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Pretreatment with Ascorbic Acid Prevents Lethal Gastrointestinal Syndrome in Mice Receiving a Massive Amount of Radiation

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Bone marrow transplantation/Apoptosis/DNA microarray.

While bone marrow or stem cell transplantation can rescue bone marrow aplasia in patients accidentally exposed to a lethal radiation dose, radiation-induced irreversible gastrointestinal damage (GI syndrome) is fatal. We investigated the effects of ascorbic acid on radiation-induced GI syndrome in mice. Ascorbic acid (150 mg/kg/day) was orally administered to mice for 3 days, and then the mice underwent whole body irradiation (WBI). Bone marrow transplantation (BMT) 24 h after irradiation rescued mice receiving a WBI dose of less than 12 Gy. No mice receiving 14 Gy-WBI survived, because of radiation-induced GI syndrome, even if they received BMT. However, pretreatment with ascorbic acid significantly suppressed radiation-induced DNA damage in the crypt cells and prevented denudation of intestinal mucosa; therefore, ascorbic acid in combination with BMT rescued mice after 14 Gy-WBI. DNA microarray analysis demonstrated that irradiation up-regulated expressions of apoptosis-related genes in the small intestine, including those related to the caspase-9-mediated intrinsic pathway as well as the caspase-8-mediated extrinsic pathway, and down-regulated expressions of these genes in ascorbic acid-pretreated mice. Thus, pretreatment with ascorbic acid may effectively prevent radiation-induced GI syndrome.

INTRODUCTION

Biomedical problems caused by radiation accidents still remain a grave concern, because we cannot rescue most of the patients exposed to critical radiation doses. After a high-dose radiation exposure, patients usually suffer from fatal damage to multiple organs, which has been referred to collectively as acute radiation syndrome.[1)] Bone marrow failure is the most common complication after acute radiation exposure, causing severe pancytopenia, which results in fatal immune dysfunction. Since the early 1950's, bone marrow transplantation (BMT) has been utilized for the reconstitution of damaged bone marrow, and recently, a more sophisticated technique, the transplantation of umbilical cord blood cells, has been developed.[2)] Such advanced therapeutic interventions effectively rescue patients from bone marrow failure caused by fatal irradiation. However, another complication has emerged among patients initially successfully rescued by stem cell transplantation, and that is radiation-induced gastrointestinal (GI) syndrome.

A nuclear criticality accident occurred in 1999 in Japan.[1,3)] Patients who were exposed to high doses of radiation developed severe bone marrow failure, and thereafter underwent hematopoietic stem cell transplantation. However, they subsequently developed severe GI damage with diarrhea and bleeding, and eventually died from multiple organ failure, despite intensive organ support therapies.[4)] Thus far there is no effective therapy for GI syndrome occurring after high-dose radiation exposure, and it is crucial to discover therapeutic tools for rescuing patients with radiation-induced GI syndrome, which is also a major complication of abdominal radiotherapy in cancer patients.[5)] In addition, if a radiation accident unfortunately occurs, rescue team mem-

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bers have to deploy in a radiation-contaminated area to rescue victims. In that situation, prevention of radiation-induced damage, in particular GI syndrome, is of vital importance to the rescue team.

Although there are some arguments about the pathogenesis of GI syndrome, radiation-induced intestinal cell death must be a major factor.[6] Radiation generates free radicals and reactive oxygen species, which damage vital cellular targets such as DNA and membranes, and cause "radiation-induced cell death". Many compounds with antioxidant activities, including neutralization of free radicals or reactions caused by free radicals, are effective radioprotectors.[7,8] Ascorbic acid, vitamin C, has been reported to be an effective antioxidant and free radical scavenger.[9] It also has been shown to protect several biological systems against ionizing radiation.[10–12] The radioprotective effect of ascorbic acid is attributed to its interaction with radiation-induced free radicals. However, it has been reported that ascorbic acid is effective at preventing cell apoptosis only at lower doses of radiation but not at lethal doses.[13] Moreover, there have been no reports regarding the effects of ascorbic acid on the development of GI syndrome after exposure to a lethal radiation dose.

In the present study, we investigated how treatment with ascorbic acid affects radiation-induced GI syndrome in mice. We found that pretreatment with ascorbic acid dramatically improved the GI syndrome, thereby rescuing mice who had undergone BMT from fatal radiation exposure.

MATERIALS AND METHODS

This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Japan.

Mice and reagents

Male C57BL/6 mice (20 ± 2 g body weight) were purchased from Japan SLC (Shizuoka, Japan) and used at 8-week-old. Ascorbic acid was purchased from Wako (Osaka, Japan).

Experimental animals and whole body X-ray irradiation

Each mouse was placed in a specially designed well-ventilated acrylic container. The mice were exposed to 6 to 14 Gy whole body irradiation (WBI), given at a dose rate of 0.45 Gy/min at 150 kV and 5 mA (HITACHI MBR-1505R2, Tokyo, Japan). The beam was filtered through a 2 mm aluminum board.

Administration of ascorbic acid

Ascorbic acid was dissolved in distilled water and administered per os (p.o.) to mice at 150 mg/kg/day for 3 days before or after irradiation. Control mice received p.o. distilled water only. In a separate experiment mice also received ascorbic acid orally (150 mg/kg) at 6 h or 12 h before irradiation.

Bone marrow transplantation (BMT)

Untreated C57BL/6 mice (8-week-old) were used as donors. As previously described,[14] the donor mice were sacrificed and their femurs removed. Bone marrow cells were obtained by injecting RPMI 1640/1% FBS into each femur using a 1 mL syringe fitted with a 26-gauge needle. After the cells were washed, they were resuspended in 33% Percoll solution and centrifuged at $500 \times g$ for 20 min at room temperature. After treatment with RBC-lysing solution, the cells were washed twice in RPMI 1640/10% FBS. Thereafter, 1×10^6 cells were intravenously injected into each recipient mouse.

Measurement of ascorbic acid levels in plasma and tissue of small intestine

A sample of small intestine (0.7 g) was removed from each mouse immediately after sacrifice. After each sample was washed with saline, it was homogenized in 5.4% metaphosphoric acid (9.8 g) and each homogenate was centrifuged at $10{,}000 \times g$ for 15 min at 4°C. Blood samples were obtained from the retro-orbital plexus in mice and then plasma samples were collected. Ascorbic acid levels in the plasma and homogenate supernatant were measured in the SRL laboratory (Tokyo, Japan) using high performance liquid chromatography (HPLC).

Measurement of free radical metabolites in the plasma

The d-ROMs test (Diacron, Grosseto, Italy) was used for the measurement of free radical metabolites in plasma.[15,16] It is a spectrophotometric method that assesses overall oxidative stress by measuring total hydroperoxide levels, given that hydroperoxides are intermediate oxidative products of lipids, peptides, and amino acids. Briefly, 0.02 mL plasma was diluted in 1 mL acetate-buffered solution. Hydroperoxide groups react with the transition metal ions liberated from the proteins in the acidic medium, and are converted to alkoxyl and peroxyl radicals according to the Fenton reaction. These newly formed radicals, the quantities of which are directly proportional to those of the peroxides, were trapped chemically with 0.02 mL chromogen (N, N-diethyl-paraphenylendiamine), leading to the formation of the radical cation of this chromogen. The purple color resulting from this reaction over time was monitored in a spectrophotometer (Wismarll FRAS4, Tokyo, Japan) at 505 nm. The results of this method were expressed in conventional units (Carratelli units [UCarr]).

