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Transition Metal Induces Apoptosis in MC3T3E1 Osteoblast: Evidence of Free Radical Release

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Transition metal ions including Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+} have been thought to disturb the bone metabolism directly. However, the mechanism for the bone lesion is unknown. In this study, we demonstrated that MC3T3E1 osteoblasts, exposed to various transition metal ions; selenium, cadmium, mercury or manganese, generated massive amounts of reactive oxygen species (ROS). The released ROS were completely quenched by free radical scavengers-N-acetyl cysteine (NAC), reduced glutathione (GSH), or superoxide dismutase (SOD). First, we have observed that selenium (10 μM), cadmium (100 μM), mercury (100 μM) or manganese (1 mM) treatment induced apoptotic phenomena like DNA fragmentation, chromatin condensation and caspase-3-like cysteine protease activation in MC3T3E1 osteoblasts. Concomitant treatment of antioxidant; N-acetyl-L-cysteine (NAC), reduced-form glutathione (GSH), or superoxide dismutase (SOD), prevented apoptosis induced by each of the transition metal ions. Catalase or dimethylsulfoxide (DMSO) has less potent inhibitory effect on the apoptosis, compared with NAC, GSH or SOD. In line with the results, nitroblue tetrazolium (NBT) stain shows that each of the transition metals is a potent source of free radicals in MC3T3E1 osteoblast. Our data show that oxidative damage is associated with the induction of apoptosis in MC3T3E1 osteoblasts following Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+} treatment.

Key Words: Heavy metal ions, Antioxidant, Catalase, Dimethylsulfoxide, Nitroblue tetrazolium, MC3T3E1 osteoblast

INTRODUCTION

Over the past several years, there have been considerable interests in the effects of chronic exposure to low levels of transition metals, especially with respect to their immunotoxic potential (Clarkson, 1997; Pollard & Hultman, 1997). In addition, the release of metal ions from biomaterials has been well documented in vitro (Leirskar, 1974; Muller, 1990; Watahae, 1991; Bumagardner Lucas, 1995) and in vivo (Berglund, 1990; Gjerdet, 1991). The released amounts of elements such as mercury may approach dietary intake and therefore, pose a potential hazard. Cadmium ingestion also induces osteomalacia or osteoporosis in experimental condition (Keiko, 1993). These phenomena have been discussed about both direct and indirect actions of cadmium on bone tissue. However, the precise mechanism about the bone disease induced by transition metals including mercury or cadmium has not yet established.

In this regard, we have initiated experiments aimed at characterizing the immunotoxic properties of various transition metals such as selenium, cadmium, mercury or manganese in osteoblasts. Findings to date indicate that selenium, cadmium, mercury, or man-

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ganese kills MC3T3E1 osteoblast; the cells exhibit signs of cell death within 24~48 hrs. We attribute the cytotoxicity to cellular injury associated with apoptosis, or programmed cell death. Apoptosis is a mode of cell death with morphological features quite distinct from those of necrosis (Kerr et al, 1972). The morphological features of apoptosis include chromatin margination along the nuclear membrane, nuclear condensation, budding, karyorrhexis, cell shrinkage, and cell fragmentation (Shenker et al, 1998). The integrity of the cellular organelles and plasma membrane is maintained until late in the process, unlike necrosis.

A family of cysteine proteases, termed caspases, which are homologous to the Caenorhabditis elegans death gene ced-3, is a common and critical component of the cell death pathway and subdivided into three major groups; caspase-1 (ICE)-, caspase-3 (CPP32)-, and caspase-2 (Ich-1)-like proteases (Yuan et al, 1993). Overexpression of these proteases leads to apoptosis of various cell types (Yuan et al, 1993). Among these caspases, caspase-3-like proteases are part of the general propagation and/or execution stages of programmed cell death (Alnemri, 1997). We now report that antioxidants like N-acetyl-L-cysteine (NAC), glutathione (GSH), or superoxide dismutase (SOD) protect MC3T3E1 osteoblast against death induced by transition metals. Our data suggest that each of the free radical scavengers can prevent transition metal-induced apoptosis in MC3T3E1 osteoblast. Clarifying the role of oxidative stress in the apoptosis could lead to novel therapeutic strategies for transition metal-induced bone toxicity.

