

Sucralfate Protects Intestinal Epithelial Cells from Radiation-Induced Apoptosis in Rats

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Radiotherapy for malignant pelvic disease is often followed by acute radiation colitis (ARC). It has been reported that sucralfate treatment has a protective effect against ARC, though the mechanisms of action are unknown. The effects of sucralfate on X-ray radiation-induced apoptosis was studied at 4 Gy in the colonic crypt cells of rats. Sucralfate enemas given prior to radiation resulted in the following: (1) reduction in number of apoptotic colonic crypt cells; (2) reduction in number of caspase-3 positive cells; (3) decreases in p53 accumulation and p21 expression; (4) decreases of Bax/Bcl-2 ratio. The protective effects of sucralfate against ARC may be partially due to the suppression of radiation-induced apoptosis by way of p53 in the colon and the protection of the colonic epithelial stem cell region.

INTRODUCTION

Exposure of the intestine to ionizing radiation results in the rapid, apoptotic death of the stem cells.¹⁾ Crypt disturbance results from the destruction of the stem cells, which are responsible for repopulating the lining of the intestine. At suitable high doses, damage to the crypt is accompanied by functional changes, such as malabsorption, which is expressed clinically as acute bowel reactions.²⁻⁴⁾ Radiotherapy for malignant pelvic disease frequently results in acute radiation-induced colitis (ARC).⁵⁻⁷⁾ The most common acute symptoms of ARC are diarrhea, tenesmus, and rectal bleeding.⁸⁾ In spite of the high incidence of these problems in a select group of patients, there is no well established therapy. Recently, it has been reported that sucralfate has a protective effect on ARC,⁹⁻¹¹⁾ but the mechanisms of action remain to be determined.

Sucralfate, an aluminium hydroxide complex of sulfated sucrose, is an anti-ulcer agent thought to act through endogenous prostaglandin production¹²⁾ and/or by an intrinsic mucosal "coating effect".¹³⁾ It has been suggested that

sucralfate increases epidermal growth factor binding to ulcerated areas, and also stimulates epithelial cell restitution and cell proliferation.¹⁴⁾ Recent study suggested that sucralfate inhibited ethanol-induced necrosis and apoptosis in primary cultures of guinea pig gastric mucosal cells,¹⁵⁾ and sucralfate prevented apoptosis occurring in the ischemia/reperfusion-induced intestinal injury.¹⁶⁾

p53 is a 393-amino acid nuclear phosphoprotein that acts as a transcription factor to control cell cycle checkpoints and induces apoptosis in response to ionizing radiation.^{17,18)} In response to DNA damage, wild-type p53 accumulates in the nucleus and arrests cell cycle progression through the cyclin-dependent kinase inhibitor p21WAF1/CIP1 (p21).¹⁹⁻²¹⁾ It is known that wild-type p53 plays a role in the control of apoptotic pathways by downregulating *Bcl-2* and upregulating *Bax*. *Bcl-2* inhibits apoptotic cell death, whereas the expression of *Bax* and subsequent formation of *Bax-Bcl-2* complex is thought to induce apoptotic cell death.²²⁾

The aim of this study was to evaluate the effects of sucralfate on acute X-ray radiation-induced apoptosis in the colon. Routine histological study with haematoxylin and eosin staining (H&E) confirmed the presence of apoptosis. Active caspase-3, which is a key effector of the apoptotic pathway,²³⁾ was then studied in the colonic crypt cells by immunohistochemistry. The effects of sucralfate on the expression of p53, p21, *Bax* and *Bcl-2* after irradiation were also examined.

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MATERIALS AND METHODS

Animals and treatment of sucralfate

Seven-week-old male Wistar rats (200–260 g) were purchased from Charles River Japan (Atsugi, Japan). 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. Food was removed one day before irradiation but water was available. 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.

Rats were given enemas of either 0.2 g of sucralfate (Chugai pharmaceutical co. Japan), in a 2 ml suspension, or 2 ml control saline 15 minutes prior to 4 Gy irradiation. Non-irradiated animals of the 0 h group were sacrificed 1.5 h after sucralfate or saline treatment. Three to four rats in each group were studied.