Expression analysis using DNA microarrays

Mice were pretreated with ascorbic acid or distilled water only for 3 days. Thereafter, they received WBI at 14 Gy or

sham treatment (unirradiated). After 24 h, samples of small intestine were obtained from the mice and stored at –80°C until assay. Details for sample preparation and processing for microarray analysis are available from Affymetrix (Santa Clara, CA). Briefly, total RNA was isolated from the intestine in order to prepare double-stranded cDNA. Biotinylated complementary RNA (cRNA) was synthesized from the cDNA and was then chemically fragmented to approximately 50 to 200 nucleotides. The fragmented cRNA was hybridized to a GeneChip HT Mouse Genome 430 microarray (Affymetrix, Santa Clara, CA), according to the manufacturer's protocols. Hybridization was carried out at the Genetic Laboratory (Sapporo, Japan). The microarray contains 45,101 probe sets representing approximately 25,000 genes. Expression profile data were prepared for analysis using Microarray Analysis Suite version 5.0 software (Affymetrix) by setting the scaling of all reference probes to a constant value of 1,000. The data were then filtered to select only those probe sets having "present" or "marginal" calls (detection $P < 0.065$) in approximately 50% of the samples.

Pathological examination of small intestine and bone marrow

Because the mice began to die from bone marrow aplasia or severe GI damage 7 days after radiation, they were euthanized 7 days after WBI to remove small intestines and bone marrow, and then the tissue damage was examined in the samples. Small intestines were immersed in 20% formalin for 2 days. The bone marrow was also immersed in 20% formalin for 2 days after 3 days of decalcification. Slides were prepared from the processed specimens, and stained with hematoxylin and eosin.

Measurement of villus height and crypt count in the small intestine

Villus height was determined by measuring the distance from the base of the crypt to the villus tip in a longitudinal section of the intestine. The number of crypts was also determined in each circumference of a transverse cross section of the intestine. For each mouse, 10–20 measurements of villus height were obtained from 3 different tissue sections to calculate a mean height value. Five circumferences per mouse were also used to calculate a mean crypt count value. These mean values were then used for statistical analysis.

Immunohistochemical staining of single stranded DNA (ssDNA)

Mice were euthanized and sacrificed before, 3, 6, and 24 h after irradiation. Small intestines were then removed from the mice. Immunohistochemical staining of ssDNA was performed on tissue sections of formalin-fixed, paraffin-embedded mouse small intestine. An indirect immunohistochemistry method was used. Briefly, after deparaffinization, specimen slides were placed in 0.3% H_2O_2 in methanol for 30 min. For antigen retrieval, the slides were incubated with 10 mg/mL proteinase K at 37°C for 10 min. After incubation with 3% normal goat serum in PBS for 20 min, the slides were incubated with polyclonal rabbit anti-ssDNA (A4506, DAKO, Glostrup, Denmark) at 1:200 dilution in PBS for 60 min in a moist chamber at room temperature. The slides were washed and then incubated with peroxidase-conjugated goat anti-rabbit IgG (P0448, DAKO) at 1:50 dilution in PBS for 30 min at room temperature. Reactions were visualized with 3, 3'-diaminobenzidine (DAB), and the slides were counterstained with hematoxylin. As a negative control, the incubation step with primary antibody was omitted.

Statistical analysis

Statistical analyses were performed using the Stat View 4.02J software package (Abacus Concepts, Berkeley, CA). Survival rates were compared by the Wilcoxon signed rank test. Statistical evaluation between two groups was performed using the Student t test, and other statistical evaluations were performed using one-way ANOVA, followed by the Bonferroni post hoc test. The data were presented as means ± SE. $P < 0.05$ was considered statistically significant.

RESULTS

BMT could not save mice after 14 Gy whole body irradiation (WBI)

Mice were irradiated with 6, 8, 10, 12, or 14 Gy and mortality and survival were monitored. Although no mice survived after WBI at doses of 8 Gy and above (Fig. 1A), BMT 24 h after radiation rescued whole body-irradiated mice from fatal disease if the doses of WBI were 8, 10, or 12 Gy (8 and10 Gy, 100% survival; 12 Gy, 75% survival). However, BMT after radiation did not rescue the mice receiving 14 Gy-WBI (Fig. 1B).

Intestinal mucosa of whole body-irradiated mice developed denudation at 14 Gy but not 8 Gy, although mice receiving either dose developed bone marrow aplasia

Mice receiving 8 Gy-WBI showed severe bone marrow aplasia 7 days after radiation compared to unirradiated controls (0 Gy) (Fig. 2A-a, B-a), while neither showed degenerative changes in the intestinal mucosa at 7 days (Fig. 2A-b, c, B-b, c). However, when mice received 14 Gy-WBI, they showed not only severe bone marrow aplasia (Fig. 2C-a), but also marked denudation of the intestinal mucosa 7 days after irradiation (Fig. 2C-b, c, indicated by arrow heads), suggesting the occurrence of radiation-induced GI damage.

Treatment with ascorbic acid before but not after radiation dramatically improved mouse survival after 14 Gy WBI in combination with BMT

We examined the effect of ascorbic acid on mouse surviv-

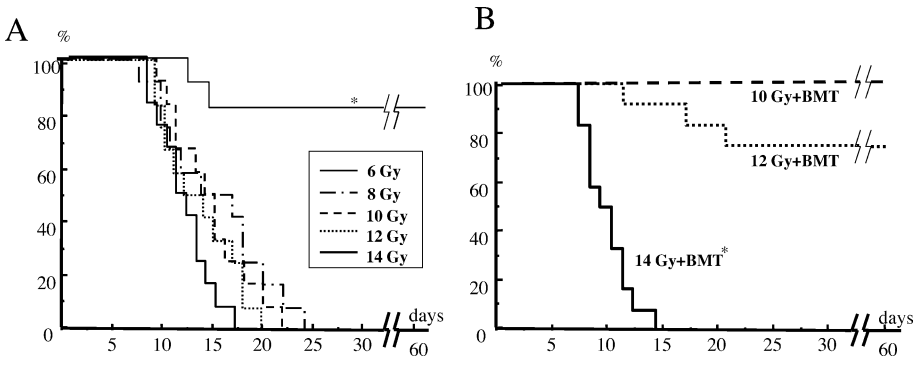

Fig. 1. Mouse survival after whole body radiation (A). Mice were irradiated at 6, 8, 10, 12, and 14 Gy. The effect of bone marrow transplantation on mouse survival after radiation (B). Mice were irradiated at 10, 12, and 14 Gy. Mice received BMT 24 h after radiation. N = 12 in each group. *$P < 0.01$ vs. other groups.

