

Adenovirus-Mediated Bcl-XL Gene Therapy Combined with Pronase Treatment Protects the Small Intestine from Radiation-Induced Enteritis in Mouse Model

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Rec date: March 27, 2014; Acc date: Sep 03, 2014; Pub date: Sep 09, 2014

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Abstract

Intestinal injury is a major side effect of radiation treatment for many malignancies. The present study investigated whether transducing Bcl-xL, a potent anti-apoptotic gene, into intestinal epithelial cells would exert protective effects against radiation-induced acute injuries.

Methods: Adenoviral vectors containing the human Bcl-xL gene (AxCABclxL) or β -galactosidase gene (AxCAlacZ) driven by the CAG promoter were generated. To increase transduction efficiency into the mucosal epithelium, the intraluminal space of the small intestine of mice was washed with buffered-saline and mucus components were digested with Pronase MS®. Gene transduction was performed by injecting 2×10^8 pfu adenoviral vector into the pre-treated small intestine. Transduction efficiency was examined by X-gal staining 24 hours after AxCAlacZ infection. Radiation-induced acute injury of the small intestine was induced by whole body irradiation (15 Gy) performed 24 hours after adenoviral vector infection. Apoptotic epithelial cells were visualized by TUNEL assay. Morphological analysis was assessed by histological examination.

Results: Successful transduction after Pronase MS® treatment was achieved in the basal crypt epithelial cells in the ileum, thought to be the location of stem cells, as determined by X-gal staining. The AxCABclxL group demonstrated significantly fewer radiation-induced apoptotic mucosal epithelial cells when compared with the other two groups at 6 hours after irradiation ($p < 0.05$). At 72 hours after irradiation, the morphological appearance of the small intestine in the AxCABclxL group showed significantly less radiation damage in terms of mucosal thickness ($p < 0.001$).

Conclusions: The present study indicates that Bcl-xL gene expression using adenoviral vector-mediated transduction is a valuable approach to prevent intestinal injury caused by radiation exposure.

Keywords: Bcl-xL; Gene therapy; Radiation; Apoptosis; Intestinal stem cell; Intestinal injuries

Introduction

Radiation therapy is widely applied as a valuable treatment for abdomino-pelvic malignancies including colon, rectum, prostate and uterine carcinomas [1-3]. Because the target area for radiation cannot be specifically limited to the malignant lesions, neighboring tissues/organs are also susceptible to the effects of irradiation. The small intestine is one of the most frequently damaged regions during pelvic irradiation. Therefore, radiation therapy, either alone or in conjunction with chemotherapy, often requires interruption or cessation due to serious acute damage in the small intestine [1-4]. Such acute injury includes severe enterocolitis, intestinal fistulas and strictures. In order to further facilitate radiation therapy, development of therapeutic approaches that provide radio protective effects in the small intestine is required [3-10].

Recent attempts to elucidate the fundamental mechanisms of radiation-related intestinal damage have found that accelerated epithelial cell death induced by apoptosis is a key event [8,9]. Ionizing radiation-induced free radicals and reactive oxygen species damage vital cellular targets such as DNA and membranes including mitochondria [3,10]. Mitochondrial damage and the subsequent release of cytochrome c into the cytoplasm lead to activation of caspase-mediated cell death pathways [9-12]. Bax facilitates the release of cytochrome c and the sequential activation of the caspase cascade [13].

Bcl-xL shows strong anti-apoptotic function by counteracting pro-apoptotic Bax [12-17]. Shinoura et al. [18] suppressed Bax-induced apoptosis in human glioma cells by adenovirus-mediated transfer of Bcl-xL. Transduction of the Bcl-xL gene in vivo was recently shown to exert protective effects against apoptosis-related cellular damage leading to phenotypic corrections in several animal disease models, including cardiac infarction and cardiac cold preservation [19,20]. However, direct protective effects by Bcl-xL gene expression in intestinal epithelial cells following irradiation have not been reported.

The present study investigated whether adenoviral Bcl-xL gene therapy could protect the small intestine from radiation-induced injuries in mouse model.

Materials and Methods

Animals

Male C57BL/6-N mice, 8 to 10 weeks of age, were purchased from Charles River Japan, Inc. (Kanagawa, Japan). All mice were placed in cages within a temperature-controlled room with a 12-h light-dark cycle and ad libitum access to food and water. All animals received humane care according to the institutional guidelines set forth by the Nara Medical University Animal Care Committee.