Irradiation

Irradiation was performed between 9:00 a.m. and 12:00 p.m. Rats received abdominal X-ray irradiation using a Toshiba EXS-300 X-ray, 200 KV, 15 mA apparatus with 0.5 mm Al filter at a dose-rate of 0.864 Gy / min. A single dose of 4 Gy was given under pentobarbital anesthesia (30 mg/kg, i.p.). Thereafter, the animals were placed in a plastic shell with lead shield (2 mm thickness) on the upper and lower sides. The abdomen region containing approximately a 5 cm length of rectum was exposed. Non-irradiated animals of the 0 h group were also anesthetized under the same conditions.

Histology and assessing apoptosis

Rats in each group were sacrificed by deep anesthesia at 1, 2 and 4 h after 4 Gy irradiation. The colon was resected and immersed in neutral-buffered formalin, and embedding in paraffin blocks from which 3- μ m sections were cut and stained with H&E. Identification of apoptosis was confirmed using a TUNEL technique (APOPTAG™, Intergen, NY) which stains the oligofragmented DNA characteristically found in apoptotic nuclei.²⁴ Active caspase-3 was detected by anti-active caspase-3 polyclonal antibody (R&D Systems, Abingdon, UK) staining. This antibody specifically recognizes amino acids 163–175 of caspase-3, but does not detect the precursor form. Immunohistochemical staining for active caspase-3 was done with a “VECTASTAIN” ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer’s instructions.

Fifty crypts per group from complete colonic crypts that had been cut in the longitudinal plane were selected for analysis. The incidence of cell death (apoptotic index) in the

colon was quantified by counting the number of dead cells in each crypt in H&E-stained sections at $\times 400$ magnification by light microscopic analysis. The distinctive morphological features of apoptosis, as described by Kerr²⁵ and Walker *et al.*²⁶ were used to recognize apoptotic cells. Small clusters of dead cell fragments were assessed as originating from one cell and given a single count and any doubtful cells were disregarded.^{27,28} Immunopositive cells for active caspase-3 were counted at least 50 crypts per animal, and the average value was calculated. Furthermore apoptosis was scored on a cell-positional basis of 50 half-crypt sections per rat with 4 rats in each group using H&E stained tissue 2 h after 4 Gy irradiation. The apoptotic index of cell position is the percentage of apoptotic cells per cell position in colonic crypt. All crypts chosen were at least 20 cells in length, with cell position 1 located at the crypt base.

Western blotting

The colon tissues of control and sucralfate-treated rats were removed at 1, 2 and 4 h after 4 Gy irradiation and frozen immediately. The tissues were then suspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% sodium deoxycholate and 0.05% SDS, pH 7.4), broken into pieces on ice and subjected to three freeze-thaw cycles.²⁹ The insoluble cell debris was removed by centrifugation. Supernatants were collected and the protein concentration was quantified using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples (30 μ g) were subjected to 10% or 15% SDS-polyacrylamide gel electrophoresis, then transferred electrophoretically to Hybond ECL Nitrocellulose Membranes (Amersham, Arlington heights, IL) as described previously.³⁰ Membranes were incubated with the following: mouse monoclonal anti-p53 (PAb 421) (Oncogene Science Inc., Uniondale, NY); rabbit polyclonal antibodies against the following: p21^{WAF1/CIP1} (Santa Cruz Biotechnology, Santa Cruz, CA); Bax (BD Pharmingen, San Diego, CA); Bcl-2 (BD Pharmingen); or actin (Sigma, St. Louis, MO). This was followed with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Zymed Labs. Inc., San Francisco, CA) or a horseradish peroxidase-conjugated anti-rabbit IgG (Amersham). Chemiluminescence (ECL Plus Amersham) was used for analyzing levels of protein according to the manufacturer's protocol. Blots were exposed to Hyperfilm ECL (Amersham). NIH image 1.61 software was used in measuring the densities of each of the protein bands. The level of protein after irradiation was determined using the level of actin as a standard.

Statistical evaluation of data

All values were expressed as the mean \pm SEM of results obtained from 3–4 animals per data point. Differences between groups were examined for statistical significance using the Mann Whitney *U*-test and Student’s *t* test for kinetics diagrams of western blot. A *P* < 0.05 value was consid-

ered to be of statistical significance.

