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Different modes of sialyl-Tn expression during malignant transformation of human colonic mucosa

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Monoclonal antibodies TKH2 and B72.3, which react with the mucin-associated sialyl-Tn(STn) antigen, preferentially bind to cancerous but not normal colonic tissues. If *O*-acetyl groups are removed by saponification of tissues, MAb TKH2 will react with normal colonocytes, whereas MAb B72.3 remains non-reactive. To explain this difference in binding specificity, we tested both MAbs against synthetic constructs of single (monomeric) or clustered (trimeric) STn epitopes by enzyme immunoassay. Both MAb TKH2 and MAb B72.3 reacted with trimeric STn, but MAb TKH2 demonstrated greater binding than MAb B72.3 to monomeric STn. This suggests that normal colonic mucosa expresses monomeric STn epitopes, but that with transformation to malignancy, clustered STn epitopes appear. The appearance of clustered STn epitopes during colonic carcinogenesis represents a novel pattern of carbohydrate antigen expression and implicates alterations at the level of apomucins and/or glycosyltransferases responsible for cluster epitope formation.

***Keywords*: mucin, sialyl-Tn, STn, cluster STn, monomeric STn, *O*-acetylation, colon cancer, immunohistochemistry, ELISA, ovine submaxillary mucin**

Introduction

Current understanding of the expression of tumor-associated sialylated antigens in the colon derives mainly from immunohistochemical studies indicating that these antigens are usually expressed in colon cancers but only weakly if ever expressed in normal colonic mucosa [1]. We have been investigating the reasons behind this cancer-associated pattern of sialylated antigen expression and reported that in the normal colon, sialyl-Tn (STn) antigen is in fact present, but is not detected by certain monoclonal antibodies such as TKH2 because the sialic acid moiety of the epitope is *O*-acetylated [2, 3]. After removal of *O*-acetyl groups by saponification of tissues, MAb TKH2 reacts with colonocytes in most normal colonic tissues.

MAb B72.3 is another antibody which also recognizes STn antigen [4, 5], and like MAb TKH2, MAb B72.3 reacts preferentially with colon cancer tissues compared to normal colonic mucosa [6, 7]. In preliminary studies, we noticed that unlike MAb TKH2, MAb B72.3 did not show enhanced binding to normal colonic mucosa after tissues were saponified. This suggested that there might be a difference in epitope recognition between these two antibodies, and prompted the present study to further investigate the reason for this difference.

There is evidence that the immunodominant epitopes of Tn and STn antigens in ovine submaxillary mucin (OSM) and glycophorin A exist in a clustered array rather than as individual (monomeric) structures [8, 9]. Little attention has been given to whether STn antigen in tissues exists as a monomer or cluster because the specificity of the various anti-STn MAbs for different forms of STn was not known. The ability to test reactivity of MAbs against clustered or monomeric STn epitopes has recently become possible by the advent of synthetic monomeric and trimeric (cluster) STn reagents [10]. Therefore, in the present study, we re-investigated the STn antigenic specificities of MAbs TKH2 and B72.3 with an aim toward defining the mode of STn expression in colon carcinogenesis.

Materials and methods

Antibodies and antigens

MAb TKH2 (mouse IgG1) hybridoma supernatant was a generous gift from Professor Sen-itiroh Hakomori (The Biomembrane Institute, Seattle, WA). MAb B72.3 (mouse IgG1) was obtained from American Type Culture

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Collection (ATCC, Rockville, MD) and was used as hybridoma supernatant.

The synthetic monomeric and trimeric STn antigens used in this study have been described previously [10]. STn-KLH consists of the STn disaccharide conjugated to KLH with a crotyl linker arm. Tri-STn-KLH consists of a cluster of three STn epitopes covalently linked through a triple serine backbone conjugated to KLH.

Tissues and cell line

Formalin-fixed paraffin-embedded surgical specimens of 25 colon cancers and paired remote normal mucosa were used for immunohistochemistry. The LS-C cell line is a clonal derivative of the human colon cancer cell line LS174T which was selected for positive expression of STn antigen by MAb TKH2 [11]. These cells strongly react with MAb TKH2, and oligosaccharides of mucin purified from LS-C cells consist only of the Tn (GalNAc-O-ser/thr) and STn (Siaα2,6GalNAc-O-ser/thr) structures.

