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Increased Intestinal Bak Expression Results in Apoptosis

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Cells in the human intestinal epithelium have a life-span of around 5 days before being lost by apoptosis at the luminal surface. We examined changes in expression of the Bcl-2 gene family which may be responsible for epithelial cell loss. In the normal and neoplastic colon, mucosal expression of immunoreactive Bak co-localized with sites of epithelial cell apoptosis. Inducing apoptosis in the human colon cancer cell line HT29 and the rat normal small intestinal cell line IEC 18 in culture was accompanied by increased Bak expression without consistent changes in expression of other Bcl-2 homologous proteins. Bak appears to be the endogenous Bcl-2 family member best correlated with intestinal cell apoptosis.

Mucosal cells lining the gastrointestinal tract undergo continuous turnover. In the small and large intestines, new cells originate from stem cells located near the base of crypts and they then differentiate as they migrate away from the crypt base over the course of 3–7 days, finally undergoing apoptosis at the mucosal surface (1). Proposed inducers of apoptosis near the luminal surface include loss of cell-substrate interaction (anoikosis), (2), autocrine production of TGFβ (3) and the effect of the colonic luminal short chain fatty acid butyrate (4). The endogenous proteins involved in the promotion of enterocyte apoptosis are not known. We have examined the enterocyte expression of the Bcl-2 gene family, an evolutionarily-conserved group of largely homologous proteins which, through homo and heterodimerization, regulate a downstream common apoptotic pathway in many cell types (5). We have found that there is increased Bak expression in areas of epithelial apoptosis and that apoptosis in intestinal epithelial cells is accompanied by increased expression of the Bcl-2 homolog Bak (6–8), without consistent changes in other members of the Bcl-2 family of proteins, both in vivo and in vitro. This implies that Bak may be the principal endogenous promoter of apoptosis in the intestinal epithelium.

MATERIALS AND METHODS

Tissue samples. Normal and neoplastic human intestinal tissues were obtained from patients undergoing surgery at St. Luke's/Roosevelt Hospital Center. Permission for tissue examination was obtained from the hospital investigational review board. Tissues were rapidly fixed in 10% formalin-buffered saline and embedded in paraffin.

Immunohistochemistry. Four μm-thick sections were immunostained using polyclonal primary antibodies specific for Bak, Bax, Bcl-2, Mcl-1 or Bcl-X and an avidin-biotin peroxidase method (ABC kit, Vector Labs, CA), following microwave pre-treatment as described previously (9–12).

Terminal deoxyuridine nick end-labeling (*TUNEL*). Apoptotic cells were identified in tissue sections using a method modified from Gavrieli (13), as previously described (14). For double-labeling, the AEC kit (Vector Labs) was used to stain TUNEL-positive nuclei red, followed by avidin-biotin peroxidase immunohistochemistry with nickel enhancement, to stain Bak black. No microwave pretreatment or counterstaining was performed for double staining.

Cell culture. HT29 and IEC-18 cell lines were obtained from American Type Culture Collection and maintained in Dulbecco's Modified Eagles medium (Gibco-BRL) with 10% fetal calf serum.

Western blotting. Blotting was performed as described previously (15). Cells were lysed in a buffer containing 1% Triton X 100, 10mM Tris-HCl, pH7.4, and protease inhibitors and the resulting insoluble material was removed by centrifugation. 100 μg samples were electrophoresed in 12% SDS-PAGE and incubated with antibody at a concentration of 1:1500 (v/v).

Assessment of apoptosis. DNA fragmentation ("laddering") was examined after DNA extraction using standard methods followed by electrophoresis on 1.5% tris-borate ethidium agarose gels. The amount of DNA fragmentation was quantitated

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by the Cellular DNA fragmentation ELISA (Boehringer Mannheim, Indianapolis, IN) and by FACS analysis, detecting a subdiploid peak after propidium iodide staining (16).

RESULTS

In human colonic epithelium, Bak expression co-localized with the distribution of apoptotic cells, as identified by TUNEL. There was a gradient of Bak expression along the colonic crypt with immunoreactivity expressed most strongly in cells located at the luminal surface of the crypt, where around 90% of the TUNEL-positive crypt cells are located (fig 1). Occasional expression of Bak and rare TUNEL-positive cells were seen in the crypt base. Bak immunoreactivity was located in the cytoplasm, below and above the nucleus. Co-localization of Bak and apoptotic epithelial cells was also seen in neoplastic colonic tissue. In adenomatous colonic polyps, both Bak expression and TUNEL-positivity were rarely seen at the polyp surface but instead were found mainly deep within elongated adenomatous crypts. In colon carcinoma, the numbers of apoptotic cells and Bak-immunoreactive cells were reduced compared with normal mucosa and apparently randomly distributed. However, foci of Bak-immunoreactivity co-localized with areas of frequent apoptotic