Adenoviral vectors

All recombinant adenoviral vectors used in the present study were derived from human adenovirus type 5, in which the E1 and E3 regions were deleted. Adenoviral vectors encoded either human Bcl-xL (AxCABclxL) or the *Escherichia coli* β -galactosidase gene (AxCALacZ) driven by the CAG promoter composed of a cytomegalovirus immediate early enhancer and a chicken β -actin promoter as described previously [18-20]. These vectors were plaque-purified twice and propagated on 293 cells cultured in DMEM/F12 (Dulbecco's modified Eagle's medium; Nutrient Mixture F12, Ham 1:1, Sigma-Aldrich, St. Louis, MO), followed by purification using Virakit for adenovirus 5 and recombinant derivatives (Virapur, San Diego, CA). All the vector stocks were stored at -80°C until use. Prior to the experiment, virus titers (plaque forming units (pfu)/ml) were determined by plaque assays using 293 cells. We had previously reported the in vitro transduction efficiency by AxCALacZ [21, 22], and Hamada, who is our coauthor, had demonstrated in vitro and in vivo gene transfer by AxCABclxL [18-20].

Vector administration procedures

All surgical procedures were performed under general anesthesia using isoflurane (Forane, Abbot, Tokyo, Japan). After laparotomy, we washed the inner lumen of the terminal ileum with Phosphate Buffered Saline (PBS). In some experiments, after the PBS wash, we injected Pronase (Pronase MS®, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan), into the ileum to remove mucous components. We then placed two clamps in the terminal ileum, 2 cm apart, followed by infusion of adenoviral vectors (2×10^8 pfu) suspended in 100 μ l PBS into the intestine between the two clamps. Thirty minutes later, we removed the clamps and closed the abdominal wound.

Irradiation procedures

Irradiation was performed as described previously [23]. Briefly, we used a 150-KVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo) with a total filtration of 0.5 mm aluminum plus 0.1 mm copper. X-ray dose rate, as measured with a thimble ionization chamber at the sample position, was about 1 Gy/min. Two different doses of radiation (10 Gy and 15 Gy) were administered for different experiments. Mice irradiated at 10 Gy were sacrificed after 10 days and mice irradiated at 15 Gy were sacrificed after either 6 hours or 72 hours.

Histological examination

Detection of lacZ-transduced epithelial cells or liver cells by AxCALacZ was performed on specimens obtained 24 hours after vector injection. Specimens were snap-frozen in Tissue Tak O.C.T. compound (Sakura, Torrance, CA). Sections (10 μ m) were fixed with 0.5% glutaraldehyde and immersed overnight in X-gal solution (X-gal, Sigma-Aldrich, St. Louis, MO) at 37°C. Cells were lightly counterstained with hematoxylin. To quantitatively measure β -galactosidase activity, a chemiluminescent reporter gene assay system using β -Gal Reporter Gene Assay (Roche, Mannheim, Germany) was used. Detection of apoptotic epithelial cells in the small intestine was performed on specimens obtained 6 hours after 15-Gy whole body irradiation. Specimens were fixed in 10% buffered-formalin and embedded in paraffin. Sections (5 μ m) were processed for TUNEL assay using the Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA), in accordance with the procedure suggested by the manufacturer. We counted apoptotic cells observed in 50 randomly selected villous crypt units in each mouse.

Histological scoring

Transfected mice were sacrificed at 72 hours after 15-Gy whole body irradiation, and the other transfected mice were sacrificed at 10 days after 10-Gy whole body irradiation. The ileum was removed and fixed in 10% formalin. Specimens were embedded in paraffin, and 5- μ m sections were cut and stained with hematoxylin/eosin (HE). Sections from experimental samples were scored [24] in terms of mucosal thickness as follows: 0, normal to 75% of normal; 1, 75% to 50% of normal; 2, 50% to 25% of normal; 3, 25% to flat ulceration; 4, frank ulceration. The number of crypts per circumference was also counted.

Statistical analysis

ANOVA and two-tailed Student's t test were used for statistical analysis of multiple groups and pairwise comparison, respectively. Values of $P < 0.05$ were considered significant.

Results

Establishment of adenoviral-mediated gene transduction for intestinal epithelium using Pronase MS®

We first investigated whether conventional vector infusion approaches could provide efficient gene transduction to the intestinal epithelium; we injected AxCALacZ into the general circulation or directly into the intraluminal space of the ileum at a dose of 2×10^8 pfu/mouse ($n=8$ in each group). No obvious gene transduction determined by X-gal staining was observed in samples taken 24 hours after the vector injection using either vector infusion approach (Figure 1 A and B). We hypothesized that the poor gene transduction observed even for direct intraluminal vector injection might be caused by mucous components within the intestinal epithelium that block viral vector infection. In order to remove the mucous component, we treated the intraluminal space of the ileum with Pronase MS® prior to vector infusion. β -galactosidase activities in small intestine after intraluminal AxCALacZ infusion were increased in the Pronase MS® pretreated group (31777 ± 9700 ($n=7$)) compared with the naïve group (8795 ± 5800 ($n=6$)) (Figure 1D). Mucous component removal followed by direct intraluminal adenovector infusion allowed efficient gene transduction into the basal epithelium of the ileum (Figure 1C).

