Polyamine depletion inhibits irradiation-induced apoptosis in intestinal epithelia

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Deng, Wenlin, Mary Jane Viar, and Leonard R. Johnson. Polyamine depletion inhibits irradiation-induced apoptosis in intestinal epithelia. Am J Physiol Gastrointest Liver Physiol 289: G599–G606, 2005. First published April 28, 2005; doi:10.1152/ajpgi.00564.2004.—Our group has previously shown that polyamine depletion delays apoptosis in rat intestinal epithelial (IEC-6) cells (Ray RM, Viar MJ, Yuan Q, and Johnson LR, Am J Physiol Cell Physiol 278: C480–C489, 2000). Here, we demonstrate that polyamine depletion inhibits γ-irradiation-induced apoptosis in vitro and in vivo. Pretreatment of IEC-6 cells with 5 mM α-difluoromethylornithine (DFMO) for 4 days significantly reduced radiation-induced caspase-3 activity and DNA fragmentation. This protective effect was prevented by the addition of 10 μM exogenous putrescine. Radiation exposure to mice resulted in a high frequency of apoptosis over cells positioned fourth to seventh in crypt-villus units. Pretreatment of mice with 2% DFMO in drinking water significantly reduced apoptotic cells from ~2.75 to 1.61 per crypt-villus unit, accompanied by significant decreases in caspase-3 levels. Further examination showed that DFMO pretreatment inhibited the radiation-induced increase in the proapoptotic protein Bax. Moreover, DFMO pretreatment significantly enhanced the intestinal crypt survival rate by 2.1-fold subsequent to radiation and ameliorated mucosal structural damage. We conclude that polyamine depletion by DFMO inhibits γ-irradiation-induced apoptosis of intestinal epithelial cells both in vitro and in vivo through inhibition of Bax and caspase-3 activity, which leads to attenuation of radiation-inflicted intestinal injury. These data indicate that DFMO may be therapeutically useful to counteract the gastrointestinal toxicity caused by chemoradiotherapy. This is the first demonstration that polyamines are required for apoptosis in vivo.

THE POLYAMINES putrescine, spermidine, and spermine are found in all eukaryotic cells (36). Their cellular concentrations are highly regulated to meet the needs for normal cell growth and differentiation (50, 54). The first rate-limiting step in polyamine synthesis is the production of putrescine from ornithine. This reaction is catalyzed by ornithine decarboxylase (ODC), which decarboxylates ornithine, the primary precursor for polyamine biosynthesis, to produce putrescine (2, 47). The addition of a propylamine group transforms putrescine into spermidine and spermine into spermine. In the gastrointestinal (GI) tract, the abundant luminal polyamines are from food, microflora, and sloughed cells. The proximal gut lumen of the rat contains 2–3 mM putrescine, and the duodenum of healthy children contains up to 95 μM spermidine and 46 μM spermine (31, 34). Infusion of putrescine directly into the ileal lumen of fasted rats significantly increases RNA, DNA, and protein synthesis (51). Ingestion of dimethylamine stimulates gastrointestinal (GI) mucosal growth (1). Polyamines are required for intestinal epithelial cell migration (29, 30). However, feeding animals a high dose of polyamines has toxic effects. These include weight loss, decreased food and water intake, convulsions, and even death (9, 56). Recent studies also suggest that polyamines are involved in apoptosis and that excessive accumulation of spermine and spermidine can activate apoptosis (42, 52, 57).

