Tumor-associated Sialylated Antigens Are Constitutively Expressed in Normal Human Colonic Mucosa

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ABSTRACT

Immunohistochemical studies have indicated that sialylated carbohydrate antigens such as sialyl-Tn, sialyl-Le\(\text{a}\), and sialyl-Le\(\text{b}\) are expressed in a tumor-associated fashion in human colon. Since sialic acid residues are O-acetylated more extensively in normal colonic epithelium than in colon cancer cells, we examined whether deacetylation of colonic tissues might enable monoclonal antibodies to recognize these tumor-associated sialylated antigens. In normal colon, deacetylation turned most cases (82%) positive with anti-sialyl-Tn mAb TKH2; and in colon cancers, it increased the number of TKH2-positive cells. Sialyl-Le\(\text{a}\) and sialyl-Le\(\text{b}\) detection was also increased after deacetylation of normal and malignant colonic tissues so that the frequency of positive cases in normal tissues was similar to that in the cancers. However, in the stomach and pancreas, the same treatment rarely increased the detection of the sialylated epitopes in normal or cancerous tissues. Thus, the same sialylated epitopes can be expressed in a tumor-associated fashion by different mechanisms in different gastrointestinal organs; in the colon, these antigens are constitutively expressed and O-acetylated, whereas in the upper gastrointestinal tract, they are rarely O-acetylated, suggesting that other mechanisms such as differences in glycosylation account for the cancer-associated expression.

INTRODUCTION

STn\(\text{3}\) antigen is a disaccharide antigen found on mucin glycoproteins. On the basis of immunohistochemical studies using monoclonal antibody TKH2, we and others have reported that STn is expressed by colonic epithelial cells in a cancer-associated fashion (1, 2). That is, STn is only rarely detected in normal colonic mucosa but becomes abundantly expressed in the majority of colon cancer tissues, where it has been associated with a poor prognosis (1–3). The same is true of STn expression in normal and malignant gastric epithelium (4). In contrast to the immunohistochemical absence of STn in normal colon, biochemical analysis has indicated that, in fact, normal colonic mucin carries oloigosaccharides with the STn structure (5, 6).

This discrepancy suggests that the lack of STn immunoreactivity may be due to an inability of anti-STn antibodies to gain access to the epitope. This might occur if long neighboring oloigosaccharides sterically hindered the ability of the antibody to gain access to the short disaccharide STn structure found immediately adjacent to the polypeptide backbone of mucin. Although possible, this hypothesis remains unproved.

Another possibility concerns the specificity of mAb TKH2 to recognize STn antigen with regard to the O-acetylation status of its sialic acid residue. Sialic acids are known to undergo a variety of modifications in nature (7). Histochemical (8–14) and biochemical (15–18) studies have demonstrated that, in normal human colonic mucosa, sialic acids often have O-acetyl substitutions of the polyhydroxyl side chain and the C4 position and that, in transformed and fetal cells, there is often a marked reduction of O-acetylation of sialic acids. However, because of the nature of the methods used in these previous studies, modification of sialic acid was demonstrated for subpopulations of sialic acids on unspecified epitopes.

We decided to revisit the issue of the expression of specific sialylated tumor-associated antigens in the gastrointestinal tract using monoclonal antibodies that recognize epitopes with different sialic acid linkages and asked whether these tumor-associated sialylated antigens were genuinely absent in normal tissues of the gastrointestinal tract.

MATERIALS AND METHODS

Antibodies. Table 1 lists the mAbs used in this study and the antigens they recognize. mAb TKH2 was generated using ovine submaxillary mucin as immunogen, and its epitope has been determined to be STn (19).

