Effects of Supplementary Seleno-L-methionine on Atopic Dermatitis-Like Skin Lesions in Mice

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Effects of selenium supplementation on atopic dermatitis (AD) were investigated by administering seleno-L-methionine (SeMet) using a mouse model of AD caused by repeated application of 2,4,6-trinitrochlorobenzene (TNCB). BALB/c mice were sensitized with TNCB to the abdomen on day −7; then, TNCB was applied repeatedly to each ear three times a week from days 0 to 23. SeMet was orally administered to the mice from days 0 to 23. The efficacy of SeMet on AD was assessed by measuring ear thickness, histologic evaluation, serum total immunoglobulin (Ig) E levels, and expression of interleukin (IL)-4 in the ear and superficial parotid lymph node. Ear thickness was remarkably increased by repeated application of TNCB, and SeMet significantly suppressed ear thickness in BALB/c mice. SeMet inhibited epidermal hyperplasia and dense infiltration of inflammatory cells. The number of TNCB-induced mast cells was significantly decreased by SeMet. Serum total IgE levels that increased by the repeated application of TNCB were significantly suppressed by SeMet. Repeated application of TNCB induced expression of IL-4, a T-helper (Th) 2 cytokine, in the ear and superficial parotid lymph node of BALB/c mice and its expression was significantly inhibited by SeMet. These results demonstrated that SeMet supplementation suppresses AD-like skin lesions in BALB/c mice and inhibits the expression of total IgE and IL-4.

Key words atopic dermatitis; seleno-L-methionine; immunoglobulin E; interleukin 4; 2,4,6-trinitrochlorobenzene

The prevalence of atopic dermatitis (AD), a chronic inflammatory skin disease, has substantially increased over the past 30 years; currently, 15–30% of children and 2–10% of adults suffer from AD, leading to a significant reduction in the QOL and increase in economic burden.1,2) The disease is characterized by intense itching and eczematous lesions, and immunoglobulin (Ig) E-mediated reactions are known to cause inflammation.3) Several reports have demonstrated that type 2 cytokine levels, including interleukin (IL)-4 levels, are increased in patients with AD.4–6) IL-4 produced by T cells has a central role in the recruitment of inflammatory cells in AD.

Selenium, an essential trace element for the health of humans and animals, is attained through consumption of various dietary components.7) Selenium is incorporated into selenoproteins, and the biological effects of selenium are exerted primarily through the function of different selenoproteins. Glutathione peroxidase (GPx) and thioredoxin reductase (TR), which are well-known selenoproteins, are important in the cellular antioxidant defense system.8) Primary dietary selenium sources include wheat and yeast, which contain seleno-L-methionine (SeMet).9,10) SeMet is one of the organic selenium compounds and is contained in various foods and is reportedly one of the main chemical forms of dietary selenium.11,12) SeMet is considered a useful chemical form for nutritional supplementation compared with inorganic selenium compounds.13–15)

Some studies have reported that patients with asthma showed significantly lower selenium concentration in nails or blood and significantly lower GPx activity in the blood.16–20) According to a cohort study on AD prevention, selenium deficiency has been reported to increase the risk of AD.21) Moreover, increased oxidative stress has been reported to be involved in the pathogenesis of AD.22,23) Based on these reports, it is speculated that the supplementation of selenium compounds might be effective for preventing AD. However, the effects of selenium supplementation on AD remain unknown.

Repeated applications of 2,4,6-trinitrochlorobenzene (TNCB) to mouse skin have been reported to induce AD-like skin lesions, which are associated with a significant increase in serum IgE and T-helper (Th) 2 cytokines, such as IL-4, at the chronic dermatitis site.24–27) In this TNCB-induced mouse model, the inflammatory response in chronic lesions has been reported to share many of the histopathological, immunological, and clinical features of human AD.24,28)

We clarified and assessed the effects of SeMet supplementation on AD in a mouse model of chronic dermatitis caused by repeated TNCB application.

MATERIALS AND METHODS

Animals Five-week-old female BALB/c mice were purchased from Japan SLC Co. (Shizuoka, Japan) and rested for 1 week after arrival. The mice were treated and kept in a specific pathogen-free room maintained at 23±1°C and 47–67% humidity, under a 12 h light–dark cycle, and given a normal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and distilled water ad libitum. The experimental protocol used was in accordance with the animal experimental guidelines of Setsunan University as modified from the guidelines of the Japanese Society for Pharmacology. This experiment was approved by

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the Committee for the Ethical Use of Experimental Animals of Setsunan University. Throughout the experimental procedures, every effort was made to minimize animal suffering as much as possible by limiting the number of animals used in this study.