Immunohistochemistry and de-acetylation

Tissue sections were stained with MAb TKH2 and B72.3 using a standard immunoperoxidase protocol [2]. De-acetylation (saponification) was accomplished by treating slides immediately after deparaffinization and rehydration with 0.1 N NaOH for 20 min at room temperature just prior to performing the immunoperoxidase procedure [2].

Immunohistochemical stains were interpreted without knowledge of treatment status. Slides were scored according to approximate percentage of normal crypts or tumor cells that stained positive.

ELISA

ELISA was performed using two synthetic versions of STn to coat the wells (25–800 ng per well). For these experiments, MAb TKH2 and B72.3 were each used at 1/10 dilution. STn-KLH, consisting of monomeric STn disaccharides conjugated to keyhole limpet hemocyanin, and

Table 1. Colonic tissue reactivity with MAbs TKH2 and B72.3 before and after saponification

Case	*Normal colon*				*Colon cancer*			
	TKH2		*B72.3*		*TKH2*		*B72.3*	
	Pre-NaOH	*Post-NaOH*	*Pre-NaOH*	*Post-NaOH*	*Pre-NaOH*	*Post-NaOH*	*Pre-NaOH*	*Post-NaOH*
1	0	0	0	0	30	60	30	60
2	0	0	0	0	10	30	10	10
3	0	30	0	0	30	30	10	15
4	0	40	0	0	30	30	20	40
5	0	50	0	0	40	80	30	70
6	0	20	0	0	60	90	60	80
7	0	10	0	0	30	70	10	30
8	0	70	0	0	60	70	60	70
9	0	30	0	0	30	50	10	10
10	0	70	0	0	0	0	0	0
11	30	100	0	0	0	40	0	0
12	0	100	0	0	10	50	0	0
13	0	100	0	0	80	80	50	60
14	0	70	0	0	20	70	0	0
15	0	80	0	0	10	90	0	40
16	0	100	0	0	70	70	0	0
17	0	80	0	0	50	100	0	100
18	0	30	0	0	80	80	80	80
19	0	80	0	0	70	70	0	0
20	0	50	0	0	N/A	N/A	N/A	N/A
21	20	50	0	0	10	10	10	10
22	N/A	N/A	N/A	N/A	0	10	0	0
23	N/A	N/A	N/A	N/A	80	90	60	60
24	N/A	N/A	N/A	N/A	60	90	0	40
25	N/A	N/A	N/A	N/A	90	100	80	80

Numbers represent approximate percentage of STn + crypts (normal colon) or STn + tumor cells (colon cancer).
N/A not available.

tri-STn-KLH, consisting of clustered trimers of STn, were prepared as described previously [10].

To determine the reactivity of MAb TKH2 and B72.3 with ovine submaxillary mucin, OSM was prepared according to Tettamanti and Pigman [12]. ELISA plates were coated overnight at 4 °C with purified OSM (40 ng per well). For whole-cell ELISA, LS-C cells were seeded at a concentration of 5×10^4 cells per well.

Results

Comparison of MAb TKH2 and MAb B72.3 reactivity in colonic tissues

Under standard staining conditions, MAb TKH2 did not react with normal colonic mucosa, except for two cases which demonstrated a small amount of reactivity (Table 1). However, after treatment of the same tissues with sodium hydroxide, practically all of the normal colonic specimens demonstrated an increase in MAb TKH2 immunoreactivity (Table 1; Figure 1). MAb B72.3 did not react with any specimen of normal colonic tissue, either before or after de-acetylation (Table 1; Figure 1). This indicates that the epitope recognized by TKH2 is *O*-acetylated in normal colon, whereas the B72.3 epitope is not expressed.

In colon cancers, MAb TKH2 reacted with all but three cases under standard staining conditions (Table 1). After de-acetylation of tissues, the percentage of TKH2-reactive tumor cells increased in most of these specimens (Figure 2). MAb B72.3 reacted with fewer cases of colon cancer than MAb TKH2 under standard conditions (Table 1). After de-acetylation, fewer cases demonstrated an increase in reactivity with MAb B72.3 and the percentage of B72.3-reactive tumor cells was only slightly increased (Figure 2).