METHODS

Culture of osteoblastic cell line, MC3T3E1

MC3T3E1 cells were cultured at 37°C in a fully humidified air atmosphere with 5% CO_2 in 100-mm plastic dishes containing an α-Minimum Essential Medium (-MEM), supplemented with 10% fetal bovine serum (FBS) (GIBCO Laboratories, Grand Isle, NY) and 100 U/ml penicillin G. A total of 5×10^4 cells/35 mm dish were cultured to 2 ml α-MEM containing 10% FBS. After incubation of cells for 3 days, the medium was removed and fresh medium containing an appropriate concentration of $SeSO_4$, $CdCl_2$, $HgCl_2$, or $MnCl_2$ was added. The cells were then cultured for 2 more days with transition metal ions.

MTT assay

For determination of cell viability, MC3T3E1 cells (1×10^4 cells/ml) were grown in 96-well plates for 24 hrs. After incubation with apoptotic stimuli for 24 hrs, cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) for 4 hrs at 37°C. The cell culture medium was removed, and cells were lysed in 2-isopropanol containing 0.04 M HCl and the amount of metabolized MTT was determined photometrically.

DNA extraction and electrophoresis

The characteristic ladder pattern of DNA break was analyzed by agarose gel electrophoresis. Briefly, DNA from the MC3T3E1 cells (1×10^6 cells/each group) was isolated by Wizard Genomic DNA purification kit (Promega Co, Wisconsin Medicine, WI, USA) and isolated by serial ethanol precipitation. Isolated genomic DNA (10 μg) was subjected on 1.5% of agarose electrophoresis at 100 V for 1 hr. DNA was visualized by staining with ethidium bromide under UV light.

Quantitation of apoptosis

Apoptosis was measured by a previously described method (Harmon et al, 1990) with some modification. Coverslips were fixed for 5 min in 3% paraformaldehyde in phosphate-buffered saline. After air-drying, coverslips were stained for 10 min in Hoechst 33258 (10 g/ml), mounted in 50% glycerol containing 20 mM citric acid and 50 mM orthophosphate, and stored at -20°C before analysis. Nuclear morphology was evaluated using a Zeiss IM 35 fluorescent microscope at an excitation and emission wavelength of 440 and 460 nm, respectively. The cells were scored and categorized into normal, apoptotic and necrotic, according to the previously described criteria (Oberhammer et al, 1992). For each heavy metal, more than five slides were prepared and more than 300 cells/slide were examined. Hoechst 33258-stained cells from the above categories were measured and expressed as a percentage of the total counted cells.

Measurement of activities of caspase-1 and caspase-3

For analysis of activities of caspase-1 and caspase-3, the heavy metals-treated cells (10^6 cells) were washed with PBS twice and suspended in 100 μl of buffer A [0.1 M HEPES (pH 7.4), 2 mM DTT, 0.1% CHAPS, 1% sucrose]. The cell lysate was centrifuged at 18500×g for 15 min, and the supernatant was obtained. The reaction was initiated by addition of 20 μM YVAD-AFC or DEVD-AFC to 50 μl of apoptotic extract at 37°C and product formation was measured using a Hitachi F-2000 spectrofluorometer with excitation at 400 nm and emission at 505 nm.

Nitroblue tetrazolium (NBT) staining

To measure whether free radical is released, NBT staining is performed as previously described (Lopez-Huertas et al, 1999). NBT (Sigma grade 3) 10 mg was dissolved in 10 ml phosphate-buffered saline at pH 7.4. MC3T3E1 cells after 2 hrs of transition metal ion treatment were incubated in this NBT solution at 37°C in a 5% CO_2 humidified atmosphere for 30 min after which they fixed in 10% phosphate-buffered formalin. Microscopic examination of MC3T3E1 cells was performed using a Nikon Diaphot phase contrast inverted microscope at lower power (×40). The total number of NBT-positive cells/well in 24 well plate was counted.

Statistical analysis

Results obtained from six independent experiments were analyzed using Student's *t*-test for paired variance and they were considered statistically significant at $p<0.05$.

