Radiation-induced gastric epithelial apoptosis occurs in the proliferative zone and is regulated by p53, bak, bax, and bcl-2

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Przemeck SM, Duckworth CA, Pritchard DM. Radiation-induced gastric epithelial apoptosis occurs in the proliferative zone and is regulated by p53, bak, bax, and bcl-2. Am J Physiol Gastrointest Liver Physiol 292: G620–G627, 2007. First published October 26, 2006; doi:10.1152/ajpgi.00391.2006.—Unlike the small intestine and colon where γ-radiation-induced apoptosis has previously been well characterized, the response of murine gastric epithelium to γ-radiation has not been investigated in detail. Apoptosis was therefore assessed on a cell positional basis in gastric antral and corpus glands from adult male mice following γ-radiation. Maximum numbers of apoptotic cells were observed in both antrum and corpus at 48 h and at radiation doses greater than 12 Gy. However, the number of apoptotic cells observed in the gastric epithelium was much lower than observed in the small intestine or colon after similar doses of radiation. Hematoxylin and eosin, caspase 3 immunohistochemistry, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling detected similar numbers and cell positional distributions of apoptotic cells, hence hematoxylin and eosin was used for subsequent studies. The highest numbers of apoptotic cells were observed at cell positions 5–6 in the antrum and cell positions 15–18 in the corpus. These distributions coincided with the distributions of PCNA-labeled proliferating cells, but not with the distributions of H1+K+ ATPase-labeled parietal cells or TFF2-labeled mucous neck cells. Decreased numbers of apoptotic gastric epithelial cells were observed in p53-null, bak-null, and bax-null mice compared with wild-type counterparts 6 and 48 h after 12 Gy γ-radiation. Significantly increased numbers of apoptotic gastric epithelial cells were observed in bcl-2-null mice compared with wild-type littermates 6 h after 12 Gy γ-radiation. Radiation therefore induces apoptosis in the proliferative zone of mouse gastric epithelium. This response is regulated by the expression of p53, bak, bax, and bcl-2.

apoptosis; stomach; p53; bcl-2 family; γ-radiation

Following a DNA-damaging stimulus, a cell may either attempt to repair its DNA and survive, or it may undergo programmed cell death, apoptosis. The failure of cells that have sustained genotoxic damage to undergo apoptosis is one mechanism that has been proposed to promote carcinogenesis. Over recent years, the apoptosis induced in murine small intestine and colon by the DNA-damaging stimulus of γ-radiation has been characterized in considerable detail (17, 18, 26). Various techniques for assessment of apoptosis in these tissues have also been systematically assessed (9). The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay has been shown to generate significant false positive results in this tissue, particularly at the tip of the small intestinal villus, where morphologically apoptotic cells are only rarely observed (9, 16). Caspase 3 immunohistochemistry and assessment of apoptosis on the basis of morphological criteria in hematoxylin and eosin (H and E) sections have therefore been considered more reliable techniques for the quantification of apoptosis in the murine intestine.

Numerous apoptotic cells are observed in the putative stem cell zone located toward the bottom of small intestinal crypts within 3–6 h of γ-radiation, and peak amounts of apoptosis are seen following radiation doses >1 Gy. Similarly, apoptotic cells can be observed in the proliferative zone of murine colonic crypts within a few hours of γ-radiation. However, the colonic epithelium is more resistant to radiation-induced apoptosis than the small intestine as peak amounts of apoptosis are not observed until doses >8 Gy are used (18). Radiation-induced intestinal apoptosis is exquisitely p53 dependent, because virtually no apoptotic cells are observed in the small intestinal and colonic crypts of p53-null mice 4.5 h following γ-radiation (2, 11). Some apoptotic cells are, however, observed in the small intestinal crypts of p53-null mice 24 h after 8 Gy γ-radiation, possibly as a result of failure of cells to progress through the G2/M cell cycle checkpoint (10). Radiation-induced intestinal apoptosis in mice has also been shown to be regulated by several members of the bcl-2 family of proteins, including bcl-2 (12), bax (21), and bcl-w (22).