RESULTS

Apoptotic index of control rats and sucralfate-treated rats after irradiation

Figure 1 shows histologic sections of colonic crypts from control and sucralfate-treated rats that were stained with H&E (Fig. 1A, B), TUNEL (Fig. 1C, D) and active caspase-3 antibody (Fig. 1E, F). At 2 h after 4 Gy irradiation, a large number of apoptotic cells was observed in the colonic crypts of control rats (Fig. 1A, C). In contrast, there was a sharp decrease in the number of apoptotic cells in the colonic crypts of sucralfate-treated rats, as determined by TUNEL staining (Fig. 1D). Positive staining for active caspase-3 was also greatly decreased in sucralfate-treated rats (Fig. 1F) when compared with control rats (Fig. 1E).

Figure 2 compares the time course of the apoptotic index detected using H&E staining and active caspase-3 expression in control and sucralfate-treated rats up to 4 h after irradiation with 4 Gy. The apoptotic index, as detected by H&E staining, increased in control rats by 4 h after irradiation. Sucralfate enema significantly reduced the number of apoptotic colonic crypt cells at 2 h and 4 h after 4 Gy irradiation to 16% ($P < 0.05$) and 55% ($P < 0.05$) of the control rat values, respectively. There was no significant difference in the background (0 h) levels of apoptosis in both groups (Fig. 2A). Figure 2B illustrates the pattern of caspase-3 activation following irradiation of the colon in control and sucralfate-treated rats. The number of active caspase-3 positive cells in control rats peaked at 2 h after irradiation. Treating with sucralfate enema resulted in a significant decrease of active caspase-3 positive cells at 2 h and 4 h after 4 Gy irradiation to 19% ($P < 0.05$) and 63% of the control rat values, respectively. The apoptotic index was highest for cell position 2 (corresponding to the putative position of the crypt stem cell) and gradually decreased at positions higher in the crypt. A significant reduction of the apoptotic index for cell positions 1–9, 11–14 and 19 was noted in sucralfate treated rats (Fig. 2C).

Western blotting for p53, p21, Bax and Bcl-2

In an effort to determine how sucralfate treatment was interfering with the pathways leading to radiation-induced apoptosis, the expression of p53, p21, Bax and Bcl-2 after 4 Gy irradiation was examined by Western blot analysis and kinetic diagrams are shown in Fig. 3 and Fig. 4. Accumulation of p53 in non-irradiated and sucralfate-treated rats was 1.4-fold higher than that of non-irradiated control rats. p53 accumulation in control rats after irradiation showed 1.9-fold increases at 1h over non-irradiated control rats. At 2h and 4 h after irradiation, the increases returned to baseline levels. Accumulation of p53 in sucralfate-treated rats was reduced to 0.7-fold at 1h and 2 h as compared to non-irra-

