Validation of the Galactose Oxidase-Schiff's Reagent Sequence for Early Detection and Prognosis in Human Colorectal Adenocarcinoma

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ABSTRACT

Based on the multistage and multifocal nature of colorectal carcinogenesis, it is likely that reduction of cancer mortality through early detection and identification of new prognostic markers is an attainable goal. Well-documented changes occur in mucin glycoconjugates during neoplastic progression in the colon, and the nonneoplastic colonic mucosa in colon cancer patients is morphologically and histochimically abnormal. In this retrospective study, 152 archival colorectal tissues from 49 patients were studied for changes in mucin secretions as detected by the galactose oxidase-Schiff's (GOS) sequence. Intensity of the stain was evaluated in histological sections by semiquantitative analysis, and the area percentage of epithelium stained was quantified by image cytometry. The correlation between gender or tumor size, location and reactivity with peanut agglutinin and quantitative expression of GOS-reactive mucins was determined as well as intratumor and interindividual variability.

Reactivity with GOS: (a) decreased during neoplastic progression and malignant conversion in the neoplasm; (b) increased in the normal colonic mucosa of patients with progressively more advanced disease; and (c) was of prognostic significance for patient survival or recurrence both in the normal colon of cancer patients and in invasive neoplasms. These data are consistent with the conclusion that GOS reactivity in the normal colonic mucosa is a dosimeter of exposure to environmental/lifestyle colorectal carcinogens rather than a marker for an oncodevelopmental cancer-associated antigen.

INTRODUCTION

CRC is the second leading cause of death from cancer in the United States population with 54,900 deaths and 131,200 new cases anticipated during 1997 (1, 2). Both inheritance and environmental/lifestyle factors (i.e., carcinogens) have a role in CRC genesis (3, 4).

Most invasive CRCs develop from intraepithelial neoplasms (adenomatous polyps); however, less than 10% of intraepithelial neoplasms show histologically malignant changes, and the adenoma to carcinoma sequence may take 10 years (5). Nearly 50% of patients with one adenomatous polyp develop additional polyps, and multiple polyps are a risk factor for synchronous and future CRC (5). Neoplastic progression is associated with mutational activation of oncogenes and loss of function of tumor suppressor genes (6–8). Based on the multistage and multifocal nature of CRC, it is likely that reduction of CRC mortality through early detection and identification of new prognostic markers is an attainable goal.

Secondary prevention of CRC through early detection requires an effective screening test for asymptomatic people. Such a test must be reproducible and allow detection of people with CRC (i.e., have a high degree of sensitivity—few false negatives) and identification of people without disease (i.e., have a high degree of specificity—few false positives). In 1988, Shamsuddin and Elsayed (9, 10) described a simple, rapid, inexpensive, and relatively noninvasive test that detects the sialic acid-free Gal-GalNac residue in rectal mucous of patients with CRC using GOS. The rectal mucous test is based on well-documented changes in mucin glycoconjugates that occur during neoplastic progression and malignant conversion in the colon (11–21) and on the observation that nonneoplastic colonic mucosa in CRC patients is morphologically and histochemically abnormal (12, 15, 17, 19–25). Because a high sensitivity (80–100%; Refs. 24 and 26–28) and a high specificity (>92%; Ref. 24) are reported, the test has the potential for use in screening and the secondary prevention of CRC through early detection. A direct correlation between expression of Gal-GalNac (detected by the GOS sequence) in a histological section of a formalin-fixed, paraffin-embedded colorectal neoplasm and the presence

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1 The abbreviations used are: CRC, colorectal cancer; Gal-GalNac, α-galactose-β-[1→3]-N-acetyl-α-galactosamine; GOS, galactose oxidase-Schiff's reagent; PNA, peanut agglutinin; TNM, tumor/lymph node metastasis/distant metastasis; A%, areal percentage; CI, confidence interval; CV, coefficient of variation.
of Gal-GalNAc in the rectal mucous of the patient is reported (24).

A major gap in our knowledge regarding the usefulness of the Gal-GalNAc marker for screening is an understanding of the stage in neoplastic progression of an intraepithelial colorectal neoplasm at which this marker is expressed. It is not known if Gal-GalNAc is found in the mucous of patients with premalignant polyps or if there is tissue expression of Gal-GalNAc in premalignant polyps. Histological expression of Gal-GalNAc has been reported for only five polyps. Two of these polyps had carcinoma in situ and three had atypia or dysplasia (24).

Here we tested the hypothesis that tissue expression of Gal-GalNAc increases during neoplastic progression and malignant conversion in human colorectal epithelium. Histological expression of Gal-GalNAc was detected with GOS and quantified by image cytometry in archival tissues representing the adenoma to carcinoma sequence. GOS reactivity was compared to the binding of PNA, a lectin known to specifically bind adenoma to carcinoma sequence. GOS reactivity was compared to expression of Gal-GalNAc was detected with GOS and quantified. Gal-GalNAc increases during neoplastic progression and malignant conversion in human colorectal tissues, thereby validating Gal-GalNAc as a very early marker for malignant potential in intraepithelial neoplasms. The correlation between gender or tumor size, location, and reactivity with PNA and quantitative expression of GOS-reactive saccharides was determined as well as intratumor and interindividual variability.

