Bisphosphonate and Taxane Effects on Osteoblast Proliferation and Differentiation

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ABSTRACT

Breast cancer is the second most commonly diagnosed cancer in women in the United States. Advanced stages of breast cancer frequently metastasize to bone, where it is difficult to diagnose and treat. Current cancer-in-bone therapeutics focus on bisphosphonates to inhibit osteolysis (bone dissolution by osteoclasts) and taxanes to impede cancer cell growth. Effects of these drugs on osteoblasts (bone forming cells) have not been well studied. The purpose of this study is to observe and quantify the effect of a bisphosphonate (zoledronic acid) and a taxane (docetaxel) on osteoblast proliferation and differentiation. Results of this research serve as preliminary data that will guide studies in an advanced three-dimensional bone tissue model.

INTRODUCTION

Breast Cancer in Bone

Breast cancer is among the most commonly diagnosed cancers in the United States. This year alone, approximately 182,460 women in the United States will develop invasive breast cancer, and 40,480 women are expected to die from the disease. The risk of a woman in the United States developing breast cancer in their lifetime is now 1 in 8[1]. Worldwide, breast cancer has reached epidemic proportions[2].

Metastatic breast cancer is the most advanced stage of breast cancer, and the most frequent site of breast cancer metastases is bone. Of the cases of breast cancer diagnosed each year, approximately 25% of those cancers metastasize, with the first site of metastasis located in bone in 46% of cases and in 70% of cases with first relapse. Once cancerous breast cells colonize bone, the cure rate is almost zero[3].

The normal bone environment is in a dynamic equilibrium – the bone undergoes constant remodeling in which osteoclasts resorb bone, whereas osteoblasts deposit bone. When breast

cancer metastasizes to bone, this balance is shifted to abnormally high osteoclastic resorption. Interactions between osteoclasts and breast-cancer cells establish a vicious cycle in which osteolysis and tumor activity both increase. Activation of osteoclasts results in pain, bone fracture, and hypercalcemia[4-6].

An In Vitro Model of Bone

Modeling the bone environment to study cancer colonization of bone is difficult because few cell culture methods simultaneously provide biological relevance and simplicity required for experimental control. The previous lack of a sophisticated model not only has hindered breast cancer metastasis research, but also has hindered the development of therapeutics for bone metastases. Dhurjati et al. recently developed a compartmentalized culture device, hereafter referred to as the bioreactor, that operates on the principle of continuous growth and dialysis[7]. The bioreactor consists of a cell growth chamber separated from a larger medium reservoir by a dialysis membrane. Waste from the growth compartment and nutrients from the medium reservoir are capable of passing through the dialysis membrane, while macromolecules synthesized by the cells as they develop are maintained in the growth chamber. The bioreactor design creates an extremely stable cellular environment that allows for the growth of three-dimensional tissue for an extended period of time. This cell-culture system allowed development of a three-dimensional multiple-cell layer of osteoblastic tissue over 10 months of continuous culture. Krishnan et al. showed that this three-dimensional tissue was a useful surrogate for studies of cancer in bone[8].

Cancer in Bone Therapy

This research extends the bioreactor model by introducing therapeutic drugs to the system. Two drug families are currently used in the treatment of bone metastases – bisphosphonates to regulate osteoclast activity and taxanes to attack cancer cells. The effects of these drugs on bisphosphonates have not been well studied.

Bisphosphonates are synthetic analogues of inorganic pyrophosphate, in which the oxygen atom connecting the two phosphates is replaced by a carbon atom. This stabilizes the molecule from biological degradation. Nitrogen-containing bisphosphonates have recently proven to be the most aggressive in targeting bone metastases. Bisphosphonates bind strongly to bone mineral, particularly in areas of increased bone activity. As bone resorption occurs, osteoclasts internalize bisphosphonates. Once internalized, the bisphosphonates inhibit an enzyme that contributes to osteoclast function and survival. Recent studies have suggested that bisphosphonates may also have antitumor effects. During bone resorption, growth factors are released that stimulate cancer cell activity; therefore decreased osteoclast activity would have negative effects on cancer cells as well. Bisphosphonates decrease cancer cell proliferation and induce apoptosis[9-13].

