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Selenite-induced apoptosis of osteoclasts mediated by the mitochondrial pathway

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Abstract

The possible effects of sodium selenite on mature osteoclasts were investigated. Incubation of osteoclast-like cells differentiated from RAW 264.7 cells with sodium selenite induced apoptosis as revealed by morphological changes, internucleosomal DNA fragmentation, and activation of caspase-3. Selenite also induced generation of the superoxide anion and reduced the number of free thiol groups in the osteoclast-like cells, suggestive of a shift to a more oxidizing intracellular environment. In addition, selenite induced protein aggregation by thiol cross-linking, loss of the mitochondrial membrane potential, and cytochrome *c* release in mitochondria isolated from the osteoclast-like cells. Finally, selenite-induced DNA fragmentation in osteoclasts was inhibited both by cyclosporin A, a blocker of the mitochondrial permeability transition pore, and by DEVD-CHO, a cell-permeable inhibitor of caspase-3. These results thus suggest that selenite induces apoptosis mediated by the mitochondrial pathway in mature osteoclasts.

Keywords: Selenium; Apoptosis; Osteoclast; Mitochondria

1. Introduction

Bone provides the body with mechanical support and physical protection as well as serves as a depository for minerals (Boyle et al., 2003; Fleisch, 1997). Bone is continuously remodeled as a result of a balance between the activities of osteoblasts and osteoclasts, both of which are present on the bone surface. Osteoblasts secrete organic matrix molecules that contribute to the formation of new bone (Suda et al., 2003), whereas osteoclasts mediate the mineralization of old bone and resorb the mineralized organic molecules (Teitelbaum, 2000). An imbalance between the activities of osteoblasts and osteoclasts results in bone diseases, such as osteopetro-

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sis and osteoporosis (Rodan and Martin, 2000; Zelzer and Olsen, 2003).

Selenium is an essential micronutrient with potent chemopreventive and inhibitory effects on cancer in mammals (Caffrey and Frenkel, 2000; Ganther, 1999; Rao et al., 2000). The biological actions of selenium depend on its concentration. At low concentrations, it exerts various effects as a result of its incorporation into selenoproteins as selenocysteine. Most selenoproteins appear to play an important role in antioxidant or hormone metabolism (Epp et al., 1983; Holben and Smith, 1999). Selenium deficiency has been associated with abnormal bone metabolism underlying osteoarthritis (Kashin–Beck disease) (Turan et al., 1997) or growth retardation (Dobbelaere et al., 2003; Moreno-Reyes et al., 2001; Rivera et al., 2001). At higher concentrations, however, selenium is cytotoxic. We and others recently showed that high concentrations of selenium trigger the opening of the mitochondrial permeability transition (MPT) pore by inducing both generation of the superoxide anion ($O_2^{\bullet -}$) and oxidation of protein thiol groups (Kim et al., 2002, 2003; Shen et al., 1999). The cancer-preventive effects of selenium are thought to be due to such cytotoxicity. Selenium cytotoxicity can also result in various pathological conditions, including blind staggers and alkali diseases, whose symptoms include loss of hair in humans or loss of hooves in animals. Such conditions are referred to collectively as selenosis (O'Toole and Raisbeck, 1995; Raisbeck, 2000). The molecular mechanisms responsible for the actions of selenium in bone-related cells are unknown, however.

The hematopoietic system has been implicated as a primary target of selenium toxicity (Raisbeck, 2000). Osteoclasts, which are multinucleated giant cells, are derived from hematopoietic progenitor cells of the monocyte-macrophage lineage (Kahn and Simmons, 1975; Udagawa et al., 1990). We have now investigated the possible effects of selenite on osteoclast-like cells derived from the mouse RAW 264.7 cell line. We found that selenite induced apoptotic cell death related to mitochondrial malfunction in the osteoclast-like cells.