Hybridoma-producing mAb CA19-9 was purchased from American Type Culture Collection (Rockville, MD). This mAb, originally raised against human colon carcinoma cell line SW1116 (20), recognizes SL&\(\text{a}\) antigen (21). mAb FH7 recognizes a disialylated Le\(\text{a}\) structure and was established using a purified ganglioside bearing this antigenic structure (22). Because mAb FH7 reacts not only with colon cancers but also with most normal colonic goblet cells, it is not considered a cancer-associated antigen (23). However, this epitope was of interest for the present study because it carries two sialic acids in different linkages.

mAb SNH3 recognizes monomeric and dimeric SL&\(\text{a}\) determinants, and mAb SNH4 specifically recognizes dimeric SL&\(\text{a}\). These antibodies were developed using lactonized sialyl-dimeric Le\(\text{a}\) as the immunogen. Lactonization of sialylated epitopes facilitates the establishment of mAbs which react with the lactonized as well as the native structure (24). We demonstrated previously that dimeric SL&\(\text{a}\) is a rather cancer-specific antigen because of its absence from normal mucosa (25).

mAb 1E3 was developed using asialo-ovine submaxillary mucin as immunogen, and this antibody recognizes Tn antigen, a nonsialylated epitope. mAb 1E3 served as a negative control antibody for each case analyzed, and the results indicated that the binding pattern did not change at all with the deacetylation procedure used in this study.

Tissues. Eleven pairs of colonic adenocarcinoma and histologically normal colonic mucosa at least 5 cm away from tumor were studied. To determine whether any changes observed in the colon were organ specific, we also analyzed normal and cancerous tissues from the stomach (n = 6) and pancreas (n = 4). To confirm that these changes were organ specific and not patient specific, gastric, pancreatic, intestinal, and colonic tissues from the same individual were obtained from two early autopsies that exhibited minimal autolysis.

Immunohistochemistry. Five-μm sections of formalin-fixed, paraffin-embedded archival tissues were placed onto poly-L-lysine-coated glass microscope slides. The removal of O-acetyl groups from sialic acid residues was performed by treating slides with 0.1 M NaOH for 20 min at room temperature (15) prior to performing immunohistochemistry (mild base treatment). Control slides were kept in PBS for the same period of time. Mild base-treated and control slides were then stained with the mAbs, according to previously described methods (1).

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3 The abbreviations used are: STn, sialyl-Tn; SL&, sialyl-Le\(\text{a}\); SL&\(\text{b}\), sialyl-Le\(\text{b}\).

4 A. Singhal and S. Hakomori, unpublished observations.
cells: therefore, only the latter score is presented.

Among colonie mucosae stained positive with mAb TKH2 before NaOH treatment, nearly all of the cases remained positive after NaOH treatment. However, it was noted that after NaOH treatment, subtle differences in staining intensity were observed, particularly in the tumors. In some cases, parietal cells and goblet cells of intestinal metaplasia reacted with the mAb. However, all of the gastric cancers reacted with TKH2 antibody. In contrast to the colon, treatment of normal or cancerous gastric tissues with NaOH did not alter their immunoreactivity with mAb TKH2, except for one cancer in which a moderate increase of staining was observed.

In normal pancreas, mAb TKH2 was completely nonreactive (Table 3; Fig. 1), whereas the antibody bound to all of the pancreatic cancers. In no case did NaOH treatment alter the staining characteristics of normal or cancerous pancreatic tissues. Thus, normal pancreatic tissues, like normal gastric mucosa, remained negative with TKH2, even after the NaOH treatment.

Since the gastric, pancreatic, and colonic tissues came from different individuals, we could not exclude the possibility that the apparent organ specificity was not in fact due to interindividual variation. To address this issue, we studied normal stomach, pancreas, and colonic tissues from the same individual using two autopsies. In both subjects, NaOH treatment caused an increase of mAb TKH2 binding in the colon only but not in the pancreas or stomach (data not shown). This corroborates the organ-specific nature of O-acetylation of sialylated antigen demonstrated above.

