after exposure to 10^{-4}M risedronate. Agarose gel, ethidium bromide staining.

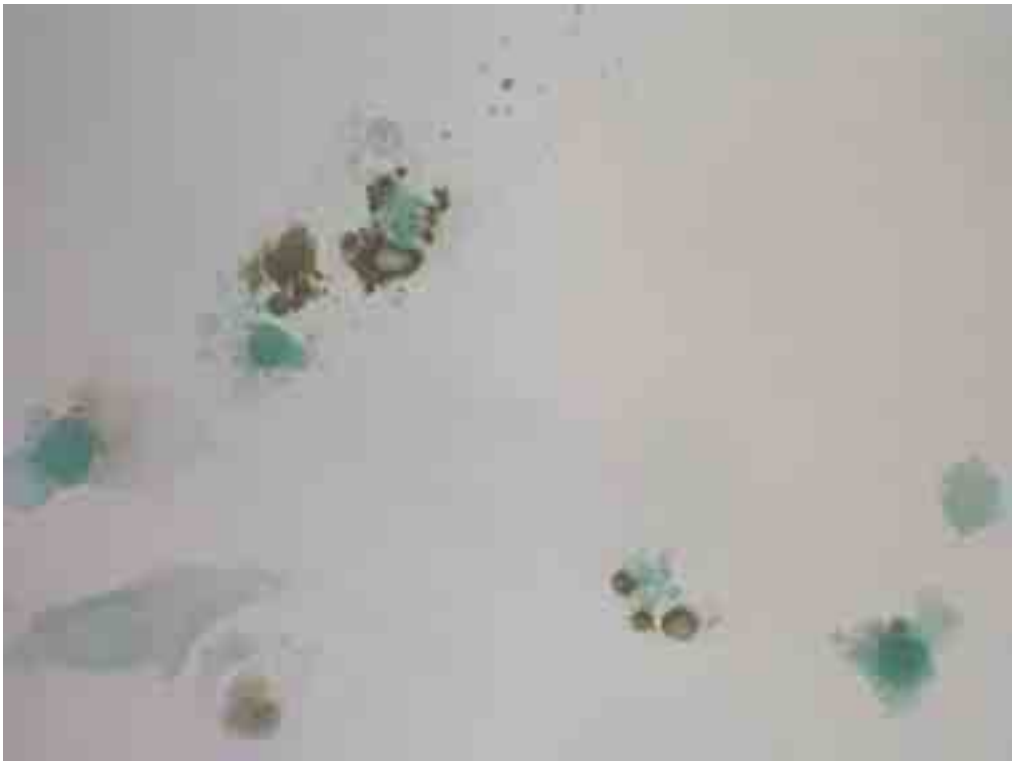


Figure 2.4: 400X Cytocentrifuge preparation; Positive TUNEL staining of apoptotic MTB-01 cells after culture in the presence of 10^{-4}M risedronate for 72 hrs.