In the Pronase MS[®] plus intraluminal injection group, no X-gal positive cells were observed in other extra-intestinal organs, such as liver (Figure 1E), while in the intravenous injection group, numerous X-gal positive cells were observed (data not shown). These findings

demonstrated that intestine-specific gene transduction could be achieved by the Pronase MS[®] plus intraluminal vector injection method.

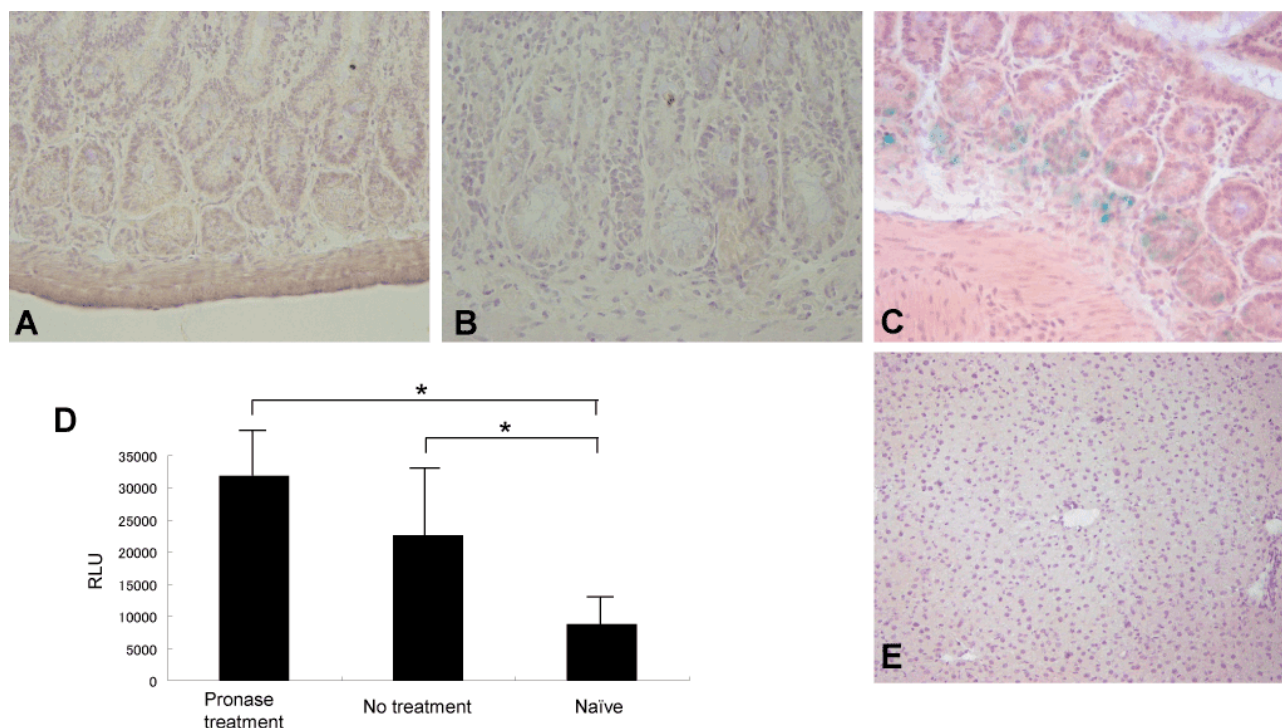


Figure 1: Adenoviral gene transduction into small intestinal crypts in mice. Mice received AxCA-lacZ (2×10^8 pfu/mouse) infused either into general circulation through the tail vein (A) or into the intraluminal space of the small intestine (B and C). In order to remove mucus components of the epithelium, mice in C received Pronase MS[®] treatment prior to vector infusion, while mice in B received saline treatment (control). (A-C) β -galactosidase expression in the small intestinal epithelium. Numerous epithelial cells in the crypts were X-gal positive in C. (D) β -galactosidase activity in the small intestine after intraluminal AxCA-lacZ infusion. * $p < 0.05$; $n = 6-8$ in each group. (E) β -galactosidase expression of the livers of mice in C. No X-gal-positive cells were observed.

