Protection by Polaprezinc Against Radiation-Induced Apoptosis in Rat Jejunal Crypt Cells

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Polaprezinc, an anti-ulcer drug, is a chelate compound consisting of zinc and L-carnosine. Polaprezinc has been shown to prevent gastric mucosal injury. The anti ulcer effects of polaprezinc have been ascribed to its antioxidative property. The effect of polaprezinc on ionizing radiation-induced apoptosis was studied in the jejunal epithelial crypt cells of rats. Seven-to eight week-old Wistar rats, which were treated with 100 mg/kg of polaprezinc orally 1h before irradiation or 2% carboxymethyl cellulose sodium in controls, were exposed to whole body X-ray irradiation at 2 Gy. The number of apoptotic cells per jejunal crypt was counted in haematoxylin and eosin stained sections at 0–6 h after irradiation. TUNEL positive cells and immunopositive cells for active caspase-3 per crypt were also counted. Accumulation of p53, p21WAF1/CIP1 and Bax expression in the jejunum after irradiation were examined by Western blot analyses. Polaprezinc treatment given prior to radiation resulted in a significant reduction in numbers of apoptotic cells, TUNEL positive cells and active caspase-3 immunopositive cells in jejunal crypt cells. Polaprezinc treatment resulted in decreases of p53 accumulation, p21WAF1/CIP1 and Bax expression after irradiation. Polaprezinc has a protective effect against ionizing radiation induced apoptosis in rat jejunal crypt cells.

INTRODUCTION

Polaprezinc \([N-(3-aminopropionyl)-L-histidinato zinc]\), a chelate compound developed as an anti-cancer drug consists of zinc ion and L-carnosine, has been commonly used in the treatment of gastric ulcer.1 Polaprezinc has been effective in preventing gastric lesions in a wide of variety of experimental models without affecting gastric acid secretion and endogenous prostaglandins.2,3 The protection of the gastric mucosa by polaprezinc may be attributed to its antioxidant activity,4 membrane-stabilizing action5 and stimulation of mucus production.6

Exposure of the intestine to ionizing radiation results in the rapid, apoptotic death of the stem cells.7 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.8,9 Apoptosis is a programmed process of active cell death that involves gross morphological alterations including condensation of nuclear chromatin, compaction of cytoplasmic organelles, membrane blebbing, and cellular fragmentation into apoptotic bodies.10 Identification and counting of apoptotic bodies in haematoxylin and eosin (H&E)-stained sections has been used extensively to study apoptosis induced by ionizing radiation and other genotoxic stimuli in the intestine.11,12

Previously, we reported that sucralfate and kefir have a protective effect on radiation-induced apoptosis, presumably in protecting the stem cell region of the rat colonic crypt cells from radiation.13,14 Sucralfate, an aluminum hydroxide complex of sulfated sucrose, is an anti-ulcer agent with a protective effect of radiation-induced apoptosis in the rat colon following decreases of p53 accumulation, p21WAF1/CIP1 expression and Bax/Bcl-2 ratio.14 The p53 tumour suppressor gene is one of the primary cellular factors which determine the nature of growth arrest and/or cell death in response to ionizing radiation.15-18 An increase in the level of the p53
protein in irradiated cells and the consequent up-regulation of p21WA F1/CIP1, the cyclin dependent kinase inhibitory protein, appear to be critical components of G1 arrest.19,20) Radiation-induced intestinal apoptosis in mice has also been shown to be regulated by several members of the bcl-2 family of proteins, including bax.21)

The aim of this study was to evaluate whether polaprezinc was capable of preventing acute X-ray radiation-induced apoptosis and to examine the mechanism by which polaprezinc exerts its effect on radiation-induced apoptosis in the jejunal crypt cells of rats. Routine histological study with haematoxylin and eosin staining (H&E) confirmed the presence of apoptosis. Identification of apoptosis was confirmed using a TUNEL technique. Active caspase-3, which is a key effector of the apoptotic pathway, was then studied in the jejunal crypt cells by immunohistochemistry. The effects of polaprezinc on the expression of p53, p21WA F1/CIP1 and Bax after irradiation were also examined.