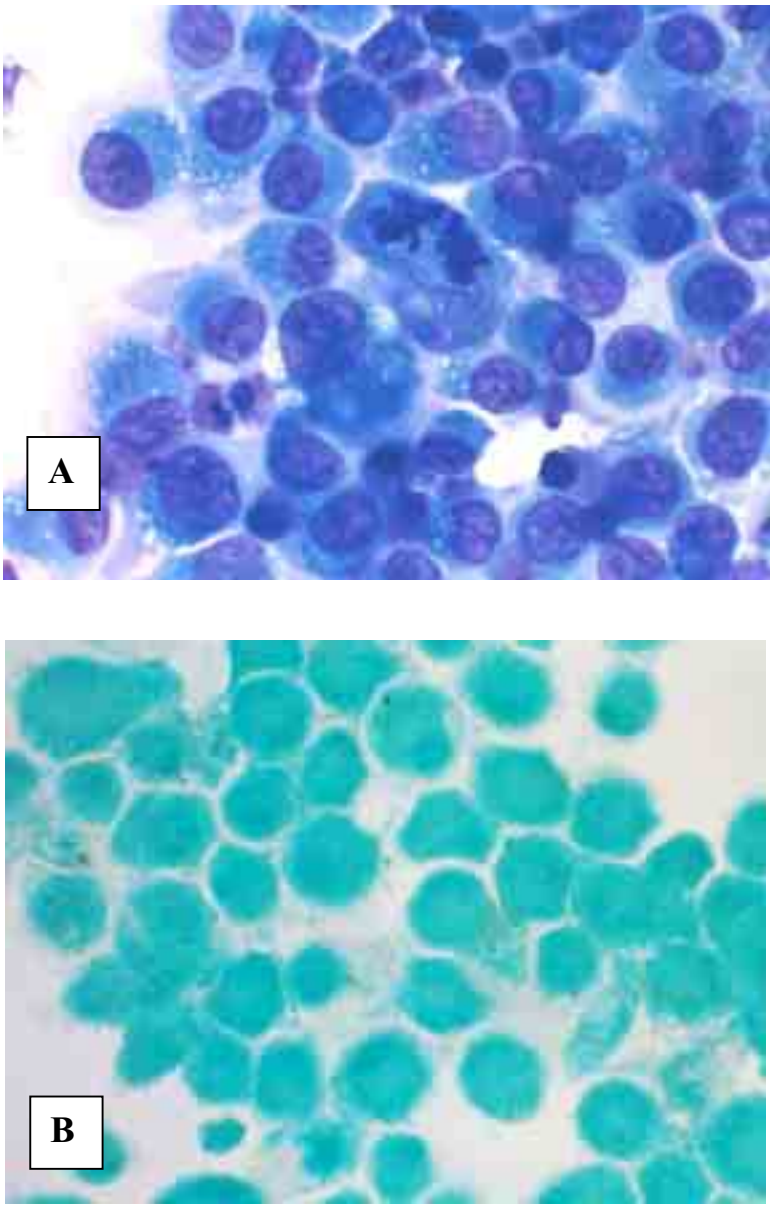


Figure 2.5: MTA cells do not show any evidence of apoptosis a) Cytocentrifuge prepartion 1000X; Diff Quik stain MTA cells after 5 days in culture with 10^{-4} risedronate. Cells are viable and mitotic figures are often observed. B) Cytocentrifuge preparation 1000X; Negative TUNEL staining of MTA cells after 96 hrs culture with 10^{-4} risedronate present.

