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

Carol Bernstein, Harris Bernstein, Harinder Garewal, Pat Dinning, Rawah Jabi, Richard E. Sampliner, Margaret K. McCuskey, Madhulita Panda, Denise J. Roe, Lawrence L’Heureux, and Claire Payne

Department of Microbiology and Immunology [C. B., H. B., R. J., L. L. C. P.], Department of Medicine [H. G. P. D., R. E. S.], Cancer Center [M. P., D. J. R.], and Department of Cell Biology and Anatomy [M. K. M.], College of Medicine, University of Arizona, Tucson, Arizona 85724, and Department of Internal Medicine, Sections of Hematology/Oncology [H. G.] and Gastroenterology [H. G., R. E. S.], Tucson, Arizona 85723

INTRODUCTION

Bile acids are important in the etiology of colorectal cancer. Bile acids induce apoptosis in colonic goblet cells at concentrations comparable to those found in fecal water after high-fat meals. Preliminary evidence indicates 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 intraindividual 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 intraindividual variation compared to interindividual 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.

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 Arizona.

Thus, this assay may prove useful as a biomarker of colon cancer risk.
ARIZONA, and informed consent was obtained from each subject. In all cases, biopsies were taken from a site 20 cm from the anal verge. To evaluate site-to-site variability, both within adjacent regions of the colon and in different anatomical regions, additional sets of biopsies were obtained from the cecum and at 40 cm from the anal verge in subsets of subjects.

Quantitation 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 m l-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 m phosphate buffer.

Processing of Tissue. For processing, the epoxy embedding procedure described by Payne et al. (22) was followed. Briefly, the tissue was postosmicated, 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 nucleus were scored. 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, quartile-quartile 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 [AI norm – 2 SDs]. Using values below [AI norm – 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 [AI norm – 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 < [AI norm – 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 < [AI norm – 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. Indicates a history of villous adenoma(s), and the designations TV of tubular adenoma(s); T a history of tubular adenomas or tubulovillous adenomas. A V average AIs at one or more locations below [AI nor categories of a history of villous adenomas or colon cancer had below [AI nor tubulovillous adenomas had one or more average AI(s) at a location in the colon). Table 1 shows that whereas 14% of normal individuals and 25% of individuals with a history of tubular or tubulovillous adenomas or colon cancer patients, we were still able to detect significant decreases in the AI of cancer patients compared with that of the normal individuals. This was because of the large magnitude of the observed decreases in the AIs of cancer patients.

The Location of the Neoplasm Compared to the Location of the Low Observed AI. There were six individuals with neoplasm(s) (three individuals with adenoma(s) and three individuals with carcinoma) who had AIs in one or more of three regions < [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 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 the 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 1. Frequency of individuals with one or more average AIs < [AI nor 2 SDs]. Values at all three locations (at 20 cm, 40 cm, and the cecum) are considered, in contrast to Fig. 1, in which only 20 cm values are shown.

<table>
<thead>
<tr>
<th>Colon history</th>
<th>No. of individuals examined</th>
<th>Individuals with one or more average AI &lt; [AI nor 2 SDs]</th>
<th>Percentage of individuals with one or more average AI &lt; [AI nor 2 SDs]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colon</td>
<td>7</td>
<td>1</td>
<td>14%</td>
</tr>
<tr>
<td>Tubular or tubulovillous adenomas</td>
<td>8</td>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Villous adenomas or colon cancer</td>
<td>6</td>
<td>4</td>
<td>67%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<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>
</tr>
<tr>
<td>History of adenomas</td>
<td>12%</td>
<td>63%</td>
<td>25%</td>
</tr>
<tr>
<td>History of cancer</td>
<td>21%</td>
<td>37%</td>
<td>42%</td>
</tr>
</tbody>
</table>

Incubation in the Absence of NaDOC. For all patients, the incubation of biopsies in the absence of NaDOC for 3 h also induced some goblet cells to undergo apoptosis. However, in the absence of NaDOC, there were no significant differences in the mean AIs between normal individuals, cancer patients, and polypos patients (data not shown).

Apoptosis at Different Colon Sites. AIs were obtained from biopsies taken in the cecum and at 40 and 20 cm from the anal verge. Table 1 shows that whereas 14% of normal individuals and 25% of individuals with a history of tubular or tubulovillous adenomas had one or more average AI(s) at a location below [AI nor 2 SDs], 67% of individuals within the higher risk categories of a history of villous adenomas or colon cancer had average AIs at one or more locations below [AI nor 2 SDs].