MATERIALS AND METHODS

Animals and treatment of polaprezinc

Seven-to eight week-old male Wistar rats (230–310 g) were purchased from Charles River Japan (Atsugi, Japan). A total 60 of rats were used for examination. 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.

Rats were given of 100 mg/kg polaprezinc (Zeria Pharmaceutical Co. Ltd., Tokyo) dissolved in 2.5 ml carboxymethyl cellulose sodium (CMC) orally 1 h before irradiation. Control rats were given only 2.5 ml 2% CMC solution.

Irradiation

Irradiation was performed between 9:00 a.m. and 12:00 p.m. Rats received whole-body X-ray irradiation using a Toshiba EXS-300 X-ray, 200 KV, 15mA apparatus with 0.5 mm Al filter at a dose-rate of 0.864 Gy/min.

Histology and assessing apoptosis

Rats in each group were sacrificed by deep anesthesia at 1 h, 2 h, 4 h and 6 h after 2 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, pH7.4), broken into pieces on ice and subjected to three freeze-thaw cycles. The insoluble cell debris was removed by centrifugation. Supernatants were collected and the protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples (30 μg) were subjected to 10% or 15% SDS-polyacrylamide gel electrophoresis, then transferred electrophoretically to Hybond ECL Nitrocellulose Membranes (Amersham, Arlington heights, IL). Membranes were incubated with mouse monoclonal anti-p53 (Pab 421) antibody (Oncogene Science Inc., Uniondale, NY), rabbit polyclonal anti-p21WA F1/CIP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), Bax (BD pharmingen, San Diego, CA), or a horseradish peroxidase-conjugated anti-mouse IgG antibody (Zymed Labs., San Francisco, CA) or rabbit polyclonal anti-actin antibody (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

This method detects apoptosis-associated DNA fragmentation by labeling of 3’-OH termini with digoxigenin nucleotides using terminal deoxynucleotidyl transferase.22) 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 performed with a DAKO LSAB-2 Kit, and peroxidase for use on rat specimens (DAKO Cytomation, Carpinteria, CA) according to the manufacturer’s instructions.

Fifty crypts per group from complete jejunal crypts that had been cut in the longitudinal plane were selected for analysis. The incidence of cell death (apoptotic index) in the jejunum and the colon was quantified by counting the number of dead cells in each crypt in H&E-stained sections at × 400 magnification by light microscopy as described previously.24) The distinctive morphological features of apoptosis, as described by Kerr et al.25) and Walker et al.26) were used to recognize apoptotic cells, and any doubtful cells were disregarded. Small dead cell fragments occurring in tightly knit groups were assessed as originating from one cell, and given a single count.25) TUNEL positive cells and immunopositive cells for active caspase-3 in at least thirty jejunal crypts per animal, and the average value was calculated.

Western blotting

The jejunal tissue of control and polaprezinc-treated rats were removed at 1 h, 2 h, 4 h and 6 h after 2 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, pH7.4), broken into pieces on ice and subjected to three freeze-thaw cycles. The insoluble cell debris was removed by centrifugation. Supernatants were collected and the protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples (30 μg) were subjected to 10% or 15% SDS-polyacrylamide gel electrophoresis, then transferred electrophoretically to Hybond ECL Nitrocellulose Membranes (Amersham, Arlington heights, IL). Membranes were incubated with mouse monoclonal anti-p53 (Pab 421) antibody (Oncogene Science Inc., Uniondale, NY), rabbit polyclonal anti-p21WA F1/CIP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), Bax (BD pharmingen, San Diego, CA) or rabbit polyclonal anti-actin antibody (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

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was determined using the level of actin as a standard.

