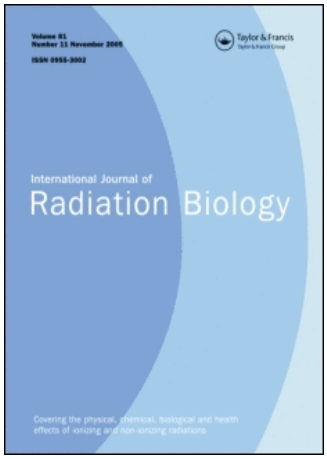

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Mechanisms underlying the radioprotective effect of histamine on small intestine

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Abstract

Purpose: To examine the protective effects of histamine on intestinal damage produced by gamma-radiation.

Materials and methods: 56 mice were divided into 4 groups. Histamine and Histamine-10 Gy groups received a daily subcutaneous histamine injection (0.1 mg/kg) starting 20 hours before irradiation and continued until the end of the experimental period; the untreated group received saline. Histamine-10 Gy and untreated-10 Gy groups were irradiated with a single dose on whole-body using Cesium-137 source (7 Gy/min) and were sacrificed 3 days after irradiation. Small intestine was removed, fixed and stained with hematoxylin and eosin. The number of intestinal crypts per circumference, and other histological characteristics of intestinal cells were evaluated. We further determined by immunohistochemistry the expression of proliferating cell nuclear antigen (PCNA), Bax, Bcl-2 (pro- and anti-apoptotic protein, respectively), antioxidant enzymes (Superoxide dismutase (SOD), Catalase and Glutathione peroxidase), histamine content and apoptosis by terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay. Cells in the S phase of the cell cycle were identified by immunohistochemical detection of 5-bromo-2′-deoxyuridine (BrdU) incorporation.

Results: Histamine treatment reduced mucosal atrophy, edema and preserved villi, crypts and nuclear and cytoplasmic characteristics of small intestine after radiation exposure. Additionally, histamine treatment increased PCNA expression and the BrdU-positive cell number, histamine content, decreased the number of apoptotic cells and significantly increased Catalase and copper-zinc-containing SOD of irradiated mice.

Conclusions: Histamine prevents radiation-induced toxicity by increasing proliferation of damaged intestinal mucosa and suppressing apoptosis that was associated with an increase in SOD and Catalase levels. This effect might be of clinical value in patients undergoing radiotherapy.

Keywords: *Histamine, ionizing radiation, radioprotectant, antioxidant enzymes, apoptosis, small intestine*

Introduction

Cancer radiotherapy relies on two essential components, killing cancer cells while sparing normal tissues. This is achieved in part by tacking advantage of the physical attributes of ionizing radiation through sophisticated planning and delivery techniques. Further therapeutic benefits can be accrued by understanding and manipulating the biological response of the microenvironment to ionizing radiation to increase tumor sensitivity to radiation or to inhibit deleterious effects (Barcellos-Hoff et al. 2005). Pharmacologic approaches to reducing radiation-induced toxicities while maintaining antitumor efficacy can be divided into radiosensitizers which ideally differentially enhance the sensitivity of tumors rather than normal tissue, and radioprotectants that reduce the detrimental effects of radiation on normal tissue while maintaining tumor sensitivity (Grdina et al. 2002).

Radiation-induced damage is introduced into genome that is the most sensitive target by either a

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direct action or indirectly via formation of reactive oxygen species (ROS) which are responsible for the resultant cell killing, mutagenesis, transformation and carcinogenesis. The latter mechanism, which accounts for about 75% of radiation-induced DNA damage by photons, can be abrogated with free radical scavengers present in the local microenvironment at the time the free radicals are formed (Saha 2003).

ROS as superoxide, hydrogen peroxide, and hydroxyl radical are highly reactive and can exert deleterious effects on cell function and viability, depending on cellular antioxidant defenses and capability to repair oxidative damage (Pani et al. 2000). ROS levels are normally controlled by the antioxidant defense system including the antioxidant enzymes: manganese-containing superoxide dismutase (MnSOD), copper-zinc-containing superoxide dismutase (CuZnSOD) that catalyze the dismutation of the superoxide radical into hydrogen peroxide, and Catalase and Glutathione peroxidase that further degrade hydrogen peroxide (Bravard et al. 2002).

We have previously reported that histamine enhances radiosentivity of breast malignant cells and protects two of the most radiosensitive tissues, small intestine and bone marrow, from high doses of gamma radiation (Medina et al. 2005, 2006).

Histamine, 2-(imidazol-4-yl)ethylamine, is a biogenic amine which is synthesized by histidine decarboxylase (HDC) and plays a key role in numerous biological processes (Falus 2004). It was previously reported that H1, H2, H3 and H4 histamine receptors are expressed in small intestine (Chand & Eyre 1975, Fargeas et al. 1989, Oda et al. 2000, Héron et al. 2001). Furthermore, in rat small intestine, histamine behaves as a growth factor accelerating repair of damaged mucosa following ischemia-reperfusion (Fujimoto et al. 1992, 1995, Yoshida et al. 2000).

The small intestine epithelium is arranged into two fundamental structures: villi and crypts. Villi are projections into the lumen covered predominantly with mature, absorptive enterocytes, along with occasional mucus-secreting goblet cells. These cells live only for a few days and, are removed by apoptosis and/or exfoliation. Proliferation is restricted to crypts of Lieberkühn that are invaginations of the epithelium around the villi and house multipotent stem cells that serve to constantly replenish all epithelial cell lineages. Therefore, protection of these stem cells is essential for long-term maintenance of the intestinal epithelium (Potten et al. 1997, Potten 1998).

The aim of the present study was to investigate the mechanisms involved in histamine radioprotective effect on small intestine. Immunohistochemistry studies were undertaken to define the cell type-specific protein expression of antioxidant enzymes MnSOD, Cu-ZnSOD, Glutathione peroxidase, Catalase; HDC and histamine content. Proliferation was determined by immunohistochemical detection of the 5-bromo-2′-deoxyuridine (BrdU, a thymidine analog) incorporation and the proliferating cell nuclear antigen (PCNA) that plays an essential role in DNA replication and repair (Kelman 1997). Additionally apoptosis and Bcl-2 family proteins expression (Bax, proapoptotic, and Bcl-2, antiapoptotic) were evaluated.