RESULTS

Initially cells were cultured in the absence or presence of various concentrations of Cd^{2+}, Se^{2+}, Hg^{2+} or Mn^{2+} for 48 hrs. To address the ability of each of the transitional metals to induce cell death, we first investigated the effect of Cd^{2+}, Se^{2+}, Hg^{2+} or Mn^{2+} on cell viability using MTT assay. Fig. 1 indicates that while the toxicity of the transition metal ions was dose dependent, a differential sensitivity to each metal ion appeared. Thus, in MC3T3E1 cells, a significant mortality was detected after incubation with 10 μM Se^{2+} or 100 μM Hg^{2+} for 18 hrs, while a significant decrease of cell viability was detected after incubation with 100 μM Cd^{2+}, or 1 mM Mn^{2+} for 36 hrs (Fig. 1B). As the morphological changes observed in apoptosis provided the most reliable markers to explore the nature of the heavy metal-induced cell death, cells were treated with Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+} and cell morphology was evaluated. In consistent, after 18 hrs incubation with 10 μM Se^{2+} and 100 μM Cd^{2+} or after 36 hrs incubation with 100 μM Hg^{2+} and 1 mM Mn^{2+}, apoptotic cells were identified in the studied cell lines by their condensed and fragmented nuclei (Fig. 2). By contrast, when cells were exposed to 50 μM Se^{2+}, 200 μM Cd^{2+}, 200 μM Hg^{2+} or 2 mM Mn^{2+}, cell disruptions, chro-

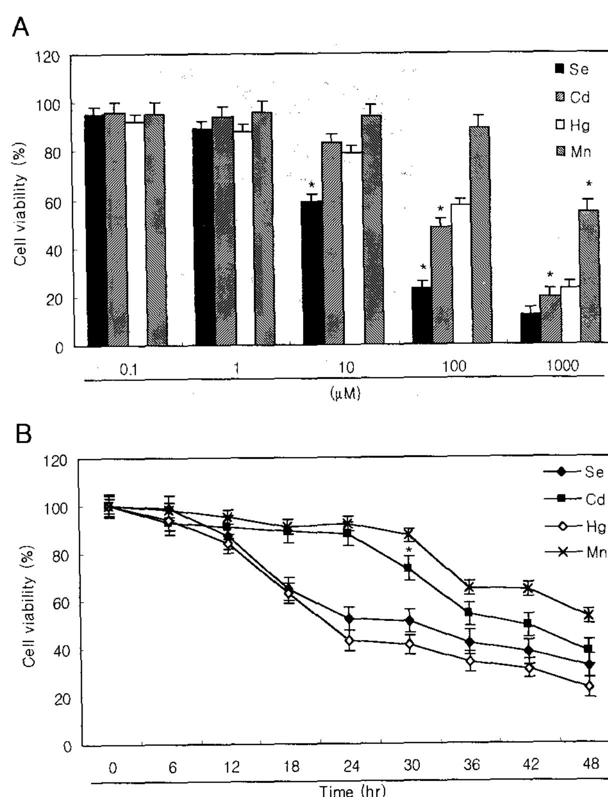

Fig. 1. Effect of selenium, cadmium, mercury, or manganese on cell viability. (A) MC3T3E1 cells were incubated with various concentrations of Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+} for 48 hrs, (B) with the indicated concentrations of 10 μM Se^{2+}, 100 μM Cd^{2+}, 100 μM Hg^{2+} or 1 mM Mn^{2+} for 0, 6, 12, 18, 24, 30, 36, 42 or 48 hrs. The number of viable cells was measured by the MTT test. Results are mean±S.E.M. of four independent experiments. Significant difference from control; $^*p<0.05$.