Contrary to our expectations, GOS reactivity decreased with neoplastic progression and malignant conversion in the neoplasm and increased in the normal mucosa of patients with progressively more advanced disease. More importantly, GOS reactivity was of prognostic significance both in the normal colon of CRC patients and in invasive neoplasms. These data suggest that GOS reactivity in the normal colon is a dosimeter of exposure to carcinogens or cytotoxicants rather than an oncodevelopmental cancer-associated antigen.

**MATERIALS AND METHODS**

**Tissues.** Archival, formalin-fixed, paraffin-embedded surgical specimens of normal colon, colorectal adenomas, and carcinomas were obtained from St. Elizabeth Medical Center (Covington/Edgewood, Kentucky). Northern Kentucky is a geographical area where there is a higher than anticipated incidence of CRC, possibly attributable to Ohio River drinking water sources (29, 30). The study was begun following approval of the Institutional Review Board of St. Elizabeth Medical Center. Specimens were from 27 female patients and 22 male patients. The average age of female patients (63.4 ± 3.2 years) was similar to that of male patients (60.6 ± 2.4 years). CRC patients did not have known predisposing conditions. Histologically normal colon from surgical margins of CRC patients and distant biopsies were analyzed as well as histologically normal colon from patients with hyperplastic polyps or adenomas. The 152 specimens of neoplastic and nonneoplastic tissues were fixed in 10% neutral buffered formalin for 24 h and routinely processed into low-melting-point paraffin. Serial 5-μm tissue sections were prepared for histochemistry.

**Pathological Staging.** The histopathology of each specimen was studied on a H&E-stained tissue section by a board-certified pathologist (H. W. C.). Tissue Consultation Reports from the Department of Laboratory Medicine, St. Elizabeth Medical Center, were used to confirm patient TNM status and to determine tumor size (31). According to this classification, stage 0 represents carcinoma in situ; stage I represents tumors that invade the submucosa or the muscularis propria (and corresponds to Dukes’ stage A); stage II represents tumors that invade through the muscularis propria into the subserosa or nonperitonealized pericolic or perirectal tissues or tumors that perforate the visceral peritoneum or directly invade other organs or structures (and corresponds to Dukes’ stage B); stage III represents any tumor with regional lymph node metastases (and corresponds to Dukes’ stage C). None of the tissues were from stage IV (tumors with confirmed distant metastases). Clinical follow-up was obtained from the Tri-State Tumor Registry database, which was given to Wood Hudson Cancer Research Laboratory by St. Elizabeth Medical Center. Five-year survival data were available for 131 specimens, and recurrence data were available for 104 specimens. The “readers” of the pathological specimens were blinded to the clinical status of the patients.

**GOS Reagent Stain.** The GOS procedure was performed as previously developed and applied (24, 32–34). The 5-μm paraffin-embedded sections were deparaffinized, rehydrated through a graded series of alcohols, and rinsed with 0.01 M potassium phosphate buffer (pH 7.0) for 10 min. Sections were incubated with t-galactose oxidase type V (Sigma Aldrich, St. Louis, MO), which was reconstituted in 0.01 M PBS (5 units/ml), and sections were incubated for 18 h at room temperature. After washing for 10 min in deionized distilled water, galactose oxidase activity was visualized by reacting with Schiff’s reagent containing pararosaniline HCl, 1% w/v, sodium bisulfate 4% w/v in HCl, 0.25 M (Accustain Schiff’s Reagent; Sigma Aldrich) for 15 min, followed by running tap water for 10 min. Sections for semiquantitative evaluation were lightly counterstained with hematoxylin and rinsed with saturated lithium carbonate for 5 s. Some adjacent sections were counterstained with 0.2 g/liter fast green in deionized water and rinsed with 1% acetic acid (35). Sections used for quantification by computerized image cytometry were not counterstained. The stained sections were dehydrated, cleared, and mounted. Sections incubated with PBS without galactose oxidase, followed by Schiff’s reagent, served as negative controls (24). The normal mucosa from one of two patients with colloid carcinomas was used as a positive control for stain reproducibility in each staining run. Sections were evaluated semiquantitatively by three observers for the presence of stain and staining intensity (0, +, ++, and +++).

**PNA Histochemistry.** Tissue sections were deparaffinized in xylene and hydrated through a graded series of alcohols to distilled water. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide in methanol. Nonspecific binding was blocked by incubating the sections, at room temperature, with normal goat serum and an avidin blocking kit (Vector Laboratories, Burlingame, CA). Sections were overlaid with biotinylated *Arachis hypogaea* (PNA; 50 μg/ml) in a humidified chamber at 27°C for 45–60 min. Binding was developed with the Vectastain ABC reagent (Vector) and diamo-
nobenzidine as the peroxidase substrate. The specificity of binding was tested by its preincubation with a 0.1 M solution of N-acetyl-D-galactosamine or D-galactose (Sigma Aldrich).

Quantification of A% of Tissue Stained by GOS with Image Cytometry. An IBAS image cytometry system (Auto-Cyte, Inc., Elon College, NC) was used to quantify the A% of epithelium and mucous secretions that were stained by GOS. The optical system consisted of a Leitz Ortholux II microscope with a ×100 oil immersion lens. Light from a voltage-regulated halogen light source (22.5 V; Ludl Electronic Products Ltd., Hawthorne, NY) was passed through an ISA monochromater (Instruments SA, Garden City, NY). Monochromatic light at 540 nm (the absorption maximum for the stain) was used to optimize detection of the stain. The microscopic image was captured by a MTI-DAGE model 68 camera with a Neuvicon tube, and the video image was digitized by the image cytometry system (36).

