Cell Death Caused by Selenium Deficiency and Protective Effect of Antioxidants*

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Selenium is an essential trace element and it is well known that selenium is necessary for cell culture. However, the mechanism underlying the role of selenium in cellular proliferation and survival is still unknown. The present study using Jurkat cells showed that selenium deficiency in a serum-free medium decreased the selenium-dependent enzyme activity (glutathione peroxidases and thioredoxin reductase) within cells and cell viability. To understand the mechanism of this effect of selenium, we examined the effect of other antioxidants, which act by different mechanisms. Vitamin E, a lipid-soluble radical-scavenging antioxidant, completely blocked selenium deficiency-induced cell death, although α-tocopherol (biologically the most active form of vitamin E) could not preserve selenium-dependent enzyme activity. Other antioxidants, such as different isoforms and derivatives of vitamin E, BO-653 and deferoxamine mesylate, also exerted an inhibitory effect. However, the water-soluble antioxidants, such as ascorbic acid, N-acetyl cysteine, and glutathione, displayed no such effect. Dichlorodihydrofluorescein (DCF) assay revealed that cellular reactive oxygen species (ROS) increased before cell death, and sodium selenite and α-tocopherol inhibited ROS increase in a dose-dependent manner. The generation of lipid hydroperoxides was observed by fluorescence probe diphenyl-1-pyrene, only in selenium-deficient cells. These results suggest that the ROS, especially lipid hydroperoxides, are involved in the cell death caused by selenium deficiency and that selenium and vitamin E cooperate in the defense against oxidative stress upon cells by detoxifying and inhibiting the formation of lipid hydroperoxides.

Selenium is an essential trace element for humans and many other forms of life, and a deficiency of this element induces some pathological conditions, such as cancer, coronary heart disease, and liver necrosis (1–5). Selenium deficiency is also accompanied by a loss of immunocompetence (6), and both cell-mediated immunity and B-cell function can be impaired (7). Supplementation with selenium has shown immunomodulatory effects, including an enhancement of activated T-cell proliferation (8). Selenium is an essential component of several enzymes such as glutathione peroxidase (GPx) (9), thioredoxin reductase (TR) (10), and selenoprotein P (SeP) (11), which contain selenium as selenocysteine. It is also well known that selenium is essential for cell culture when a serum-free medium is used (12). Serum-free media, especially for immune cells and neurons, contain insulin, transferrin, and sodium selenite. Without selenium, cells can neither proliferate nor survive. However, the underlying mechanism for the role of selenium in cell proliferation is still unknown.

Vitamin E, a generic term for tocopherols and tocotrienols, is one of the most potent lipid-soluble antioxidants (13). Vitamin E occurs in nature in at least eight different isomers: α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols (14). Tocotrienols differ from the corresponding tocopherols only in their aliphatic tail. Vitamin E deficiencies have been implicated in some pathologic conditions, such as cancer, coronary heart disease, and liver necrosis (15, 16) and are also accompanied by a loss of immunocompetence (17). It is well known that selenium and vitamin E show compensative effects and that a deficiency of both elements causes massive injury in some cases (18–20).

In the present study, we characterize the nature of cell death caused by selenium deficiency and the cell death inhibitory effect of antioxidants including vitamin E. We also demonstrate the involvement of reactive oxygen species (ROS), especially lipid hydroperoxides, on the cell death.

EXPERIMENTAL PROCEDURES

Chemicals—Sodium selenite, bovine serum albumin fraction V, tert-buty1 hydroperoxide, and GSH were obtained from Nacalai, Kyoto, Japan; GSH reductase from Oriental Yeast Co., Ltd., Tokyo, Japan; RPMI 1640 medium, seleno-DL-cystine, seleno-DL-methionine, and seleno-L-methionine from Sigma-Aldrich Co.; recombinant human insulin and human transferrin from Wako, Osaka, Japan; 3,4,5-dimethyldihydroxybenzaldehyde; SeP, selenoprotein P. From the §Human Stress Signal Research Center (HSSRC), National Institute of Advanced Industrial Science and Technology (AIST), 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan and the ¶Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12 Nishi 6, Kita-ku, Sapporo, 060-0812, Japan

Experimental Procedures

Cell Culture and Determination of Cell Viability—Jurkat E6–1 cells, human T-leukemia (American Tissue Type Collection) were maintained in RPMI 1640 medium containing 100 units/ml penicillin G, 100 μg/ml
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Effect of Selenium Deficiency on the Viability of Jurkat Cells—To determine the effect of selenium on viability, Jurkat cells were cultured with serum-free RPMI 1640 medium (ITA-RPMI). When cultured with a selenium-deficient medium, the viability of Jurkat cells decreased with incubation time (Fig. 1). The cell viability started to decrease after 24 h, and a higher than 95% loss was observed within 60 h. The viabilities as measured by MTT and TPB assay were in close agreement. In contrast, Jurkat cells cultured with serum-free RPMI 1640 medium containing 100 nM sodium selenite did not show any significant loss of viability.

