Altered Glycosylation of the MUC-1 Protein Core Contributes to the Colon Carcinoma-associated Increase of Mucin-bound Sialyl-Lewis\textsuperscript{x} Expression\textsuperscript{1}

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ABSTRACT

The mucin carbohydrate epitope sialyl-Le\textsuperscript{x}, detected with the monoclonal antibody AM-3, is strongly overexpressed in >90% of human colon carcinomas. We show here that in colon carcinoma one of the mucin cores bearing the sialyl-Le\textsuperscript{x} group is MUC-1, whereas sialyl-Le\textsuperscript{x} present in normal colon is not detectable on MUC-1. The amounts of MUC-1 core detectable with the monoclonal antibody BC3 in extracts of tumor tissue are 60–180% of those in normal tissue. Two other carbohydrate epitopes located on MUC-1 in mucins from normal and tumor tissue have also been characterized. In contrast to sialyl-Le\textsuperscript{x}, their expression on MUC-1 is variable and does not correlate with the malignant transformation of colonic mucosa. The transfer of the sialyl-Le\textsuperscript{x} group onto the MUC-1 core contributes to the colon carcinoma-associated overexpression of the sialyl-Le\textsuperscript{x} epitope.

INTRODUCTION

Colon carcinogenesis is accompanied by consistent increase in expression of several mucin carbohydrate antigens. These antigens comprise the core antigens Tn and T and their sialylated counterparts sialyl-Tn and sialyl-T, as well as the peripheral structures representing blood group antigens Le\textsuperscript{a}, Le\textsuperscript{b}, Le\textsuperscript{r}, and Le\textsuperscript{o} and their derivatives (1, 2). Although it is known that these structures may occur on the same mucin molecules, we have no systematic knowledge regarding how their expression is related to the expression of the mucin protein cores carrying them. The observed cancer-related expression of carbohydrate structures on mucins can be due to (a) increased biosynthesis of preexisting “normal” molecules, which in normal tissue are present in small or even undetectable amounts, or (b) emergence of new mucin molecules not synthesized by normal colonic tissue. These new mucins might arise either through altered glycosylation of the preexisting mucin core or through biosynthesis of new cores which are glycosylated in an aberrant manner. Systematic study of this question has been performed with TAG 72 antigen (3) and its carbohydrate epitopes B72.3, CC 49, and CC 119. The expression of TAG 72 mucin is increased in colonic cancer. The cancer-related alteration of expression of the epitopes present on this mucin is not, however, a concerted process but rather an independent change for each epitope. Similar observations have been made recently with several B-cell lines transfected with the same MUC-1 gene which expressed the cognate protein or carbohydrate epitopes in an incongruent manner (4). By contrast, the ectopic expression of four carbohydrate epitopes of the small intestinal mucin antigen in colonic cancer was reported to follow in a coordinate and sequential manner, corresponding to the gradual alterations of the adenoma-carcinoma sequence (5).

In the present work, the influence of malignant transformation on the expression of different mucin epitopes of the same molecule has been investigated based on the example of colon carcinoma-associated mucin carrying the sialyl-Le\textsuperscript{x} epitope, which is detectable with the monoclonal antibody AM-3 (6). The overexpression of sialyl-Le\textsuperscript{x} is an established carcinoma-associated property (7, 8).

We show here that, while sialyl-Le\textsuperscript{x} epitope is present regularly on MUC-1 in colon carcinoma but not in normal colonic tissue, the epitopes AM-4 and AM-7 occur on MUC-1 in tumor and in normal tissue with a variable frequency.

MATERIALS AND METHODS

Generation and Selection of Monoclonal Antibodies. Monoclonal antibodies were generated with mucins isolated from human colon carcinomas and selected as reported previously (8). The antibody BC3 was generated by immunization with human milk fat globule membrane and reacts with the variable number of tandem repeats on the MUC-1 protein core (9, 10).

Purification of AM-3-positive Mucins. Fresh colonic carcinoma or normal mucosa tissue was frozen and pulverized under liquid nitrogen in a mortar. The powder was extracted for 24 h at 4°C in a buffer containing 4 M guanidine hydrochloride, 0.1 M Tris, and 5 mM EDTA, pH 8.0, and was centrifuged at 100,000 × g for 30 min, and the mucins were purified in three CsCl gradients as described previously (11). The gradient fractions (1 ml) were collected and their AM-3 epitope content was determined in slot-blot. The purified, DNA-free, AM-3-positive mucin fractions were used as a source of AM-3 antigen.