The threshold for positive staining was determined for each histological slide. Image cytometry permitted quantification of GOS reactive area as a percentage of the total area viewed in a microscopic field. A% positive staining was calculated by the image cytometry system from:

\[
A% = \frac{\text{Number of pixels in the threshold range}}{\text{Number of pixels in the video image}} \times 100
\]

A% positive staining was measured in each of 25 fields per slide. The mean A% positive staining (and SE) for each slide was calculated by the IBAS statistical analysis program. The 25 microscopic fields analyzed were chosen randomly from the epithelial component of the tissue excluding lamina propria. More than 95% of each field contained epithelium (21). The A% of epithelium stained in the section of normal mucosa from a patient with a colloid carcinoma that had been used as the positive control for the staining protocol was determined daily.

Statistical Analysis. Tissues were grouped according to histopathology and TNM pathological stage, colonic region (proximal colon: cecum and ascending and transverse segments; or distal colon: splenic flexure, descending, and sigmoid and rectum segments), gender and age of the patient, and whether the patient had CRC. ANOVA was used to determine differences between these covariates and the continuous outcome variable, A% of epithelium stained by GOS. The usual assumptions of ANOVA (normally distributed data with equal variance) were checked. Because the data exhibited skewness, all values were log transformed. The assumptions were then satisfied. Hence geometric means and 95% CIs are reported. With factors having multiple levels (e.g., tissue groups), Tukey multiple comparisons were performed. Interactions between covariates were also considered. Because several tissue samples could have come from the same tumor and because several tissues could have come from the same patient, mixed linear models were also considered to take into account the between and within patient and slide variation. Usual t tests were used when comparing covariates on small subsets of tissues. Sampling error was evaluated in multiple samples from the same neoplasm. The CV between multiple samples from the same tumor or patient was calculated from:

\[
CV = \frac{SD}{\text{Mean}} \times 100
\]

Correlations were computed to assess the relationship between the GOS stain and markers quantified previously (21, 25). Clinical follow-up information was available for 5 years for most patients.

RESULTS

Determination of the Stage in the Progression of Adenomas to Carcinomas at which GOS Stain Becomes Detectable: Semiquantitative Evaluation of Stain Intensity. Histological staining characteristics include intensity of stain and area of tissue stained. Intensity of stain was evaluated in microscopic images without instrumentation, whereas the area of tissue stained was quantified by computer-assisted image cytometry. The semiquantitative evaluation of the stain intensity in GOS-stained and hematoxylin or fast green counterstained sections was made by three observers. A board certified pathologist (H. W. C.) confirmed the histopathological diagnosis in each section. Stain intensity varied from no apparent stain (0), to light pink (+), to very pink (++), to intense magenta (+++). Sections not pretreated with galactose oxidase were negative. The prevalence of staining by the GOS sequence and stain intensity at the histological and clinical stages of CRC development and progression are summarized in Table 1.

Overall, stain intensity was greatest in the normal colon from patients with invasive or metastatic CRC (Fig. 1; Table 1), but most normal mucosa from patients with intraepithelial neoplasms (i.e., tubular adenomas, tubulo-villous adenomas, villous adenomas, or carcinoma in situ) also had increased staining compared to patients without CRC. Reactivity with the GOS sequence was found in 77.8% of the specimens from normal mucosa of patients with intraepithelial neoplasms (Table 1, Normal B), with 83% (11 of 12) of patients having one or more positive specimens. The GOS sequence stained the normal mucosa of all CRC patients (17 of 17) in one or more specimens from the adjacent ("transitional") mucosa, from the surgical margins, or from distant biopsy sites. Only 1 of 41 specimens from CRC patients (2.4%) was considered negative, with 97.6% of the specimens demonstrating reactivity (Table 1, Normal C; Fig. 1, A and G). These data confirmed that the GOS stain sequence in normal colon has a high degree of sensitivity (98–100%) for detecting patients with CRC and suggested that most patients with intraepithelial colorectal neoplasms (83%) can also be detected by the technique.

"Normal" colonic specimens from patients without colorectal neoplasms were from patients biopsied because of clinical symptoms (chronic diarrhea and abdominal pain). Of these patients, 6 of 10 had nonspecific inflammation or ulcerative colitis, but none of the patients developed CRC for 5 years (Table 1). Of the five positive specimens, four were from patients with nonspecific inflammation or ulcerative colitis (Fig. 1E). The fifth specimen stained intensely and was from a patient that subsequently had two 5–6-cm thyroid adenomas surgically removed. Thus in this limited series, the specificity of the GOS stain sequence for identification of patients without CRC was good (i.e., the stain was negative) when ulcerative colitis or nonspecific inflammation was not present. Although the normal mucosa of both patients with ulcerative colitis and two of four patients with nonspecific inflammation stained positively with
the GOS sequence, the average stain intensity was significantly less in the normal mucosa from patients without colorectal neoplasms (including those with ulcerative colitis and nonspecific inflammation) than in the normal mucosa from patients with CRC (Table 1, Normal A versus Normal C; P < 0.001; Fig. 1) or patients with intraepithelial neoplasms (Table 1, Normal A versus Normal B; P < 0.05; Fig. 1). Staining by the GOS sequence occurred in 86% of the hyperplastic polyps but with weak intensity (Table 1). These results in nonneoplastic colonic mucosa suggested that increased staining by GOS indicated an adaptive response.