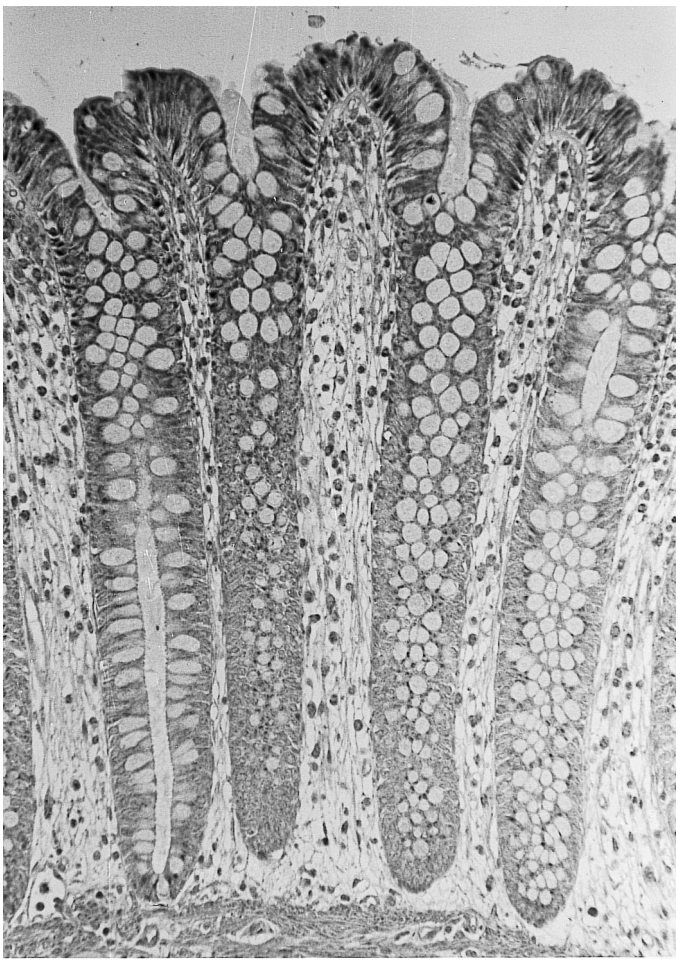

FIG. 1. Bak expression in normal colonic crypts. Bak immunostaining is evident in the supra and infranuclear cytoplasm of colonic epithelial cells, with maximal expression toward the luminal surface of the crypts (top). Peroxidase immunohistochemistry, hematoxylin counterstain. Original magnification ×200.

cells, and in these areas doubly-labelled cells with both nuclear TUNEL-positivity and with Bak expression in the nucleus could be seen (fig 2).

To determine whether this co-localization has functional implications, we examined the effect of inducing apoptosis in vitro upon Bak expression in intestinal cell lines. In HT29 cells, derived from a human colon carcinoma, apoptosis was induced by incubation with 5mM sodium butyrate (4), resulting at 48 hours in a 49% reduction in total cell number and an approximately three-fold increase in genomic DNA fragmentation, consistent with apoptosis. Western blot analysis of cell lysates at 48, 72 and 144 hours demonstrated that the induction of apoptosis by butyrate was accompanied by increased expression of Bak and a small increase in Bcl-2 but no change in the expression of Bax, Bcl-X_L and Mcl-1. (fig 3) Bcl-X_S was not expressed in these cells.

In IEC 18 cells, which are derived from normal rat small intestine, apoptosis was induced by incubation with either of the active metabolites of the non-steroidal anti-inflammatory drug sulindac, sulindac sulfide or sulindac sulfone (17). At 72 hours, the number of floating cells was increased over 10-fold compared with medium only controls and apoptosis was confirmed by DNA