Reactivity of MAbs TKH2 and B72.3 with various STn-positive targets

In order to elucidate the cause of the differential staining in normal colonic mucosa by the two MAbs, both MAbs were tested for reactivity against various STn-positive targets by

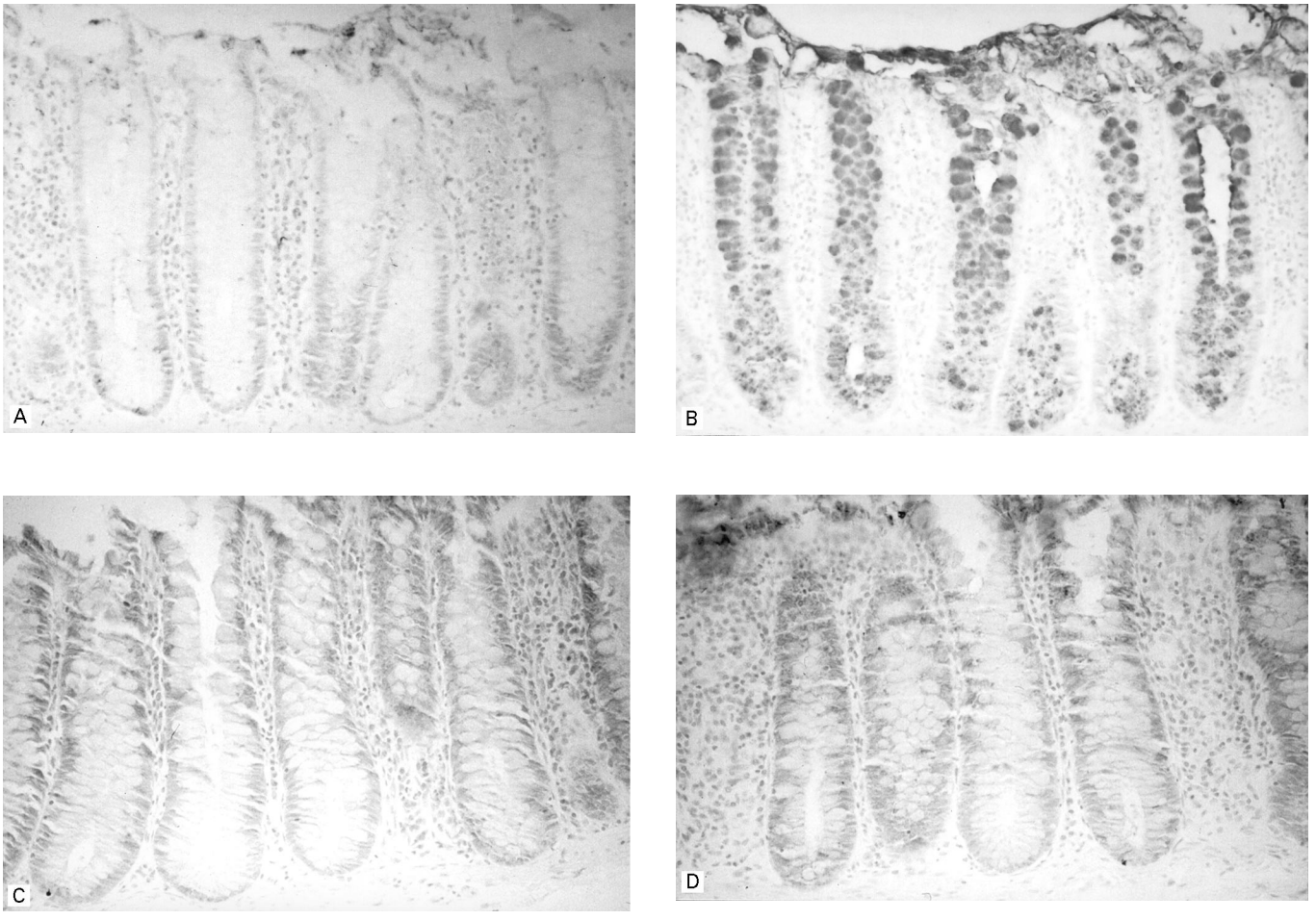

Figure 1. Normal colonic mucosa stained with MAb TKH2 before (A) and after (B) deacetylation of the tissue. Note the marked increase in STn expression in goblet cells after de-acetylation. Normal colonic crypts stained with MAb B72.3 before (C) and after (D) de-acetylation showing a complete lack of immunoreactivity under both conditions.