Materials and methods

Treatment and irradiation

Fifty six nude mice (NIH nu/nu) were purchased from the Division of Laboratory Animal Production, Faculty of Veterinary Sciences, University of La Plata, Buenos Aires and were randomly separated into 4 groups ($n = 14$ each). Mice were maintained in our animal healthcare facility at 22 to 24°C and 50 – 60% humidity on a 12 h light/dark cycle with food and water available *ad libitum*.

Histamine and Histamine-10 Gy groups received a daily subcutaneous histamine injection (0.1 mg/kg) starting 20 h before irradiation and continued till the end of experimental period and untreated groups received saline. Histamine-10 Gy group and untreated-10 Gy group were irradiated using Cesium-137 source (IBL 437C type H) of 189 TBq (dose rate: 7 Gy/min) with a single dose of 10 Gy on whole-body and were killed 3 days after irradiation by cervical dislocation.

Four animals of each group received an intraperitoneal injection of BrdU (100 mg/kg in saline; Sigma Chemical Co., St Louis, MO, USA) 1 h before sacrifice.

Animal procedures were in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA, 1996.

Histopathological studies

Small intestine was opened along the mesenteric border and samples were pinned flat on cork board, mucosal side up, to allow immediate fixation with 10% neutral buffered formalin and comparable samples. Tissue samples were embedded in paraffin and cut into serial sections of 3 μm thick. Tissue morphology was examined on tissue sections after hematoxylin-eosin staining.

Parameters analyzed in the proximal part of the small intestine were: (a) Mucosal trophism (Normal: normal appearance of mucosal villi and crypts;

Mild atrophy: slight reduction in the number of villi and crypts only confirmable through crypt comparative counting but conserving histological normal appearance; Marked atrophy: severe reduction in the number of villi and crypts with morphological changes in shape and structure); (b) number of intestinal crypts per circumference; (c) mucosal ulceration (Absent: total continuity of cells layers covering the mucosal villi; Mild: occasional loss of continuity in the epithelial covering of villi with mild inflammatory response; Severe: significant number of mucosal ulcers with important inflammatory response and hemorrhages); (d) nuclear and cytoplasmic changes (Absent: adequate nucleus/cytoplasmic ratio; Mild anisocariosis: slight alteration in the nucleus/cytoplasmic ratio and different nuclear size between mucosal cells; Anisocariosis/anisocytosis: marked alteration of the nucleus/cytoplasmic ratio, important difference in the nuclear and cytoplasmic size between mucosal cells); (e) villous edema (Absent: total absence of edematous sectors; Present: edematous separation of the mucosal layers by clear sectors in the histological preparation); and (f) vascular damage (Absent: normal appearance of the vascular structures; Present: vascular congestion, proliferation and edema of endothelial cells, obliteration and thrombosis).

Immunohistochemical staining

After deparaffinization, specimens were placed in citrate buffer (10 mM, pH 6.0) and heated in a microwave oven twice for 2 min at boiling temperature for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2 in distillated water. After blocking, tissues were incubated with primary mouse anti Catalase, rabbit anti histamine (1:100, Sigma Chemical Co., St Louis, MO, USA), sheep anti MnSOD, sheep anti Cu-ZnSOD, rabbit anti Glutathione peroxidase (1:100, Calbiochem, San Diego, CA, USA), mouse anti Bcl-2, rabbit anti Bax (1:100, Santa Cruz, Santa Cruz, CA, USA), mouse anti PCNA (1:40, DakoCytomation, Glostrup, Denmark), guinea pig anti HDC (1:100, Euro-Diagnostica AB, Mälmo, Sweden), and mouse anti BrdU (1:150, Sigma Chemical Co., St Louis, MO, USA) antibodies overnight in a humidified chamber at 4°C. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-mouse, anti-rabbit, anti-guinea pig, or anti-sheep IgG, as appropriate, and visualized by diaminobenzidine staining (Sigma Chemical Co., St Louis, MO, USA). To evaluate subcellular localization of these proteins, nuclei were stained with hematoxylin. Light microscopy was performed on an Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken at 630 × magnification using a Canon PowerShot G5 camera (Tokyo, Japan). The immunostaining assessment was performed blind to the data in all tests by consensus agreement of 2 observers (V.M. and M.C.). An overall examination of staining was carried out at 10 × magnification, and representative area of intestine specimen was then viewed at 1000 × magnification. To control the signal specificity, serial sections were made from five selected positive cases which were subjected to the same staining procedure, with either a normal mouse or rabbit IgG or phosphate buffered saline (PBS) to replace the first antibody. This control staining did not give rise to a signal. For histamine content, HDC, MnSOD, CuZnSOD, Catalase, Glutathione peroxidase, and Bax, a score based on the intensity of positive homogeneous staining was assigned as: − (undetectable), + (very low), ++ (low), +++ (medium), ++++ (high), +++++ (very high). For PCNA and Bcl-2, a percentage score based on the number of stained cells was assigned as: − (undetectable), + (1–20%), ++ (21–40%), +++ (41–60%), ++++ (61–80%) and +++++ (81–100%). These scoring systems were published elsewhere (Blancato et al. 2004, Erbil et al. 2005). Proliferation was evaluated by assessing BrdU incorporation and results were expressed as the number of BrdU-positive cells per crypt. Determinations were made in cells of crypts and villi and at least 10 fields were examined.

Determination of apoptosis

Apoptotic cells were determined by terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay. Fragmented DNA in cells undergoing apoptosis was detected using Apoptag^{TM} plus peroxidase in situ apoptosis Detection Kit (CHEMICON International, Temecula, CA, USA) according to the manufacturer's instructions. Tissues were visualized using Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken at 630 × magnification using a Canon PowerShot G5 camera (Tokyo, Japan). Results were expressed as the number of TUNEL-positive cells per crypt or villus. Determinations were made in cells of crypts and villi and at least 10 fields were examined.