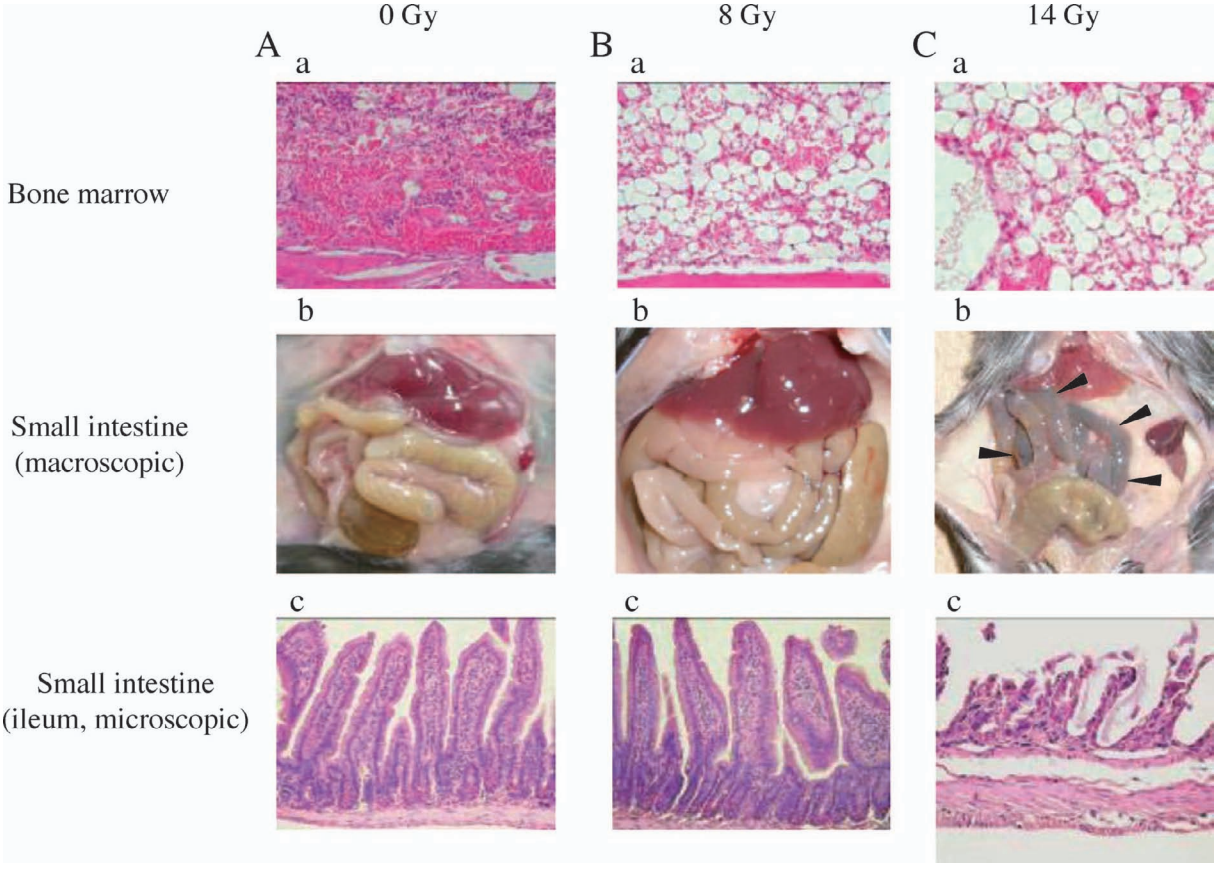

Fig. 2. Radiation-induced damage to the bone marrow and small intestine in mice 7 days after radiation. The mice were irradiated at 0, 8, and 14 Gy. Seven days after whole body radiation, they were euthanized to remove the bone marrow and small intestine, and radiation-induced damage to these organs was examined. Arrows indicate severe radiation-induced degeneration of the small intestine (C-b). Images shown are representative of each mouse group (n = 5).

al after 14 Gy WBI. We preliminarily studied dose response curve of treatment with ascorbic acid in the irradiated mice. The mice were pretreated with 1.5, 15, 150, and 1,500 mg/kg/day of ascorbic acid for 3 days before 14 Gy WBI. They then received BMT 24 h after radiation. Pretreatment with 150 mg/kg/day of ascorbic acid rescued some subjected mice, although pretreatment with other doses of ascorbic acid rescued no mice (data not shown), thereby studying 150 mg/kg/day of ascorbic acid in the following experiments. Oral intake of 1,500 mg/kg/day ascorbic acid induced potent

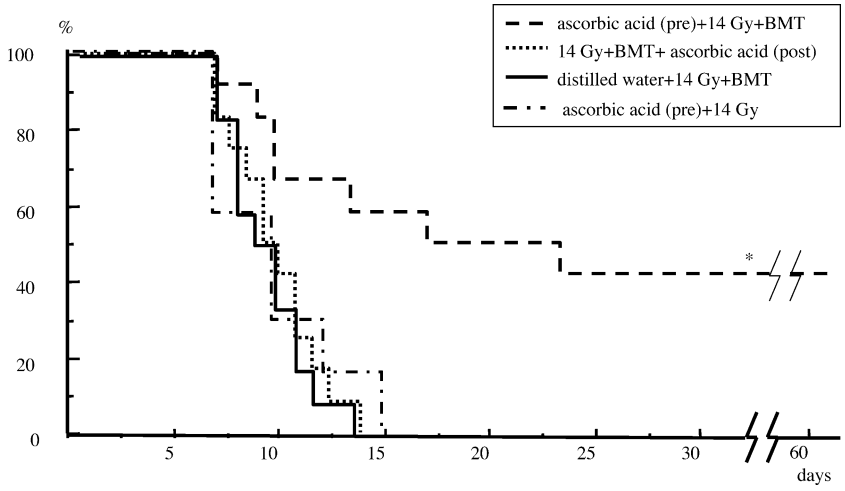

Fig. 3. The effect of treatment with ascorbic acid on mouse survival after radiation. The mice were irradiated at 14 Gy and received BMT 24 h after radiation. They received ascorbic acid p.o. for 3 days either before or after radiation, or received distilled water only before radiation. The mice pretreated with ascorbic acid were also irradiated at 14 Gy without BMT. N = 12 in each group, $*P < 0.01$ vs other groups.

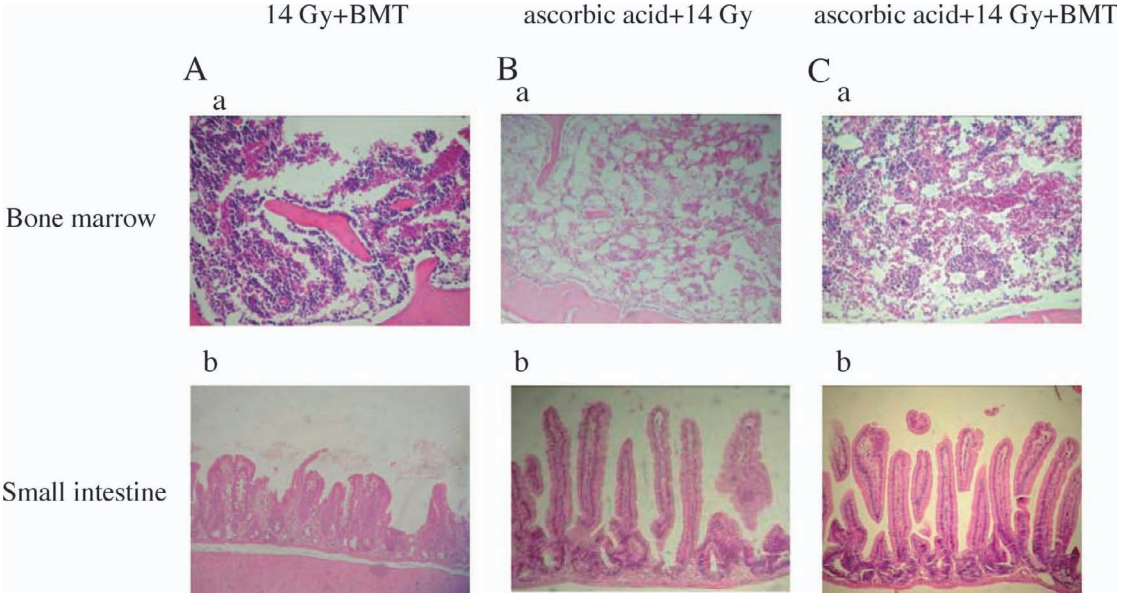

Fig. 4. The effect of pretreatment with ascorbic acid or BMT following WBI on the bone marrow or small intestine in the irradiated mice. The mice were pretreated with ascorbic acid p.o. or distilled water for 3 days and then irradiated at 14 Gy. One day after radiation, they received BMT or did not. The mice were euthanized 7 days after radiation to assess radiation-induced damage to the bone marrow and small intestine. Data shown are representative of each mouse group (n = 5).

diuretic effect in mice, leading to severe dehydration and thereby exacerbating mortality. Next, we examined the effect of pretreatment or post-treatment with ascorbic acid on the irradiated mice. Mice were treated with 150 mg/kg/day of ascorbic acid for 3 days before or after 14 Gy WBI and they also received BMT at 24 h after radiation. Pretreatment with ascorbic acid before 14 Gy WBI dramatically improved mouse survival (42% survival), whereas treatment with ascorbic acid after radiation did not affect survival (no mice survived) (Fig. 3). When the mice did not receive BMT following 14 Gy WBI, pretreatment with ascorbic acid rescued no subjected mice (Fig. 3). The rescued mice that were pretreated with ascorbic acid and received BMT after 14 Gy WBI were healthy 30 days after radiation, although they showed marked radiation-induced depigmentation in the fur (data not shown).