2. Materials and methods

2.1. Cell culture and osteoclast differentiation

RAW 264.7 cells were maintained under an atmosphere of 5% CO_2 at 37 °C in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics. The differentiation of osteoclasts from RAW 264.7 cells (2×10^5 per 60-mm dish) was induced in α-minimum essential medium (α-MEM; Gibco) containing receptor activator of NF-κB ligand (RANKL) at a concentration of 300 ng/ml, as described previously (Takami et al., 2002); after 3 days, the cells were supplied with fresh medium, and incubated for an additional 12 h. The osteoclast-like cell cultures were then treated for 6 h in the additional presence of 5 or 10 sodium selenite (Na_2SeO_3; Sigma, St. Louis, MO).

2.2. Tartrate-resistant acid phosphatase (TRAP) staining

Cells were fixed with 10% formaldehyde for 10 min and washed with a 1:1 (v/v) mixture of ethanol and acetone. The cells were then stained with Fast Red Violet LB salt (500 μg/ml, Sigma) dissolved in TRAP buffer [120 mM sodium acetate, 66 mM sodium tartrate, naphthol AS phosphate (100 μg/ml), pH 5.2], washed with tap water, air-dried, and examined with a light microscope. TRAP-positive cells with three or more nuclei per cell were counted as osteoclasts.

2.3. DNA fragmentation test

Cells were grown on 60-mm culture dishes at a density of 2×10^5 cells/dish. For detection of DNA fragmentation, total cells (floating and adherent) were harvested after treatment with selenite and lysed in a solution containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 4% Triton X-100, and 0.5% deoxycolate. Extranuclear DNA was then extracted with an equal volume of neutral phenol:chloroform (1:1, v/v). The DNA was precipitated with two volumes of 100% ethanol, washed with 70% ethanol, air-dried, and suspended in 40 μl of DNase-free RNase A (50 μg/ml). 20 microliters of the resuspended DNA was resolved by electrophoresis on a 1.2% agarose gel. The gel was stained with ethidium bromide and observed under ultraviolet illumination.

2.4. Assay of caspase activity

After exposure of osteoclasts to selenite for 6 h, total (floating and adherent) cells were washed with ice-cold PBS, maintained on ice for 15 min in a solution containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin, and homogenized by repeated passage through a 26-gauge needle. Caspase activity was determined by measurement of absorbance at 405 nm with a microplate reader (Bio-Rad, Richmond, CA), as described (Jeong et al., 2001).

2.5. Measurement of superoxide anion and free thiol content

For measurement of $O_2^{\bullet -}$ generation, RAW 264.7 cells (2×10^3 cells/well) were transferred to 96-well culture plates and induced to differentiate into osteoclasts. They were then incubated for 4 h in the additional presence of 1.5 mM nitroblue tetrazolium (NBT, Sigma) with or without 5 or 10 μM selenite. The cells were washed with PBS and then lysed in 200 μl of isopropanol by repeated passage through the tip of a pipette. The formation of formazan from the reaction of NBT with $O_2^{\bullet -}$ was evaluated by measurement of absorbance at 595 nm (Kim et al., 2003).

For measurement of total thiol groups, RAW 264.7 cells (2×10^5 per 60-mm dish) were induced to differentiate into osteoclasts, treated with selenite for 6 h, and washed with ice-cold PBS. After homogenization and centrifugation, the resulting supernatant (cytosolic fraction) was incubated for 15 min at room temperature with 500 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma), as described previously (Kim et al., 2002). Absorbance at 405 nm was then measured, with glutathione as a standard for calibration.

2.6. Analysis of mitochondrial protein aggregation

Mitochondria were isolated from mature osteoclasts as described previously (Kim et al., 2002). They (1 mg/ml) were incubated for 1 h at room temperature with 1 mM selenite in a solution containing 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and 0.5 μM rotenone and then harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The mitochondrial pellet was dissolved by the addition of 100 μl of SDS sample buffer [50 mM Tris–HCl (pH 6.8), 0.1% bromophenol blue, 10% glycerol, 2% SDS], with or without 160 mM DTT, and incubation for 5 min at 80 °C. Portions (10 μl) of the resulting solution were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel, which was then stained with Coomassie brilliant blue.