Characterization of Cell Death Caused by Selenium Deficiency—To identify the type of cell death caused by selenium deficiency, PS exposure and caspase activity were analyzed. The selenium-deficient cells cultured with ITA-RPMI for 36 h were incubated with annexin V-FITC and propidium iodide and then subjected to flow cytometry analysis. Selenium-deficient cells showed not only signs of PS exposure but also uptake of propidium iodide (Fig. 2B). The dead cells in the selenium-
deficient medium did not show any exclusion of propidium iodide for the time tested (24–40 h, data not shown). Caspase activity in the selenium-deficient cells was also measured using DEVD peptide conjugated to the chromophore pNA. In the selenium-deficient cells cultured for 36 h, caspase activity was below the background level seen in control and selenium-sufficient cells (Fig. 2C), while activity was detected in cells treated with 50 μM hydrogen peroxide for 6 h (apoptotic condition), but not in those treated with 500 μM hydrogen peroxide (necrotic condition), as previously reported (28). Selenium-deficient cells did not show caspase activation for the time tested (24–36 h, data not shown), suggesting that this cell death is necrotic rather than apoptotic.

**Inhibitory Effect of Selenium-containing Protein and Compounds on Cell Death Caused by Selenium Deficiency**—A dose-dependent study of the inhibitory effects of selenium revealed that sodium selenite at levels higher than 10 nM protected cells almost completely (Fig. 3). The ED_{50} of sodium selenite was 3.3 ± 1.5 nM. SeP, which functions as a selenium supply protein (22), also demonstrated an inhibitory effect, with the ED_{50} being 0.066 nM. Selenium-containing amino acids, such as seleno-DL-cystine, seleno-L-methionine, and seleno-DL-methionine, also inhibited cell death (Table I). Ebselen, which is a mimic of GPx (29), did not have an inhibitory effect. To clarify temporally the site of selenium action in selenium deficiency-induced cell death, sodium selenite was added to cells at various time points after culturing with the selenium-deficient medium. Almost complete protection of cell death was observed even when sodium selenite was added at 24 h after selenium deficiency (Fig. 4).

**Effects of Selenium Deficiency on the Enzyme Activity of Selenoproteins**—We next examined the effects of selenium deficiency on the enzyme activity of cellular selenoproteins in Jurkat cells. As shown in Fig. 5, cellular GPx (cGPx), phospholipid hydroperoxide GPx (PH-GPx), and thioredoxin reductase (TR) activities were reduced in selenium-deficient cells grown in a selenium-deficient medium for 24 h. cGPx, PH-GPx, and TR activities were reduced to 36, 36, and 39%, respectively, of those in control cells grown in a medium containing serum. Selenoenzyme activities of cells cultured with a selenium-deficient medium for 48 and 72 h could not be measured because of the lower cell recovery rates (Fig. 5).

**Inhibitory Effect of Vitamin E and Other Antioxidants on Cell Death Caused by Selenium Deficiency**—As described above, a marked decrease of selenoenzyme activities was observed in the selenium-deficient cells. It is well known that these selenoenzymes play an important role in the antioxidative defense system. To understand the underlying mechanism of the protective effect of selenium, we examined the effect of other types of antioxidants. Water-soluble antioxidants, such as ascorbic acid, N-acetylcysteine, and glutathione, did not inhibit cell death caused by selenium deficiency even at 1 mM (Table I), whereas the lipid-soluble antioxidant α-tocopherol completely blocked cell death (Fig. 6), although α-tocopherol
did not produce any decrease of selenoenzyme activities (Fig. 5). Almost complete protection of cells was also observed even when \( \alpha \)-tocopherol was added at 24 h after selenium deficiency (Fig. 4). We also observed the inhibitory effect of other forms of vitamin E, such as \( \beta \), \( \gamma \), and \( \delta \)-tocopherols and \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-tocotrienols (Fig. 6 and Table I). The \( ED_{50} \) values for tocotrienols were smaller than those of the corresponding tocopherols, suggesting that tocotrienols are more potent inhibitors than the corresponding tocopherol isomers. The cellular uptake of \( \alpha \)-tocotrienol was found to be 2.2-fold higher than that of \( \alpha \)-tocopherol after incubation for 72 h (data not shown), which corresponds well with the difference in their \( ED_{50} \) values, the ratio being 2.8-fold (Table I). We also examined the inhibitory effect of vitamin E derivatives, such as PMC and Trolox, the former being a short-chain homolog of \( \alpha \)-tocopherol and the latter a water-soluble analog of PMC. These compounds also blocked cell death (Table I), and the large difference in the \( ED_{50} \) between them suggests that the lipid-soluble antioxidant retained in the membranes exerts a higher level of activity than does the hydrophilic antioxidant. BO-653, a synthetic radical-scavenging antioxidant (21), showed a similar inhibitory effect to tocopherols (Table I). Deferoxamine mesylate, which has metal chelating properties, also completely blocked the cell death caused by selenium deficiency (Table I).

