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Preclinical evaluation of zoledronate using an in vitro mimetic cellular model for breast cancer metastatic bone disease

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ABSTRACT

Background: The interactions between metastatic breast cancer cells and host cells of osteoclastic lineage in bone microenvironment are essential for osteolysis. In vitro studies to evaluate pharmacological agents are mainly limited to their direct effects on cell lines. To mimic the communication between breast cancer cells and human osteoclasts, a simple and reproducible cellular model was established to evaluate the effects of zoledronate (zoledronic acid, ZOL), a bisphosphonate which exerts antiresorptive properties.
Methods: Human precursor osteoclasts were cultured on bone-like surfaces in the presence of stimuli (sRANKL, M-CSF) to ensure their activation. Furthermore, immature as well as activated osteoclasts were co-cultured with MDA-MB-231 breast cancer cells. TRAP5b and type I collagen N-terminal telopeptide (NTx) were used as markers. Osteoclasts' adhesion to bone surface and subsequent bone breakdown were evaluated by studying the expression of cell surface receptors and certain functional matrix macromolecules in the presence of ZOL.
Results: ZOL significantly suppresses the precursor osteoclast maturation, even when the activation stimuli (sRANKL and M-SCF) are present. Moreover, it significantly decreases bone osteolysis and activity of MMPs as well as precursor osteoclast maturation by breast cancer cells. Additionally, ZOL inhibits the osteolytic activity of mature osteoclasts and the expression of integrin β3, matrix metalloproteinases and cathepsin K, all implicated in adhesion and bone resorption.
Conclusions: ZOL exhibits a beneficial inhibitory effect by restricting activation of osteoclasts, bone particle decomposition and the MMP-related breast cancer osteolysis.
General significance: The proposed cellular model can be reliably used for enhancing preclinical evaluation of pharmacological agents in metastatic bone disease.

1. Introduction

Bone microenvironment provides a fertile ground for metastatic breast cancer cells to migrate, proliferate and finally colonize on bone surface, establishing skeletal metastases. The consequences are severe skeletal disorders due to stimulation of bone cells, which in turn lead to imbalanced bone remodeling that may be osteolytic, osteoblastic or a combination of both, causing mixed lesions [1]. The processing steps in the development of bone metastasis involve primary tumor cells' invasion by modification and breakdown of the normal surrounding extracellular matrix (ECM), favored by proteolytic enzymes such as matrix metalloproteinases (MMPs) [2]. Tumor cells traverse the walls of both small normal and tumor-induced blood vessels to enter circulation and travel to distant target organs including skeletal tissue, where they establish respective metastases [3–7]. Each of these consecutive steps involves important molecular interactions between the tumor cells and the host cells, and is considered a potential target for the development of drugs that are designed to abrogate the metastatic process [8].

The most crucial process in breast cancer metastasis to bone is the molecular communication between tumor and bone cells [9]. Interactions between tumor cells and osteoclasts in bone microenvironment cause, not only osteoclast activation and subsequent bone resorption, but also aggressive growth and behavior of the tumor cells.

Bisphosphonates (BPs), compounds based on a P–C–P spine similar to endogenous pyrophosphate, are able to inhibit osteoclast-induced

Abbreviations: BPs, bisphosphonates; ZOL, zoledronate; RANKL, receptor activator of nuclear factor-κB ligand; NTx, N-terminal telopeptide; CATK, cathepsin K; OPG, osteoprotegerin; PTHrP, parathyroid hormone-related peptide; M-CSF, macrophage colony-stimulating factor; ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane type-MMP; TIMP, tissue inhibitors of MMPs

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bone destruction. Zoledronic acid (ZOL), a third generation nitrogen-containing BP (N-BP) found to be the most effective, is approved by the FDA for breast cancer patients with osteolytic lesions to reduce the skeletal complications of malignancy. N-BPs inhibit farnesyl bisphosphate (FPP) synthase, a key enzyme in the mevalonate pathway [10–12]. The result is the loss of FPP and geranylgeranyl diphosphate (GGPP), which are required for the post-translational lipid modification (prenylation) of small GTPases such as Ras, Rho and Rac. Failure to activate small GTPases appears to disrupt downstream signaling pathways, resulting in apoptosis of osteoclasts and tumor cells via a caspase-dependent pathway [13]. Preclinical and preliminary clinical data suggest that ZOL alone exerts direct or indirect antitumoral effects on a variety of cancers including breast cancer or when used in combination with known neoadjuvant chemotherapy, common in clinical settings for breast cancer improves its therapeutic effects [14–16]. Recently, we have shown [17] that ZOL inhibits the functional properties of breast cancer cells, as a result of its ability to modulate the expression of key matrix molecules implicated in breast cancer progression [16]. In addition, ZOL prevents the invasion of malignant cells and cell adhesion of osteoclasts on bone tissue, thus reducing bone metastatic status [18]. Furthermore, osteoprotegerin (OPG) production of human osteoblasts is stimulated by ZOL [19].

