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Expression of matrix macromolecules and functional properties of breast cancer cells are modulated by the bisphosphonate zoledronic acid

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ABSTRACT

Background: The extracellular matrix (ECM) components play key roles in the multistep process of cancer growth and progression. Preclinical and clinical data show that bisphosphonates (BPs) may exert direct or indirect antitumoral effects. Despite proven efficiency in cancer treatment, the mechanism by which BPs can interfere with cancer progression remains elusive.
Methods: We investigated the effects of the third generation BP, zoledronate (zoledronic acid, Zometa®), in the expression of ECM macromolecules as well as the functional properties (proliferation, adhesion, migration and invasion) in two breast cancer cell lines (MDA-MB-231 and MCF-7) with different metastatic potentials.
Results: The data highlight that zoledronate effectively inhibits growth of breast cancer cells, functional invasion migration and adhesion to various matrices. At the level of ECM interacting molecules, the expression of specific heparan sulfate proteoglycans implicated in cancer progression, such as syndecan-1, -2 and glypican-1 is downregulated, whereas syndecan-4 expression is upregulated upon treatment with zoledronate. The levels of integrins $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ were significantly reduced following treatment with zoledronate which is in accordance with the reduced cell adhesion on various ECM matrices. The expression of hyaluronan and its receptor CD44 was also significantly suppressed. Moreover, ZOL suppressed the expression of metalloproteinases MMP-2, -9, the membrane type MT1- and MT2-MMP, whereas it increased the expression of their endogenous tissue inhibitors.
Conclusions and general significance: The obtained results demonstrate that zoledronate is a critical modulator of ECM gene expression and powerful anticancer agent inhibiting growth, migration and the matrix-associated invasion of breast cancer cells.

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1. Introduction

Breast cancer is the most common malignancy and the second leading cause of death observed in women [1,2]. Solid tumors, such as breast cancer, have a great avidity for colonizing in the bone and in fact more than 80% of patients with advanced disease exhibit such metastasis. Therefore, inhibition of tumor growth as well as invasiveness and metastatic potential of breast cancer cells is of critical importance for cancer treatment [3].

The underlying premise during cancer growth and progression is the highly coordinated interplay between cancer cells and their surrounding extracellular matrix microenvironment. Cancer cell proliferation, migration, invasion, metastasis, and angiogenesis are dependent on unique ECM properties, involving alterations in ECM components' structure and/or expression as well as their activity modifications that take place during this process [4,5]. An equally important role in cancer pathogenesis has the ECM–interacting cell surface molecules including proteoglycans (PGs). Syndecans and glypicans represent two major subfamilies of heparan sulfate PGs, the type I transmembrane and glycosyl–phosphatidylinositol anchored to cell membrane, respectively. They are mediators of cell–cell, cell–ECM interactions and interact with various factors regulating cell signaling activity and subsequently various cellular properties like proliferation, adhesion and motility [6]. Such diverse cellular functions important in cancer progression are accomplished among other through CD44 signaling; a transmembrane receptor that interacts with stromal ECM hyaluronan (HA) [7–9]. It is postulated that HA

Abbreviations: BPs, bisphosphonates; ZOL, zoledronate; ECM, extracellular matrix; HA, hyaluronan; MMP, matrix metalloproteinase; PG, proteoglycan; MT-MMP, membrane-type MMP; TIMPs, tissue inhibitors of MMPs; COL I, collagen I; COL II, collagen II; COL-IV, collagen IV; FN, fibronectin; LN, laminin; TN, tenascin; VN, vitronectin; PKCa, protein kinase C-alpha; FAK, focal adhesion kinase

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through its binding to CD44 not only protects cancer cells against the immune system, but also facilitates their adherence to endothelial cells of bone marrow [10,11]. Integrins constitute a family of cell surface glycoproteins that are heterodimers composed of non-covalently associated alpha and beta subunits. They exert the adhesive properties of cancer cells by cross-linking the molecules of ECM and growth factor receptors with the cytoskeleton [12]. Apart from their signaling activity, integrins contribute to the interplay of cancer cells with matrix molecules and host tissue cells [13,14]. Last but not the least, the zinc-dependent endopeptidases, matrix metalloproteinases (MMPs), play a crucial role in tumor growth and the multistep processes of invasion and metastasis, as they contribute to the proteolytic degradation of ECM, thereby altering cell–cell and cell–ECM interactions, migration and angiogenesis [5]. Their activity is regulated at various levels by their endogenous tissue inhibitors of metalloproteinases (TIMPs). It has been suggested that the balance between MMPs and TIMPs is an important factor in the proteolysis of matrix within the tumor microenvironment and that disruption of this balance may alter cancer phenotype to a more aggressive one [15].

Bisphosphonates are established agents widely clinically used for the prevention of the complications (SREs) associated with excessive osteoclast-mediated bone resorption, such as hypercalcaemia, Paget's disease, multiple myeloma, as well as bone metastasis [16,17]. Structurally, they resemble endogenous pyrophosphate, where the oxygen is replaced by carbon atom (P–C–P). The nitrogen-containing bisphosphonates, such as zoledronate, are the newest generation of bisphosphonates and are suggested to have the most potent antiresorptive effects tested [18,19]. Mounting evidence elucidates the highly antitumor activity of zoledronate on the growth and dissemination against a variety of cancer cell lines including breast cancer [20]. Preclinical and preliminary clinical data suggest that BPs exert direct or indirect antitumoral effects on growth factor release by cancer cells, cell adhesion, invasion and angiogenesis [21]. There is also ample evidence to suggest a possible direct antitumor effect of zoledronate when used in combination with known neoadjuvant chemotherapy, common in clinical settings for breast cancer [22,23].