**Table I**

Effect of selenium and vitamin E on cell death induced by selenium deficiency

| Compound        | \( ED_{50} \) \( \mu M \) | Compound | \( ED_{50} \) \( \mu M \) |
|-----------------|---------------------------|----------|---------------------------|
| Sodium selenite | \( 3.2 \pm 1.5 \)         | \( \alpha \)-T | \( 36 \pm 8.1 \)         |
| SeP             | \( 0.066 \pm 0.027 \)      | \( \alpha \)-T3 | \( 13 \pm 6.5 \)         |
| Se-DL-Met       | \( 63 \pm 33 \)           | \( \beta \)-T       | \( 31 \pm 2.3 \)         |
| Se-L-Met        | \( 17 \pm 12 \)           | \( \beta \)-T3       | \( 24 \pm 2.9 \)         |
| Se-DL-Cys       | \( 0.62 \pm 0.42 \)       | \( \gamma \)-T       | \( 40 \pm 17 \)         |
| Ebselen         | —                         | \( \gamma \)-T3      | \( 23 \pm 5.0 \)         |
| Ascorbic acid   | —                         | \( \delta \)-T       | \( 46 \pm 9.3 \)         |
| GSH             | —                         | \( \delta \)-T3      | \( 39 \pm 10 \)         |
| N-acetyl cysteine| —                        | PMC        | \( 65 \pm 9.5 \)         |
|                 |                           | Trolox     | \( 630 \pm 320 \)       |
|                 |                           | BO-653     | \( 50 \pm 9.6 \)         |
|                 |                           | DFOM       | \( 390 \pm 85 \)        |

\( ^a \) \( ED_{50} \): the concentration to inhibit 50% cell death. Means and S.E. of three experiments are shown.

\( ^b \) \( \alpha \)-T, \( \beta \)-T, \( \gamma \)-T, and \( \delta \)-T, tocopherol; T3, tocotrienol; DFOM, deferoxamine mesylate.

\( ^c \) Not effective up to 10 \( \mu M \).

\( ^d \) Not effective up to 1 mm.

**Fig. 4.** Effect of sodium selenite and \( \alpha \)-tocopherol at various time intervals after selenium deficiency. 100 \( \mu M \) sodium selenite or 2 \( \mu M \) \( \alpha \)-tocopherol were added at the indicated times after cells were cultured in a selenium-deficient medium. After 3 days from selenium deficiency, the viability was measured by MTT assay, and the means \( \pm \) S.E. of three experiments are shown. \( ^* \), \( p < 0.05 \) when compared with the time 0.

**Fig. 5.** Effect of selenium deficiency on the enzyme activity of selenoproteins. Cells were cultured in a selenium-deficient medium (open circles), containing 100 \( \mu M \) sodium selenite (closed circles) or 2 \( \mu M \) \( \alpha \)-tocopherol (open squares) for the indicated times, and selenoenzyme activities (A, cGPx; B, PH-GPx; C, TR) in the cytosol were measured by coupled enzyme assay as described under “Experimental Procedures.” Enzyme activities of cells cultured in each medium relative to those of cells cultured in the serum medium are shown as means \( \pm \) S.E. (\( n = 3 \)). These enzyme activities in the serum medium were as follows: cGPx, 18; PH-GPx, 13; and TR, 16 nmol/min/mg protein.
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of selenium and α-tocopherol on intracellular ROS levels revealed that these compounds prevented the increase of DCF fluorescence in a dose-dependent manner (Fig. 7C). Water-soluble antioxidants, such as ascorbic acid, N-acetyl cysteine and glutathione, did not prevent the accumulation of intracellular ROS despite the addition of as much as 1 mM (data not shown).