Staining by the GOS sequence was found in 77% of the intraepithelial neoplasms from patients without CRC and in 100% of the intraepithelial neoplasms in patients with CRC. Stain intensity was also significantly higher in the intraepithelial neoplasms from patients with synchronous CRCs (2.00 ± 0.21 versus 1.39 ± 0.19; P < 0.05). Of 41 specimens of intraepithelial neoplasms, 83% were stained by the GOS sequence (Table 1). Staining was found in adenomas without dysplasia or carcinoma in situ (Fig. 1, B and D). Staining intensity was significantly higher in intraepithelial neoplasms than in the normal mucosa of patients without colorectal neoplasms (P < 0.05) and lower than in the normal mucosa of patients with CRC (Table 1).

The semiquantitative data failed to confirm the hypothesis that tissue GOS reactivity would increase during neoplastic progression and malignant conversion in human colorectal neoplasms. In contrast to the progressive increase in staining in normal colon from patients with increasingly advanced disease, the prevalence of stain did not increase significantly in invasive and metastatic CRCs relative to intraepithelial neoplasms, and there was a downward trend in staining intensity (Table 1). Some CRC patients had positive stain with GOS in the normal mucosa, but their CRC did not stain by the GOS sequence (Fig. 1, G and H). Whereas 98% of the normal specimens from CRC patients had reactivity with the GOS sequence, only 86% of the invasive neoplasms and 84% of the metastatic neoplasms were stained by the technique (Table 1). These data confirm those of Xu et al. (24), who found 81% of the CRCs examined were stained by GOS. Xu et al. (24) also found that staining was most intense in mucinous and signet-ring cell CRC and that well to moderately differentiated CRCs exhibited less frequent reaction or weak (+) staining. In the present study, all of the invasive neoplasms and 91% of the metastatic neoplasms were moderately differentiated, and none were mucinous or signet-ring cell CRC. Stain intensity was significantly less in invasive or metastatic neoplasms than in intraepithelial neoplasms in patients with CRC (2.00 ± 0.21; P < 0.05). Stain intensity in invasive or metastatic neoplasms was significantly less than in normal mucosa from CRC patients (Table 1; Fig. 1).

Quantification of the Area of Tissue Stained by the GOS Sequence: Reproducibility and Intra- and Interindividual Variability. Quantification of the area of tissue stained by the GOS sequence was based on computer-assisted image cytometry. Although measuring another parameter of histological staining, these quantitative data were consistent with the semiquantitative evaluation of intensity of stain. The reaction product absorbed maximally at 540 nm. Therefore, to increase the specificity of stain detection, the A% of tissue stained (i.e., the ratio of area of stained tissue to total tissue area × 100) was determined at this wavelength in sections that had not been counterstained. When stain was quantified at 540 nm in 13 tissues apparently negative (0) by semiquantitative analysis, the A% of stained tissue was 3.2 ± 0.47%.

The normal mucosa from one of two patients with colloid carcinomas was used as a positive control for GOS stain reproducibility (Fig. 1A). The CV of repeat measurements of the same field by image cytometry using the IBAS system was 1.66% (36, 38). Reproducibility of the GOS stain and the image cytometry procedure was tested by three to eight repetitive stains and image analyses of four tissue blocks. Average CV of these analyses was 15.37%.

Intraindividual variability was tested by comparison of three to six blocks from each of 11 individual tumors or normal mucosa from individual patients. The average CV of analyses of multiple blocks of tissue from the same patient or tumor was 53.61%. Interindividual variability was greater (CV = 74.16%) than intraindividual variability. Thus, biological variability was
Fig. 1 Staining by the GOS sequence in colonic tissues (no counterstain). Note intense magenta stain (×1050): A, the normal colonic mucosa of a patient with a colloid carcinoma that was used as a positive control for staining and quality control in image cytometry; B, a tubular adenoma from a patient that does not have CRC; C, normal mucosa from a patient without cancer but with a villous adenoma; D, villous adenoma from the patient seen in C; E, colonic mucosa from a patient with ulcerative colitis; F, villous portion of a neoplasm that is stage I CRC from a patient that did not survive 5 years; G, normal mucosa from a patient with stage II CRC; H, carcinoma (clinically stage II and measuring 6 × 4 × 0.5 cm) without reactivity with the GOS sequence from same patient as seen in G. These photomicrographs were made with a ×40 apochromatic objective; however, image cytometry used a ×100 oil immersion objective so that more than 95% of the image analyzed was epithelium.
Tissue Group

<table>
<thead>
<tr>
<th>Tissue Group</th>
<th>A%</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal w/o Cancer</td>
<td>4.38</td>
<td></td>
</tr>
<tr>
<td>Normal w/ Cancer</td>
<td>16.01</td>
<td>1.74</td>
</tr>
<tr>
<td>Intraepi/Neoplasms</td>
<td>9.69</td>
<td></td>
</tr>
<tr>
<td>Invasive Neoplasms</td>
<td>7.84</td>
<td></td>
</tr>
<tr>
<td>Metastatic Neoplasms</td>
<td>4.65</td>
<td></td>
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</table>

Area % Epithelium with GOS Reactivity (Geometric Mean)