Taxanes are widely used chemotherapeutic agents. Taxanes are microtubule interfering agents that bind to β tubulin, causing abnormal assembly of microtubules and preventing disassembly. This arrests the cell cycle in the G_2M phase and induces apoptosis. Taxanes also cause programmed cell death by inducing phosphorylation of Bcl-2, an anti-apoptotic protein[14-15].

Recent work has suggested that the most effective therapy for metastatic breast cancer to bone is combinatorial therapy with a bisphosphonate and a taxane. Bisphosphonates have been shown to enhance the antitumor effects of taxanes *in vitro* on cancer cell invasion, adhesion, and apoptosis[16-17].

The purpose of this research was to assess the effects of a bisphosphonate (zoledronic acid) and taxane (docetaxel) on osteoblast proliferation and differentiation. The goal was to determine an appropriate concentration for use in the bioreactor model. Low concentrations (0.05-0.50 μM) of zoledronic acid enhanced both osteoblast proliferation and differentiation, while high concentrations of docetaxel (10.0 μM) had positive effects on osteoblast differentiation.

MATERIALS AND METHODS

Cells and Cell Culture

Osteoblast Proliferation

Murine calvaria pre-osteoblast (MC3T3-E1) cells were plated at $1x10^4$ cells/cm^2 in a 24-well plate and grown in alpha minimum essential medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin, hereafter referred to as growth medium. Cultures were incubated at 37°C. After 24 hours, zoledronic acid was added to the growth medium in 0.05, 0.5 and 5 μM concentrations. Medium was changed after 24 hours to allow for acute exposure (pulse dose) and chronic exposure (chronic dose).

Osteoblast Differentiation

MC3T3-E1 cells were plated at $1x10^4$ cells/cm^2 in a 24-well plate and grown in α-MEM supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, 10mM β-glycerophosphate and 50 μg/mL ascorbic acid, hereafter referred to as differentiation medium. Cells were allowed to differentiate for 17 days prior to the addition of zoledronic acid in 0.05, 0.5 and 5.0 μM concentrations or docetaxel in 0.1, 1.0 and 10.0 μM concentrations. After 24 hours, the supernatant was collected for cytokine assays and a media change was performed to allow for acute and chronic dosing. Medium was collected and replenished again after three days.

Zoledronic Acid and Docetaxel

A 5 μM stock solution of zoledronic acid was prepared in 0.1N sodium hydroxide. The stock solution was diluted with growth medium and differentiation medium for measures of cell proliferation and differentiation, respectively. A 10 μM stock solution of docetaxel was prepared in 100% ethanol and diluted with differentiation medium.

Assessment of Cell Proliferation

Four day osteoblasts grown in varying concentrations of zoledronic acid were rinsed twice with phosphate buffered saline (PBS) and detached with three rinses of 0.002% pronase in PBS. Upon detachment, the pronase was neutralized with growth medium. The cell suspension was diluted with 0.4% trypan blue dye and both viable and apoptotic cells were counted with a hemocytometer.

Differentiated osteoblasts grown in varying concentrations of zoledronic acid and docetaxel were also counted with a hemocytometer and trypan blue stain. Cells were initially rinsed twice with PBS and detached with three rinses of 0.002% pronase in PBS. The differentiated cells detached as an aggregate accumulated in the collagen matrix. In an attempt to further detach the osteoblasts from the collagen matrix, the pronase solution was neutralized with growth medium and then placed in a centrifuge. The solution was removed and replaced with accutase.