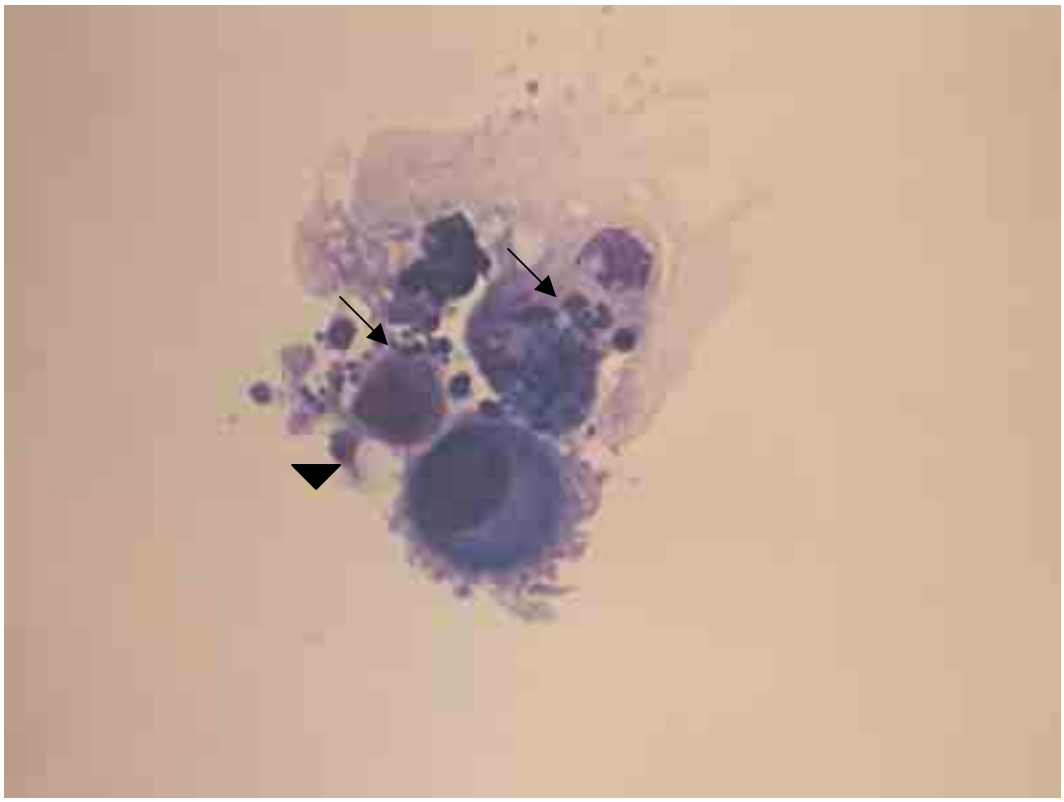


Figure 2.6: Cytocentrifuge preparation 1000X; Diff Quik stain, MTB-01 cells after 72 hrs culture in the presence of 10^{-4}M risedronate. Nuclear condensation and fragmentation, (arrow) cell shrinking and apoptotic bodies(arrowhead).

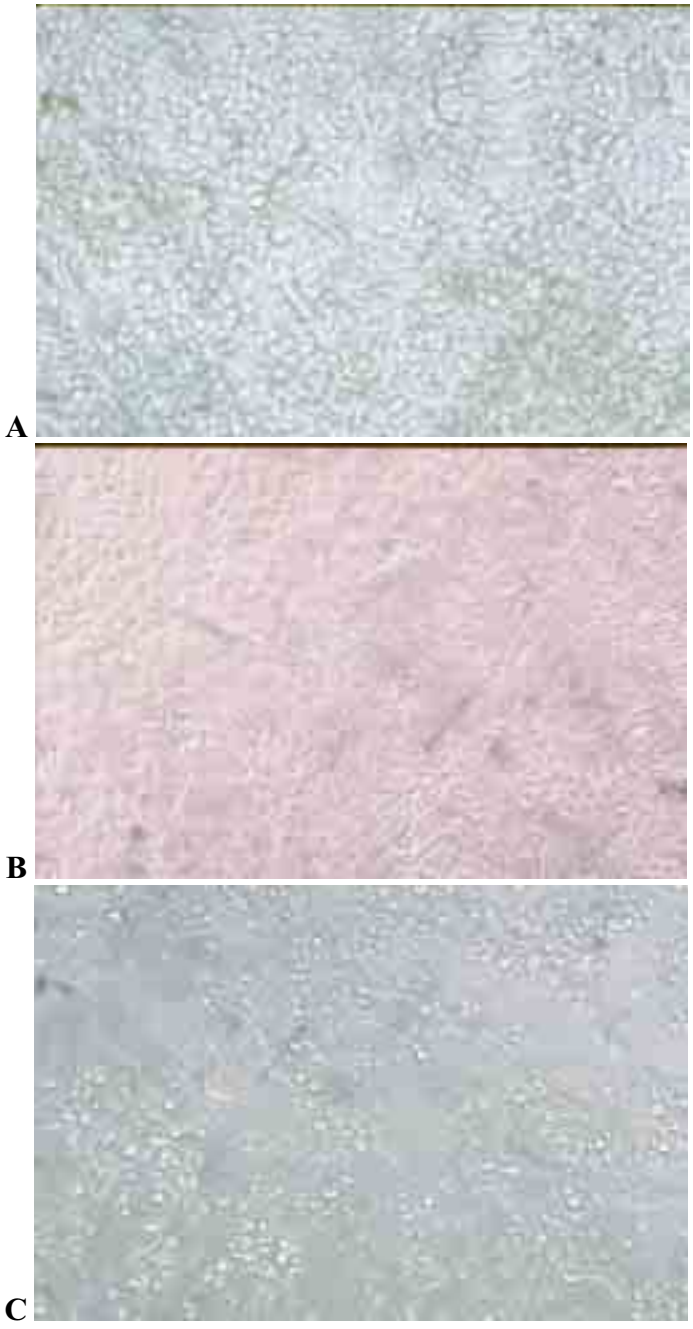


Figure 2.7: Phase contrast pictures of MTB-01 cells at 72 hrs a) untreated cells; b) cells treated with 10^{-4}M risedronate c) cells treated with 10^{-5}M risedronate. Adhering cells consistent with viable cells were markedly decreased in cells exposed to 10^{-4}M risedronate.