**Statistical evaluation of data**

All values were expressed as the mean ± SEM of results obtained from three to five animals per data point. Differences between groups were examined for statistical significance using the Student’s t-test. A $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Apoptotic index of control rats and polaprezinc-treated rats after irradiation**

Figure 1 shows histologic sections stained with H&E of jejunal crypts from control and polaprezinc-treated rats (Fig. 1A, B), TUNEL (Fig. 1C, D) and active caspase-3 antibody (Fig. 1E, F). At 2 h after 2 Gy irradiation, a large number of apoptotic cells were observed in the base of the jejunal crypts of control rats in H&E-stained sections and with TUNEL staining (Fig. 1A, C). In contrast, there was a sharp decrease in the number of apoptotic cells in the jejunal crypts of polaprezinc-treated rats (Fig. 1B, D). Active caspase-3 immunoreactivity was largely confined to apoptotic bodies towards the base of the intestinal crypts in the area where apoptotic bodies are most often seen in H&E-stained sections (Fig. 1E). Active caspase-3 immunoreactivity was also greatly decreased in polaprezinc-treated rats (Fig. 1F) when compared with control rats.

Figure 2 compares the time course of the apoptotic index detected using H&E staining, TUNEL staining and active caspase-3 expression in the jejunum and the colon in control and poraprezinc-treated rats up to 6 h after irradiation with 2 Gy. The apoptotic index of the jejunum in control rats increased by 2 h after irradiation, then decreased gradually at 4 h and 6 h. Polaprezinc treatment significantly reduced the number of apoptotic jejunal crypt cells at 2 h, 4 h and 6 h after 2 Gy irradiation to 13% ($p < 0.001$), 44% ($p < 0.05$) and 41% ($p < 0.01$) 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 shows the pattern of TUNEL positive cells following irradiation of the jejunum in control and polaprezinc treated rats. The number of TUNEL positive cells in control rats increased at 2 h and 4 h, and then decreased slightly at 6 h after 2 Gy irradiation. The number of TUNEL positive cells in control rats was not higher than the apoptotic index at each sampling time after irradiation. Polaprezinc treatment significantly reduced the number of TUNEL positive cells at 2 h, 4 h and 6 h after irradiation to 17% ($p < 0.001$), 49% ($p < 0.05$) and 47% ($p < 0.05$) of the control rat values, respectively. Figure 2C illustrates the pattern of caspase-3 activation following irradiation of the jejunum in control and polaprezinc treated rats. The number of active caspase-3 immunopositive cells in control rats peaked at 2 h, and then decreased sharply at 4 h after irradiation. Treating with polaprezinc resulted in a significant decrease of active caspase-3 positive cells at 2 h and 6 h after irradiation to 17% ($p < 0.001$) and 33% ($p < 0.001$) of the control rat values, respectively. Figure 2D shows the apoptotic index following irradiation of the colon in control and polaprezinc treated rats.
Fig. 2. Apoptotic Index (A), TUNEL (B) and active caspase-3 (C) positive cells per jejunal crypt cells, and apoptotic index of colonic crypt cells (D) in control rats (□) and polaprezinc-treated rats (●) at 0 to 6 h after 2 Gy irradiation. Data are the mean ± SEM values of 3–5 rats per data point. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control rats.

Fig. 3. Western blotting analysis and kinetics of p53 (A), p21WAF1/Cip1 (B) and Bax (C) expression in the jejunum of control and polaprezinc-treated rats. The amounts of p53, p21WAF1/Cip1 and Bax were quantified by densitometric analysis. Each of the protein levels is expressed as ratios to non-irradiated rats (0 h) in control (□) or polaprezinc-treated rats (●). Data represent mean ± SEM values of three to four separate experiments from two rats. *p < 0.05 and **p < 0.01 vs. control rats.
The apoptotic index of the colon in both groups increased by 4 h and then decreased. There was no significant difference between control rats and polaprezinc treated rats.