Pretreatment with ascorbic acid markedly improved radiation-induced intestinal damage, thereby rescuing mice receiving WBI at 14 Gy in combination with BMT from fatal disease

When the mice received BMT after 14 Gy WBI, bone marrow aplasia was remarkably improved in the irradiated mice 7 days after radiation, whereas they showed severe degenerative changes in the intestinal mucosa (Fig. 4A-a, b). In contrast, when the mice were pretreated with ascorbic acid before 14 Gy WBI but not received BMT, they showed severe bone marrow aplasia but markedly improved mucosal degeneration in the intestine (Fig. 4B-a, b). However, when the mice were pretreated with ascorbic acid and received BMT following 14 Gy WBI, they remarkably improved both bone marrow aplasia and intestinal mucosal degeneration (Fig. 4C-a, b).

Pretreatment with ascorbic acid prevented decreases in villus height and crypt counts in the small intestines of mice receiving 14 Gy WBI followed by BMT

We evaluated the villus height and crypt counts in the small intestines of treated mice 7 days after radiation. The mice receiving 14 Gy WBI but not 8 Gy showed significantly lower villus height and crypt counts per circumference in the intestine (Fig. 5A, B). Although treatment with BMT alone following 14 Gy WBI did not affect these degenerative changes, pretreatment with ascorbic acid markedly improved these changes in the mice receiving 14 Gy WBI (Fig. 5A, B). The mice that were pretreated with ascorbic acid and received BMT following 14 Gy WBI also showed significant improvements (Fig. 5A, B).

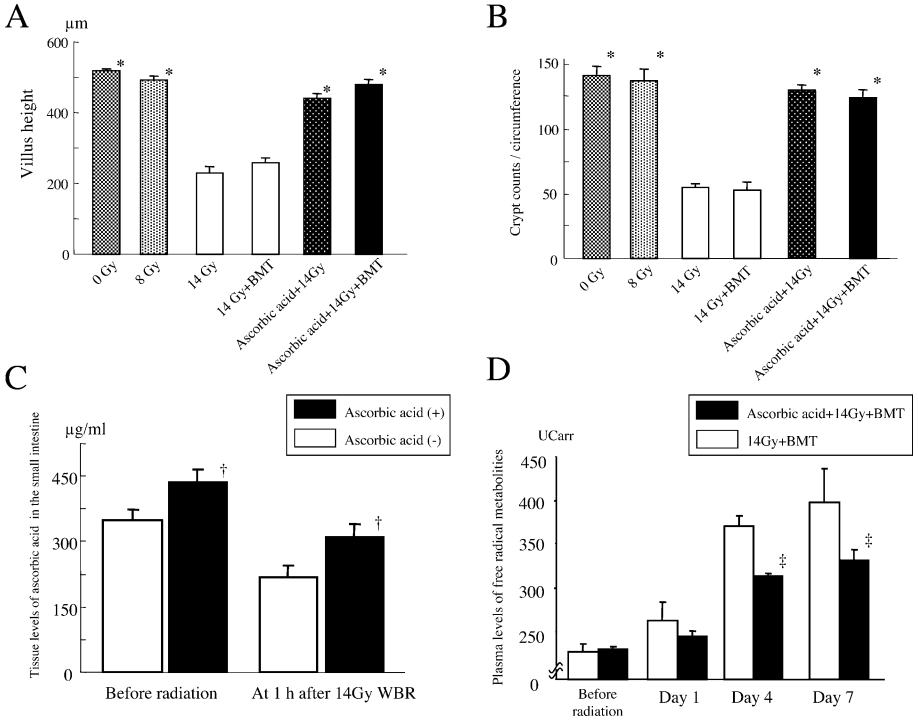

Fig. 5. The effect of pretreatment with ascorbic acid on villus height (A) and crypt counts (B) of the small intestine in mice irradiated with 14 Gy followed by BMT. The mice were irradiated with 0, 8, or 14 Gy. The mice receiving 14 Gy WBI were pretreated with ascorbic acid or distilled water, and they were also treated with or without BMT. At 7 days after radiation, villus height (A) and crypt counts per circumference (B) of the small intestine were measured as described in the Materials and methods. Data are means ± SE from 5 mice in each group. *$P < 0.01$ vs. '14 Gy' and '14 Gy + BMT' groups. Tissue levels of ascorbic acid in mouse small intestine (C). The mice received ascorbic acid or distilled water only p.o. for 3 days. They were then irradiated at 14 Gy. Just before and 1 h after radiation, they were euthanized to remove the small intestine. The tissue concentrations of ascorbic acid in the small intestine were determined. Data are means ± SE and are pooled from 2 independent experiments with 3 mice per group. †$P < 0.05$ vs. other group. The effect of pretreatment with ascorbic acid on the production of free radical metabolites in mice irradiated at 14 Gy followed by BMT (D). The mice were pretreated with ascorbic acid or distilled water only for 3 days. They were then irradiated at 14 Gy followed by BMT. Blood samples were obtained from the mice at indicated times to measure free radical metabolite levels. Data are means ± SE and pooled from 2 independent experiments with 3 mice per group. ‡$P < 0.05$ vs. 14 Gy + BMT group.

Oral administration of ascorbic acid increased ascorbic acid tissue concentration in the small intestine

We confirmed that oral administration of ascorbic acid for 3 days significantly increased ascorbic acid concentration in plasma of treated mice compared to untreated controls (57 ± 11 vs. 30 ± 8 μg/mL, $P < 0.05$). We examined the ascorbic acid concentration in small intestine samples of 14 Gy-WBI mice. Pretreatment with ascorbic acid significantly increased ascorbic acid concentrations in the tissues of small intestine just before radiation (Fig. 5C). Interestingly, tissue concentrations of ascorbic acid significantly decreased 1 h after radiation in both ascorbic acid-pretreated and water-only control mice (Fig. 5C), indicating consumption of tissue ascorbic acid by X-irradiation. Nevertheless, the mice pretreated with ascorbic acid still showed significantly higher tissue ascorbic acid concentrations than the untreated control mice (Fig. 5C).

Pretreatment with ascorbic acid suppressed a radiation-induced increase in free radical metabolites in mouse plasma

We examined the effect of pretreatment with ascorbic acid on the production of free radical metabolites in WBI mice. Mice with or without pretreatment with ascorbic acid received 14 Gy WBI followed by BMT. Although free radical metabolites gradually increased in the plasma of untreated mice after radiation, pretreatment with ascorbic acid significantly suppressed the increase in free radical metabolites at Days 4 and 7 (Fig. 5D). Treatment with ascorbic acid after radiation did not suppress the increase in free radical metabolites in mice (data not shown).

Pretreatment with ascorbic acid suppressed radiation-induced DNA damage in crypt epithelial cells of mouse small intestine

We examined the effect of pretreatment with ascorbic acid on DNA damage in the mouse small intestine after 14 Gy WBI, using immunohistochemical staining with anti-ssDNA. In mice without ascorbic acid pretreatment, positive-staining cells for ssDNA increased in the epithelial crypts of the small intestine 6 h after radiation, and further increased at 24 h (indicated by arrow heads, Fig. 6). By contrast in irradiated mice, pretreatment with ascorbic acid apparently suppressed the increase in ssDNA positive-staining cells (Fig. 6). These results suggest that radiation-induced DNA damage of mouse intestinal crypt cells is effectively inhibited by ascorbic acid.