Variability between Biopsies and Its Implications. Fig. 2 shows the individual AIs for the 15 patients who had duplicate sets of biopsies at 20 and 40 cm and at the cecum. The mean difference between the pairs of AIs was as follows: (a) at 20 cm, 6.2 ± 6.0% SD; (b) at 40 cm, 8.9 ± 8.9% SD; and (c) at the cecum, 11.0 ± 6.2% SD.

To assess potential sources of variation between individuals, between locations within the same individual, and between biopsies within the same location, a components of variance analysis was performed using data from the 21 patients with either single or duplicate biopsies at the three sites. If there were substantial interindividual variability relative to intraindividual variability (due to location and biopsies), this would suggest a greater homogeneity of AI values within each individual. If this were true, this would be the ideal for surrogate biological end point biomarkers. On the other hand, if greater intraindividual variability were found, this would reflect physiological differences from area to area.

In our data, among normal individuals, the largest source of variation was between subjects (Table 2). That is, within a single normal individual, the AI was relatively constant, both with respect to location (at 20 cm, 40 cm, or the cecum) and with respect to adjacent biopsies. In contrast, for individuals with colonic adenomas or cancer, location and biopsy were greater sources of variation (Table 2). This suggests that for the nonnormal subjects, variation within an individual was greater than variation between individuals, which is consistent with the “patchiness” of the susceptibility of regions of the colon to bile acid-induced apoptosis (a patchy field effect).

However, despite the patchiness found in adenoma and cancer patients, we were still able to detect significant decreases in the AI of cancer patients compared with that of the normal individuals. This was because of the large magnitude of the observed decreases in the AIs of cancer patients.
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 \( < \text{AI}_{\text{norm}} - 2 \text{SDs} \) 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 poly 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 0.33% 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
1), some cells will receive damage (perhaps DNA damage) that remains unrepaired. Apoptosis serves to protect the colon from cancer by deleting cells with unrepaired DNA damage (34), as indicated in Fig. 5, level 2. Thus, a consistent high-fat diet causing high concentrations of bile acids in the colon can result in high frequencies of cell death due to apoptosis. As noted in the “Introduction,” when cells are cleared, there is rapid epithelial restitution by the migration of cells from neighboring regions of the epithelium (18, 19). Over time, this may select for the survival of mutant cells or cells with a survival phenotype that are resistant to the induction of apoptosis (Fig. 5, level 3). Over a period of years of consumption of a high-fat diet, apoptosis-resistant mutant goblet cells could repopulate the colonic mucosa. Perhaps consistent with this, after the first 15 weeks of a high-fat diet, the mice in the study of Risio et al. (33) no longer had greatly increased levels of apoptosis. Then, if dietary carcinogens are ingested, the protective apoptosis pathway would be deficient (Fig. 5, level 4). An increasing accumulation of clones of apoptosis-resistant cells would result in a fertile field for cancer development (16, 17). Consistent with this, in mice fed a high-fat Western-type diet, cells of the colonic epithelium had much increased frequencies of atypical nuclei at later ages (33). DNA damage would remain in cells within this field without triggering apoptosis. If the cells with unrepaired DNA damage were to replicate, mutations would tend to arise at the sites of damage because of replication errors at these sites. Some of these mutations could then lead to cancer (Fig. 5, level 5).

Our finding of large contributions of variation in biopsies or location relative to intersubject variability for nonnormal subjects in the present study suggests that there is patchiness of the susceptibility to bile acid-induced apoptosis within the colon. This would indicate that different regions of the colon may reach the stage indicated in Fig. 5, level 5, whereas other regions are less affected. We note that our results are based on small sample sizes; thus, there is substantial variability in the variance components estimates. However, the larger intraindividual variability observed in subjects with neoplasia indicates that multiple biopsies must be obtained for reliable results.

The concentrations of DOC found in the fecal water of individuals consuming a high-fat diet range up to 0.78 nm (13). However, DOC constitutes only about 40% of the bile acid bile. An additional 25–50% of the bile acids in the fecal water (up to about 0.68 nm) 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 AIs, 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.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/10/2353

Cited articles
This article cites 30 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/10/2353.full.html#ref-list-1

Citing articles
This article has been cited by 18 HighWire-hosted articles. Access the articles at:
/content/59/10/2353.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.