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Vitamin C protects against ionizing radiation damage to goblet cells of the ileum in rats

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Summary

The aim of the present study was to evaluate the radioprotective effect of vitamin C on gamma-radiation-induced damage to goblet cells of the ileum. Thirty male Wistar albino rats weighing between 250 and 300 g were randomized into the following study groups: I, control; II, single dose radiation treated; III, two dose radiation treated with a 4-day interval between doses; IV, single dose radiation treated with vitamin C; V, two dose radiation treated with vitamin C. Each group contained six animals. The rats in groups IV and V were given a daily dose of 100 mg/kg of vitamin C for 14 and 18 days, respectively. During the vitamin C administration period, the rats in group IV were exposed in the abdominal area to a gamma-ray dose of 5 Gy on day 10 and group V was exposed to same dose of radiation on days 10 and 14. Irradiation and treatment groups were decapitated 4 days after exposure to single or two dose irradiation and ileum tissues were removed for light and electron microscopic investigation. Single or two dose gamma-irradiation caused a marked intestinal mucosal injury in rats. Radiation produced increases in the number of goblet cells. Using transmission electron microscopy, extensions in the area between the cells, disorders in the microvilli, mitochondrial damage and endoplasmic reticulum (ER) cisternae dilatation were observed. Antioxidant treatment with vitamin C prior to irradiation provided protection against intestinal damage.

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Introduction

In organisms exposed to long-term ionizing radiation for acute and chronic diseases, accom-

panying tissue and cell damage may develop, depending on the dose and exposure time (Weiss et al., 1990). More than half of all cancer patients receive radiation therapy at some period during the course of their disease. However, radiation therapy of cancer continues to be dose-limited by the tolerance of critical surrounding normal tissues.

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The intestine is an important dose-limiting organ during radiation therapy of tumors in the pelvis or abdomen. Intestinal radiation toxicity (radiation enteropathy) is, by convention, classified as early or delayed, depending on when it occurs relative to the time of radiation therapy (Wang et al., 2006). It was previously believed that the severity of intestinal radiation toxicity depended directly on cell death in intestinal crypts. This view has been supplanted by the recognition that radiation-induced changes in cellular function and alterations secondary to cell death contribute substantially to the intestinal radiation response (Denham et al., 2001; Denham and Hauer-Jensen, 2002). Because these functional and secondary changes develop over time after radiation exposure, they are particularly promising targets for interventions aimed at preventing or reducing intestinal radiation toxicity.

The gastrointestinal tract is covered by a mucous layer secreted by goblet cells, which arise from pluripotent stem cells present at the base of crypts (Cheng and Leblond, 1974). Goblet cells reside throughout the length of the intestine and are responsible for the production and maintenance of the protective mucous blanket by synthesizing and secreting high molecular weight mucins (Cheng, 1974; Cheng and Leblond, 1974; Forstner, 1978). Other components within the mucus gel include water, electrolytes, sloughed epithelial cells and secreted immunoglobulins. This produces a physical and chemical barrier that protects the epithelium from physical damage by luminal contents, guards against bacterial invasion, regulates epithelial hydration and interacts with secreted immunoglobulin A to produce antibody and antitoxin effects. Much is known about the biochemical nature of mucins and the qualitative distribution of goblet cells in the small intestine (Kemper and Specian, 1991; Specian and Oliver, 1991). However, a kinetic analysis of goblet cell dynamics of rat small intestine in the process of restitution of surface epithelium subjected to radiation injury has to our knowledge not so far been reported.

