Research Article

Stathmin is involved in the cooperative effect of Zoledronic acid and gefitinib on bone homing breast cancer cells in vitro

Miki Oda\(^a,\)\(^b\), Keiichi Iwaya\(^b,\)\(^*\), Ryoko Kikuchi\(^b\), Takayuki Kobayashi\(^b\), Toshiyuki Yoneda\(^d\), Kahoko Nishikawa\(^c\), Osamu Matsubara\(^b\), Norio Kohno\(^a\)

\(^a\) Department of Breast Oncology, Tokyo Medical University, Shinjuku, Tokyo, Japan
\(^b\) Department of Basic Pathology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan
\(^c\) Department of Traumatology and Critical Care Medicine, National Defense Medical College, Tokorozawa, Saitama, Japan
\(^d\) Department of Molecular and Cellular Biochemistry, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan

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A B S T R A C T

Zoledronic acid (Zol) is the most potent inhibitor of bone resorption among the bisphosphonates and is commonly used for inhibiting bone metastasis. However, it remains unclear whether Zol provides a survival benefit. Recent findings indicate that epidermal growth factor (EGF) signaling is an important mediator of bone metastasis. Thus, we examined the combined effects of Zol and an EGF receptor-tyrosine kinase inhibitor, gefitinib, on the proliferation and invasion of a bone-seeking clone and the breast cancer cell line MDA-MB-231. Combined treatment with Zol and gefitinib synergistically inhibited both invasion and cell proliferation of the bone-seeking clone, but not those of the MDA-MB-231 cells. Two-dimensional difference gel electrophoresis and mass spectrometry demonstrated that stathmin was down-regulated during these cooperative effects. Stathmin is a signal transduction regulatory factor which plays an important role in cell division and malignant tumor development. Our data suggest that stathmin may be a promising target molecule for blocking bone metastasis of breast cancer.

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1. Introduction

Approximately 75% of patients with advanced breast cancer develop bone metastasis [1]. Patients with such bone metastasis suffer skeletal-related events (SREs) such as pathologic fracture, bone pain, spinal cord compression, and hypercalcemia [2]. As pathologic fracture is associated with a significantly increased risk of death, prevention of bone metastasis improves not only the quality of life but also the survival rate of patients with advanced breast cancer [3–5].

Zoledronic acid (Zol), which contains two nitrogen atoms within an imidazole ring structure, is the most potent inhibitor of bone metastasis-related osteolysis and effectively reduces the incidence of SREs [2]. Zol inhibits the mevalonate pathway in osteoclasts, thus inhibiting the synthesis of farnesyl pyrophosphate synthase and geranylgeranyl pyrophosphate, which are required for prenylation of signaling GTPases. Recent preclinical studies have suggested that Zol exerts specific anti-tumor effects including inhibition of cancer cell proliferation and/or invasion [6–8]. Bisphosphonates significantly reduce or prevent SREs. The use of Zol for patients with bone metastases is currently the gold standard treatment [4]. However, it is still unclear whether Zol contributes to a survival benefit [9]. Niikura et al. recently reported that Zol did not prolong progression-free survival or overall survival of breast cancer patients with bone-only metastasis [10]. We aimed to enhance the anti-tumor effects on bone metastasis, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor, gefitinib was used with Zol treatment.

Epidermal growth factor (EGF) signaling is also an important mediator of bone metastasis. The EGFR is overexpressed in a wide variety of cancer types including ~30% of breast cancers [11]. In addition to direct stimulation to cancer cell proliferation, EGF signaling has been implicated in the modulation of stromal cells in the tumor microenvironment [12,13]. Bone metastasis of the breast cancer clonal cell line MDA-MB-231, which overexpresses EGFR but lacks a proliferative response to EGF, is markedly suppressed by inhibition of EGFR-tyrosine kinase. In fact, significant relief of bone pain in breast cancer patients with bone metastases has been observed in a clinical trial of the EGFR-tyrosine kinase inhibitor gefitinib [14,15].
We have studied a bone-seeking clonal cell line that was established by repeated sequential passages of metastatic cells from bone in nude mice and also in vitro, and shown that it metastasizes exclusively to bone, producing larger osteolytic lesions than the MDA-MB-231 parental cells. In contrast, MDA-MB-231 cells target other organs such as brain, ovary, liver, and adrenal gland. The biological properties of this bone-seeking clone were shown to differ from those of MDA-MB-231, the former producing more parathyroid hormone-released protein than the latter. Activation of insulin-like growth factor I occurred, and anchorage-independent growth was not inhibited by transforming growth factor β [16].

