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Involvement of Ras/extracellular signal-regulated kinase, but not Akt pathway in risedronate-induced apoptosis of U937 cells and its suppression by cytochalasin B

Hirofumi Fujita [a,b,*], Toshihiko Utsumi [c], Shikibu Muranaka [a], Tetsuya Ogino [d], Hiromi Yano [e], Jitsuo Akiyama [f], Tatsuji Yasuda [b], Kozo Utsumi [a]

[a] *Institute of Medical Science, Kurashiki Medical Center, Kurashiki 710-8522, Japan*
[b] *Department of Cell Chemistry, Institute of Molecular and Cell Biology, Okayama University Medical School, Okayama 700-8558, Japan*
[c] *Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
[d] *Department of Pathological Research, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan*
[e] *Department of Health and Sports Science, Kawasaki University of Medical Welfare, Kurashiki 701-0193, Japan*
[f] *Doonan Institute of Medical Science, Ishikawa-cho, Hakodate 041-8508, Japan*

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Abstract

Although risedronate, a nitrogen containing bisphosphonate (BPs), strongly inhibits bone resorption by enhanced apoptosis of osteoclasts, its mechanism remained unclear. In this study, we investigated the molecular mechanism of risedronate-induced apoptosis of U937 cells, with a focus on extracellular signal-regulated kinase 1/2 (ERK 1/2) and protein kinase B (Akt) pathways, mitochondria-mediated apoptosis, and the effect of disruption of the actin cytoskeleton. Risedronate facilitated the relocation of Ras from membrane to cytosol through inhibited isoprenylation. Accordingly, risedronate suppressed the phosphorylation of ERK 1/2, a downstream survival signaling kinase of Ras, affected the intracellular distribution of Bcl-xL, and induced the mitochondrial membrane depolarization, cytochrome c release, activated caspase cascade and DNA fragmentation. The risedronate-induced apoptosis was effectively suppressed with cyclosporine A plus trifluoperazine, potent inhibitors of mitochondrial membrane permeability transition (MPT). The risedronate-induced apoptosis was independent of Akt, another cAMP-dependent survival signaling kinase. Risedronate facilitated dephosphorylation of Bad at Ser112, an ERK phosphorylation site, but not at Ser136, an Akt phosphorylation site. All of these apoptosis-related changes induced by risedronate were strongly suppressed by cytochalasin B, an inhibitor of actin filament polymerization. These results indicate that risedronate-induced apoptosis in U937 cells involves Ras/ERK, but not Akt signaling pathway, and is dependent on MPT, and that disruption of the actin cytoskeleton inhibits the risedronate-induced apoptosis at its early step.

Keywords: Apoptosis; Bisphosphonate; Cytochalasin B; ERK1/2; Bad; Membrane permeability transition

1. Introduction

Bisphosphonates (BPs) are potent inhibitors of bone resorption and are used for the therapy of osteoporosis, Paget's disease, bone metastasis, and other bone diseases [1]. They are structurally similar to pyrophosphate and preferentially bind to hydroxyapatite of the bone. Bisphosphonates can be divided into two groups with distinct molecular mechanisms of action depending on the R2 side chain. One group is the BPs that lack nitrogen, such as clodronate and etidronate. This group does not inhibit protein prenylation and show weak inhibition of bone resorption activity [2,3]. The other group is nitrogen-containing BPs, such as alendronate, zoledronate and risedronate. This group strongly inhibits bone resorption and causes apoptosis of osteoclasts and other cell lines in vitro.

Abbreviations: ERK, extracellular signal-regulated kinase; PS, phosphatidylserine; BPs, bisphosphonates; AMC, 7-amino-4-methyl-coumarin; Ac-IETD-CHO, acetyl-Ile-Glu-Thr-Asp-CHO; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide; FPP, farnesyl pyrophosphate; GGOH, geranylgeraniol; s-GTP-bp, small GTP-binding proteins; MPT, membrane permeability transition; pCPT-cAMP, 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate

* Corresponding author. Tel.: +81 86 426 8616; fax: +81 86 426 8616.
E-mail address: fujita01@mx3.kct.ne.jp (H. Fujita).

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The nitrogen containing BPs inhibit FPP synthase, thereby inhibiting the biosynthesis of farnesyl diphosphate and/or geranylgeranyl diphosphate [4–6]. These compounds are important membrane-anchoring molecules of small GTP-binding proteins (s-GTP-bp) such as Ras. Their shortage facilitates dissociation of Ras from the inner surface of the membrane, and decreases Ras-mediated growth signal, thereby inhibiting cell proliferation [7,8]. Indeed, zoledronate induced delocalization of p21ras (Ras) from the cell membrane in cancer cells [9,10], and the downstream kinase that mediates survival signal, namely ERK 1/2, has been reported to be suppressed [10,11]. In this context, geranylgeraniol (GGOH) which bypasses inhibition of FPP synthase and replenishes the cells with a substrate for protein geranylgeranylation blocked the BP-induced apoptosis of osteoclasts [2]. Moreover, the protein kinase B (Akt) pathway has also been suggested to be involved in zoledronate-induced apoptosis in human pancreatic cancer cells and endothelial cells [10,12], but not in other experimental model using HL-60 cells [11]. These results indicate that BP-induced apoptosis might depend on the cell type and the structure of R2 moiety in BPs.