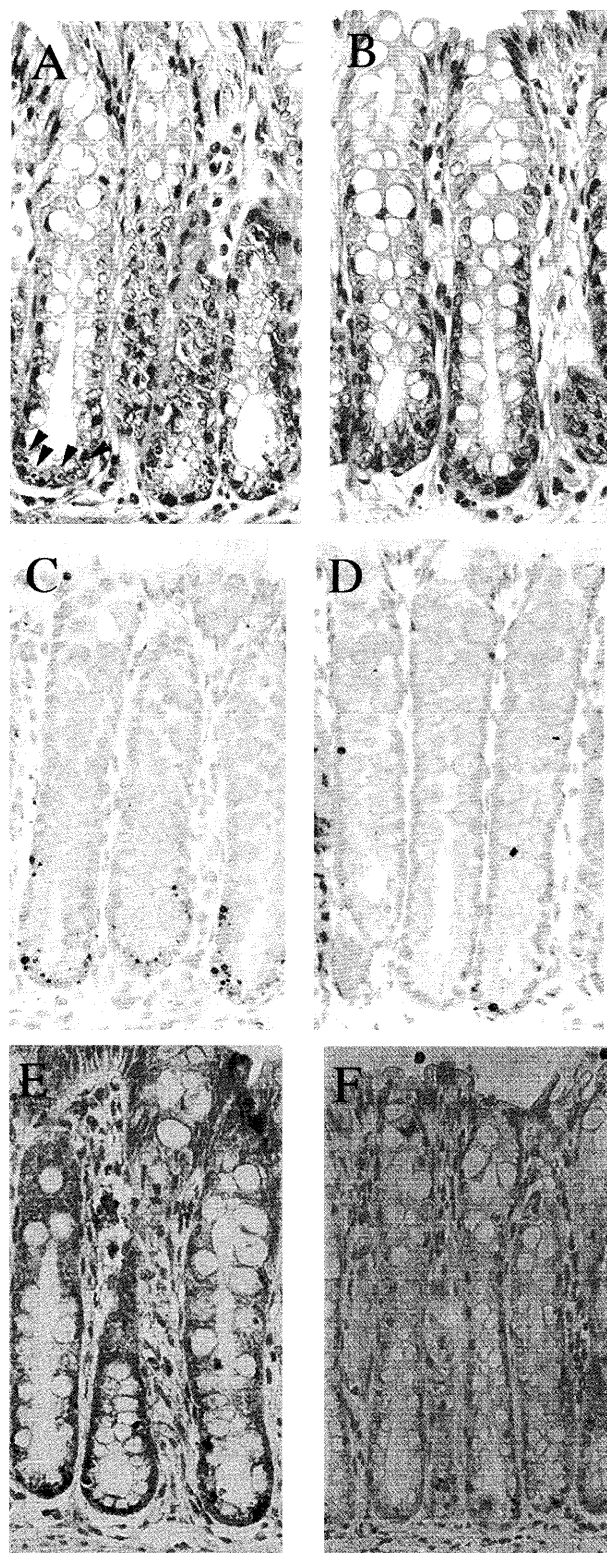


Fig. 1. Histologic sections of the colon in control rats (A, C and E) and sucralfate-treated rats (B, D and F) at 2 h after 4 Gy irradiation, stained by the H&E (A, B), and the TUNEL method (C, D) to make visible the cells containing DNA fragments and active caspase-3 (E, F) ($\times 400$).

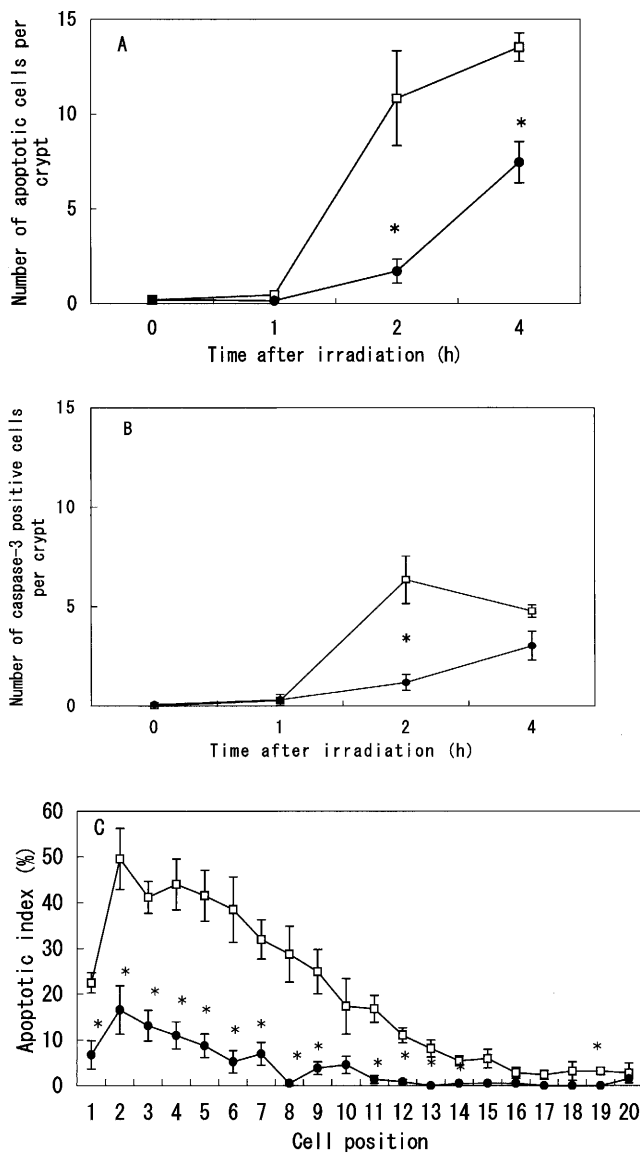


Fig. 2. Apoptotic Index (A) and caspase-3 activation (B) of colonic crypt cells in control rats and sucralfate-treated rats at 0, 1, 2 and 4 h after 4 Gy irradiation. The cell-positional distribution of apoptosis in the crypts was scored at 2 h after 4 Gy irradiation (C). (□) control rats, (●) sucralfate-treated rats. Data are the mean \pm SEM values of 3–4 rats per data point. * $P < 0.05$ vs control rats. .