Clustering analysis of altered gene expression in the small intestine of ascorbic acid-pretreated mice after 14 Gy WBI

We examined the effect of pretreatment with ascorbic acid on gene expression in the mouse small intestine after irradiation. Mice pretreated with or without ascorbic acid received 14 Gy WBI or non-WBI sham treatment. Total RNA was extracted from mice small intestines 24 h after radiation/sham treatment. Gene chip microarray analysis was performed to identify possible mechanisms underlying the increased resistance to WBI in ascorbic acid-pretreated mice. First, we selected 1,347 apoptosis-related genes from the Gene Ontology database (http://www.geneontology.org). After prefiltering, we analyzed the differences in these gene expressions among four mouse samples by hierarchical clustering. The initial data analysis of fluorescence intensity in the tissue samples by global hierarchical clustering showed limited correlation among them. To detect genes differentially expressed in each mouse, we further selected 279 genes which showed > 2- or < ½-fold changes among the 4 samples. Then, we identified 9 clusters (Fig. 7A). In cluster 1, which was the largest group containing 132 genes, gene expressions were up-regulated by radiation but slightly down-regulated by pretreatment with ascorbic acid in both irradiated and unirradiated mice (Fig. 7B). Genes in cluster 1 include *Bax*, *Bid*, *Fas*, and *Fas ligand*, all of which are involved in the induction of apoptosis. Interestingly, in cluster 4, which contained 26 genes, gene expressions were evidently up-regulated by radiation but down-regulated by pretreatment with ascorbic acid in both irradiated and unirradiated mice (Fig. 7B). Cluster 4 includes *Bcl-2 related protein A1* gene, *Bcl-2-like 1* gene, and *Hip1*, all of which are related to the mitochondria-mediated intrinsic apoptotic pathway. Genes related to caspase-8-mediated extrinsic apoptotic pathway (CFLAR) are also in cluster 4. These two apoptotic cascades are representative but distinct apoptotic signaling pathways. In cluster 5, which also includes apoptosis-related protein genes such as bcl-2 like protein gene and the Fas-associated death domain, gene expressions were also evidently suppressed in mice pretreated with ascorbic acid (Fig. 7B).

Pretreatment with ascorbic acid suppressed gene expressions related to the mitochondria-mediated apoptotic pathway in small intestines of WBI mice

BH3-only proteins, the members of the proapoptotic Bcl-2 family, have been recognized as essential initiators of apoptosis in mice.[17] Signals originating from these proteins activate their downstream cascade of mitochondria-mediated intrinsic apoptotic pathways, such as the Apaf 1-Caspase 9-Caspase 3 pathway. Therefore, we selected these genes for microarray analysis and demonstrated expressions of these genes in the small intestine (Fig. 8). Interestingly, the mice pretreated with ascorbic acid showed significantly lower expression of the *Bim* gene 24 h after 14 Gy WBI (Fig. 8A). Also, the expression of *Puma* tended to be lower compared to the untreated control mice (Fig. 8B). Both Bim and Puma are representative BH3-only proteins, and *Bim* notably belongs to cluster 1 in Fig. 7, which is up-regulated after irradiation. Mice pretreated with ascorbic acid also showed

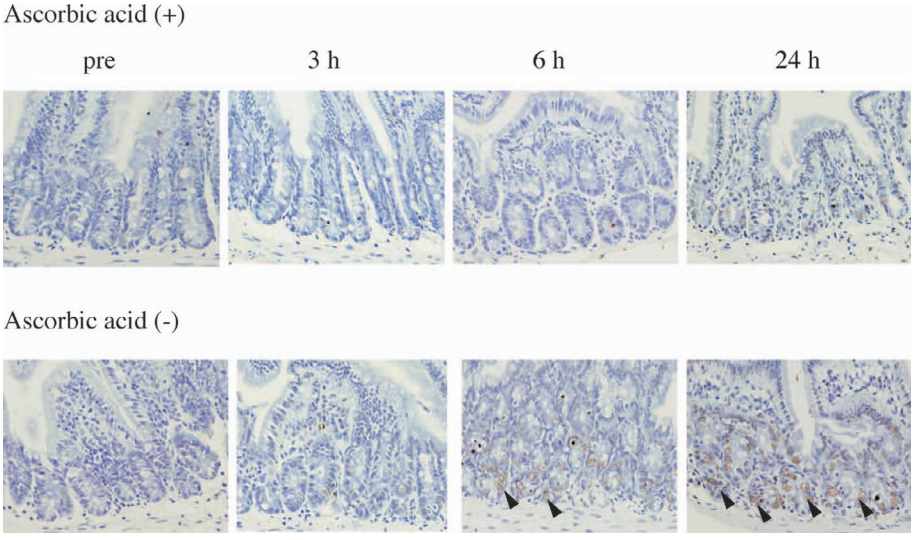

Fig. 6. Immunohistochemical findings of ssDNA staining in the small intestine of mice irradiated with 14 Gy with and without ascorbic acid pretreatment. The mice were pretreated with ascorbic acid or distilled water for 3 days and then irradiated with 14 Gy. Crypt cells in small intestines were examined for ssDNA staining at the indicated times. Arrows indicate positive staining for ssDNA. Data shown are representative of each mouse group (n = 5).

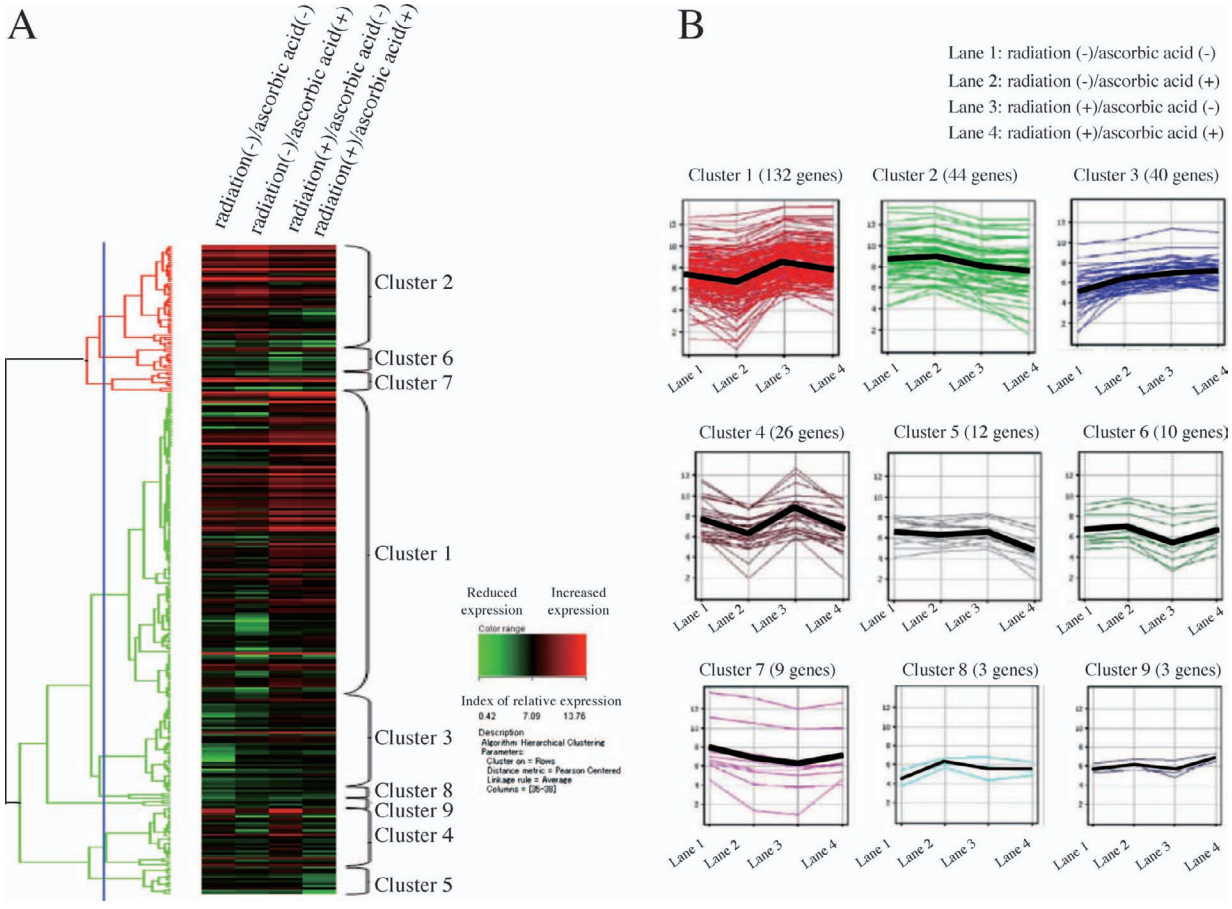

Fig. 7. Hierarchical clustering of gene expression data in mouse small intestine. Mice were pretreated with ascorbic acid or distilled water only for 3 days and then received whole body irradiation at 14 Gy or unirradiated sham treatment. Small intestines were removed from the mice 24 h after radiation for DNA microarray analysis. Thick lines indicate the mean expression values (B).