Apoptosis, or programmed cell death, is an important physiological process in epithelia for eliminating senescent, damaged, redundant, or deleterious cells (44). Intestinal epithelia have rapid cell turnover rates, accompanied by an equally high rate of apoptosis for maintaining mucosal homeostasis (10). However, intestinal epithelial cells, especially rapidly proliferating cells in the crypts, are highly sensitive to cancer chemotherapy and γ-irradiation. Exposure to cytotoxic agents or γ-irradiation leads to DNA damage, which initiates either a self-repairing process or apoptosis to eliminate injured cells (17, 53). In the latter situation, cytotoxic treatment results in excessive epithelial apoptosis or dysregulation of apoptosis. Studies have demonstrated that excessive apoptosis is one of the main etiological factors that contribute to the GI syndrome after use of cytotoxic agents and/or γ-irradiation (16, 19, 41), which are therapeutic mainstays for leukemia and lymphoma or complementary remedies for other cancers. This GI syndrome includes diarrhea, general malabsorption, and infection. These severe complications inflict not only discomfort on patients undergoing chemoradiotherapy but also limit the dose of therapeutic agents. Therefore, preventing apoptosis in intestinal epithelia is an important therapeutic strategy.

The rate-limiting enzyme for polyamine biosynthesis, ODC, can be specifically inhibited by α-difluoromethylornithine (DFMO, a structural analog of ornithine), which binds competitively and irreversibly to the activated enzyme. Recently, in an in vitro model with intestinal epithelial IEC-6 cells, a nontransformed crypt cell line derived from rat small intestine with intact p53 (45), we demonstrated that depletion of polyamines by DFMO delays apoptosis in response to the cytotoxic agent camptothecin (DNA topoisomerase inhibitor) (46). That this effect was caused by polyamine depletion was shown by preventing it by the addition of exogenous polyamines in the presence of DFMO (46, 64). At this time, the mechanism underlying the DFMO-induced antia apoptotic activity remains largely unclear. Pretreatment of IEC-6 cells with DFMO significantly decreases translocation of Bax from the cytosol to mitochondria, increases cellular Bcl-xl and Bcl-2 levels, and inhibits the conversion of procaspase-3 into its active unit in

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response to camptothecin treatment, leading to the inhibition of DNA fragmentation (64). New evidence also shows that polyamine depletion induces sustained activation of ERK in response to TNF-α plus cycloheximide, which correlates with the protection from apoptosis (4).

The purpose of the present study is twofold. First, we wanted to determine whether polyamine depletion would prevent apoptosis in vivo. Second, we wished to examine whether polyamine depletion might have beneficial effects on the small intestine mucosa following γ-irradiation. We found that DFMO significantly attenuated γ-irradiation-induced DNA fragmentation and inhibited caspase-3 activity in IEC-6 cells. Pretreatment of mice with DFMO for 4 days reduced apoptosis in the intestinal crypt by 51% following γ-irradiation, leading to significantly higher rates of crypt survival 4 days after exposure. Furthermore, polyamine depletion inhibited the γ-irradiation-induced increase in the level of Bax, indicating that the DFMO-elicited antiapoptotic activity was mediated at least in part through the mitochondrial pathway. Together, these data suggest that polyamine depletion by DFMO may have therapeutic potential in ameliorating the GI toxicity caused by chemoradiotherapy.

MATERIALS AND METHODS

Reagents. DFMO was a gift from Merrell Dow (Cincinnati, OH). Rabbit anti-caspase-3, anti-Bax, and mouse anti-Bcl-2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). N-acetyl-Asp-Glu-His-Asp-p-nitroanilide (Ac-DEVD-pNA) colorimetric caspase substrate was purchased from BioMol Laboratories (Plymouth Meeting, PA). 5-Bromo-2'-deoxyuridine (BrdU), 5-fluoro-2'-deoxyuridine (FdU), and horseradish peroxidase-conjugated secondary antibody were purchased from Sigma (St. Louis, MO). The BrdU staining kit was purchased from Zymed Laboratories.