**DISCUSSION**

The essential role of selenium in nutrition has been well established. It is also well known that selenium is necessary for cell culture when using a serum-free medium. Insulin (as a growth factor), transferrin (as an iron source), and selenite are added to the serum-free media for immune and neuronal cells. Although the effects of selenium on cell viability and the cell-cycle progression has been reported (12, 32), the underlying mechanism of the protective effect of this element has not yet been elucidated. In the present study using Jurkat cells, a model of proliferating T lymphoma cells, the decrease in cell viability was observed when applying a serum-free medium without selenium. This cell death was completely blocked by selenium-containing materials, except for ebselen, in a dose-dependent manner. It has been reported that these selenium-containing materials are incorporated and can be the cellular source of selenium used for synthesis of selenoprotein (22). SeP, which is a selenium-rich extracellular glycoprotein (11, 33, 34) that functions as selenium transport protein (22, 35, 36), was the most effective of the materials tested (Table I).

Although we observed a loss of cell viability after 24 h, Jurkat cells duplicated for 20 h under the serum-free culture conditions. This observation suggests that cell death occurred after a single division. We speculate that the proliferating cells became selenium-deficient, and the divided cells contained almost half of the selenoenzyme activities, such as cGPx, PH-GPx, and TR. In the presence of sodium selenite, these enzyme activities were retained or up-regulated in cells. The decrease in TR activity was lower than that of cGPx and PH-GPx activities in the Jurkat cells cultured with selenium-deficient medium for 72 h (Fig. 5). Selenoproteins have been proposed to follow a hierarchy for selenium supply in that the amounts of certain selenoproteins decrease more rapidly under selenium-deficient conditions (37). A previous study suggested that this was due in part to differences in the SECIS elements. Gadsada et al. (38) demonstrated that the element of TR was highly active; therefore, TR levels would be better preserved when the selenium supply was limited as in a selenium-deficient medium. It has been reported that these selenoproteins play an important role in the defense against oxidative stress (1, 39). In the case of lipid hydperoxides, it is known that PH-GPx, but not cGPx, is able to reduce lipid hydperoxides, including phospholipid hydperoxide and cholesterol hydperoxide, directly (40, 41). It has also been proved that overexpression of PH-GPx suppresses cell death due to oxidative damage induced by radical initiator and lipid hydperoxide (42, 43). Moreover, Lewin et al. (44) reported that TR also plays a role in preventing oxidative damage induced by tert-butyl hydperoxide and oxidized LDL, but the mechanism of the protection afforded by this selenoprotein against oxidative stress induced by lipid hydperoxides is still unclear. At present, these selenoproteins are assumed to play an important role in the defense against oxidative stress related to lipid hydperoxides. Under selenium-deficient conditions, the decrease in these selenoproteins is speculated to cause peroxidation in the lipid layer inside cells.

It is noteworthy that radical-scavenging antioxidants, such as α-tocopherol, completely blocked the cell death caused by selenium deficiency, although α-tocopherol did not affect the enzyme activity of selenoproteins. α-Tocopherol-supplemented Jurkat cells did not show any loss of cell viability despite the undetectable levels of cGPx and PH-GPx activity. Other isoforms of vitamin E, such as β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols, were also effective. Tocotrienols were more effective than the corresponding tocopherols (Table I), which may be ascribed primarily, if not solely, to the differences in the rate of cellular uptake. A higher uptake of α-tocotrienol than α-tocopherol into culture cells has been reported (45, 46). Such a difference was also observed for liposomal membranes (47). Vitamin E derivatives, such as PMC and its water-soluble analogue Trolox, also showed an inhibitory effect on cell death, but their higher ED₅₀ values (Table I) suggest that the antioxidant incorporated into cell membranes is more effective than that localized outside the membranes. Interestingly, a synthetic radical-scavenging antioxidant, BO-653, also showed an inhibitory effect, suggesting the importance of the inhibition of lipid peroxidation in the cell membranes. The fact that selenium deficiency, which results in a decrease in the capacity to reduce lipid hydperoxides, induced cell death and
that this cell death could be inhibited by radical-scavenging antioxidants that suppress the formation of lipid hydroperoxides strongly indicates the causative role of lipid hydroperoxides in cell death.

