Inhibition of Apoptosis during Development of Colorectal Cancer

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Abstract

Colorectal tumorigenesis proceeds through an accumulation of specific genetic alterations. Studies by the mechanism by which these genetic changes effect malignant transformation have focused on the deregulation of cell proliferation. However, colorectal epithelial homeostasis is dependent not only on the rate of cell production but also on apoptosis, a genetically programmed process of autonomous cell death. We investigated whether colorectal tumorigenesis involved an altered susceptibility to apoptosis by examining colorectal epithelium from normal mucosa, adenomas from familial adenomatous polyposis, sporadic adenomas, and carcinomas. The transformation of colorectal epithelium to carcinomas was associated with a progressive inhibition of apoptosis. The inhibition of apoptosis in colorectal cancers may contribute to tumor growth, promote neoplastic progression, and confer resistance to cytotoxic anticancer agents.

Introduction

The development of colorectal cancer proceeds through a series of genetic alterations involving the activation of oncogenes and loss of tumor suppressor genes (1). Most colorectal carcinomas arise from benign adenomas that gradually increase in size, dysplasia, and villous (finger-like) morphology. The progressive accumulation of genetic alterations (e.g., APC, p53, DCC, and ras) governs the transition of normal colorectal epithelium to adenomas and their malignant transformation to adenocarcinomas (2). Although genetic events responsible for this histopathological conversion have been well described, it remains unclear how these alterations result in the development and growth of colorectal tumors. Previous investigations of colorectal tumorigenesis have focused on the deregulation of cell proliferation (2). However, the size of a continuously renewing cell population is determined not only by the rate of cell production but also by the rate of cell loss. Normal tissue homeostasis requires the physiological deletion of cells by activation of apoptosis, a genetically determined program of autonomous cell death (3, 4). Inhibition of apoptosis by the deregulation of certain oncogenes results in clonal expansion (3). Therefore, we investigated the possibility that transformation of normal colorectal epithelium to cancer involves the inhibition of apoptosis. We measured the rate of spontaneous apoptosis in colorectal epithelium from four discrete histopathological sources representing various stages in the neoplastic transformation to cancer: normal mucosa, flat mucosa and adenomas from patients with FAP, sporadic adenomas, and sporadic colorectal carcinomas.

Materials and Methods

In Situ (TUNEL) Labeling of Apoptotic DNA Fragmentation in Histopathological Sections of Colorectal Tissue. Apoptotic cells were visualized in histopathological sections which maintained colorectal crypt architecture by use of TUNEL of apoptotic DNA strand breaks (5). Frozen endemic colorectal mucosal biopsy tissue sections (4–6 µm) were fixed in cold 4% buffered formaldehyde (pH 7.4), followed by cold 70% ethanol. Tissue sections were incubated with 20 µg/ml proteinase K (Sigma Chemical Co.), washed in cold PBS, and immersed in 100 µl reaction buffer [0.2 µM potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 0.25 mg/ml BSA, and 2.5 mM cobalt chloride] supplemented with terminal deoxynucleotidyl transferase (0.3 e.u./µl) and biotinylated dUTP. Each experiment was performed with a negative control (without b-dUTP) and a positive control (10 min pretreatment with 1 µg/ml DNase dissolved in reaction buffer; Sigma). Following incubation at 37°C for 30 min, the sections were washed in PBS and incubated with 100 µl FITC-avidin solution [4X saline-sodium citrate buffer (Sigma), 2.5 µg/ml fluoresceinated avidin, 0.1% Triton X-100, and 5% w/v nonfat dry milk] at room temperature for 30 min in the dark. The sections were washed and incubated with 0.5 ml PI solution (5 µl PI, 5 µl RNase, and 1 ml PBS) for 30 min at room temperature in the dark. The viable (PI+, FITC+ nuclei-red) and apoptotic (FITC+ nuclei-yellow) cells in each tissue section were visualized by dual-color fluorescence microscopy using a dual-band filter.