Western blot analysis

Mice were treated and euthanized as described under 'Treatment and irradiation'. The small intestine was isolated, washed with PBS, homogenized in lysis buffer (100 mM Tris/HCl buffer, pH 8, containing 1% Triton X-100 and protease inhibitors), and incubated for 15 min on ice. Homogenates were

cleared by centrifugation for 10 minutes at 6000 rpm, 6× loading buffer (100 mM Tris/HCl buffer, pH 8, containing 1.7% sodium dodecyl sulfate (SDS), 0.02% bromophenol blue, 1.5% dithiotreitol, and 5% of glycerol) was added to supernatants and they were heated at 95°C for 10 min. Proteins were then separated by SDS-polyacrylamide gels (12%) and blotted onto a nitrocellulose membrane (Sigma Chemical Co., St Louis, MO, USA). Membranes were blocked and probed overnight at 4°C with primary mouse anti-Catalase, mouse anti-β-actin (1:1000, Sigma Chemical Co., St Louis, MO, USA), or sheep anti Cu-ZnSOD (1:1000, Calbiochem, San Diego, CA, USA) antibodies. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-mouse or anti-sheep IgG, as appropriate, and visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). Densitometric analyses were performed using the software Image J 1.32J (NIH, Bethesda, MA, USA).

Statistical analysis

Data shown are mean ± standard error of the mean (SEM). Statistical evaluations were made by analysis of variance (ANOVA) that was followed by Newman-Keuls' Multiple Comparison Test. P values < 0.05 were considered significant. All statistical analyses were performed with GraphPad Prism Version 4.00 software (San Diego, CA, USA).

Results

Histamine protects small intestine from ionizing radiation damage

As we have previously described (Medina et al. 2005), histamine reduced mucosal atrophy, edema, vascular damage and preserved villi, crypts and nuclear and cytoplasmic characteristics of small intestine after ionizing radiation exposure. The number of crypts per circumference in histamine-treated and irradiated mice was comparable to that of the control animals (132 ± 9 vs. 153 ± 6 in controls) (Table I).

Histamine increases PCNA expression and BrdU-positive cells while reduces ionizing radiation-induced apoptosis in irradiated small intestine

PCNA is a well documented indicator of active proliferation being an essential component of the DNA replication machinery (Kelman 1997). In small intestine derived from untreated mice, PCNA expression was moderate and restricted to crypts and no significant difference was observed after histamine treatment in non-irradiated mice (Figure 1A, Table II). Additionally, proliferation was evaluated by assessing the incorporation of BrdU, a thymidine analog. Interestingly, histamine treatment slightly increased the number of BrdU-positive cells per crypt (3.4 ± 0.4 vs. 1.9 ± 0.4 in untreated mice, $p < 0.05$) and in both untreated and histamine-treated mice, BrdU-positive cells were found to be located in the crypts with the highest occurrence near to the base (Figure 1A, 1B). Conversely, apoptosis was low and preferentially occurred in villi and was not varied by histamine in non-irradiated mice (Figure 1C, 1D).

Ionizing radiation produced the complete disappearance of PCNA expression and a marked decrease in BrdU-positive cells indicating the lack of proliferation while increased considerably the number of apoptotic cells in crypts. In contrast, a significant increase in PCNA expression and in BrdU-positive cells per crypt (2.0 ± 0.3 vs. 0.1 ± 0.1 in untreated, $p < 0.01$) was observed in small intestine of histamine-treated and irradiated mice. This outcome was accompanied by a reduction in the number of apoptotic cells per crypt (0.2 ± 0.1 vs. 2.0 ± 0.7 in untreated, $p < 0.01$) (Figure 1, Table II). The different immunoreactivity observed between Brd-U incorporation after 1 h of administration and PCNA (Figure 1A) might reflect differences in the relative concentrations of a detectable PCNA form that persists in the cell as it progresses

Table I. Histopathological characteristics of small intestine.

Group	Mucosal trophism	No. of crypts/ circumference*	Mucosal ulceration	Nuclear and cytoplasmic changes	Villous edema	Vascular damage
Untreated[a]	Normal	153 ± 6	Absent	Absent	Absent	Absent
Histamine[b]	Normal	159 ± 7	Absent	Absent	Absent	Absent
Untreated-10 Gy[c]	Marked atrophy	85 ± 5[e,f]	Severe	Anisocariosis/anisocytosis	Present	Present
Histamine-10 Gy[d]	Mild atrophy	132 ± 9	Mild	Mild anisocariosis	Absent	Absent

*Mean value of the experimental group calculated from the average number of crypts of 10 fields examined; [a]Representative of small intestines from at least eight saline-treated mice; [b]Representative of small intestines from at least eight 0.1 mg/kg.day histamine-treated mice; [c]Representative of small intestines from at least eight saline-treated and 10 Gy-irradiated mice; [d]Representative of small intestines from at least eight histamine-treated and 10 Gy-irradiated mice; [e]$p < 0.01$ and [f]$p < 0.01$ compared with the untreated and the histamine-10 Gy groups, respectively. (ANOVA and Newman-Keuls test).