In the present study, we examined the combined effect of Zol and gefitinib on both cellular proliferation and invasion of the bone-seeking clone, in comparison with those of the parental MDA-MB-231 cells. The key protein responsible for the cooperative effects was identified by 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE) and mass spectrometry.

2. Materials and methods

2.1. Cell cultures and drug treatment

The human breast cancer cell line MDA-MB-231 and its bone-seeking clone were studied. The bone-seeking clone had been established by repeated cardiac inoculation of the MDA-MB-231 parental cells into nude mice [16]. These cells were cultured in RPMI 1640 medium (Life Technologies Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (GE Healthcare Japan, Hino, Japan) and 1% penicillin (GE Healthcare Japan) at 37°C under 5% CO₂.

Zol was provided by Novartis Pharma (Basel, Switzerland). Gefitinib (Iressa®) was purchased from Tocris Bioscience (St. Louis, MO, USA). Zol powder was dissolved in 0.1 N NaOH, and gefitinib powder was dissolved in dimethyl sulfoxide (DMSO). Cells were treated with 10 μM Zol and/or 1 μM gefitinib for 24 h or 72 h. The final concentration of NaOH or DMSO was less than 0.01% in all experiments.

2.2. Proliferation assay

Effects of Zol or gefitinib on cell proliferation were determined using the WST-8 assay (Dojindo, Kumamoto, Japan). This cell counting assay is sensitive for the determination of cell viability in cell proliferation assay. WST-8 is reduced by dehydrogenase activities in cells to give a yellow color formazan dye. The amount of formazan dye is directly proportional to the number of living activities in cells to give a yellow color formazan dye. The amount

2.3. Invasion assay

Invasion was estimated using a 24-well Boyden chamber (BD BioCoat™ Matrigel™ Invasion Chamber, BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer’s recommendations. After hydration of the matrigel inserts for 2 h, 5.0 × 10⁴ cells in 500 μl RPMI medium containing 10 μM Zol, 1 μM gefitinib, or Zol plus gefitinib were added to the upper chambers. The lower chambers were filled with 750 μl RPMI medium containing drugs at the same concentrations as those in the upper chambers. After 24 h of incubation at 37°C, non-invasive cells were removed from the membranes, and the membranes were fixed with methanol and stained with crystal violet. Only cells that had penetrated the membranes were counted in 10 randomly chosen high-power fields (HPFs) (×400), and the mean number per HPF was adopted as the number of cells that had invaded through the membrane. The above experiments were performed in triplicate and repeated at least three times.

2.4. Protein extraction

The bone-seeking clone was cultured to 70% confluency and treated with each drug (Zol, gefitinib, or Zol plus gefitinib) for 24 h. The cells were then washed twice with ice-cold PBS, followed by fixation with 10% trichloroacetic acid, and scraped off into a tube and centrifuged at 3000 rpm for 10 min. The cell pellet was treated with lysis buffer (30 mM Tris, 7 M urea, 4% CHAPS, dilute HCl, and D₂O), and the resulting cell lysate was separated from debris by centrifugation and sonicated for 1.5 min at 250 W (Bioruptor, COSMO BIO, Yokohama, Japan). Protein concentration was measured with a 2-D Quant kit (GE Healthcare, Milwaukee, WI, USA).