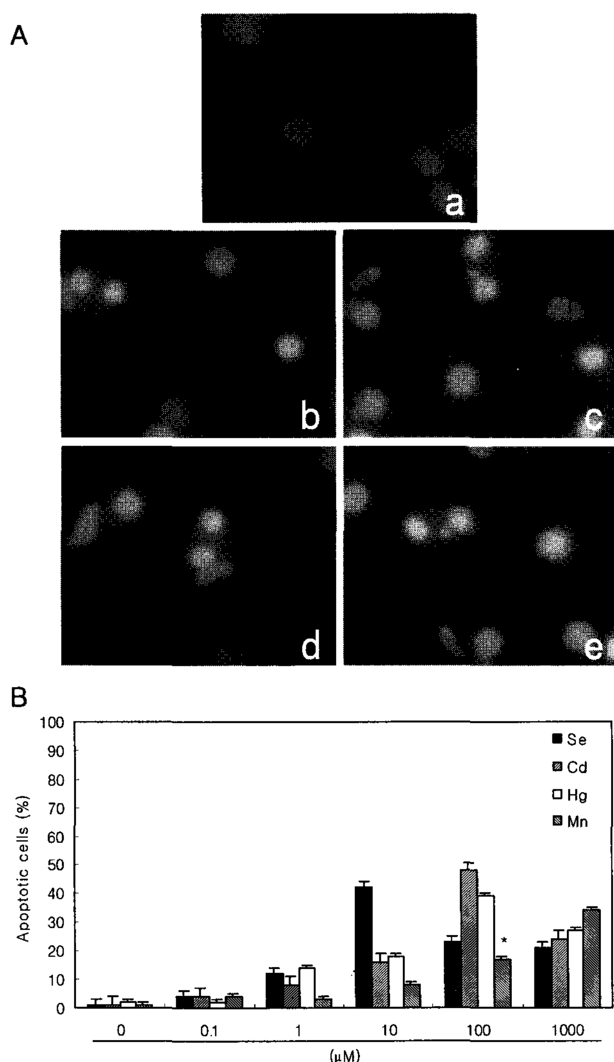

Fig. 2. Morphological evaluation by fluorescene microscopy of selenium, cadmium, mercury or manganese-induced cell death. (A) MC3T3E1 cells were incubated for 24 hrs with the medium alone (a), with selenium at 10 μM (b), or with mercury at 100 μM (c), or for 36 hrs with the medium alone , with cadmium at 100 μM (d), or with manganese at 1 mM (e). And then the cells were stained with Hoechst 33258 (10 μM), the nucleus condensation of the cells was analyzed with a fluorescence microscope. (B) Apoptotic cells were quantified by fluorescence microscopy. Results are mean $\pm$ S.E.M. of four independent experiments. Significant difference from control; $^*p<0.05$.

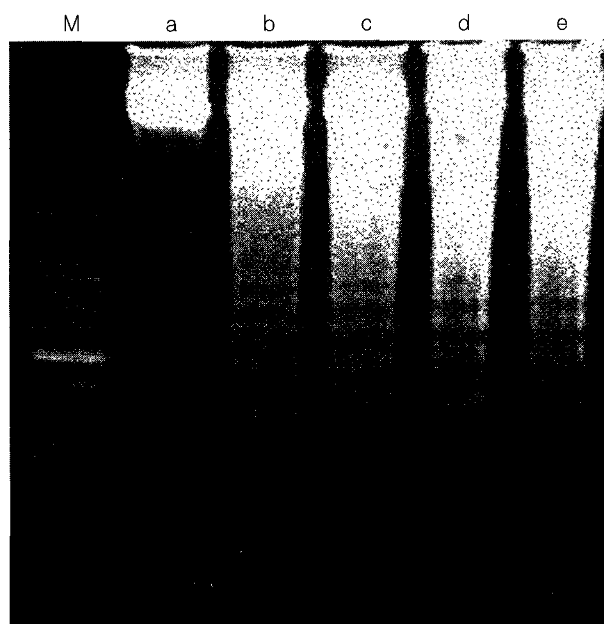

Fig. 3. DNA fragmentation in selenium, cadmium, mercury or manganese-treated cells. Agarose gel electrophoresis of DNA from MC3T3E1 cells treated with medium alone (a), with selenium at 10 μM (b) or with mercury at 100 μM (c) for 24 hrs, or with cadmium at 100 μM (d) or manganese at 1 mM (e) for 36 hrs. M: marker.

matin lysis, and diffusion (characteristics of necrosis) were observed (data not shown).

As the biochemical hallmark of apoptosis is the cleavage of DNA into oligonucleosome-length fragments, cells were finally treated with 10 μM Se^{2+} or 100 μM Hg^{2+} for 18 hrs or with 100 μM Cd^{2+} or 1 mM Mn^{2+} for 36 hrs and DNA was extracted and electrophoresed. As shown in Fig. 3, DNA from heavy metal ion-treated cells was degraded into discrete fragments. Oligosomal DNA fragmentation consistent with the onset of apoptosis, was evident after 18 hrs in Se^{2+} or Hg^{2+} and after 36 hrs in Cd^{2+} or Mn^{2+}-treated cells.