diated sucralfate-treated rats, and 1.4-fold increases at 4 h were noted over non-irradiated sucralfate-treated rats. There was significant difference at 1h ($p < 0.05$) between control and sucralfate-treated rats (Fig. 3A). The expression of p21 in non-irradiated sucralfate-treated rats was 3-fold higher than that of non-irradiated control rats. The expression of p21 in control rats increased 1.8, 1.9 and 2.2-fold at 1 h, 2h and 4 h respectively over non-irradiated control rats, while such increases after irradiation were not observed in sucralfate-treated rats. There was significant difference at 2h ($p < 0.01$) and 4h ($p < 0.05$) between control and sucralfate-treated

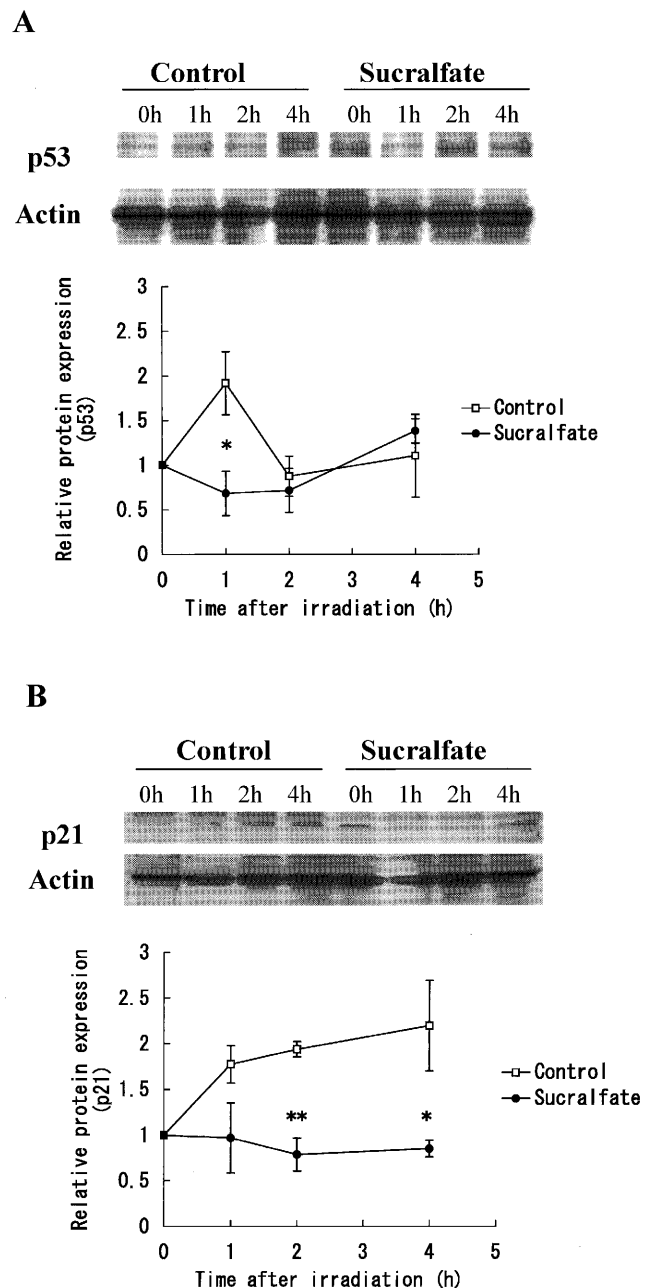


Fig. 3. Western blotting analysis and kinetics of p53 (A) and p21^{WAF1/Cip1} (B) expression in the colon of control rats and sucralfate-treated rats at 1, 2 and 4 h after 4 Gy irradiation. (□) control rats, (●) sucralfate-treated rats. The amounts of p53 and p21 were quantified by densitometric analysis. Each of the protein levels is expressed as ratios to non-irradiated rats (0h) in control or sucralfate-treated rats. Data represent mean \pm SEM values of three separate experiments from two rats. * $P < 0.05$ and ** $P < 0.01$ vs control rats.

ed rats (Fig. 3B).

