The Protective Effect of Fermented Milk Kefir on Radiation-induced Apoptosis in Colonic Crypt Cells of Rats

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To evaluate the effect of fermented milk kefir on X-ray-induced apoptosis in the colon of rats, we examined the apoptotic index, the mean number of apoptotic cells detected by H&E staining per crypt in the colon, in control rats and kefir-pretreated rats drinking kefir for 12 days before irradiation. Apoptotic cells were confirmed by TUNEL staining, and active caspase-3 expression was studied by immunohistochemistry. The cell position of apoptotic cells and active caspase-3 positive cells were examined. The apoptotic index of kefir-treated rats was significantly ($p < 0.05$) decreased 2 h after 1 Gy irradiation in comparison with control rats at crypt cell positions 1–3, 5–7, 13, and 15. Active caspase-3 expression in the kefir-treated rats was also significantly ($p < 0.05$) reduced in comparison with control rats 2 h after 1 Gy irradiation at crypt cell positions 1–4, 13, and 15. This study indicated that kefir protects colonic crypt cells against radiation-induced apoptosis, which was most pronounced in the stem cell region of the crypt. The antiapoptotic effect of fermented milk kefir was due to the inhibition of caspase-3 activation.

INTRODUCTION

The exposure of the intestine to ionizing radiation results in the rapid apoptotic death of the stem cells[1]. Crypt disturbance results from cell loss by apoptosis and the destruction of the stem cells in the crypts that are responsible for repopulating the lining. At suitably high doses, damage to the crypt is accompanied by functional changes, including malabsorption, that are expressed clinically as acute bowel reactions, including radiation proctitis[2–5]. Radiotherapy for malignant pelvic disease is usually followed by acute radiation proctitis. Some available therapeutic agents for acute radiation proctitis or radiation-induced apoptosis have been reported[6–9].

Kefir is fermented milk originally derived from the Caucasus, including various species of lactobacilli, lactococci, leuconostocs, aceterobacteria, and yeasts (both lactose-fermenting and nonlactose-fermenting). Kefir differs from yogurt and other fermented milks in that kefir grains (small clusters of microorganisms held together in a polysaccharide matrix or mother cultures from grains) are added to milk and cause fermentation. It has been reported that kefir has antibacterial and antitumor effects in animals[10,11]. Kefir and sphingomyelin isolated from the lipids in kefir have been reported to stimulate the immune system in both *in vitro* and *in vivo* studies[12–14]. Recently kefir has been reported to have a protective effect on ultraviolet-induced apoptosis in human melanoma cells[15]. Moreover, it has been reported that the intake of fermented milk products can decrease chronic bowel discomfort following the irradiation of pelvic malignancies[16].

Caspase-3 activation has been identified as a key effector of the apoptotic pathway in intestinal cells following irradiation[17]. Caspase-3 is formed by a cleavage of procaspase 3 by caspase-8 or caspase-9. In this active form, caspase-3 plays a role in the proteolytic cleavage of proteins, such as the nuclear DNA repair enzyme poly (ADP-ribose) polymerase and inhibitor of caspase-activated DNase that promotes the activation of DNA-cleaving caspase-activated DNase[18–20].

In this study to explore whether fermented milk kefir has a protective role in regard to acute radiotherapy-induced intestinal damage, the effects of kefir on X-ray radiation-induced apoptosis and cell position of apoptotic cells in the colon of rats were examined. Moreover, the expression of active caspase-3 was studied by immunohistochemistry.

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MATERIALS AND METHODS

Animals and treatment with kefir

Seven-week-old male Wistar rats were purchased from Charles River Japan (Atsugi, Japan) for use in this study. The rats were housed in groups of 2 to 3 per cage in an air-conditioned room at 24°C (lights on from 7 a.m. to 9 p.m.), and allowed free access to food (laboratory chow F2, Japan CLEA, Tokyo) and tap water at the Laboratory Animal Center of Nagasaki University. All animals were kept in a specific pathogen-free facility at the Animal Center in accordance with the rules and regulations of the Institutional Animal Care and Use Committee.

Kefir was supplied by Japan Kefir Co. Ltd. (Kanagawa, Japan). The kefir-pretreated rats consumed kefir *ad libitum* instead of water and the standard diet for 12 days before irradiation. The control rats consumed only tap water and the standard diet. We set the treatment period with kefir to be 12 days, longer than the report of Kubo *et al.*, in which 10 days of treatment with kefir had an antitumor effect[10].