The effects of radiation are caused mainly by the generation of reactive oxygen species (ROS). These ROS interact with biological molecules producing toxic free radicals leading to lipid peroxidation and DNA damage (Jagetia and Reddy, 2005). Lipid peroxidation has important effects on biological membranes and studies on lipid peroxidation can provide important information about detrimental effects of gamma-radiation. Apart from the lipid peroxidation, ROS can also alter the balance of endogenous protective systems, such as glutathione and enzymic antioxidant (SOD, CAT and

GPx) defence systems (Prasad et al., 2005). The endogenous antioxidant defences are inadequate to reduce the radiation-induced free radicals. Appropriate antioxidant intervention may inhibit or reduce free radical toxicity and thus offer protection against radiation. Several dietary antioxidants have been reported to decrease free radical attack on biomolecules (El-Habit et al., 2000). Numerous studies have examined the radioprotective effects of antioxidant free radical scavengers, which protect the cell and its organic constituent molecules from free radical damage.

Vitamin C (ascorbic acid) has been reported to be an effective antioxidant and free radical scavenger and, *in vivo* and *in vitro* conditions, reduce oxidative and free radical-induced damage to DNA and membranes in biological systems (Wilson, 1983). Vitamin C functions as a free radical scavenger of active and stable oxygen radicals and has been shown to protect several biological systems against ionizing radiation. The radioprotective effect of ascorbic acid seems to be due to its interactions with radiation-induced free radicals (Duschesne et al., 1975). Ascorbic acid pre-treatment inhibited the radiation-induced elevation in lipid peroxidation (Jagetia, 2004). It protected the mice against radiation-induced sickness, reduced the mortality and improved the healing of wounds after exposure to whole body gamma-radiation (Mallikarjun Rao and Jagetia, 2004).

Radioprotective effects of vitamin C have been restricted to pro-oxidant properties in high doses. No study has been reported so far on the radioprotective activity of vitamin C on ileum goblet cells. The aim of the present study was to evaluate the radioprotective effect of vitamin C on gamma-radiation-induced damage to ileum goblet cells.

Material and methods

Animals

Thirty male Wistar albino rats weighing between 250 and 300 g were randomized into the following study groups: I control; II, single dose radiation treated; III, two dose radiation treated; IV, single dose radiation treated with vitamin C; V, two dose radiation treated with vitamin C. Each group contained six animals. All animals were kept under standardized conditions: temperature between 22 and 24 °C, relative humidity 50–60%, and 12 h light:dark cycle. They were fed a standard laboratory diet with free access to food and water. Animal care was performed according to the Guide for the

Care and Use of Laboratory Animals published by the National Institutes of Health. The experimental protocols for the study were approved by the Institutional Animal Ethical Committee of Trakya University, Edirne, Turkey.

Irradiation

The rats were anesthetized by intraperitoneal (i.p.) administration of 90 mg/kg ketamine and 10 mg/kg xylazine. Irradiation was delivered by a ^{60}Co teletherapy unit (Cirrus, *cis-Bio Int.*, Gif Sur Yvette, France) at a source-skin distance of 100 cm. Five Gray radiations were given at a depth of 1.5 cm (half thickness) with a dose rate of 62.41 cGy/min to the whole abdominal area in a supine position. Correct positioning of the fields was controlled for each individual rat using a therapy simulator (Mecaserto-Simics, Paris, France). The ^{60}Co unit was calibrated with an Exradin Farmer type ionization chamber (Keithley 35040 radiation dosimeter, Cleveland, OH, USA). There was an estimated uncertainty of $\pm 3\%$ in the absorbed dose. Animals in the single dose radiation treated groups II and IV, and two dose radiation treated groups III and V (on day 4 following the first dose) were exposed to 5 Gy gamma-radiation. The control rats (group I) were treated but they were not irradiated. The initial and final body weight changes of the various groups were recorded.

Drug preparation and sample collection

Groups I-III received 1 ml physiological saline. Group IV were given vitamin C (at a dose of 100 mg/kg body weight) once a day by i.p. injection for 14 days (10 days before and 4 days after irradiation) and group V for 18 days (14 days before and 4 days after the two dose irradiation). Irradiation and vitamin C-treated groups were decapitated on day 4 after exposure to single or two dose irradiation and ileum tissues were removed for light and electron microscopical investigation.

