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Original Research Communication

Contribution of Thioredoxin Reductase to T-Cell Mitogenesis and NF-κB DNA-Binding Promoted by Selenite

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ABSTRACT

Although the essential role of selenium for cellular immune responses is obvious, delineation of the functions is lacking because selenium can either promote or inhibit cell growth, cytokine production, and activation of transcription factor nuclear factor-κB (NF-κB). Studies with human thioredoxin-1 (Trx-1)-transgenic (Tg) mice were conducted to evaluate the relationship between stimulation of T-cell mitogenic response by sodium selenite and the intracellular Trx-1 levels, and the activities of selenoenzymes and NF-κB–DNA binding. Concanavalin A-induced mitogenesis of wild-type mouse splenic cells was stimulated by exposure to low levels of selenite (0.02–0.1 μ*M*), with augmentation of NF-κB–DNA binding activity. Treatment with NF-κB nuclear translocation inhibitor SN50 or thioredoxin reductase (TR) inhibitor aurothioglucose depressed this stimulatory action. The mitogenic response of Trx-1-Tg mouse splenic cells was enhanced by exposure to relatively high levels of selenite ($ 0.05 μ*M*), compared with the wild-type mouse. Selenite also augmented TR activity but not cellular glutathione peroxidase activity in the Trx-1-overexpressed cells. These results suggest that the stimulation of T-cell mitogenic response by the physiological levels of selenite is predominantly caused by increased TR activity, which may lead to reduction of Trx-1 dependent on the intracellular expression level and promotion of DNA binding of NF-κB. *Antioxid. Redox Signal.* 9, 115–121.

INTRODUCTION

SELENIUM IS AN ESSENTIAL TRACE element for mammals and many other forms of life (35). Selenium has been shown to regulate many intracellular functions by being a chemical component of selenoproteins (1). The well-known selenoproteins are selenium-dependent enzymes such as cellular glutathione peroxidase (cGPx) and thioredoxin reductase (TR), which have selenocysteine residues in the catalytic centers and function as antioxidant enzymes (1). Selenium deficiency induces pathological conditions such as cancer, cardiovascular disease, and viral infections, some of which may be caused by a loss of immunological competence (40). In fact, selenium deficiency has been implicated in accelerated disease progression and poorer survival among populations infected with human immunodeficiency virus (2). Although the essential role of selenium for cellular immune reactions has thus been reported, delineation of the functions and underlying mechanisms is lacking. Selenium has been shown to cause cell growth promotion (30, 46) and cytokine production (31), and in contrast, cell growth inhibition (6, 38), induction of cell death (37) or apoptosis (47), and inhibition of transcription factor nuclear factor-κB (NF-κB) (10, 18). At higher concentrations than nutritional requirements, selenium has anticancer effects (42), which may be mediated through cell cycle arrest, apoptosis, and/or the toxic effect. These abilities are presumably exhibited by acting on the functions of many intracellular proteins important for signal transduction, depending on the chemical form and concentration of selenium.

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NF-κB plays an important role in regulation of the gene expression of cytokines and other immune response factors. In contrast to most transcription factors that are activated by a restricted number of extracellular stimuli, NF-κB is activated by widely different stimuli, including inflammatory cytokines, mitogens, and bacterial products (27). The actual activation cascade of NF-κB involves phosphorylation of a bound inhibitor IκBa by Iκ kinases (IKK) with subsequent ubiquitination, release, and proteolysis of IκBa, translocation from the cytosol to the nucleus, and binding of NF-κB to the promoter region of a target gene (18). Reactive oxygen species (ROS) enhances this activation in the cytoplasm (15). Thioredoxin-1 (Trx-1) and the TR system also serve as critical regulators for activation of NF-κB and promote the DNA binding by converting the essential cysteine residues in the protein into the active thiol forms (13, 33). Such redox regulation of transcription factors including NF-κB seems to be dependent on an intracellular redox balance between ROS production and their scavenging by glutathione (GSH)/cGPx and/or Trx-1/peroxiredoxin/TR systems. As supplementation of the most popular selenium compound, sodium selenite may not only induce selenoenzymes such as cGPx and TR, but may also consume endogenous GSH (36) and produce ROS (20). The action of selenite in NF-κB activation may depend on the selenium status and can be a tricky issue. The present study was thus designed to evaluate relationships between stimulation of T-cell mitogenic responses by sodium selenite and intracellular Trx-1 levels, and activities of selenoenzymes and NF-κB–DNA binding, compared with splenic cells which were prepared from human Trx-1-overexpressing transgenic (Tg) C57BL/6 mice and the wild-type mice as the control.

