Vitamin E-Deficiency Did Not Exacerbate Partial Skin Reactions in Mice Locally Irradiated with X-rays

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We previously showed that free radicals and oxidative stress are involved in radiation-induced skin reactions. Since vitamin E (VE) is a particularly important lipophilic antioxidant, VE-deficient mice were used to examine its effects on radiation-induced skin damage. The VE content of the skin was reduced to one fourth of levels of normal mice. Neither the time of onset nor the extent of the reactions quantified with a scoring system differed between normal and VE-deficient mice after local X-irradiation (50 Gy). Similarly, there was no difference in the levels of the ascorbyl radical between the groups, although they were higher in irradiated skin than non-irradiated skin. X-irradiation increased the amount of Bax protein in the skin of normal mice both in the latent and acute inflammatory stages, time- and dose-dependently. The increase was associated with an increase in cytochrome c in the cytosolic fraction, indicating that apoptosis was also promoted by the irradiation. The increase in Bax protein correlated well with the thickness of the skin. Although a deficiency in VE should lower resistance to free radicals in the mitochondrial membrane and thus enhance radiation-induced Bax expression and apoptosis, it actually attenuated the increase in Bax protein caused by irradiation.

INTRODUCTION

Most case of skin damage from ionizing irradiation involve accidental exposure or radiotherapy for cancer. Although the damage is known to be caused by an inflammatory response, the molecular mechanisms underlying it remain unclear. We previously demonstrated that iNOS-derived NO was produced during acute inflammatory reactions. Moreover, levels of lipid peroxidation and the ascorbyl radical were found to increase in the early stage of skin reactions. These findings suggest that oxidative stress and related proteins contribute to the pathological changes, and free radicals and reactive oxygen species (ROSs) are produced in the irradiated skin.

Vitamin E (VE) is an important lipophilic antioxidant that protects tissues and cells from oxidative damage by scavenging the peroxyl radical. Its concentration in tissues may therefore affect the pathogenesis of diseases caused by free radicals. However, the effects of VE on skin reactions induced by X-irradiation have not been examined properly. Since VE is lipophilic and difficult to dissolve in water, it is not easy to control its content via the i.p. or i.v. route in experimental animals. Instead, it is usually controlled through supplementation in foods. Using an experimental model in which the concentration of VE in the body of mice is decreased, namely VE-deficient mice, it is possible to examine the effects of VE on the occurrence and progression of radiation-induced skin-damage. In the present study, we examined effects of VE on skin reactions induced by local X-irradiation in hairless mice. For this purpose, we used VE-deficient mice in addition to normal mice.

MATERIALS AND METHODS

Reagents

HPLC-grade reagents were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Leupeptin, aprotinin, pepstatin, sodium orthovanadate and sodium fluoride were products of Sigma. The anti-Bax polyclonal antibody and anti-active Caspase-3 antibody were products of Santa Cruz Biotechnology Inc., Santa Cruz, USA. The 5–20% resolving mini gels were purchased from Biorad Laboratories. The peroxidase-linked anti-mouse and anti-rabbit secondary
antibodies were products of Amersham Biosciences Co.,
Piscataway, USA. Water was double distilled and treated
with an ultra-pure water apparatus (Simpli Lab, Nihon
Millipore K.K., Tokyo, Japan). Other reagents were of an-
alytical grade and used without further purification.

Mice
All animal experiments conformed to institutional guide-
lines and were approved by the Institutional Animal Care
and Use Committee of the National Institute of Radiological
Sciences. Four-week-old male hairless mice (Hos:HR-1)
were purchased from Japan SLC Co. (Hamamatsu, Japan)
and kept in the animal facility of the National Institute of
Radiological Sciences, with water and food freely available.
A VE-free or VE-containing (normal) food was fed for 8
weeks to prepare VE-deficient or normal mice, respectively,
as described below. The mice were housed in a tempera-
ture- and humidity-controlled room at 23 ± 1°C and 55 ± 5%,
respectively, and maintained on a 12-h light-dark cycle. The
mice were kept five per cage, and received acidified water
and food ad libitum during the experimental period.

Preparation of VE-deficient mice
To establish VE-deficient mice, hairless mice (Hos: HR-
l, male) were fed a VE-free diet from age four to twelve
weeks. The VE-free diet was prepared according to the spec-
ifications of Eisai Co. Ltd., with 20% vitamin-free casein,
0.3% dl-methionine, 25% glucose, 25% sucrose, 15% α-
cornstarch, 5% cellulose powder, 3.5% mineral mixture
(AIN-76), 1% vitamin mixture (without VE), 0.2% choline
hydrogen tartrate, and 5% stripped corn oil. The VE-con-
taining diet (normal diet) contained 0.015% VE in addition
to the components described above. These diets were
obtained from Funabashi Farm Co., Funabashi, Japan.