Cell culture and induction of apoptosis in vitro. IEC-6 cells were obtained from the American Type Culture Collection (Manassas, VA) at passage 13 and grown in DMEM supplemented with 5% fetal bovine serum, insulin (10 μg/ml), and gentamycin sulfate (50 μg/ml) at 37°C in a humidified 90% air-10% CO2 atmosphere. The medium was changed every other day. Cells between passages 15 and 21 were used for the experiments. Subconfluent cells were washed and incubated in DMEM without serum the night before experiments unless indicated otherwise. For induction of apoptosis by γ-irradiation, IEC-6 cells were subjected to 25-Gy Cs137 source (Mark I model 25 Gamma Irradiator, J. L. Shepherd & Associate, San Fernando, CA) at a rate of 4.84 Gy/min (×5.16 min). DNA fragmentation and caspase-3 activity were measured 16-h postirradiation.

Caspase-3 activity and DNA fragmentation ELISA. We measured caspase-3 activity in IEC-6 cells by ELISA following the procedures described previously (7, 46). For analysis of caspase-3 activity in intestine, tissue samples were washed thoroughly with PBS, cut into very small pieces, mixed with lysis buffer, and homogenized. After a 2-h incubation on ice, the lysates were centrifuged at 10,000 g at 4°C for 20 min. Caspase-3 activity in the supernatants was measured by using the Ac-DEVD-pNA chromogenic substrate. We measured DNA fragmentation in IEC-6 cells by ELISA, following a modified procedure provided with the cell death detection kit from Boehringer (Indianapolis, IN), which has been described previously (7, 46). DNA absorbance was read at 405 nm in a microplate reader. Duplicates of the samples were used to quantify protein, using the bicinchoninic acid kit from Pierce (Rockford, IL). DNA fragmentation was expressed as absorbance units per microgram protein per minute.

Western blot. Caspase-3 was measured in the cell lysates prepared for caspase-3 activity. Bax and Bcl-2 were measured in total cellular protein lysates. Twenty micrograms of protein were mixed with an equal volume of 2× Laemmli sample buffer (Bio-Rad, Hercules, CA), denatured by boiling for 5 min; this was followed by separation in SDS-PAGE gels and transfer to nitrocellulose membranes. Membranes were blocked with 5% (vol/vol) milk in PBS-Tween 20 for 30 min at room temperature and probed with primary antibody overnight. After thorough washing with PBS-Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, followed by four washes, and developed with the enhanced chemiluminescence Western blotting detection system (New England Nuclear Life Science Products, Boston, MA).

Animal treatment and irradiation. Our whole-body irradiation protocol (no. 1058) was been reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Sciences Center. Male C57BL/6 mice aged 8 wk were purchased from Harlan (Indianapolis, IN) and maintained on a 12:12-h light-dark cycle and fed standard laboratory mouse chow and water ad libitum. After 2 wk of acclimatization, mice were given either vehicle or 2% DFMO in drinking water (pH was corrected with sodium hydroxide to the pH of normal drinking water) for 4 days and were then fasted overnight before whole-body γ-irradiation (Cs137 source at a rate of 4.84 Gy/min) to analyze intestinal apoptosis. For intestinal apoptosis analysis, mice were killed 4 h after irradiation by isoflurane inhalation. For the clonogenic assay, mice were killed 4 days after irradiation. BrdU (120 mg/kg) and 5-fluoro-2'-deoxyuridine (12 mg/kg) were given intraperitoneally 2 h before death to label the S-phase cells. Small intestines were flushed of their contents, washed, and weighed. Proximal jejunum segments (10 cm from pylorus) from each mouse were fixed in 10% neutralized formaldehyde (pH 7.4) buffer and processed for histological evaluation of apoptosis and crypt survival. Distal jejunal segments from each mouse were immediately frozen for measurement of caspase-3 activity or Bax and Bcl-2 protein levels.

HPLC analysis of mucosal putrescine content. The mucosal putrescine content was determined by the method of Tsai and Lin and Wang and Johnson (58, 61). Briefly, mucosal scrapings were collected and frozen immediately at −80°C until samples were ready for dansylation, extraction, and HPLC. The standard curve encompassed the range from 0.31 to 10 μM. Putrescine content was expressed as nanomoles per milligram of protein.