Fig. 2  A% of epithelium showing reactivity with the GOS sequence during neoplastic progression in the colon (Geometric Mean). The threshold for positive staining, as detected at 540 nm, was determined for each histological slide. Image cytometry permitted quantification of the GOS-reactive area as a percentage of the total area viewed in a microscopic field using a ×100 oil immersion objective. A% positive staining was calculated by the image cytometry system from the equation: [(number of pixels in the threshold range/number of pixels in the video image) × 100]. A% positive staining was measured in each of 25 fields/slide. The mean A% positive staining (and SE) for each slide was calculated by the IBAS statistical analysis program. The 25 microscopic fields analyzed were chosen randomly from the epithelial component of the tissue excluding lamina propria. The A% of epithelium stained in the section of normal mucosa from a patient with a colloid carcinoma that had been used as the positive control for the staining protocol was determined daily (see Fig. 1A). Numbers in parentheses, the number of tissues evaluated. Tissues included normal from patients without or with CRC; intraepithelial neoplasms (tubular, tubulo-villous and villous adenomas, and carcinoma in situ); invasive neoplasms (stages I and II); and metastatic neoplasms (stage III and lymph node metastases). All of the invasive neoplasms were moderately differentiated; 91% of the metastatic neoplasms were moderately differentiated, and 9% were poorly differentiated. Normal tissues from patients without CRC include hyperplastic polyps and mucosa from patients with nonspecific inflammation, ulcerative colitis, and intraepithelial neoplasms. *, significantly different from normal mucosa without CRC, invasive neoplasms, or metastatic neoplasms (see text).

3.5–5-fold greater than variability due to staining and image cytometry procedures. Therefore, for purposes of analysis, individual samples rather than the average of multiple samples from the same patient or tumor were evaluated.

Heterogeneity of staining with GOS from cell to cell is evident even in the most intensely stained specimens (Fig. 1), indicating heterogeneity in the terminal nonreducing saccharides of the oligosaccharide portion of mucins detected by GOS. This intratumor variability found with the GOS sequence is not unique to this marker. Similar intratumor and intraindividual variability was found in these specimens in studies of lectin reactivity (21). Hakomori (39) found a distinctive pattern of antigen distribution in each serial tumor section that was sequentially dissected and stained with five antibodies to tumor antigens and periodic acid-Schiff’s reagent.

Quantification of Area of Tissue Stained by the GOS Sequence in Normal Colonic Mucosa and During Neoplastic Progression: Effects of Gender and Location. The A% of tissue stained by GOS was significantly greater in the normal colonic mucosa of patients with CRC than in patients without CRC (Fig. 2). Hyperplastic polyps did not differ significantly from normal colonic mucosa from patients without CRC but did differ significantly from the normal mucosa from patients with CRC. Therefore, hyperplastic polyps and normal mucosa from patients without CRC are grouped together. A one-way ANOVA was performed based on tissue group, which resulted in an F of 5.69 and a P of 0.0003 for the log-transformed data and an F of 6.21 and a P of 0.0001 for the nontransformed data. Tukey multiple comparison of the five tissue groups given in Fig. 2 found that the A% of epithelium showing reactivity with GOS in the normal epithelium from CRC patients was significantly different from either patients without CRC or metastatic tumors but not significantly different from intraepithelial neoplasms. When tissue groups were considered individually, the A% of tissue with GOS reactivity in the normal colon of CRC patients was significantly greater than either intraepithelial neoplasms, invasive neoplasms, or metastatic neoplasms. Regression analysis indicated that there was a significant decreasing trend in A% of epithelium with GOS reactivity in normal mucosa from CRC patients > intraepithelial neoplasms > invasive neoplasms > metastatic neoplasms (P = 0.0001). The A% of epithelium with GOS reactivity in metastatic CRC was not significantly different from the A% of epithelium with GOS reactivity in normal colon from patients without CRC. The A% of epithelium with GOS reactivity in normal colon from patients without CRC was significantly lower than the GOS reactivity in intraepithelial neoplasms (Fig. 2).
Consistent with the semiquantitative analysis of stain intensity (Table 1), the A% of normal mucosa stained by GOS in patients with intraepithelial neoplasms but without invasive or metastatic neoplasms was intermediate between patients without any neoplasm and patients with CRC (geometric means, 3.66, 5.54, and 16.01%, P for trend = 0.0001).

In an ANOVA with tissue group and gender as main effects, we found no interaction; there was a significant difference between tissue groups (F_{4,142} = 8.91; P ≤ 0.0001), and there was no significant difference between genders (F_{1,142} = 0.04; P = 0.8386).

The A% of tissue stained by GOS was not significantly higher in intraepithelial neoplasms from patients with synchronous CRC compared to patients without CRC (P = 0.0942). Intraepithelial neoplasms from patients with and without synchronous CRC were similar in size and also in all other biomarkers examined, including lectin reactivity, nuclear size, and proliferating cell nuclear antigen expression (21, 25).

In an ANOVA with tissue group and region as main effects, we found no interaction; there was a significant difference between tissue groups (F_{4,142} = 8.02; P ≤ 0.0001) and a significant difference between regions (F_{1,142} = 13.08; P = 0.0004; Table 2). As in the case with other differentiation or cell proliferation markers (21, 25), intraepithelial neoplasms demonstrated a regional difference in GOS reactivity (Table 2). The A% of tissue stained by GOS was significantly higher in intraepithelial neoplasms in the proximal colon compared to the distal colon (Table 2; P = 0.0199). Stage III CRC in the proximal colon and their lymph node metastases also had significantly greater GOS reactivity (Table 2; P = 0.0224). Although hereditary colon CRC syndromes have a proximal colon prevalence (40), to the best of our knowledge, all patients in this study had CRC without hereditary colon CRC syndromes.