Western blotting for p53, p21\(^{WAF1/CIP1}\) and Bax

In an effort to determine how polaprezinc treatment was interfering with the pathways leading to radiation-induced apoptosis, the expressions of p53, p21\(^{WAF1/CIP1}\) and Bax after 2 Gy irradiation were examined by Western blot analyses and kinetic diagrams are shown in Fig. 3. p53 accumulation in control rats after irradiation increased 1.2-fold, 2.8-fold, 2.9-fold and 2.6-fold at 1 h, 2 h, 4 h and 6 h respectively over non-irradiated control rats. p53 accumulation of polaprezinc treated rats after irradiation increased 1.2, 1.5 and 1.4-fold at 1 h, 2 h and 4 h respectively, and then decreased to 0.9-fold at 6 h over non-irradiated polaprezinc treated rats. There was a significant difference at 6 h (p < 0.05) between control and polaprezinc-treated rats (Fig. 3A). The expression of p21\(^{WAF1/CIP1}\) in control rats after irradiation increased 1.3, 2.4, 3.0 and 3.4-fold at 1 h, 2 h, 4 h and 6 h respectively over non-irradiated control rats, while that in polaprezinc treated rats increased 1.4, 1.8 and 2.3-fold at 1 h, 2 h and 4 h respectively, and then decreased to 1.3-fold at 6 h over non-irradiated polaprezinc treated rats. There was a significant difference at 6 h (p < 0.05) between control and polaprezinc-treated rats (Fig. 3B). The expression of Bax in control rats after irradiation increased 1.3, 2.5, 1.9 and 3.7-fold at 1 h, 2 h, 4 h and 6 h respectively over non-irradiated control rats, while that in polaprezinc treated rats increased 1.5 and 1.3-fold at 1 h and 4 h respectively, and decreased to 0.9 and 1.0-fold at 2 h and 6 h over non-irradiated polaprezinc treated rats. There was a significant difference at 2 h (p < 0.05), 4 h (p < 0.01) and 6 h (p < 0.05) between control and polaprezinc-treated rats (Fig. 3C).

DISCUSSION

Intestinal stem cells in the crypts are rapidly proliferating cells in vivo which are most sensitive to radiation-induced damage. Apoptosis is the main cause of irradiation-induced intestinal damage responsible for many of the side effects of aggressive cancer therapy. Nonalimentary pelvic tumors are usually treated with 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 frequently results in acute radiation-induced colitis (ARC). The most common acute symptoms of ARC are diarrhea, tenesmus, and rectal bleeding. In spite of the high incidence of these problems in a select group of patients, there is no well established therapy. Polaprezinc is an anti-ulcer agent that is comprised of zinc and carnosine. Zinc is an antioxidant that probably acts by preventing oxidation of a specific sulfhydryl group and by substituting for pro-oxidant metals, such as Cu and Fe, at membrane binding sites. Clinically, it has been suggested that zinc supplementation used in conjunction with radiotherapy could postpone the development of severe mucositis and dermatitis for patients with cancers of the head and neck, and can also alleviate the degree of mucositis and dermatitis. The aim of this study was to evaluate whether polaprezinc was capable of preventing X-ray radiation-induced apoptosis in the jejunum.