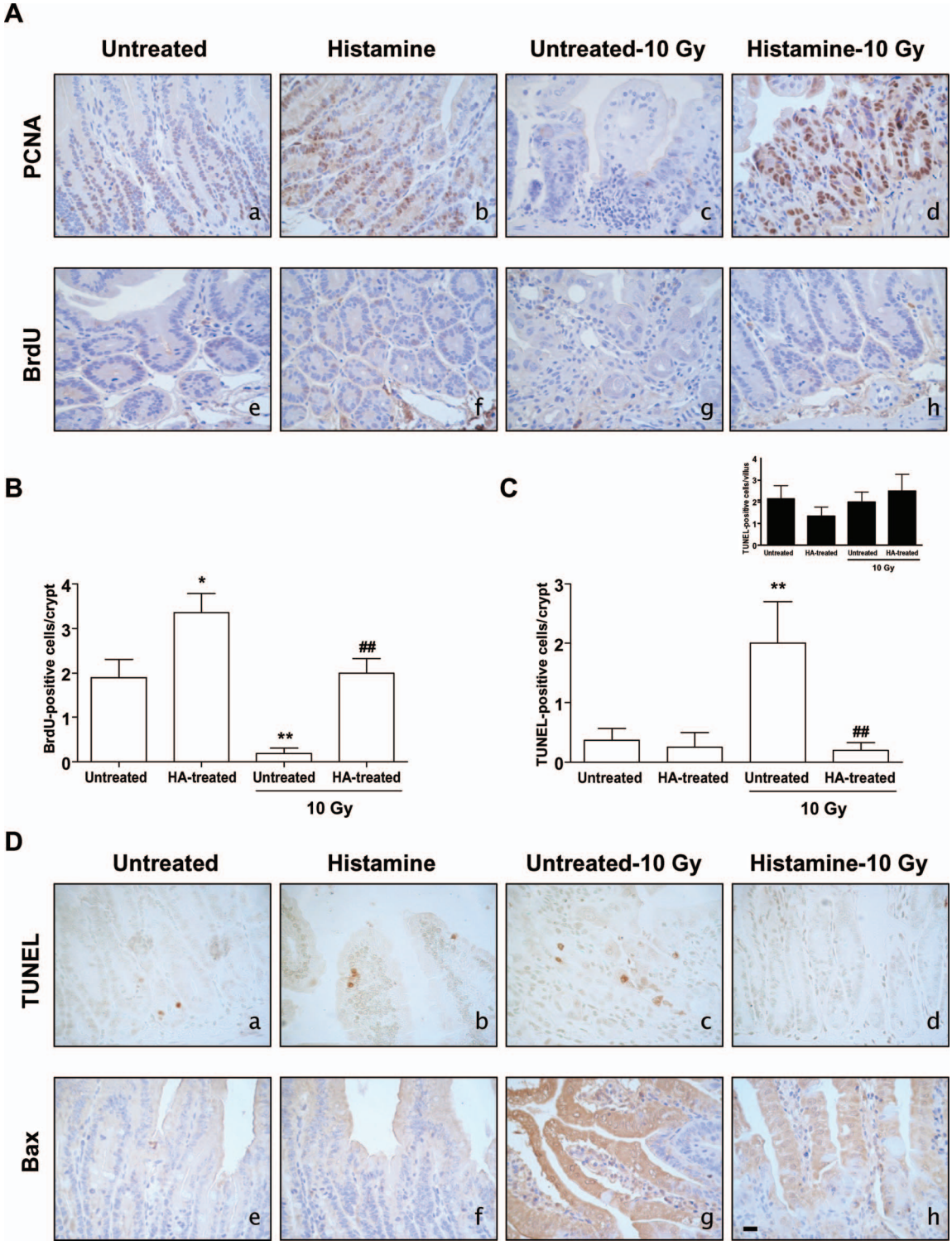

Figure 1. Effect of histamine and ionizing radiation on epithelial cell proliferation and apoptosis in the mouse small intestine. (A) Representative intestinal mucosal sections from untreated (a,e), histamine (HA) treated (b,f), untreated and 10 Gy irradiated (c,g) and 10 Gy irradiated and histamine treated mice (d,h). a,b,c,d illustrate PCNA immunoreactivity; e,f,g,h show BrdU immunoreactivity. (B) Proliferation was evaluated by BrdU immunohistochemistry. The number of BrdU positive cells per crypt are expressed as mean ± SEM ($n = 4$ mice). $^{*}p < 0.05$, $^{**}p < 001$ compared with the untreated group; $\#\#p < 0.01$ compared with the untreated and 10 Gy irradiated group. (C) Apoptotic cells were determined using TUNEL assay. The number of TUNEL positive cells per crypt are expressed as mean ± SEM ($n = 10$ mice). $^{**}p < 001$ compared with the untreated group; $\#\#p < 0.01$ compared with the untreated and 10 Gy irradiated group. Inset: Line graph indicates the number of TUNEL positive cells per villus. Values are expressed as mean ± SEM ($n = 10$ mice). (D) Representative intestinal mucosal sections from untreated (a,e), histamine treated (b,f), untreated and 10 Gy irradiated (c,g) and 10 Gy irradiated and histamine treated mice (d,h). a,b,c,d immunohistochemical detection of apoptosis by TUNEL assay; e,f,g,h exemplify Bax immunoreactivity. Scale bar 20 μm.

through the cell cycle. PCNA increases through G1, peaks at the G1/S-phase interface and decreases through G2, reaching low levels, which are virtually undetectable by immunocytochemical methods in M-phase and quiescent cells. In contrast, BrdU is incorporated only during the S-phase (Coltrera & Gown 1991).

Furthermore, the reduction in ionizing radiation-induced apoptosis exerted by histamine was associated with a diminution of the immunoreactivity levels of the pro-apoptotic protein Bax in both villous and crypt sections. (Figure 1D, Table II). The anti-apoptotic protein Bcl-2 was undetectable in small intestine and remained unaffected by histamine treatment. Conversely, only ionizing radiation slightly augmented Bcl-2 immunoreactivity particularly in goblet cells (Table II).

Modulation of antioxidant enzymes in small intestine of irradiated and histamine treated mice

Results indicated that intestines of the untreated group expressed MnSOD only in villi; Catalase, CuZnSOD, and Glutathione peroxidase in both villi and crypts. The only change exerted by histamine treatment was the appearance of MnSOD expression in crypts. On the other hand, ionizing radiation produced a marked decrease in Glutathione peroxidase and CuZnSOD and MnSOD expression (Table III). Interestingly, histamine treatment prevented the effect of ionizing radiation on CuZnSOD increasing its expression in crypts, and significantly augmented Catalase protein expression in both crypts and villi (Figure 2A, 2B, Table III). Additionally, histamine increased the expression of MnSOD in crypts of irradiated mice (Table III). These results suggest that the final balance evoked by histamine treatment in irradiated mice is the decrease in hydrogen peroxide levels that is a potent oxidant and may be highly toxic to the cells.

Table II. Immunohistochemical detection and localization of PCNA and Bcl-2 family proteins in small intestine. See key for groups in Table I.

	PCNA[e]		Bax[f]		Bcl-2[e]	
Group	V	C	V	C	V	C
Untreated[a]	−	+++	++	++	−	−
Histamine[b]	−	+++	++	++	−	−
Untreated-10 Gy[c]	−	−	+++++	+++	+	++
Histamine-10 Gy[d]	−	++++	++++	++	+	++

[e]Percent positivity: − (undetectable), + (1–20%), ++ (21–40%), +++ (41–60%), ++++ (61–80%), and +++++ (81–100%); [f]Staining intensity: − undetectable, + very low, ++ low, +++ moderate, ++++ high, +++++ very high; V, Expression in villi; C, Expression in crypts.

Histamine augments histamine intracellular content in irradiated small intestine

Table IV and Figure 2C show the results from the immunohistochemical analysis of histamine and histamine-synthesizing enzyme, HDC. Small intestine expressed HDC and its expression was reduced by histamine administration. However, histamine intracellular levels were not modified suggesting that histamine treatment is also altering histamine-catalyzing enzymes or histamine uptake. On the other hand, ionizing radiation reduced HDC immunoreactivity but histamine levels remained unaffected in villi while even increased in crypts. Finally, histamine treatment significantly increased histamine intracellular levels in both villi and crypts of irradiated-mice and this was associated with an enhanced expression of HDC (Figure 2C, Table IV). Further studies of enzymes responsible for histamine catabolism such as diaminooxidase (DAO) whose activity is high in the gastrointestinal tract of all investigated species (Bieganski 1983), need to be performed to fully understand histamine effect on histamine intracellular levels.