**Area of Colorectal Neoplasm Stained by the GOS Sequence Is Negatively Correlated with Neoplasm Size.** The size of the neoplasm was evaluated in relation to A% of tissue stained by the GOS sequence. Intraepithelial neoplasms were significantly smaller (P = 0.0001) than invasive or metastatic neoplasms (1.48 ± 0.22 cm versus 4.23 ± 0.32 cm versus 3.96 ± 0.27 cm). Metastatic neoplasms from the distal colon were significantly larger than those from the proximal colon (4.47 ± 0.31 cm versus 3.11 ± 0.38 cm; P = 0.0130).

Consistent with the data in Figs. 1 and 2, larger CRCs had less staining by GOS. The size of invasive colorectal neoplasms was negatively correlated with A% of tissue stained by GOS (r = -0.51679; P = 0.0337). When intraepithelial, invasive, and metastatic neoplasms were considered, GOS reactivity remained negatively correlated with neoplasm size (P = 0.0170). Staining by GOS did not differ significantly between metastatic neoplasms and primary stage III CRCs.

**Prognostic Significance of the Area of Tissue Stained by the GOS Sequence.** The prognostic significance of GOS staining in the normal mucosa of CRC patients was tested by evaluation of 5-year survival and tumor recurrence data from the Tri-State Tumor Registry (Table 3). Patients who survived were followed 58 ± 4 months (48–64 months). Patients who died lived 19 ± 16 months (1–43 months).

In an ANOVA with tissue group and survival as main effects, there was no significant interaction between tissue group and survival, but there was a significant difference between tissue groups (F_{4,92} = 11.67; P < 0.0001) and a significant difference between patients that did or did not survive 5 years (F_{1,92} = 7.23; P = 0.0085). Metastatic neoplasms were significantly different in GOS reactivity than either normal mucosa from CRC patients or intraepithelial neoplasms. Although there was no interaction, it is clear from the data in Table 3 that for metastatic neoplasms there was no difference in GOS reactivity related to prognosis. The A% of normal mucosa stained by GOS in CRC patients that did not survive 5 years was twice as great as those with a 5-year survival (25.28% versus 12.61%; P = 0.0161; Table 3). Importantly for prognostication, less than 5 years of survival was negatively correlated with the A% of invasive tumor stained by GOS (r = -0.53409; P = 0.0331).

The A% of CRC from patients that did not survive 5 years was 18.53% versus 6.34% from those who did survive (Table 3). When tumor recurrence was considered, there was a significant difference between tissue groups (F_{3,71} = 5.75; P = 0.0014) and a significant difference between patients that did or did not develop a recurrence (F_{1,71} = 4.87; P = 0.0305).

**Table 2** Regional differences in area of tissue stained by the GOS sequence during neoplastic progression in the colon: Geometric means of A% of tissue stained, number of specimens (N), and 95% CI

<table>
<thead>
<tr>
<th>Tissue group</th>
<th>Nonneoplastic</th>
<th>Intraepithelial neoplasms</th>
<th>Invasive neoplasms</th>
<th>Metastatic neoplasms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pts. without CRC</td>
<td>Geo mean (N)</td>
<td>4.20 (6)</td>
<td>19.04 (22)</td>
<td>16.51 (12)</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>(0.89, 19.85)</td>
<td>(13.72, 26.42)</td>
<td>(11.19, 24.37)</td>
</tr>
<tr>
<td>Pts. with CRC</td>
<td>Geo mean (N)</td>
<td>4.44 (17)</td>
<td>12.33 (19)</td>
<td>7.33 (23)</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>(2.69, 7.34)</td>
<td>(7.80, 19.49)</td>
<td>(4.64, 11.58)</td>
</tr>
</tbody>
</table>

Tissues included nonneoplastic (normal + hyperplastic polyps); intraepithelial neoplasms (tubular, tubulo-villous and villous adenomas, and carcinoma in situ); invasive neoplasms (stages I and II); and metastatic neoplasms (stage III and lymph node metastases).
Table 3 Prognostic significance of area of tissue with GOS reactivity: geometric means of A% tissue stained, number of specimens (N), and 95% CI

<table>
<thead>
<tr>
<th>Tissue group</th>
<th>Nonneoplastic patients with CRC</th>
<th>Intraepithelial neoplasms</th>
<th>Invasive neoplasms</th>
<th>Metastatic neoplasms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival/No</td>
<td>25.28 (13)</td>
<td>26.52 (3)</td>
<td>18.53 (5)</td>
<td>4.58 (20)</td>
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<tr>
<td>Geo mean (N)</td>
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<td>(11.19, 62.87)</td>
<td>(8.60, 39.90)</td>
<td>(2.95, 7.12)</td>
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<td>Survival/Yes</td>
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<td>13.32 (6)</td>
<td>6.34 (11)</td>
<td>4.77 (12)</td>
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<td>(4.82, 17.37)</td>
<td>(3.43, 12.45)</td>
<td>(2.08, 10.92)</td>
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<tr>
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<td>7.45 (4)</td>
<td>5.17 (13)</td>
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<td>(0.88, 63.38)</td>
<td>(2.78, 9.62)</td>
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<tr>
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<td>4.77 (12)</td>
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<tr>
<td>Geo mean (N)</td>
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<td>(1.76, 52.77)</td>
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<td>(2.08, 10.92)</td>
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<td>4.58 (20)</td>
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<td>Geo mean (N)</td>
<td>(6.75, 17.62)</td>
<td>(1.76, 52.77)</td>
<td>(3.49, 15.23)</td>
<td>(2.08, 10.92)</td>
</tr>
</tbody>
</table>

* Tissues included nonneoplastic (normal + hyperplastic polyps); intraepithelial neoplasms (tubular, tubulo-villous and villous adenomas, and carcinoma in situ); invasive neoplasms (stages I and II); and metastatic neoplasms (stage III and lymph node metastases). Rec/surv: if recurrence = Yes and survival = No, then Rec/Surv = 1; if recurrence = No and survival = Yes, then Rec/Surv = 0.