The ED$_{50}$ value of $\alpha$-tocopherol was as low as 36 nM (Table I). One may argue that this concentration is quite low compared with the physiological concentration; for example, 30 nM in human plasma. It should be pointed out, however, that the concentration of $\alpha$-tocopherol in the membrane is of more importance than that in the bulk phase. In the present study, the lipid concentrations were measured as follows; FC, 4.4; PC, 12; PE, 5.2 nmol/10^6 cells. Thus, the molar ratio of $\alpha$-tocopherol to total lipids in the cell culture system (10^6 cells/ml) was 36 x 10^-9/22 x 10^-8 M = 1/610 mol/mol, which is similar to that in human plasma; that is, 30 x 10^-6/11 x 10^-3 M = 1/370 mol/

Fig. 7. Generation of intracellular reactive oxygen species and its inhibition by sodium selenite and $\alpha$-tocopherol. Cells were cultured in a selenium-deficient medium, without and with 100 nM sodium selenite or 2 $\mu$M $\alpha$-tocopherol for indicated times, and the intracellular ROS production was measured using a fluorescence probe DCFH-DA as described under “Experimental Procedures.” A, cells were cultured in each medium for indicated times. *, $p < 0.05$ when compared with selenium-sufficient and $\alpha$-tocopherol-sufficient cells. B, cells were cultured for 24 h in a selenium-deficient medium in the presence of variable amounts of sodium selenite and $\alpha$-tocopherol. Mean values of DCF fluorescence per total protein relative to that of DCFH-DA-labeled control cells (time 0; 3,600 fluorescence/mg of protein) are shown with S.E. (n = 3).

Fig. 8. Formation of lipid hydroperoxide in Jurkat cells cultured in a selenium-deficient medium. Cells were cultured in a selenium-deficient medium, without and with 100 nM sodium selenite or 2 $\mu$M $\alpha$-tocopherol for the indicated times, and the intracellular lipid hydroperoxides were measured using a fluorescence probe DPPP as described under “Experimental Procedures.” A, cells were cultured in each medium for the indicated times. *, $p < 0.05$ when compared with selenium-sufficient and $\alpha$-tocopherol-sufficient cells. B, cells were cultured for 24 h in a selenium-deficient medium in the presence of variable amounts of sodium selenite and $\alpha$-tocopherol. Mean values of DPPP oxide fluorescence per total protein relative to that of DPPP-labeled control cells (time 0; 3,600 fluorescence/mg of protein) are shown with S.E. (n = 3).
mol. It may be noted that the micromolar ranges of α-toco-
pherol applied to many cell culture systems are not always
physiological, but that the concentration in the membranes
should be considered. Deferoxamine mesylate, a well-known
iron chelator, was also found to be a potent inhibitor of
cell death induced by selenium deficiency. It has been suggested
that iron plays an important role in oxidative damage to cells
(48, 49), by reacting with hydrogen peroxide or lipid hydroper-
oxides to form reactive oxygen radicals.

It was found that the removal of selenium from the culture
medium induced ROS production as measured by DCF fluo-
rescence and also lipid hydroperoxides as measured by DPPF
fluorescence and HPLC chemiluminescence. We are aware of
the inherent drawbacks of DCFH, but it can be a useful probe
for estimating semi-quantitatively the generation of ROS un-
der specific conditions (50). Both selenium and α-tocopherol
suppressed ROS by apparently different mechanisms, the for-
ermer by enhancing the reduction of hydroperoxides, while the
latter by inhibiting their formation. FC-OOH was detected as
a major lipid hydroperoxide, which was unexpected since poly-
unsaturated lipids in PC and PE are more susceptible to oxi-
dation than free cholesterol. One possible reason could be that
the reduction of FC-OOH by PH-GPx is at least six times slower
than that of phospholipid hydroperoxides (41).

Many reports have shown significant correlations between
selenium deficiency and the incidence of cancer (51, 52). It has
been also reported that selenium supplementation prevents the
generation of cancer (2, 53). As shown in this study, selenium
deficiency caused a significant increase in ROS and peroxida-
tion inside cells. It is therefore considered that selenium-defi-
cient conditions cause oxidative DNA damage that eventually
leads to cancer formation.

In conclusion, the present study clearly shows that selenium
deficiency decreased the activities of GPx, PH-GPx, and TR,
increased lipid peroxidation in the membranes, and eventually
induced cell death. The cell death was inhibited by other types of
antioxidants with different functions, such as tocopherols and
deferoxamine, which inhibit lipid peroxidation in the mem-
branes and sequester redox-active iron, respectively. These
results strongly indicate that the lipid hydroperoxides play a
causal role in the oxidative damage to cells induced by selenium
deficiency.

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