Determination of the Mucin-bound Carbohydrates through Periodic Acid-Schiff’s Reagent Staining. Aliquots of mucin solutions were blotted in a slot-blot apparatus (Bio-Rad, Munich, Germany) onto polyvinylidene difluoride membrane and stained with periodic acid-Schiff’s reagent as described by Thornton et al. (12). The signals were evaluated by means of a reflectance scanning densitometer (Hoefer, San Francisco, CA) and compared with a standard.

Purification of Antibodies from Supernatants and Conjugation to Peroxidase. The antibodies were precipitated from fetal calf serum-free supernatants through addition of ammonium sulfate to 50% saturation. Then, AM-3 or AM-7 (both IgM) were separated from the smaller proteins through fractionation on a Sephacryl-300 column (2.5 cm x 80 cm) and collection of the void volume peak, containing the IgM fraction. The AM-4 antibody (IgG) was purified by affinity chromatography on a Protein A-Sepharose (Pharmacia, Freiburg, Germany) column (5 ml). The purity of the antibodies was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and was >95%. The conjugation to peroxidase was carried out as described (13).

Sandwich ELISA with Peroxidase-conjugated Antibodies. Sandwich ELISA\textsuperscript{3} for mucins was carried out on Immulon ELISA microtiter plates (Dynatech, Denkendorf, Germany) under conditions which were previously optimized (14). Unless explicitly mentioned, for all ELISA tests purified AM-3-positive mucins were applied in amounts saturating the catcher antibody. The peroxidase-conjugated tracer antibodies (AM-3-peroxidase, AM-4-...
peroxidase, or AM-7-peroxidase) were added in excess (10 ng/ml) and were incubated for 5 h at 4°C. The development followed with benzamidine for 10 min and was stopped with 4 N H2SO4. If the sensitivity of the assay was not sufficient a BLAST amplification system (DuPont, Dreieich, Germany) was applied according to the recommendations of the manufacturer. The evaluation of the ELISA signal was carried out with a Dynatech ELISA reader at 405 nm.

**Characterization of the Epitopes AM-4 and AM-7.** Oxidation of sugar moieties with periodate, sialidase digestion, mild acid hydrolysis, and alkaline hydrolysis were carried out as described previously by Hanisch et al. (6).

**Immunohistochemical Detection of AM-3, AM-4, and AM-7 Epitopes.** Deparaffinized 5-µm-thick tissue sections of colorectal carcinoma and adjacent, morphologically normal, colonic tissue from five patients were sequentially incubated for 30 min with the hybridoma supernatant, rabbit anti-mouse immunoglobulin, mouse anti-alkaline phosphatase/alkaline phosphatase complex (Dianova, Hamburg, Germany), and naphthol AS biphosphate (Sigma, Munich, Germany) as the substrate. Alternatively, if the anti-mouse antibody conjugate was labeled with peroxidase then the amplification was carried out with the avidin-biotin system (Daco, Hambour, Germany). The detection followed with benzamidine as the substrate. As a negative control, NS-1 cell supernatant was used. The sections were scored as positive when 10% or more of the cells in the investigated tissue were stained.

**Immunoblotting.** For determination of the relative content of AM-3, AM-4, AM-7, and BC3 epitopes in tissue extracts from colorectal carcinoma or from normal colonic mucosa by slot-blotting, serial dilutions of each preparation were applied to polyvinylidene difluoride membranes (Millipore, Bedford, MA) in a Bio-Rad slot-blot chamber, incubated with the tested antibody, and detected with β-galactosidase conjugate as described (15). The intensity of the signal was evaluated with a Hoefer reflectance scanning densitometer (Hoefer, San Francisco, CA) and plotted against the amount of protein applied to the slot.

**Visualization of Mucins by Rotary Shadowing.** The rotary shadowing of mucins or mucin-antibody complexes was carried out as described previously (16). Electron microscopy was performed with a JEOL 100 CX scanning transmission instrument at 100 keV.