$P = 0.0020$; Table 3). Although the number of synchronous intraepithelial neoplasms in CRC patients was limited, a similar trend was noted. The A% of synchronous intraepithelial neoplasms stained by GOS was 26.06% in patients whose CRCs recurred or who died and 9.64% in those that survived and did not have a recurrence. Thus, when the area of normal mucosa, synchronous intraepithelial neoplasms, or invasive neoplasm stained by the GOS sequence was approximately 20% or greater, the prognosis for the CRC patient was poor.

Relationship of GOS Staining to Reactivity with PNA.

The lectin from *Arachis hypogaea*, PNA, binds the disaccharide (Gal-GalNAc) of the T-antigen (13-16). Capping of the T-antigen with sialic acid eliminates PNA reactivity. Because the GOS sequence was approximately 20% or greater, the prognosis for the CRC patient was poor.


discussion

We concluded from semiquantitative analyses that GOS reactivity in normal colon or invasive neoplasms is an intermediate biomarker in colorectal carcinogenesis that could be used for identification of patients with either CRC or intraepithelial neoplasms (41). Quantification of GOS staining in colonic mucosa by image cytometry enhanced the objectivity of the data, permitted hypothesis testing by statistical methods, and demonstrated the prognostic significance of GOS reactivity. The A% of normal mucosa in CRC patients stained by GOS was 36.9% of that stained by GOS in patients without CRC, including patients with nonspecific inflammation or ulcerative colitis. Regression analysis indicated that there was a highly significant decreasing trend in A% of epithelium with GOS reactivity during neoplastic progression with normal mucosa from CRC patients > invasive neoplasms > normal mucosa from CRC patients > normal mucosa from CRC patients > normal mucosa from CRC patients.

Staining of colorectal mucins by the GOS sequence was thought to represent detection of Gal-GalNAc, the Thomsen-Friedenreich (T) antigen (27). The T-antigen is an oncodevelopmental cancer-associated antigen in the colon (20). We found that there was a significant difference in GOS reactivity between tissues with or without PNA reactivity, a lectin that is specific for the T-antigen. Discrepancies between PNA reactivity and GOS staining in human colon and several rodent tissues have been reported previously (24, 33). Here, only 64% of the specimens evaluated were stained by both PNA and GOS, suggesting that the GOS sequence is detecting terminal saccharides in addition to the terminal disaccharide Gal-GalNAc specifically detected by PNA (34). Because tissue reactivity with GOS can indicate changes in terminal N-acetylgalactosamine or galactose residues on mucins, the stain could be detecting changes in blood groups A or B determinants as well as changes in expression of the T-antigen (32, 34, 45, 46). Schulte and Spicer (34) found that 90% of the...
mucous tubule cells of the human trachea are stained intensely by GOS as well as lectins specific for N-acetylgalactosamine in blood group A individuals (or the lectin specific for α-D-galactose in AB individuals), whereas only 10% of these cells are stained by PNA. Thus, GOS staining, particularly in the 36% of the specimens without PNA reactivity, may be due to reactivity with blood group antigens or other oligosaccharides with terminal galactose or N-acetylgalactosamine residues. GOS reactivity is greater in the proximal colon, where expression of blood group substances is found (47), than in the distal colon. The 98% sensitivity of GOS for detecting patients with CRC found here in histological sections of normal colonic mucosa or in the rectal mucous test (26–28) could be explained if the GOS sequence is detecting any one of several possible alterations in cellular glycoconjugates.

The near 100% correspondence between increased GOS reactivity in the normal colonic mucosa and the presence of a colonic neoplasm (or a predisposing condition such as inflammatory bowel disease) also suggests that alterations in colonic mucin resulting in increased GOS reactivity are closely associated with the carcinogenic process. Increased GOS reactivity in the normal colon: (a) could be secondary to the presence of a neoplasm; (b) could represent a “field effect”; or (c) could be an adaptive response reflecting the level of exposure to endogenous or exogenous toxicants or carcinogens (10, 27, 39, 48, 49, 51).

