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Research Article

Zoledronic acid and atorvastatin inhibit αvβ3-mediated adhesion of breast cancer cells

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ABSTRACT

Bone metastases represent common long term complications of patients with breast cancer. Zoledronic acid, an amino-bisphosphonate and mevalonate pathway inhibitor, is an established agent for the treatment of bone metastases. Direct antitumor effects of zoledronic acid have been proposed in breast cancer. Statins are another group of mevalonate pathway inhibitors that have been repeatedly discussed for potential anti-tumor activity. In this study, we tested the hypothesis, whether these agents regulate adhesion of breast cancer cells to extracellular matrix components. Treatment of breast cancer cells with zoledronic acid and atorvastatin, significantly impaired MDA-MB-231 breast cancer cell adhesion on the αvβ3 ligands gelatin and vitronectin, but had no effect on collagen type 1 (α2β1-ligand) and fibronectin (α5β1-ligand). Anti-adhesive effects of zoledronic acid were fully reversed by geranylgeranyl pyrophosphate (GGPP), but not by farnesylpyrophosphate (FPP). Furthermore, effects of zoledronic acid and atorvastatin were mimicked by a specific inhibitor of geranylgeranylation GGTI-298. Functional (using integrin array) and quantitative (using FACS) integrin analyses on MDA-231 cells following zoledronic acid exposure revealed decreased levels of αv and αvβ3 expression. In addition to its effects on integrin mediated adhesion of breast cancer cells, the presence of zoledronic acid caused pronounced morphological changes in MDA-231 cells as seen by F-actin and vinculin rearrangement. Furthermore, phosphorylation of the focal adhesion kinase was inhibited by zoledronic acid. In both cases, changes were fully reversed by GGPP. These results emphasize the role of mevalonate pathway mediated impairment of geranylgeranylation in the anti-adhesive effects of zoledronic acid in breast cancer cells.

1. Introduction

Breast cancer is the most prevalent malignancy in women, and the occurrence of bone metastases is a common long-term complication of this disease [1]. The pathophysiology of bone metastases is complex and a number of steps have to be surpassed by the cancer cell to successfully establish a metastatic lesion, including the adhesion of cancer cells at their metastatic site [2]. Bisphosphonates represent a standard therapy for patients with malignant bone lesions [3]. In addition to their established anti-resorptive effects, direct effects of amino-bisphosphonates on tumor biology have been proposed [4]. Established *in vitro* and *in vivo* effects of bisphosphonates on cancer cells include an induction of apoptosis, inhibition of proliferation, migration and invasion as well as anti-angiogenic effects [4–6]. In contrast to first generation bisphosphonates, which act by forming toxic ATP analogs, amino-bisphosphonates are inhibitors of the mevalonate pathway that block the farnesyl pyrophosphate (FPP) synthase [7]. FPP synthase inhibition leads to a decreased formation of isoprenoid lipids such as FPP and geranylgeranyl pyrophosphate (GGPP) and thereby impairs posttranslational protein prenylation [8]. In fact, many of the observed anti-tumor effects of bisphosphonates have been proposed to be mediated by inhibited protein geranylgeranylation [9]. Statins are the second major class of clinically approved compounds that act by mevalonate pathway inhibition [10]. Statins are widely used for their cholesterol-lowering

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effects, but have also been associated with potential direct anticancer effects [11].

Cancer cell adhesion is an important step of the metastatic cascade that is essential for the cancer to establish persistence at the site of metastasis. Mevalonate pathway inhibition may impair the adhesive abilities of circulating cancer cells and thereby impact their metastatic potential. Indeed, anti-adhesive effects of bisphosphonates have been described for several cell types, including breast cancer and HUVEC [12,13], but the underlying molecular mechanisms have not been studied in detail. In this study we investigate the effects of zoledronic acid on integrin mediated adhesion of breast cancer cells in the context of mevalonate pathway inhibition.

2. Materials and methods

2.1. Cells and reagents

Human MDA-MB-231 breast cancer cells were purchased from ATCC (Manassas, VA). MDA-BONE cells (also known as MB-231-TxSA) were obtained from the University of Texas (San Antonio, USA). All cell lines were cultured in DMEM/Ham's F-12 (PAA, Pasching, Austria) with 10% fetal calf serum supreme (Lonza, Cologne, Germany) and 1% penicillin/streptomycin (PAA, Pasching, Austria). Cell line authenticity was determined by short tandem repeat profiling and by matching with the known profiles at DSMZ (German Collection of Microorganisms and Cell Cultures). Zoledronic acid, atorvastatin, mevalonate, geranylgeranyl-pyrophosphate (GGPP), farnesyl pyrophosphate (FPP), GGTI-298 and FTI-277 were obtained from Sigma-Aldrich (Munich, Germany). Zoledronic acid was solved in PBS. Mevalonate, FPP and GGPP were solved in methanol:NH_4OH solution and atorvastatin, GGTI-298 and FTI-277 were solved in DMSO. Appropriate controls were added to untreated control cells. Breast cancer cell lines were treated with zoledronic acid, atorvastatin, FTI-277 and GGTI-298 for 24 h (unless otherwise indicated). Mevalonate substrates (GGPP, FPP and mevalonate) were supplemented 2 h prior to zoledronic acid treatment to reverse specific pathway inhibition.