**RESULTS**

**Localization of AM-4 and AM-7 Epitopes on Sialyl-Leα-bearing Mucins**

Sandwich ELISA with AM-4, AM-7, or BC3 (anti-MUC-1) as catcher antibodies and AM-3-peroxidase conjugate as tracer indicated that sialyl-Leα-bearing mucins purified from several pooled colorectal carcinomas contained molecules sharing these epitopes. In the normal mucin preparation, no sialyl-Leα could be detected on MUC-1. The signal in the plateau part of the ELISA curve for AM-4 or AM-7 antibodies was severalfold higher in tumor tissue-derived than in normal tissue-derived mucins (Fig. 1). Firm conclusions regarding the relative amounts of AM-7 or AM-4 epitopes in tumor versus normal tissue cannot be drawn, since the results are affected by the higher avidity of the tracer (AM-3-peroxidase conjugate) for the tumor-derived, relative to the normal tissue-derived, mucins.

**Localization of AM-3, AM-4, and AM-7 Epitopes on the MUC-1 Core**

Separately purified mucins from colon carcinoma of six patients revealed, in sandwich ELISA, the presence of AM-3, AM-4, or AM-7 epitopes on the MUC-1 core when anti-MUC-1 (BC3) antibody was used as catcher. In mucins from normal colonic mucosa, the AM-4 and AM-7 epitopes, but not the AM-3 epitope, were found on the MUC-1 molecule (Fig. 2).

**Carbohydrate Nature of AM-4 and AM-7 Epitopes**

Both epitopes were insensitive to pronase digestion and heat treatment but were significantly affected by mild periodate oxidation. This indicates that both antibodies AM-4 and AM-7 recognize carbohydrate moieties, as shown previously also for AM-3 (6). In contrast to the AM-3 antibody, partial hydrolysis with Vibrio cholerae sialidase increased AM-4 and AM-7 binding activities to 209% and 249%, respectively, indicating that sialic acid may partly mask these epitopes. Since mild acid hydrolysis of sialic acid and fucose abolished the binding of both antibodies, these epitopes may contain fucose.

Binding studies on neoglycoproteins and neoglycolipids corroborated that both antibodies define Leα-antigen-related epitopes, but with distinct fine specificities. While AM-4 binding was strictly dependent on a 4-linked fucose of the Leα trisaccharide, AM-7 cross-reacted with Leα-derived type 1 chain on lacto-N-tetraose and with a related epitope on the Thomsen-Friedenreich-(α,β) antigen (data not shown).

**Influence of Malignant Transformation on Carbohydrate and Peptide Epitope Accessibility on MUC-1 Molecules**

**Immunohistochemistry.** The AM-3 epitope, as reported previously, was detected only in carcinomatous colonic tissue and not in normal tissue (Fig. 3, A and B). Both AM-4 and AM-7 epitopes were immunohistochemically detectable in all normal (five patients) and in 60% of malignant (three of five patients) colonic tissue samples. Both were detectable in the secretory material of normal crypts. In addition, AM-4 antibody stained dense cytosolic granules in the perinuclear area of epithelial cells (Fig. 3C). In tumor tissue, AM-3, AM-4, and AM-7 antibodies reacted with cell membranes and the secretory material. Antibodies AM-3 and AM-4 also detected material present in...
ColoN CANCer-AssOCIAted Mucins

Fig. 3. Immunohistochemical detection of epitopes AM-3 (A and B), AM-4 (C and D), and AM-7 (E and F) in normal (A, C, and E) and carcinomatous (B, D, and F) colonic tissue. Arrows, positive staining. Thick arrow in C, granular cytosolic staining in normal epithelial cells. Bar, 0.1 mm.

the cytosol of the tumor cells (Fig. 3, B and D). Normal colonic tissue, positive for the AM-4 or AM-7 epitopes, was negative for AM-3 (Fig. 3A), indicating that it contains AM-4- or AM-7-positive molecules which have no or very few AM-3 epitopes.

The BC3 antibody stained the normal and carcinomatous colonic tissue, indicating the presence of the core protein in both nonmalignant and malignant tissues (Fig. 4, A and B). AM-3 staining of the parallel sections showed that, while in normal tissue sialyl-Le* was immunohistochemically undetectable, in carcinoma it yielded a strong signal (Fig. 4, C and D). This result is in agreement with the sandwich ELISA data (Fig. 2) and supports the notion that MUC-1 carries more sialyl-Le* moieties in tumor than in normal tissue.