Preventive effect of Bcl-xL gene transduction on acute phase radiation injuries of the small intestine

Twenty-four hours before irradiation, mice were treated with one of the following combinations: Pronase MS[®]-PBS ($n = 6$), Pronase MS[®]-AxCALacZ ($n = 5$), or Pronase MS[®]-AxCABclxL ($n = 6$). When mice received 15-Gy whole body irradiation, apoptotic changes in the intestinal epithelial cells were observed at 6 hours, indicative of radiation-induced acute phase injuries (Figure 2A). The number of

apoptotic cells at 6 hours identified by positive TUNEL staining per 50 villous crypt units were 205 ± 65.4 in the Pronase MS[®]-PBS and 174 ± 24.1 in the Pronase MS[®]-AxCALacZ groups, respectively (Figure 2D). In contrast, the number of apoptotic cells was significantly reduced in the Pronase MS[®]-AxCABclxL group (90 ± 25.2) compared with the other two groups (Figure 2D). These results indicated that transducing Bcl-xL genes reduced apoptotic damage of intestinal epithelial cells caused by radiation.

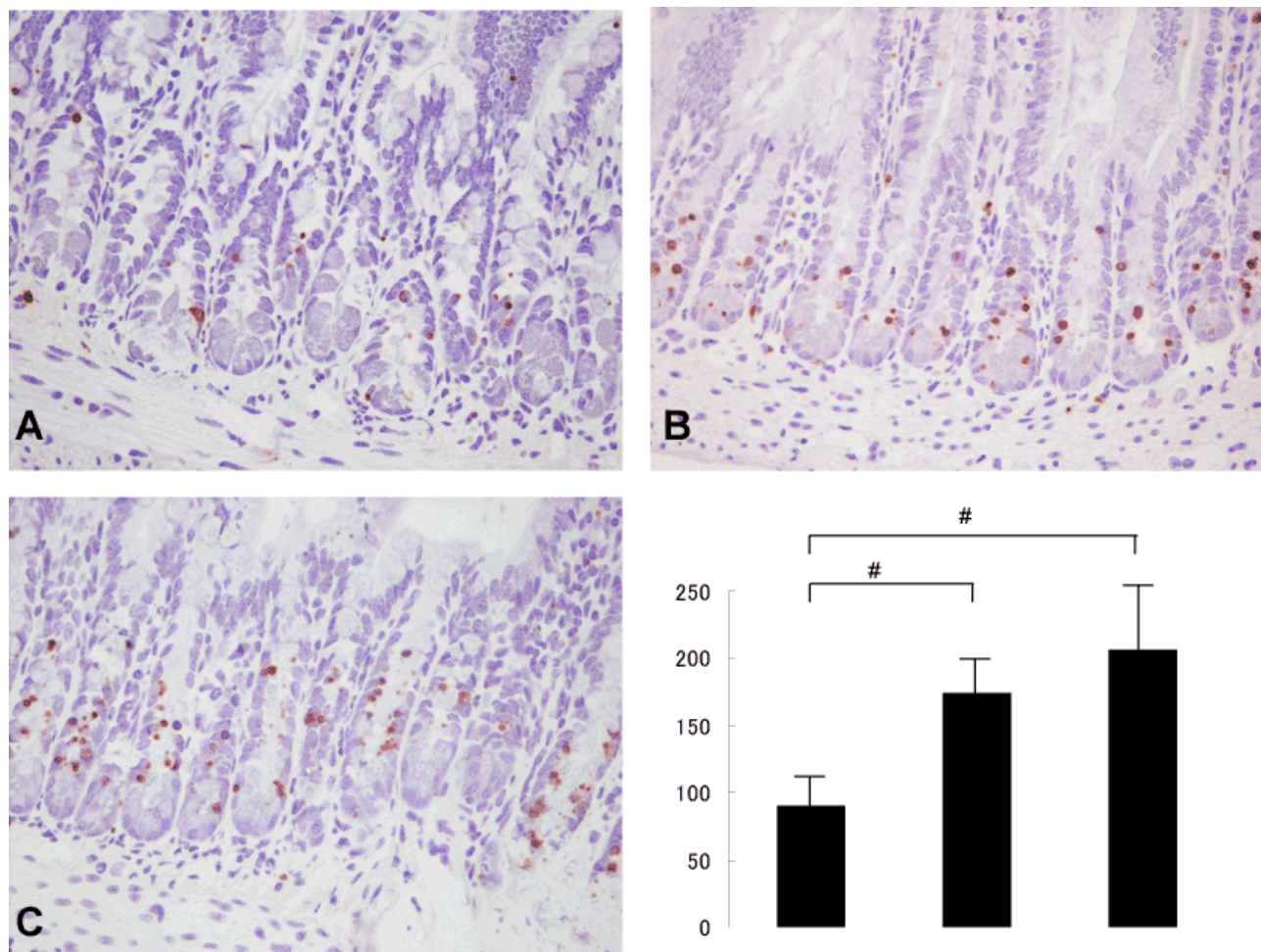


Figure 2: Detection of radiation-induced apoptotic epithelial cells in the small intestine, as determined by TUNEL staining. Mice received intraluminal infusion of AxCA-Bcl-XL (A), AxCA-lacZ (B) or PBS (C) with Pronase MS[®] treatment. Twenty four hours later, mice underwent 15-Gy whole body irradiation and samples were obtained at 6 hours after irradiation. (A-C) TUNEL staining. Original magnification $\times 400$. (D) Number of TUNEL staining-positive apoptotic epithelial cells among 50 crypts. * $p < 0.01$ versus the other two groups; $n = 5-6$ in each group.

