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Advances in Brief

Reduced Bile Acid-induced Apoptosis in "Normal" Colorectal Mucosa: A Potential Biological Marker for Cancer Risk[1]

Harinder Garewal,[2] Harris Bernstein, Carol Bernstein, Richard Sampliner, and Claire Payne

Department of Internal Medicine, Section of Hematology/Oncology [H. G.] and Section of Gasteroenterology [H. G., R. S.], Tucson Veteran Affairs Medical Center and Arizona Cancer Center, Tucson, Arizona 85723, and Department of Microbiology and Immunology, University of Arizona Health Sciences Center, Tucson, Arizona 85724 [H. B., C. B., C. P.]

Abstract

Dietary factors, including bile acids, are important in the causation of colorectal cancer (CRC). We have previously shown that *in vitro* exposure of colorectal mucosal biopsies to low concentrations of bile acids produces apoptosis selectively in goblet cells. Apoptosis is an important mechanism for clearing DNA-damaged cells. Inhibition of apoptosis would result in increasing accumulation of DNA-damaged cells, resulting in increased cancer risk. We compared the percentage of apoptosis induced by bile acids in mucosal biopsies from CRC patients with that of noncancer subjects.

Mucosal biopsies from 15 to 20 cm from the anal verge were incubated in 1 mM sodium deoxycholate, and the percentage of goblet cells undergoing apoptosis was quantitated. Seven patients with a history of CRC within the previous 5 years were compared with 18 noncancer subjects [4 neoplasia free and 14 with small (≤9 mm) polyps only].

The CRC patients had a significantly lower percentage of apoptosis than noncancer subjects; the mean for CRC was 10.7% (range, 0.9–26%) and for noncancer subjects was 55.9% (range, 20.3–71%; $P \leq 0.001$). Two other noncancer patients had very high-risk lesions, *i.e.*, large villous adenomas and multiple large polyps during several colonoscopies over the previous 6 years. Their percentage of apoptosis was in the cancer range, *i.e.*, 6.2 and 10.7%.

Reduced apoptotic ability may imply increased cancer risk. By applying a quantitative bile acid-induced apoptosis assay to colorectal mucosal biopsies, the percentage of apoptosis was found to be significantly reduced in CRC patients. This assay may prove to be a useful intermediate biological marker for identifying subjects at increased risk of cancer.

Introduction

There is intense interest in developing intermediate biological markers that would be useful in identifying subjects and populations at increased risk of cancer. In addition to their potential use in strategies for screening programs, these markers may serve as end points in cancer prevention trials. The "field cancerization" hypothesis suggests that diffuse biological abnormalities are present in an epithelium in which cancers occur, which are probably produced by continual exposure to carcinogens over time (1). In the colon, long-standing exposure to carcinogens, such as high-fat diets, may result in the entire mucosa accumulating abnormalities that make it a "field" in which cancers can develop.

To be clinically useful, it is important that a putative biological marker for colorectal cancer risk be detectable in normal-appearing mucosa obtained relatively easily for analysis. Although many markers, including genetic, enzymatic, and proliferative, have been proposed and are the subjects of ongoing study, none has thus far been fully validated (2). Perhaps the best known marker is increased proliferation rate in colonic crypts, often accompanied by altered distribution of proliferating cells along the length of the crypt (3). A large number of studies have been reported using this marker, measured by a variety of methods, but it still remains controversial and unproven as a valid marker for assessing the risk of malignancy. More recently, genetic changes have been identified in colorectal cancer and its precursor lesions, large adenomatous polyps, but, other than in the relatively rare familial syndromes, genetic changes have thus far not been identified in normal-appearing colorectal mucosa. This limits their utility as intermediate markers for the majority of subjects at risk for colorectal cancer.

Naturally occurring cell death, or apoptosis, is an important mechanism for maintaining a continuously renewing population of cells and for eliminating cells with DNA damage (4). A reduction in apoptotic ability would result in the retention of cellular damage, including DNA damage, with a consequent increase in the possibility of neoplasia formation.

Considerable epidemiological evidence suggests that diet is an important factor in colon carcinogenesis (5, 6). Although a number of dietary components have been identified as possible carcinogens, fecal bile acids have long been strongly implicated in this process (7, 8). High fecal bile salt concentrations accompany a high-fat, low-fiber Western-type diet (7, 8). The precise mechanism(s) of bile acid-induced colon carcinogenesis is not known. Bile acids have been shown to cause DNA damage in mammalian cells (9, 10). *In vitro*, at high concentrations, bile salts are cytotoxic and produce necrotic cell death. We have studied the effect of lower concentrations of bile salts on human colonic mucosa and have shown that, at these lower concentrations, bile acids cause increased apoptotic cell death primarily in immature and mature goblet cells (11, 12).