MATERIALS AND METHODS

Chemicals and animals

Sodium selenite (Na_2SeO_3, 99.999%), concanavalin A (Con A), and aurothioglucose were purchased from Sigma-Aldrich Japan (Tokyo). Male Trx-1-Tg mice were established as described previously (39) and the wild-type C57BL/6 mice were maintained at the same condition of 25 ± 1°C, 50 ± 2% relative humidity, and a light/dark cycle of 12 h each, and had access to sterilized water and pelleted rodent chow containing 135 ± 47 ng Se/g. These mice were used for experiments at 6 weeks of age.

T-cell mitogenesis assay

T-cell mitogenic response was assayed as described previously (32), with a slight modification. The splenic cells were prepared by flushing with filter-sterilized RPMI-1640 medium containing cysteine instead of cystine as a component, in order not to add 2-mercaptoethanol. The cells were dispensed into wells of a microtiter 96-well solid plate, white (Thermo Labsystems Oy, Helsinki, Finland) at 2 3 10^5 cells/well in 200 μL of the medium which was supplemented by 5 m*M* HEPES, 50 mg/L potassium benzylpenicillin, 50 mg/L streptomycin sulfate, and 10% fetal calf serum (FCS). The cells was then incubated for 72 h at 37°C in 5% CO2 after addition of a T-cell mitogen Con A at 2 μg/mL, selenite and the other additives at the specified concentration. Total DNA amount of the cultured cells was determined fluorometrically with ethidium bromide. Cell growth ratio was represented as percentage of cell number grown by the mitogen alone, which was estimated from a calibration curve between cell number and fluorescence intensity based on the DNA amount.

Determination of selenium status

Cultured cells were washed once with PBS after centrifugation at 900 *g* for 10 min at 4°C. The packed cells were suspended in 0.2% Triton-X- containing 50 m*M* phosphate buffer (pH 7.0), followed by one cycle of freezing and thawing. After centrifugation at 13,000 *g* for 15 min at 4°C, the supernatant was dialyzed with 8000 MW-cut-off membrane (Pierce, Biotechnology, Inc., Rockford, IL) against 50 m*M* phosphate buffer (pH 7.0) to remove endogenous thiolic substances. Activity of cGPx was determined according to the coupled enzymatic method (45) with a slight modification. The assay mixture contained 0.2 m*M* NADPH, 0.5 m*M* EDTA, 0.2 m*M* NaN_3, 2 m*M* GSH, 1 unit/mL GSH reductase, 10 m*M* phosphate buffer (pH 7.0), and 0.015 m*M* hydrogen peroxide as the substrate in a total volume of 1 mL, including a dialyzed sample. To measure TR activity, the dialyzate was heated at 55°C for 10 min to inactivate heat-instable thiol-reduction enzymes. The sample was cooled and centrifuged at 13,000 *g* for 30 min at 4°C. TR activity was determined by a spectrophotometric insulin-reduction assay, as described previously (12), with a slight modification. The assay mixture contained 0.8 m*M* NADPH, 0.34 m*M* insulin, 4.5 m*M* EDTA-2Na, 90 m*M* HEPES buffer (pH 7.0), and with or without 4.5 μ*M* *E. coli* Trx in 200 μL. The mixture was started by the addition of 50 μL of the supernatant sample at 37°C, incubated for 20 min, and stopped by the addition of 750 μL of a solution containing 0.4 mg/mL DTNB, 6 *M* guanidine hydrochloride, and 0.2 *M* Tris-HCl (pH 8.0). TR activity was calculated by measuring absorbance at 412 nm of duplicate reactions with and without Trx, as suggested by Holmgren and Björnstedt (14). Both activities of cGPc and TR were expressed as nmol NADPH oxidized per min per mg protein of the dialyzate and the heated supernatant, respectively. Selenium content in the diets and tissues was determined by fluorometry using 2,3-diaminonaphthalene (44).