Irradiation

Irradiation was performed from 9:00 a.m. to 12:00 p.m. The rats received whole-body X-ray irradiation by means of a Toshiba EXS-300 X ray, 200 kV, 15 mA apparatus with a 0.5 mm Cu + 0.5 mm Al filter at a dose-rate of 0.461 Gy/min. Two rats being simultaneously treated were held in a paper box. A single dose of 0.25, 0.5, 1, 2, and 4 Gy was given. A nonirradiated control group was otherwise handled identically.

Tissue preparation and apoptosis assessment

Four to eight animals in each group of rats were sacrificed by deep anesthesia 1, 2, 4, and 6 h after irradiation, and the colon was immediately resected and immersed in neutral-buffered formalin. This tissue was processed for embedding in paraffin blocks, from which 3 μm sections were cut and stained with hematoxylin and eosin (H&E).

A TdT-mediated digoxigenin-labeled dUTP nick end labeling (TUNEL) technique[21] was performed to detect DNA fragmentation by using an ApopTag Kit (Intergen Co., New York, NY). Fifty crypts per group from complete colonic crypts cut in the longitudinal plane were selected for analysis. The incidence of cell death in the colon was quantified by counting the number of apoptotic cells in each crypt by a microscopic examination of H&E-stained sections at ×400 magnification. The distinctive morphological features of apoptosis, as described by Kerr[22] and Walker *et al.*[23], were used to recognize apoptotic cells. Small clusters of dead cell fragments were assessed as originating from one cell and given a single count. Doubtful cells were disregarded[24]. The mean number of apoptotic cells per crypt is presented as the apoptotic index. The distribution of apoptotic cells at each position per crypt was obtained.

Immunostaining for active caspase-3

Active caspase-3 staining was detected by antiactive caspase-3 polyclonal antibody (R&D Systems, Abingdon, UK) staining. This antibody specifically recognizes amino acids 163–175 of caspase-3, but it does not detect the precursor form. Active caspase-3 immunohistochemical staining was done with a "VECTASTAIN" ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Immunopositive cells for active caspase-3 were counted in at least 50 crypts per animal, and the average value was used for the representative data. The localization of immunopositive cells for each position per crypt was obtained.

Statistical evaluation of data

All values were expressed as the mean ± SEM of results obtained from at least four animals per data point. The differences between groups were examined for statistical significance by using a Student's *t*-test. A $p < 0.05$ value was considered to be of statistical significance.

RESULTS

The consumption of kefir was 39.1 ± 0.9 ml/d in kefir-treated rats, and the consumption of water was 43.3 ± 1.8 ml/d in control rats. The histological sections of irradiated colonic crypts in control rats and kefir-treated rats were stained with H&E (Fig. 1, A and B), TUNEL (Fig. 1, C and D), and active caspase-3 antibody (Fig. 1, E and F). A higher frequency of apoptosis was observed in colonic crypt cells in vehicle-treated control rats by H&E staining (Fig. 1A) and TUNEL staining (Fig. 1C) 2 h after 1 Gy irradiation. Active caspase-3 immunoreactivity was largely identified at the base of colonic crypts in the area where apoptotic bodies are most often seen in H&E-stained sections (Fig. 1E). In kefir-treated rats, the number of apoptotic cells was greatly decreased in the colonic crypt detected by H&E staining (Fig. 1B) and TUNEL staining (Fig. 1D) compared to control rats 2 h after 1 Gy irradiation. Positive active caspase-3 staining was also greatly decreased in kefir-treated rats compared with control rats 2 h after 1 Gy irradiation (Fig. 1F).
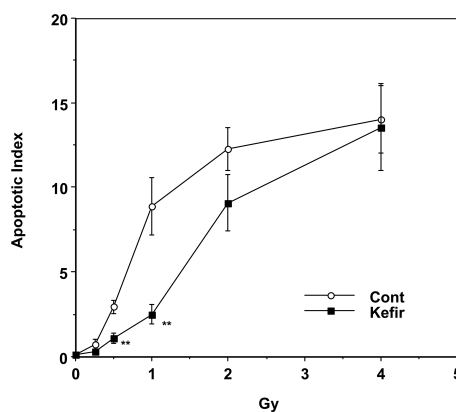
Apoptotic frequency in control rats and kefir-treated rats at 2 h after 0.25, 0.5, 1, 2, and 4 Gy detected by H&E staining is shown in Fig. 2. The apoptotic index increased in a dose-dependent manner to 4 Gy, from 0.25 Gy, in control rats. The apoptotic index in the colon of kefir-treated rats was reduced significantly, to 37% and 28% at 0.5 Gy ($p < 0.01$) and to 1 Gy ($p < 0.01$) of the control values. The apoptotic index of kefir-treated rats following irradiation with 2 Gy was reduced to 74% of the control values, but there was no statistical difference between control rats and kefir-treated rats. At 4 Gy, the apoptotic index of kefir-treated rats was similar to that of control rats.