Slot-Blot. To verify and extend the immunohistochemical data, slot-blot detection was carried out using 100,000 × g supernatants, obtained after guanidine hydrochloride extraction, from 10 patients with colorectal carcinoma. AM-3, AM-7, and BC3 epitopes were detectable in all the investigated extracts. AM-4 epitope was detectable in all tumor extracts and in nine of 10 normal tissue extracts, i.e., the detection by all the antibodies in cell extracts was more frequent than in tissue sections. Several pairs of extracts were then quantitatively analyzed in slot-blot (Fig. 5). As reported previously, the AM-3 curve indicated a larger number of AM-3 epitopes per mg protein in tumor extracts and a higher avidity of the antibody for tumor-derived mucins. The AM-4 and AM-7 antibodies yielded parallel slot-blot curves for tumor and normal tissue extracts, i.e., malignant transformation did not alter the avidity of mucin binding by either of these two antibodies. The BC3 antibody yielded parallel slot-blot curves in five of eight extract pairs; in three cases the avidity for tumor mucins was higher than that for normal tissue-derived mucins. The distance between the parallel curves was variable and indicated that the amount of the BC3 epitope in tumor extract was between 60% and 180% of that in normal tissue extract of the same protein content.

Electron Microscopy. Human colonic mucins are long elastic thread-like molecules without globular structures which were observed in rat gastric mucins (17) (Fig. 6). The distribution of the AM-4 and AM-7 epitopes was investigated after rotary shadowing of immune complexes obtained with the antibodies. Both AM-4 and AM-7 antibodies frequently bound in regular intervals of about 120 nm,
COLON CANCER-ASSOCIATED MUCINS

Fig. 4. Immunohistochemical detection of BC3 (A and B) and AM-3 (C and D) epitopes in parallel sections of normal (B and D) and carcinomatous (A and C) colonic mucosa, carried out with peroxidase-conjugated second antibody. Bar, 0.2 mm.

Fig. 5. Slot-blot detection of AM-3, AM-4, AM-7, and BC3 epitopes in 100,000 X g supernatants after guanidine hydrochloride extraction of normal colonic mucosa () or colon carcinoma ( ). The curves shown were obtained after evaluation of slot-blot and are representative of three to eight investigated patients. The parallelism of the curves for AM-4 and AM-7 was always observed; the distance between them was variable in different patients. The parallelism of curves for BC3 was observed in five of eight cases.

Possibly due to a repetitive distribution of these epitopes (Fig. 6). For AM-4 antibody the slot-blot data were additionally verified through determination of the number of bound antibodies per 1000 nm of mucin length. The evaluation of AM-4 complexes with tumor mucins (120 molecules) or normal colonic mucins (156 molecules) showed that the number of bound AM-4 molecules was 8.7 ± 3.0/1000 nm of the mucin molecule length for normal tissue-derived mucins and 7.00 ± 3.1/1000 nm of the mucin molecule length for tumor tissue-derived mucins.

CsCl Gradient Centrifugation. To address the question of whether sialyl-Lea is present on mucin cores other than MUC-1, the AM-3-positive mucins from normal and malignant colonic tissue from six patients were separately purified through threefold centrifugation on CsCl gradients. The distribution of the investigated epitopes was measured along the gradient by slot-blot or ELISA. The profiles of two patients are shown in Fig. 7 and Fig. 8. Mucins from normal colonic tissue were usually distributed as a symmetric peak and the profiles of AM-3, AM-4, AM-7, and BC3 epitopes overlapped in five of six patients. In one patient the overlapping of AM-3 and BC3 profiles was not complete (Fig. 7), indicating that at least in normal colon a second protein core carrying sialyl-Lea is present. By using sandwich ELISA AM-4 and AM-7 epitopes, but not the AM-3 epitope, were detected on MUC-1 in these preparations (Fig. 2).

In mucins from tumor tissues the periodic acid-Schiff's reagent-determined peak was broader and the monoclonal antibodies detected mucin populations of different densities and different epitope contents (Fig. 8). Neither normal tissue- nor tumor-derived mucins reacted with the monoclonal antibody CCP58 (data not shown), which detects the MUC-2 core (18), after the third CsCl gradient.

In sandwich ELISA tumor mucin preparations revealed the presence of sialyl-Lea on the MUC-1 core in all patients. The epitopes AM-4 and AM-7 were detectable on MUC-1 in these mucins with lower frequency than in the profiles used for the test (Table 1), i.e., in some patients they were present on molecules other than MUC-1.

DISCUSSION

The present data indicate that in normal colonic tissue sialyl-Lea is accessible on mucin molecules other than MUC-1, whereas in colon carcinoma MUC-1 is one of the mucin molecules bearing the epitope sialyl-Lea. In contrast to the regular expression of sialyl-Lea on MUC-1 in cancer, two other carbohydrate epitopes are variably expressed on MUC-1 in cancer, two other carbohydrate epitopes are variably expressed on MUC-1, in a manner independent of malignant transformation.