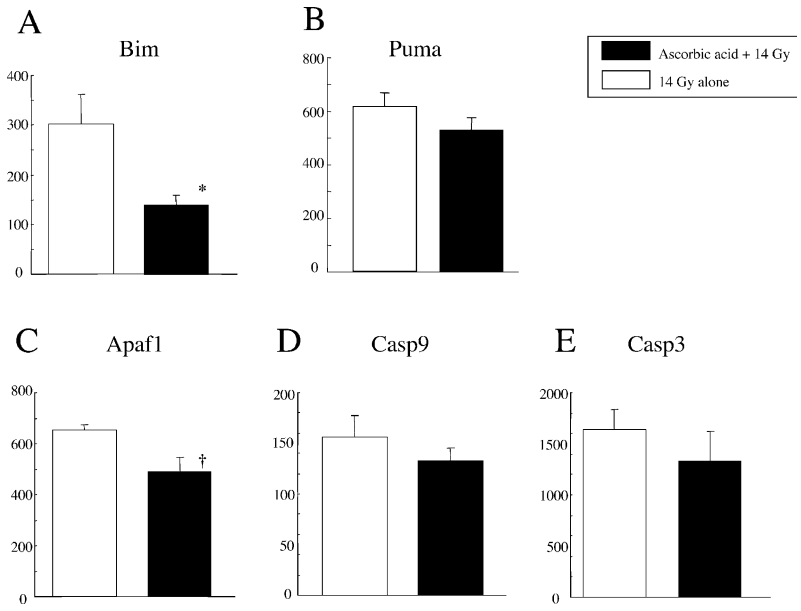

Fig. 8. The effect of pretreatment with ascorbic acid on the expression of genes involved in the mitochondria-mediated apoptotic pathway in the irradiated mice. The mice were pretreated with ascorbic acid or distilled water only for 3 days, and then irradiated at 14 Gy. Small intestines were removed from the mice 24 h after radiation to examine gene expression of *Bim* (A), *Puma* (B), *Apaf-1* (C), *Caspase-9* (D), and *Caspase-3* (E). Data are means ± SE from 3 mice in each group. *$P < 0.01$, †$P < 0.05$ vs. other group.

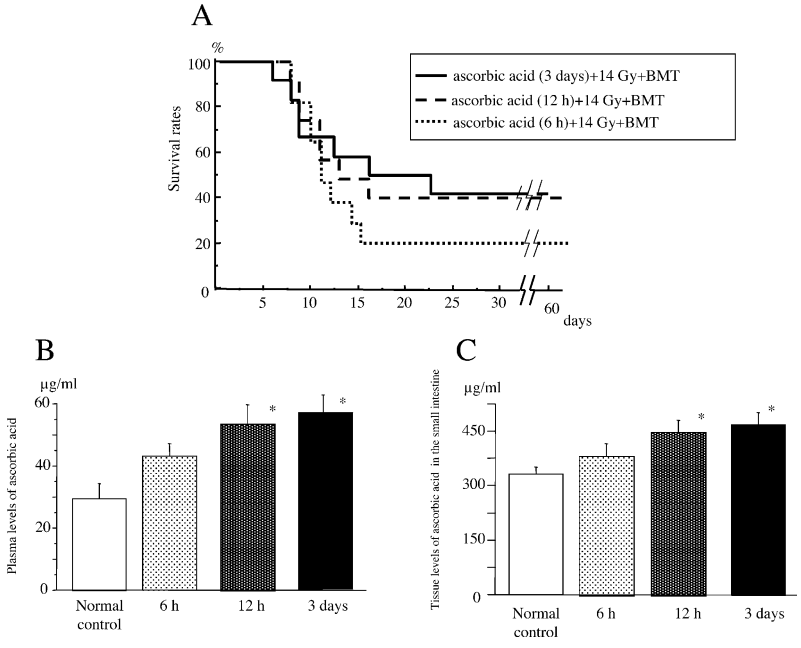

Fig. 9. The effect of different pretreatment times with ascorbic acid on mouse survival after14 Gy WBI followed by BMT (A). The mice were pretreated with ascorbic acid at 6 h or 12 h before radiation, and for 3 days. The mice then received 14 Gy WBI followed by BMT and survival rates were monitored. N = 12 in each group. The effect of ascorbic acid pretreatment times on plasma ascorbic acid concentrations (B) and small intestine tissue ascorbic acid concentrations (C) in mice. Blood samples and small intestines were obtained from mice pretreated with ascorbic acid just before radiation, and also obtained from the untreated normal mice as a control, to measure ascorbic acid concentrations. Data are means ± SE and pooled from 2 independent experiments with 3 to 4 mice per group. *$P < 0.05$ vs. normal control.

a significant reduction in *Apaf-1* expression as well as lower tendencies of expression of *caspase-9* and *caspase-3* 24 h after irradiation, compared to mice without ascorbic acid pretreatment (Fig. 8C, D, E).

Oral intake of ascorbic acid for 12 h before 14 Gy WBI induced a substantial effect on survival of mice with GI syndrome

Finally, we determined the minimal period of ascorbic acid pretreatment which induced a therapeutic effect on mice after 14 Gy WBI. The mice received oral intake of ascorbic acid at 6 h or 12 h before radiation. Another group of mice received ascorbic acid for 3 days before radiation. They were then irradiated at 14 Gy followed by BMT. Ascorbic acid concentrations in plasma and small intestine were measured just before radiation. The intake of ascorbic acid only at 12 h improved mortality, comparable to mortality seen by ascorbic acid intake for 3 days (both treatments for 12 h and 3 days showed 42% survival) (Fig. 9A). Even at 6 h intake of ascorbic acid produced a significant effect on mouse survival (25% survival). The mice receiving oral intake of ascorbic acid at 12 h also showed ascorbic acid concentrations in plasma and small intestine similar to concentrations seen after 3 day oral intake (Fig. 9B, C). We also examined the effect of oral intake of ascorbic acid for 7 days before radiation, and did not demonstrate additional ameliorative effects on survival and in ascorbic acid concentrations in plasma and small intestine compared to effects seen with 3 day pretreatment with ascorbic acid (data not shown).

DISCUSSION

Radiation-induced tissue damage is involved in the production of free radicals and their metabolites, which are formed by the ionization of molecules in cells. DNA damage caused by free radicals and metabolites leads to the formation of single- and double-stranded breaks in DNA.[18] This radiation-induced cell damage may alter intracellular signaling cascades, which results in the activation of responsive genes related to apoptosis signals. In the present study, tissue concentrations of ascorbic acid significantly decreased in the small intestine after radiation. In bone marrow, ascorbic acid also is reported to decrease after radiation, accompanied with a marked increase in dehydroascorbic acid, an oxidized form of ascorbic acid,[19] suggesting that ascorbic acid acts as a hydrogen donator to scavenge radiation-induced free radicals in both GI tract and bone marrow. Hydrogen donation is an important step toward chemical repair of damaged DNA,[20] and ascorbic acid is believed to strongly contribute to this process.