Detection of SLe\(^\alpha\) and SLe\(^\alpha\) Antigens

Since we were able to detect STn antigen in normal colonic epithelium by the deacetylation treatment, we applied the same method to other sialylated, non-STn antigens in order to reinvestigate their tumor-associated expressions.

\textbf{SLe\(^\alpha\) Antigen Detected by mAb CA19-9}

Colonic Tissues. As with mAb TKH2, most of the normal colonic mucosae (82\%) were negative with mAb CA19-9 under control conditions, but the number of positive cases markedly increased after NaOH treatment (64\%; Table 2). Unmasked SLe\(^\alpha\) antigen was observed primarily in goblet cell vacuoles of the upper crypt in normal mucosa (Fig. 2, A and B). Among the tumors, 82\% of the cases were positive with mAb TKH2 under control conditions. NaOH treatment of colon cancers turned one more case positive (total, 91\%) and also enhanced the percentage of positive cells in 7 of 9 originally positive cases. One cancer specimen that was STn(-) under control conditions remained STn(-) after NaOH treatment.

Gastric and Pancreatic Tissues. CA19-9 antibody did not stain any mucus-secreting cells of normal gastric mucosa, and the negative staining was not changed by the alkaline treatment. In some cases, the antibody reacted with parietal cells and goblet cells of intestinal metaplasia. Two of the six stomach cancer tissues stained positive with this antibody under control conditions. NaOH treatment turned only one more cancer tissue positive and left the others unchanged. In contrast to the colon and stomach, all cases of both normal and cancerous pancreatic tissues stained positive with CA19-9. In normals, the antibody reacted with ducts and ductules. In no case did NaOH treatment alter the staining characteristics of any of the pancreatic tissues.
Fig. 1. Summary of reactivity of mAbs against sialylated tumor-associated antigens in the colon, stomach (Stom), and pancreas (Panc). Reactivity is expressed as the percentage of positive cases stained by mAbs TKH2, CA19-9, FH7, SNH3, and SNH4 in colon, gastric, and pancreatic tissues derived from data shown in Tables 2 and 3. Ni, normal; Ca, cancer.

**Di-SLe" Antigen Detected by mAb FH7**

**Colonic Tissues.** mAb FH7 immunoreactivity is very prevalent in normal colonic mucosae (Table 2). Since all cases showed strong staining under control conditions, little or no change in binding pattern was discernible after NaOH treatment. In colon cancers, FH7 reactivity was less prevalent, and the frequency of positive cases increased from 55 to 64% after NaOH treatment.

**Gastric and Pancreatic Tissues.** In the stomach and pancreas, FH7 showed almost exactly the same staining pattern as CA19-9 both before and after deacetylation treatment. That is, all of the normal gastric specimens were negative, and 2 gastric cancers which were positive with CA19-9 were also positive with FH7 under control conditions. None (except one normal tissue) was altered after NaOH treatment; the majority of cells in all of the pancreatic tissues, both normal and cancer, stained positive and remained unchanged after NaOH treatment.

**SLe" Antigen Detected by mAb SNH3**

**Colonic Tissues.** mAb SNH3, which recognizes both monomeric and dimeric SLe" epitopes, reacted with only a few cases (27%) of normal colonic mucosa under control conditions, usually in cells at the crypt base (Fig. 2E). However, like mAbs TKH2 and CA19-9, mAb SNH3 became positive in most cases of normal mucosa (73%) after NaOH treatment. Unmasked SLe" was observed in goblet cell vacuoles of both the upper and lower crypt (Fig. 2F). The majority of colon cancers (91%) bound mAb SNH3 under control conditions. NaOH treatment did not alter the number of positive cases, although 55% of the cancers exhibited a slight increase in immunoreactivity.

**Gastric and Pancreatic Tissues.** Mucus-secreting cells of only one normal gastric mucosal specimen bound mAb SNH3, whereas parietal cells in a couple of cases and some cells of the deep gastric glands in three other cases reacted with this antibody. mAb SNH3 was not very reactive with gastric cancers either, as only two of the six
gastric cancers bound the antibody. Treatment with NaOH did not alter the immunoreactivity of this antibody to either normal gastric mucosa or gastric cancer cells.