Discussion

Radiation side-effects are inevitable, even with localized radiotherapy. Radiation enteritis occurs during the radiotherapy for many intraabdominal and pelvic cancers such as cervix, endometrium, ovary, bladder, prostate, and rectum. Although

Table III. Immunohistochemical detection and localization of antioxidant enzymes in small intestine. See key for groups in Table I.

	MnSOD[e]		CuZnSOD[e]		Catalase[e]		Glutathione Peroxidase[e]	
Group	V	C	V	C	V	C	V	C
Untreated[a]	+++	−	+++	+	+	+	+	+
Histamine[b]	+++	++	+++	+	+	+	+	+
Untreated-10 Gy[c]	++	−	++	+	+	+	−	−
Histamine-10 Gy[d]	++	+	+++	++	+++	+++	−	−

[e]Staining intensity: − undetectable, + very low, ++ low, +++ moderate, ++++ high, +++++ very high. V, Expression in villi; C, Expression in crypts.

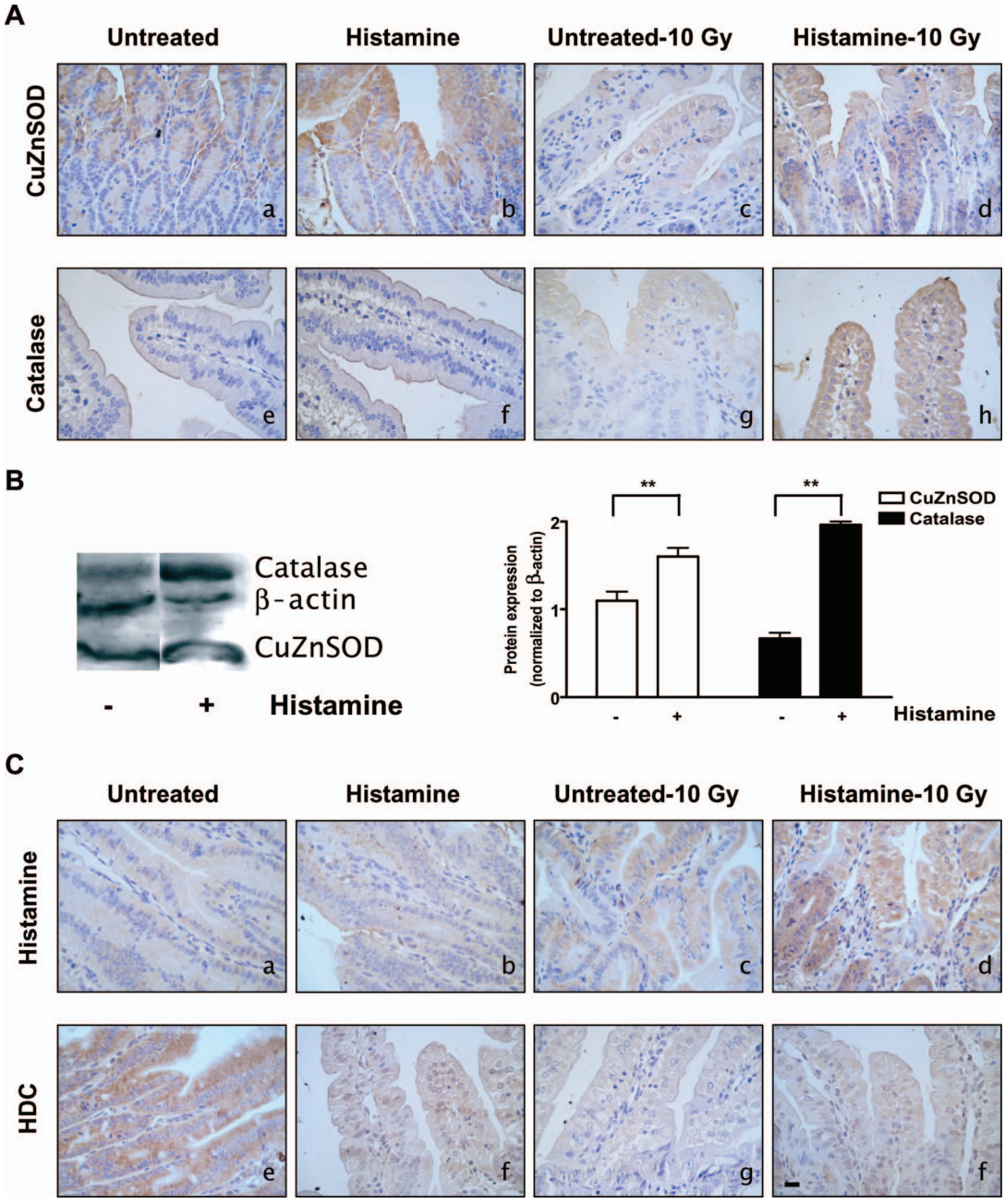

Figure 2. Effect of histamine and ionizing radiation on antioxidant enzymes (A,B) and histamine and HDC immunoreactivity (C) in the mouse small intestine. Representative intestinal mucosal sections from untreated (a,e), histamine treated (b,f), untreated and 10 Gy irradiated (c,g) and 10 Gy irradiated and histamine treated mice (d,h). (A) a,b,c,d illustrate CuZnSOD immunoreactivity; e,f,g,h show Catalase immunoreactivity. (B) Protein level validation of histamine-affected antioxidant enzymes in 10 Gy irradiated mice by western blot of total intestinal proteins. β-actin (42 kDa) was used to normalize the expression levels of CuZnSOD (16 kDa) and Catalase (60 kDa). Results are expressed as mean $\pm$ SEM ($n = 4$ mice). $^{**}p < 0.01$ compared with the untreated and 10 Gy irradiated group. (C) a,b,c,d demonstrate histamine immunoreactivity; e,f,g,h exemplify HDC immunoreactivity. Scale bar 20 μm.

ionizing radiation affects other intraabdominal organs, the most radiosensitive is the small intestine (Emami et al. 1991, Erbil et al. 2005). Ionizing radiation causes mucosal damage in the gastrointestinal epithelium that comprises destruction of crypt cells, decrease in villous height and number, ulceration and necrosis (Yeoh and Horowitz 1987, Erbil et al. 1998, 2005, Bismar & Sinicrope 2002).