In the present study, we showed that pretreatment with ascorbic acid evidently suppressed DNA damage in the crypt cells of irradiated small intestine, indicating the important role of ascorbic acid in preventing radiation-induced crypt cell apoptosis. However, apoptosis after radiation also may be a protective mechanism to remove cells with damage by radiation before they divide to form neoplasia. Although ascorbic acid might suppress the apoptosis, it also might scavenge free radicals and metabolites immediately after radiation. These free radicals/metabolites induce severe DNA damage that may cause radiation-induced oncogenesis. Therefore, treatment with ascorbic acid might not increase a risk of neoplasia despite its suppressive effect of apoptosis.

Paries *et al.* have suggested that radiation-induced apoptosis of microvascular endothelial cells may be the primary cause of GI syndrome,[21] although other reports has indicated that apoptosis was observed in crypt epithelial cells[22,23] but not in vascular endothelial cells.[24] Pretreatment with ascorbic acid may thus either directly reduce damage in crypt cells or indirectly reduce the damage to crypt cells via protection against vascular endothelial injury in the GI tract.

There are many reports on ascorbic acid's radioprotective effect on various cell types.[10,25–27] Nevertheless, despite its significant efficacy in GI syndrome, pretreatment with ascorbic acid does not appear to effectively improve radiation-induced bone marrow aplasia compared to the BMT therapy. Both the GI tract and bone marrow continually renew tissues that contain highly ordered progenitors and stem cells. In a previous report, bone marrow stem cells were shown to be more radiosensitive than the intestinal progenitor cells found in the crypts.[28] In addition, Harapanhalli *et al*. have observed that ascorbic acid does not effectively protect hematopoietic tissue against a lethal radiation dose, whereas it can effectively protect at sublethal doses.[13] Therefore, we consider that exposure to a certain but substantial dose of irradiation may cause irreversible damage to the bone marrow cells, but may only induce reversible damage to the crypt cells. Combination therapy of ascorbic acid with BMT may be important to rescue the host exposed to a high-dose radiation.

Metabolites of free radicals produced by radiation gradually increased/accumulated after radiation exposure even in the mice pretreated with ascorbic acid. Because all of the mice pretreated with ascorbic acid could not survive from 14 Gy WBI followed by BMT (42% survival), we think that pretreatment with ascorbic acid did not completely inhibit radiation-induced free radical production and thereby did not entirely quench an increase in free radical metabolites after radiation. However, there is a possibility that protection of crypt cells with ascorbic acid may have another mechanism than the hydrogen donation by ascorbic acid. Further study will be required on this issue.

Treatment with ascorbic acid after radiation exposure could not rescue mice from lethal GI syndrome, even if they received BMT. Therefore, it may be important to maintain high tissue concentrations of ascorbic acid before and at the time of radiation exposure, because DNA damage occurs during exposure. It is notable that oral intake for only 12 h

might be sufficient to cause the ascorbic acid therapeutic effect, suggesting that if a mouse orally receives a substantial amount of ascorbic acid one time, the GI syndrome is prevented. Therefore, when we undertake the rescue of victims from a radiation-contaminated area just after a radiation accident or terrorism, it is important for rescue team members to promptly take ascorbic acid orally. It has been reported that pharmacokinetic curve of ascorbic acid shows plateau steady-state plasma level at 80 μM (approximately 14 μg/ml) in human healthy volunteers, which level is lower than that of mice.[29] Ascorbic acid is unstable in water and is readily oxidized under atmosphere. Therefore, we are now trying to examine the radioprotective effect of ascorbic acid using a new compound form of ascorbic acid, which is stable in a drinking water.[30,31]

Regarding the radiotherapy of human abdominal cancers, the prophylactic use of ascorbic acid can now be a possible option to reduce damage to the GI tract. To confirm this, we have verified that abdominal radiation exposure causes radiation-induced GI damage without bone marrow dysfunction in mice, and pretreatment with ascorbic acid is markedly effective for the prevention of GI damage (unpublished data). It should also be noted that serum ascorbic acid concentrations are inversely related to the incidence of stroke (both cerebral infarction and hemorrhage), indicating ascorbic acid's beneficial effect on vascular endothelial cells.[32]

Radiation-induced damage triggers apoptosis through two major pathways.[33–35] One is a mitochondria-mediated intrinsic pathway that is regulated by pro- and anti-apoptotic members of the Bcl-2 family; the release of cytochrome *c* from mitochondria promotes the activation of caspase-9 by its adaptor Apaf-1.[36,37] In our experiments, *Bcl-2 related protein A1*, *Bcl-2-like 1*, and *Hip 1* genes, which belong to cluster 4, were up-regulated by irradiation but suppressed by pretreatment with ascorbic acid. The other pathway is a cell surface death receptor-mediated extrinsic pathway that stimulates intracellular activators. This pathway includes Fas/Fall molecules, and CFLAR (caspase-8 and FADD-like apoptosis regulators) which corresponds to c-FLIP (cellular-FADD-like interleukin-1β-converting enzyme inhibitory protein) in humans.[38] CFLAR also belongs to cluster 4. Pretreatment with ascorbic acid thus is shown to suppress both mitochondria-mediated intrinsic and receptor-mediated extrinsic pathways in the small intestine of irradiated mice. Also, the expression of *Bim*, a Bcl-2 family protein gene, was significantly reduced in cluster 1 by pretreatment with ascorbic acid, suggesting an important role for the mitochondria-mediated intrinsic pathway in the execution of radiation-induced apoptosis.[33] Although *Puma*, *Apaf-1*, *caspase-9*, and *caspase-3* are all involved in the mitochondria-mediated intrinsic pathway, they did not belong to the nine clusters studied, because these four genes were excluded from the initial 279 gene selection.

REFERENCES

1. Hirama, T. and Akashi, M. (2005) Multi-organ involvement in the patient who survived the Tokai-mura criticality accident. B.J.R. **Suppl 27**: 17–20.
2. Flomenberg, N. and Keever, C. A. (1992) Cord blood transplants: potential utility and potential limitations. Bone Marrow Transplant. **10 Suppl 1**: 115–120.
3. Ishii, T., Futami, S., Nishida, M., Suzuki, T., Sakamoto, T., Suzuki, N. and Maekawa, K. (2001) Brief note and evaluation of acute-radiation syndrome and treatment of a Tokai-mura criticality accident patient. J. Radiat. Res. **42 Suppl**: S167–S182.
4. Igaki, H., Nakagawa, K., Uozaki, H., Akahane, M., Hosoi, Y., Fukayama, M., Miyagawa, K., Akashi, M., Ohtomo, K. and Maekawa, K. (2008) Pathological changes in the gastrointestinal tract of a heavily radiation-exposed worker at the Tokai-mura criticality accident. J. Radiat. Res. **49**: 55–62.
5. Bolis, G., Zanaboni, F., Vanoli, P., Russo, A., Franchi, M., Scarfone, G. and Pecorelli, S. (1990) The impact of whole-abdomen radiotherapy on survival in advanced ovarian cancer patients with minimal residual disease after chemotherapy. Gynecol. Oncol. **39**: 150–154.
6. Brown, M. (2008) What causes the radiation gastrointestinal syndrome?: overview. Int. J. Radiat. Oncol. Biol. Phys. **70**: 799–800.
7. Toklu, H. Z., Sehirli, O., Ozyurt, H., Mayadagli, A. A., Eksioglu-Demiralp, E., Cetinel, S., Sahin, H., Yegen, B. C., Ulusoylu Dumlu, M., Gokmen, V. and Sener, G. (2009) Punica granatum peel extract protects against ionizing radiation-induced enteritis and leukocyte apoptosis in rats. J. Radiat. Res. **50**: 345–353.
8. Weiss, J. F. and Landauer, M. R. (2003) Protection against ionizing radiation by antioxidant nutrients and phytochemicals. Toxicology **189**: 1–20.
9. Mathew, D., Nair, C. K., Jacob, J. A., Biswas, N., Mukherjee, T., Kapoor, S. and Kagiya, T. V. (2007) Ascorbic acid monoglucoside as antioxidant and radioprotector. J. Radiat. Res. **48**: 369–376.
10. Adaramoye, O., Ogungbenro, B., Anyaegbu, O. and Fafunso, M. (2008) Protective effects of extracts of Vernonia amygdalina, Hibiscus sabdariffa and vitamin C against radiation-induced liver damage in rats. J. Radiat. Res. **49**: 123–131.
11. Ueno, A., Vannais, D., Lenarczyk, M. and Waldren, C. A. (2002) Ascorbate, added after irradiation, reduces the mutant yield and alters the spectrum of CD59- mutations in A(L) cells irradiated with high LET carbon ions. J. Radiat. Res. **43 Suppl**: S245–S249.
12. Waldren, C. A., Vannais, D. B. and Ueno, A. M. (2004) A role for long-lived radicals (LLR) in radiation-induced mutation and persistent chromosomal instability: counteraction by ascorbate and RibCys but not DMSO. Mutat. Res. **551**: 255–265.
13. Harapanhalli, R. S., Yaghmai, V., Giuliani, D., Howell, R. W. and Rao, D. V. (1996) Antioxidant effects of vitamin C in mice following X-irradiation. Res. Commun. Mol. Pathol. Pharmacol. **94**: 271–287.
14. Kinoshita, M., Shinomiya, N., Ono, S., Tsujimoto, H.,