2.7. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondria (1 mg/ml) were suspended in a solution containing 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and 0.5 μM rotenone and were incubated at 4 °C first for 5 min in the additional presence of 5 mM succinate and then for 30 min in the additional presence of 1 mM selenite and 5 μM rhodamine 123 (Sigma). The $\Delta\Psi_m$ of the mitochondria was then determined by flow cytometry with excitation at 488 nm and emission monitored at 530 nm, as described previously (Kim et al., 2002).

2.8. Immunoblot analysis of cytochrome c release

Mitochondria (1 mg/ml) were suspended in a solution containing 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and 0.5 μM rotenone and were incubated at 4 °C first for 5 min in the additional presence of 5 mM succinate with or without 5 μM EGTA and then for 30 min in the additional presence of 1 mM selenite. The mitochondria were removed by centrifugation at 12,000 × *g* for 10 min at 4 °C, and the resulting supernatant was fractionated by SDS-PAGE on a 12% gel. The separated proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) and probed with a mouse monoclonal antibody to cytochrome *c* (BD Bioscience Pharmingen, San Diego, CA). Immune complexes were detected with horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin G (Calbiochem, San Diego, CA) and enhanced chemiluminescence reagents (Pierce, Rockford, IL).

3. Results

3.1. Effects of selenite on RAW 264.7 cell-derived osteoclasts

To examine the cytotoxic effects of selenite on mature osteoclasts, we first induced to differentiate RAW 264.7 cells into osteoclast-like cells by culture in the presence of RANKL (Fig. 1A). The differentiated cells were exposed to selenite for 6 h and then examined by phase-contrast microscopy. Selenite-treated osteoclasts exhibited morphological changes, such as membrane blebbing, in the cells in a concentration-dependent manner, as a result of apoptotic events (Fig. 1B). Selenite also induced internucleosomal DNA fragmentation in the osteoclasts in a concentration-dependent manner (Fig. 1C). These findings thus show that selenite indeed induces apoptosis in osteoclasts.

3.2. Generation of the superoxide anion and thiol oxidation in selenite-treated osteoclasts

We next examined whether selenite induces oxidative stress in osteoclasts. The superoxide anion is generated intracellularly as a result of electron leakage from the mitochondrial electron transport chain (Jacobson, 1996) as well as by the action of several enzymes, such as NADPH oxidase, cyclooxygenase, nitric oxide synthase, and xanthine oxidase. It is also formed by the reaction