Measurement of VE in the skin of mice by HPLC
Skin homogenate (10%) was prepared from the back of
the mice (12 weeks, male, VE-deficient or normal) as
described previously.15 The homogenate (1.5 mL) was cen-
trifuged (2,000 rpm, 10 min, 4°C) with a TL-100 (Beckman)
and the supernatant (100 μL) was put into a screw-capped
test tube, to which 500 μL of 1% NaCl, 2 mL of 6% pyrogallol
in ethanol, and 1 mL of 35% KOH were added. The test tube
was heated at 100°C for 45 min after mixing. It was then
cooled to room temperature and 2 mL of 10% ethyl acetate/n-hexane and 20 μL of 25 μM dl-tocotrienol were added and mixed vigorously for 3 min. After centrifugation
(2,000 rpm, 10 min, 4°C) with the TL-100, the upper phase
was removed to a new test tube. The remaining lower phase
was extracted with 2 mL of n-hexane again and the new
upper phase was combined with the previous upper phase.
To this solution were added the same volume of water and
5 drops of 1% NaCl, and the sample was centrifuged
(2,000 rpm, 10 min, 4°C). The upper phase was treated by
the same procedure again and the resultant upper phase was
transferred to a brown tube and evaporated with a centrifugal
evaporator (RD-21, Yamato, Tokyo, Japan). The residue was
dissolved in 100 μL of methanol (HPLC grade, Wako Ltd.,
Osaka, Japan) and passed through a membrane filter (PTFE,
0.02 μm, Millipore). The filtrate (10 μl) was applied to an
HPLC system (Nanospace SI-2, Shiseido, Tokyo, Japan)
with an electrochemical detector (720 mV). The HPLC
system consists of a high-pressure pump, a hand injector
(Rheodyne 7161) with a 100-μL injection loop, and a C18
UG-120 column (2.0 mm Φ × 250 mm, Shiseido, Japan).
The elution was at 140 μL/min and the mobile phase con-
tained 0.7% NaClO₄·H₂O/methanol.

X-irradiation of mice
An X-ray generator (200 kV and 20 mA, Pantak HF-320,
Shimadzu) with a filter (0.5 mm Cu and 0.5 mm Al) was
used for the experiments. The dose of radiation was deter-
mined using a dose meter. Mice (12 weeks, male, VE-
deficient or normal) were anesthetized with pentobarbital
at a dose of 50 mg/kg body weight (bw). Adhesive tape
was used to immobilize the animals on a Lucite plate and posi-
tion their right hind legs for local irradiation with an X-ray
generator (Pantak HF-320, Shimadzu, Kyoto, Japan) at a
rate of 1.27 Gy/min. A combination of toroidal and circular
lead plates (> 30 mm thick) with a 30-mm gap was used to
protect the other organs of the mice.

Scoring of skin reactions
The acute skin reaction following 50 Gy of local X-ray
irradiation at the right hind leg was scored every day for
about 7 weeks using the arbitrary scale shown in Table 1.7)

Measurement of ascorbyl radical in the skin
Skin was stripped from the hind legs of each mouse; the
right leg for the irradiated sample and the left leg for the
control. The skin was placed into a tissue cell and ESR spec-
tra were measured with an X-band ESR spectrometer (LFR-
30, JEOL, Tokyo, Japan) according to a previous paper. Signal intensity is shown as the ratio of signal height to a manganese marker placed in a cavity of the instrument.

**Western blot analysis**

Skin tissue was removed from the right-hind leg at 10 d after irradiation, immediately frozen in liquid nitrogen, and stored at −80°C. The frozen skin samples were pulverized into a powder in liquid nitrogen as described previously. The skin tissue powder was homogenized in an ice-water bath with 8 volumes of a lysis buffer containing 50 mM Hepes (pH 7.4), 100 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM ethylene glycol bis (2-aminoethyl ether)-tetraacetic acid (EGTA), 1% Tween 20, 1% Triton X-100, 1 μg/mL each of pepstatin, leupeptin and aprotinin, 1 μM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate and 1 mM sodium fluoride. The mixture was centrifuged at 10,000 × g for 10 min at 4°C, and the protein content of the supernatant was determined by the Bradford method using a protein assay kit (BioRad Laboratories). The supernatant was mixed with a sample buffer containing 1.6% sodium dodecyl sulphate, 5% glycerol, 0.1 M dithiothreitol (DTT), 0.002% bromophenol blue and 62.5 mM Tris-HCl (pH 6.8) and the mixture was heated to 100°C for 5 min. Fifty-five micrograms of protein was applied to each lane of a gradient gel. After electrophoresis at a constant current of 40 mA/gel, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad Laboratories, 0.2 μm), and briefly stained with Ponceau S (Sigma) to verify efficient transfer. The PVDF membrane was incubated for 30 min at 37°C with rabbit polyclonal anti-Bax antibody and subsequently with peroxidase-linked anti-rabbit secondary antibody diluted in the blocking solution. Protein bands were detected and quantified with a lumino-image analyzer (LAS-1000, Fuji Film, Tokyo, Japan) after staining with ECL plus chemiluminescence detection reagents (Amersham Biosciences Co., Piscataway, U.S.A). The membranes were reprobed with anti-actin antibody to verify equal loading of proteins. The exposure time for Bax and actin bands with the Bradford method was 30 and 8 min, respectively.