Analysis of mucosal ODC activity. The activity of ODC was assayed by a radiometric technique (36). Mucosal scrapings were collected and placed immediately in a pH 7.4 buffer containing 1 mM Tris, 1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM diithiothreitol. Samples were immediately frozen at −80°C until analyses. Samples were homogenized, they were centrifuged and ODC activity was determined in the 12,000-g supernatant. Samples were incubated at 37°C in the presence of L-[1-14C]ornithine, and the 14CO2 liberated by the decarboxylation of ornithine was trapped on filter paper impregnated with NaOH; this was then measured by liquid scintillation counting. Enzymatic activities were expressed as picomoles of CO2 per milligram of protein per hour.

Detection of apoptosis and crypt survival in intestine. Fixed proximal jejunum was subjected to routine histological procedures for paraffin embedding. Paraffin cross sections (5 μm) were cut perpendicular to the long axis of the small intestine. To detect apoptosis, cross sections were stained with hematoxylin and eosin (H&E). A minimum of 100 half crypt-villi units from each experimental group were scored, and the frequencies of apoptotic cells along the crypt-villi unit were plotted against their cell positions. The number of surviving crypts per jejunal circumference was counted. A surviving crypt was defined as a regenerative crypt that contained a cluster of 10 or more H&E-stained cells. The viability of each surviving crypt was confirmed by strong positive immunohistological staining of BrdU incorporation into five or more crypt cells.

Immunohistological staining. We confirmed surviving intestinal crypts by BrdU labeling of S-phase cells using a BrdU staining kit. Briefly, prelabeled sections were deparaffinized in xylene, rehydrated
from endogenous endonuclease cleavage. Exposure to using specific antibodies against DNA fragments generated induced apoptosis by measuring genomic DNA fragmentation we followed the same polyamine depletion model and inves-
tigation 16 h later (data not shown). Exposure to γ-irradiation at 10 Gy resulted in ~20% apoptotic cells with pyknotic nuclei and a significant threefold increase in DNA fragmentation 16 h later (data not shown). Exposure to γ-irradiation at 25 Gy increased DNA fragmentation by sevenfold compared with control (Fig. 1). Pretreatment of IEC-6 cells with 5 mM DFMO for 4 days significantly inhibited the γ-irradiation-induced apoptosis by more than 60% (*P < 0.001). The addition of 10 μM putrescine prevented the protection observed in the DFMO group. These data indicate that

RESULTS

Polyamine depletion prevents γ-irradiation-induced apoptosis in IEC-6 cells. Our previous polyamine depletion studies showed that treatment of IEC-6 cells with 5 mM DFMO for 4 days was sufficient to deplete cellular putrescine and spermine and ~50% of cellular spermine (29). In the present study, we followed the same polyamine depletion model and investigated the effect of polyamine depletion on γ-irradiation-induced apoptosis by measuring genomic DNA fragmentation using specific antibodies against DNA fragments generated from endogenous endonuclease cleavage. Exposure to γ-irradiation at 10 Gy resulted in ~20% apoptotic cells with pyknotic nuclei and a significant threefold increase in DNA fragmentation 16 h later (data not shown). Exposure to γ-irradiation at 25 Gy increased DNA fragmentation by sevenfold compared with control (Fig. 1). Pretreatment of IEC-6 cells with 5 mM DFMO for 4 days significantly inhibited the γ-irradiation-induced apoptosis by more than 60% (*P < 0.001). The addition of 10 μM putrescine prevented the protection observed in the DFMO group. These data indicate that

DFMO specifically inhibits γ-irradiation-induced apoptosis through polyamine depletion.