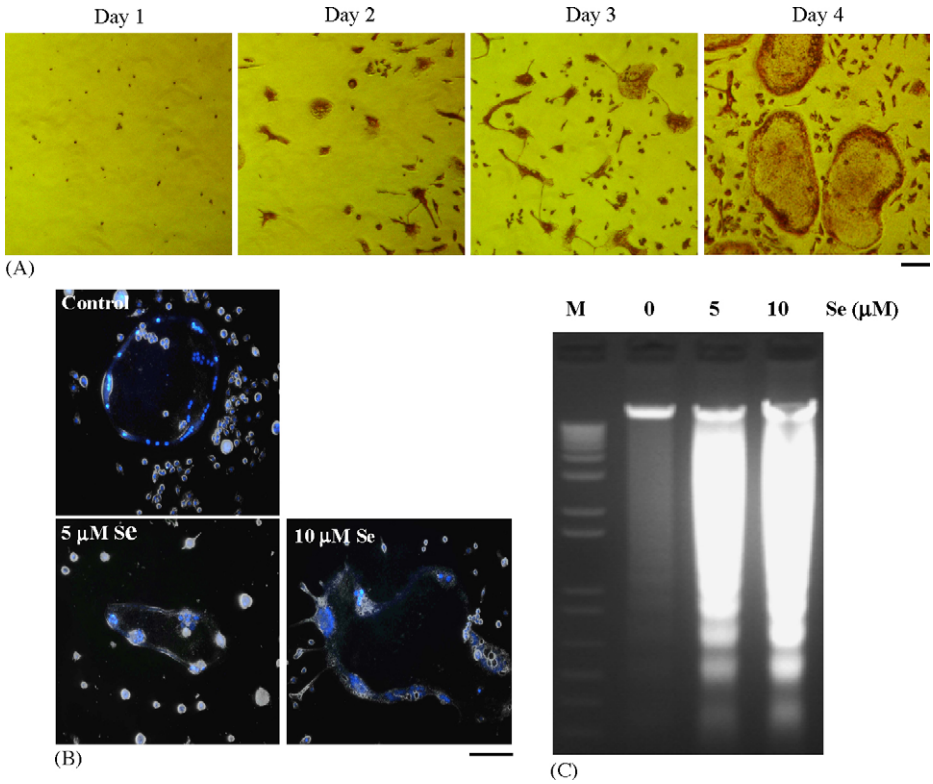

Fig. 1. Effects of selenite on the apoptotic cell death of RAW 264.7 cell-derived osteoclasts. RAW 264.7 cells were completely differentiated into osteoclasts in the presence of RANKL (A). Mature osteoclasts were incubated for 6 h in the absence (control) or presence of selenite (5 or 10 μM). Cells were then observed with a light microscope (B). Scale bar, 50 μm. DNA fragmentation was evaluated by agarose gel electrophoresis (C).

of selenite with the reduced form of glutathione (Kim et al., 2003; Shen et al., 1999). To investigate the effect of selenite on generation of the superoxide anion in osteoclasts, we treated the cells with selenite in the presence of NBT and measured the formation of formazan, the product of the reaction between the superoxide anion and NBT. Exposure of osteoclasts to selenite induced a concentration-dependent increase in the amount of the superoxide anion in the cells (Fig. 2A). We also examined whether $O_2^{\bullet -}$ is generated by the reaction of selenite with glutathione in mitochondria isolated from mature osteoclasts. The generation of $O_2^{\bullet -}$ by mitochondria was increased in a concentration-dependent manner by the addition of either selenite, reduced glutathione, or both agents (data not shown).

Selenite reacts with cysteine residues of proteins, resulting in thiol oxidation (Kim et al., 2003). We therefore examined whether such oxidation also occurs in osteoclasts treated with selenite. Selenite indeed induced thiol oxidation in osteoclasts in a concentration-dependent manner (Fig. 2B). These results thus suggest that selenite increases the production of the superoxide anion and induces thiol oxidation in osteoclasts.

3.3. Selenite-induced protein aggregation, loss of $\Delta\Psi_m$, and cytochrome c release in osteoclast mitochondria

Mitochondria play a central role in the regulation of apoptosis (Green and Reed, 1998). The opening of the MPT pore, which is induced by apoptotic stimuli, results in loss of the $\Delta\Psi_m$, swelling of the mitochondrial matrix, and consequent rupture of the outer mitochondrial membrane and the release of proapoptotic proteins, such as cytochrome *c* and apoptosis-inducing factor (Madesh and Hajnoczky, 2001; Saelens et al., 2004). To assess the effects of selenite on mitochondria isolated from osteoclasts, we first examined whether this agent might induce protein aggregation by acting as a thiol cross-linker. Incubation of isolated mitochondria with 1 mM selenite for 1 h resulted in the formation of protein aggregates of high molecular weight (Fig. 3A). This effect of selenite was reversed by treatment of the mitochondria with DTT, indicating that it was the result of thiol cross-linking. We next examined the effects of selenite on mitochondrial events associated with apoptosis. Selenite induced a decrease in the $\Delta\Psi_m$ of isolated mitochondria