All pancreatic tissues, both normal or cancer, reacted with SNH3 to various extents. Alkaline treatment produced no change in any specimen.

Dimeric SLe* Antigen Detected by mAb SNH4

Colonic Tissues. This antigen was detected in a strict tumor-associated fashion in colonic tissues in that no normal colon specimens and 64% of colon cancer cases were positive. Like other antigens tested, this antigen was unmasked by NaOH treatment in 36% of normal colonic tissues, but in colon cancer specimens, the staining pattern barely changed.

Gastric and Pancreatic Tissues. mAb SNH4 did not bind to any specimens of normal or cancerous stomach, even after NaOH treatment. The same staining results were obtained with the pancreas specimens, except for one case where cancer cells weakly bound the antibody and slightly increased the binding after alkaline treatment.

DISCUSSION

Sialylated tumor-associated antigens, such as STn, SLe*, and SLe*, have achieved clinical importance because of their potential usefulness in cancer diagnosis and prognosis. Most previous studies, includ-
ing our own, have reported that normal colonic epithelia do not express these antigens, whereas the majority of cancer specimens stain positive (1, 26). In addition, several studies have indicated that cancers which lack expression of STn (3, 4) or SLLe\(^a\) (27) are associated with a much better prognosis than those which express these antigens.

The tumor-associated pattern of expression of these sialylated antigens relies upon the specificities of the mAbs used and has been primarily determined by immunohistochemical means. However, despite an awareness for several decades that sialic acid residues are O-acetylated in normal human colonic mucosa and that this modification is markedly reduced in transformed and fetal cells, very little attention has been paid to the effect of O-acetylation of sialic acids on specific epitopes in determining the specificities of the mAbs against sialylated epitopes. This issue has been partially investigated in melanoma, where MAb that react with 9-O-acetylated G\(_{03}\) gangoside but not with nonacetylated G\(_{03}\) selectively bind to melanomas (28, 29).

In a preliminary report, we noted that O-acetylation of sialic acid influenced the immunohistochemical detection of the most commonly studied sialylated tumor-associated antigens in the gastrointestinal tract (30). Others have also described that reactivity of mAb TKH2 with normal colorectal goblet cells is affected by O-acetylation (31). The saponification method used in the present study has been widely used in histochemical studies to remove O-acetyl esters from sialic acid residues. Although this deacetylation method (0.1 M NaOH at 20 min at room temperature) is not specific to the cleavage of O-acetyl groups from sialic acid residues, to our knowledge and under the conditions used, the O-acetyl group is the only structure that could be released from the glycoconjogates of human cells.

Perhaps the most striking finding was that deacetylation enabled TKH2 antibody to positively stain normal colonic mucosa in most of the cases (82%). This indicates that this epitope was indeed constitutively synthesized with its sialic acid O-acetylated in the normal colonic epithelium and, therefore, escaped detection by the antibody which was incapable of binding to the antigen in its O-acetylated form. We would conclude, therefore, that the tumor-associated nature of STn expression in the colon is not caused by aberrant glycosylation in tumor cells as has been postulated (32) but may instead result from O-acetylation of sialic acid in the normal colonic mucosa with the consequential inability of the antibody to bind to the epitope involving O-acetylated sialic acid residue.

In the stomach and pancreas, STn antigen was also expressed in a strict tumor-associated fashion under control conditions. However, in contrast to the colon, alkaline treatment did not unmask STn antigen in normal gastric or pancreatic tissues. It seems, therefore, that in the upper gastrointestinal tract, STn oligosaccharide is not synthesized in normal tissues but becomes synthesized without O-acetylation in cancers, presumably by means of altered glycosyltransferase activities. Thus, the same STn epitope can be expressed in a cancer-associated fashion by two different mechanisms.