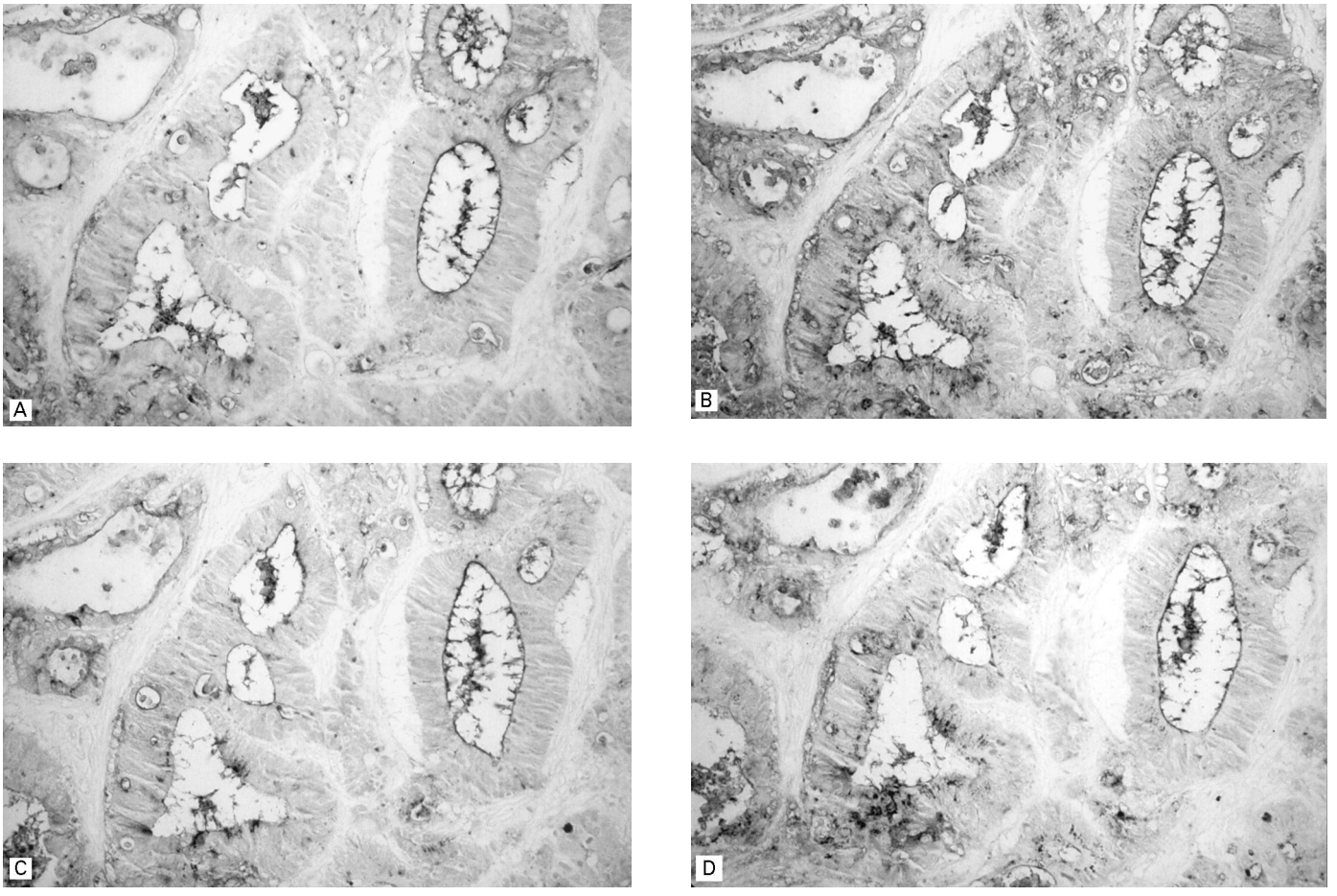

Figure 2. Colon cancer specimen stained with MAb TKH2 before (A) and after (B) deacetylation. MAb TKH2 mainly stains apical membranes and luminal secretions (A), but after de-acetylation of the tissue there is an increase in cytoplasmic staining as well (B). Same malignant glands stained with MAb B72.3 before (C) and after (D) de-acetylation. Apical membranes and luminal secretions stain positive (C), but after de-acetylation there is no increase in reactivity (D).