Figure 3 gives a comparison of the time course of the apoptotic index detected by using H&E staining and active caspase-3 expression in control rats and kefir-treated rats up to 6 h following 1 Gy irradiation in the colonic crypt cells. The apoptotic
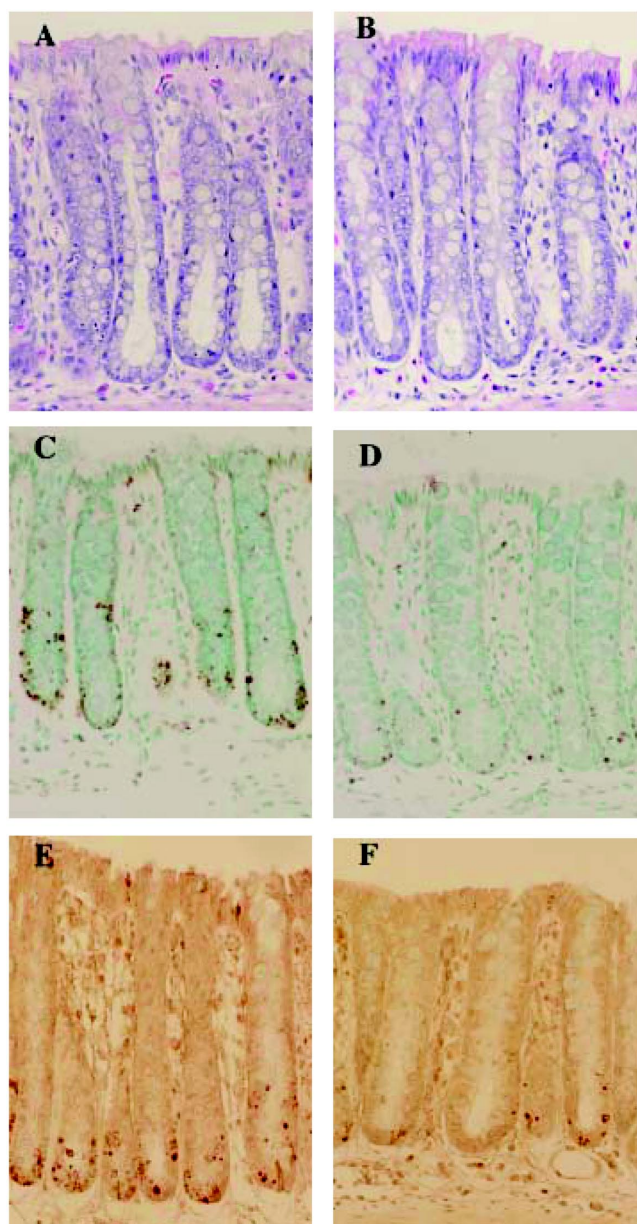
Fig. 1. Histological sections of irradiated colonic crypts (2 h after 1 Gy). (A), (C), and (E) are control rats, and (B), (D), and (F) are kefir-treated rats. (A) and (B) H&E staining; (C) and (D) TUNEL staining to allow visualization of cells containing fragmented DNA; (E) and (F) Active caspase-3 expression (×400).