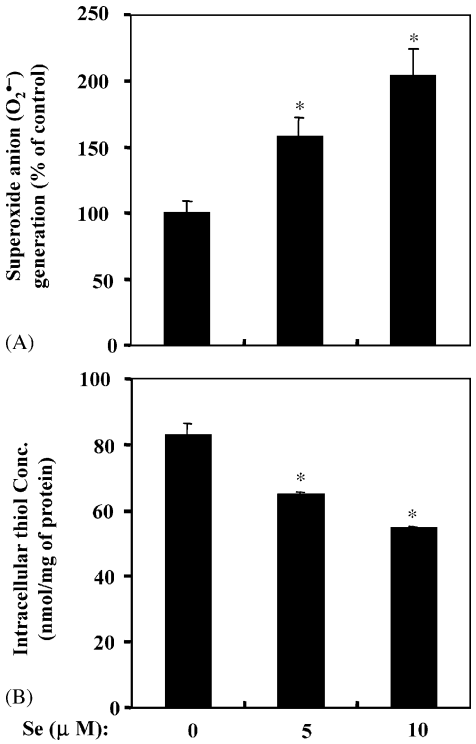

Fig. 2. Generation of the superoxide anion and thiol oxidation in selenite-treated osteoclasts. (A) Superoxide anion generation. Osteoclasts were incubated for 4 h with NBT in the absence or presence of 5 or 10 μM selenite, after which the intracellular accumulation of formazan was determined. Data are expressed as a percentage of the value for control cells and are means ± S.D. from three experiments. (B) Thiol oxidation. Osteoclasts were incubated in the presence of 0, 5, or 10 μM selenite for 6 h, after which a cytosolic fraction was prepared and assayed for the total number of free thiol groups. Data are expressed as nanomoles of thiol groups per milligram of protein and are means ± S.D. of triplicates from a representative experiment. $^{*}p < 0.01$ vs. control.

(Fig. 3B) as well as the release of cytochrome *c* from these organelles (Fig. 3C). Selenite-induced cytochrome *c* release was inhibited by the Ca^{2+} chelator EGTA, consistent with the proposed role for Ca^{2+} in opening of the MPT pore (Fagian et al., 1990). These data thus indicate that selenite is able to induce mitochondrial dysfunction, characterized by the formation of protein aggregates due to thiol cross-linking, collapse of the $\Delta\Psi_m$, and cytochrome *c* release, in osteoclast-like cells.

3.4. Selenite-induced caspase-3 activation

To determine whether selenite indeed induced apoptosis in osteoclasts, we examined the activation status of caspase-3 and caspase-8, members of a family of cysteine proteases that play a key role in the morphological changes and DNA fragmentation associated with apoptosis (Grutter, 2000). Selenite induced a concentration-dependent increase in caspase-3 activity but had no effect on the activity of caspase-8 (Fig. 4A). We finally examined the effects of an MPT pore blocker (cyclosporin A) and a caspase-3 inhibitor (DEVD-CHO) on selenite-induced apoptosis in osteoclasts. Both cyclosporin A and DEVD-CHO inhibited selenite-induced DNA fragmentation in the osteoclasts (Fig. 4B), implicating both opening of the MPT pore and activation of capase-3 in selenite-induced apoptosis in these cells.

4. Discussion

With the exception of bisphosphonates, which are potent inhibitors of bone resorption and are used in the treatment of osteoporosis (Reszka et al., 1999), minerals or metabolites have not previously been shown to induce apoptosis in osteoclasts. We have now shown that selenite induces apoptosis in mature osteoclasts.

Bone, which is continuously remodeled as a result of the activities of bone-forming osteoblasts and bone-resorbing osteoclasts, is composed of organic (collagen and noncollagenous proteins) and inorganic components, with the mineral constituents including calcium and hydroxyapatite (Rho et al., 2004). Some minerals and vitamins, such as Vitamins C, D, and K, are thought to contribute to bone metabolism (Schaafsma et al., 2001). The target cells of one such osteotropic factor, 1α,25-dihydroxyvitamin D_3, are osteoblasts, which express RANKL and macrophage colony stimulating factor and are thus capable of stimulating osteoclast formation (Lacey et al., 1998; Yasuda et al., 1998).