Unlike the small intestine and colon, the apoptosis induced in murine gastric epithelium by DNA damage has not previously been studied in detail. There are significant differences between the epithelia of the stomach and intestine. For example, in the gastric corpus, the putative stem cell zone and proliferative zone are located at the isthmus of the gland (rather than toward the base of small intestinal and colonic crypts), with bidirectional migration of differentiated cell types (4–6). We therefore hypothesize that γ-radiation-induced apoptosis in the stomach may be regulated differently from radiation-induced intestinal apoptosis. Several previous studies have shown increased apoptosis in the gastric epithelium following infection with the carcinogenic bacterium Helicobacter pylori (3, 24, 25). It has been proposed that an organism’s susceptibility to undergo gastric epithelial apoptosis following H. pylori infection is one factor that determines the likelihood of it developing gastric adenocarcinoma as a consequence of long-term infection with this bacterium. Understanding the genetic regulation of gastric epithelial apoptosis in vivo may therefore provide important insights into gastric carcinogenesis. Techniques that have previously been validated in the murine intestine have therefore been used to assess the kinetics, cell positional distribution, and genetic regulation of gastric epithelial apoptosis following γ-radiation.
MATERIALS AND METHODS

Animals. Outbred CD1, inbred Balb/c, and inbred C57BL/6 mice were purchased from Charles River UK (Margate, UK), and inbred FVB/N mice were purchased from Harlan UK (Bicester, UK). p53 Wild-type and null mice (10, 11, 20, 23), bcl-2 wild-type and null mice (12, 14, 21), bax wild-type and null mice (7, 21), and bak-null mice (purchased from Jackson Laboratories) (8) were bred in house and genotyped by PCR-based techniques as previously described. Mice were housed under routine animal house conditions and were fed a commercially prepared pelleted diet and given water ad libitum. The animals were maintained on a 12:12-h light-dark cycle, and all irradiations were performed between 0900 and 1100. Experiments were conducted with UK Home Office approval.

Assessment of apoptosis. Groups of 10- to 12-wk-old male mice were subjected to γ-radiation using a 137Cs source at a dose rate of 2.6 Gy/min and were killed by cervical dislocation at various times afterward. Ligatures were tied around the distal esophagus and proximal duodenum, and the stomach lumen was infiltrated with 4% formal saline. This procedure distended the stomach and fixed gastric glands in a reliable manner for histological assessment. Following fixation, the tissue was paraffin embedded and 3- to 5-μm sections were cut and either stained with H and E or processed for TUNEL or immunohistochemistry. TUNEL and caspase 3 immunohistochemistry were performed as previously described (9). The TUNEL kit was from R&D Systems, catalog no. TA4626. Twenty antral and 40 corpus half glands per mouse were then scored on a cell positional basis for morphologically apoptotic or labeled cells as previously described in detail for the small intestine and colon (13, 21). Briefly, cells were numbered from the base of the gland, designated cell position 1, and scored as normal, apoptotic (containing one or more apoptotic fragments) or mitotic, up to the gastric lumen. Data are presented as mean apoptotic index percentage for a group of mice or as plots of apoptotic index percentage against cell position along the gland.

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded sections as previously described (27). The primary antibodies used were rabbit polyclonal anti-PCNA (FL-261) by Santa Cruz (sc-7907) dilution 1:300; rabbit polyclonal anti-H+/K+-ATPase, α-subunit, by Calbiochem (119101) dilution 1:1,000; mouse monoclonal anti-human spasmolytic factor (TFF2) by Novo Castra (NCL-HSP) dilution 1:20; and rabbit polyclonal anti-active caspase-3 by R&D Systems (AF835) dilution 1:750. All primary antibodies were applied overnight at 4°C. Secondary antibodies were either biotin-labeled goat anti-rabbit immunoglobulins (Dako, EO432) or biotin-labeled goat anti-mouse immunoglobulins (Dako, EO433) at a dilution of 1:200 for 30 min at room temperature. Microwave antigen retrieval in a citrate buffer was used for all antibodies other than anti-H+/K+-ATPase. Glands were scored on a cell positional basis according to whether cells showed positive staining as previously described (27).

Statistical analysis. Two statistical tests were used as previously described: a one-way ANOVA test and a modified median test to assess significant differences at individual cell positions (19, 21). A statistically significant difference between two groups was defined by P < 0.05 in the ANOVA test along with more than three consecutive cell positions being significantly different in the modified median test.