Fig. 2. Dose dependent effect on apoptotic index in colonic crypt cells of control rats and kefir-treated rats at 2 h following irradiation with 0.25, 0.5, 1, 2, and 4 Gy. (solid squares) kefir treated rats, (open circles) control rats. Data are the mean ± SEM for 4 - 8 rats per data point. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control rats, respectively.
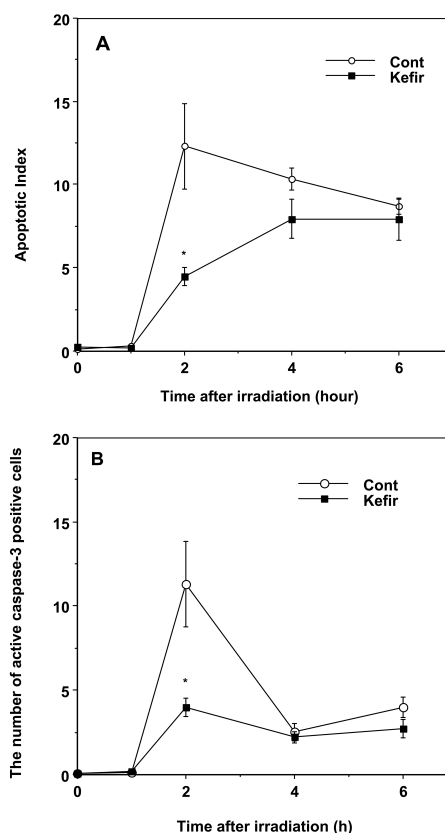
Fig. 3. (A) Apoptotic index and (B) caspase-3 activation of colonic crypt cells in control rats and kefir-treated rats up to 6 h following 1 Gy irradiation. (solid squares) kefir-treated rats, (open circles) control rats. Data are the mean ± SEM for 4–5 rats per data point, $^{*}p < 0.05$ vs. control rats.

index of the colonic crypts in control rats peaked at 2 h after irradiation, and by 6 h a decline in the index was evident (Fig. 3A). The apoptotic index of kefir-treated rats was significantly reduced to 36 % of the control rat values ($p < 0.05$) at 2 h. Although at 4 h and 6 h, the apoptotic index of kefir-treated rats was reduced to 77% and 91% of the control rat values, there was no significant difference between control rats and kefir-treated rats. The background levels of apoptosis in both groups were
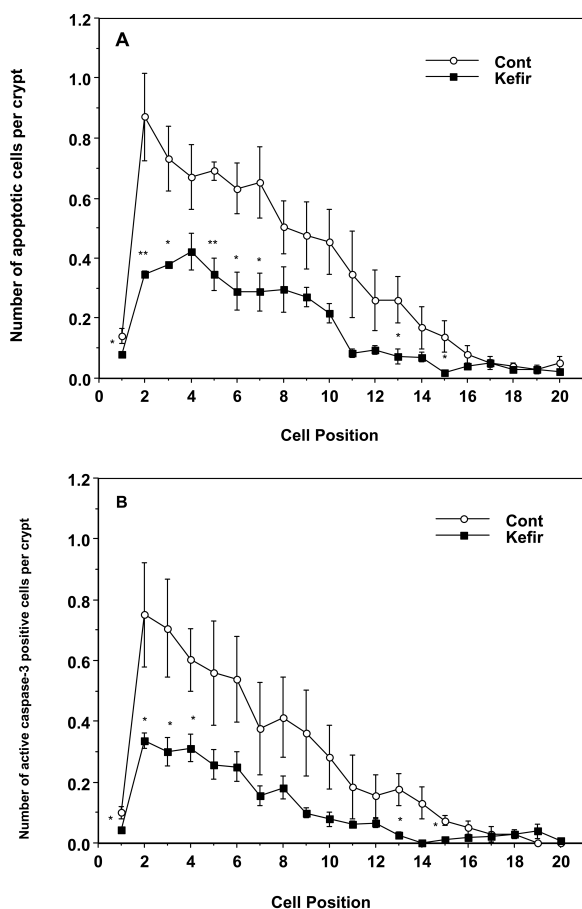
identical. Figure 3B illustrates the pattern of caspase-3 activation at a number of time points following the irradiation of the colon of control rats and kefir-treated rats. At 2 h after irradiation, an

Fig. 4. Cell position distribution of (A) apoptotic cells detected by using H&E staining and (B) active caspase-3 expression in colonic crypts 2 h after 1 Gy irradiation (solid squares) kefir-treated rats, (open circles) control rats. Data are the mean ± SEM for 4 rats per data point. $^*p < 0.05$ and $^{**}p < 0.01$ vs. control rats.

active caspase-3 expression in the colon of control rats peaked, but that in kefir-treated rats was reduced to 35% of the control rat values ($p < 0.05$). At 4 h and 6 h after irradiation, the active caspase-3 expression was decreased in both groups with no difference between control rats and kefir-treated rats.