The selenium concentration of various tissues of the human body increases according to the rank order liver < spleen < pancreas < heart < brain < lung < bone < skeletal muscle (Zachara et al., 2001). With regard to the total body content of selenium, skeletal muscle contains the greatest amount (25–50%) followed by bone (16%). Selenium circulates through the food chain. The geographic distribution of selenium in soil varies greatly, with volcanic regions commonly possessing selenium-deficient soil. A diet deficient in selenium has been linked to various medical conditions, including Kashin–Beck disease (osteoarthritis), Keshan's disease (cardiomyopathy), cretinism, adverse mood states (depression), and white muscle disease (Rayman, 2000). It is therefore important to maintain sufficient levels of selenium in the body through consumption of selenium sources, such as plants, meat, and water.

The cancer-preventive effects of selenium in humans and animals are thought to be mediated by three distinct

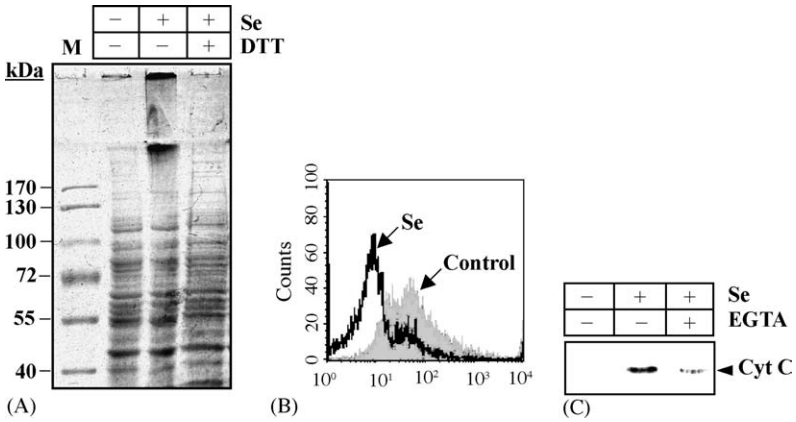

Fig. 3. Selenite-induced protein aggregation, loss of the $\Delta\Psi_m$, and cytochrome *c* release in osteoclast mitochondria. (A) Protein aggregation. Mitochondria isolated from osteoclasts were incubated in the absence or presence of 1 mM selenite for 1 h, after which the organelles were solubilized with SDS sample buffer with or without 160 mM DTT. Mitochondrial proteins were then resolved by SDS-PAGE and stained with Coomassie brilliant blue. The positions of molecular size standards (in kilodaltons) are indicated on the left. (B) Collapse of $\Delta\Psi_m$. Isolated mitochondria were incubated as described in Section 2. The $\Delta\Psi_m$ was then analyzed by flow cytometry. (C) Cytochrome *c* release. Mitochondria were treated as in (B) with the exception that the incubations with succinate and selenite were performed in the absence or presence of 5 μM EGTA (without rhodamine 123). The mitochondria were removed by centrifugation, and the resulting supernatant was subjected to immunoblot analysis with a monoclonal antibody to cytochrome *c*.

mechanisms: (1) many selenoproteins, including glutathione peroxidase and thioredoxin reductase, in which selenium is specifically incorporated as selenocysteine, function as scavengers of reactive oxygen species, which are recognized as carcinogenic (Ganther, 1999). (2) Selenium regulates intracellular signaling related to cell survival through oxidation of essential thiol groups of proteins, such as the transcription factors AP-1 and NF-κB and the protein kinase JNK (Handel et al., 1995; Kim and Stadtman, 1997; Jeong et al., 2002; Park et al., 2000a,b). (3) Selenium inhibits cell cycle progression and regulates apoptosis through thiol modification and activation of caspase-3 (Ip et al., 2000; Kaeck et al., 1997; Park et al., 2000a,b; Sinha et al., 1996). Our present results suggest that selenite-induced apoptosis in osteoclasts is mediated by the mitochondrial pathway. Selenite-induced apop-