Reagents SeMet was purchased from Acros Organics (Geel, Belgium). TNCB (purchased from Tokyo Kasei Co., Tokyo, Japan) was dissolved in acetone–olive oil (3:1) as a 0.1% (w/v) solution, and used for sensitization of mice and challenge of AD-like skin lesions.

Experimental Procedure Mice were sensitized by a single epicutaneous application of 50 µL TNCB solution to the shaved abdomen on day −7. Then, 10 µL/ear TNCB solution was applied repeatedly to each ear three times a week through days 0 to 23. As a negative control, vehicle (acetone–olive oil [3:1]) was applied to both ears instead of TNCB. SeMet (TNCB+SeMet group) or saline (vehicle and TNCB-only group) was administered orally once daily on days 0 to 23. Ear thickness was measured using a digital thickness gage (Ozaki MFG. Co., Tokyo, Japan) at 0, 3, 6, and 24 h after each challenge. The specimens were homogenized in 0.5 mL of ice-cold phosphate buffered saline containing 0.1% Tween 20 by rapid homogenization using Micro Smash MS-100 (TOMY SEIKO Co., Ltd., Tokyo, Japan), and then centrifuged for 10 min at 12000 g. Ear thickness was measured using a digital thickness gage (Ozaki MFG. Co., Tokyo, Japan) at 0, 3, 6, and 24 h after each challenge. The serum was collected from the mice on days 0, 6, 13, 20, and 24. On day 24, 24 h after the final TNCB challenge, the serum was collected from the mice on days 0, 6, 13, 20, and 24. On day 24, 24 h after the final TNCB challenge, the mice were sacrificed and their ears, superficial parotid lymph nodes, and liver were collected. The design of the experimental schedule is summarized in Fig. 1.

Measurement of Selenium Concentration Liver tissues were digested at 80°C for 1 h, then at 140°C for 2 h, and finally at 170°C for 30 min in 1 mL of a 1:2 mixture of nitric acid and perchloric acid. Selenium concentration was measured by the fluorometric method using 2,3-diaminonaphthalene.

Histological Evaluation A piece of skin was removed, fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), embedded in paraffin, and cut into 5-µm thick sections. One of the sections was stained with hematoxylin (Mayer’s Hematoxylin Solution; Merck, Darmstadt, Germany) and eosin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), whereas the other one was stained with toluidine blue (Wako Pure Chemical Industries, Ltd.) for the detection of mast cells. The number of mast cells was expressed as previously described. Briefly, punched ear (8 mm in diameter) and one superficial parotid lymph node were collected 24 h after the final TNCB challenge. The specimens were homogenized in 0.5 mL of ice-cold phosphate buffered saline containing 0.1% Tween 20 by rapid agitation for 1 min in the presence of 2-mm zirconia beads using Micro Smash MS-100 (TOMY SEIKO Co., Ltd., Tokyo, Japan), and then centrifuged for 10 min at 12000×g in a cold microfuge. Each supernatant was quantified using an ELISA kit for IL-4 (BioLegend). Assays were performed according to the manufacturer’s instructions.

Measurement of IL-4 Protein IL-4 was measured as previously described, but with a slight modification. Briefly, punched ear (8 mm in diameter) and one superficial parotid lymph node were collected 24 h after the final TNCB challenge. The specimens were homogenized in 0.5 mL of ice-cold phosphate buffered saline containing 0.1% Tween 20 by rapid agitation for 1 min in the presence of 2-mm zirconia beads using Micro Smash MS-100 (TOMY SEIKO Co., Ltd., Tokyo, Japan), and then centrifuged for 10 min at 12000×g in a cold microfuge. Each supernatant was quantified using an ELISA kit for IL-4 (BioLegend). Assays were performed according to the manufacturer’s instructions.

Statistical Analysis Statistical analysis was performed using one-way ANOVA with a Bonferroni post hoc test. In the figures, ** and * indicate statistical significance at p values of 0.01 and 0.05, respectively.

RESULTS

Effects of SeMet on Ear Thickness in an AD Model with Repeated Application of TNCB Mice were sensitized with TNCB on day −7, and then TNCB solution was applied repeatedly to each ear on days 0, 2, 5, 7, 9, 12, 14, 16, 19, 21,
and 23 (Fig. 1). SeMet was orally administered to mice on days 0 to 23 (Fig. 1). As shown in Table 1, selenium concentration in liver increased following SeMet supplementation in a dose-dependent manner. Ear thickness was measured at 0, 3, 6, and 24 h after each challenge (Fig. 2). Peak ear thickness appeared at 24 h after TNCB application on days 0 to 5, and at 6 h on days 7 to 23 (Fig. 2A). These results showed a shift in the time course of peak ear thickness from 24 h after TNCB application to 6 h by repeated application of TNCB. SeMet supplementation suppressed ear thickness in a dose-dependent manner. Ear thickness at 6 h after the each TNCB application (early-type response) gradually increased with repeated application (Fig. 2B). Although ear thickness in mice administered 10 µmol/kg/d SeMet also gradually increased with repeated application, the degree of the increase was suppressed over the whole period. Similarly, ear thickness at 24 h after each application (delayed-type response) also gradually increased.

