Thus, this assay may prove useful as a biomarker of colon cancer risk. Bile acids may induce apoptosis in colonic goblet cells at concentrations comparable to those found in fecal water after high-fat meals. Preliminary evidence indicated that cells of the normal-appearing (nontumorous) portion of the colon epithelium of colon cancer patients are more resistant to bile salt-induced apoptosis than are cells from normal individuals.

In the present study, 68 patients were examined, and biopsies were taken at 20 cm from the anal verge, cecum, and descending colon. The patients included 17 individuals with a history of colorectal cancer, 37 individuals with adenomas, and 14 individuals who were neoplasia free. The mean bile salt-induced apoptotic index among normal individuals was 57.6 ± 3.47 (SE), which differed significantly (P < 0.05) from the mean value of 36.41 ± 3.12 in individuals with a history of colon cancer.

The correlation between independent observers was 0.89 (P < 0.001), indicating good interobserver reliability. Components of variance comparing interindividual versus intranidividual sources of variation suggested that site-to-site variability, both between regions of the colon and for adjacent biopsies, was larger than the interpatient variability for individuals with a history of neoplasia. Therefore, there was “patchiness” of the susceptibility of regions of the colon to bile acid-induced apoptosis in individuals with a history of neoplasia (a patchy field effect). There was no obvious correlation of low-apoptotic index regions with regions in which previous neoplasias had been found and removed. On the other hand, for normal, i.e., neoplasia-free, individuals, there was relatively less intrinidividual variation compared to interindvidual variation.

Our assay shows an association between resistance to bile acid-induced apoptosis, measured at 20 cm from the anal verge, and colon cancer risk. Thus, this assay may prove useful as a biomarker of colon cancer risk.

INTRODUCTION

Bile acids have been implicated as important etiological factors in colon cancer (1–4). The concentrations of the two major fecal bile acids, DOC and LCA, correlate positively with the level of dietary fats such as corn oil, safflower oil, or linoleic acid, but not with olive oil or stearic acid (5, 6). Fecal concentrations of DOC and LCA, on the other hand, correlate negatively with the level of dietary fiber such as wheat bran or whole wheat plus oat fiber, but not with pectin (7, 8). DOC and LCA are cytotoxic to colon-derived cells (9, 10).

It has been hypothesized (11, 12) that large regions of colonic epithelium may be abnormal in individuals who are at increased risk for cancer and that these biological abnormalities may have been caused by long-term exposure to damaging agents. Bile acids may be one such damaging agent in the colon. At the increased concentrations present in the colon after high fat meals (13, 14), bile acids can cause apoptosis, especially among goblet cells at all levels within the crypts (15–17). Thus, high concentrations of bile acids in solution in the colonic contents after high-fat meals may induce apoptosis in colonic epithelial cells. After cells in an area are killed by a cytotoxic agent (including the bile acid DOC), surviving cells from nearby areas migrate in to produce new epithelium (18, 19). Cells are more likely to survive and repopulate an area of the colon if they are mutated and have a survival phenotype that renders these cells resistant to the induction of apoptosis. Thus, when individuals have high-fat meals over several decades, large areas of their colonic epithelium may become populated by cells with an abnormally high resistance to bile acid-induced apoptosis.

The ability to undergo apoptosis is an important mechanism for maintaining control over a population of cells under continual renewal, as seen in the colonic epithelium. In particular, the ability to undergo apoptosis is important for the elimination of cells with unrepaired DNA damage (16, 17). A reduction in this apoptotic ability would result in the retention of cells with DNA damage and a consequent increased risk of mutations, including those that are carcinogenic.

Recent reports have indicated that patients with a previous history of colon cancer or with familial adenomatous polyposis have normal-appearing colonic mucosa in which cells have a reduced ability to undergo induced apoptosis compared to individuals with no history of colon neoplasia (20). More recently, Chang et al. (21) presented evidence that measurements of apoptosis had a much greater prognostic value, on a population basis, as an intermediate marker for colon tumorigenesis than did measurements of proliferation.

We have previously reported the development of an assay for measuring bile salt-induced apoptosis. Preliminary results involving a small number of subjects showed that patients with a cancer history had significantly less induced apoptosis than did those free of neoplasia (16). The present study extends the previous study to 68 patients, uses improved criteria for identifying apoptotic cells, and addresses issues of importance to the quality control and reliability of the assay, such as interobserver variability and regional differences within the colon.