In the present study, we clearly demonstrated that histamine treatment significantly protects small intestine against radiation-induced toxicity ameliorating histological injury and improving trophism of enterocytes. Histamine completely prevented the decrease in the number of crypts evoked by ionizing radiation which is vital for small intestine restoration since the intestinal crypt contains a hierarchy of stem

Table IV. Immunohistochemical detection and localization of histamine and histidine decarboxylase in small intestine. See key for groups in Table I.

	HDC[e]		Histamine[e]	
Group	V	C	V	C
Untreated[a]	+++	+++	++	+
Histamine[b]	++	++	++	+
Untreated-10 Gy[c]	+	+	++	++
Histamine-10 Gy[d]	++	++	+++	+++

[e]Staining intensity: + very low, ++ low, +++ moderate, ++++ high, +++++ very high; V, Expression in villi; C, Expression in crypts.

cells that preserve the potential to regenerate the stem cell population and the tissue after cytotoxic exposure (Potten et al. 1997, 2002, Potten 1998). Our results also revealed that histamine radioprotective effect on small intestine was associated with an increased rate of proliferation as evidenced by the enhanced PCNA protein expression and BrdU incorporation in crypts. Furthermore, histamine treatment also augmented cell growth of non-irradiated intestinal cells. In accordance, Héron et al. demonstrated that epithelial cells lining the Lieberkühn crypts of adult intestinal mucosa that could be identified as stem cells, expressed histamine H3 receptor transcripts and histamine treatment increased their proliferation (Héron et al. 2001). In addition, it was reported that histamine via H3 receptor not only did increase cell proliferation and migration in rat fundic mucosa, but also exerted a long lasting growth-promoting effect on the stomach, distal small intestine and distal colon of rats (Morini et al. 2002, Grandi et al. 2006). Besides it functions in the replication of DNA, PCNA is involved in both nucleotide excision repair and base excision repair being an indispensable component in the process of double-strand breaks repair, critical for cell survival following exposure to ionizing radiation (Amorino et al. 2003). Our results indicate that histamine by increasing proliferation and possible by accelerating repair of damaged intestinal mucosa, may lead to small intestinal radioprotection. In agreement, Fujimoto et al. demonstrated that histamine and HDC contribute to mucosal repair in small intestine subjected to ischemia-reperfusion (Fujimoto et al. 1992).

In rapidly proliferating tissues, such as the small intestine epithelium, the stringent control of cell proliferation and cell death by apoptosis is central to the maintenance of tissue homeostasis (Potten et al. 1997, Potten 1998). Furthermore, the ultimate stem cells appear to have an exquisite radiosensitivity such that a single hit anywhere in their DNA molecule can trigger an altruistic apoptotic cell deletion (Potten et al. 1994, 2002). In this light, we decided to investigate whether histamine could influence ionizing radiation-induced apoptosis. Our study indicates that histamine significantly reduced the number of TUNEL positive apoptotic cells induced by ionizing radiation in crypt epithelial lineages. Coincidently, previous studies showed that increased histamine level due to a treatment with aminoguanidine, a suppressor of DAO activity, attenuated intestinal mucosal apoptosis induced by ischemia-reperfusion. This result might be partly supported by the fact that histamine, working as a growth factor, accelerated repair of damaged mucosa in the rat small intestine (Yoshida et al. 2000, Fujimoto et al. 2001).

To further investigate the role of histamine in apoptosis, we determined the expression of the Bcl-2 family proteins Bax and Bcl-2. The former is a well known inducer and the latter is a suppressor of apoptosis (Sedlak et al. 1995, Lee et al. 1999). Bax interacts with the Bcl-2 protein and this interaction results in acceleration of cell death rate, probably through altering the ratio of Bax/Bcl-2 (Sedlak et al. 1995). Our results are in accordance with previous studies that indicate that Bcl-2 protein is not or barely expressed in mice small intestine whereas Bax protein is expressed in villi and crypts (Potten et al. 1997, Potten 1998, Coopersmith et al. 1999). We were not able to detect a significant modification in either apoptosis or apoptotic-related protein expression exerted by histamine in the non-irradiated small intestine. Following irradiation, there is a considerable enhancement of Bax immunoreactivity in crypts and villi and a slight increase in that of Bcl-2 only in crypts that leads to an imbalance of Bax/Bcl-2 ratio. Interestingly, histamine decreased Bax immunoreactivity reducing the Bax/Bcl-2 ratio which is related to the attenuation of apoptosis in irradiated mice.

Radiation is a recognized producer of ROS originating a pro-oxidant state which contributes to cell radiation injury and can activate apoptosis (Jacobson 1996, Das 2002). The net intracellular concentration of ROS is the result of their production and the ability of antioxidants to remove them. In order to investigate whether histamine-induced reduction in apoptosis was associated with a variation in the antioxidant enzymes levels, we further examined their expression. In non-irradiated and histamine treated mice, we did not observe modifications in the expression of the antioxidant enzymes except for an increase in MnSOD level in crypts. On the other hand, in histamine-treated and irradiated mice, we observed an increased expression of SOD in crypts and also in Catalase in both villi and crypts compared to the untreated and irradiated mice. The increase in both superoxide degrading enzyme SOD and hydrogen peroxide catabolizing

enzyme Catalase in histamine treated and irradiated mice, indicates a regulation in the oxidant/antioxidant balance toward a more reduced state in the correct subcellular location that is compatible with a less radiation-induced damage. It was reported that superoxide is associated with the induction of Bax (Ueta et al. 2001) therefore, histamine-induced reduction of Bax immunoreactivity might we related to the increase in SOD expression.

Histamine radioprotective effect may be mediated by the increase in cell proliferation, the reduction in apoptosis due to producing the optimum ratio of Bax/Bcl-2.