Preventive effect of Bcl-xL gene transduction on semi-acute phase radiation injuries of small intestine

Since Bcl-xL gene transduction demonstrated preventive effects against radiation-induced acute intestinal injury, we then assessed the effects in the semi-acute phase 3 days after 15-Gy irradiation. Mice received irradiation after pretreatments with one of the following combinations: Pronase MS[®]-PBS ($n=6$), Pronase MS[®]-AxCALacZ ($n=5$), or Pronase MS[®]-AxCABclxL ($n=6$). Histological findings of the irradiated ileum revealed mucosa flattening, crypt numbers reduction, and submucosal edema in both Pronase MS[®]-PBS and Pronase MS[®]-AxCALacZ groups (Figure 3B and C). In marked contrast, these

phenotypic epithelial alterations were less associated in the Pronase MS[®]-AxBclxL group (Figure 3A). Based on the radiation-damage scoring system, significantly less damage was observed in the Pronase MS[®]-AxCABclxL group as compared with the other two groups (Figure 3D). Morphologic preservation of the intestinal epithelium in the Pronase MS[®]-AxCABclxL group was also observed in terms of the crypt number per circumference (Figure 3E). These findings clearly demonstrated that transducing the Bcl-xL gene could provide protective effects against semi-acute phase radiation-induced injuries leading to morphological maintenance.