Using antioxidants such as N-acetyl-L-cysteine (NAC), glutathione (GSH), superoxide dismutase (SOD), catalase, or dimethylsulfoxide (DMSO), we analyzed DNA from these cells by agarose gel electrophoresis for evidence of fragmentation (Fig. 4C). By the treatment of NAC (10 mM), GSH (10 mM) or SOD (300 IU), a substantial DNA fragmentation was inhibited in each transition metal-treated cells. However treatment of other antioxidants such as catalase (200 IU) or DMSO (0.001%) did not suppress subsequent occurrence of apoptosis induced by each of the transition metals. In line with the results, NAC, GSH, or SOD significantly reduced the number of apoptotic cells, while the extent of apoptotic cell death was reduced to only 23% in catalase or DMSO-

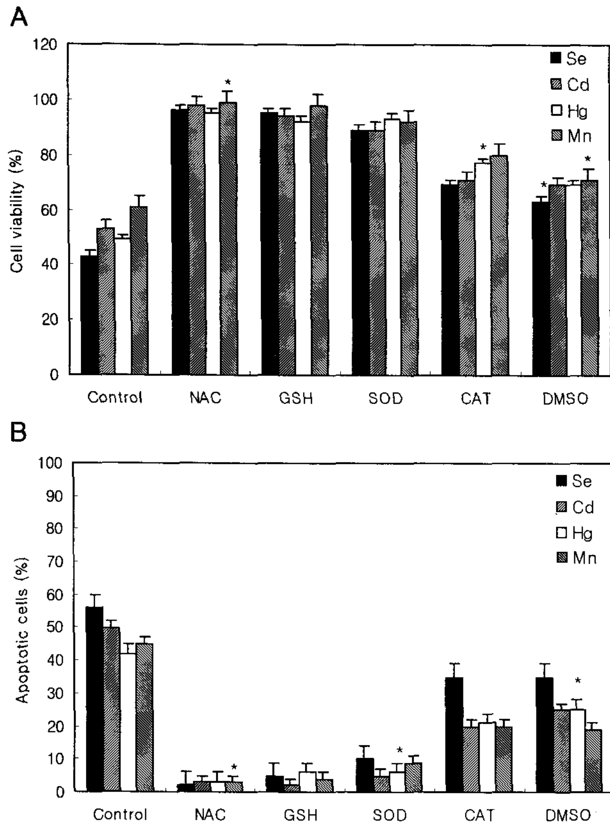

Fig. 4. Effect of antioxidants on selenium, cadmium, mercury or manganese-induced apoptosis in MC3T3E1 osteoblasts. MC3T3E1 cells were incubated with the medium alone, selenium at 10 μM, mercury at 100 μM, cadmium at 100 μM, or manganese at 1 mM in the presence or absence of 10 mM NAC, 10 mM GSH, 200 IU SOD, 200 IU catalase or 0.001% DMSO. The number of viable cells was measured by the MTT test (A). Apoptotic cells were quantified by a fluorescence microscopy (B). Results of (A) and (B) are mean$\pm$ S.E.M. of four independent experiments.