RESULTS

Radiation induces apoptosis in murine gastric antrum and corpus. Time course and dose response experiments for induction of apoptosis by γ-radiation in male CD1 mouse stomach were initially performed. Very few apoptotic cells (assessed by morphological criteria in H and E-stained sections) were observed in control unirradiated CD1 mice (mean apoptotic index 0.15% in antrum and 0.2% in corpus). However, apoptosis was induced in murine gastric antrum 6 h after 12 Gy γ-radiation, and maximum numbers of apoptotic cells were observed at 48 h (Fig. 1A). In murine corpus, there was little induction of apoptosis at 6 h, maximum numbers of apoptotic cells were observed at 72 h, and the mean apoptotic index was lower than in the antrum. A plateau in the amount of apoptosis induced was observed in the antrum at radiation doses >8 Gy and in the corpus at doses >12 Gy (Fig. 1B). A radiation dose of 12 Gy and time points of 6 and 48 h were therefore used in subsequent experiments to compare different strains and genotypes of mice.

TUNEL, caspase 3 immunohistochemistry, and morphological assessment of H and E sections detect equivalent levels of gastric radiation-induced apoptosis. Three methods were compared for the assessment of radiation-induced apoptosis in murine stomach. Assessment by morphological criteria on H and E-stained sections, caspase 3 immunohistochemistry, and TUNEL produced the same yield of apoptotic cells in murine antrum and corpus 48 h after 12 Gy γ-radiation (no significant differences were detected between the groups by ANOVA) (Fig. 2A). Little nonspecific staining was observed with TUNEL as has previously been demonstrated in small intestinal crypts. Moreover, the cell positional distributions of apoptotic cells detected by these three independent techniques were similar (no significant differences at any cell position detected between the three techniques using the modified median test) (Fig. 2, B and C). Assessment of apoptosis by morphological criteria on H and E sections was therefore used for subsequent experiments.

Radiation induces apoptosis in the proliferative zone of murine gastric antrum and corpus. Cell positional assessment of H and E-stained sections showed that apoptosis was induced...
maximally at cell position 5 in antral glands and at cell position 15 in corpus glands (Fig. 2, B and C). This coincides with the zone above the base of the antral gland and the neck zone of the corpus gland. Immunohistochemical markers specific for various cell types within the gastric epithelium were therefore used to investigate further which cell types within the epithelium were undergoing apoptosis following \(^\gamma\)-radiation. In view of the location of the observed apoptotic cells, we assessed the distribution of cell types known to be located in these zones, namely H\(^+\)-K\(^+\)-ATPase-labeled parietal cells, trefoil factor-2 (TFF-2)-labeled mucous neck cells and proliferating cell nuclear antigen (PCNA)-labeled proliferating cells. We did not assess the distribution of chief cells (because these are located near the base of the gland) or the distribution of surface mucous cells. In the antrum, maximum numbers of apoptotic cells and proliferating cells were both observed at cell position 5 (Figs. 3A and 4, A and B). In the corpus, maximum numbers of apoptotic and PCNA-labeled cells were both seen at cell positions 15–16, whereas maximum numbers of both parietal cells and mucous neck cells were observed at cell position 12 (Figs. 3B and 4, C and D). Moreover, whereas only very few morphologically apoptotic cells stained positively with antibodies toward H\(^+\)-K\(^+\)-ATPase and TFF-2, almost 80% stained positively with anti-PCNA antibody (data not shown).

**Inbred mouse strains show differential sensitivities to radiation induced gastric apoptosis.** The experiments described above were all performed on outbred CD1 mice. However, inbred mouse strains have previously been shown to have different small intestinal and colonic apoptotic responses to \(^\gamma\)-radiation (18). Three inbred mouse strains were therefore compared regarding induction of apoptosis 6 and 48 h after 12 Gy \(^\gamma\)-radiation. Balb/c mice showed significantly less antral apoptosis compared with FVB/N and C57BL/6 at 6 h when assessed by ANOVA (\(P < 0.05\)), but no significant differences were demonstrated between any of these groups by the modified median test. Similarly, no significant differences were observed in antral apoptosis between C57BL/6, FVB/N, and Balb/c mice after 48 h (Fig. 5A). After 48 h C57BL/6 mice showed significantly increased numbers of apoptotic cells in the corpus compared with both FVB/N (\(P < 0.05\) by ANOVA and significant differences at cell positions 13–16 by modified median test) and Balb/c (\(P < 0.05\) by ANOVA and significant differences at cell positions 11–16 by modified median test) (Fig. 5B). No significant differences in corpus apoptosis were
observed between FVB/N and Balb/c mice by ANOVA or modified median test after 48 h.

