Isoprenoid-independent pathway is involved in apoptosis induced by risedronate, a bisphosphonate, in which Bim plays a critical role in breast cancer cell line MCF-7

KEIKO SUYAMA, YOSHIHIKO NOGUCHI, TOMOAKI TANAKA, TOMOHIKO YOSHIDA, TAKAHISA SHIBATA, YASUSHI SAI TO and ICHIRO TATSUNO

Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Received July 26, 2007; Accepted August 28, 2007

Abstract. Bisphosphonates cause apoptosis to various types of cancer cells including breast cancer. Inhibition of the mevalonate pathway was reported to be involved in the apoptosis induced by bisphosphonates, but its precise mechanism has not been unveiled. In the present study, we investigated the molecular mechanism of risedronate, a bisphosphonate, in the apoptosis of the breast cancer cell line MCF-7 in comparison with that of cerivastatin, an HMG CoA reductase inhibitor (statin), since statin has been known to induce apoptosis through an isoprenoid-dependent pathway in these cells. We found that i) risedronate induced MCF-7 cells into apoptosis in a manner similar to cerivastatin with the activation of caspase-9 followed by caspase-6 and -7, that ii) bisphosphonate-induced apoptosis was significantly, but not fully, recovered by the addition of GGOH, an isoprenoid, which completely rescued in case of cerivastatin-induced apoptosis, that iii) risedronate induced G2 arrest with the induction of Bim (BH3-only protein), but that statin induced G1 arrest without it, and that iv) the down-regulation of Bim protein by siRNA significantly attenuated the risedronate-induced apoptosis. These data clearly indicate that both isoprenoid-dependent and -independent pathways might be involved in the apoptosis induced by bisphosphonate, and Bim might be a critical component for the isoprenoid-independent apoptotic pathway.

Introduction

Breast cancer is one of most common and serious neoplasms for women in the western world. The prevention of metastasis is critical to the improvement of the prognosis of patients with breast cancer. Bone is one of most frequent metastatic sites of breast cancer, with metastatic bone disease causing substantial morbidity, leading to major skeletal events such as fracture. Although anti-estrogen therapies are effective for controlling metastasis in breast cancers, not all breast cancers possess estrogen receptors. Anticancer chemotherapies are also effective, but they usually have serious side effects that often require their cessation (1,2).

Accumulating evidence showing the anti-tumor effects of bisphosphonates has provided the rationale for their use as an adjuvant therapy in patients at risk for bone metastases. In fact, bisphosphonates have been shown to decrease bone metastasis and improve the prognosis of breast cancer patients with bone metastasis (3-7).

Bisphosphonates inhibit osteoclastic bone resorption, which occurs at an accelerated pace due to the presence of tumor cells in the bone microenvironment (8). In addition to their inhibitory effect on osteoclasts (9), there is increasing preclinical data to indicate that bisphosphonates have direct antitumor activity (10-12). These include the inhibition of tumor cell growth and the induction of cancer cell apoptosis.

The molecular mechanism of apoptosis induced by bisphosphonates is not fully understood. Nitrogen-containing bisphosphonates affect osteoclast action by the inhibition of enzymes of the mevalonate pathway (13). Target enzymes include farnesyl pyrophosphate synthase and/or geranylgeranyl pyrophosphate synthase, leading to a lack of formation of farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These isoprenoids are requisite for post-translational lipid modification (i.e., farnesylation and geranylgeranylation) of signaling GTPases, such as Ras, Rho and Rac (14,15). As these control a variety of important cell functions, their loss ultimately leads to apoptosis through the activation of several caspases in breast cancer. Actually, bisphosphonate-induced apoptosis in breast cancer is significantly restored by the addition of farnesyl pyrophosphate and geranylgeranyl pyrophosphate. However, the molecular mechanism of apoptosis in breast cancer induced by bisphosphonate has still to be fully revealed.