In this study, we compared the degree of apoptosis produced in normal-appearing colorectal mucosa from patients with a history of colon cancer *versus* subjects with either no cancer or small polyps only. The cancer patients had markedly reduced bile acid-induced goblet cell apoptosis in this assay.

Materials and Methods

Patients. Endoscopic biopsies were obtained from patients undergoing colonoscopy for a clinical indication. The protocol was approved by the Human Subjects Committee. Two study biopsies were obtained from each subject from a site 15–20 cm from the anal verge. One biopsy was processed for routine histology while the other was used for the apoptosis assay.

Quantitation of Bile Acid-induced Apoptosis. The biopsies were immediately placed in cold Eagle's MEM, with an α modification (catalogue no. M4526; Sigma Chemical Company) containing heat-inactivated FCS, nonessential amino acids (Sigma Chemical Company), penicillin, streptomycin, and HEPES buffer (pH 7.2). They were kept on ice and brought to the laboratory where they were incubated in the same MEM medium containing 1.0 mM

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[2] To whom requests for reprints should be addressed, at Section of Hematology-Oncology (111D), Tucson Veterans Affairs Medical Center, 3601 South 6th Avenue, Tucson, AZ 85723. Phone: (520) 792-1450, Ext. 6410; Fax: (520) 629-1861.

sodium deoxycholate. The incubation was at 37°C for 3 h, at which time the MEM was removed and 2 ml cold 3% glutaraldehyde in 0.1 M phosphate buffer was added. The tissue was kept in the refrigerator at 0–4°C overnight and then transferred to 0.1 M phosphate buffer.

For processing, the epoxy embedment procedure of Payne *et al.* (13) was followed. In brief, the tissue was postosmicated, dehydrated in a graded series of ethanols, and embedded in Spurr's epoxy resins. One-μm semithin epoxy sections were prepared using glass knives and firmly heat attached to a slide for 5 min on a hot plate maintained at 80°C. The sections were then stained with methylene blue-azure II-basic fuchsin (polychrome stain) and rinsed with distilled water (14). The stain intensity was checked under the microscope after the initial methylene blue-azure II staining (2–7 min). Stain intensity was adjusted with a second staining with basic fuchsin (1–4 min), if necessary, by reheating the slides on the hot plate and flooding with the staining solution. The slides were then rinsed again with distilled water.

As previously described, the only cells induced to undergo apoptosis under the above conditions are the goblet cells (11, 12). The number of darkly stained (apoptotic) and lightly stained (nonapoptotic) goblet cells were quantitated by light microscopy under a ×100 oil immersion lens (Fig. 1). Only goblet cells whose nuclei were observed in the plane of the section were scored. This ensured that one of the hallmarks of apoptosis, chromatin condensation, was evident in the affected cells. At least 200 goblet cells obtained from 10 or more different crypts were scored, and the percentage of apoptotic goblet cells was determined.

Statistical Analysis. An unpaired Student's *t* test was used for statistical analysis.

Results

Seven patients had a history of colorectal cancers diagnosed and resected within the past 5 years. Their mean age was 64.5 (range, 50–71) years. Four subjects had normal, neoplasia-free colonoscopies, with no previous history of polyps or cancer, and 14 had small adenomas (<9 mm) only, either on a previous colonoscopy or during the present one. The mean age of these 18 subjects was 63.7 (range, 46–80) years.

Fig. 2 displays the percentage of bile acid-induced apoptosis of the cancer cases *versus* the normal or small polyp-only group. The mean percentage of apoptosis for the cancer patients was 10.7% (range, 0.9–26%) and for the noncancer patients was 55.9% (range, 20.3–71%; $P < 0.001$).

As shown in Fig. 2, one patient in the normal small polyp group had a low percentage of apoptosis (20.3%). He is a 48-year-old man with a history of microcytic anemia, previous small polyp removal, and multiple foci of adenomatous changes on histological evaluation of biopsies from grossly normal-appearing mucosa.