Histology and electron microscopy

All samples were immediately fixed in Bouin's solution. The tissue was then processed for embedding in paraffin wax, sectioned serially into 5 μm thick sections, and sections stained with hematoxylin and eosin (H&E), Masson's trichrome (Halberg et al., 1991), and periodic acid-Schiff and haemalum (PAS&H) (Tarladacalisir et al., 2008), all using routine protocols.

For transmission electron microscopy (TEM), samples of ileum were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 1.5 h. After rinsing three times in the buffer, samples were then stored in the buffer for 24 h, postfixed in 1% osmium tetroxide in sodium phosphate buffer (pH 7.2) for 1 h, dehydrated in increasing ethanol concentrations, cleaned in propylene oxide and embedded in araldite. Ultrathin sections (40-60 nm) were cut from the blocks using an ultramicrotome (RMC-MTX Ultramicrotome, USA), mounted on 300-mesh copper grids and double-stained with uranyl acetate and lead citrate before examination with a Jeol-JEM 1010 transmission electron microscope (Jeol, Tokyo, Japan) at an accelerating voltage of 80 kV.

Histopathological analysis

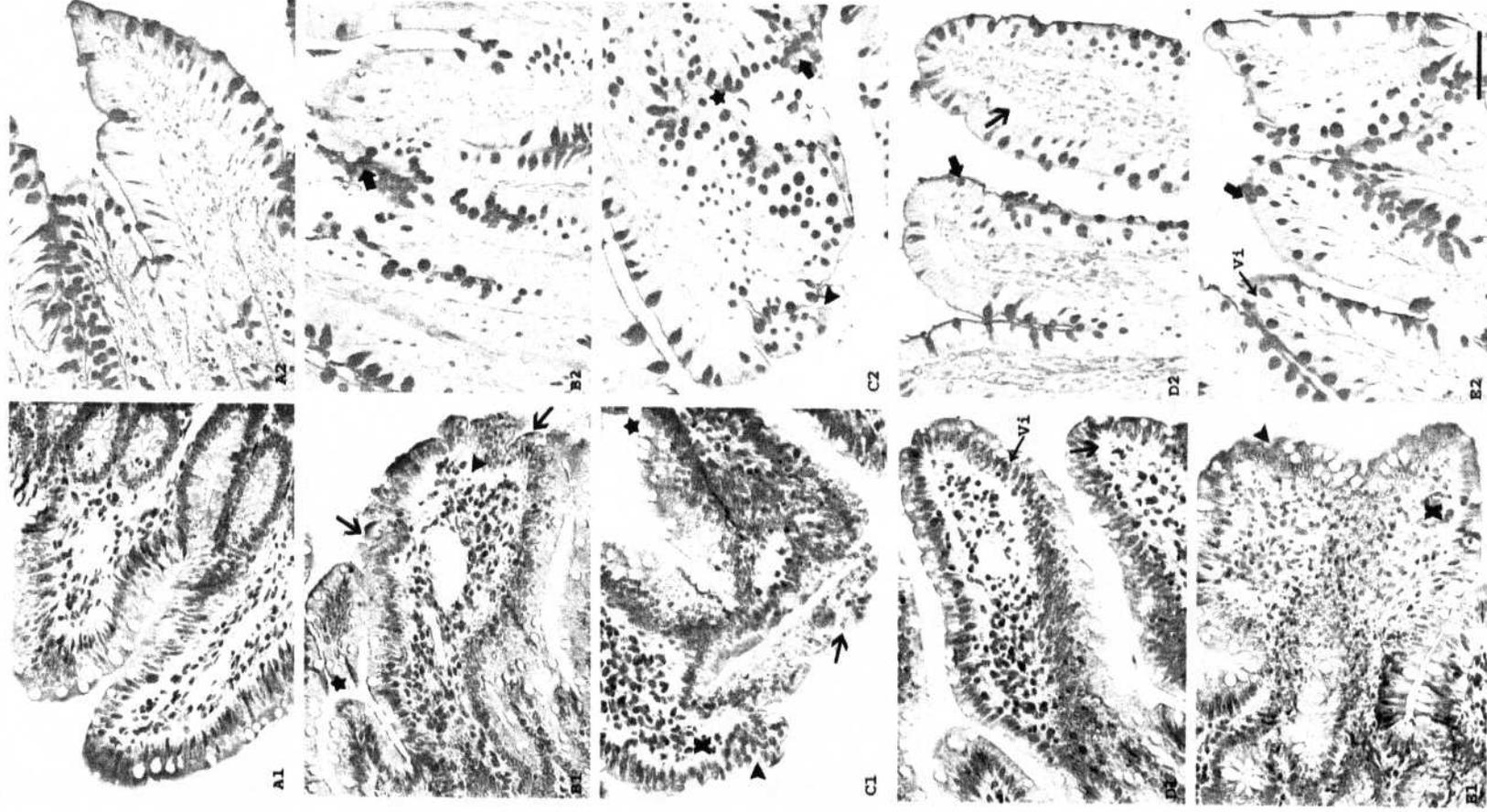
Ileum sections were stained with H&E, and mucosal injury, inflammation and hyperemia/hemorrhage were assessed and graded by a histologist blinded to sample identity and using the histological injury scale previously defined by Chiu et al. (1970). Mucosal damage was graded as: 0, normal mucosa; 1, development of subepithelial Gruenhagen space; 2, lifting of epithelial layer; 3, few tips denuded; 4, completely denuded villi; 5, digestion and disintegration of lamina propria, hemorrhage, and ulceration.