Assessment of Cell Differentiation – Alkaline Phosphatase

Differentiated osteoblasts were stained for alkaline phosphatase (ALP) activity. Cells were rinsed once with PBS and then fixed for 10 minutes with 10% formaldehyde. Cells were then rinsed three times for five minutes each with PBS. The cells were stained with an ALP stain consisting of pre-warmed dH_2O, 0.2M Tris, napthol and Fast Blue RR Salt and incubated at 37°C for 30 minutes. They were then rinsed three times for five minutes each with dH_2O and the dish was set to dry. The cell culture dish was then scanned into a computer. The stain intensity was quantified using ImageQuant software.

MCP-1 and IL-6 Expression

MCP-1 and IL-6 were quantified using a sandwich ELISA. Flat-bottom 96-well plates were coated with antibody at 0.4 μg/ml for MCP-1 and 2 μg/ml for IL-6. Plates were incubated overnight at 4°C. The plates were washed four times with PBS with 0.05% Tween 20 and blocked for 2 hours with PBS and 1% BSA. After three washes, samples and standards were added and incubated overnight at 4°C. The plates were then washed four times and incubated with detection antibody for 2 hours at room temperature. The plates were washed 6 times and incubated with NeutrAvidin horseradish peroxidase conjugate for 30 minutes at room temperature. The plates were washed 8 times and then incubated with ABTS peroxidase substrate at room temperature for 90 minutes. Plates were read at 405 nm in an ELISA reader.

RESULTS

Osteoblast Proliferation

Drug effects on osteoblast proliferation was assessed by means of cell counting and a trypan blue stain. Low concentration zoledronic acid (0.05 μM) enhanced osteoblast growth. As zoledronic acid concentration increased, cell growth declined. Results were the same for both acute and chronic exposures (Fig. 1).

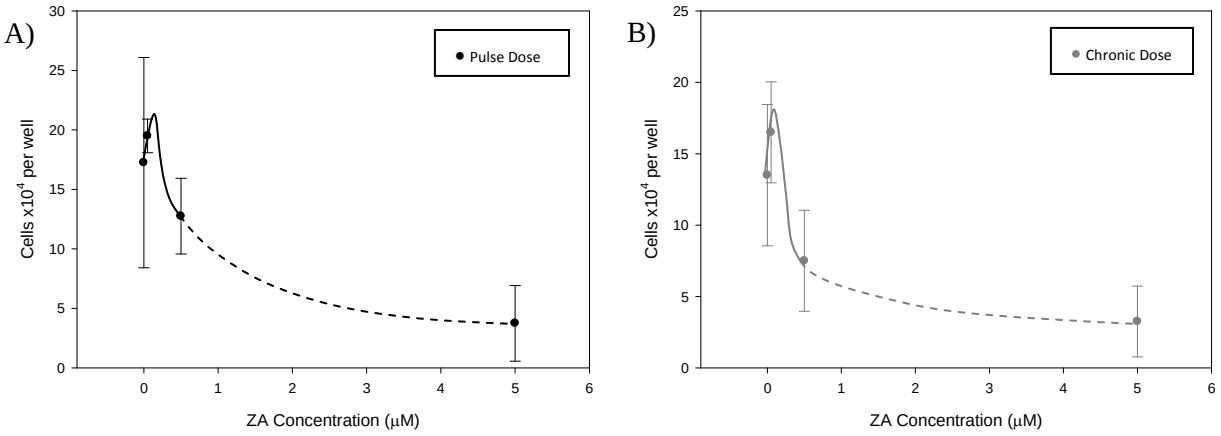

FIGURE 1. Effect of zoledronic acid (ZA) on osteoblast proliferation. Osteoblasts were plated at $1x10^4$ cells/cm^2 and ZA was added in concentrations of 0.00, 0.05, 0.50 and 5.00 μM. After 24 hours, medium was removed and replaced according to a dosing regimen. Wells designated for acute exposure (A) were replenished with osteoblast growth medium, while those for chronic exposure (B) received another drug dose. Proliferation was assessed with a trypan blue stain. Lines are guides to the eye.