Table 1. Selenium Concentration in the Liver

<table>
<thead>
<tr>
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<th>Selenium concentration (µg/g)</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>1.59±0.11</td>
</tr>
<tr>
<td>TNCB-only</td>
<td>1.62±0.13</td>
</tr>
<tr>
<td>TNCB+SeMet (5 µmol/kg/d)</td>
<td>2.34±0.63*</td>
</tr>
<tr>
<td>TNCB+SeMet (10 µmol/kg/d)</td>
<td>3.88±0.33**</td>
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The results are presented as mean±standard deviation (S.D.) (n=6). **p<0.01, *p<0.05, compared with the TNCB-only group.

Fig. 2. Effects of SeMet on Ear Thickness after Repeated Application of TNCB

Ear thickness was measured at 0, 3, 6, and 24 h after each TNCB challenge on days 0 to 23. (A) 0, 3, 6, and 24 h after each TNCB challenge. (B) At 6 h after each TNCB challenge. (C) At 24 h after each TNCB challenge (○, vehicle; △, TNCB; ▲ and ▼, TNCB+SeMet 5 and 10 µmol/kg/d, respectively). The results are presented as mean±S.D. (n=12). **p<0.01, *p<0.05, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.
with repeated applications (Fig. 2C). Ear thickness in mice administered $10 \mu\text{mol/kg/d}$ SeMet (TNCB+SeMet group) was less than that in mice administered TNCB only. On the contrary, repeated treatment with vehicle (negative control group) had no detectable effect.

**Morphologic Ear Changes** The inflammatory features in the ear were analyzed histologically (Fig. 3). Increased ear thickness, epidermal hyperplasia, and significant inflammatory cell infiltration were observed in TNCB-treated ears compared with vehicle. SeMet supplementation markedly reduced TNCB-induced increased ear thickness, epidermal hyperplasia, and significant inflammatory cell infiltration. The number of mast cells in the ear (measured with toluidine blue staining; Fig. 4) was significantly increased in the TNCB-only compared with the negative control (vehicle) groups (Fig. 4B). The number of TNCB-induced mast cells was significantly decreased by SeMet supplementation (TNCB+SeMet groups).

**Serum Total IgE Levels** To evaluate the changes in total IgE level associated with progression of TNCB-induced AD, we collected serum on days 0, 6, 13, 20, and 24. Serum total IgE levels, measured by ELISA, increased with repeated TNCB application (Fig. 5). A relatively steep increment in total IgE levels was observed from days 6 to 13, almost reaching a plateau at day 13. In the TNCB+SeMet group, total IgE levels were lower at any point compared with the TNCB-only group, increased until day 13, and then decreased until day 24. TNCB-induced IgE elevation was suppressed significantly by SeMet in a dose-dependent manner.

**IL-4 Expression in Ear and Superficial Parotid Lymph Node** Expression of mRNA for IL-4 in ear and superficial parotid lymph node at 24 h after the final TNCB challenge on day 24 was measured using real-time quantitative PCR. IL-4 mRNA levels in ear and superficial parotid lymph node were significantly increased by repeated TNCB application. TNCB-induced IL-4 mRNA expression in the ear was inhibited significantly in mice administered $10 \mu\text{mol/kg/d}$ SeMet (Figs. 6A, B). IL-4 mRNA expression in superficial parotid lymph node was inhibited significantly in mice administered 5 or $10 \mu\text{mol/kg/d}$ SeMet.

We also measured the production of IL-4 in ear and superficial parotid lymph node at 24 h after the final TNCB challenge on day 24 using ELISA (Figs. 7A, B). IL-4 protein production in ear and superficial parotid lymph node was increased significantly by repeated application of TNCB compared with that in the vehicle group. IL-4 production was inhibited significantly at both locations in mice given $10 \mu\text{mol/kg/d}$ SeMet. These results corresponded with results of IL-4 mRNA expression in ear and superficial parotid lymph node.

**DISCUSSION**

Using a TNCB-induced AD mouse model, we demonstrated
that SeMet supplementation suppresses AD-like skin lesions, and inhibits IgE and IL-4.