The expression of the other (non-STn) sialylated antigens detected by mAbs CA19-9, FH7, SNH3, and SNH4 were characteristic to each gastrointestinal organ (Fig. 1). In the normal colon, only a few cases were stained positive by these non-STn antibodies (except FH7, which stained all specimens). NaOH treatment of normal colonic tissues, however, revealed the epitopes in the majority of cases, making the pattern of positive cases in normal and cancer specimens very similar (Fig. 1). This implies that, in normal colon, all of the sialylated antigens examined, except disialosyl-Le\(^a\), were subject to O-acetylation of sialic acid, much in the way that STn was. In contrast, deacetylation did not alter the detection of the non-STn sialylated antigens in gastric and pancreatic tissues, indicating that in the upper gastrointestinal tract, these antigens are only rarely O-acetylated. Nevertheless, in the stomach, all of the mAbs except SNH4 reacted in a cancer-associated fashion. This suggests that the cancer-associated expression pattern of the same antigen may be due to different mechanisms, depending upon the organ studied. In the colon, O-acetylation of sialic acid influences this pattern, whereas in the stomach, other mechanisms such as altered glycosylation play a role. In the pancreas, only STn behaved in a cancer-associated fashion, because most other antigens were highly prevalent in normal pancreatic tissues.

Although O-acetylation of sialic acids appears to account for much of the STn-negative immunophenotype of normal colonocytes, it does not completely explain all instances in which STn is not expressed. For example, in a few cases of normal colonic mucosa, the tissue remained negative for STn, SLLe\(^a\), and/or SLLe\(^b\) even after deacetylation, while their cancer counterparts were positive. In these cases, altered glycosyltransferase activities may be involved. In addition, because of the significance of the STn-negative phenotype in the prognosis of colon cancer patients, we also examined four other STn-negative colon cancer specimens (a total of six cases, including the two shown in Table 2; data not shown). Interestingly, four of the six cancers did not turn positive after deacetylation although the normal mucosa in five cases was positive, indicating that the STn-negative phenotype in colon cancer is not simply caused by masking with O-acetyl groups.

Although the majority of colon cancer specimens were stained positive by most of the antibodies under control conditions, NaOH treatment usually enhanced the number of positive cells. In some individuals, only one or two antigens were O-acetylated, whereas in others, several antigens were O-acetylated. Therefore, in an individual person, O-acetylation of sialic acid residues can be differentially regulated in colon cancer cells. This suggests that there may be differences in O-acetyltransferase activities responsible for O-acetylation.
Although the current study indicates that normal colonic mucosa contains O-acetylated STn antigen, the same oligosaccharide identified by biochemical analysis in normal human colonic mucin was not reported to have such modification on the sialic acid residue (5, 6). This discrepancy can be explained by the methods used for purification of oligosaccharides. When oligosaccharides are released from mucin by alkaline/borohydride treatment, this can totally remove O-acetyl groups from sialic acid residues. In fact, many methods commonly used for structural analysis of glycoconjugates [such as base hydrolysis during purification of glycolipids, alkaline borohydride release of O-linked chains, hydrazinolysis, methylation analysis, and strongly basic anion-exchange columns (such as Dowex 1 and Dowex 2)] can destroy sialic acid modifications (7).

Our data suggest the following conclusions: (a) in the normal colon, sialylated antigens are usually cryptic because of the inability of mAbs to bind to O-acetylated sialic acid; (b) in the colon, sialylated antigens are O-acetylated in an epitope-specific manner, whereas in the upper gastrointestinal tract, these antigens are rarely if ever O-acetylated; and (c) STn antigen is the only sialylated antigen expressed in a cancer-associated fashion in all three gastrointestinal organs studied.

When interpreting results of immunohistochemical studies that use antibodies to sialylated antigens, the possibility that negative expression may be due to modification of sialic acid residues should be considered.

REFERENCES

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