ELISA. Figure 3 illustrates the reactivity of each MAb with synthetic monomeric or clustered STn structures and represents the results of one typical experiment which was repeated four times. MAb TKH2 reacted much more strongly than MAb B72.3 with monomeric STn-KLH (Figure 3A). However, both antibodies reacted equally well with trimeric, clustered STn-KLH (Figure 3B).

Figure 4 demonstrates the reactivity of the two MAbs to naturally occurring STn epitopes and represents one typical experiment which was repeated four times. Ovine submaxillary mucin was tested as an example of a natural mucin molecule with clustered STn epitopes (Figure 4A). MAbs TKH2 and B72.3 bound to OSM in an identical manner. To test the MAb reactivity against naturally occurring STn epitopes on human cancer cells, LS-C colon cancer cells were used in whole-cell ELISA (Figure 4B). MAb TKH2 showed greater reactivity than MAb B72.3 at antibody concentrations where both MAbs showed the same reactivity to OSM.

Discussion

The STn antigen has gained attention as a cancer-associated antigen because its expression is restricted to only a few normal tissues but is quite prevalent in a variety of adenocarcinomas [6, 7, 13, 14]. In addition, STn expression by cancer cells has been associated with cancer progression and a poor prognosis in patients with cancers of the colon, ovary, and stomach [15–19]. Previous studies using MAb TKH2 indicated that the apparent lack of STn expression in normal colonic mucosa was due to masking of the sialic acid moiety of the epitope by *O*-acetylation [2, 3]. Thus, the preferential reactivity of MAb TKH2 with colon cancer cells reflects, at least in part, a decrease in *O*-acetylation of sialic acids during colon carcinogenesis [20–23]. However, in other organs such as stomach and pancreas, the lack of STn expression in normal epithelium and the increased expression in cancerous cells appears to be unaffected after deacetylation [2]. Therefore, mechanisms other than *O*-acetyl

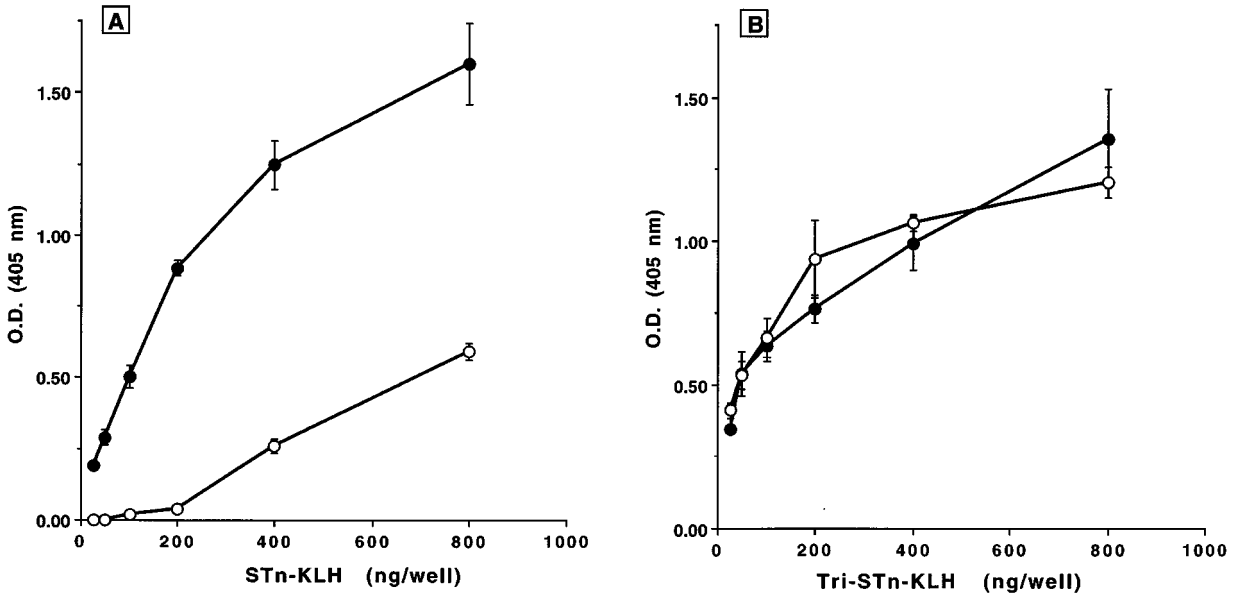

Figure 3. ELISA of MAbs TKH2 and B72.3 binding to monomeric STn-KLH (A) and clustered, trimeric STn-KLH (B). MAb TKH2, solid circles; MAb B72.3, open circles. Each data point represents mean O.D. ± SD of triplicates.