Our data demonstrated that polaprezinc treatment before whole body X-ray irradiation resulted in a significant suppression of apoptotic cells in the rat jejunal crypt at 2 Gy. The increased incidence of apoptosis detected by H&E staining compared with background (0 h) levels peaked at 2 h and then decreased gradually by 6 h in the jejunum. The significant suppression of apoptotic cells by polaprezinc treatment was observed at 2 h, 4 h and 6 h using H&E and TUNEL staining methods in jejunal crypt cells (Fig. 2A, B). TUNEL positive cells in controls reached a plateau at 4 h after irradiation, but the sensitivity of TUNEL assays in the controls was not greater than that seen in H&E staining. This may be because the TUNEL assay is greatly dependent on technical details, mostly related to DNA strand-breaks associated with excessive levels of protease digestion, with fixation and processing procedures, or with the action of section cutting or other various pretreatments. Caspase-3 activation is a major effector in the apoptotic process following the irradiation of intestinal cells. In this study, the apoptosis index in H&E-stained sections of the control groups increased up to 2 h and decreased gradually by 6 h. In contrast, caspase-3 activation peaked at 2 h and then was sharply reduced at 4 h, with no further increase at 4 h and 6 h. A significant suppression of active caspase-3 positive cells was observed in the polaprezinc treated rats at 2 h and 6 h after irradiation (Fig. 2C). These results suggest that polaprezinc can protect radiation-induced apoptosis and inhibit the caspase-3 activation. The number of active caspase-3 positive cells was lower as compared to the apoptotic index in control groups at 4 h and 6 h. The difference in the numbers of apoptotic cells and active caspase-3 positive cells may reflect the time lag of caspase activation, apoptotic body processing, and degradation because caspase-3 was activated earlier in the apoptotic process than DNA cleavage and cell fragmentation, or it may be due to a caspase-independent apoptosis pathway. Irradiation induces DNA damage leading to the activation of p53, p21\(^{WAF1/CIP1}\) and Bax in the murine intestinal epithelium. To examine the radiation-induced apoptosis pathways suppressed by polaprezinc treatment, p53, p21\(^{WAF1/CIP1}\) and Bax expressions after X-ray irradiation were examined. p53 accumulation, p21\(^{WAF1/CIP1}\) and Bax expression in polaprezinc-treated rats showed significant decreases compared to control rats after X-ray irradiation at 6 h (Fig. 3).

The antioxidant ability of polaprezinc has also been demonstrated by in vivo and in vitro studies. Yoshikawa et al. suggested that the increase in area of the erosions on the
stomach after ischemia-reperfusion and the increase in lipid peroxides in the gastric mucosa were significantly inhibited by the oral administration of zinc N-(3-aminopropionyl)-L-histidine (Z-103). In addition Z-103 inhibits superoxide generation by polymorphonuclear leukocytes, and hydroxyl radical production by the Fenton reaction. Z-103 is also found to scavenge superoxide as well. On the other hand, it was reported in an in vitro study that polaprezinc inhibits apoptosis via inhibition of caspase-3 activation in indomethacin treated RGM1 cells, a rat gastric mucosal cell line but did not prevent mitochondrial cytochrome c release. That paper indicated that the molecular target of polaprezinc lies downstream of cytochrome c efflux, yet upstream of caspase-3 activation in indomethacin induced apoptosis. In our study the suppression of p53 accumulation, p21WAF-1/CIP1 and Bax expression in polaprezinc-treated rats occurred after the peak of the apoptotic index. Our data demonstrated that the mechanism of the protective effect of polaprezinc treated rats from radiation-induced apoptosis at 2 h and 4 h may be dependent on a p53 independent pathway and the possibility that polaprezinc reacts as an anti-oxidant in vivo and its anti-ulcer action may be considered in part by the protective effect on the radiation-induced apoptosis mechanism. But in our study, we didn’t investigate the effect of polaprezinc on antioxidant activity in rat intestine after irradiation. Further studies are required to elucidate the mechanism of antiapoptotic effect induced by polaprezinc treatment.

A recent study suggested that polaprezinc protects the small intestine (in either animals or humans) from a non-steroidal anti-inflammatory drug (NSAID) induced injury, and stimulated cell migration and proliferation in vitro. Furthermore, it is suggested that zinc L-carnosine administration protects the colonic mucosa against acetic acid by induction of HSP72 and suppression of NF-kappa B activation. In this study, polaprezinc administered orally 1 h before irradiation did not suppress apoptotic cells in the colon (Fig. 2D). This may be because polaprezinc was not present in the colon during radiation in this study design. It may be important the polaprezinc mechanism of membrane-stabilizing action and stimulation of mucus production in the protection of radiation-induced apoptosis in the intestine. Pretreatment with polaprezinc and its presence in the lower digestive tract during radiation may be important for the therapy. It may be possible to prevent acute radiation proctitis with polaprezinc pretreatment during radiotherapy for pelvic malignancies, but further studies are needed. In conclusion, polaprezinc has shown a protective effect of radiation-induced apoptosis in the rat jejunum crypt cells, and decreases of p53 accumulation, p21WAF-1/CIP1 and Bax expression were demonstrated.

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