The prognostic significance of GOS reactivity argues against hypothesis (a), because patients with the same stage of disease but with subsequently different clinical courses have significantly different production of GOS detectable mucins in the normal colonic mucosa. Because the changes in human colonic mucosa of CRC patients resemble those found during experimental colon carcinogenesis in the rat (48, 51), it has been proposed that as a result of carcinogen exposure, there are random biochemical alterations in the genome of the colonic epithelial cells throughout the entire large intestine (“field effect”; Ref. 10). A “field carcinization” model was first proposed by Slaughter et al. (49) for oral squamous epithelium, and a “field effect” is consistent with the multifocal nature of CRC. Altered glycosylation of glycolipids and glycoproteins as well as changes in the core proteins of mucins are well-documented findings in most human carcinomas including CRCs (39, 52, 53). Our finding that GOS reactivity decreases with neoplastic progression in CRC argues against the proposal that GOS is detecting an oncodevelopmental antigen associated with neoplastic progression. Hypothesis (c), that GOS is an adaptive response that is a dosimeter of exposure to carcinogens or toxicants, is most consistent with the data from this study: (I) the A% of epithelium expressing GOS reactivity in the normal mucosa of patients with CRC was 365% of that in the normal mucosa of patients without CRC; (2) the A% of normal mucosa expressing GOS in patients with intraepithelial neoplasms was intermediate between those patients with CRC and those patients without CRC; and (3) the A% of normal mucosa, synchronous intraepithelial neoplasms and invasive neoplasms stained by GOS was twice as great in patients that did not survive 5 years compared to those who did survive. Specimens in this study came from an area of higher than anticipated CRC that has been associated with the drinking water source (29, 30).

Mucins in the gastrointestinal tract represent the major barrier between the internal and external environment of the intestinal epithelial cell (51). Altered synthesis of mucins, as in the case of ulcerative colitis, may represent a nonspecific response to injury or oxidative stress. Synthesis and secretion of mucins is also regulated by irritants, drugs (notably anti-inflammatory drugs and nicotine), hormones, vitamins, metals, bacterial toxins, and neurotransmitters (51). Because alteration in mucin synthesis and secretion can represent an adaptive response to a toxic environment, cellular adaptation is a mechanism to explain the apparent loss of GOS reactivity during progression of CRC. Colorectal carcinogenesis in patients without hereditary colon cancer syndromes may be similar to hepatocellular carcinogenesis, where adaptation to a toxic environment results in the appearance of “resistant” hepatocytes (54, 55). Farber has proposed that progressive cellular adaptation to a cytotoxic chemical, rather than cell proliferation per se, is one component of the mechanism for hepatocellular carcinogenesis associated with chronic exposure to cytotoxicants (54–56). He suggests that carcinogenesis is associated with a new state of differentiation in which the few isolated cells with a new phenotype have a selective growth advantage relative to their surrounding cells (54). Maronpot (57) has also suggested that carcinogenesis can be considered a form of toxicity wherein cells achieve a different steady state from normal and do not respond normally to homeostatic mechanisms. In this view, “initiated” cells could result from either adaptation or mutation. Clonal expansion of initiated cells could occur either because initiated cells are: (a) more responsive than normal cells to growth stimuli; (b) less susceptible to the toxicity of the chemical; or (c) less susceptible to regulatory signals for apoptosis (58). Consistent with this cellular adaptation theory, we have recently shown by image cytometry in a rodent model that adaptive cellular changes occur in the liver following exposure to a carcinogenic by-product of drinking water chlorination, dichloroacetic acid (59).

In the present study, the A% of epithelium showing reactivity with GOS in the normal epithelium from CRC patients was significantly different by Tukey multiple type comparison from either the normal mucosa of patients without CRC or metastatic tumors but not significantly different from intraepithelial or invasive neoplasms (and these stage III neoplasms were similar to the normal mucosa of patients without CRC). If increased synthesis of GOS-reactive mucins by colonic epithelial cells is an adaptive response to carcinogens or cytotoxicants, it would be predicted that with selection of cells “resistant” to the cytotoxic effects of the environment, cellular production of GOS reactive mucins would decrease, resulting in the highly significant progressive loss of GOS reactivity during neoplastic progression seen in Fig. 2. Again, from the perspective of cellular adaptation, GOS reactivity in the normal colon would be a dosimeter of exposure to an endogenous or exogenous carcinogen or cytotoxicant. Studies of humans and marmosets with ulcerative colitis further illustrate this point. Reactive oxygen radicals generated by inflammatory cells are an example of endogenous cytotoxicants. In three separate prospective studies of humans and marmosets with colitis, greater degrees of inflammation were associated with both increased expression of Gal-GalNAc and subsequent development of CRC, leading Boland et al. (60), Pihl et al. (61), and Boland and Clapp (62) to
suggest an etiological linkage between acute inflammatory activity and neoplasia. In a chemical carcinogen rat model, Gal-GalNAc-positive mucin was found in carcinogen-treated, normal-appearing colonic mucosa preceding the appearance of neoplasia (63). Shamsuddin et al. (22, 48) also observed changes in colonic mucin in ulcerative colitis patients and in carcinogen-treated rodents prior to the development of neoplasia, suggesting that these mucin changes are primary responses to carcinogenic/cytotoxic stimuli. The highly significant correlation between GOS reactivity in the normal colonic mucosa of CRC patients and tumor recurrence and/or poor survival found here is consistent with chronic exposure to environmental/lifestyle carcinogens such as drinking water chemicals, cigarette smoking, or alcohol consumption.

If GOS reactivity in the normal colonic mucosa is a dosimeter of exposure to endogenous or exogenous colorectal carcinogens rather than a secondary effect due to the presence of a neoplasm, it could be a highly effective screening tool for determining relative risk in pre (or non)-CRC patients as well as an aid to making therapeutic decisions in patients with CRC.

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REFERENCES


Validation of the galactose oxidase-Schiff's reagent sequence for early detection and prognosis in human colorectal adenocarcinoma.


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