Kawabata, T., Matsumoto, A., Hiraide, H. and Seki, S. (2006) Restoration of natural IgM production from liver B cells by exogenous IL-18 improves the survival of burn-injured mice infected with Pseudomonas aeruginosa. J. Immunol. **177**: 4627–4635.
15. Cesarone, M. R., Belcaro, G., Carratelli, M., Cornelli, U., De Sanctis, M. T., Incandela, L., Barsotti, A., Terranova, R. and Nicolaides, A. (1999) A simple test to monitor oxidative stress. Int. Angiol. **18**: 127–130.
16. Buonocore, G., Perrone, S., Longini, M., Terzuoli, L. and Bracci, R. (2000) Total hydroperoxide and advanced oxidation protein products in preterm hypoxic babies. Pediatr. Res. **47**: 221–224.
17. Huang, D. C. and Strasser, A. (2000) BH3-Only proteins-essential initiators of apoptotic cell death. Cell **103**: 839–842.
18. Scholes, G. (1983) Radiation effects on DNA. The Silvanus Thompson Memorial Lecture, April 1982. Br. J. Radiol. **56**: 221–231.
19. Umegaki, K., Aoki, S. and Esashi, T. (1995) Whole body X-ray irradiation to mice decreases ascorbic acid concentration in bone marrow: comparison between ascorbic acid and vitamin E. Free Radic. Biol. Med. **19**: 493–497.
20. Prise, K. M., Davies, S. and Michael, B. D. (1992) A comparison of the chemical repair rates of free radical precursors of DNA damage and cell killing in Chinese hamster V79 cells. Int. J. Radiat. Biol. **61**: 721–728.
21. Paris, F., Fuks, Z., Kang, A., Capodieci, P., Juan, G., Ehleiter, D., Haimovitz-Friedman, A., Cordon-Cardo, C. and Kolesnick, R. (2001) Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science **293**: 293–297.
22. Matsuu-Matsuyama, M., Shichijo, K., Okaichi, K., Ishii, K., Wen, C. Y., Fukuda, E., Nakayama, T., Nakashima, M., Okumura, Y. and Sekine, I. (2006) Sucralfate protects intestinal epithelial cells from radiation-induced apoptosis in rats. J. Radiat. Res. **47**: 1–8.
23. Matsuu-Matsuyama, M., Shichijo, K., Okaichi, K., Nakayama, T., Nakashima, M., Uemura, T., Niino, D. and Sekine, I. (2008) Protection by polaprezinc against radiation-induced apoptosis in rat jejunal crypt cells. J. Radiat. Res. **49**: 341–347.
24. Schuller, B. W., Rogers, A. B., Cormier, K. S., Riley, K. J., Binns, P. J., Julius, R., Hawthorne, M. F. and Coderre, J. A. (2007) No significant endothelial apoptosis in the radiation-induced gastrointestinal syndrome. Int. J. Radiat. Oncol. Biol. Phys. **68**: 205–210.
25. el-Nahas, S. M., Mattar, F. E. and Mohamed, A. A. (1993) Radioprotective effect of vitamins C and E. Mutat. Res. **301**: 143–147.
26. O'Connor, M. K., Malone, J. F., Moriarty, M. and Mulgrew, S. (1977) A radioprotective effect of vitamin C observed in Chinese hamster ovary cells. Br. J. Radiol. **50**: 587–591.
27. Witenberg, B., Kletter, Y., Kalir, H. H., Raviv, Z., Fenig, E., Nagler, A., Halperin, D. and Fabian, I. (1999) Ascorbic acid inhibits apoptosis induced by X irradiation in HL60 myeloid leukemia cells. Radiat. Res. **152**: 468–478.
28. Biedermann, K. A., Sun, J. R., Giaccia, A. J., Tosto, L. M. and Brown, J. M. (1991) scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc. Natl. Acad. Sci. USA **88**: 1394–1397.
29. Levine, M., Conry-Cantilena, C., Wang, Y., Welch, R. W., Washko, P. W., Dhariwal, K. R., Park, J. B., Lazarev, A., Graumlich, J. F., King, J. and Cantilena, L. R. (1996) Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc. Natl. Acad. Sci. USA **93**: 3704–3709.
30. Nakamura, S. and Oku, T. (2009) Bioavailability of 2-O-alpha-D-glucopyranosyl-L-ascorbic acid as ascorbic acid in healthy humans. Nutrition **25**: 686–691.
31. Chandrasekharan, D. K., Kagiya, T. V. and Nair, C. K. (2009) Radiation protection by 6-palmitoyl ascorbic acid-2-glucoside: studies on DNA damage in vitro, ex vivo, in vivo and oxidative stress in vivo. J. Radiat. Res. **50**: 203–212.
32. Yokoyama, T., Date, C., Kokubo, Y., Yoshiike, N., Matsumura, Y. and Tanaka, H. (2000) Serum vitamin C concentration was inversely associated with subsequent 20-year incidence of stroke in a Japanese rural community. The Shibata study. Stroke **31**: 2287–2294.
33. Hosokawa, Y., Sakakura, Y., Tanaka, L., Okumura, K., Yajima, T. and Kaneko, M. (2005) Radiation-induced apoptosis is independent of caspase-8 but dependent on cytochrome c and the caspase-9 cascade in human leukemia HL60 cells. J. Radiat. Res. **46**: 293–303.
34. Green, D. R. (1998) Apoptotic pathways: the roads to ruin. Cell **94**: 695–698.
35. Strasser, A., O'Connor, L. and Dixit, V. M. (2000) Apoptosis signaling. Annu. Rev. Biochem. **69**: 217–245.
36. Wang, X. (2001) The expanding role of mitochondria in apoptosis. Genes Dev. **15**: 2922–2933.
37. Choi, S. A., Kim, S. J. and Chung, K. C. (2006) Huntingtin-interacting protein 1-mediated neuronal cell death occurs through intrinsic apoptotic pathways and mitochondrial alterations. FEBS Lett **580**: 5275–5282.
38. Chandrasekaran, Y., McKee, C. M., Ye, Y. and Richburg, J. H. (2006) Influence of TRP53 status on FAS membrane localization, CFLAR (c-FLIP) ubiquitinylation, and sensitivity of GC-2spd (ts) cells to undergo FAS-mediated apoptosis. Biol. Reprod. **74**: 560–568.

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