Polyamine depletion inhibits caspase-3 activity and activation in IEC-6 cells. Programmed cell death or apoptosis requires a group of effector caspases to dismantle the cells. The activation of caspase-3 is an essential step leading to cleavage of the DNA repair enzyme, poly(ADP-ribose) polymerase, resulting in genomic DNA fragmentation (27, 33). In the control group, only negligible amounts of spontaneous caspase-3 activity and activation were detected. Exposure to 25-Gy γ-irradiation increased caspase-3 activity five-fold compared with control (Fig. 2A). Pretreatment of IEC-6 cells with 5 mM DFMO for 4 days significantly inhibited γ-irradiation-induced caspase-3 activity by more than 80% (*P < 0.001). The effect of DFMO was prevented by the addition of 10 μM putrescine (Fig. 2A). Further analysis of caspase-3 activation by Western blot showed that DFMO caused a significant reduction in the conversion of the 32-kDa procaspase-3 into its 20-kDa active units compared with the control group (Fig. 2B). Consistently, addition of 10 μM putrescine almost totally prevented the DFMO-induced inhibition of caspase-3 activation (Fig. 2B).

Polyamine depletion prevents γ-irradiation-induced apoptosis in the intestine of mice. The inhibitory effects of DFMO on ODC activity and subsequent depletion of polyamines have been described previously in rats and cell lines. Consistent with our data in rats (60), oral DFMO almost totally eliminated ODC activity in mice and significantly (*P < 0.001) reduced mucosal putrescine levels (Fig. 3). Because putrescine is the product of the ODC reaction, our control group to show that the results of DFMO were due to polyamine depletion consisted of animals given DFMO in combination with putrescine.
To establish an optimal dose for studying apoptosis in the intestinal epithelia of mice, we tested 6-Gy, 8-Gy, 12-Gy, and 15-Gy γ-irradiation exposures. Consistent with previous reports (13, 38), we found that 6 Gy resulted in severe and significant apoptosis in the crypt-villus unit 4 h postirradiation. A mild but not significant increase in the apoptotic index was observed when doses were gradually increased to 15 Gy (two mice in each group, preliminary data not shown). Four hours after 15-Gy γ-irradiation exposure, the apoptotic bodies in crypt-villus units increased sixfold compared with control (Fig. 4A). Apoptotic bodies were manifested by condensation of chromatin and nuclear fragmentation (Fig. 5). In this study, pretreatment of mice with 2% DFMO significantly reduced the number of apoptotic cells from 2.75 to 1.61 per crypt-villus unit (Fig. 4; *P < 0.01). Analysis of the correlation of apoptosis and cell position along the crypt-villus unit showed that γ-irradiation caused apoptosis preferentially over the stem cell zone, four to seven cell positions from the crypt base, whereas the paneth cells over the first to third cell positions from the base were relatively resistant (Figs. 4B and 5). These findings were consistent with previous studies (16, 37, 38). Pretreatment with DFMO nonpreferentially decreased the frequency of apoptosis at cell positions having a regenerative or clonogenic potential. After regenerative cells are eliminated, crypts largely disappear by the day 2 postirradiation. Those retaining one or more regenerative cells regrow and form crypt-like structures over a period of 3 days (37). In a preliminary study, we used a series of γ-irradiation doses ranging from 6 to 18 Gy and examined crypt survival 4 days after irradiation exposure. We found that 6 Gy was insufficient to affect small intestinal crypt survival, as evaluated by histological H&E staining. At 8 Gy, γ-irradiation caused <10% loss of crypts compared with control (an average of 105 intact crypts per cross section), and the number of crypt losses increased with higher doses (two mice in each group, preliminary data not shown). In the 15-Gy group, only 9.6 crypts per cross section survived γ-irradiation. Polyamine depletion significantly increased the number of surviving crypts in the proximal jejunum to 20.2 crypts per cross section (Fig. 7, *P < 0.05). Similar protection was observed in the 12.5-Gy groups (Fig. 7). The H&E staining results (Fig. 8, A–C) were corroborated by immunostaining for BrdU-labeled S-phase cells (Fig. 8, D–F). Both 12.5 Gy and 15 Gy resulted in definitive pathology 4 days postirradiation. This included a decrease in small intestinal weight and severe morphological damage, which was manifested by shortened or disrupted villi (Fig. 8). In addition to preventing crypt loss, polyamine depletion significantly ameliorated the structural damage to the mucosa. BrdU staining also demonstrated the decrease in crypts capable of proliferative ability, approximately the 3rd to 14th cell position along the crypt-villus unit (Fig. 4; *P < 0.01). Western blot analysis confirmed that the DFMO-elicited inhibition of caspase-3 activity correlated with the reduction of its activation, as indicated by the decrease in γ-irradiation-induced conversion of procaspase-3 into its 20-kDa active units (Fig. 6B). Next, we evaluated the effect of polyamine depletion on the level of Bax and Bcl-2, which regulate caspase-3 activation by affecting cytochrome c release from the mitochondria. We found that γ-irradiation induced a dramatic increase in Bax, which was prevented by DFMO pretreatment (Fig. 6C). Neither γ-irradiation nor DFMO had effects on Bcl-2 (data not shown).