HMG CoA reductase inhibitor (statin) was reported to induce apoptosis in various types of cancer cell lines through inhibiting the production of isoprenoids in a mevalonate pathway (16). Statins, cholesterol-lowering drugs, exert
pleiotropic functions by preventing the synthesis of mevalonic acid. Additional evidence suggests that both lovastatin and cerivastatin exert a cytostatic effect on mesothelioma (17), glioma (18), neoplastic thyroid (19), acute myeloid leukemia (20), and multiple myeloma cells (21) by directly promoting apoptosis. It seems likely that defective isoprenylation of proteins involved in the cell cycle (22-24), namely Ras, Rac and Rho A, results in their inappropriate localization and functions, triggering apoptosis. It is reported that geranylgeranyl pyrophosphate can restore the effect of statin (25).

In the present study, we examined the molecular mechanism of apoptosis induced by bisphosphonate, using risedronate in comparison with cerivastatin in a human breast cancer cell line, MCF-7. We found that i) bisphosphonate-induced apoptosis was significantly, but not entirely, recovered by the addition of isoprenoids, that ii) bisphosphonate induced G2 arrest with the induction of Bim protein, but that statin induced G1 arrest without it, and that iii) the down-regulation of Bim by siRNA significantly attenuated the apoptosis induced by bisphosphonate. These data clearly indicated that both isoprenoid-dependent and -independent pathways might be involved in the apoptosis induced by bisphosphonate, and that Bim is a critical component for the isoprenoid-independent apoptotic pathway.

Materials and methods

Reagents. Cerivastatin was kindly provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). Risedronate was obtained from LKT Laboratories, Inc (MN, USA). Mevalonate (MVA), farnesol (FOH) and geranylgeraniol (GGOH) were purchased from Sigma Chemical Co (St. Louis, MO, USA) and stock solutions were prepared in 100% ethanol. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. Cell culture media and reagents [Dulbecco's modified Eagle's medium, penicillin-streptomycin solution, fetal bovine serum (FBS), trypsin 0.25% with EDTA (Dulbecco's modified Eagle's medium, penicillin-streptomycin)] were purchased in 100% ethanol. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. Cell culture media and reagents, Dulbecco's modified Eagle's medium, penicillin-streptomycin, fetal bovine serum (FBS), trypsin 0.25% with EDTA 1 mM, and nitrocellulose [Dulbecco's modified Eagle's medium, penicillin-streptomycin] were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Dulbecco's phosphate buffered saline (PBS) was purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Anti-caspase-9, -6 and -7 antibodies were purchased from BioWhittaker Molecular Applications (Rockland, ME, USA). Anti-Bim (Santa Cruz, CA, USA) and Lipofectamine was purchased from Invitrogen. DNA fragmentation assay. To determine apoptosis, DNA fragmentation was assessed using a PCR kit for DNA ladder assay (Maxim Biotec, Inc.) as suggested by the manufacturer. Terminal deoxynucleotidyltransferase-mediated biotin-dUTP nick-end labeling (TUNEL) assay was also performed (data not shown). Briefly, the culture medium in 2-chamberslide glass was removed, and cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. Then, the cells were washed twice with PBS and stained using the ApopTag Fluorescein Direct In Situ Apop-Tag Detection Kit (Intergen, NY, USA) according to the manufacturer’s protocol. Vectashield mounting medium for fluorescence with DAPI (Vector, CA, USA) was applied and the coverslips were mounted on the slides.

Cell cycle analysis. Cell cycle analysis was performed by measuring the DNA content of cells using FACS analysis after nuclear staining with propidium iodide. Cells harvested by trypsinization were washed with PBS and stained with propidium iodide using Cycle Test Plus DNA Reagent Kit (Becton Dickinson Immunocytometry System, San Jose, CA, USA) according to the manufacturer’s instructions. The cell cycle profiles of samples were analyzed by Cell Quest software (BD Biosciences).

Western blot analysis. Western blot analyses were performed as previously reported (26). Briefly, samples from each experiment were treated with 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 7% glycerol, and 0.01% bromophenol blue for 3 min in boiling water, and 20 μg of each sample was subjected to SDS-PAGE (12.5%). Western blotting was performed with the specific antibodies against the cleaved caspase-9/-6/-7 and Bim using an ECL Western blotting kit (Amersham Biosciences, UK).