Morphometric analysis

For morphometric analysis, four whole circumference sections, 5 μm thick and 20 μm apart, were cut per animal and stained with PAS&H. Tissue sections were examined and the number of the villus goblet cells counted within random standard high-power fields using an Olympus BX51 light microscope incorporating a square graticule in the eyepiece (eyepiece $\times 10$, objective $\times 40$, a total side length of 25 μm). Villus goblet cell density in each site was calculated and recorded as the number of goblet cells/ μm^2 .

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) software (SPSS, Chicago, IL). All data were presented as mean (\pm) standard deviations (S.D.). Differences in measured parameters among the five groups were analyzed with a nonparametric test (Kruskal-Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a



Mann-Whitney *U*-test; *p* values <0.05 were considered to be significant.

Results

Histology

Single and two dose radiation caused mucosal injury. Four days after irradiation, the tips of the villi were denuded, villous tips covered by goblet cells were observed, and villous height was diminished (Figure 1; B₁-B₂, C₁-C₂). Concomitant with cell depletion, additional alterations in cell morphology were observed in the epithelium. The atrophic mucosa was lined with a continuous layer of grossly abnormal racket-shaped epithelial cells at the luminal side of the ileum. The epithelial cell damage in the single dose radiation treated group was not widespread when compared to the two dose radiation treated group (Figure 1; B₁-B₂, C₁-C₂). In the single dose radiation with vitamin C (Figure 1; D₁-D₂) and two dose radiation with vitamin C-treated (Figure 1; E₁-E₂) rat ileum, the intensity of epithelial cell damage was less than in the radiation-only treated groups. In the vitamin C-treated groups, the detachment of the goblet cells partly continued and villous shape, epithelial continuity, and epithelial cell shape showed partial recovery (Figure 1; D₁-D₂, E₁-E₂). The number of goblet cell profiles increased significantly in both radiation-only treated and radiation with vitamin C-treated rats when compared to control rats (Table 1).

Table 2 summarises mucosal histopathologic changes. The radiation injury score was significantly higher in all the irradiated rats when compared to the control animals. The radiation injury score reflects the global level of injury.

Table 3 shows body weight changes on the day 0 before irradiation, and on the day of sacrifice. Group II-V rats developed diarrhoea, characterized by loss of pellet formation and faecal adherence to the perianal region between days 3 and 4 after

Table 1. Goblet cells per unit area (0.625 μm²) in the intestinal villi of I (control), II (single dose radiation treated), III (two dose radiation treated), IV (single dose radiation treated with vitamin C) and V (two dose radiation treated with vitamin C) groups

Groups	Goblet cell number Mean ± SD
I	62.7 ± 8.8
II	120.4 ± 12.7 ^a
III	154.4 ± 17.9 ^b
IV	117.8 ± 12.1 ^a
V	135.4 ± 14.6 ^c

^a*p* < 0.01 compared to control group.