2.5. 2-D DIGE (two-dimensional fluorescence difference gel electrophoresis)

Proteins were labeled with fluorescent cyanine dyes (GE Healthcare) in accordance with the manufacturer’s instructions. In brief, 50 μg of a pair of samples extracted from the bone-seeking clone treated or untreated with an anti-cancer drug was adjusted to pH 8.5 with 50 mM NaOH and labeled with 400 pmol Cy3 or Cy5, while another pair of samples was labeled inversely with Cy5 or Cy3. The fluorescence labeling was performed on ice in the dark for 30 min, and then quenched with 1 μl of 10 mM lysine (Sigma-Aldrich, St. Louis, USA) for 10 min. Each preparation was treated with 2 × sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% immobilized pH gradient (IPG)-buffer, pH range 4–7, and 2% dithiothreitol (DTT)), and the final volume was adjusted to 260 μl with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG-buffer, pH range 4–7, 0.2% DTT). The proteins were applied to Immobiline DryStrips (pH 4–7, 13 cm) and focused on an Ettan IPGphor II (GE Healthcare).

Four focused IPG strips were equilibrated, and then loaded onto 12.5% SDS-polyacrylamide gels (30% acrylamide, 1.5 M Tris–HCl pH 8.8, 10% SDS, 10% APS, 10% TEMED) using low-fluorescence glass plates on an Ettan DALT II system (GE Healthcare). All electrophoresis procedures were performed in the dark. After SDS-PAGE, a pair of gels were scanned with a Typhoon 9410 scanner (GE Healthcare) with appropriate excitation/emission wavelengths specific for Cy3 (532/580 nm) and Cy5 (633/670 nm) to generate protein spot maps. The above procedure was repeated three times.

DeCyder 7.0 software (GE Healthcare) was used for 2-D DIGE analysis in accordance with the manufacturer’s recommendation. The DeCyder differential in-gel analysis (DIA) module was used for pairwise comparisons of each sample. Differential protein spots (ratio > 2, P < 0.05) that showed consistent alteration were selected.

2.6. In-gel digestion and mass spectrometric analysis

Peptide mass fingerprinting (PMF) was performed for mass spectrometric analysis. To extract the differential protein spots,
500 μg of unlabeled sample was loaded onto SDS gel as described above. The gel was then stained with Coomassie blue for 1 h, and destained for 1 h in 10% acetic acid and 40% methanol. After washing with distilled water overnight, each spot was excised from the gel using a 1-mm pipet and kept in a PCR plate with water. In-gel digestion was performed as described by Kondo et al. [17]. Briefly, reduction was done using 45 mg DTT in 10 mM ammonium bicarbonate (ABM) and alkylation using 40 mg iodoacetamide in 50 mM ABM. After addition of 50% methanol, the gel plugs were washed in an ultrasonic water bath with ice for 10 min, then dehydrated with 50–100% acetonitrile (ACN) and incubated in vacuo for 10 min. Each procedure was performed 4–6 times. After drying for 1 h, the gel plugs were digested overnight in 0.5% trypsin in 50 mM ABM. The trypsin digests were recovered with 45% ACN and 0.1% trifluoroacetic acid (TFA), and the gel plugs were subjected to speedvac at room temp for 20 min. Finally, 15 μl of 0.1% TFA was added.

The trypsin digests were subjected to mass spectrometry [JMS-S3000 MALDI-spiral TOF-TOF, Japan Electron Optics Laboratory, Akishima, Japan] and a database search was done using the Mascot search engine at http://www.matrix-science.com. Proteins that scored greater than 62 were judged to be significant (P < 0.05).

2.7. Western blot analysis

We further validated the identification of proteins using Western blot analysis. Samples from MDA-MB-231 cells and the derivative bone-seeking clone treated with 1 μM gefitinib and/or 100 μM Zol were extracted with cell lysis buffer (1 M Tris pH 7.5, NP-40, 5 M NaCl, 0.5 M EDTA pH 7.5–8.0, 2 mM Na3VO4, 100 mM NaF, 10 mM NaP2O7). Fifty micrograms of each sample was separated using 12% SDS-PAGE gels, and transferred to a membrane using a Trans-Blot® Turbo™ Blotting System (Bio-Rad, Hercules, CA USA). After blocking with 5% non-fat milk powder in PBS overnight at 4 °C, the membranes were incubated with mouse monoclonal antibody against stathmin diluted 1:500 (BD Biosciences, San Jose, CA) or an anti-β-actin antibody (dilution 1:2000) (Sigma) for 1 h at room temperature. After washing with PBS containing 0.1% Tween 20, the membranes were incubated with the secondary antibody, anti-mouse IgG diluted at 1:2000 (GE Healthcare), for 30 min at room temperature and then washed for 30 min 4 times. The bands were reacted with chemiluminescence reagent (SuperSignal®, Thermo, Rockford, IL, USA) and visualized using a luminescence image analyzer (LAS-3000, Fuji Film, Kanagawa, Japan). The level of protein expression was evaluated using Multi Gauge ver. 3.0 (Fuji Film).