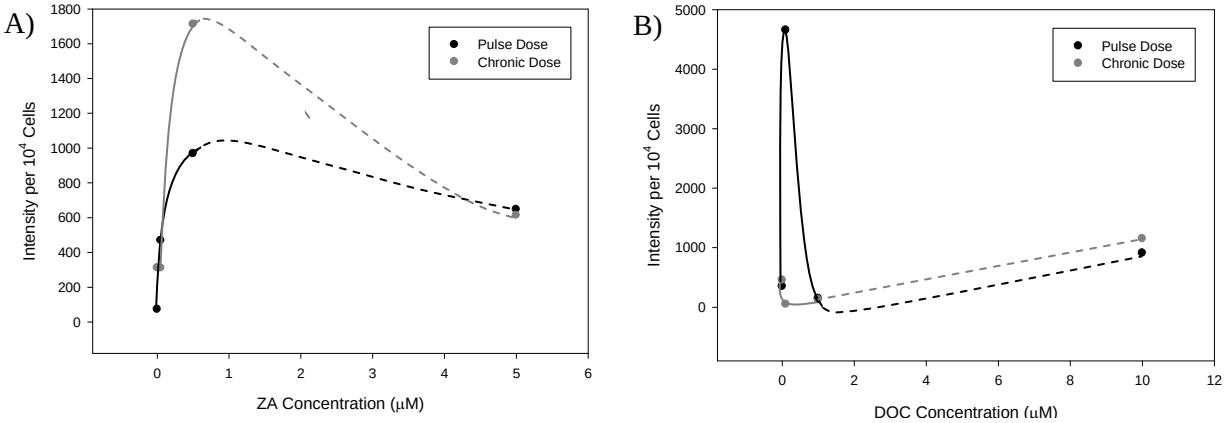

FIGURE 2. Drug effects on alkaline phosphatase production. Osteoblasts were plated at $1x10^4$ cells/cm^2 and grown in differentiation medium for two weeks. Cells were treated with either zoledronic acid (ZA) or docetaxel (DOC). ZA (A) or DOC (B) was added in low, medium, and high concentrations (ZA – 0.00, 0.05, 0.50 and 5.00 μM; DOC – 0.00, 0.10, 1.00 and 10.00 μM). After 24 hours, medium was removed and replaced according to a dosing regimen. Wells designated for acute exposure were replenished with osteoblast differentiation medium, while drug doses were added to wells for chronic exposure. Cells were stained for alkaline phosphatase. The intensity of the stain was quantified using ImageQuant software, and intensity was normalized to viable cell number (cell counts obtained using trypan blue stain). Lines are guides to the eye.

Osteoblast Differentiation

Drug effects on osteoblast differentiation were assessed by measuring alkaline phosphatase production. Alkaline phosphatase (ALP) production increased with low and medium (0.05 and 0.50 μM, respectively) zoledronic acid concentrations, while ALP production declined at the higher concentration. Chronic exposure to zoledronic acid caused a more dramatic rise in ALP than the acute exposure. Docetaxel treated cells showed different responses to pulse and chronic

dosing. Acute exposure to docetaxel resulted in a sharp increase in ALP production for low concentration docetaxel (0.1 μM), followed by a rapid decrease to the medium concentration (1.0 μM) and a slight increase with the higher concentration. Chronic exposure to docetaxel produced an immediate decline in ALP production followed by a slight increase with concentration (Fig. 2).

Osteoblast Inflammatory Response

Drugs effects on MCP-1 and IL-6 expression were analyzed to determine whether either drug elicited an inflammatory response from the osteoblasts. Acute exposure of zoledronic acid decreased expression of MCP-1 as a function of concentration. Chronic exposure, however, increased MCP-1 expression through low and medium concentrations of zoledronic acid (0.05 and 0.50 μM, respectively), followed by a decline in MCP-1 expression (Fig. 3). Most zoledronic acid treated cells produced IL-6 below the level of detection, therefore the data were inconclusive (Table 1).