The distribution of apoptotic bodies in colon positions detected by using H&E staining and active caspase-3 expression 2 h after 1 Gy irradiation is shown in Fig. 4. The localization of apoptotic bodies along the crypt in kefir-treated rats was significantly reduced at cell positions 1–3, 5–7, 13, and 15 (Fig. 4A). An active caspase-3 expression of kefir-treated rats gave significantly lower values compared with control rats at cell positions 1–4, 13, and 15, which is almost the same position as for H&E staining (Fig. 4B).

DISCUSSION

Intestinal epithelial stem cells are rapidly proliferating cells *in vivo* and are the most sensitive to irradiation-induced damage[25].

Apoptosis is the main cause of irradiation-induced intestinal damage[3,26,27], which is responsible for many of the side effects of aggressive cancer therapy. Nonalimentary pelvic tumors are usually treated by doses in the range 50–70 Gy over 6–7 weeks, administered 5 days per week, once-daily, in about 2 Gy fractions, which entail significant acute morbidity that includes diarrhea, abdominal cramps, and hematochezia[4]. The aim of our study was to evaluate whether fermented milk kefir was capable of preventing X-ray radiation-induced colonic apoptosis *in vivo* in a rat model.

The results of this study have clearly demonstrated that kefir ingestion before irradiation had a protective effect on radiation-induced apoptosis in the colon of rats. The greatest suppression was observed 2 h after 1 Gy irradiation detected by apoptotic morphological changes (H&E staining) and by labeling DNA strand breaks (TUNEL) in colonic crypt cells (Fig. 1, A–D, Fig. 2, and Fig. 3A). In the graph showing dose-dependent effects at 2 h following irradiation, the apoptotic index of control rats closed to the plateau at 2 Gy. On the other hand, the apoptotic index of kefir-treated rats didn't reach the plateau of 4 Gy (Fig. 2). Kefir treatment may increase the dose of X rays needed to reach plateau, but it was unable to protect the apoptotic cells that have already reached plateau. At 4 h and 6 h after 1 Gy irradiation, although the apoptotic index of kefir-treated rats was lower than control rats, there was no significant difference between control rats and kefir-treated rats (Fig. 3A).

Caspase-3 activation is a major effector in the apoptotic process following an irradiation of intestinal cells[17]. In this study, kefir treatment significantly suppressed active caspase-3 expression in the colonic crypt at 2 h after 1 Gy irradiation (Fig. 1, E and F, and Fig. 3B). At 4 h and 6 h, the number of active caspase-3 positive cells was lower than the apoptotic index in both groups (Fig. 3, A and B). Recently, Ohara *et al.* suggested a caspase-3-independent pathway in radiation-induced intestinal epithelial cell apoptosis[28]. We considered that the difference in the apoptotic index value and the number of active caspase-3 positive cells in control and kefir-treated rats 4 h and 6 h after irradiation might be due to a caspase-independent pathway. Kefir may protect radiation-induced apoptosis by inhibition of the caspase-3 dependent pathway, but not of the caspase-3 independent pathway. The reduction of apoptotic bodies and active caspase-3 expression by kefir was mostly observed in the lower region, including the stem cell region, which is at cell 1–2 positions at the base of the crypt[29] (Fig. 4, A and B). Nagira *et al.* have suggested that kefir treatment could decrease thymine dimers by stimulating the DNA repair activity in HMV-1 cells after UV-irradiation[15]. We speculate that kefir treatment may decrease DNA damage by enhancing DNA repair activity, or kefir may block a step in the apoptotic pathways[30]. Further studies are required to elucidate the mechanisms of antiapoptotic effect induced by kefir treatment.

Our results clearly demonstrated that kefir protects the colonic epithelial stem cell region, which is the region most sensitive to DNA damage, and is extremely important for regeneration[31] fol-

lowing radiation-induced apoptosis; this antiapoptotic effect of kefir was mediated through the inhibition of caspase-3 activation. Kefir treatment may have possibilities to diminish side effects in the intestinal epithelium of patients undergoing irradiation therapy for malignancy.

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