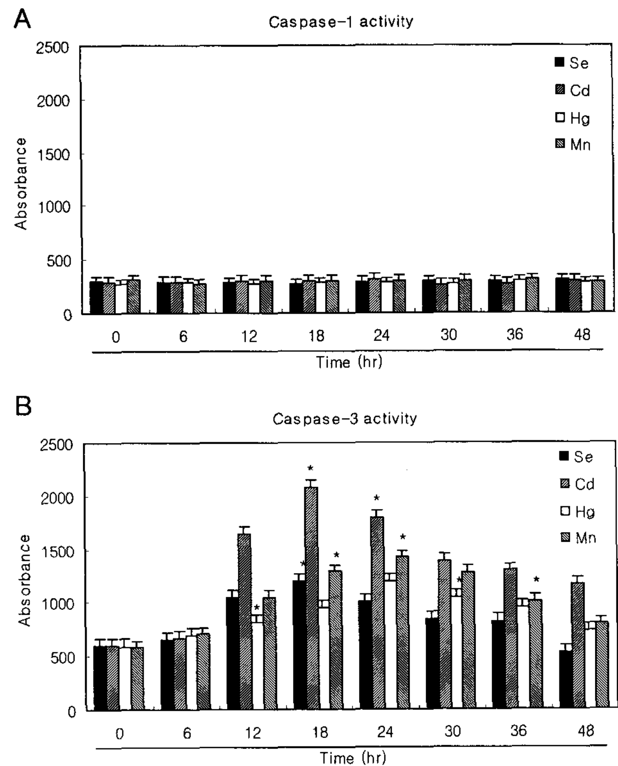

Fig. 5. Effect of antioxidants on selenium, cadmium, mercury or manganese-activated caspase in MC3T3E1 osteoblasts. Caspase-1 and -3 activities were measured by cleavage of YVAD- and DEVD-AFC respectively. (A) MC3T3E1 cells (1×10^6 cells/ml) were incubated with the medium alone, selenium at 10 μM, mercury at 100 μM, cadmium at 100 μM, or manganese at 1 mM. (B) in the presence or absence of 10 mM NAC, 10 mM GSH, 200 IU SOD, 200 IU catalase or 0.001% DMSO for the indicated times. After the treatment, samples were assessed for YVAD- or DEVD-AFC to the cell extract. Product formation (protease activity) was monitered by measuring the fluorescence. Results are mean$\pm$S.E.M. of four independent experiments. Significant difference from control; *$p<0.05$.

treated cells (Fig. 4B). These results show that the release of reactive oxygen species is an important signal of heavy metal-induced apoptosis in MC3T3E1 osteoblasts.

It has been well documented that caspase-1 and its related cysteine proteases such as caspase-3 play important roles in apoptosis. Activation of caspase-1 and caspase-3 in Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+}-treated cells was measured using a fluorometric assay with the substrates YVAD-AFC and DEVD-AFC, respectively. In MC3T3E1 cells, maximal caspase-3 activity was observed after treatment with Se^{2+} or Hg^{2+} for 12 hrs (Fig. 4A). In Cd^{2+} or Mn^{2+}-treated cells, maximal caspase-3 activity was observed at 32 hr or 36 hr after treatment of the heavy metals. Caspase-1 was not activated by treatment with Se^{2+}, Cd^{2+}, Hg^{2+}, or Mn^{2+}-induced apoptosis. We also observed that the inhibition of apoptosis by NAC, GSH, or SOD was associated with suppressed caspase-3-like activity. As shown in Fig. 5B, NAC, GSH, or SOD efficiently blocked the heavy metals-induced caspase-3-like protease activation. For catalase or DMSO-treated cells, the activity of caspase-3 was reduced to 25%. These results indicate that caspase-3 is involved

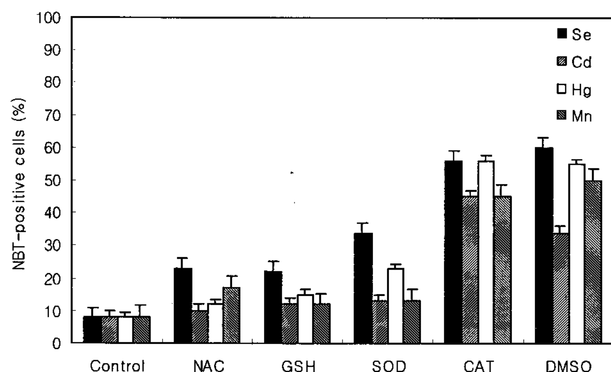

Fig. 6. NBT staining in selenium, cadmium, mercury or manganese-treated cells. MC3T3E1 cells were incubated with the medium alone, selenium at 10 μM, mercury at 100 μM, cadmium at 100 μM, or manganese at 1 mM, in the presence or absence of 10 mM NAC, 10 mM GSH, 200 IU SOD, 200 IU catalase or 0.001% DMSO for 2 hrs. And then NBT-positively stained cells were counted as described in Materials and Methods. Results are mean $\pm$S.E.M. of four independent experiments.

in Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+}-induced apoptosis and that DNA ladder formation follows the activation of caspase-3, suggesting involvement of reactive oxygen species in the heavy metal-mediated apoptosis.

To investigate whether reactive oxygen species are released in each the heavy metal-treated condition, the cells were examined using the inverted microscope at low power ($\times$40) after staining with NBT (NitroBlue Tetrazolium). NBT-positive cells were seen in the cells exposed to the agents. In the heavy metal-exposed cells pretreated with NAC (10 mM), GSH (10 mM), or SOD (200 IU), NBT-positive cells were so few as to be difficult to detect (Fig. 6). However, hydroxyl radical scavengers like dimethylsulfoxide (DMSO) or catalase partially reduced the number of NBT-positive cells, compared with other antioxidants.

These data indicate (that) when MC3T3E1 osteoblasts were treated to various heavy metals, free oxygen radicals were generated and resulted in apoptosis.