Expression of Bax in non-irradiated sucralfate-treated rats was 2.3-fold higher than that of non-irradiated control rats. Bax expression in control rats after irradiation was increased

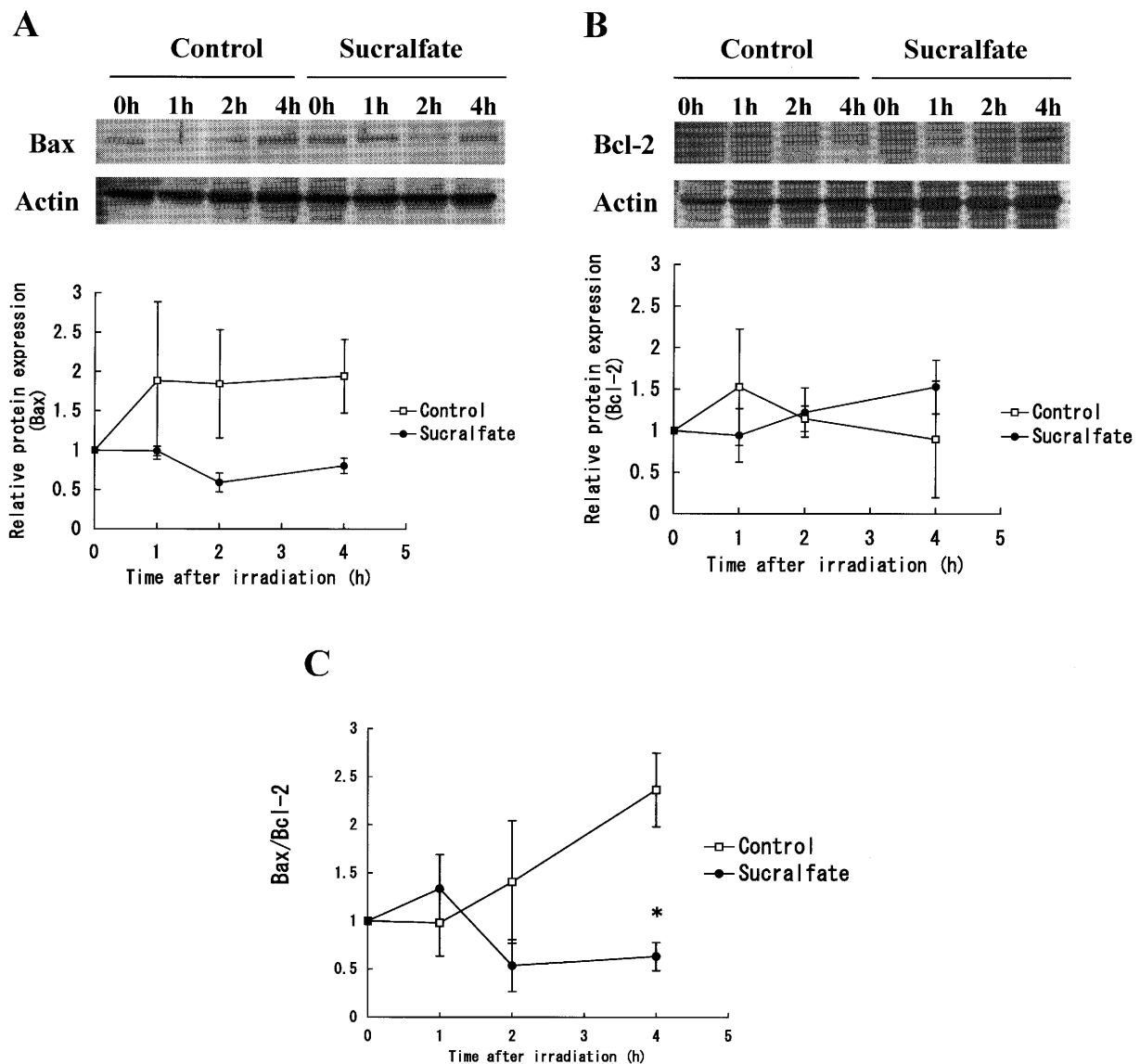


Fig. 4. Western blotting analysis and kinetics of Bax (A) and Bcl-2 (B) expression, and Ratio of Bax to Bcl-2 (C) in the colon of control rats and sucralfate-treated rats at 0, 1, 2 and 4 h after 4 Gy irradiation. (□) control rats, (●) sucralfate-treated rats. The amounts of Bax and Bcl-2 were quantified by densitometric analysis. Each of the protein levels is expressed as ratios to non-irradiated rats (0h) in control or sucralfate-treated rats. Data represent mean \pm SEM of three separate experiments from two rats. * $P < 0.05$ vs control rats.

1.9, 1.8 and 1.9-fold at 1h, 2h and 4h respectively over non-irradiated control rats, while no such increases were detected in sucralfate-treated rats. However there was no statistical difference between control and sucralfate-treated rats (Fig. 4A). Expression of Bcl-2 in non-irradiated sucralfate-treated rats was 1.5-fold higher than that of non-irradiated control rats. Bcl-2 expression of control rats after irradiation was increased 1.5-fold at 1h over non-irradiated control rats and then decreased 0.9-fold at 4h, while that of sucralfate-treated rats was increased 1.2-fold and 1.5-fold at 2h and 4h respectively as compared to non-irradiated sucralfate-treated rats. However there was no significant difference between control

and sucralfate-treated rats (Fig. 4B). The Bax/Bcl-2 ratio (an index of cell susceptibility to apoptosis) in control rats after irradiation was increased 1.4 and 2.4-fold at 2h and 4h respectively over non-irradiated control rats, while that in sucralfate-treated rats was reduced to 0.5 and 0.6-fold at 2h and 4h respectively as compared to non-irradiated sucralfate-treated rats. There was a significant difference at 4h ($p < 0.05$) between control and sucralfate-treated rats (Fig. 4C).