Western blot analysis

Expressions of human and murine Trx-1 proteins were determined using anti-human Trx mouse monoclonal antibody and anti-murine Trx rabbit polyclonal antibody (Fujirebio, Tokyo, Japan) as described previously (39). Briefly, prepared splenic cells and the cultured cells were washed once with PBS after centrifugation at 900 *g* for 10 min at 4°C. The packed cells were suspended in a lysis buffer containing 0.5% Nonidet P-40, 10 m*M* Tris·HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride, and 0.111 units/mL aprotinin, and the extracts were cleared by centrifugation. Equal amounts of protein estimated using BCA Protein Assay Reagent Kit (Pierce) were electrophoresed on a 15% SDS-polyacrylamide gel, and then electrophoretically transferred to a poly(vinylidene difluoride) membrane (ATTO Co., Tokyo). After blocking with 1%

skim milk in Tris-HCl-buffered saline containing 0.05% Tween 20 at 4°C overnight, the membrane was incubated with the first antibody, and then with the peroxidase-linked anti-imouse IgG and the anti-rabbit IgG as the second antibodies (Cell Signaling Technology, Inc., Danvers, MA). b-Actin was used as a loading control with the anti-b-actin (Novus Biologicals, Inc., Littleton, CO). Chemiluminescence was detected with an ECL Western blot detection kit (GE Healthcare UK Ltd., Buckinghamshire, England). Immunoreactive bands were quantified by volume densitometry using Light-Capture and CS Analyzer (ATTO Co.,) and normalized to b-actin.

Measurement of NF-kB DNA binding activity

A couple of cell-permeable peptides carrying the hydrophobic sequence for intracellular translocation and the nuclear localization sequence (NLS) from NF-kB, p50, SN50, and the mutated sequence of NLS, SM (24) were supplied by Funakoshi (Tokyo). Cells after 24 h-cultivation of mitogenesis assay in the presence of the peptide were harvested and washed once with PBS after centrifugation, and the nuclear fraction was extracted using Nuclear Extract Kit (Active Motif, Tokyo, Japan). DNA-binding activity of NF-kB in the nuclear fractions was assayed using a chemiluminescence-based sandwich-type ELISA (Oxford Biomedical Research, Inc., Oxford, MI) that employed an oligonucleotide containing the DNA-binding NF-kB consensus sequence bound to a 96-well plate. The activity was expressed as pg NF-kB per mg protein in the sample.

RESULTS

Relevance between selenite-stimulated T-cell mitogenesis and NF-kB activity

The effect of supplementary sodium selenite on Con A-induced T-cell mitogenesis was tested using splenic cells prepared from wild-type C57BL/6 mouse. Con A-induced cell growth was promoted by the co-addition of selenite at the concentrations of 0.02–0.1 µ*M*, with an increase of NF-kB DNA-binding activity (Fig. 1). Thus the promoting effect by selenite was observed at relatively low exposure levels. Treatment with SN50, which is the cell-permeable peptide with the nuclear localization sequence (NLS) of NF-kB, and competitively inhibits the nuclear translocation, dose-dependently depressed both NF-kB activity and T-cell growth rate stimulated by 0.1 µ*M* selenite with Con A (Fig. 2). When the peptide carried the mutated NLS, SM was treated at 10 µg/mL that is the maximum concentration of SN50, neither NF-kB activity nor cell growth rate was significantly affected regardless of exposure to selenite. As shown in Fig. 3, treatment with the TR inhibitor aurothioglucose resulted in dose-dependent suppression of TR activity and T-cell growth rate, with inhibition of NF-kB activity at 25 m*M*. These results indicate that there is relevance among promotion of Con A-induced T-cell mitogenesis by relatively low levels of selenite, increase in NF-kB DNA-binding activity and TR activity.