Isolation and Culture of Colorectal Epithelial Cells. Epithelial cell suspensions were prepared from endoscopic mucosal biopsies as described (2). Colorectal mucosal samples obtained by endoscopic biopsies were rinsed with sterile saline, cut into <0.5-cm² pieces, treated with 0.25% collagenase, and dissociated into single-cell suspensions with a stainless steel mesh and repeated trituration. Adherent cells (fibroblasts and macrophages) were removed from the cell suspensions by plastic adherence depletion for 1 h at 37°C. The nonadherent cells were subject to multiple centrifugation rinses (eight times), dispersed through 60 µm mesh, and resuspended in serum-free and protein-free hybridoma media (Sigma) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), and gentamicin (25 µg/ml) at a concentration of 2 X 10^7/ml. The colonocyte content of the cell suspensions was confirmed by indirect immunofluorescent labeling of cells with an anti-cytokeratin antibody; 92 ± 8% of cells were positively stained for cytokeratin.

Gel Electrophoretic Analysis of Epithelial Cells from Normal Colorectal Mucosa, FAP, and Colorectal Carcinoma for Detection of Oligonucleosomal DNA Fragmentation. Apoptosis is identified by nuclear chromatin condensation and a typical endonuclease-mediated degradation of DNA into discrete oligonucleosomal (190-base pair) fragments. Cells (5 X 10^6) were harvested immediately after isolation (0 h) and after 2, 4, and 8 h of culture. Total genomic DNA was isolated after SDS lysis and proteinase K digestion. Oligonucleosomal DNA fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide as described (6). Determination of Cell Cycle Distribution and Apoptosis of Colorectal Epithelial Cells by DNA Content Analysis of PI-Labeled Cells. Cells (5 X 10^6) were harvested immediately after isolation (0 h) and again after 8, 16, and 24 h in ex vivo culture, fixed in 50% ethanol, permeabilized with 0.1% Triton-X-100, treated with 5 µg/ml RNase, and incubated at 37°C for 15 min before staining with 50 µg/ml PI for 60 min at 4°C (6). The fraction of subdiploid cells with oligonucleosomal DNA degradation characteristic of apoptosis was quantified by flow cytometric analysis as described (7). The cell cycle distribution of viable cells (mean ± SEM in the G0/G1, S, and G2-M cell cycle phases) was also determined. The percentages of apoptotic cells in...
colorectal epithelium from normal subjects \((n = 8)\), nonadenomatous colon polyps \((n = 4)\), flat mucosa of patients with FAP \((n = 8)\), adenomas from patients with FAP \((n = 8)\), sporadic adenomas \((n = 10)\), and sporadic colorectal carcinomas \((n = 11)\) at each time point and the mean for each group were determined. The significance of the difference in apoptosis between tissue groups was determined by calculating the two-tailed \(P\) using the Mann-Whitney unmatched two-sample test.

**Immunohistochemical Analysis of Bcl-2 Protein Expression in Normal Colonic Mucosa and Colon Carcinoma.** Frozen tissue sections \((4-6 \mu \text{m})\) obtained from mucosal biopsies were subjected to immunoperoxidase \((3\text{-amino-9-ethylcarbazole})\) staining with a monoclonal mouse anti-human Bcl-2-specific antibody using a modification of the method of Hockenbery et al. \((8)\). Tissue sections were fixed in acetone at 4°C for 20 min and treated with 0.3% \(\text{H}_2\text{O}_2\) in methanol for 45 min. The sections were blocked with diluted goat serum \((\text{Vector Laboratories})\) for 30 min. The sections were incubated with 100 \(\mu\text{l}\) monoclonal mouse anti-human Bcl-2 \((\text{dilution of 1:40}; \text{DAKO, Car}