2.2. Western blot

Western blot analyses were performed as previously described [14]. Briefly, cells were washed and scraped in a lysis buffer and quantified. Twenty μg of protein were loaded on a SDS–PAGE and transferred onto a 0.2 μm nitrocellulose membrane. Following blocking for 1 h with 5% non-fat dry milk in Tris-buffered saline with 1% tween-20 (TBS-T), membranes were incubated with the primary antibody overnight. After washing, the membrane was incubated for 1 h with the HRP-conjugated secondary antibody. Membranes were washed 3 times with TBS-T again, and proteins were visualized with Super Signal (Pierce, Bonn, Germany) enhanced chemiluminescence. Antibody for RAP1A (sc-1482) was from Santa Cruz (Heidelberg, Germany) and the RAS (610001) antibody was from BD Biosciences (Heidelberg, Germany). Antibodies for FAK and phosphoFAK (Tyr397) were from Cell Signaling Technology (Boston, USA).

2.3. Adhesion assay

The adhesion assay was performed using 96-well microplates coated with vitronectin (R&D Systems, Wiesbaden, Germany), gelatin, collagen or fibronectin (all from BD, Heidelberg, Germany). The microplates were rehydrated with 200 μl PBS/well for 30 min at room temperature prior to use and the PBS removed before adding the cells. MDA-231 breast cancer cells were treated with different inhibitors of the mevalonate pathway for 24 h as indicated. Cells were then stained with the fluorescent dye $DilC_{12}(3)$ (from BD Biosciences, Heidelberg, Germany) for 1 h and washed twice with PBS. $DilC_{12}(3)$ is a fluorescent tracer specifically designed to label viable cells for tumor cell invasion or migration assays. After carefully harvesting the cells with 0.0015 M EDTA/PBS, they were washed again with PBS, counted and reconstituted in DMEM. Cells (125,000/well) were then given on 96-well microplates coated with different surfaces and incubated at 37 °C for 1 h in a CO_2 incubator allowing them to adhere. Afterwards the plates were washed gently 3 times with PBS to remove non adherent cells and 100 μl of DMEM added into the wells. The adherent cells were then quantified by their relative fluorescence signal with Fluostar Omega plate reader at 544/590 nm.

2.4. Integrin array

We used the Alpha/Beta (α/β) Integrin-Mediated Cell Adhesion Array Combo Kit (Chemicon/Merck Millipore, Schwalbach, Germany) to examine the adhesion of MDA-231 breast cancer cells in relation to their cell surface integrin subunit expression. This assay contains microplates coated with monoclonal antibodies against the human integrins α1–α5, αv, αvβ3, β1–β4, β6, αvβ5 and α5β1. The attachment to the different integrin subunits is used as an indirect indicator to assess functioning integrin cell surface expression. MDA-231 breast cancer cells were treated as indicated and harvested with 0.0015 M EDTA/PBS, counted and diluted separately to the final concentration of 1×10^6 cells/ml. Thereafter, 100 μl of the cell suspension was added to each of the anti-integrin coated wells and the control wells and incubated for 1 h at 37 °C. After incubation the wells were washed gently twice with assay buffer. Next, 150 μl of assay buffer and 50 μl of Lysis Buffer/Dye solution provided in the kit were adjoined to each well and incubated for 15 min at room temperature. Finally, 150 μl of the mixture were transferred to a 96-well plate and absorbance was determined with a Fluostar Omega plate reader at 485/530 nm.

2.5. Immunofluorescence

Immunofluorescence (IF) was performed as previously described [15]. Briefly, MDA-231 cells were disseminated on glass slides and treated with zoledronic acid, atorvastatin, GGTI-298, FTI-277 and GGPP as indicated. After washing the cells with PBS, they were fixed for 15 min with 4% paraformaldehyde/PBS and permeabilized after a triple washing step with PBS for 20 min using 0.1% Triton X-100/PBS. Afterwards they were washed again with PBS. Cells were then blocked with 1% BSA, 0.05% Tween/PBS for 1 h. After a triple washing step with PBS, 200 μl of 10 μg/ml Alexa-Fluor-488-Phalloidin (from Cell Signaling Technology, Boston, USA) and 2.5 μg/ml Vinculin (from Sigma-Aldrich, Munich, Germany) in 1% BSA, 0.05% Tween/PBS were added per well and incubated for 1 h at room temperature. Afterwards the cells were washed again three times with PBS and incubated for 1 h at room temperature with Alexa-Fluor-594 antibody (from Life Technologies, Darmstadt, Germany). Following a brief washing step with PBS, the cells were stained with 0.2 μg/ml DAPI for 5 min and then washed several times. The fluorescence-preserving mounting medium Dako was added to the slides to assess the cells thereafter using digital microscopy.

2.6. Flow cytometry

MDA-231 breast cancer cells were treated with zoledronic acid for 24 h, harvested with 0.0015 M EDTA/PBS and resuspended in FACS buffer (1% BSA/PBS). Cells were stained with an αvβ3-FITC antibody or with an αv (CD51) antibody (both from Chemicon/