Recent studies showed that mitochondria have an important role in the induction of apoptosis. It was reported that forced expression of the anti-apoptotic protein Bcl-2 attenuated BP-induced loss of cell viability and induction of DNA fragmentation in MDA-MB-231 cells and that zoledronate-mediated apoptosis was associated with a time and dose-related release of mitochondrial cytochrome c into the cytosol in two cell lines [13]. Furthermore, BP-induced mitochondrial membrane depolarization in osteoclasts and hematopoietic tumor cells was also reported [14,15]. However, it is not clear whether the BP-induced apoptosis depends on mitochondrial membrane permeability transition (MPT).

Nitrogen containing BPs cause disruption of the ruffled border and actin cytoskeleton of osteoclasts and induce apoptosis, because nitrogen containing BPs reduce geranylgeranylated proteins which regulate the cytoskeleton [16,17]. However, it is currently unknown whether actin cytoskeleton regulates nitrogen containing BPs-induced apoptosis. In this context, cytochalasins, inhibitor of actin filament polymerization, has an ability to stimulate or suppress cell apoptosis through different mechanisms [18–22]. Thus, it is interesting to study how cytochalasin B affect on BP-induced apoptosis of U937 cells.

In this study, therefore, molecular mechanism of risedronate-induced apoptosis on U937 cells and its sensitivity to cytochalasin B were investigated. Our results indicated that the risedronate-induced apoptosis involved Ras/ERK, but not Akt pathway, and depended on mitochondrial MPT. We also found that cytochalasin B strongly suppressed the apoptosis-related changes in U937 cells by risedronate.

2. Materials and methods

2.1. Chemicals

Cyclosporine A, 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate (pCPT-cAMP), cytochalasin B, demecolcine, farnesol and GGOH were obtained from Sigma. TACS^{TM} Annexin V-FITC kit is obtained from Trevigen. 5,5′6,6′-Tetrachloro-1,1′,3,3′-tetraethyl- benzimidazolylcarbo-cyanine iodide (JC-1) was from Molecular Probes. Polyclonal antibody of Bid was from Genzyme-Techne. Monoclonal antibody of actin was from CHEMICON. Bcl-2 (N-19), Bax (Δ21) and Bcl-xL (S-18) polyclonal antibodies were from Santa Cruz Biotechnology. Polyclonal antibodies against Akt, ERK, Bad, phospho-Akt (p-Akt) (Ser473), phospho-ERK1/2 (p-ERK) (Thr202/Tyr204), phospho-Elk-1 (p-Elk-1) (Ser383), phospho-GSK-3β (p-GSK-3β) (Ser9), phospho-FKHR (p-FKHR) (Ser256), phospho-Bad (p-Bad) (Ser112) and p-Bad (Ser136) were from Cell Signaling Technology. Polyclonal antibody of Bad for intracellular localization was from BD Transduction Laboratories. Polyclonal antibody of cytochrome c was from Pharmingen. Monoclonal antibody of Ras was from CALBIOCHEM. Can Get Signal^{TM} was from TOYOBO. Fluorogenic tetrapeptide substrates, such as Ac-DEVD-MCA (for caspase-3), Ac-IETD-MCA (for caspase-8) and Ac-LEHD-MCA (for caspase-9), and Ac-IETD-CHO (for caspase-8 inhibitor) were from the Peptide Institute. Risedronate was donated from Ajinomoto.

2.2. Cell line

U937 was obtained from RIKEN Cell Bank and was maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37 °C under 5% CO_2/95% air. Cells were plated onto 35 mm and 100 mm dishes (0.2×10^5 cells/ml) in 2 ml and 20 ml of RPMI 1640 containing 10% fetal bovine serum, respectively, and incubated for 16 h before treatment with various reagents.

2.3. Analysis for PS exposure

PS exposed on the outside of the cells was determined by TACS^{TM} Annexin V-FITC kit. Briefly, cells were washed with cold PBS, pelleted and resuspended in 100 μl annexin V-FITC diluted 3:100 in binding buffer (10 mM Hepes, 100 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) containing propidium iodide (1:10). Cells were incubated for 15 min at room temperature, then the stained cells were observed by fluorescence microscopy. Total cells (1000–1500 cells) and FITC positive cells were counted in the same field, and annexin V positive rate (%) was calculated.