Patients with allergic disease, such as AD and asthma, showed significantly lower selenium concentration in the blood or nails. Thus, we speculated that supplementation of selenium compounds might be effective in preventing AD. However, the effects of selenium supplementation on AD remain unknown. In this study, we examined the effects of SeMet on AD-like skin lesions in BALB/c mice.

Fig. 5. Total IgE Levels in Serum

Total IgE levels were measured by ELISA on days 0, 6, 13, 20, and 24 (○, vehicle; ●, TNCB; ▼, TNCB + SeMet at 5 and 10 µmol/kg/d, respectively). The results are presented as mean±S.D. (n=6). **p<0.01, *p<0.05, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

Fig. 6. Effects of SeMet on Expression of IL-4 mRNA in Ears and Superficial Parotid Lymph Nodes

Ears (A) and superficial parotid lymph nodes (B) were collected 24 h after the final challenge on day 24, homogenized, and subjected to ELISA. The results are presented as mean±S.D. (n=6). **p<0.01, *p<0.05, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

Fig. 7. Effects of SeMet on Production of IL-4 in Ears and Superficial Parotid Lymph Nodes

Ears (A) and superficial parotid lymph nodes (B) were collected 24 h after the final challenge on day 24, homogenized, and subjected to ELISA. The results are presented as mean±S.D. (n=6). **p<0.01, *p<0.05, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

In this study, SeMet was orally administered to mice on days 0 to 23. The intake of selenium from diet was 1.0 µg/mouse/d on average (data not shown). The dose of selenium administered to mice by SeMet administration was 7.4 µg/mouse/d (5 µmol/kg/d) or 14.9 µg/mouse/d (10 µmol/kg/d).
on average. It has been reported that oral administration of SeMet at a dose of 80.5 µg selenium/mouse/day for 28 d is safe and nontoxic. Therefore, the dose used in this study is considered safe and non-toxic for mice. As we have previously reported, the increase in selenium concentration in the liver by SeMet administration is correlated with the increase in selenium concentration in other tissues, such as skin and blood. Because other tissues and blood were reserved for real-time quantitative PCR or ELISA, we measured the selenium concentration in the liver as a representative. In this study, the selenium concentration in the liver increased following SeMet supplementation in a dose-dependent manner, and there was no significant difference in body weight, coat appearance, and general grooming between the SeMet-supplemented and control groups (data not shown).

Histological evaluation with hematoxylin and eosin staining showed that supplementation of SeMet inhibited epidermal hyperplasia and dense infiltration of inflammatory cells. Moreover, toluidine blue staining showed increased numbers of mast cells in the dermis after repeated TNCB application and this increase was suppressed by SeMet supplementation. Accumulations of mast cells have been associated with several cutaneous diseases, including AD. Natsuki et al. reported that a local increase in mast cells by repeated application of a hapten is involved in early-type responses induced by repeated hapten application in mice. Therefore, inhibition of increased mast cell number in the ear due to SeMet supplementation is considered to be involved in the suppression of ear thickness by SeMet, especially in the early-type response.

Total IgE level was increased by repeated TNCB application, and this increase was suppressed significantly by SeMet. Furthermore, increased IgE on day 13 was gradually decreased by SeMet supplementation. Accumulations of mast cells have been associated with several cutaneous diseases, including AD. Natsuki et al. reported that a local increase in mast cells by repeated application of a hapten is involved in early-type responses induced by repeated hapten application in mice. Therefore, inhibition of increased mast cell number in the ear due to SeMet supplementation is considered to be involved in the suppression of ear thickness by SeMet, especially in the early-type response.

In our study, the expression of IL-4 mRNA and the production of IL-4 in the ear and superficial parotid lymph node on day 24 increased following repeated TNCB application, and these increases were inhibited by SeMet supplementation. IL-4 is produced in the skin of AD patients and is considered to play an important role in the pathogenesis of atopic dermatitis. IL-4 is effective in stimulating the expression of IgE. These studies indicate that the reduction of IL-4 by SeMet is also involved in the suppression of AD. Repeated application of TNCB has been reported to induce Th2-polarized inflammation and the production of IL-4 in the regional lymph node involves Th2 cell development; thus suggesting that SeMet might be involved in the differentiation of Th2 cells.

We concluded that SeMet supplementation suppresses AD-like skin lesions and inhibits expression of total IgE and IL-4.

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Conflict of Interest The authors declare no conflict of interest.

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19) Misso NL, Pkers KA, Gillon RL, Stewart GA, Thompson PJ. Reduced platelet glutathione peroxidase activity and serum selenium concentration in the liver as a representative. In this study, the selenium concentration in the liver increased following SeMet supplementation in a dose-dependent manner, and there was no significant difference in body weight, coat appearance, and general grooming between the SeMet-supplemented and control groups (data not shown).

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