MATERIALS AND METHODS

Patients. Study biopsies were obtained from sequentially available patients who were undergoing colonoscopy because of clinical indications. The patients included: (a) individuals known to have had a previous resection for colon cancer; (b) individuals who were not known to have had previous polyps (and thus were possibly in the group of patients with no current or former neoplasia); and (c) individuals present on the same day as members of the other two groups who were willing to have biopsies taken from their colons. We used a protocol approved by the Human Subjects Committee of the University of...
Quantification of Bile Acid-induced Apoptosis. Medium was prepared containing Eagle’s MEM (α modification; catalogue number M4526; Sigma Chemical Co.) and 10% heat-treated FCS, 1% nonessential amino acids (catalogue number M7145; Sigma), a 1% solution of penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml), a 0.5% solution of 1 m HEPES buffer, and a 2% solution of 200 mM t-glutamine (which was prepared and kept frozen until used). The medium was adjusted to pH 7.3 and filter sterilized. This medium was made fresh every 3 weeks because of the instability of glutamine in the liquid medium. The medium was placed in tubes on ice, and the biopsies were placed in the tubes immediately upon removal and brought to the laboratory. There, the biopsies were removed from the tubes. Each biopsy was cut in half, and each half was placed in one of two vials containing media prewarmed to 37°C and equilibrated with CO₂ for 30 min; one of these vials also contained 1.0 mM NaDOC. These biopsies were then incubated at 37°C for 3 h, after which the MEM was removed, and 2 ml of cold, half-strength Karnovsky’s fixative were added. The tissue was kept in the refrigerator at 0°C–4°C overnight and then transferred to 0.1 mM phosphate buffer.

Processing of Tissue. For processing, the epoxy embedding procedure described by Payne et al. (22) was followed. Briefly, the tissue was postfixi
cated, dehydrated in a graded series of ethanols, and embedded in Spurr’s epoxy resin. Epoxy sections (1 µm) were prepared using glass knives, and the sections were heat-attached to slides 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 (23). The stain intensity was checked under the microscope after the initial staining procedure (2–7 min). Stain intensity was adjusted with a second staining with basic fuchsin (1–4 min; polychrome method) by reheating the slides on the hot plate and flooding with the staining solution. The slides were then rinsed again with distilled water.

Measurement of Apoptosis. As described previously, the goblet cells of the colon epithelium tend to undergo apoptosis upon treatment with NaDOC (15, 17). The number of darkly stained (apoptotic) and lightly stained (non-apoptotic) goblet cells was quantitated by light microscopy under a ×100 oil immersion lens. Only goblet cells in which the goblet cell cytoplasm (distinguishable by the presence of mucin granules) was clearly connected to the nucleus were scored. This ensured that hallmarks of early apoptosis (24, 25), such as chromatin condensation or margination and increased density of the nucleoplasm were scored in the quantitation of apoptotic cells. At least 100 goblet cells obtained from more than 10 different crypts were scored; the percentage of goblet cells that were apoptotic was determined, and this was called the AI.

Statistical Analysis. The observed values of the AI had a Gaussian distribution, as assessed by a box plot, quantile-quantile plot, and histogram. Mean values of the AI were compared among normal, colorectal cancer, and adenomatous polyp patients using a one-way ANOVA; pairwise comparisons were obtained using Tukey’s multiple range test. A decision rule was defined based on the mean – 2 SDs of the normal individuals; assuming a Gaussian distribution of the AI values implies that 97.73% of normal individuals should have measurements above this value.

A comparison of the AI values in the presence and absence of bile salts was performed using a paired t test. Components of variance were used to assess the relative variabilities of subjects, the location within subject, and the biopsy within the location. Restricted maximum likelihood estimates of the variance components were obtained using SAS PROC MIXED software (SAS Institute Inc.). Finally, the correlation between measurements made by different observers on the same specimens was assessed using the Pearson correlation coefficient.

RESULTS

Controls without Colon Neoplasia. Fourteen subjects with no previous history of polyps or cancer had normal, neoplasia-free colonoscopies. The AIs are shown in Fig. 1. These biopsies had a mean NaDOC-induced AI of 57.64 ± 3.47% SE with a SD of 13.0%. All AIs for this group fell within 2 SDs of the mean (31.6–83.6%). In Fig. 1, a dashed line is drawn at 31.6%, 2 SDs below the mean AI for neoplasia-free (normal) individuals, which represents [AIₙor – 2 SDs]. Using values below [AIₙor – 2 SDs] to indicate those at higher risk results in 100% specificity; i.e., all normal individuals are correctly classified. All normal individuals had AIs above [AIₙor – 2 SDs].

Colorectal Cancer Patients. Seventeen patients had a history of colorectal cancer that had been diagnosed and resected. The biopsies taken 20 cm from the anal verge of these individuals showed a mean NaDOC-induced AI of 36.41 ± 3.12% SE. The mean AIs of the normal and cancer groups differ significantly at the level of P < 0.05 by Tukey’s test. A total of 47% of the patients with a history of colorectal cancer had AIs < [AIₙor – 2 SDs].

Adenoma Patients. Fig. 1 also shows that the 37 biopsies from individuals with adenomas had a mean AI of 51.68 ± 2.15% SE. Among the individuals with adenomas, the majority (29 of 37 individuals) had tubular adenomas, 5 of 37 individuals had tubulo-villous adenomas, and 3 of 37 individuals had villous adenomas. Three individuals (8%) with adenomas had AIs < [AIₙor – 2 SDs]; (a) two individuals with villous adenomas; and (b) one individual with a large tubular adenoma of greater than 1.5 cm in diameter. The mean AIs of the cancer and polyp patients differed significantly (P < 0.05), but there was not a significant difference between the mean AIs of normal and polyp patients.
sets of biopsies from the same individual taken 2 months apart; a C indicates a history of resected colon cancer. V1ad indicates a history of villous adenoma(s), and the designations of tubular adenoma(s);

average AIs at one or more locations below [AI_{nor} - 2 SDs]. In Fig. 3, we show the locations of the neoplasm(s), which are indicated by black dot(s), and the value and location of the AIs obtained for each of these six individuals. Values of AI < [AI_{nor} - 2 SDs] are circled. For the individual designated C2s, one AI was obtained within the transverse colon rather than the cecum because the individual had a prior right hemicolectomy that removed the region of the colon from the cecum to midway through the transverse colon.