^b*p* < 0.001 compared to control group.

^c*p* < 0.01 compared to control group.

Table 2. Mucosal histopathologic change scores of I (control), II (single dose radiation treated), III (two dose radiation treated), IV (single dose radiation treated with vitamin C) and V (two dose radiation treated with vitamin C) groups

Groups	Minimum	Maximum	Mean ± SD
I	0.00	0.00	0.00 ± 0.00
II	2.00	4.00	3.00 ± 0.43 ^a
III	3.00	4.00	3.50 ± 0.47 ^b
IV	1.00	3.00	1.62 ± 0.39 ^c
V	2.00	4.00	3.00 ± 0.41 ^a

^a*p* < 0.001 compared to control group.

^b*p* < 0.0001 compared to control group.

^c*p* < 0.01 compared to control group.

radiation. The body weight of the all irradiated rats was significantly decreased on the 4th day after irradiation.

Electron microscopy

Four days after single and two dose irradiation, there were ultrastructural changes in ileal mucosa. The mucus granules in goblet cells were seen to have an irregular honeycomb shape and abnormal

Figure 1. Histology of rat ileum stained with Masson's trichrome (A₁, B₁, C₁, D₁, E₁,) and PAS&H (A₂, B₂, C₂, D₂, E₂,). (A₁, A₂) Control ileum; showing normal morphology. (B₁, B₂) Single dose radiation treated rats; degenerative changes in the epithelial cells (arrow), lifting of epithelial layer from the lamina propria (arrowhead), epithelial lifting down the sides of villi (asterisk) and increase in the goblet cells and mucins showing strongly positive PAS staining (thick arrows). (C₁, C₂) Two dose radiation treated rats; thickened and irregular villi (arrowhead), breakage in the epithelial cells (arrow), and capillary congestion (x) in the villus, epithelial lifting down the sides of villi (asterisk) and increase in the goblet cells and mucins showing strongly positive PAS staining (thick arrows). (D₁, D₂) Single dose radiation treated with vitamin C rats; villi were partly normal (Vi), apical regions of some villi were lightly subepithelial lifting (arrows), generally normal scattered goblet cells (thick arrows). (E₁, E₂) Two dose radiation treated with vitamin C rats; capillary congestion (x) usually at the apex of the villus, occasional occurrence of thickened villi (arrowhead) along with normal villi (Vi), partly normal scattered goblet cells showing strongly positive PAS staining (thick arrows). Scale bar: 50 μm.

Table 3. Body weight (g) on the day (day 0) before irradiation, and on the day of sacrifice of I (control), II (single dose radiation treated), III (two dose radiation treated), IV (single dose radiation treated with vitamin C) and V (two dose radiation treated with vitamin C) groups

Groups	Day 0	Day of sacrifice
I	257.9 ± 2.7	260.1 ± 3.1
II	305.5 ± 2.3	284.5 ± 1.2 ^a
III	317.2 ± 2.0	296.3 ± 1.9 ^b
IV	275.3 ± 5.2	267.3 ± 4.1 ^c
V	301.2 ± 3.2	280.0 ± 1.7 ^d

^a*p* < 0.05 compared to group II.

^b*p* < 0.05 compared to group III.

^c*p* < 0.05 compared to group IV.

^d*p* < 0.05 compared to group V.

spread and condensation (Figure 2; B₁-B₂). Radiation led to the dilatation of cisternae of the rough endoplasmic reticulum (ER). Some mitochondria showed enlargement of the cristae (intracristal swelling or ballooning) (Figure 2; B₁-B₂). The nuclear chromatin condensed and marginated onto the nuclear lamina. Microvilli of epithelial cells also showed changes in length and frequency (Figure 2; B₁, C₁-C₂). Mitotic figures were seen in the crypts of Lieberkühn for both regimes of irradiation. Widening of intercellular spaces following irradiation was observed in intestinal epithelium (Figure 2; C₁).