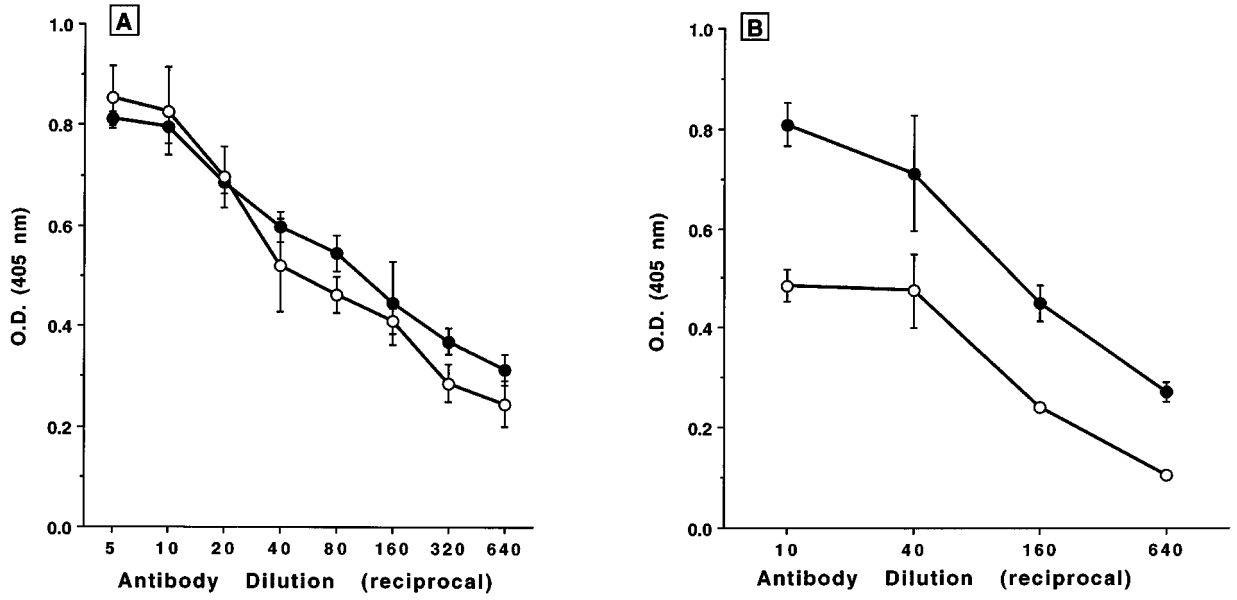

Figure 4. ELISA of MAbs TKH2 and B72.3 binding to ovine submaxillary mucin (40 ng/well) (A) and LS-C human colon cancer cells (5×10^4 cells/well) (B). MAb TKH2, solid circles; MAb B72.3, open circles. Each data point represents mean O.D. ± SD of triplicates.

modification of sialic acid residues can also account for the appearance of STn epitopes in those cancer cells.

After completion of the study on the effect of saponification of MAb TKH2 binding [2], we noticed that another widely used anti-STn antibody, MAb B72.3, did not bind to de-acetylated normal colonic mucosa, leading us to investigate the differences between the two MAbs. This investigation was facilitated by the recent development of synthetic reagents carrying monomeric and trimeric STn epitopes that can be used to test the fine specificity of anti-STn antibodies [10]. The ELISA data presented herein revealed that MAb B72.3 preferentially recognized trimeric STn, whereas MAb TKH2 reacted with both trimeric and monomeric STn. When the MAbs were applied to OSM, a nearly identical pattern of reactivity was revealed, in accordance with previously reported data that OSM carries mostly clustered STn structures.