Previous reports suggested that histamine synthesized by HDC may facilitate healing of the gut mucosa and inhibit the further generation of ROS by neutrophils; however the role of HDC activation and histamine generation in the response to oxidant stress of the gastrointestinal tract remains unclear (Höcker et al. 1998). Moreover, intracellular HDC and histamine content in regenerating bone marrow populations in HDC+/+ mice increased in all days after total-body irradiation and a faster bone marrow repopulation was observed in wild type in comparison with HDC−/− mice (Horvath et al. 2006). In addition, there are compounds that have a relatively low specific antioxidative activity but when present at high concentrations, can contribute significantly to the overall ROS scavenging activity. Practically all aminoacids can serve as targets for oxidative attack by ROS, although some of them, such as histidine are particularly sensitive to ROS (Dröge 2002). In our study we also evaluated histamine content and its localization. We observed that histamine treatment increased histamine intracellular content especially in small intestine crypts of irradiated mice by enhancing HDC expression. Our results suggest that histamine can also be acting as a free-radical scavenger in small intestine. In this line, it was previously described that the H2 receptor antagonist cimetidine is a very powerful hydroxyl radical scavenger and that the methylated imidazole with a sulfur and amino group containing side chain is the part of the molecule responsible for this activity (Ching et al. 1993). Furthermore, it was reported that imidazole is a radioprotective agent (Prasad 1995) and also other biogenic amines as polyamines, have antioxidant properties (Weiss & Landauer 2000).

The clinical use of radioprotectors in radiation therapy continues to be plagued by issues relating to possible tumor protection and diminution of therapeutic gain. Amifostine is today the only radioprotective drug approved by the Food and Drug Admnistration. However, this phosphorothioate exhibits a dose-limiting toxicity and is used only for the reduction of xerostomia in patients treated for head and neck cancer (Weiss and Landauer 2000, Hall & Giaccia 2006). On the contrary, histamine dihydrochloride (developed as a subcutaneous formulation known as Maxamine) is being used in several clinical trials as an adjuvant with interleukin-2 or Interferon α therapy for the potential treatment of different types of cancer as metastatic melanoma, acute myelogenous leukemia and renal cell carcinoma. In all cases, histamine dihydrochloride was generally well-tolerated and no unexpected or irreversible side effects were reported, demonstrating that histamine dihydrochloride can be safely administered (Mitchell 2003, Agarwala et al. 2004, Galmarini 2004).

Conclusions

Data presented here show that histamine has the potential to prevent ionizing radiation-induced toxicity by increasing proliferation of damaged intestinal mucosa and additionally, by suppressing apoptosis. The latter effect is associated with a modification of antioxidant enzymes levels that could lead to enhance the antioxidant capacity of intestinal cells. Furthermore, histamine might act as a ROS scavenger.

Current studies are aimed to determine the effect of histamine on the radiosensitivity of human mammary and pancreatic tumors induced in nude mice in order to evaluate whether histamine behaves as a valid radioprotector reducing radiation-induced toxicities while maintaining antitumor efficacy. Previous results indicate that histamine *in vitro* enhances the radiosensitivity of breast cancer cells (Medina et al. 2006) while does not modify that of melanoma (Medina et al. 2005) and pancreatic carcinoma cells (data not shown).

Present results suggest that histamine is a selective radioprotector and may be of clinical value in reducing radiation toxicity to the intestine of patients undergoing radiotherapy.

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References

Agarwala SS, Hellstrand K, Gehlsen K, Naredi P. 2004. Immunotherapy with histamine and interleukin 2 in malignant melanoma with liver metastasis. Cancer Immunology Immunotherapy 53:840–841.

Amorino GP, Mikkelsen RB, Valerie K, Schmidt-Ullrich RK. 2003. Dominant-negative cAMP-responsive element-binding protein inhibits proliferating cell nuclear antigen and DNA repair, leading to increased cellular radiosensitivity. Journal of Biological Chemistry 278:29394–29399.

Barcellos-Hoff MH, Park C, Wright EG. 2005. Radiation and the microenvironment-tumorigenesis and therapy. Nature 5:867–874.

Bieganski T. 1983. Acta Physiologica Polonica 34:139–154.

Bismar MM, Sinicrope FA. 2002. Radiation enteritis. Current Gastroenterology Reports 4:361–365.

Blancato J, Singh B, Liu A, Liao DJ, Dickson RB. 2004. Correlation of amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, *in situ* hybridization and immunohistochemical analyses. British Journal of Cancer 90:1612–1619.

Bravard A, Ageron-Blanc A, Alvarez S, Drane P, Le Rhun Y, Paris F, Luccioni C, May E. 2002. Correlation between antioxidant status, tumorigenicity and radiosensitivity in sister rat cell lines. Carcinogenesis 23:705–711.

Chand N, Eyre P. 1975. Classification and biological distribution of histamine receptor sub-types. Agents and Actions 5(4): 277–295.

Ching TL, Haenen GR, Bast A. 1993. Cimetidine and other H2 receptor antagonists as powerful hydroxyl radical scavengers. Chemico-Biological interactions 86:119–127.

Coltrera MD, Gown AM. 1991. PCNA/Cyclin expression and BrdU uptake define different subpopulations in different cell lines. The Journal of Histochemistry and Cytochemistry 39:23–30.

Coopersmith CM, O'Donnell D, Gordon JI. 1999. Bcl-2 inhibits ischemia-reperfusion-induced apoptosis in the intestinal epithelium of transgenic mice. American Journal of Physiology 276:G677–686.

Das UN. 2002. A radical approach to cancer. Medical Sciences Monitor 8:79–92.

Dröge W. 2002. Free radicals in the physiological control of cell function. Physiological Reviews 82:47–95.

Emami B, Lyman J, Brown A, Coia L, Goitein M, Munzenrider JE, Shank B, Solin LJ, Wesson M. 1991. Tolerance of normal tissue to therapeutic irradiation. International Journal of Radiation Oncology, Biology, Physics 21:109–122.

Erbil Y, Dibekoglu C, Türkoglu U, Ademoglu E, Berber E, Kizir A, Mercan S, Toker G. 1998. Nitric oxide and radiation enteritis. European Journal of Surgery 164:863–868.

Erbil Y, Oztezcan S, Giris M, Barbaros U, Olgac V, Bilge H, Kücücük H, Toker G. 2005. The effect of glutamine on radiation-induced organ damage. Life Sciences 78:376–382.

Falus A. 2004. Histamine: Biology and medical aspects. Budapest: SpringMed Publishing Ltd.

Fargeas MJ, Fioramonti J, Bueno L. 1989. Involvement of different receptors in the central and peripheral effects of histamine on intestinal motility in the rat. The journal of Pharmacy and Pharmacology 41:534–540.