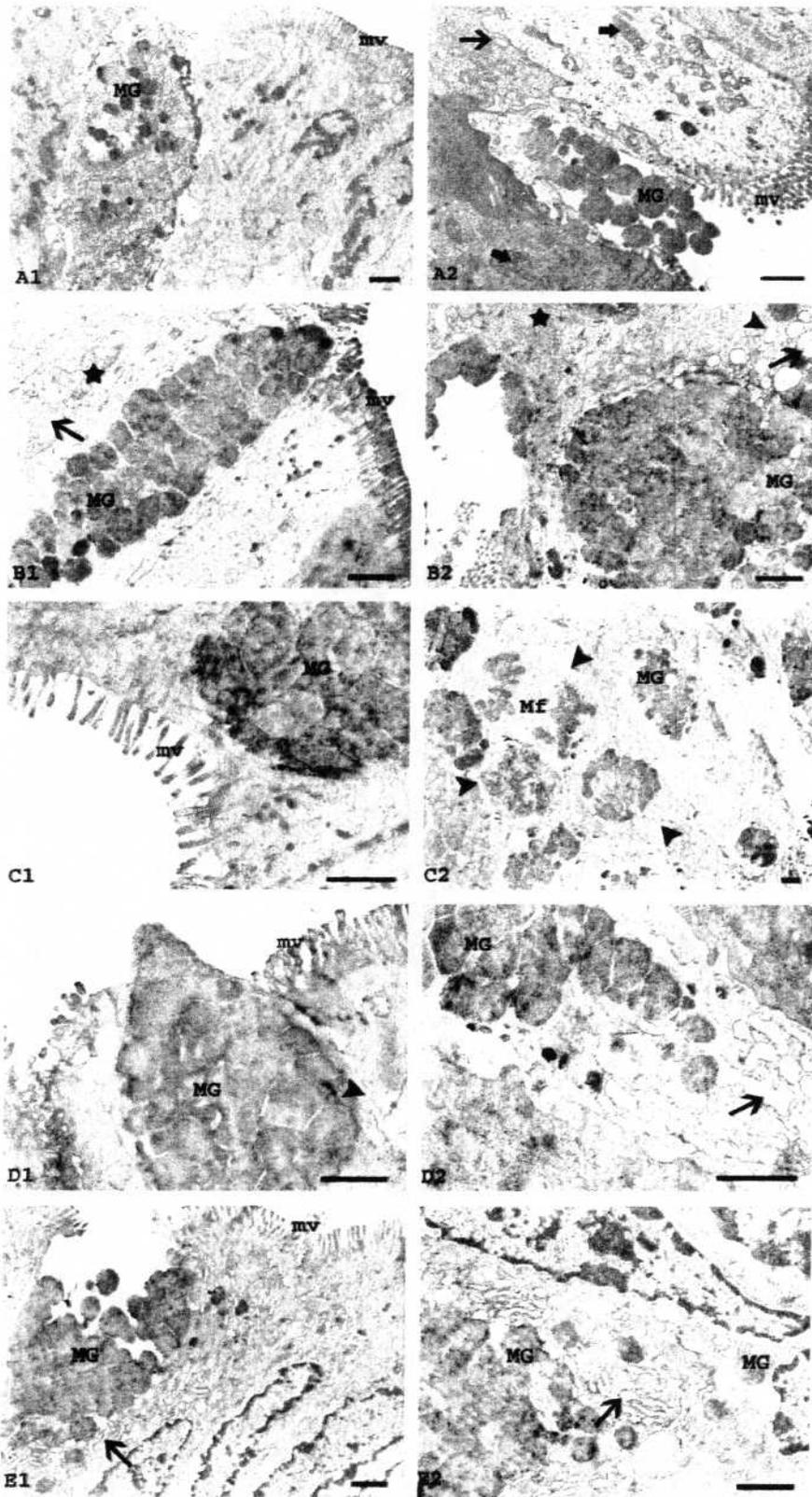
Vitamin C treatment was partly effective in preventing the dilatation of ER, mitochondrial degeneration and formation of irregularly shaped nuclei. In the vitamin C-treated groups, the severity of degenerative changes in the cytoplasm of cells was less than that observed in the radiation-only treated groups (Figure 2; D₁-D₂, E₁-E₂).