DISCUSSION

Intestinal stem cells in the crypts are rapidly proliferating

cells *in vivo* which are most sensitive to irradiation-induced damage. Apoptosis is the main cause of irradiation-induced intestinal damage^{4,31,32} responsible for many of the side effects of aggressive cancer therapy. Nonalimentary pelvic tumors are usually treated to doses in the range 50–70 Gy, over 6–7 weeks, administered 5 days per week in about 2 Gy fractions on a once-daily basis. Such a treatment schedule has been known to result in significant acute morbidity, including diarrhea, abdominal cramps, and hematochesia.⁷ A number of therapeutic measures have been tried in the treatment of radiation proctitis. These include derivatives of 5-aminosalicylic acid, formalin, topical sucralfate,^{9,10,33} topical amifostine^{34,35} and topical sodium butyrate.³⁶ With respect to clinical applications, several trials have suggested that sucralfate in oral and enema forms appear to be effective in the treatment of chronic radiation enteritis, though some reports have suggested that sucralfate was not effective in reducing the risk of acute and late rectal toxicity due to radiotherapy.^{37,38} According to Kochhar *et al.* the topical administration of sucralfate in patients exerted a protective effect which was able to heal mucosal lesions and to stop rectal bleeding from radiation-induced mucosal proctosigmoiditis.⁹ The protective mechanisms of sucralfate for radiation proctitis are still unknown. The aim of this study was to evaluate whether sucralfate was capable of preventing X-ray radiation-induced apoptosis and to determine the mechanism of sucralfate effect on radiation-induced apoptosis in the colonic crypt cells of rats.