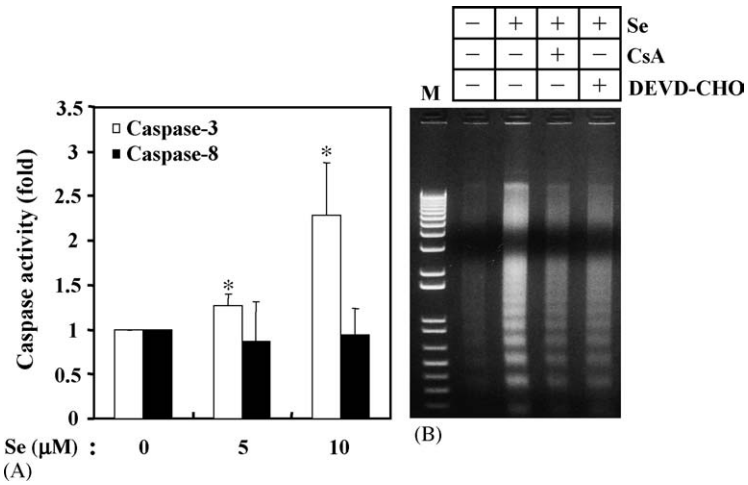

Fig. 4. Activation of caspase-3 and inhibitory effects of an MPT pore blocker (cyclosporin A) and a caspase-3 inhibitor (DEVD-CHO) on selenite-induced DNA fragmentation in osteoclasts. (A) Caspase activity. Mature osteoclasts were incubated for 6 h in the presence of 0, 5, or 10 μM selenite, after which cytosolic extracts of the cells were assayed for caspase-3 and caspase-8 activities. Data are expressed as fold increase in activity relative to the control cells and are means ± S.D. from three independent experiments. $^{*}p < 0.05$ vs. control. (B) Inhibition of selenite-induced DNA fragmentation by cyclosporin A and DEVD-CHO. Cells were incubated first for 1 h in the absence or presence of 2 μM cyclosporin A or 2 μM DEVD-CHO and then for 6 h in the additional absence or presence of 5 μM selenite. Extracted DNA was then fractionated by agarose gel electrophoresis.

tosis in osteoclasts was inhibited by cyclosporin A, a blocker of the MPT pore, as well as by DEVD-CHO, a cell-permeable inhibitor of caspase-3. We thus showed that selenite induced the generation of the superoxide anion in these cells, thiol modification both in intact cells and in isolated mitochondria, opening of the MPT pore, and consequent loss of the $\Delta\Psi_m$, cytochrome *c* release, and caspase-3 activation. Moreover, a primary target of selenium in the body has been suggested to be hematopoietic progenitors (Raisbeck, 2000). We have now shown that intraperitoneal injection of mice with sodium selenite (10 or 20 mg/kg) resulted in inhibition of the differentiation of isolated bone marrow-derived monocytes (BMMs) into osteoclasts in vitro and culture of BMMs from naive mice with selenite (5 or 10 μM) also inhibited osteoclastogenesis (Supplementary data). These inhibitory effects of selenite on osteoclastogenesis is resulted from growth retardation.

In conclusion, we have shown that selenite both inhibits the differentiation of BMMs into osteoclasts and induces apoptosis by the mitochondrial pathway in mature osteoclasts. Pathological bone loss, osteoporosis, is caused by either menopause or bone-related tumors. Compounds, such as estrogens, tamoxifen, and bisphosphonates, have been reported to induce bone-resorbing osteoclastic apoptosis (Arnett et al., 1996; Halasy-Nagy et al., 2001; Hughes et al., 1996), which plays a critical determinant in life time of its cells. Bone resorption has been a target for pharmaceutical therapy. Although the mechanism by which selenite inhibits osteoclastogenesis remains to be determined, our data might provide a basis for the design of novel selenium-based therapeutic strategies for the treatment of osteoclast-related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2005.06.019.

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