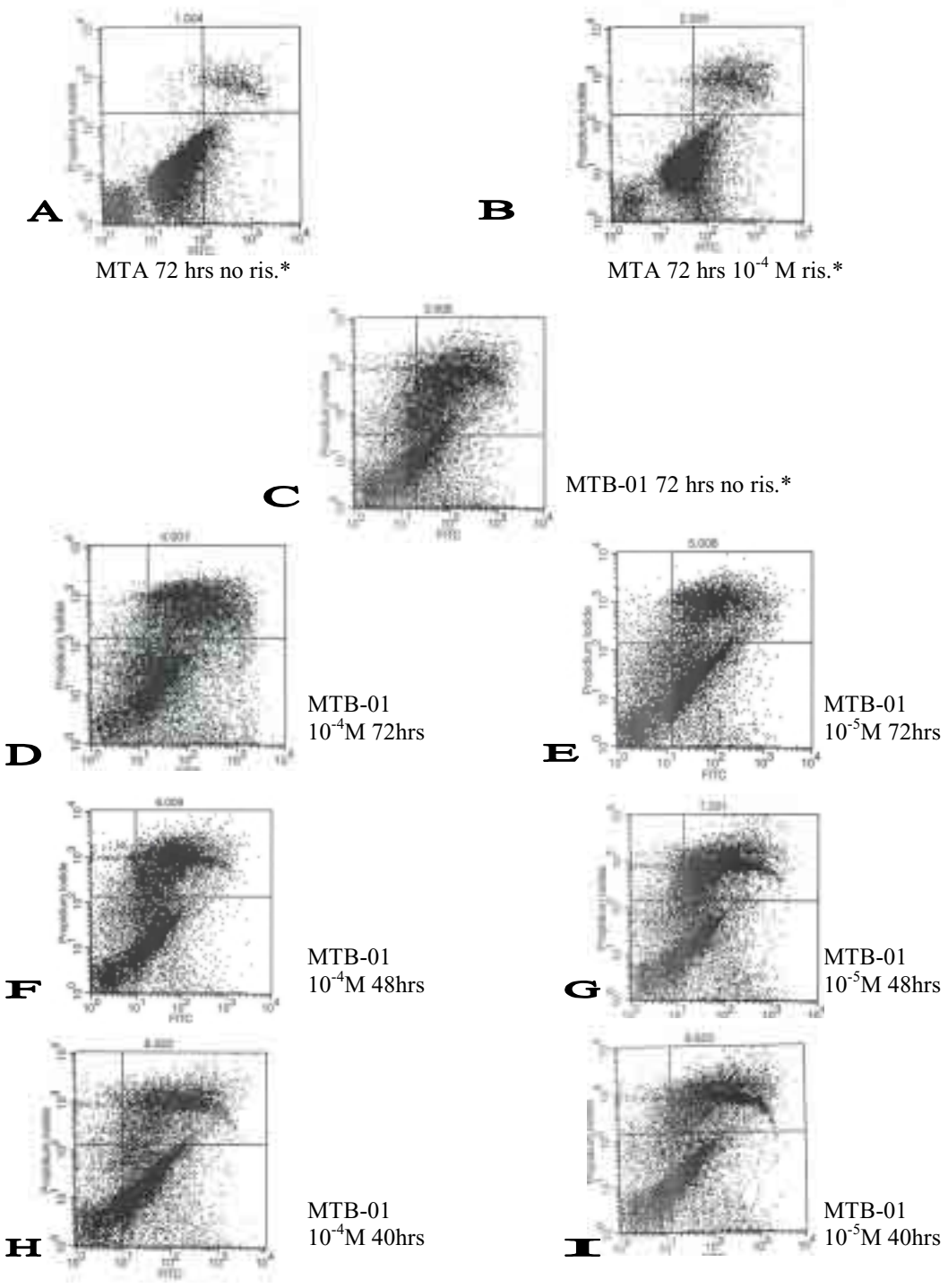


Figure 2.8: Annexin-V binding of the MTA and MTB-01 cell line analyzed by flow cytometry. In the MTA cell line, risedronate causes an increase in the number of cells in the lower right quadrant indicating apoptosis. In the MTB-01 cells, the highest percentage of apoptosis was observed at 40 hrs with 10^{-4}M risedronate. As time progresses note the shift of cells from the lower right to the upper right quadrant indicating a shift away from apoptosis toward necrosis. (*ris= risedronate)

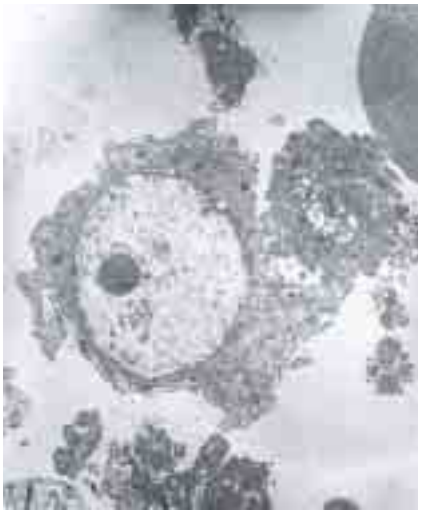
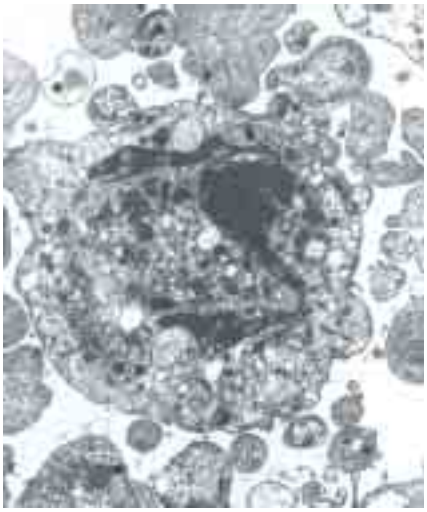
A  B 