In addition to the patients shown in Fig. 2, two other patients had a low percentage of apoptosis in the range of the cancer group. Both had very high-risk lesions. The first was a 70-year-old man with a history of large villous adenomas and multiple large (and small) polyps on several colonoscopies over the previous 6 years. In addition, he had a family history of colon cancer in first-degree relatives. His percentage of apoptosis was 10.7%. The second patient was a 63-year-old man with three large villous adenomas in addition to other large tubulovillous adenomas plus large (and small) tubular adenomas found during four colonoscopies over the previous 2 years. His percentage of apoptosis was 6.2%.

Discussion

Apoptosis is a mechanism for natural cell death, which, in addition to other roles, has the important function of eliminating damaged cells. For example, cells with genetic damage caused by exposure to carcinogens may be deleted by undergoing apoptosis, thereby preventing their replication and accumulation of clones of abnormal cells. Bile acids are known promoters of colon carcinogenesis and can cause DNA damage. Given a Western diet, exposure to bile acids probably occurs continually over life, beginning at an early age. We hypothesize that initially efficient apoptotic mechanisms may help clear most, if not all, cells damaged by bile acids. Cells with efficient apoptotic mechanisms would therefore get cleared with a selective survival of apoptosis-resistant cells as time passes. This results in increasing accumulation and retention of clones of apoptosis-resistant damaged cells, providing a field for cancer development. Fig. 3 illustrates this hypothesis.

Based on our studies of bile acid-induced goblet cell apoptosis, we have developed and applied a quantitative assay to assess apoptosis in

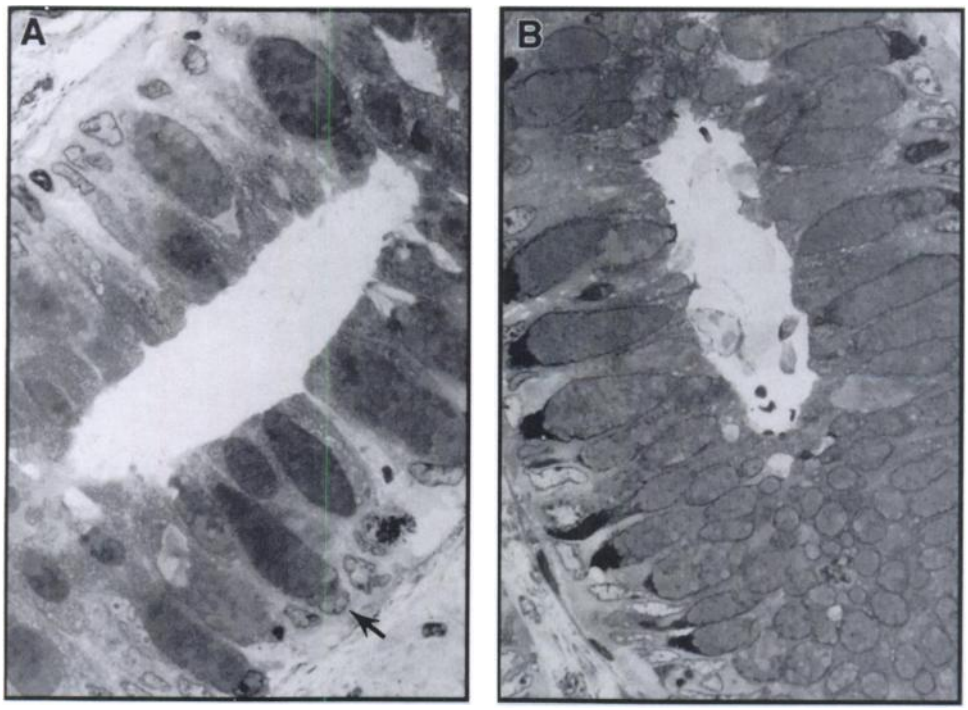

Fig. 1. Biopsies of the normal mucosa of a non-colon cancer patient with adenomatous polyps (low-risk group). The polychrome stain allows for the easy identification of the mucin granules and the quantitation of the normal and apoptotic goblet cells. *A*, tissue incubated for 3 h at 37°C in the absence of sodium deoxycholate. *Arrow*, a goblet cell nucleus with a normal-appearing diffuse chromatin pattern. *B*, tissue incubated for 3 h at 37°C in the presence of 1.0 mM sodium deoxycholate. Numerous darkly stained goblet cell nuclei can be easily identified. Polychrome stain; ×100 oil immersion objective.