Results and Discussion

Normal colorectal mucosa was obtained by endoscopic biopsy from eight normal subjects. *In situ* TUNEL assays of normal epithelium immediately after biopsy revealed numerous apoptotic cells along the entire crypt epithelial column with extensive apoptotic death in the upper third of each crypt (Fig. 1). Gel electrophoretic analysis of DNA isolated from normal epithelial cell suspensions prepared immediately following biopsy \((0 \text{ h})\) exhibited characteristic apoptotic ladders; oligonucleosomal DNA fragmentation became more extensive over 8 h in culture (Fig. 2). Flow cytometric analysis of normal colorectal epithelial cell suspensions immediately after isolation \((0 \text{ h})\) showed

Fig. 2. Gel electrophoretic analysis of epithelial cells from normal colorectal mucosa, FAP, and colorectal carcinoma for detection of oligonucleosomal DNA fragmentation. Molecular size markers \(C_1, 100 \text{ base pairs}; C_2, 1 \text{ kilobase}\) are indicated.

<table>
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<th>Normal</th>
<th>FAP</th>
<th>Carcinoma</th>
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<td>(C_1)</td>
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Fig. 1. *In situ* labeling of apoptotic DNA fragmentation in TUNEL-stained histopathological sections of colorectal tissue: A, colonic mucosa \((\times 10)\); B, regions of colonic crypts exhibiting maximal apoptotic nuclei \((\times 100)\). In each tissue section, the viable cells show \(\text{PI}^+, \text{FITC}^-\) nuclei \((\text{red})\) and apoptotic cells exhibit \(\text{FITC}^+\) nuclei \((\text{yellow})\). The transition from adenoma \((\text{Ad})\) to carcinoma \((\text{Ca})\) is indicated.
the following cell cycle distribution: G1, 81.4 ± 2.7%; S, 7.9 ± 3%; and G2, 10.3 ± 2% with 27.1 ± 1.5% cells present in the apoptotic subdiploid fraction (Fig. 3). The subdiploid fraction progressively increased over time in cell culture with apoptotic loss of 58.4 ± 7.7% and 85.6 ± 5% of the entire population of normal colonocytes at 8 and 24 h after biopsy, respectively (Fig. 3).

FAP is an inherited autosomal dominant disease characterized by the formation of hundreds of colorectal adenomas in young adults. Virtually all patients with FAP develop colorectal cancer by the fifth decade of life. Patients with FAP have a germline mutation of the APC tumor suppressor gene (9). APC gene mutations are also involved in the early development and possibly the initiation of sporadic colorectal tumors (10). Therefore, the colorectal epithelium of patients with FAP represents an early preneoplastic stage in the evolution of colorectal cancer. Endoscopic biopsy specimens of eight tubular adenomas and adjoining flat colorectal mucosa was obtained from eight patients with FAP. Germline mutation of the APC gene was confirmed in the patients by protein and allele-specific expression assay.
(11). In contrast to normal colorectal epithelium, in situ assays of FAP epithelium immediately after biopsy showed a reduced apoptotic fraction with TUNEL-stained epithelial cells restricted to the luminal surface of the mucosa (Fig. 1). DNA fragmentation assays failed to show the typical DNA ladders observed in normal epithelium at 0 h, but apoptotic DNA fragmentation became evident after 4 h of culture (Fig. 2). Flow cytometry analyses showed a normal cell cycle distribution with no appreciable difference between adenomas (G1, 78.6 ± 3.6%; S, 10.1 ± 3.9%; G2, 12 ± 2%) or adjoining flat epithelium (G1, 82 ± 3.5%; S, 7.5 ± 2.9%; G2, 10.5 ± 2.4%). Compared to normal epithelium, the subdiploid apoptotic fraction was reduced in FAP epithelium at the time of isolation (flat, 11.6 ± 3.9%, P = 0.01; adenoma, 12.8 ± 2.9%, P = 0.006) as well as after 8 h of ex vivo culture (flat, 27 ± 2.2%, P = 0.006; adenoma, 29 ± 2.6%, P = 0.016) (Fig. 3B). However, extensive apoptosis of FAP epithelial cells occurred after 24 h in culture (flat, 74 ± 7%, P = 0.25 compared with normal; adenoma 73 ± 6.6%, P = 0.11 compared with normal) (Fig. 3B). Interestingly, there was no difference in the susceptibility to apoptosis between adenomatous and flat FAP mucosa.