DISCUSSION

Transition metal consists of a group of environmental threats with strongly toxic effects. The mechanism producing cellular damage depends on the type of metal, its subcellular distribution and also on the concentration of the exposure (Cherian & Ferguson, 1997). In addition, the release of metals at high concentrations during their transport into the cell can cause rupture of the cell membrane, resulting in loss of homeostatic control, impairment of energy metabolism and cell death by necrosis. On the other hand, during that accumulation the metal can directly influence various inflammatory processes, affecting certain enzymes or producing indirect effects such as the formation of free radicals, peroxides or cytokines (Kane et al, 1993; Thompson, 1995; Kasprzak, 1997).

We studied transition metals such as Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+}-induced apoptosis in MC3T3E1 osteoblast. Each of the heavy metals promoted apoptosis, which was manifested by DNA fragmentation and the typical morphological changes of apoptosis. A dramatic reduction in cell viability without substantial DNA fragmentation was observed in high concentrations, as indicated by the morphological changes of necrotic cell death (data not shown).

In this study, we have observed that reactive oxygen species are released in Se^{2+}, Cd^{2+}, Hg^{2+} or Mn^{2+}-exposed MC3T3E1 cells. As shown in nitroblue tetrazolium (NBT) staining (Fig. 6), all of these agents rapidly release reactive oxygen species. NBT is a useful marker that is reduced to a blue insoluble formazan by free radical intermediates. It has been widely used to investigate radical production in various cells (Zhang et al, 1996; Lopez-Huertas et al, 1999). It is recently reported that osteoclasts produce reactive oxygen species (ROS) at the interface between the bone surface and the osteoclast membrane. Superoxide anion or other ROS resulting from superoxide production stimulate bone resorption. Khalkhali' report (1997) indicated a possible osteoblast-dependent control of superoxide dismutase related glycoprotein expression and in turn H_2O_2 production by the osteoclast. Since osteoblasts reside in the direct vicinity of osteoclasts, these reports indicate the need for a H_2O_2 scavenging enzyme system in osteoblasts. Furthermore, the observation that $HgCl_2$, one of transition metal ions, perturbs both mitochondria membrane potential and pH_i suggests that the metal binds to and damages the inner mitochondrial membrane (Tai, 1998). While mitochondrial-dependent depletion of ATP would by itself damage the cells due to the failure to provide energy for membrane pumps, a greater hazard is the generation of ROS. Our results show that osteoblasts have the capacity to reduce NBT, and suggest when osteoblasts are the

cells in which free radical generation occurs when exposed to Se^{2+}, Cd^{2+}, Hg^{2+}, or Mn^{2+}. Antioxidants such as N-acetyl-L-cysteine (NAC), glutathione (GSH), superoxide dismutase (SOD), catalase or dimethylsulfoxide (DMSO) conferred significant decrease of NBT positively stained cell numbers.

Next, the ability of antioxidants to inhibit each heavy metal-mediated apoptosis has been observed in MC3T3E1 osteoblast (Fig. 4). Much evidence that apoptosis can be induced by oxidative stress, has been provided by studies in which mediators of apoptosis either induced free radicals, or were inhibited by the addition of antioxidants (Hirose et al, 1993). These data are the first to demonstrate that free radical release is required in the apoptosis in MC3T3E1 osteoblast exposed to each of the transition metals-Se^{2+}, Cd^{2+}, Hg^{2+}, or Mn^{2+}. Recently, it has been reported that Cd^{2+} interferes with mitochondrial function (Koizumi et al, 1994) and causes lipid peroxidation (Manca et al, 1991), resulting in oxidative stress to the cell, which may ultimately lead to apoptosis (Slater et al, 1995).

Previous studies have shown that cytochrome c, which is released from mitochondria during the induction of apoptosis, can initiate cleavage of procaspase-3 and activation of caspase-3 (Guoha et al, 1998). It has been also reported that the activated caspase-3 cleaved DNA fragmentation factor, which is a human homologue of the mouse inhibitor of caspase-activated deoxyribonucleosomal sites, and led to DNA fragmentation at internucleosomal sites (Alnemri, 1997).

In agreement with these reports, this present study suggests that the transition metals induce the generation of free radicals, and subsequently initiate the activation of caspase-3, resulting in apoptosis in MC3T3E1 osteoblast. As a consequence, the inhibition of heavy metal-induced apoptosis by antioxidants may constitute a single and critical target for therapeutic intervention in those pathologies associated with abnormal bone resorption associated with transition metals.

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