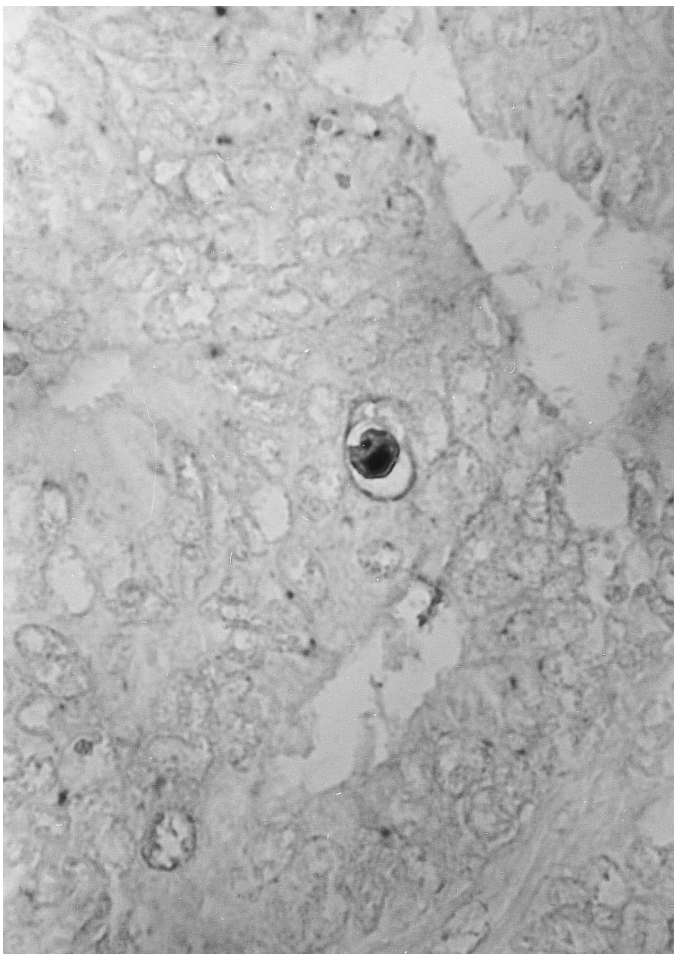

FIG. 2. Double staining with nickel-enhanced peroxidase immunohistochemistry for Bak and TUNEL with AEC. In this poorly differentiated colon adenocarcinoma, Bak is expressed at low levels in the cytoplasm of a minority of cells, for example at top. In contrast, at center in a cell undergoing apoptosis, with nucleus stained positively by TUNEL, there is also strong nuclear expression of Bak. Original magnification ×400.

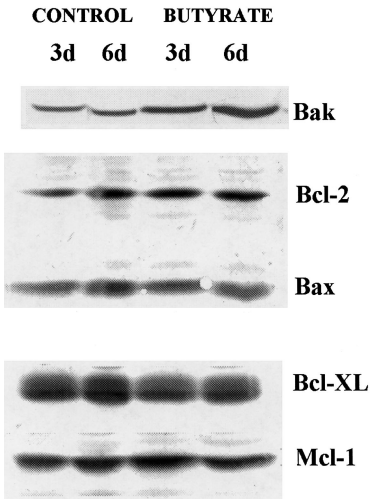

FIG. 3. Western blot demonstrating expression of Bcl-2 family proteins 3 and 6 days after exposing HT29 cells to butyrate (lanes 3 & 4) or medium only as a control (lanes 1 & 2).

laddering in agarose gel electrophoresis experiments as well as the appearance of a subdiploid peak on FACS analysis of DNA content. Western blotting demonstrated that the induction of apoptosis by sulindac sulfide was accompanied by a time-dependent increase in Bak expression, as early as 24 hours after exposure to the drug (fig 4). No changes in expression of other Bcl-2 homologous proteins were detected.

DISCUSSION

We have demonstrated increased Bak expression in enterocytes during apoptosis in-vitro. The geographical co-localization of Bak immunoreactivity in areas of enterocyte apoptosis in both normal and neoplastic intestinal tissue in-vivo further supports the concept that Bak is the Bcl-2 homolog most likely to be responsible for promoting apoptosis in the intestine. The observation of decreased Bak expression in colon neoplasia (18) is therefore consistent with findings of reduced apoptosis in colon cancer (19) and suggests that reduced Bak expression contributes to cell accumulation during carcinogenesis in the colon.

In the normal human colon, Bak immunoreactivity was evident in cells that were TUNEL-positive and also in some cells at positions 1–2 days away from terminal differentiation and apoptosis. Thus, increased Bak expression may occur before apoptosis and may not necessarily be the stimulus for apoptosis, but rather would presumably render terminally differentiated colonic enterocytes highly susceptible to apoptotic stimuli that occur in the bowel lumen such, as butyrate or bile salts.

We found increased Bak expression during apoptosis in HT29 cells, which have mutant p53 (20),

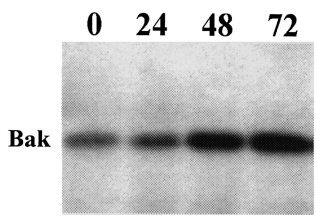

FIG. 4. Western blot showing that in IEC-18 cells Bak expression increases in a time-dependent manner during apoptosis. Time after exposure to sulindac sulfide is indicated in hours.

implying that the regulation of Bak in these cells is p53-independent. Examining the factors responsible for regulating Bak expression in enterocytes will enable us to understand the control of intestinal growth in health and disease.

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