2.8. Statistical analysis

The significance of differences between the mean values of triplicate experiments in the proliferation and invasion assays was calculated using one-way analysis of variance (ANOVA) test. Differences were considered to be statistically significant at P < 0.05.

3. Results

3.1. Cell proliferation

Dose–response studies of Zol and gefitinib for both cell lines were shown in Figs. 1 and 2, respectively. Treatment with 10 μM Zol significantly reduced the absorbance of the bone-seeking clone from 0.77 nm (without any treatment) to 0.28 nm (reduction rate: 64%; 0.77–0.28/0.77; P < 0.001 indicated by *), and also that of MDA-MB-231 from 0.61 nm (without any treatment) to 0.17 nm (reduction rate: 72%; P < 0.001 indicated by **) (Fig. 3). In the bone-seeking clone, combined treatment with 10 μM Zol and 1 μM gefitinib reduced the absorbance by 50% to 0.14 nm, in comparison with a reduction to 0.28 nm by treatment with Zol alone (P < 0.01 indicated by **). In contrast, the reduction rate
was 18% in MDA-MB-231, and no significant cooperative effect of gefitinib with Zol was detected.

3.2. Invasion assay

The effects of Zol and/or gefitinib on invasion by the bone-seeking clone and MDA-MB-231 are shown in Fig. 4. The median number of cells that invaded through the membrane was significantly reduced by treatment with 10 μM Zol from 113.7 (without any treatment) to 35.0 (69%) for the bone-seeking clone (*P < 0.001 indicated by *) and from 80.0 (without any treatment) to 36.7 (54%) for MDA-MB-231 cells (P < 0.05 indicated by **). Combined treatment with 10 μM Zol plus 1 μM gefitinib markedly reduced the number of invading cells from 35.0 (treatment with Zol alone) to 4.3 (88%) for the bone-seeking clone (*P < 0.01 indicated by ***) but no such significant effect was detected for MDA-MB-231 cells.

3.3. 2-D DIGE

2-D DIGE identified four spots whose expression levels were significantly changed by treatment with Zol (Fig. 5a). All four spots were up-regulated more than 3-fold. Treatment with 1.0 μM gefitinib alone induced differential up-regulation of the expression of one spot (indicated by the arrowhead in Fig. 5c and d), but the other spots showed no significant change in expression though many spots were differentially expressed within 2-folds up- or down-regulation.

When the bone-seeking clone was treated with both Zol and gefitinib, these four spots were confirmed to show a change in protein level. Moreover, three new spots, numbered ①–③, appeared and showed significant down-regulation of their expression (Fig. 5d). These three spots, whose expression levels were not changed by treatment with either Zol or Gef alone (Fig. 5b and c, respectively), were located within a pH range of 5–6 and had molecular masses of 17–20 kDa. They were then subjected to identification by mass spectrometry.

3.4. Mass spectrometric analysis

We focused on the three protein spots (Fig. 5b–d ①–③) whose expression was significantly down-regulated by combined treatment with the two drugs. Fig. 6 shows the peak flow of the digested peptide derived from spot ③, which was identified as stathmin by a Mascot search. Among the three spots, ① and ③ shared a common sequence of 16 peptides (P = 0.005). There was no evident difference in the amino acid sequences that could
explain their different electrophoretic mobilities. Spot A could not be identified in this study.