The epitopes AM-4 and AM-7 are O-linked carbohydrate structures that are detectable in nearly all tissue extracts of normal and malignant transformed colonic mucosa. The slot-blot data show that the amounts of AM-4 and AM-7 epitopes per mg protein of tumor or normal colon extract are similar. Further, the identical slopes of the ELISA curves for tumor and normal tissue mucins and the regular antibody distribution on rotary-shadowed immune complexes indicate that the expression of AM-4 and AM-7 epitopes is less affected by malignant transformation than is that of the AM-3 epitope. The data obtained with the BC3 antibody implied that the amounts of the
Colon carcinoma

Normal colon

MUC-1 protein core are moderately altered (60–180% of the normal value) in colonic tumors. The results of sandwich ELISA revealed, however, that, while the AM-3 epitope was present on MUC-1 molecules from all investigated tumor tissues and was not detectable on MUC-1 molecules from normal tissues, the AM-4 and AM-7 epitopes were present on MUC-1 in about 50% of the investigated specimens, irrespective of their origin. The CsCl gradients and the subsequent results of sandwich ELISA showed further that in tumor tissue AM-4 or AM-7 epitopes may also be present on molecules other than MUC-1. The nature of the mucin bearing sialyl-Leα in normal tissue is not yet known, but the material did not react with anti-MUC-2 antibody CCP58.

The ELISA signal of AM-3-, AM-4-, or AM-7-peroxidase conjugates with tumor-derived mucins was 4–6-fold higher than that with normal tissue-derived mucins if AM-3 was also used as catcher antibody. This ratio does not necessarily reflect the relationship of the epitope number in tumor and normal mucins but is due to bound IgM or IgG covering more than one epitope (15) and to the higher avidity of AM-3 for tumor-associated mucins.

The MUC-1 molecule, also called polymorphic epithelial mucin, is synthesized in normal breast and in breast carcinoma, where several of its peptide-containing epitopes (e.g., 115D8) (19) are overexpressed. Data obtained with serum from healthy donors and from patients with breast carcinoma (20) indicated that the density of the protein epitope DF3 is higher on mucins in tumor patient serum than on mucins in normal serum, possibly because the MUC-1-bound carbohydrate

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and malignant colon mucosa, i.e., in these cases this region is not equally accessible in normal tissue (10), is in five of eight cases equally accessible in normal breast carcinoma the MUC-1 polypeptide epitope Pro-Asp-Thr-Arg-Pro, detectable with the SM-3 antibody, is more accessible in tumor than in normal tissue (26). Indeed, in MUC-1-transfected B-cells the epitopes BC3 and SM-3 differ in their modulation by glycosylation; whereas the SM-3 epitope was strongly expressed after inhibition of MUC-1 glycosylation, the BC3 epitope was hardly affected (4).

A mucin molecule produced in leukocytes and called leukosialin has recently been shown to carry the sialyl-Le^a^ moiety on a carbohydrate chain with core 2 (27). In normal colonic epithelial cells only core 3 has been detected (28). Colon carcinoma cells (CaCo-2), however, lack the B1-3 N-acetylglucosaminyltransferase synthesizing carbohydrate core 3 (29). Therefore, the present data are compatible with the hypothesis that a switch from core 3 in normal epithelial cells to core 2 in carcinoma cells results in the biosynthesis of sialyl-Le^a^ on MUC-1. Indirect evidence that in colon carcinoma cells (COLO 205) MUC-1 is modified by sialyl-Le^a^ has been reported (30).

The abundant presence of sialyl-Le^a^ groups on the membrane-located MUC-1 molecule is of particular interest in view of the functional role of sialyl-Le^a^ as an ELAM-1 (endothelial cell-leukocyte adhesion molecule 1) ligand. The high density of sialyl-Le^a^ epitopes on a long elastic molecule protruding from the cells would be expected to considerably increase the adhesion of those cells to cells possessing the appropriate receptor.

In conclusion, the presented data indicate that after malignant transformation of the colon there is a consistent expression of sialyl-Le^a^ epitopes on the MUC-1 protein core, while two other carbohydrate epitopes are expressed in an irregular manner, irrespective of cell transformation. Determination of whether the regularity of sialyl-Le^a^ expression on MUC-1 in colon carcinoma is due to the alteration of particular glycosyltransferases or their modified distribution in cancer cells requires further study.

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