Both pulse and chronic doses of docetaxel elicited similar osteoblast responses. Low concentration docetaxel (0.1 μM) increased MCP-1 expression, followed by a decline through medium and high concentrations of the drug (Fig. 3). IL-6 concentrations were below the level of detection in both dosing methods. After 24 hours exposure to docetaxel, IL-6 expression was detectable, with IL-6 concentrations decreasing with increasing docetaxel concentration (Table 2).

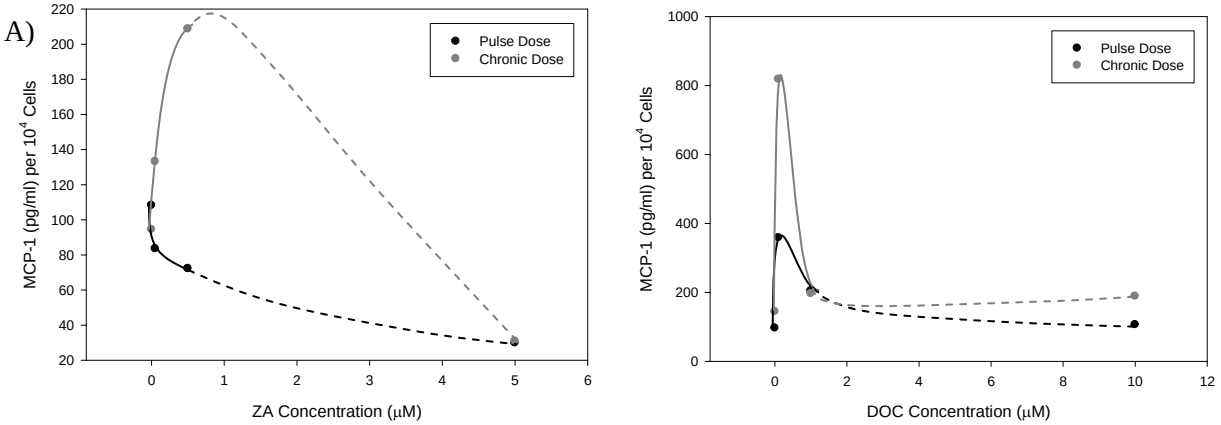

FIGURE 3. Drug effects on MCP-1 expression. Osteoblasts were plated at $1x10^4$ cells/cm^2 and grown in differentiation medium for two weeks. Cells were treated with either zoledronic acid (ZA) or docetaxel (DOC). ZA (A) or DOC (B) was added in low, medium, and high concentrations (ZA – 0.00, 0.05, 0.50 and 5.00 μM; DOC – 0.00, 0.10, 1.00 and 10.00 μM). After 24 hours, medium was removed and replaced according to a dosing regimen. Supernatant was collected after an additional 3 days of culture. Supernatant was assayed for MCP-1 using a sandwich ELISA. MCP-1 concentration was normalized to viable cell number (cell counts obtained using trypan blue stain). Lines are guides to the eye.

TABLE 1. Effects of zoledronic acid (ZA) on IL-6 expression.

	IL-6 Concentration (pg/ml)		
ZA Concentration	**24 Hours**	**Pulse Dose**	**Chronic Dose**
Control - 0	54.0	*	*
Low - 0.05	*	*	22.0
Medium - 0.5	*	*	*
High - 5.0	26.0	18.0	*

Osteoblasts were plated at $1x10^4$ cells/cm^2 and grown in differentiation medium for two weeks. Cells were treated with ZA in control, low, medium and high concentrations (0.00, 0.05, 0.50 and 5.00 μM, respectively). After 24 hours, supernatant was collected and replaced according to a dosing regimen. Supernatant was collected after an additional 3 days of culture. Supernatant was assayed for IL-6 using a sandwich ELISA.

TABLE 2. Effects of docetaxel (DOC) on IL-6 expression.