3.5. Western blot analysis

Expression of stathmin in the bone-seeking clone was increased 6-fold in comparison with that in the parental line, MDA-MB-231 (Fig. 7a). In the bone-seeking clone, it was confirmed that the expression level of stathmin was halved by combined treatment with Zol and gefitinib, in comparison with Zol alone (Fig. 7b). This kind of down-regulation was not clearly detected in MDA-MB-231 because MDA-MB-231 expressed stathmin lower than bone-seeking clone without the treatment of Zol or gefitinib. (data not shown).

4. Discussion

Zol acts against osteolytic bone metastasis by inducing isoprenylation of proteins that are required for osteoclast survival. It also induces apoptosis in several types of cancers, including primary breast cancer, through the pathway responsible for prenylation of small GTP-binding proteins [18]. It is well known that EGF is an anti-apoptotic factor, and it is conceivable that gefitinib might enhance the susceptibility of cancer cells to apoptosis. In fact, a combination of Zol and gefitinib with SC-234 was reported to elicit a cooperative anti-tumor effect in a non-bone metastatic model of breast and prostate cancer [19]. Such combined treatment has also been reported to elicit a tumor-suppressive effect, which is significantly more effective and less toxic in vitro and in vivo, on EGFR-mutated non-small cell lung carcinoma cells, in comparison with the individual anti-tumor effect of gefitinib or Zol alone [20]. In the present study using a bone-seeking breast cancer clonal cell line in vitro, we demonstrated an enhanced inhibitory effect on bone metastasis using Zol plus gefitinib in combination.

The bone-seeking clone used in this study showed higher expression of stathmin than the parental MDA-MB-231 cell line. Similarly, using 2-D DIGE and mass spectrometry, Xu et al. have demonstrated that stathmin-1 was up-regulated in a highly metastatic variant of MDA-MB-231 [21]. A proteomic analysis designed to examine the time course of neoplastic transformation of human mammary epithelial cells showed that stathmin was up-regulated in parallel with an early and progressive increase in metastatic potential [22]. Moreover, overexpression of stathmin has been detected in different types of human malignant tissue, such as sarcoma [23], gastric cancer [24], endometrial cancer [25], colon cancer [26], nasopharyngeal cancer [27], and breast cancer [28,29]. All these data suggest that overexpression of stathmin is significantly correlated with clinically aggressive behavior such as lymph node metastasis, poor histologic differentiation, an advanced clinical stage, or poor prognosis.

In the present study, we found that stathmin was significantly down-regulated upon treatment with a combination of Zol and gefitinib. The down-regulation of stathmin, which was not detected by 2-D DIGE after treatment with either agent alone,
was considered to be attributable to cooperative effect of Zol and gefitinib. Down-regulation of endogenous stathmin by transfection with an adenovirus expressing stathmin small interfering RNA (siRNA) was reported to decrease the migration of HT1080 (human fibrosarcoma) cells through fibronectin-coated Transwells [23]. Silencing of stathmin by siRNA has also been shown to decrease the proliferation, viability, or invasion of cancer cells derived from malignancies of the prostate [30], breast [31], stomach [24], and colon [26]. These findings are in accord with our present data indicating that down-regulation of stathmin paralleled the inhibition of both cell proliferation and invasion of the bone-seeking clone upon combined treatment with Zol and gefitinib, suggesting that stathmin could be a potential candidate for therapeutic target for inhibition of bone metastasis.

Finally, our data allow us to propose a potentially promising strategy for cancer therapy. Down-regulation of stathmin has been reported to enhance the sensitivity to anti-microtubule drugs [32–34]. Because the combined treatment of Zol and gefitinib down-regulates the expression of stathmin, a further inhibitory effect against breast cancer might be expected if anti-microtubule drugs were to be added. Although a preclinical study would be needed to investigate this triple strategy, synergistic anti-angiogenic effects of stathmin inhibition and taxol exposure have already been reported [35]. Combination of these three agents might be indicated for patients with aggressive breast cancers such as those with the triple negative phenotype.

In summary, we have demonstrated that combined treatment with Zol and gefitinib, which down-regulate stathmin, produces enhanced anti-tumor effects on bone homing breast cancer cells in vitro. Thus, co-administration of Zol and gefitinib may constitute a basis for developing a more effective therapy for patients with bone metastasis.

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