Radiation-induced gastric apoptosis is genetically regulated. To investigate the genetic regulation of γ-radiation-induced apoptosis in murine stomach, various knockout mice were used.

p53-Null mice showed significantly less apoptosis than p53 wild-type littermates in both antrum and corpus at 6 and 48 h after 12 Gy γ-radiation (Fig. 6) ($P < 0.05$ by ANOVA in both tissues and at both time points and significant differences were detected at cell positions 3–9 in antrum at 6 h, 4–5 in antrum at 48 h, 11–24 in corpus at 6 h, 8–18 in corpus at 48 h by modified median test).

Bak-knockout mice showed significantly less apoptosis than their wild-type C57BL/6 counterparts in the antrum at 6 h after 12 Gy γ-radiation ($P < 0.05$ by ANOVA and significant differences at cell positions 8–11) and in the corpus 48 h after 12 Gy γ-radiation ($P < 0.01$ by ANOVA and significant differences at cell positions 12–14 by modified median test) (Fig. 7).

Bax-null mice showed significantly less apoptosis than wild-type littermates in both antrum and corpus at 6 and 48 h after 12 Gy γ-radiation (Fig. 8) ($P < 0.05$ by ANOVA in both tissues at both time points and significant differences were detected at cell positions 3–6 in antrum at 6 h, 4–6 in antrum at 48 h, 15 in corpus at 6 h, and 9–17 in corpus at 48 h by the modified median test).

Bcl-2-knockout mice showed significantly increased numbers of antral and corpus apoptotic cells compared with their wild-type littermates 6 h after 12 Gy γ-radiation ($P < 0.01$ by ANOVA in both tissues and significant differences at cell positions 4–12 in antrum and 8–22 in corpus by modified median test) (Fig. 9). However, at 48 h, no significant differences were observed in the antrum or corpus of bcl-2−/− mice compared with their wild-type littermates.

DISCUSSION

In summary, we have demonstrated that γ-radiation induces apoptosis in the gastric antrum and corpus, and our data suggest that cells within the proliferative zone are most susceptible to undergoing apoptosis following this stimulus. We have demonstrated differential susceptibility to the induction of gastric epithelial apoptosis between inbred mouse strains and have shown that murine gastric apoptosis is regulated by p53 and by three members of the bcl-2 family.

The kinetics of γ-radiation-induced gastric epithelial apoptosis (Fig. 1) are different from the apoptosis observed in the
small intestine and colon following the same stimulus. Following γ-radiation, maximum numbers of apoptotic cells were observed in the gastric epithelium at later time points (48–72 h) than in the small and large intestines, where apoptosis peaks at 4–6 h (18). Whereas maximum numbers of apoptotic cells have been reported to occur in the small intestine following 1 Gy and in the colon following 8 Gy γ-radiation, maximum numbers of apoptotic cells were not observed in the gastric corpus until doses >12 Gy were used, suggesting that the murine stomach is more resistant to the induction of apoptosis than the intestine. In addition, the maximum mean apoptotic indexes observed in the antrum and corpus of wild-type mice were ~4 and 1%, respectively, whereas the maximum mean apoptotic index previously reported in small intestinal crypts is ~10% and in colonic crypts ~6% (15, 18). This confirms that the gastric epithelium, especially the corpus, is relatively resistant to γ-radiation-induced apoptosis.

We have demonstrated that analysis of gastric tissue sections stained with TUNEL, caspase 3 immunohistochemistry, and H and E all showed approximately equal numbers and distributions of apoptotic cells (Fig. 2). This justifies the use of analysis of H and E sections in future studies and shows that TUNEL can be used for accurate analysis of γ-radiation-induced gastric epithelial apoptosis, unlike the small intestine and colon (9, 16). The reason why the TUNEL assay gives fewer false positive cells in the stomach compared with the small intestine is not currently known, but our results highlight the need for assessing the validity of apoptosis assays in specific tissues.

The distribution of radiation-induced gastric apoptotic cells coincided almost exactly with the distribution of PCNA-labeled proliferating cells in the stomach (Figs. 3 and 4). Although it is not surprising that cells within the proliferative zone in the stomach are more likely to undergo apoptosis following γ-radiation than terminally differentiated cells, the stomach again differs from the small intestine in the cell position of peak apoptosis. In the small intestine, most apoptotic cells are observed a few cell positions nearer the base of the crypts than the cell position of peak cellular proliferation (18). Thus, in the small intestine, it has been proposed that apoptosis occurs predominantly in the putative stem cell zone. Our data suggest that in the stomach the cell positions of maximum radiation-induced apoptosis and maximum cellular...
proliferation are coincident. It is therefore possible that murine gastric stem cells are located within the zone of maximum proliferation.