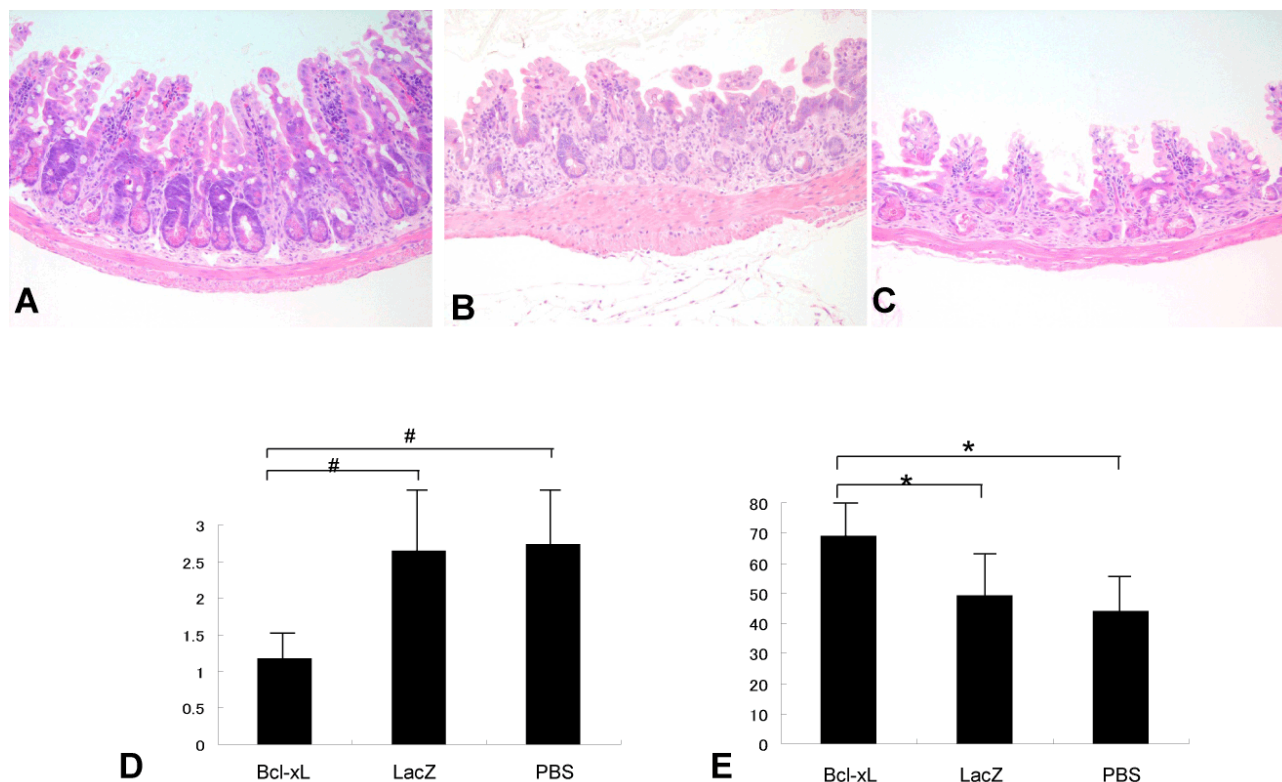


Figure 3: Histological analysis of mouse small intestine after irradiation (acute phase). Mice received intraluminal infusion of AxCA-Bcl-XL (A), AxCA-lacZ (B) or PBS (C) with Pronase MS[®] treatment. Twenty four hours later, mice underwent 15-Gy whole body irradiation and samples were obtained at 3 days after irradiation. (A-C) Hematoxylin/eosin staining. (D) Evaluation of small intestinal epithelial cell damage as determined using the radiation injury scoring system. (E) Number of crypts per circumference. #p<0.01 and *p<0.05 versus the other two groups; n=5-6 in each group.

Preventive effect of Bcl-xL gene transduction on recovery phase radiation injuries of small intestine.

We performed a further long-term experiment (10 days) in order to assess the effect of Bcl-xL gene transduction on the recovery phase after irradiation. Since we found more than 80% mortality by day 10 in mice that received 15-Gy irradiation, we reduced the radiation dosage to 10-Gy in this experiment. Mice received 10-Gy whole body irradiation 24 hours after pretreatment with one of the following combinations: Pronase MS[®]-PBS, Pronase MS[®]-AxCALacZ, or Pronase MS[®]-AxCABclxL (n=3 in each group). Ten day survival of all mice was obtained only in the Pronase MS[®]-AxCABclxL group. Two mice in the Pronase MS[®]-AxCALacZ group and one mouse in the Pronase MS[®]-PBS group died within 5 days after irradiation. For samples taken 10

days after irradiation, histological examination revealed that the height of villi of the Pronase MS[®]-AxCABclxL group was almost identical to normal ileum, while reduced height of villi was observed in the Pronase MS[®]-AxCABclxL group and the Pronase MS[®]-PBS group (Figure 4A-C). Because only one mouse of the Pronase MS[®]-AxCALacZ group survived at 10 days after irradiation, regarding crypt number per circumference of the ileum, we show the chisel in Figure 4D as the results of the Pronase MS[®]-AxCABclxL group and the Pronase MS[®]-PBS group. Crypt number per circumference of the Pronase MS[®]-AxCABclxL group was significantly higher than that of the Pronase MS[®]-PBS group (Figure 4D). This study confirms the therapeutic potential of the Bcl-xL gene therapy approach to provide rapid recovery of the intestinal epithelium from radiation injury.