Zoledronate is a pharmaceutical agent discovered 15 years ago and used in the treatment of cancer. Despite proven efficiency in cancer treatment, the mechanism by which this pharmacological agent can affect ECM molecules implicated in breast cancer growth and progression remains elusive. In respect with the above emerging data on the antitumor role of zoledronate and the importance of specific ECM macromolecules in cancer progression, we evaluated the direct effect of zoledronic acid on the proliferation and functional cell properties (migration, adhesion, invasion) of two breast cancer cell lines with different invasive potentials as well as its putative role in the regulation of the expression of ECM macromolecules implicated in breast cancer progression.

2. Materials and methods

2.1. Chemicals, biochemicals and reagents

Zoledronate active substance (zoledronic acid) was supplied by Novartis Pharma AG. Stock solutions of zoledronate were prepared by dissolving zoledronate in DMSO. Eagle's minimal essential medium (EMEM), fetal bovine serum (FBS), sodium pyruvate, sodium bicarbonate, L-glutamine, nonessential amino acids, penicillin, streptomycin, amphotericin B and gentamycin were all obtained from Biochrom KG (Berlin, Germany). Insulin was obtained from Sigma Chemicals (Steinhelm, Germany). All other chemicals used were of the best commercially available grade.

2.2. Cell lines and cell culture conditions

MCF-7 (low metastatic), MDA-MB-231 (highly invasive) mammary breast cell lines were obtained from the American Type Culture Collection (ATCC) and cultured as monolayers at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 and 95% air. All cancer cells were cultured in EMEM supplemented with 10% 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 of insulin and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 mg/mL streptomycin, 10 mg/mL gentamicin sulfate and 2.5 mg/mL amphotericin B). To ensure cancer cell viability, the effect of zoledronate was studied in the presence of 5% serum. Primary cultures of human breast fibroblasts were developed from surgically removed cancer and adjacent normal breast tissue as described previously [24]. Human breast fibroblasts were isolated from normal tissues after an overnight treatment with collagenase. Then the cells were routinely cultured in DMEM supplemented with 10% FBS.

2.3. Cell proliferation

In order to evaluate the effects of the zoledronate on breast cancer cell proliferation, cells were seeded in the presence of serum into 48-well plates at a density of 15,000 cells/well. Twenty-four hours after plating, new medium supplemented with zoledronate was added. After 48 h or 96 h incubation, WST-1 (water-soluble tetrazolium salt) was added at a ratio 1:10. The assay is based on the reduction of WST-1 by viable cells, producing a soluble formazan salt absorbing at 450 nm (reference wavelength at 650 nm).

2.4. RNA isolation and RT-PCR

Breast cancer cells were first grown in serum-containing media up to a 60–65% confluence and then zoledronate in 5% FBS culture medium was added for 96 h. Total cellular RNA was isolated after cell lysis using NucleoSpin RNA II Kit (Macherey-Nagel, Germany). The amount of isolated RNA was quantified by measuring its absorbance at 260 nm. All total RNA preparations were free of DNA contamination as assessed by RT-PCR analysis.

Total RNA was reverse transcribed using the PrimeScript 1st strand cDNA synthesis kit (TAKARA) and DyNAzyme II DNA Polymerase kit (Finnzymes). Semi-quantitative analysis of cDNA sequences was carried out based on simultaneous amplification of a "house-keeping gene", glyceraldehyde-3-phosphate dehydrogonase (GAPDH). All amplification products were separated by electrophoresis in a 2% agarose gel, containing Gel Star® stain (BioWhittaker, Rockland, ME, USA). Bands were visualized on a UV lamp and gels were photographed with a CCD camera. The sequences of primers as well as their prime characteristics for the genes of interest are provided in Table 1. For semi-quantitative analysis, gene expression was determined as relative fluorescence obtained for each molecule as compared to the reference gene (GAPDH). Image analysis was performed using the program UNIDocMv version 99.03 for Windows (UVI Tech, Cambridge, UK).

2.5. Expression of ECM molecules by Western blotting

MCF-7 and MDA-MB-231 cells used for this experiment were harvested at the same time intervals used for harvesting RNA. Cells were washed (2 times) with cold PBS and lysed with lysis buffer RIPA (50 mM Tris–HCl pH 7.3, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Cell lysates were separated using a 10% SDS-PAGE and transferred overnight to polyvinylidene difluoride membranes (Macherey-Nagel, Germany). Membranes were blocked and incubated for 16–20 h at 4 °C with primary antibody recognizing the certain ECM molecule each time. The immune complexes were detected after incubation with the appropriate peroxidase-conjugated secondary antibody with the SuperSignalWest Pico Chemiluminescent substrate (Pierce). Protein expression of each molecule was expressed as relative intensity, normalized to α-tubulin. The antibodies used in the current study were sc-7099 (polyclonal goat anti-Syndecan-1), sc-12766 (monoclonal mouse anti-Syndecan-4) from Santa Cruz (Santa Cruz Biotechnology Inc., USA), M5808 (rabbit anti MMP-14), T9026 (monoclonal

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