Discussion

The results of the present study indicate that treatment with vitamin C may protect ileal goblet cells from the damaging effects of ionizing radiation as determined using histological and ultra-

structural indices. The degree of radiation-induced cell damage depends on several factors including the radiation dose, stage of the cell within the cell cycle, levels of cellular antioxidant defence, time of administration and the availability of oxygen in tissues during irradiation (Weichselbaum et al., 1997). Radiation-induced damage to cells and tissues involves generation of ROS and reactive nitrogen species (RNS), which in turn cause alterations in DNA, membrane-lipids and proteins eventually leading to cellular dysfunction or cell death (Greenstock, 1993). The radiation-induced cell damage may be manifest as clonogenic cell death or alterations in cell-signaling cascades resulting in activation of responsive genes inducing apoptosis. Apart from DNA, other major targets of radiation damage are the membranes of cytoplasmic organelles and plasma membranes (Maurya et al., 2006). Vitamin C has been shown to be a potent water-soluble antioxidant. Extensive animal, clinical and epidemiological studies have confirmed the role of vitamin C in the prevention of disease (Naidu, 2003). Vitamin C is an important dietary factor that has been reported to decrease the adverse effects of ROS and RNS generated *in vivo* in animals by scavenging radicals or by neutralizing an array of these radicals (Halliwell and Gutteridge, 1999). Dietary antioxidants such as vitamins E, C and beta-carotene have previously been reported as effective in protecting normal tissue during radiation therapy (Prasad et al., 2002). Vitamin C has been reported to have a protective effect against radiation-induced cell death and cytogenetic damage (El-Nahas et al., 1993). The radioprotective effect of vitamin C has been ascribed to its interactions with radiation-induced free radicals (Nair et al., 2001); however, higher doses of vitamin C may produce some toxicity. Radioprotective effects of vitamin C have been restricted to a pro-oxidant property at high doses. There is some indication that vitamin C at low concentrations could protect DNA from radiation-induced damage in mouse bone marrow cells, but at high concentrations may enhance the radiation-induced effects

Figure 2. Transmission electron micrographs of rat ileum. (A₁, A₂) Control rat ileum; the mucus granules (MG) have a typical honeycomb shape, well-packed in the goblet cell. The goblet cell has regular cisternae of ER (arrow). Mitochondria (thick arrows) have a typical cylindrical shape with organized cristae. Regular microvilli (mv) of enterocytes. (B₁, B₂) Single dose radiation treated rats; abnormal spread and condensed mucus granules (MG), the dilatation of ER cisternae (arrow) and degranulation of RER membranes (arrowhead) in the goblet cell. Disordered mitochondrial shape (asterisks) and shortening in the microvilli of enterocytes (mv). (C₁, C₂) Two dose radiation treated rats; microvilli have irregular shape (mv). Separation of cell contact region (arrowhead), condensation of mucus granules (MG) in the goblet cell. Mitotic figure (Mf). (D₁, D₂) Single dose radiation treated with vitamin C rats; the mucus granules (MG) have a partly honeycomb shape in the goblet cell. The goblet cell has partly disorganized cisternae of ER (arrow). Cell contact regions show some disintegration (arrowhead). Microvilli have a slightly irregular shape (mv). (E₁, E₂) Two dose radiation-treated with vitamin C rats; the goblet cell has irregular cisternae of ER (arrow), slight spread of mucus granules (MG). Microvilli have a lightly irregular shape (Mv). Scale bar: 1 μm.