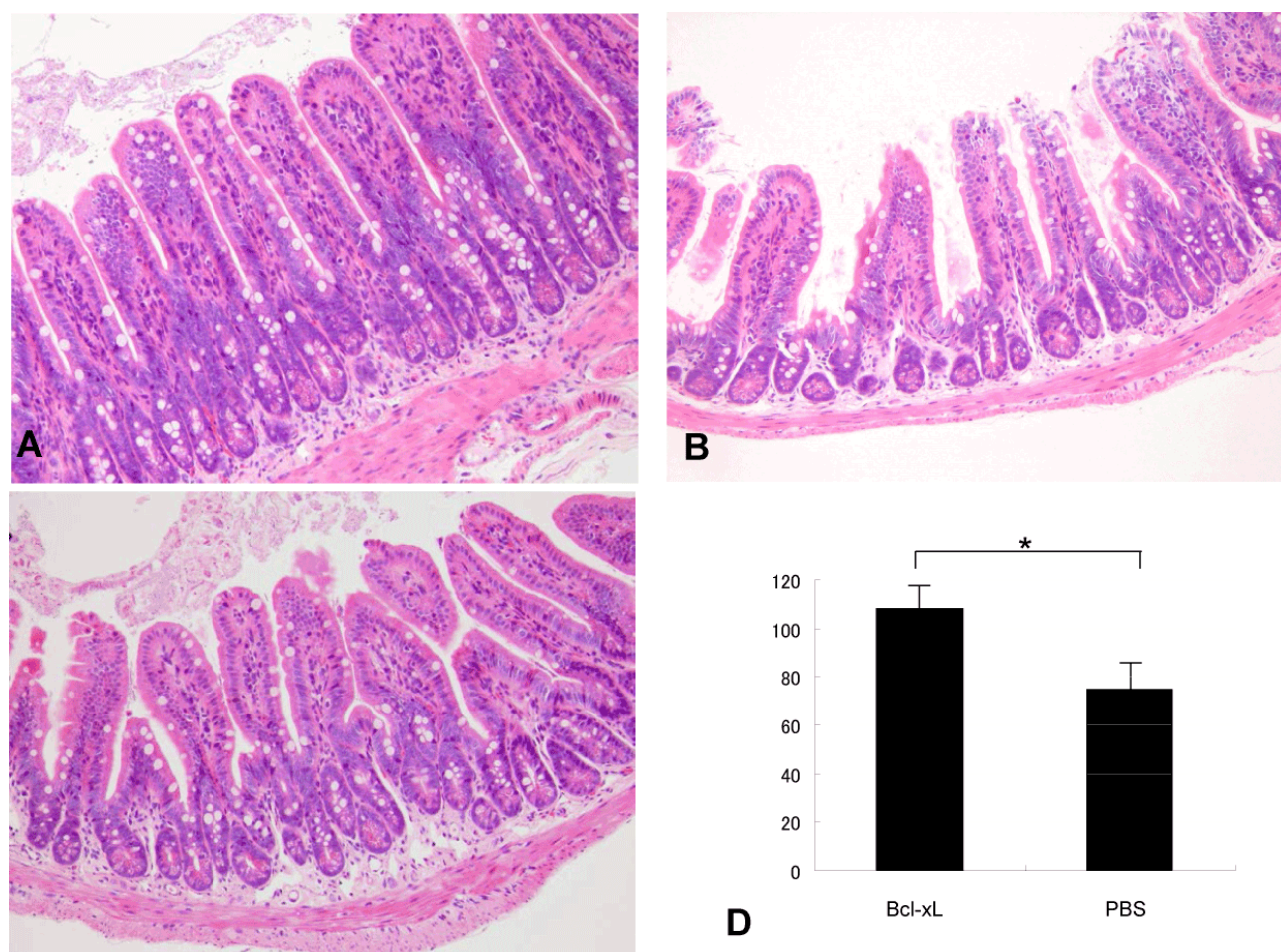


Figure 4: Histological analysis of mouse small intestine after irradiation (recovery phase). Mice received intraluminal infusion of AxCALacZ (A), AxCALacZ (B) or PBS (C) with Pronase MS[®] treatment. Twenty-four hours later, mice underwent 10-Gy whole body irradiation and samples were obtained at 10 days after irradiation. (A-C) Hematoxylin/eosin staining. (D) Number of crypts per circumference *p<0.05. The data of the Pronase MS[®]-AxCALacZ group were not shown because two mice died within 7 days after irradiation.

Discussion

Radiation-induced Bax up regulation and the subsequent translocation of Bax from the cytosol to the mitochondria results in the release of cytochrome c into the cytoplasm and the resultant activation of the caspase-mediated apoptotic cell death pathway [9,12,13,25,26]. Kitada et al. [15] found Bax induction and apoptosis in the small intestinal crypt cell by radiation. Bcl-xL was reported to inhibit Bax translocation to the mitochondria, decrease the release of cytochrome c from the cytosol and protect retinal cell apoptosis in transgenic mice [26]. Huang et al reported that the adenoviral transfer of the Bcl-xL gene could inhibit Bax translocation from the cytosol to the mitochondria and could inhibit cardiac cell apoptosis in a rat reperfusion injury model [20]. Therefore, our hypothesis was that adenoviral Bcl-xL gene therapy could protect the small intestine from radiation-induced injuries in mouse model. We hypothesized that the critical issue for protection was sufficient and specific gene transduction into the intestinal crypt cell.

We demonstrated that intraluminal adenoviral injection after removing mucus components allowed a high level of gene

transduction specifically to crypt epithelial cells in the ileum. Adenoviral vector-mediated Bcl-xL gene transduction into the small intestine exerted anti-apoptotic effects immediately after and during the recovery phase of radiation injury. In particular, the successful transduction of Bcl-xL into the bottom of the crypt appeared to reduce apoptosis in intestinal stem cells, resulting in diminished mucosal destruction, preserved villi, and preserved crypt number after radiation exposure.

The intestinal tract has numerous features that make it an attractive target for therapeutic gene transfer: (a) easy accessibility via the intestinal lumen; (b) large surface area of the epithelium; (c) possibility of in situ gene transfer by endoscopy; (d) known locations of stem cells within the intestinal crypt; and (e) ability of intestinal cells to secrete foreign proteins into the circulation [27].

Systemic injection of viral vectors into the circulation results mainly in transduction of hepatocytes and spleen cells, and does not result in efficient transduction of the intestinal epithelium [28,29]. In 1995, Lau et al. [30] directly administered a retrovirus into the intestinal tract to achieve genetic expression in mucosal epithelia, and in 1997, Cheng et

al. [31] achieved the same by directly administering an adenoviral vector into the intestinal tract. Adenovirus-mediated gene expression is only transient and declines sharply after 2-3 days due to the high turnover of gut epithelium [27,28,31]. For perpetuation of gene expression, repeated administration without systemic antibody response or other enhanced immune reaction is possible [29].