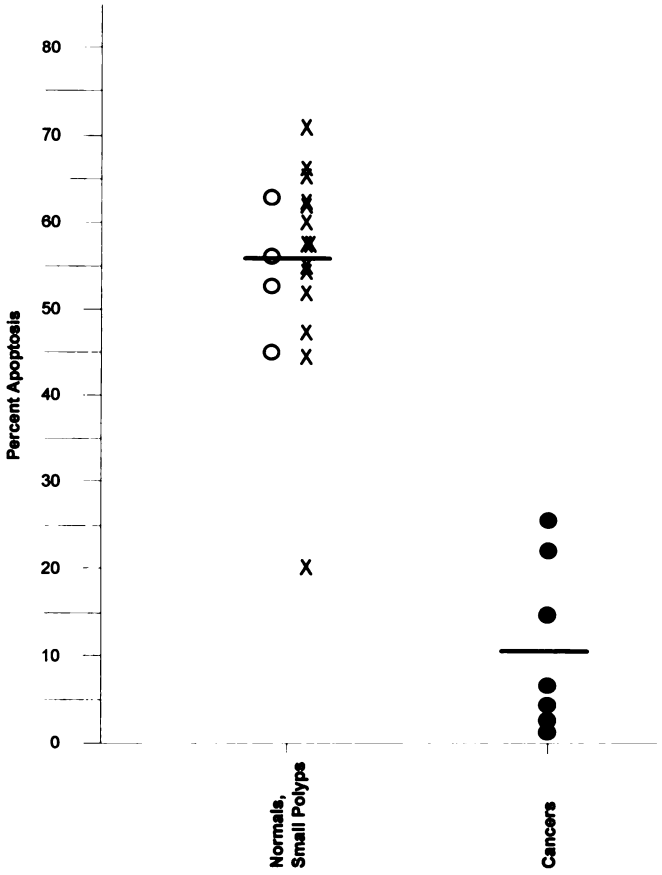

Fig. 2. Percentage of apoptosis in goblet cells induced by sodium deoxycholate in "normal" mucosal biopsies from patients with cancer (●) compared with noncancer controls. The latter consist of those with no neoplastic lesions (○) or only small (≤9 mm) polyps (×).

normal-appearing mucosa from cancer *versus* noncancer subjects. The results demonstrate that apoptosis is reduced in the cancer cases. The assay uses mucosal biopsies from a site easily reached via a flexible sigmoidoscope, and thus has the potential for clinical applicability.

Although the hypothesis shown in Fig. 3 would suggest that decreased apoptotic ability should characterize all colonic epithelial cells in a setting of increased cancer risk, we have previously shown that immature and mature goblet cells, irrespective of location within the crypt, are preferentially induced to undergo apoptosis by the bile salt treatment compared to other cell types present in the colonic mucosa (11, 12). The reason for the sensitivity of goblet cells specifically to sodium deoxycholate treatment during a relatively short incubation *in vitro* is unknown at the present time, and may relate to specific membrane characteristics, intracellular antioxidant defenses, or signal transduction mechanisms that result in endonuclease activation (11, 15). It is possible that other epithelial cell types may undergo apoptosis under other conditions, *e.g.*, an extended period of time in culture. Bedi *et al.* (16) have recently compared the levels of apoptosis that occur after incubation of tissue *in vitro* from normal mucosa from normal subjects, normal mucosa from familial polyposis patients, adenomas, and carcinomas. They found that normal individuals had the highest level of apoptosis, normal mucosa from familial polyposis patients had less, and adenomas and carcinomas the least of all. More recently, Pasricha *et al.* (16) reported that sulindac, a nonsteroidal anti-inflammatory drug, which reduces polyp formation in subjects with familial polyposis, caused no change in proliferative indices after 3 months of treatment, but increased the apoptotic fraction of colonocyte cell suspensions. Based on these initial studies, and our own findings, apoptosis-based bioassays may indeed prove to be very useful in assessment of cancer risk. Our bile acid-induced apoptosis assay is a relatively simple and rapid procedure, and this report is the first demonstration of a measurable reduction in apoptotic ability in subjects with sporadic cancers.

We have previously described the development of this assay, initially using the "gold standard" of apoptosis assays, *i.e.*, electron microscopy. The method was then adapted for light microscopy as was done in this study. Our initial developmental work used a range of concentrations of sodium deoxycholate over various incubation times (12). The 1 mM concentration over a 3-h incubation period produced the best level of apoptosis. Lower concentrations produced significantly less apoptosis, while longer incubations and/or higher concentrations resulted in necrotic death.