For two of the six individuals (V2d and C2s), the low AIs seen were distant from the known locations of prior neoplasms. For the other four individuals, the sites of at least one prior neoplasm were close to a low observed AI value. These data could be consistent with patches of colon epithelium with low AI from which neoplasms would tend to arise, but the patches may not be large enough to see a clear association of neoplasms with an AI taken at a location some distance away.

Observer Variability. Observer variability of AI, as determined by two independent observers (H. B. and C. P.) with expertise in the evaluation of apoptotic cells in the colon (17), is shown in Fig. 4. From these data, the correlation between the observers is 0.89 (P < 0.001), with 1.0 representing complete agreement.

Table 2. Source of variation as determined by components of variance test.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Patient history</th>
<th>Subjects</th>
<th>Location</th>
<th>Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No history of colonic neoplasia</td>
<td>52%</td>
<td>33%</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>History of adenomas</td>
<td>12%</td>
<td>63%</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>History of cancer</td>
<td>21%</td>
<td>37%</td>
<td>42%</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

In this study, we extend our earlier observations on using bile acid-induced AI as a potential biomarker. Whereas patients with a history of colon cancer had histologically and clinically normal-appearing colonic epithelium at 20 cm from the anal verge, there was a statistically significant difference in AIs between them and normal individuals, with 47% of individuals with a history of cancer having AIs < [AI_{nor} - 2 SDs] or lower than 97.7% of normal individuals. Such apoptosis resistance would presumably prevent cellular death and would permit the accumulation of cells with DNA damage. Although the number of cases is small, follow-up is planned to determine whether the subjects with low AI have a different clinical outcome than the others. In addition, the utility of this assay could be tested as a part of future large multicenter polyp or cancer recurrence trials to access larger numbers of patients.

The majority of polyp patients had AIs similar to those of the normal individuals, which is consistent with the clinical observation that not all patients with polyps progress to cancer. Thus, it would be of interest to follow the three patients with polyps who had low AIs. Interestingly, two patients had villous polyps and one patient had a large polyp, factors that are clinically associated with greater cancer risk.

Apoptosis has a major role in the elimination of DNA damaged cells (26). For example, Potten et al. (27) showed that damage in mouse colon crypts caused by any of four mutagenic/carcinogenic alkylating agents increased the apoptosis frequency from about 0.3% to 10–20% at 5–8 h after injection. Park et al. (28) showed that upon replication, surviving mutagen-damaged cells may lead to the emergence of crypts that are composed wholly of cells with a different, mutated phenotype. Thus, apoptotic deletion of cells with DNA damage prevents their replication into an abnormal clone of cells.

Bile acids are promoters of colon carcinogenesis (29, 30) and are known to cause DNA damage (31, 32). The typical Western high-fat, low-fiber diet causes a relatively high exposure of the colonic epithelium to bile acids. Feeding mice such a Western-type diet causes significantly increased frequencies of apoptosis at all levels of their colon crypts during the first 15 weeks after the diet is initiated (33). We recently proposed a role for apoptosis in colon carcinogenesis, based on the results of our studies (16, 17), which are also consistent with the study of Bedi et al. (20). Our provisional explanation of these results is given in the flow chart (Fig. 5) showing a hypothesized sequence of bile salt-induced events in colon carcinogenesis. When high concentrations of bile salts are present in the colon (Fig. 5, level

![Image](image-url)
11. Rafter, J. J., Child, P., Anderson, A. M., Alder, R., Eng, V., and Bruce, W. R. Cellular toxicity of fecal water (up to about 0.68 mM ) may be contributed by LCA, which is even more cytotoxic (14). Van Munster et al. (10) showed that mixtures of cytotoxic bile acids are additive in their cytotoxic effects on colon-derived cells. Thus, the 1.0 mM level of NaDOC that we used in our in vitro assay may have a cytotoxic effect roughly comparable to the additive bile acid cytotoxic concentration present in the colon after the consumption of a high-fat meal.

Our assay shows an association of resistance to bile acid-induced apoptosis (measured at 20 cm from the anal verge) with colon cancer risk. Thus, it may prove to be useful as a biomarker of cancer risk, either in individuals or in populations. Additional quality control studies are needed to assess changes within an individual in assays done at different times. Finally, clinical follow-up is needed, especially of those subjects displaying low Al, to correlate this with the development of serious pathology such as large villous polyps or cancer.

REFERENCES
A Bile Acid-induced Apoptosis Assay for Colon Cancer Risk and Associated Quality Control Studies

Carol Bernstein, Harris Bernstein, Harinder Garewal, et al.


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