In vitro experiments have shown that intestinal mucus can be solubilized by a variety of agents, including proteases, detergents, and sulfhydryl compounds [2]. We found that intraluminal adenoviral injection after removing mucus components with Pronase MS® allowed a high level of gene transduction specificity in the basal crypt epithelial cells in the ileum, thought to be the location of stem cells [32,33]. Pronase MS® is used as a premedication for gastro endoscopy to remove the gastric mucus [34]. In vivo pilocarpine pretreatment followed by phosphate buffered saline flush may also effectively reduce the mucus barrier in the crypts to facilitate gene transfer by adenoviruses or other vectors [35]. Radiation-induced apoptosis occurs predominantly within the stem cell region [8,36,37]. Gene transduction into areas with stem cells by directly administering a vector into the intestinal tract after removing intestinal mucus appears to be a very useful technique for gene therapy during radiation therapy. In this study, apoptosis can be detected within 2 hours after irradiation. Apoptosis is most frequently seen in the small intestine at positions 4-6, which is thought to be the location of stem cells [8,37,38]. Tessner et al. [38] delivered doses of 12 Gy of radiation and detected apoptosis six hours later. Surviving crypts were about 10-1 at 4 days with 10 Gy and 10-3 with 15 Gy [37], and Guo et al. [4] delivered 15 Gy of radiation into the small intestine of mice and conducted histological analysis 72 hours later. Based on the results of that study, we irradiated the entire bodies of mice with 15 Gy, detected apoptosis by TUNEL staining six hours later and conducted histological analysis 72 hours later. The number of apoptotic cells in the Bcl-xL group was significantly lower than that in the other groups. This suggests that transduced Bcl-xL suppressed the radiation induced Bax translocation from the cytosol to the mitochondria to suppress apoptosis. Because apoptosis was suppressed during the early stages of irradiation, histological analysis conducted at 72 hours after irradiation showed that villous height and crypt density were maintained.

In order to assess the long-term course following irradiation, whole body irradiation was carried out at 10 Gy, and histological analysis was conducted 10 days later. At 15 Gy, whole body irradiation was lethal and histological analysis could not be conducted 10 days later (data not shown). At 10-Gy whole body irradiation, all three mice in the Pronase MS®-AxCABclxL group survived for 10 days, while 2 of 3 mice in the Pronase MS®- AxCalacZ group and 1 mouse in the Pronase MS®- AxCalacZ group did not survive for 5 days after irradiation. When compared to normal mice, villous height had almost recovered, but the number of surviving crypts was low in the survived mice in all groups. When compared to the PBS group, the number of surviving crypts in the Bcl-xL group was significantly higher. Suppressed apoptosis at 6 hours after irradiation led to higher numbers of surviving crypts at 72 hours after irradiation and at 10 days after irradiation. Villous height became transiently lower, but recovered at ten days due to surviving stem cells. These results suggest that Adenoviral Bcl-xL gene therapy combined with Pronase MS® pretreatment succeeded in saving the mice from death by severe radiation enteritis through decrease of apoptosis of small intestinal stem cells.

The present study used murine ileum, as the small intestine is highly sensitive to ionizing radiation injury. Radiation enteritis is likely to occur, and the severity of inflammation can be easily assessed. In addition, the terminal ileum can easily be identified. In clinical settings, radiotherapy is most often performed for pelvic malignancies, and in this therapy, the rectum poses the greatest challenge. Gene therapy in the rectum can be administered anally, and when compared to the small intestine, administration is more convenient [39]. Migaly et al. [40] administered an adenoviral vector (TGF-β) trans-anally and achieved successful gene transduction in the colon. In normal radiation therapy for uterine cancer and prostate cancer, the irradiation schedule is set beforehand, and gene transduction can be performed before irradiation. In the present study, favorable results were obtained by performing gene transduction 24 hours before irradiation.

The results of the present study clarified that when an adenoviral vector was injected directly into the intestinal tract after Pronase MS® pretreatment, gene transfer occurred primarily in the area of the intestinal tract with stem cells. In addition, apoptosis due to irradiation was suppressed by Bcl-xL gene transduction. In the future, when applying radiation to the pelvis for the treatment of uterine or prostate cancer or when performing intraoperative irradiation for the treatment of pancreatic cancer, gene therapy combined with endoscopic mucus removal before irradiation appears to be useful for preventing radiation enteritis in the duodenum or rectum. This therapy may also be used in other diseases resulting in apoptosis, such as enteritis caused by chemotherapy or ischemic reperfusion injury.

Grant support

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and the Japanese Society for the Promotion for Science.

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