The increased incidence of apoptosis from background (0 h) levels was first observed at 2 h, peaked at 4 h, and then decreased gradually by 24 h in the large intestine after 2 Gy whole-body irradiation (data not shown). Arai *et al.* had similar observations about radiation-induced apoptosis in the colonic crypt cells.³⁹ The apoptotic index of control rats showed gradual increases at 2 h and 4 h after 4 Gy irradiation (Fig. 2A). The significant suppression of apoptotic cells by sucralfate enema was observed at 2 h using H&E, TUNEL and active caspase-3 staining methods in colonic crypt cells (Fig. 1, Fig. 2). Caspase-3 activation is a major effector in the apoptotic process following the irradiation of intestinal cells. Caspase-3 was activated earlier in the apoptotic process than DNA cleavage and cell fragmentation.²¹ In this study, apoptosis in H&E-stained sections increased steadily up to 4 h, while caspase-3 activation peaked at 2 h and then was reduced at 4 h in the control group. A significant suppression of active caspase-3 positive cells was observed in the sucralfate treated group at 2 h after irradiation (Fig. 2B). These results suggest that sucralfate can protect radiation-induced apoptosis by inhibition of the caspase-3 activation. The number of active caspase-3 positive cells was lower as compared to the number of apoptotic cells determined by H&E staining in both groups. The difference in the apoptotic cells number with H&E staining and active caspase-3 positive cells may be due to a caspase-indepen-

dent apoptosis pathway.⁴⁰ The reduction of apoptotic bodies by sucralfate was mostly observed in the lower region of the crypt, including the stem cell region, which is at the 1-2 cell positions at the base of the crypt⁴¹ (Fig. 2C). We have reported that fermented milk kefir has a protective effect on radiation-induced apoptosis, presumably in protecting the stem cell region of the colonic crypt cells from radiation.⁴² Sucralfate appears to have a similar protective effect as kefir.

Irradiation induces DNA damage leading to the activation of p53, Bax and caspase-3 effector mechanisms. To determine the radiation-induced apoptosis pathways suppressed by sucralfate treatment, p53, p21, Bax and Bcl-2 expression after X-ray irradiation were examined. p53 accumulation in sucralfate-treated rats showed an early decrease at 1h ($p < 0.05$) when compared with control rats after irradiation (Fig. 3A), and then p21 expression in sucralfate-treated rats showed decreases when compared with control rats at 2 h ($p < 0.01$) and 4 h ($p < 0.05$) after irradiation (Fig. 3B). The Bax/Bcl-2 ratio is considered to be an index of cell susceptibility, and in sucralfate-treated rats this ratio showed decreases at 2 h and 4 h ($p < 0.05$) after irradiation. This pattern was similar with that of the suppression of apoptosis in the colon of sucralfate-treated rats (Fig. 4C). The protective effect of sucralfate in rats from radiation-induced apoptosis may be dependent on the p53 pathway, and the decreased expression of the Bax/Bcl-2 ratio may be partially involved in the protective effect of sucralfate on radiation-induced apoptosis in the colon.

Included among the effect of sucralfate are its binding capacity to mucosal surfaces especially when inflamed or denuded, and the stimulating action on mucus production and bicarbonate secretion together with its effects on surface phospholipids, prostaglandin synthesis, mucosal blood flow, cell proliferation, and availability of epidermal growth factor.⁴³ Sencan A. B. *et al.* suggested that sucralfate is beneficial in the prophylaxis and treatment of hypoxia/reoxygenation-induced intestinal injury,¹⁶ and they supported the idea that sucralfate has an anti-oxidant effect.⁴⁴ The anti-oxidant effect of sucralfate may effect the reduction of p53 accumulation, p21 expression, Bax/Bcl-2 ratio after irradiation.

In this study, sucralfate treatment showed upregulation of p53 accumulation. Because of p53 induction by sucralfate, sucralfate-treated rats may show reduced p53 responses to X-rays, and therefore suppression of p53 accumulation might be seen at 2 h and delayed appearance of p53 accumulation might occur at 4 h after irradiation (Fig. 3A). This is the first report that sucralfate upregulates the p53 pathway, though it is not known how this is achieved. One idea proposed is that sucralfate acts as an anti-oxidant and may influence the p53 pathway, which however, remains to be confirmed.

In conclusion, sucralfate has shown a protective effect of radiation-induced apoptosis in the rat colon following decreases in p53 accumulation, p21 expression and Bax/Bcl-

2 ratio, and protects the colonic epithelial stem cell region, which is the region most sensitive to DNA damage. Pretreatment with sucralfate and its presence in the lower digestive tract during radiation may be important for the therapy and prevention of acute radiation proctitis during radiotherapy for pelvic malignancies.

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