The osteoclast is a tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursor cells at or near the bone surface. It is well established that the two hematopoietic factors necessary and sufficient for osteoclastogenesis, are the TNF-related cytokine RANKL and the macrophage colony-stimulating factor (M-CSF) [20–23]. Together, M-CSF and RANKL are required to induce expression of genes that typify the osteoclast lineage, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), β_3-integrin and MMPs, functional macromolecules of mature osteoclasts. TRAP5b is specifically released by active mature osteoclasts and activated macrophages [24]. In blood serum samples, TRAP can be found in two isoforms alternatively glycosylated, known as TRAP5a and 5b. TRAP5b is of osteoclast origin and 5a of active macrophage origin [25]. An important step in bone resorption is the adhesion of activated osteoclasts on bone surface, a procedure mainly mediated by integrins such as $\alpha v\beta 3$ and $\beta 3$ [26,27]. Collagen type I N-terminal peptides (NTx) are secreted during bone lysis as stable products of catabolism and are considered to be among the most trusted osteolysis markers, due to their unique amino acid sequence and the specific a2 (I) N-telopeptide orientation.

Therefore, NTx is one of the byproducts of proteolytic enzyme catabolic activity, produced during bone collagen breakdown by active osteoclasts. The most accurate quantitative detection method of NTx is, to date, competitive ELISA, approved by the FDA, since it uses target-specific monoclonic antibodies against NTx. The tumor-induced activation of osteoclasts results in secretion of well-known proteolytic enzymes that may drive bone resorption e.g. MMP-1, -2, -9, MT1-MMP and CATK. A lysosomal cysteine protease, CATK, degrades the triple helix of collagen I at multiple sites and thus increases its susceptibility to collagenolytic enzymes such as MMP-1 [28]. Furthermore, membrane type 1-MMP (MT1-MMP or MMP-14) expressed in tumor cells activates proMMP-2 produced by stromal cells in the primary focus and at the metastatic site [29]. MMP-9 and MT1-MMP seem to play an important role in the process of osteoclasts' migration toward bone breakdown sites [30,31]. Moreover, MMP-9 was found to localize to bone resorbing osteoclasts in human breast to bone metastases [32].

Several studies, in *in vitro* and murine models, have demonstrated that breast cancer cells influence osteoclastic maturation and activity either directly or by stimulating osteoblast-derived osteoclastogenic factors [33,34]. In a murine osteoblast–spleen cell co-culture system, MDA-MB-231, MDA-MB-435 and MCF-7 cells were found to induce osteoclast formation by promoting host IL-11 production and down-regulating M-CSF [35]. In addition, osteoclast precursors are sensitive to MDA-MB-231-released factors, leading to increased expression of key osteoclastogenic transcription factor NFATc1 after a short treatment with RANKL, and final mature osteoclast formation with subsequent release of CATK and MMP-9 [36]. Lau et al. [37] also showed that osteoclast formation and lacunar resorption took place, by a RANKL-independent mechanism, when the conditioned medium from MDA-MB-231 and MCF-7 cells was added (with M-CSF) to monocyte cultures.

It was, therefore, motivating to evaluate the effect of zoledronate on the function and expression of important ECM macromolecules derived from osteoclasts cultured on bone-like surfaces to facilitate a mimetic local bone microenvironment. More specifically, the present study involves the impact of ZOL on three subsequent levels: 1) on the maturation/activation of precursor (immature) osteoclasts, cultured in the presence of bone microenvironment growth factors, 2) on the maturation/activation of immature osteoclasts co-cultured with the highly invasive breast cancer cells MDA-MB-231, in the presence or absence of growth factors, and 3) on the activity of mature osteoclasts on bone destruction and the expression of certain matrix effective macromolecules.

2. Materials and methods

2.1. Chemicals and reagents

FBS (Fetal Bovine Serum), EMEM, sodium pyruvate, sodium bicarbonate, L-glutamine, nonessential amino acids, penicillin, streptomycin, amphotericin B and gentamycin were all obtained from Biochrom (Berlin, Germany). Osteoclast Precursor Basal Medium (OPBM) and OsteoAssay ™ Human Bone Plate were obtained from Lonza (Walkersville, USA). Bovine insulin and p-aminophenylmercuric acetate (APMA) were obtained from Sigma (Steinhelm, Germany). MMP-2 and MMP-9 were obtained from Chemicon (Harrow, UK). Zoledronate was supplied by Novartis Pharmaceuticals (Basel, Switzerland). All other chemicals used were of the best commercially available grade.

2.2. Cell cultures

MDA-MB-231 [ATCC HTB 26; human breast adenocarcinoma of high metastatic potential, estrogen receptor α (ERα)-negative] cell line was obtained from the American Tissue Culture Collection (Rockville, MD). MDA-MB-231 cells were cultured in EMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 0.01 mg/mL insulin and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamicin sulfate and 2.5 μg/mL amphotericin B). Cells were routinely grown at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. Culture medium was changed every 48–72 h and the cultures were not left to become confluent. Cells were harvested by trypsinization with 0.05% (w/v) trypsin in PBS containing 0.02% (w/v) Na_2EDTA.

Poietics™ Osteoclast Precursor Cell System was obtained from Lonza (Walkersville, USA). Precursor osteoclasts were cultured in OPBM supplemented with 10% (v/v) FBS in the presence of 2 mM L-glutamine. It is important to mention that pre- and mature osteoclasts are not able to be re-cultured. In order to achieve differentiation to mature and active osteoclasts, M-CSF and soluble RANKL were added in final concentrations of 33 ng/mL and 66 ng/mL, respectively for 7 days.

2.3. Pre-osteoclasts' culture and co-culture with MDA-MB-231 cells on bone substrate

OsteoAssay™ Human Bone Plate remained at room temperature for 1 h. This plate provides a thin layer of adherent human bone particles for the culture of primary human or non-human osteoclasts [38], osteoclast precursors and osteoclast cell lines. Osteoclast precursors (1×10^4 cells) were placed in a 48-well plate and were cultured,