We have demonstrated that three inbred mouse strains show differential susceptibility to the induction of gastric epithelial apoptosis, particularly in the corpus (Fig. 5). Similar differential susceptibility among inbred mouse strains has previously been reported in the intestine (18). In a previous study, more apoptotic cells were observed in C57BL/6 intestinal crypts than Balb/c 4.5 h after 8 Gy γ-radiation (18), suggesting that similar factors may be regulating the sensitivity to apoptosis induction throughout the gastrointestinal tract. Wang and colleagues (25) have previously demonstrated that C57BL/6 mice are more susceptible than Balb/c to Helicobacter felis-induced gastric apoptosis and carcinogenesis. This is consistent with our observation of C57BL/6 being more susceptible to apoptosis induction following γ-radiation than Balb/c. Similar genetic factors may therefore regulate radiation and Helicobacter-induced gastric epithelial apoptosis in these mouse strains.

To investigate genetic factors potentially involved in the regulation of gastric epithelial apoptosis, we used four different genotypes of knockout mice. Our data suggest that similar genetic factors are involved in the regulation of gastric and intestinal epithelial apoptosis. Radiation-induced gastric epithelial apoptosis was almost completely abolished in p53-null mice at 6 h (Fig. 6), in a similar fashion to that previously

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**Fig. 7.** Cell positional distribution of apoptosis in antrum (A and C) and corpus (B and D) of **bak-null** mice and their wild-type C57BL/6 counterparts 6 h (A and B) and 48 h (C and D) following 12 Gy γ-radiation (n = 6 per experimental group). Black line C57BL/6 wild-type; dashed line **bak-null**.

**Fig. 8.** Cell positional distribution of apoptosis in antrum (A and C) and corpus (B and D) of **bax** wild-type and null mice 6 h (A and B) and 48 h (C and D) following 12 Gy γ-radiation (n = 6 per experimental group). Black line **bax** wild-type; dashed line **bax-null**.
reported in the intestine after 4.5 h (2, 11). Some apoptotic cells were, however, observed in the stomach 48 h after 12 Gy γ-radiation, analogous to the p53-independent apoptosis observed in p53-null mouse small intestine 24 h after 8 Gy irradiation (10). Radiation-induced gastric epithelial apoptosis was also reduced in mice deficient in the proapoptotic bcl-2 family members bak and bax (Figs. 7 and 8). We have previously demonstrated that radiation-induced intestinal apoptosis was also reduced in bax-null mice, although to a lesser extent than reported here in the stomach (21). Radiation-induced intestinal apoptosis has not previously been reported in bak-knockout mice, although others have suggested that this protein may play an important role in the regulation of Helicobacter-induced gastric epithelial apoptosis (1). The magnitude of the differences in radiation-induced gastric apoptosis observed between bak-null mice and their wild-type counterparts was less than between bax wild-type and null mice. The biological significance of these differences is currently being studied by assessing the consequences of long-term Helicobacter felis infection in these animals. Mice in which the antiapoptotic gene bcl-2 was deleted showed a large increase in gastric epithelial apoptosis 6 h following 12 Gy γ-radiation (3-fold in antrum and 10-fold in corpus) (Fig. 9). These mice have previously been reported to show a similar large increase in colonic epithelial apoptosis 4.5 h following γ-radiation (12). However, small intestinal radiation-induced apoptosis was previously shown to be largely bcl-2 independent (12). Bcl-2 expression may therefore normally contribute to the relative resistance to apoptosis induction shown by the stomach and colon compared with the small intestine. Rather surprisingly, bcl-2 wild-type and null mice showed similar numbers of apoptotic gastric epithelial cells 48 h after γ-radiation. It will therefore be interesting to investigate whether bcl-2-null mice have a different response to chronic gastric injury such as long-term Helicobacter infection.

Our studies have therefore demonstrated that γ-radiation-induced gastric epithelial apoptosis shows many similarities to radiation-induced intestinal apoptosis. In particular, apoptosis at both sites appears to be regulated by the same genes. However, our data have also highlighted important differences in apoptosis induction at different sites within the murine gastrointestinal tract. For example, we have shown that the gastric epithelium is more resistant to the induction of apoptosis than the intestine and that apoptotic cells are observed later in the stomach than in the intestine following γ-radiation. It is hoped that these studies will provide a useful framework for proposed future investigations of the kinetics and genetic regulation of Helicobacter-induced apoptosis in the stomach, particularly addressing the question of the importance of apoptosis induction during the development of gastric adenocarcinoma.

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REFERENCES