Table 2. Human mucin genes: potential *O*-glycosylation sites

Mucin gene	*Chromosome location*	*Tandem repeat structure*	*Predominant tissue expression*
MUC1	1q21	GV**TS**APD**T**RPAPG**ST**APPAH	Breast, pancreas
MUC2	11p15	P**TTT**PI**TTTTTT**V**T**P**T**P**T**G**T**Q**T**	Colon, small intestine, trach.
MUC3	7q22	H**ST**P**S**F**TSS**I**TTT**E**TTS**	Colon, small intestine, trach., GB
MUH4	3q29	**TSS**A**ST**GHA**T**PLPV**T**D	Trachea, colon
MUC5AC	11p15	**TTSTTS**AP	Trachea, stomach
MUC5B	11p15	**SST**PG**T**AH**T**L**T**VL**TTT**A**TT**P**T**A**T**GS**T**A**T**P	Trachea, colon
MUC6	11p15	**S**PF**SST**GPM**T**A**TS**FQ**TTTT**YP**T**P**S**HPQ **TT**LP**T**HVPPF**STS**LV**T**P**ST**G**T**V**I**T**PP**T**H AAQMA**TS**A**S**IH**ST**PTG**T**IPPP … (169 aa)	
MUC7	4	**TT**AAPP**T**PSA**TT**PAPP**SSS**APPG	Salivary gland

Bold type: posible *O*-glycosylation sites.

The fact that MAb TKH2 recognized STn after de-acetylation of tissues whereas MAb B72.3 remained non-reactive even after de-acetylation suggests that normal colonocytes express monomeric but not cluster STn. In cancer tissues, however, both MAbs were highly reactive, suggesting that during malignant transformation in the colon, cluster STn structures emerge along with endogenous de-acetylation of sialic acids. Since MAb TKH2 can recognize STn as a monomer or in the context of a cluster, it is difficult to known whether the STn expression in cancer tissues is due to only cluster STn or a combination of both clustered and monomeric STn. The observation that MAb TKH2 exhibited a higher reactivity than MAb B72.3 to LS-C cells at antibody concentrations with which both MAbs exhibited identical reactivity against OSM suggests that, at least in these colon cancer cells, both cluster and monomeric STn structures co-exist.

Although the mechanism(s) by which the configuration of STn expression may change with malignant transformation in the colon is currently unexplained, two possibilities seem most likely. First, the ability to synthesize monomer or cluster STn structures on mucin polypeptide backbones should depend in part on the sequence of *O*-glycosylation sites of the apomucin. Mucin genes share the property of having tandem repeat regions that contain many glycosylation sites, although the amino acid sequence of each repeat unit is unique (Table 2). For *MUC1*, there are only two regions within a tandem repeat where consecutive serine-threonine residues might permit cluster STn formation. For all other mucins, however, there is a greater potential to form clustered arrays of STn due to the frequent occurrence of consecutive serine and/or threonine residues. Therefore, one can speculate that in normal colonocytes STn may reside on mucins like MUC1 which has a sparse array of glycosylation sites, whereas during malignant transformation, STn might also reside on other apomucins that have more clustered glycosylation sites. This mechanism is plausible, because despite earlier immunohistochemical studies suggesting that normal colon is MUC1-negative [24–26], other studies using northern blot hybridization [27] and immunohistochemistry following sodium periodate treatment to unmask MUC1 apomucin [28] clearly document the presence of MUC1 in normal colon. What is less clear is which of the other apomucins might carry cluster STn in colon cancer cells. MUC2 and MUC3 might be considered good candidates because they are quite prevalent in normal colonic mucosa, but ironically, these two threonine-rich apomucins are markedly decreased in colon cancers [27, 29, 30], suggesting that other apomucins are more likely to carry cluster STn.

A second mechanism for cluster STn formation relates to the repertoire and specificity of glycosyltransferases required for STn synthesis that exist in normal *vs* cancerous colonic cells. The initial transfer of GalNAc to the apomucin peptide can be performed by several different polypeptidyl:GalNAc transferases, some of which have recently been cloned and their acceptor specificities described [31–40]. Studies using MUC1 peptide acceptors indicate that in breast and pancreatic cancer cells, GalNAc is primarily synthesized on single threonine residues [31, 32] although there may be some threonine-serine dimers that both receive GalNAc [33]. At present, the pattern of GalNAc addition to other apomucins is not known, but it is possible that certain polypeptidyl-GalNAc transferases might preferentially glycosylate single threonine (or serine) residues, whereas others might prefer to glycosylate multiple threonines (or serines). Further studies will be required to explore the mechanism(s) underlying cluster STn formation and the association between this configuration and carcinogenesis.

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