Effect of selenite on T-cell mitogenesis and selenoenzyme activities in Trx-1-overexpressing splenic cells

To investigate the effect of intracellular Trx-1 on T-cell mitogenesis by sodium selenite, male Trx-1-Tg and the wild-type C57BL/6 mice were first acclimated under the same conditions until a consistent selenium status was kept. Although selenium levels in liver, testes, erythrocytes, muscle, and heart of both mice were almost the same at 6 weeks of age, there were slight differences between them in spleen, kidney, and plasma (data not shown). Indeed, selenium content in spleen was 9.4 ± 0.7 and 6.1 ± 1.2 nmol/g tissue in Trx-1-Tg and the wild-type mice, respectively. When splenic cells from both were prepared, the selenium level in the former cells was three times higher than that in the latter cells, as the level was 2.7 ± 0.7 and 0.9 ± 0.2 pmol/10^6 cells. As shown in Figure 4, however, there was almost no effect of selenite exposure on the expression of Trx-1 in Con A-induced T-cells. When both expressions of human and murine Trx-1 were normalized to b-actin, the relative expression of total Trx-1 in splenic cells of Trx-1-Tg mice was estimated

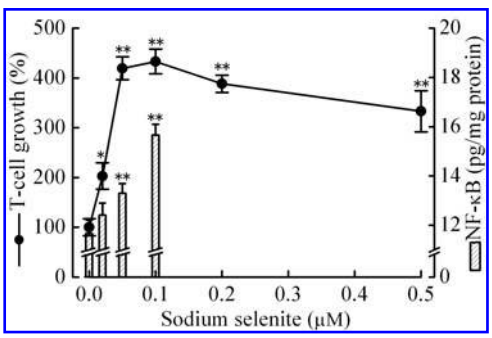

FIG. 1. Growth rate and NF-kB-DNA binding activity in T-cell mitogenesis of splenic cells exposed to selenite. The wild-type mouse splenic cells were exposed to 2 µg/mL Con A and 0–0.5 µ*M* selenite for 72 h to determine the cell growth (•), and NF-kB DNA-binding activity (▨) was measured after the initial 24 h. Data are given as means ± SD (n = 4). *p < 0.05; ** p < 0.01, compared with the control.

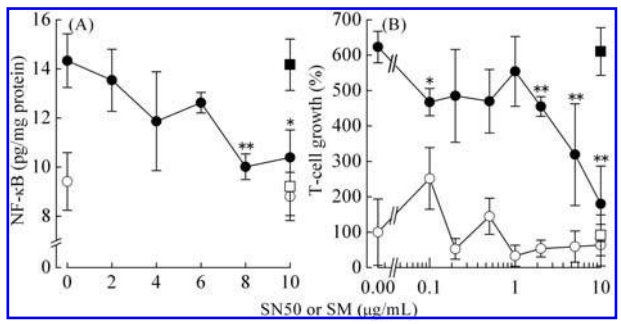

FIG. 2. Effect of SN50 on NF-kB-DNA binding activity (A) and growth rate (B) in selenite-promoted T-cell mitogenesis. The wild-type mouse splenic cells were exposed to 0–10 µg/mL SN50 (p), 10 µg/mL SM (m), 0.1 µ*M* selenite + 0–10 µg/mL SN50 (P) or 0.1 µ*M* selenite + 10 µg/mL SM (M) for 24 h with 2 µg/mL Con A. Data are given as means ± SD (n = 4). *p < 0.05; **p < 0.01, compared with the control.

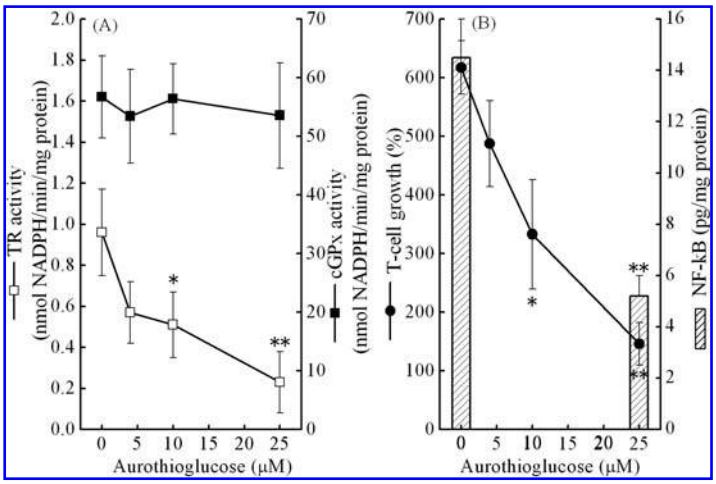

FIG. 3. Effect of aurothioglucose on TR and cGPx activities (A), growth rate and NF-kB-DNA binding activity (B) in selenite-promoted T-cell mitogenesis. The wild-type mouse splenic cells were exposed to 0–25 μ*M* aurothioglucose with 0.1 μ*M* selenite and 2 μg/mL Con A. TR (m) and cGPx (M) activities and cell growth rate (P) were determined after the T-cell mitogenesis for 72 h, and NF-kB DNA-binding activity (▨) was measured after the initial 24 h. Data are given as means ± SD ($n = 4$). *$p < 0.05$; **$p < 0.01$, compared with the control.

to be about five times higher than that of the wild-type mice.