	IL-6 Concentration (pg/ml)		
DOC Concentration	**24 Hours**	**Pulse Dose**	**Chronic Dose**
Control - 0	139.0	*	*
Low - 0.1	47.0	*	*
Medium - 1.0	84.0	*	*
High - 10.0	91.0	*	*

Osteoblasts were plated at $1x10^4$ cells/cm^2 and grown in differentiation medium for two weeks. Cells were treated with DOC in control, low, medium and high concentrations (0.00, 0.10, 1.00 and 10.00 μM, respectively). After 24 hours, supernatant was collected and replaced according to a dosing regimen. Supernatant was collected after an additional 3 days of culture. Supernatant was assayed for IL-6 using a sandwich ELISA.

DISCUSSION AND FUTURE WORK

The bisphosphonate zoledronic acid and the taxane docetaxel have pronounced effects on osteoblast proliferation and differentiation. Low concentrations of zoledronic acid enhanced both osteoblast proliferation and differentiation. However, low concentrations also elicit the most pronounced inflammatory response. High concentrations of docetaxel incur the lowest inflammatory response and have the most positive effects on osteoblast differentiation. From these observations, zoledronic acid at 0.05 μM and docetaxel at 10 μM are candidates for use in the bioreactor model. Choice of ideal concentrations will depend on the effect on breast-cancer cells from future work. Future experiments include assessing the effects of these drugs on breast-cancer cells and evaluating combinatorial therapy in standard tissue culture.

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REFERENCES

1. American Cancer Society. *Breast Cancer Facts & Figures 2007-2008.* Atlanta: American Cancer Society, Inc.
2. *Nature Medicine*. Special Web Focus: Breast Cancer. 2001(4).
3. Landis SH, Murray T, Bolden S, Wingo PA. Cancer Statistics. CA Cancer J Clin 1999:49:8-31.

4. Rubens RD. Bone Metastases – The Clinical Problem. Eur J Cancer 1998:34:210-213.
5. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2002:2:584-593.
6. Coleman RE. Clinical Features of Metastatic Bone Disease and Risk of Skeletal Morbidity. Clin Cancer Res 2006:12(20):6243-6249.
7. Dhurjati R, Liu X, Gay CV, Mastro AM, Vogler EA. Extended-Term Culture of Bone Cells in a Compartmentalized Bioreactor. Tissue Engineering 2006:12:3045-3054.
8. Fleisch H. Development of bisphosphonates. Breast Cancer Res 2002:4(1):30-34.
9. Dhurjati R, Krishnan V, Shuman LA, Mastro AM, Vogler EA. Metastatic breast cancer cells colonize and degrade three-dimensional osteoblastic tissue in vitro. Clin Exp Metastasis 2008:
10. Coleman RE. Bisphosphonates in breast cancer. Annals of Oncology 2005:16:687-695.
11. Green JR. Bisphosphonates: Preclinical Review. The Oncologist 2004:9(4):3-13.
12. Coxon FP, Thompson K, Rogers MJ. Recent advances in understanding the mechanism of action of bisphosphonates. Current Opinion in Pharmacology 2006:6:307-312.
13. Russell RGG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 2008:19:733-759.
14. Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering Agents Stimulate the Transcription of Cyclooxygenase-2. The Journal of Biological Chemistry 2000:20:14838-14845.
15. Olsen SR. Taxanes and COX-2 inhibitors: from molecular pathways to clinical practice. Biomedicine and Pharmacology 2005:59:306-310.
16. Magnetto S, Boissier S, Delmas PD, Clezardin P. Additive antitumor activities of taxoids in combination with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. Int J Cancer 1999:83:263-269.
17. Inoue K, Karashima T, Fukata S, Nomura A, Kawada C, Kurabayashi A, Furihata M, Ohtsuki Y, Shuin T. Effect of Combination Therapy with a Novel Bisphosphonate, Minodronate (YM529), and Docetaxel on a Model of Bone Metastasis by Human Transitional Cell Carcinoma. Clin Cancer Res 2005:11(18):6669-6677.