**Measurement of skin thickness**

The mice were killed by decapitation and the skin of the upper third of the right leg on the dorsal side was sampled and immediately measured using a micrometer (Shinwa Sokutai Company, Osaka, Japan). For the non-irradiated controls, the thickness of the skin at the same position of the left leg was measured.

**Immunohistochemistry**

For immunohistochemical staining, fresh skin was embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in hexane at −60°C. Frozen sections (10 μm thick) were prepared with a Jung MC 1900 cryostat (Leica, Nussloch, Germany), placed on coated glass slides, and allowed to cool for half an hour. The sections were rehydrated in PBS before immunostaining. Streptavidin-biotin peroxidase immunostaining was carried out with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, U.S.A.) according to the manufacture’s instructions. In brief, endogenous peroxidase activity was inactivated with a 0.3% H2O2/methanol solution for 30 min at room temperature. The sections were washed twice with PBS for 5 min, then blocked using normal serum. Next, the sections were incubated with the primary antibody for 60 min and then with biotinylated secondary antibody for 30 min. The antibodies were visualized with 3,3’-diaminobenzidine tetrahydrochloride as a chromogen. The sections were also counterstained with a lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 μg/mL each of aprotinin, leupeptin, and pepstatin, 1 mM DTT, 2 mM sodium orthovanadate, and 10 μg/mL PMSF) on ice for 20 min, then the lysate was centrifuged at 15,000 × g for 5 min at 4°C. The resultant supernatant is the solubilized mitochondria-enriched fraction. Protein concentrations of the cell fractions were assayed with the Bradford method using a protein assay kit (BioRad Laboratories).

**Fig. 1.** Vitamin E levels in the skin of hairless mice fed a normal diet (Normal) and VE-free diet (VE-Def). The diets were fed to mice from the age of 4 to 11 weeks and VE content was measured by HPLC as described in the text. n = 5 for the Normal group and 8 for the VE-Def group. p < 0.001.
with hematoxylin, dehydrated, and mounted with a mounting reagent (O. Kindler, Germany).

Statistical analysis

Student’s t-test was used to test the significance of differences between the groups. The level of significance was taken as $p < 0.05$.

RESULTS

VE Content of the skin of normal and VE-deficient mice

The amount of VE in the skin of normal and VE-deficient mice was measured by HPLC. As shown in Fig. 1, the amount was significantly (about 20%) lower in the mice fed the VE-deficient diet, 0.47 pmol/mg skin, than in the mice fed the normal diet, 2.4 pmol/mg skin.

Skin reaction induced by X-ray irradiation

Figure 2 shows the time-course of skin reaction for hairless mice after local irradiation of the right hind leg at 50 Gy. The reaction began at 6 days and reached a maximum at 14 days post-irradiation. In total, it lasted more than 8 weeks. There was no significant difference between normal mice and VE-deficient mice, either in the latent period or in the
time taken to reach the maximum. The maximum score was similar between the two groups.

**Change in the amount of ascorbyl radical in the skin after X-irradiation**

Figure 3 shows the time-course of the production of the ascorbyl radical in irradiated and non-irradiated skin of normal and VE-deficient mice. In irradiated skin, levels of the ascorbyl radical gradually increased after irradiation and reached maximum at 10–14 days. A significant increase compared to the non-irradiated sample was observed from day 5 post-irradiation in the skin of both normal and VE-deficient mice. However, in the irradiated skin, no significant difference was observed between the two groups of mice. No change was observed in the non-irradiated skin in either normal or VE-deficient mice.

**Expression of Bax protein in the skin after irradiation**

The amount of Bax proteins in the skin of normal mice increased with time after X-irradiation at 50 Gy. Figure 4 shows that the expression increased in whole cell lysate 48 h after irradiation. When the lysate was fractionated into cytosolic and mitochondrial components, most of the protein was observed in the cytosolic fraction. An increase however was observed in both fractions. The expression of cytochrome c was also increased by the irradiation. Similar to Bax, cytochrome c was mainly observed in the cytosolic fraction though an increase was detected in both fractions.