Fig. 4. Polyamine depletion inhibits apoptosis in small intestinal epithelia of mice after γ-irradiation. Mice (C57BL/6) received vehicle (control) or 2% DFMO in drinking water for 4 days and were then subjected to 15-Gy γ-irradiation. Animals were killed 4 h after γ-irradiation for evaluation of apoptosis in the small intestine by hematoxylin and eosin (H&E) staining. A: apoptotic cells per crypt-villus unit. B: correlation between apoptotic cells and their positions along the crypt-villus unit; n = 6 in each group, and a minimum of 100 half crypt-villus units were scored in each group. *P < 0.001 compared with irradiation alone.
of producing new cells due to irradiation depicted in Fig. 7 and the protective effects of DFMO (Fig. 8).

DISCUSSION

The tolerance of the intestinal mucosa to chemoradiotherapy has been a major concern in the use of cytotoxic agents and/or γ-irradiation for treatment of cancer patients. Drug intervention to ameliorate the GI toxicity or enteropathies accompanied by chemoradiotherapy has been a therapeutic strategy for decades. We previously demonstrated that polyamine depletion by DFMO inhibited camptothecin (DNA topoisomerase inhibitor, which results in cell death by stabilizing topoisomerase-DNA complexes)-induced apoptosis in intestinal epithelial (IEC-6) cells (4, 46, 64). This identified DFMO as a promising therapeutic agent to prevent enteropathies resulting from chemoradiotherapy. In this study, we examined the effects of DFMO on γ-irradiation-induced apoptosis in intestinal epithelial cells both in vitro and in vivo, and we demonstrated that polyamine depletion produced a strong antiapoptotic effect against γ-irradiation in IEC-6 cells. Further in vivo experiments found that supplying DFMO to mice for 4 days in their drinking water significantly reduced γ-irradiation-induced apoptosis in intestinal crypts, leading to higher crypt survival rates and decreased mucosal damage. This is the first demonstration that polyamine depletion protects cells from apoptosis in vivo. These results provide further evidence that polyamine depletion by DFMO could counteract γ-irradiation-induced gastrointestinal toxicity.

The crypt-villus unit in the small intestine is functionally organized into two compartments: the crypts of Lieberkühn and the villi. Proliferation occurs in stem cells near the base of the crypt. Although some cells remain anchored as new stem cells, others divide rapidly, migrate upward, and give rise to terminally differentiated cells that populate the villi. The villus cells are extruded into the lumen after an average residence on the villus of 3 days (5, 6). Because the crypt cells proliferate

Fig. 5. H&E sections showing apoptotic cells from experiments described in Fig. 4. Note the rare frequency of apoptosis in control (left), the high frequency of apoptosis induced by γ-irradiation (middle), and the lower frequency of apoptosis in mice pretreated with 2% DFMO for 4 days before γ-irradiation exposure (right). Photographs shown are representatives of 6 mice in each group.