Sporadic adenomas also show a high prevalence of somatically acquired APC mutations (10). However, as sporadic adenomas increase in size and degree of dysplasia, they usually acquire additional genetic changes, such as ras gene mutations or inactivation of the DCC and p53 tumor suppressor genes. Therefore, sporadic adenomas represent an intermediate stage in the evolution of colorectal cancer from normal colorectal epithelium. Ten sporadic colorectal adenomas of varying size (0.8 to 3.8 cm; average, 1.64 cm) and histopathology (five tubular and five tubulovillous) were examined. Sporadic adenomas also showed a reduction in apoptotic epithelial cells using in situ TUNEL assays (Fig. 1). Quantification of the subdiploid apoptotic fraction by flow cytometry showed a substantive reduction in cell death not only at 0 h (13 ± 2%, P = 0.001 compared with normal) but also at 8 h following the initiation of culture (24.7 ± 4.3%, P = 0.002 compared with normal) (Fig. 3B). At 24 h, epithelial cells from the five tubular sporadic adenomas (size: mean, 1.1 cm; range, 0.8–1.6 cm) mimicked the survival of normal and FAP epithelium, with loss of 77.4 ± 10% of the cell population (P = 0.13, compared with normal) (Fig. 3B). However, in contrast to FAP or sporadic tubular adenomas, four of five sporadic tubulovillous adenomas showed a more prolonged inhibition of apoptotic cell death in culture; colorectal epithelial cells from the five tubulovillous adenomas (size: mean, 2.2 cm; range, 1.0–3.8 cm) exhibited only 35.6 ± 10% apoptotic death after 24 h in culture (P = 0.005, compared with normal; P = 0.016, compared with sporadic tubular adenomas) (Fig. 3B). The cell cycle distribution of viable cells from sporadic adenomas was normal (G1, 82.8 ± 6%; S, 6.4 ± 2.3%; G2, 10.9 ± 4%). In contrast to adenomatous polyps, the survival of colonic epithelium from four nonneoplastic polyps (two hyperplastic, lipoma, inflammatory polyps) was similar to normal epithelium, with extensive apoptosis at 0 h (29.5 ± 5%), after 8 h (61 ± 15%), and after 24 h (77.5 ± 10%) in culture (Fig. 3B). These results suggest that the inhibited apoptosis of adenomatous colorectal epithelium may be a consequence of the genetic alterations which result in neoplastic growth and tumor progression. However, only the subset of sporadic adenomas with tubulovillous histopathology appear to have acquired the changes that allowed sustained in vitro survival over 24 h.

Colorectal carcinomas (n = 11) exhibited the most dramatic reduction of apoptosis. Apoptosis could not be detected by TUNEL staining (Fig. 1) and DNA fragmentation assays (Fig. 2) at 0 h or after 8 h of ex vivo culture. As observed in adenomas of patients with FAP and sporadic adenomas, carcinomas displayed an abnormally reduced fraction of spontaneous apoptosis at 0 h (11.4 ± 1.8%, P = 0.0003 compared with normal) and after 8 h of ex vivo culture (13.4 ± 1.9%, P = 0.0002 compared to normal) (Fig. 3). However, in contrast to most adenomas, 9 of 11 carcinomas exhibited sustained survival in culture; only 29 ± 3% of the entire population was apoptotic after 24 h in culture (P = 0.0002 compared to normal; P = 0.03 compared to sporadic adenomas) (Fig. 3B). Colorectal carcinomas were also capable of more extended survival with only 43.7 ± 5% apoptosis after 42 h in culture. Therefore, malignant transformation to carcinomas is associated with prolonged in vitro survival conferred by the sustained inhibition of apoptosis. In addition, flow cytometry of single-cell suspensions showed variable cell cycle distributions (G1, 75 ± 15.7%; S, 6.4 ± 2.8%; G2, 17.9 ± 17%), with 3 of 11 tumors displaying an abnormal increment in the size of the G2 fraction.