Gene silencing with small interfering RNAs. MCF-7 cells were cultured in six-well plates until 60% confluent. Cells in 2 ml of culture medium were transfected with 4 μg of annealed oligonucleotides using Lipofectamine (Invitrogen) according
to the manufacturer's instructions. Twenty-four hours after transfection the cells were treated with 80 μM cerivastatin or 800 μM risedronate. After 48 h of treatment, both the suspension and the adherent cells were collected for either Western blot analysis, or trypan blue staining. The Bim siRNA sequences were the same as previously reported (27). Bim sense 5'-CAAUUGUCUACCUUCUCGG (dTdT)-3'; Bim antisense 5'-CCGAGAAGGUAGACAAUUG (dTdT)-3'; control sense 5'-UUCUCCGAACGUGUCACGU (dTdT)-3'; and control antisense 5'-ACGUGACACGUUCGGAGAA (dTdT)-3'.

Statistical analysis. Statistical differences within each experiment were determined by analysis of variance, and differences between groups were calculated by Fisher's PLSD. A rejection level of p<0.05 was considered significant. This analysis was carried out on a personal computer using the 'StatView J4.02' software statistical package (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Both cerivastatin and risedronate induced apoptosis in MCF-7 cells in a dose- and time-dependent manner. The time course for the cell death of MCF-7 induced by cerivastatin (80 μM) and risedronate (800 μM) is shown in Fig. 1A. The live cell number was determined by MTT assay. Both cerivastatin and risedronate induced cell death into MCF-7 cells in a time-dependent manner. The number of live cells started to decrease after 24 h in the case of risedronate and after 48 h in the case of cerivastatin after their addition, and progressively continued to decrease during the following 72 h. Nearly 75% of cells had died by 72 h (Fig. 1A). In some experiments, we performed the cell counting assay by trypan blue exclusion assay in parallel with the MTT assay to determine the number of live cells. There was linear correlation between the MTT assay and the number of live cells (r2=0.987, p<0.05; data not shown).

The dose-dependent effect of cell toxicity by cerivastatin and risedronate over a 72-h period is shown in Fig. 1B. MCF-7 cells were incubated with various doses of cerivastatin and risedronate for 72 h. The cell survival was determined by MTT assay as shown in Fig. 1B. Cerivastatin induced cell death at concentrations over 5 μM in a dose-dependent manner, and >75% of MCF-7 cells died at 80 μM. Risedronate also induced cell death in MCF-7 cells at concentrations over 300 μM in a dose-dependent manner, and >75% of MCF-7 cells died at 800 μM. We used DNA ladder and TUNEL assays to analyze the types of cell death induced by cerivastatin and/or risedronate. Both cerivastatin (80 μM) and risedronate...
(800 μM) had evident DNA ladders for 72 h (Fig. 1C), and the dead cells were TUNEL positive (data not shown), indicating that their death was apoptotic.

Both cerivastatin and risedronate increased the expression of cleaved caspase-9, -6 and -7. In order to investigate the molecular mechanism of apoptosis induced by cerivastatin and risedronate in MCF-7 cells, the expressions of caspases, the key enzymes in the apoptotic cascade, were analyzed by Western blotting. After the addition of cerivastatin (80 μM) and risedronate (800 μM), the expression of cleaved caspase-9, -6 and -7 increased in a time-dependent manner, peaking at 48 h after the stimulation (data not shown). Then, the dose-dependent effects of cerivastatin and risedronate on the expression of cleaved caspases in MCF-7 cells were examined at 48 h after the addition (Fig. 2A and B). MCF-7 cells were treated with various concentrations of cerivastatin and risedronate for 48 h. Both cerivastatin and risedronate increased the expression of cleaved caspase-9, -6 and -7 in a dose-dependent manner.

Cerivastatin (5 μM) induced cell cycle arrest at G1 phase; however, risedronate (200 μM) induced cell cycle arrest at G2 phase. There was an apparent difference in the apoptotic mechanism between cerivastatin and risedronate, since cerivastatin induced cell death in a GGOH-dependent manner, but risedronate did so only partially. In order to study the molecular mechanism of risedronate, we performed FACS analysis of MCF-7 cells (Fig. 3). Cerivastatin at 5 μM increased the cell numbers in the G1 phase from 51 to 63%, and decreased the numbers in the S phase from 22 to 6%, demonstrating G1-phase cell cycle arrest (Fig. 3). Risedronate at 200 μM increased cells in S phases from 22 to 40%, indicating that risedronate induced G2 arrest (Fig. 3).