We then examined the effect of Trx-1-overexpression on selenite-stimulated T-cell mitogenesis by comparing cell numbers after the mitogenic responses of both mouse splenic cells treated with physiological to toxic levels of selenite (Fig. 5). There was no significant difference between the mitogenic responses of both cells at the low selenite level of 0.02 μ*M*. However, mitogenesis was greatly enhanced in splenic cells of Trx-1-Tg mouse by the addition of relatively high levels of selenite ($ 0.05 μ*M*), compared with those of the wild-type mouse. The Trx-1-overexpressing splenic cells also lowered the toxicity of selenite that was observed at the concentration more than 2 μ*M* in the wild-type splenic cells. Figure 6 shows the selenoenzyme activities in these cells. Although TR activity was dose-dependently increased in both the wild type and Trx-1-overexpressed splenic cells by exposure to selenite at the levels up to around 0.1 μ*M*, cGPx activity was not enhanced in the latter cells under the same exposure conditions. These results therefore suggest that the selenoenzyme correlating with the promoting effect of T-cell mitogenesis by selenite in both cells is TR rather than cGPx.

DISCUSSION

In a preliminary study, we found that sodium selenite showed the most enhancing effect for T-cell mitogenesis at less than 0.1 μ*M* in ICR mouse splenic cells, of inorganic and organic selenium compounds tested. The stimulating effect of selenite on the mitogenesis using the wild-type C57BL/6

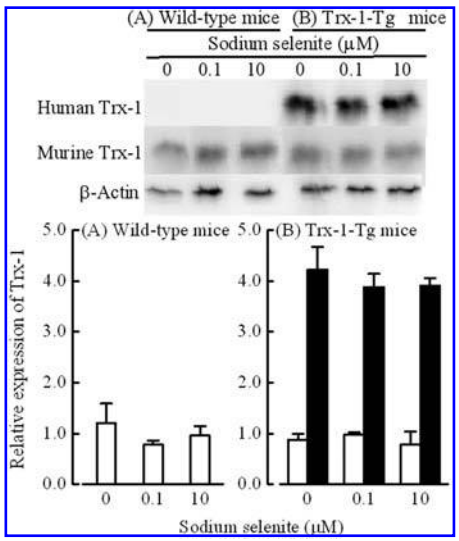

FIG. 4. Expressions of murine and/or human Trx-1 after selenite-promoted T-cell mitogenesis in the wild-type (A) and Trx-1-Tg mouse splenic cells (B). The wild-type and Trx-1-Tg mouse splenic cells were exposed to 2 μg/mL Con A and 0–10 μ*M* selenite for 72 h, and both expressions of murine Trx-1 (O) and human Trx-1 (O) were determined and normalized to b-actin. Data are given as means ± SE ($n = 3$).

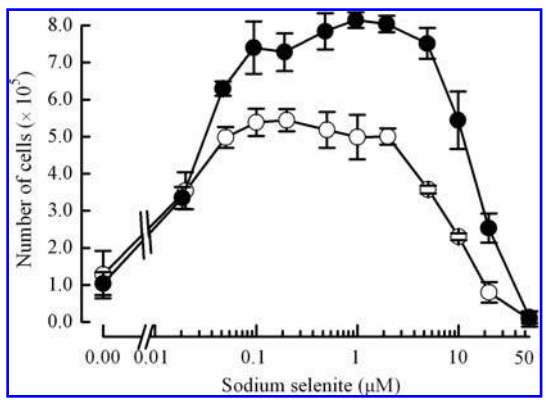

FIG. 5. Effect of selenite on cell number after T-cell mitogenesis of the wild-type and Trx-1-Tg mouse splenic cells. The T-cell mitogenic responses of splenic cells prepared from the wild-type (p) and Trx-1-Tg mouse (P) were performed as shown in Fig. 3 and compared by cell number. Data are given as means ± SD ($n = 4$).