In addition to morphology, other biochemical procedures have been used to identify apoptosis; these include agarose gel electrophoresis (DNA ladder), field inversion gel electrophoresis to identify high molecular weight DNA fragments and *in situ* DNA fragmentation assays (15). It has recently been established that the formation of a DNA ladder may be an epiphenomenon, seen only in certain cell types and/or under certain conditions, and may even be seen in necrotic cell death or represent an artifact of DNA isolation (18–20). Similarly high molecular weight DNA fragments can be seen in both apoptosis and necrosis (21), and the *in situ* DNA fragmentation assay can only be interpreted as positive for apoptosis if morphology is also taken into consideration (22). In our assay, on the other hand, we have relied on standard morphology, as defined by Kerr and colleagues (23–26),

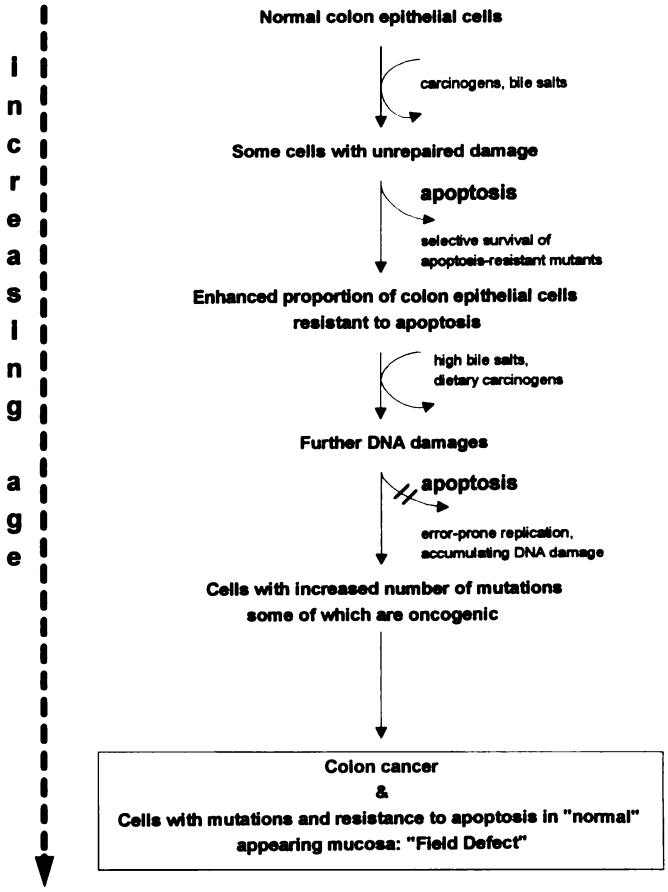

Fig. 3. Hypothesis illustrating selection of apoptosis-resistant clones and increasing accumulation of DNA damage with age. Ongoing exposure to carcinogens, such as bile salts, produces DNA damage and selective survival of apoptosis-resistant clones eventually resulting in neoplasia and malignancy.

which does not have the drawbacks of the other methods mentioned. As stated previously, in developmental work published elsewhere, the present light microscopy method is based on the gold standard, which is ultrastructural identification of apoptotic cells (11). We have shown that the classic ultrastructural features of apoptotic cells, such as condensation and margination of chromatin, increased cellular electron density, and the presence of cytoplasmic vacuoles is induced in the goblet cells by the bile salt sodium deoxycholate (11).

The hypothesis illustrated in Fig. 3 would suggest a decreasing ability for apoptosis with age in a Western population exposed to bile acids. We are in the process of conducting a study assessing bile acid-induced apoptosis during different decades of life. Since not all subjects are destined to get colon cancer, such a study will require a large number of participants to demonstrate a statistically significant finding.

In this initial study, we compared patients with a known history of cancer with subjects that were either normal or had small polyps only. Although polyps are considered to be precursor lesions for colon cancer, most of this risk is confined to patients with large or villous lesions. Clearly every patient with a polyp will not get cancer, since polyps occur in 40% or more of the population over the age of 60 years while cancer is far less frequent. Interestingly, in our study population, the only two patients with large villous adenomas had a percentage of apoptosis that was in the range for cancer patients. Only four of our patients were absolutely normal, with no polyps at all during the study colonoscopy or anytime previously. The high frequency of polyps was because the colonoscopies were being performed for clinical indications, such as known polyp surveillance. These initial findings are very promising, and this assay procedure of bile acid-induced apoptosis in goblet cells may be a useful marker to assess the risk of colon cancer, thereby making it feasible to do more targeted screening.

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