Risedronate, but not cerivastatin, increased the expression of Bim. Since risedronate induced G2 arrest, and the apoptosis induced by cerivastatin was completely dependent on GGOH while that induced by cerivastatin only partially, we supposed that risedronate induced apoptosis through several pathways. One is GGOH-dependent in a manner similar to cerivastatin. Another is GGOH-independent and is related to G2 arrest.

Since Bim was reported to be involved in G2 arrest, we analyzed the protein expression of Bim, which has three isoforms, EL, L, and S, by Western blotting. In MCF-7 cells, risedronate increased the expression of Bim in a time-dependent manner, peaking at 48 h after the stimulation, in which EL isoform was dominantly expressed (data not shown). But cerivastatin resulted in no change to the expression of Bim (data not shown).

When MCF-7 cells were treated with various concentrations of cerivastatin or risedronate for 48 h, risedronate increased the expression of Bim in a dose-dependent manner, but cerivastatin did not (Fig. 4).

Risedronate-induced apoptosis was partially recovered by GGOH. We investigated the effects of intermediates of the mevalonate pathway in the apoptosis induced by risedronate in comparison with that induced by cerivastatin. Apoptosis induced by cerivastatin was restored >90% by mevalonate

---

**Figure 2.** Expression of cleaved caspase-9, -6 and -7 in MCF-7 cells treated with cerivastatin or risedronate for 48 h. (A) Dose-effect of cerivastatin. (B) Dose-effect of risedronate. Cell lysates (20 μg) were analyzed by immunoblotting with antibodies against cleaved caspase-9, -6 and -7 as described in Materials and methods. The data shown are representative of three independent experiments.

**Figure 3.** Effect of cerivastatin or risedronate on cell cycle progression determined by flow cytometry in MCF-7 cells. MCF-7 cells were incubated with cerivastatin (5 μM) and risedronate (200 μM) for 48 h. Cells were stained with propidium iodide and analyzed by FACScan as described in Materials and methods. The data shown are representative of three independent experiments.
(MVA) or geranylgeraniol (GGOH), whereas the effect of farnesol (FOH) recovered only partially.

On the other hand, apoptosis induced by risedronate was recovered by GGOH, but only partially. There was no effect of MVA or FOH (Fig. 5A), and no additional effect by FOH with GGOH compared to GGOH alone (data not shown). GGOH restored the apoptosis induced by risedronate in a dose-dependent manner, reaching a plateau at concentrations over 10 μM (data not shown).

The effect of GGOH on the expression of cleaved caspase-9, -6, and -7 and Bim was examined in the apoptosis induced by cerivastatin and risedronate for 48 h (Fig. 5B). Although GGOH reduced the expression of cleaved caspase-9, -6 and -7 induced by cerivastatin to the control level, the expression of caspase-9, -6 and -7 induced by risedronate was suppressed partially by GGOH. There was no increase of Bim expression after cerivastatin treatment. In the case of risedronate, Bim expression increased and there was little effect on Bim expression by GGOH (Fig. 5B).

Silencing of Bim by siRNA resulted in a reduction in risedronate-induced apoptosis in MCF-7 cells. We clarified the role of Bim in the apoptosis induced by risedronate by its silencing with siRNA (27). The transfection of siRNA specific for Bim caused a distinct decrease in the expression of Bim, and significantly reduced risedronate-induced apoptosis (Fig. 6A), although control siRNA did not have any effect on Bim expression and risedronate-induced apoptosis. In addition, the treatment of siRNA for Bim inhibited the expression of cleaved caspase-6 induced by risedronate, although the expression of cleaved caspase-7 and -9 were not significantly changed (Fig. 6B).

**Discussion**

In breast cancers, it has been reported that bisphosphonates decrease bone metastasis and improve the prognosis of patients with bone metastasis (2,4-7). These favorable effects of bisphosphonate in breast cancers might be involved in the apoptosis of cancer cells, since nitrogen-containing bisphosphonates induce significant apoptosis in many types of cancer cell lines (10,28-30).