Figure 5 shows the radiation-dependent change in Bax protein and skin thickness in normal mice. Both parameters increased in parallel with the dose of irradiation, indicating that the amount of Bax protein is a good marker for skin inflammatory reactions. Immunohistochemical staining of

![Fig. 5. Local X-irradiation-dependent increase in skin thickness and Bax protein. The normal mice (12 weeks, male) were locally irradiated with doses of 5, 15 and 50 Gy in the right hind leg. For the 0 Gy point, non-irradiated mice were used. Skin thickness was measured with a micrometer 10 days after X-irradiation. Bax was measured by Western blotting. Skin thickness increased in parallel with the Bax protein after irradiation. The number of mice used for each point was 4.](image)

![Fig. 6. Immunohistochemical localization of Bax protein in the skin of normal mice. A) and C): Non-irradiated sample. B) and D): Irradiated sample. In the non-irradiated skin, Bax was distributed only in some cells of the dermis. After irradiation, Bax staining appeared in both the epidermis and the dermis. The scale bars are 100 μm for A, B and D and 50 μm for C.](image)
skin sections revealed Bax to be present in both the dermis and the epidermis, especially in the basal layer of the epidermis, after the irradiation (Fig. 6). In contrast, no staining was observed in the epidermis of the control skin, and only some cells in the dermis were positive for Bax.

**Effect of VE on Bax protein expression in the skin after X-irradiation**

Since Bax seemed to be a good marker for skin reactions after irradiation, we measured the change in the amount of the protein in the skin of VE-deficient mice. The amount increased with time in both the VE-deficient mice and the normal mice as shown in Fig. 7A. The increase was unexpectedly lower in the former mice. Significantly lower levels of Bax in VE-deficient skin compared to normal skin at 48 h after irradiation were observed for both 18 Gy and 50 Gy (Fig. 7B).

**DISCUSSION**

VE-deficient hairless mice can indeed be established using a VE-free diet. The VE content of their skin was less than one fourth of the normal amount. This decrease in the skin was similar to that observed in blood (unpublished result). The low levels of VE in the skin did not enhance inflammatory reactions caused by X-irradiation or the production of the ascorbyl radical.

The amount of Bax in the skin of normal mice was increased by X-irradiation in both the latent and acute inflammatory stages, time- and dose-dependently. The increase was associated with an increase in cytochrome c in the cytosolic fraction, indicating that apoptosis was also promoted by the irradiation. The increase in Bax protein correlated well with the thickness of the skin. Since skin thickness is a parameter for inflammation of the skin, Bax expression might also be a parameter for skin inflammation.

The mitochondria-dependent pathway of apoptosis is important to the pathogenesis of radiation-induced skin damage. Since the Bax protein was localized in the basal layer cells (Fig. 6) where an active caspase 3 was also detected (data not shown), epidermal apoptosis may be a major event in the early stages of skin inflammation. Though its connection with inflammation is not very clear, apoptosis may not only function as a signal for the migration of leukocytes and clearance of cells, but also play roles in the resolution of inflammatory conditions. The finding that more Bax-positive-staining was observed in the proliferating basal layer cells, among which stem cells reside, may suggest that these cells are more sensitive to ionizing radiation.

It is reasonable to assume that a deficiency of VE would lower resistance to free radicals in the mitochondria membrane and, thus, enhance the increase in Bax protein and apoptosis caused by irradiation. However, it actually attenuated the increase in Bax protein caused by irradiation. It was reported that VE deficiency decreased oxidative stress in the brain and protected rats from X-ray-induced cataracts, which are pathological processes associated with the generation of ROS and oxidative stress. Although the mechanisms underlying the reduced response to X-irradiation in the skin of VE-deficient mice are still unclear, several explanations are possible. First, VE is an important antioxidant in the membranes of mitochondria and lowering its level may affect the integrity of these membranes, thereby blocking the activation of Bax. Second, VE also has a non-antioxidant role, regulating Bax protein expression by modulating the expression of related genes. Third, a deficiency of VE would impair Bax-mediated cell death, and enhance other cell death pathways, i.e. necrosis, an explanation supported by the regulation of caspase-independent apoptosis by VE.

In the present study, we clearly showed that reducing the amount of VE to about one fourth of normal levels in mouse
skin did not exacerbate the inflammation reactions caused by X-irradiation. This finding indicates that a normal concentration of VE is not enough to protect the skin from X-ray-induced damage. To explore the protective roles of VE and related compounds, larger amounts may be necessary. For such experiments, the development of VE analogs that are easy to administer is desirable.

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