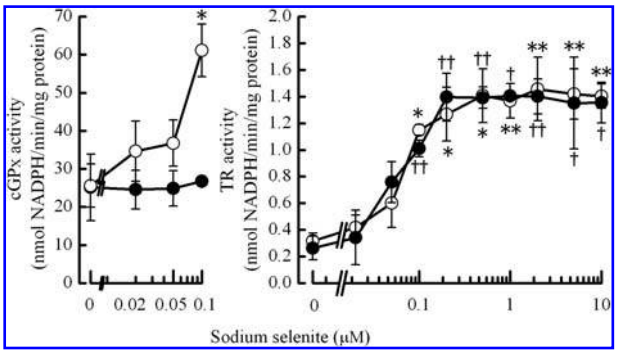

FIG. 6. Effect of selenite on TR and cGPx activities after T-cell mitogenesis in the wild type and Trx-1-Tg mouse splenic cells. The selenoenzyme activities were determined after the T-cell mitogenesis of the wild-type (p) and Trx-1-Tg mouse splenic cells (P) shown in Fig. 3. Data are given as means ± SD ($n = 4$). *$p < 0.05$; **$p < 0.01$ (wild-type), †$p < 0.05$; ††$p < 0.01$ (Trx-1-Tg), compared with the control.

mouse as the control of Trx-1-Tg was observed at almost the same response as ICR strain. The cell growth rate induced by Con A alone was time-dependently increased until 72 h-cultivation of mitogenesis, while NF-kB DNA-binding activity showed the maximum after the initial 24 h (data not shown). In this experimental condition, low levels (0.02–0.1 μ*M*) of selenite apparently promoted the transcription factor activity of NF-kB with the cell growth stimulated by Con A (Fig. 1), although the effect of selenite on the basal NF-kB activity in splenic cells cultured without the mitogen was not able to be specified on account of no cell growth. The cell growth-promoting effect by selenite was also connected with an augmentation of NF-kB DNA-binding activity because the nuclear translocation inhibitor SN50 dose-dependently depressed both NF-kB activity and T-cell growth at the concentration of more than 1 μg/mL, whereas with the peptide carrying the mutated NLS, SM had no affect (Fig. 2). The selenite level was adjusted to 0.1 μ*M*, which was the maximum concentration showing dose-dependency of the cell growth as well as NF-kB activity (Fig. 1) and almost the same level as that in FCS (0.18 μ*M*). Aurothioglucose, which inhibited TR activity, also suppressed the T-cell growth and NF-kB activity stimulated at the selenium level, with no inhibition of cGPx activity (Fig. 3). The gold compound is a potent inhibitor of selenocysteine-containing enzymes (26) and causes selective inhibition toward TR by suitable dosage. These results therefore suggest that the physiological level of selenite may promote Con A-induced T-cell mitogenesis and increase NF-kB DNA-binding activity through induction of TR activity.

However, a number of reports have shown that selenium compounds inhibit binding of NF-kB to nuclear responsive elements (18), nuclear translocation of NF-kB (10, 34), and/or IkBa phosphorylation by IKK (7). This inconsistency may result from differences between the target cell lines, redox status including ROS production, or exposure levels of selenium compounds used. For example, cells are often treated with them at higher concentrations than the immunological and/or nutritional requirements in anticipation of cancer chemotherapy (7), or protective effects for asthma (16) and inflammation (19). This may give a different redox status, depending on the selenium dosage. Another reason may be due to tumor necrosis factor a (TNF-a) (7,, 16, 25)), hydrogen peroxide (22), or quinolinate (34) used for activation of NF-kB, which may cause stronger activation of NF-kB than Con A used as a mitogen because selenium supplementation dose-dependently enhances release of cytokines, including TNF-a under the stimulation of lipopolysaccharide (31). Selenium also causes phosphorylation of Ser^{536} of the RelA/p65 subunit of NF-kB, which induces nuclear translocation of NF-kB, even though the ratio of the phospho-NF-kB to total NF-kB has been attenuated by selenium (41). On the other hand, NF-kB activation and the nuclear translocation are stimulated by oxidized glutathione, while the DNA binding is inhibited by the oxidizing conditions due to the redox-sensitive cysteine residue (4). Hence, this evidence suggests that modulation of NF-kB activity by selenium supplementation is dependent upon the experimental conditions that are subject to redox status including ROS production as a trigger and intracellular thiol levels.