These results demonstrate that the inhibition of apoptosis is an integral component of the genesis of colorectal adenomas and carcinomas. The inhibition of apoptosis by bcl-2 in follicular lymphomas or BCR-ABL in chronic myeloid leukemia is known to provide a selective growth advantage (6, 12), as well as confer resistance to anticancer agents (13). Aberrant cell survival could also promote the accumulation of secondary genetic changes that lead to neoplastic progression. Therefore, the inhibition of apoptosis in colorectal neoplasms may contribute to tumor growth, clonal evolution, and inherent resistance to chemotherapeutic agents. What are the specific mechanisms responsible for the inhibition of apoptosis in colorectal neoplasia? The base of the colonic crypt harbors the proliferative compartment of epithelial cells. These cells differentiate as they ascend the crypt-epithelial column and are deleted via activation of apoptosis and exfoliation at the luminal surface. Expression of the bcl-2 proto-oncogene is known to prolong cell survival by inhibition of apoptosis (12). In normal colonic crypts, bcl-2 expression is restricted to the proliferative zone at the base and lower third of the vertical crypt-epithelial column (8). The loss of bcl-2 expression as cells differentiate and migrate toward the luminal surface correlates with commitment to programmed cell death. This topographic distribution of bcl-2 expression may confer prolonged survival in proliferative cells and yet allow the programmed death of terminally differentiated cells (8). Recent studies indicate that bcl-2 expression is deregulated in human primary colorectal tumors and cell lines (14). We studied the topographic distribution of bcl-2 expression in the normal colonic epithelium, seven sporadic adenomas (five tubular, two tubulovillous), and six sporadic carcinomas, already characterized for apoptosis, by immunoperoxidase staining of frozen tissue sections with a monoclonal mouse anti-human bcl-2 antibody (DAKO-bcl-2). The bcl-2 protein was restricted to the crypt base in normal colonic epithelium (Fig. 4B), FAP adenomas, and five tubular adenomas studied. However, bcl-2 was expressed along the entire neoplastic gland axis in the two tubulovillous adenomas studied. The most striking expression of bcl-2 was observed in the colorectal carcinomas; the protein was detected throughout the malignant epithelium of all six carcinomas studied, with no discernible topographic restriction of expression (Fig. 4, C and D). The ability to sustain prolonged ex vivo colonocyte survival over 24 h also became manifest during the late stages of tumor progression to carcinomas, raising the possibility that abnormal expression of the bcl-2 gene is a potential mechanism for the inhibition of apoptosis during colorectal tumorigenesis. Since several other proteins in addition to bcl-2 can alter the susceptibility to apoptosis (3), it is possible that the observed inhibition of apoptosis could also be attributed to other genetic alterations associated with colorectal tumorigenesis. Mutations of the APC gene occur early in the development of sporadic colorectal cancers. Since the APC gene product has been shown to be associated with an adherens junction protein called β-catenin (15), the loss of APC function could deregulate cell growth or survival by alteration of contact inhibition. In our study, germline and somatic APC gene mutations were associated with a
decreased fraction of apoptotic cells at the time of biopsy and after 8 h in culture. Other genetic alterations including either p53 (16) or DCC (17), which occur late in tumor progression (1), could also conceivably alter the susceptibility to apoptosis. In this context, wild-type p53 has been shown to be a component of the apoptotic response to DNA damage, and the induction of wild-type p53 expression in a human colon cancer cell line has been shown to result in apoptosis (18). Moreover, a wild-type p53-binding repressor element has been recently identified on the promoter of the bcl-2 gene (19, 20). Therefore, the inhibition of apoptosis in colorectal carcinomas may result from APC mutations, bcl-2 overexpression, p53 inactivation, or other genetic changes that accumulate during colorectal tumorigenesis. The identification of the mechanism responsible for the inhibited apoptosis could provide a biomarker of malignant transformation in colorectal adenomas as well as a novel target for therapeutic strategies against colorectal cancer.

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References

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