Fig. 6. Polyamine depletion inhibits γ-irradiation-induced caspase-3 activation and Bax in the small intestine of mice. Mice (C57BL/6) received 2% DFMO in drinking water for 4 days and were then subjected to 15-Gy γ-irradiation. Mice were killed 4 h after γ-irradiation. A: caspase-3 activity in the mucosa of small intestine was measured by ELISA. *P < 0.001 compared with irradiation alone. Data shown are means ± SE of 3 independent measurements. B: procaspase-3 and its active units were evaluated by Western blot. C: Western blot analysis of the level of Bax in the mucosa of small intestine. A total of 20 μg of lysate protein was loaded in each lane. Treatment conditions for A–C were identical. Data shown are representative of 3 independent blots; n = 6 in each group.

Fig. 7. Polyamine depletion enhances intestinal crypt survival in mice following γ-irradiation. Mice (C57BL/6) received vehicle or 2% DFMO in drinking water for 4 days and were then subjected to 12.5- or 15-Gy γ-irradiation. Mice were killed 4 days after γ-irradiation. Crypt survival was evaluated by H&E staining. Viability of surviving crypts was confirmed by immunostaining for bromo-2′-deoxyuridine (BrdU) labeling. Data are means ± SE of the mean number of surviving crypts per cross section. *P < 0.05 compared with γ-irradiation.
Fig. 8. H&E-stained (A–C) and BrdU-stained (D–F) sections of intestine from animals described in Fig. 7. A and D: mice received vehicle without γ-irradiation. B and E: mice received vehicle and 15-Gy γ-irradiation. C and F: mice received 2% DFMO for 4 days before γ-irradiation. Note that the mucosal disruption and disappearance of crypts and loss of regenerative crypts cells in irradiated mice (B and E) were significantly reversed by DFMO pretreatment (C and F). Photographs shown are representatives of 6 mice in each group.

rapidly, they are extremely sensitive to cytotoxic agents and γ-irradiation. An early event that follows γ-irradiation is apoptosis, which can be observed within 2 h and peaks within 3–6 h (8, 37). In agreement with previous reports (16), we found that γ-irradiation (6–15 Gy) resulted in severe apoptotic cell death over the fourth to seventh cell positions along the crypt-villus unit. Polyamine depletion nonselectively inhibited apoptosis in these cell positions (Fig. 4). No significant differences were observed between 6-Gy and 15-Gy γ-irradiation-induced apoptotic cells in the crypts. On the other hand, IEC-6 cells, a nontransformed cell line derived from rat intestinal crypts, were relatively resistant to γ-irradiation; 10-Gy γ-irradiation caused mild damage, but 25 Gy resulted in severe DNA fragmentation, which was significantly greater than that inflicted by 10 Gy. The discrepancy in radiosensitivity of crypt cells between in vitro and in vivo models has not been explained.

Similar to cytotoxic agents, γ-irradiation inflicts direct DNA damage. Sensing DNA damage initiates either a self-repairing process or apoptosis. In the latter case, apoptotic signal cascades ultimately activate a group of effector caspases, including caspases-3, -6, and -7, which lead to the systematic dismantling of cells and DNA fragmentation (15, 48, 55). In this study, we found a fivefold increase in caspase-3 activity in IEC-6 cells and a threefold increase in the small intestine of mice following γ-irradiation. The increased caspase-3 activity in IEC-6 cells was correlated with a sevenfold increase in DNA fragmentation (Figs. 1 and 2). Polyamine depletion significantly inhibited the caspase-3 activation and subsequent DNA fragmentation (Figs. 1 and 2). Although the exact mechanism underlying caspase-3 inhibition by DFMO is not clear, we examined the apoptosis-related Bcl-2 protein family members. Bax, a proapoptotic protein abundantly expressed in the small intestine and colon (40), mediates apoptosis by affecting the mitochondrial permeability transition pore and subsequently the release of cytochrome c (23, 28). Cytochrome c complexes with Apaf-1 and procaspase-9, resulting in the activation of caspase-9. Caspase-9 subsequently activates caspase-3 (26), leading to DNA fragmentation. In contrast to Bax, the antiapoptotic protein Bcl-2 inhibits caspase-3 activation by preventing cytochrome c release (22). Although Bcl-2 is expressed at a much lower level than Bax in the small intestine (14), a definite role of Bcl-2 in regulating intestinal apoptosis has been demonstrated since the epithelial apoptotic index is increased in the small intestine of Bcl-2-null mice (43). A high ratio of Bax to Bcl-2 in the epithelium of small intestine relative to the colon positively correlated with their apoptotic sensitivity (40). We found that γ-irradiation elicited a significant increase in the level of Bax in the small intestine of mice and that polyamine depletion significantly inhibited this increase (Fig. 6). These data suggested that the antiapoptotic effect of polyamine depletion was mediated, at least in part, through the mitochondrial pathway.