Fujimoto K, lmamura I, Granger DN, Wada H, Sakata T, Tso P. 1992. Histamine and histidine decarboxylase are correlated with mucosal repair in rat small intestine after ischemia-reperfusion. The Journal of Clinical Investigation 89:126–133.

Fujimoto K, Gotoh Y, Ogata S, Tsunada S, Ohyama T, Ootani A, Okamoto K, Sakata T. 1995. Histaminergic control of mucosal repair in the small intestine. Obesity Research 3(Suppl. 5): 795S–799.

Fujimoto K, Iwakiri R, Yoshida T, Noda T, Kojima M, Utsumi H, Wu B, Okada K, Sakata Y, Ootani A. 2001. Histaminergic effect on apoptosis of small intestinal mucosa after ischemia-reperfusion in the rat. In: Watanabe T, Timmerman H, Yanai K, editors. Histamine research in the new millennium. Amsterdam: Elsevier Science B.V. pp 185–190.

Galmarini CM. 2004. Histamine dihydrochloride (subcutaneous) Maxim. Current Opinion in Investigational Drugs 5: 1298–1310.

Grandi D, Schunack W, Morini G. 2006. Epithelial cell proliferation is promoted by the histamine H3 receptor agonist (R)-α-methylhistamine throughout the rat gastrointestinal tract. European Journal of Pharmacology 538:141–147.

Grdina DJ, Murley JS, Kataoka Y. 2002. Radioprotectans: Current status and new directions. Oncology 63:2–10.

Hall EJ, Giaccia AJ. 2006. Radioprotectors. In: Hall EJ, Giaccia AJ, editors. Radiobiology for the radiologist. 6th ed. Philadelphia: Lippincott Williams & Wilkins. pp 129–134.

Héron A, Rouleau A, Cohois V, Pillot C, Schwarts JC, Arrang JM. 2001. Expression analysis of the histamine H3 receptor in developing rat tissues. Mechanisms of Development 105:167–173.

Höcker M, Rosenberg I, Xavier R, Henihan RJ, Wiedenmann B, Rosewicz S, Podolsky DK, Wang TC. 1998. Oxidative stress activates the human histidine decarboxylase promoter in AGS gastric cancer cells. Journal of Biological Chemistry 273:23046–23054.

Horvath Z, Pallinger E, Horvath G, Jelinek I, Falus A, Buzas EI. 2006. Histamine H1 and H2 receptors but not H4 receptors are upregulated during bone marrow regeneration. Cellular Immunology 244:110–115.

Jacobson MD. 1996. Reactive oxygen species and programmed cell death. Trends in Biochemical Sciences 21:83–86.

Kelman Z. 1997. PCNA: structure, functions and interactions. Oncogene 14:629–640.

Lee JU, Hosotani R, Wada M, Doi R, Kosiba T, Fujimoto K, Miyamoto Y, Tsuji S, Nakajima S, Nishimura Y, Imamura M. 1999. Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. European Journal of Cancer 35:1374–1380.

Medina VA, Cricco GP, Mohamad NA, Crocci M, Nuñez M, Martin G, Cocca C, Bergoc RM, Rivera ES. 2005. Histamine is a selective protector against cellular damage produced by ionizing radiation. Inflammation Research 54:17–18.

Medina V, Cricco G, Nuñez M, Martín G, Mohamad N, Correa-Fiz F, Sanchez-Jimenez F, Bergoc R, Rivera E. 2006. Histamine-mediated signaling processes in human malignant mammary cells. Cancer Biology Therapy 5:1462–1471.

Mitchell MS. 2003. Immunotherapy as part of combinations for the treatment of cancer. International Immunopharmacology 3:1051–1059.

Morini G, Grandi D, Schunack W. 2002. Ligands for histamine H3 receptors modulate cell proliferation and migration in rat oxyntic mucosa. British Journal of Pharmacology 137:237–244.

Oda T, Morikawa N, Saito Y, Masuho Y, Matsumoto S. 2000. Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. Journal of Biological Chemistry 275:36781–36786.

Pani G, Bedogni B, Anzevino R, Colavitti R, Palazzotti B, Borello S, Galeotti T. 2000. Deregulated manganese superoxide dismutase expression and resistance to oxidative injury in p53-deficient cells. Cancer Research 60:4654–4660.

Potten CS, Merritt A, Hickman J, Hall P, Faranda A. 1994. Characterization of radiation-induced apoptosis in the small intestine and its biological implications. International Journal of Radiation Biology 65:71–78.

Potten CS, Wilson JW, Booth C. 1997. Regulation and significance of apoptosis in the stem cells of the gastrointestinal epithelium. Stem Cells 15:82–93.

Potten CS. 1998. Stem cells in gastrointestinal epithelium: Numbers, characteristics and death. Philosophical Transactions of the Royal Society of London Series B 353:821–830.

Potten CS, Owen G, Booth D. 2002. Intestinal stem cells protect their genome by selective segregation of template DNA strands. Journal of Cell Science 115:2381–2388.

Prasad KN. 1995. Radiation syndromes and their modifications. In: Prasad KN, editor. Handbook of radiobiology. 2nd ed. Florida: CRC Press, Inc. pp 123–152.

Saha GP. 2003. Physics and radiobiology of nuclear medicine, 2nd ed. New York: Springer-Verlag Inc.

Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, Korsmeyer SJ. 1995. Multiple Bcl-2 family members demonstrate selective dimerization with Bax. Proceedings of the National Academy of Sciences of the United States of America 92:7834–7838.

Ueta E, Yoneda K, Kimura T, Tatemoto Y, Doi S, Yamamoto T, Osaki T. 2001. Mn-SOD antisense upregulates *in vivo* apoptosis of squamous cell carcinoma cells by anticancer drugs and γ-rays regulating expression of the Bcl-2 family proteins, Cox-2 and p21. International Journal of Cancer 94:545–550.

Weiss JF, Landauer MR. 2000. Radioprotection by antioxidants. Annals New York Academy of Sciences 899:44–60.

Yeoh EK, Horowitz M. 1987. Radiation enteritis. Surgery, Gynecology & Obstetrics 165:373–379.

Yoshida T, Iwakiri R, Noda T, Okamoto K, Kojima M, Fukuyama K, Fujimoto K. 2000. Histaminergic effect on apoptosis of rat small intestinal mucosa after ischemia-reperfusion. Digestive Diseases and Sciences 45:1138–1144.