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Figure 2.9: Transmission electron microscopy of MTB-01 cells after 72 hrs culture with 10^{-4}M risedronate. a) Viable cell next to condensed apopotic cell with membrane blebbing; b) Cellular and nuclear condensation consistent with apoptotic cell death; c) Cytoplasmic,organelle,and nuclear swelling and loss of membrane integrity characteristic of cell death by necrosis.

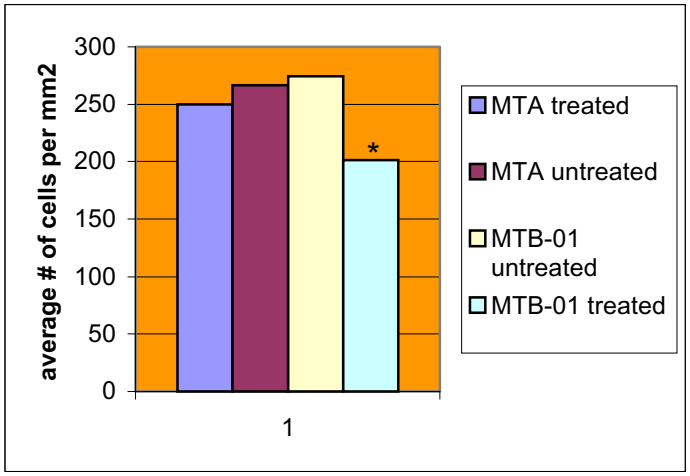

Figure 2.10: Bar graph illustrating the average number of MTA and MTB-01 adhering to risedronate treated bone. MTB-01 cells adhering to risedronate treated bone is significantly lower than MTB-01 cells adhering to untreated bone and MTA cells adhering to treated or untreated bone. ($p < 0.05$)