In the present study, we confirmed that both cerivastatin and risedronate induce apoptosis in the breast cancer cell line MCF-7 by DNA ladder assay and TUNEL assay. Both reagents induced apoptosis in a time- and dose-dependent manner as reported by others (12,16-19,21,31). Since it has been reported that the activation of caspases is involved in the apoptosis of breast cancer cells (16,25), we compared risedronate with cerivastatin in terms of the activation of caspases. In the MCF-7 cells, the activations of caspase-6 and -7 are thought to be critical for the final step of the caspase cascade, since it is known that MCF-7 cells do not express caspase-3 (32). Our results show that both cerivastatin and risedronate increased the expression of cleaved caspase-6, -7 and -9 time-dependently, reaching a maximum at 48 h after their addition. Therefore, we examined the dose-dependency of risedronate in the activations of caspases at 48 h post addition; we found that it activated caspase-6, -7 and -9 in a dose-dependent manner.
Enzymes that catalyze the covalent attachment to farnesyl and were reported to be major products of this pathway (22-25). FPP, two components of isoprenoids, synthesis. Geranylgeranyl-pyrophosphate (GGPP) and isoprenylation of proteins rather than interrupting cholesterol apoptosis can be attributed to its activity in suppressing the proliferation through cell cycle progression and induce HMG-CoA reductase inhibitor (statin) to interfere with apoptosis in various types of cancer cell lines.

Isoprenoids are also involved in bisphosphonate-induced apoptosis, as bisphosphonates are known as inhibitors of FPP synthase and GGPP synthase (14,15), and this kind of apoptosis was attenuated by the addition of geranylgeranol (GGOH) or farnesol (FOH), which are thought to be converted to GGPP or FPP, respectively, in cells. However, the precise molecular mechanism is yet to be unraveled.

We compared the effects of isoprenoids on the restoration of the apoptosis induced by cerivastatin and risedronate. Mevalonate and GGOH completely restored that induced by cerivastatin, whereas FOH had only a partial restorative effect. There was no additional effect of FOH in the presence of GGOH. These results are very similar to those of previous reports. Notably, the apoptosis by risedronate was partially restored by GGOH, and FOH had little effect. These results indicated that risedronate-induced apoptosis is partially GGOH-dependent, different from that by cerivastatin.

We used FACS to analyze the effect of risedronate on MCF-7 cells in order to clarify the molecular mechanism involved. There was an apparent difference in terms of the cell cycle, as cerivastatin was observed to induce G1 arrest while risedronate induced G2 arrest. This means that risedronate has an additional mechanism to cause cell growth arrest, which may be involved in apoptosis.

It was reported that anti-cancer drug paclitaxel causes G2 arrest to induce apoptosis, in which Bim, a member of the Bcl2-family, has an important role (27,41,42). In addition, bisphosphonate was reported to potentiate the apoptosis induced by paclitaxel (11). Therefore, we hypothesized that Bim is one of the key regulators for risedronate-induced apoptosis. As expected, risedronate increased the expression of Bim both time- and dose-dependently, whereas cerivastatin had no such effect. This means that the induction of Bim is independent to isoprenoid synthesis. Effects of GGOH and FOH on Bim expression showed the same result. The addition of GGOH or FOH to risedronate-induced apoptosis had no direct effect on Bim expression. It was proved by the result that GGOH has no effect on Bim induction by risedronate. To confirm our hypothesis that Bim is involved in the apoptosis induced by risedronate, we chose siRNA for down-regulation of Bim (27). siRNA successfully inhibited the protein expression of Bim, resulting in 40% inhibition of apoptosis induced by risedronate, but not by cerivastatin (data not shown). This result shows that Bim is not only involved, but in fact plays an important role in the apoptosis induced by risedronate, but not much in that by cerivastatin. The protein expression of cleaved caspase-6 by risedronate, not by cerivastatin, was suppressed by siRNA for Bim. Among the caspases, little effect was found in the expression of cleaved caspase-7 and -9, suggesting that Bim is linked to the activation of caspase-6 specifically.
In summary, bisphosphonate induces apoptosis through isoprenoid-dependent and -independent pathways. In the isoprenoid-independent apoptosis induced by bisphosphonate, Bim is a key molecule in the induction of G2 arrest and apoptosis, which specifically might activate caspase-6.

References