In the present study, the selenium level in splenic cells of Trx-1-Tg mice was higher than in the wild-type mice, even though both groups were acclimated under the same conditions and there were no significant differences in selenium contents of the other tissues, excluding kidney. This might mean that a nutritional requirement for selenium is high in immunologically competent cells overexpressing Trx-1. However, this difference in selenium level can be negligible, compared with the selenium level in the cell culture medium containing 10% FCS (0.018 μ*M*). We also found that selenite little affected both expressions of murine and human Trx-1 that were normalized to b-actin (Fig. 4). These relative expressions were less changed than when they were normalized to glyceraldehydes-3-phosphate dehydrogenase (G3PDH) (data not shown), as the intracellular G3PDH level is shown to be influenced by a variety of redox modulators including Trx (21) and *S*-glutathiolation (9). Human Trx-1 is identical with murine Trx-1, as most of the amino acid residues are conserved within mammalian Trxs, and acts as a substrate for selenium-containing murine TR in an insulin assay (17). However, there was neither significant difference of cell numbers nor TR activity after mitogenesis between the wild-type and Trx-1-Tg mouse T-cells when selenite was untreated (Figs. 5 and 6). This suggests that the overexpression of Trx-1 without selenium supplementation does not affect the cell growth stimulation by Con A and cellular amounts of TR. Therefore the intracellular amount of reduced Trx-1, which is dependent upon TR activity and its total expression, seems to be critical because of providing deoxyribonucleotides by ribonucleotide reductase for DNA synthesis (28).

It is proposed that DNA binding activity of NF-kB is mainly subject to a redox-controlled mechanism dependent on the availability of reduced Trx-1 rather than being controlled by its normal regulator IKK (8). We also recognized that low levels of selenite caused suppression of ROS produced by Con A (unpublished data), even though NF-kB-DNA binding activity was preferably increased within the dose range. However, we estimated that the contribution of cGPx was low because the enzyme activity was not enhanced

in the Trx-1-Tg mouse cells after mitogenesis (Fig. 6). This might be due to selenium being preferably utilized for TR synthesis with the higher growth rate, or overexpression of Trx-1 suppresses ROS production until no induction of cGPx. Although there is relevance between cGPx activity and the immune response in selenium deficiency (3), changes in immune function by selenium compounds might not be mediated by induction of this enzyme activity because supplementation of selenite to adult subjects increased some aspects of immune function with no significant increase in cGPx (5).

More than 5 μ*M* of selenite showed the toxic response and inhibited cell growth, even in splenic cells of Trx-1-Tg mouse. At such high doses, selenite induces apoptosis through the activation of caspase-3 (11) and/or ROS production (37). Therefore the rise in surviving cells in T-cell mitogenesis of Trx-1-overexprsssing cells probably resulted from strong resistance against oxidative stress. Selenite is also a substrate for mammalian TR at high concentration and can cause oxidation of NADPH and production of ROS such as hydrogen peroxide (23). The latter can stimulate cell proliferation at low concentrations and cause inhibition at high concentration. Thus selenium is directly involved in both antioxidant systems and an induction of oxidative stress, depending on the dose. This suggests that the nutritional levels of selenium also may cause suppression of apoptosis during Con A-induced mitogenesis by acting as an antioxidant in addition to the promotion of NF-κB-DNA binding, as the mitogen produces significant levels of ROS and activates NF-κB (27, 29). NF-κB may induce production of several cytokines including TNF-α, which also improves cell survival at low levels (43).

In conclusion, the stimulation of T-cell mitogenic response by the physiological levels of selenite is caused by increased activity of TR rather than cGPx, which may lead to reduction of Trx-1 dependent on the intracellular expression level and promote DNA binding of NF-κB.

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ABBREVIATIONS

cGPx, cellular glutathione peroxidase; Con A, concanavalin A; FCS, fetal calf serum; GSH, glutathione; G3PDH, glyceraldehydes-3-phosphate dehydrogenase; IKK, IκB kinases; NF-κB, nuclear factor-κB; NLS, nuclear localization sequence; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α; Tg, transgenic; Trx-1, thioredoxin-1; TR, thioredoxin reductase.

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