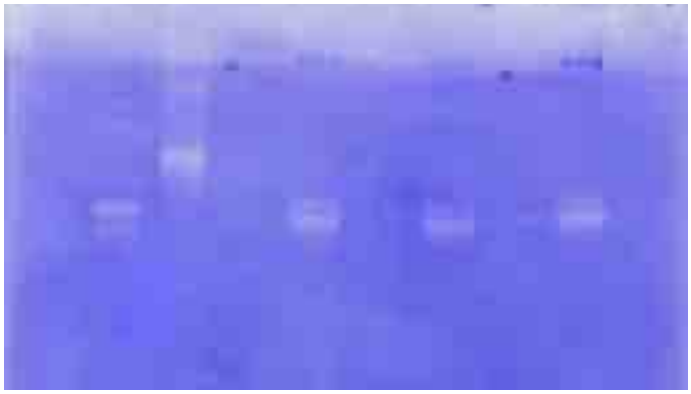

Figure 2.11: Zymography gel with MTA conditioned media samples; Clear bands in lanes 1 & 2 represent purified human MMP-2, 72kD, and MMP-9, 92 kD, respectively. Lane 4: conditioned media from cells cultured without risedronate present; Lane 6 conditioned media from cells exposed to 10^{-4}M risedronate; Lane 5 conditioned media from cells exposed to 10^{-5}M risedronate. Note clear bands at 72 kD and absence of bands at 92 kD in MTA samples.

Figure 2.12: Zymography gel with MTB-01 conditioned media samples; Clear bands in Lanes 1 and 2 represent purified human MMP-2, 72kD and MMP-9, 92kD, respectively. Lanes 3-12 were loaded with MTB-01 serum free cell conditioned media with or without risedronate. Note the absence of clear bands in all lanes indicating lack of MMP-2 or MMP-9 production.

CONCLUSION

These experiments have characterized the MTA rat mammary carcinoma cell line as a nonresponder to the third generation bisphosphonate, risedronate. Initially, this cell line was shown to reliably produce widespread metastasis following accurate injection into the left cardiac ventricle. Next, rats were inoculated with MTA cells and divided into treated and untreated groups. Treated animals received daily subcutaneous injections of risedronate and untreated animals received a daily saline placebo. At day 14 the animals were euthanized and bone metastatic lesions were compared by light microscopy. The size and number of lesions were found to be essentially equal in the two groups, thus establishing the MTA cell line as a nonresponder to risedronate.

Although human patients with bone metastatic cancers that are poorly responsive to bisphosphonate therapy have been identified clinically, until this point there has been little investigation into potential mechanisms by which this may occur. To our knowledge this is the first report of a rodent model of bone-metastatic breast cancer that has been characterized specifically as a nonresponder to bisphosphonate therapy.

The second part of this project characterized in vitro cellular effects of risedronate on the nonresponding MTA cell line and a related responding rat mammary carcinoma cell line MTB-01 in an effort to identify potential factors that make the MTA line a poor responder to risedronate therapy. Apoptosis detection systems, gelatin zymography, and adhesion assays were compared. We discovered that the nonresponding cell line possessed characteristics that were not observed in the responding cell line, such as

decreased susceptibility to risedronate-induced apoptosis, the ability to produce MMP-2 in the presence or absence of risedronate, and the ability to adhere to risedronate-treated bone matrix with the same affinity as to untreated bone matrix. These in vitro differences represent three possible mechanisms by which the MTA cell line is able to colonize and proliferate in the risedronate-treated bone microenvironment.

The identification of factors that could potentially account for poor responses of bone metastatic neoplasms to bisphosphonate therapy opens the door for the creation of new treatment modalities targeted to specific steps in the metastatic process and specific characteristics possessed by each neoplasm. Future work with this cell line could involve evaluating the effects of combining bisphosphonates with other drugs such as cytotoxic drugs, inhibitors of MMP's, or antibodies to adhesion molecules such as integrins. In addition, the effects of risedronate on other factors vital to the process of metastasis such as invasion and angiogenesis at the metastatic site could be investigated and could reveal additional factors that contribute to the poor response of the MTA cell line to risedronate.