(Konopacka et al., 1998). The authors hypothesized that high concentrations of vitamin C could participate in the production of hydroxyl free radicals through a Fenton reaction.

A major function of the intestine is to prevent the absorption of toxins, antigens, proteases and microorganisms across the intestinal wall (Kemper and Specian, 1991; Specian and Oliver, 1991). Epithelial cells cover the surface of the gastrointestinal tract, serving as a barrier between the luminal and tissue compartments. Maintenance of this barrier depends on the integrity of cellular plasma membranes and tight junctions as well as the elaboration of endothelial and epithelial secreting products. Ionizing radiation induces acute morphological changes of the intestine within 24–48 h (Dalla, 1968). Early radiation enteropathy develops during radiation therapy as a result of intestinal crypt cell death, disruption of the epithelial barrier and mucosal inflammation (Wang et al., 2006). After ionizing irradiation, epithelial cells frequently lose contact with each other and possess many lateral and basal projections (Carr, 1981; Fatemi et al., 1985; Wartiovaara and Tarpila, 1977). Our results indicate that gamma-irradiation induced disorganization of the adherent junctions. The altered interactions between epithelial cells and their micro-environment may be critical for the maintenance of normal homeostasis.

The importance of the goblet cells in the production of mucins for the maintenance of the mucous blanket is widely acknowledged (Cheng, 1974; Cheng and Leblond, 1974; Forstner, 1978). Goblet cells reside throughout the length of the intestine and are responsible for the production and maintenance of the protective mucous blanket by synthesizing and secreting high-molecular-weight glycoproteins. The involvement of goblet cell mucins in the pathophysiology of intestinal neoplasia and inflammatory bowel disease has been reported (Filipe and Fenger, 1979; Podolsky and Isselbacher, 1983, 1984). The present study provides detailed evidence of the contribution of goblet cells to the process of restitution and repair in cases of radiation injury in the ileum.

In the present study, radiation exposure caused severe degenerative changes, such as dilation of ER cisternae and degranulation of ER membranes, degeneration of mitochondrial cristae and marginal condensation of chromatin in the nuclear matrix of epithelial cells. Microvilli of epithelial cells also showed changes in length and frequency and alteration of the plasma membranes. Decreased length and surface of villi, as well as the microvillar changes, caused a reduction of surface area of the altered ileum. These data corroborate previous

studies reported by other investigators on radiation induced intestinal injury in animals (Carr, 1981; Carr et al., 1992a; Fatemi et al., 1985; Somosy, 2000). Our results also indicate that abdominal exposure of rats to a single or two doses of 5 Gy gamma-irradiation results in a disruption in the ileum mucosa on day 4 following irradiation. Both radiation regimes produced an increase in the number of goblet cell profiles, resulting in an increase of the total production of mucin. Previous acute radiation studies indicate that the goblet cell response is complex. Some reports describe decreases or no change in cell number at 72 h after irradiation (Becciolini et al., 1985; Carr et al., 1991, 1992a,b) while others demonstrate increases, particularly after several weeks and months (Lewicki et al., 1975; Van Dongen et al., 1976). The histological and TEM studies here clearly demonstrate that goblet cells show resistance to injury compared with enterocytes. Matovelo et al. (1989) reported features of goblet cells covering the villous tips subjected to deoxycholic acid exposure in the rat small intestine and speculated that these cells are less sensitive to noxious effects than are absorptive cells, perhaps because of their more limited exposure. There are differences in the integrin family of cell surface receptors between goblet cells and absorptive cells (Perreault et al., 1995). In our study, in the vitamin C-treated rat ileum, the severity of degenerative changes in the cells was less than that observed in the radiation-only treated groups.

In conclusion, goblet cells appear to be intimately involved in restitution of the epithelium of the rat small intestine subjected to radiation injury. Antioxidant treatment with vitamin C prior to irradiation provides a partly effective protection against intestinal damage.

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