γ-irradiation results in crypt cell loss, but the process of cell migration onto villi is radiation resistant (13, 18, 37). At the same time, crypt cell proliferation dramatically slows down (25). These factors together contribute to crypt and villus shrinkage. Exposure to γ-irradiation at >8 Gy can eliminate proliferative cells by day 2 postirradiation, leading to crypt losses, depletion of villus cells, disruption of mucosal integrity, and finally clinical enteropathies. Crypts that retain one or more surviving regenerative or clonogenic cells regrow and form new crypt-like structures, and mucosal integrity is maintained if sufficient new crypts are generated (37). We observed that 8-Gy γ-irradiation did not damage the intestinal mucosa significantly, other than causing apoptosis, but 15 Gy caused severe mucosal structural injury, including loss of more than 90% of crypts and severe disruption of mucosal integrity. Both were significantly prevented by polyamine depletion. Polyamine depletion after DFMO treatment initiated antiapoptotic activity and protected crypt cells, which contributed to intestinal crypt regeneration and subsequently increased crypt survival rates.
The effects of polyamines on apoptosis depend somewhat on the cell type involved and the nature of the agent stimulating cell death. The controversies in this field have been reviewed in detail by Schipper et al. (49). Various studies with different cell systems showed a fast and marked increase in ODC activity after induction of apoptosis, but the increase was not sustained for long and polyamine levels usually decreased. The effects of polyamine analogs on apoptosis are varied (49). The most consistent observations, however, are that the inhibition of ODC with DFMO inhibits apoptosis. In 10 different reports, DFMO decreased apoptosis eight times and had no significant effects the other two times (49). Thus our findings in both IEC-6 cells and mice are consistent with these reports.

No effective protector against intestinal toxicity from chemoradiation therapy has been developed. An ideal strategy should be affordable, effective, administered orally, and without side effects. Interleukins, stem cell factor, amifostine, and keratinocyte growth factor enhance intestinal crypt survival postirradiation (11, 12, 20, 39, 62). However, amifostine itself has undesirable side effects at the dose required for protection. These include vomiting, hypocalcemia, and inhibition of parathyroid hormone secretion (21, 24). Cytokines may cause anaphylaxis and hypotension (59), and growth factors have the danger of stimulating neoplastic growth (3). In addition, most of the above-mentioned potential agents are not effective when given orally. These disadvantages limit their therapeutic value. In this study, we demonstrated the effectiveness of DFMO against radiation-induced gastrointestinal toxicity by the oral route and with no obvious side effects observed. Moreover, recent studies have shown that polyamine depletion by DFMO successfully prevents or inhibits cancer growth (32, 35, 63). Thus exploiting the double effects of DFMO could have significant clinical benefits.

Together, these data indicate that polyamine depletion by DFMO protects the intestine from radiation-induced apoptosis through the inhibition of Bax and caspase-3 activity. DFMO initiated antiapoptotic activity and reduced the severity of intestinal crypt cell loss postirradiation, leading to higher crypt survival rates and the amelioration of GI toxicity.

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REFERENCES