Increased Number of Accessible Sugar Epitopes Defined with Monoclonal Antibody AM-3 on Colonic Mucins Is Associated with Malignant Transformation of Colonic Mucosa

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ABSTRACT

Monoclonal antibody AM-3 detects a mucin sugar epitope (AM-3 epitope) the expression of which increases in the course of human colon carcinogenesis parallel to the gradual morphological alterations (so-called adenoma-carcinoma sequence). In the present report the AM-3-positive mucin has been purified from human normal and neoplastic colon tissue. About 300-fold enrichment of the epitope per protein from both sources was achieved after ultracentrifugation, gel filtration on Sepharose CL-6B and isopycnic gradient centrifugation. Slot-blot and enzyme-linked immunosorbent assays of the purified preparation indicated not only different amounts of the mucins but also a consistent qualitative difference between the molecules from both sources. The qualitative difference could be obliterated by a partial removal of the AM-3 epitope from the tumor-derived mucin with neuraminidase. The visualization of the molecules by rotary shadowing indicated that the mucins from both sources have similar length distribution, 80% of the molecules being 100-600 nm long. The reaction with AM-3 antibody followed by rotary shadowing showed that in the purified preparations more than 95% of the tumor-derived molecules and 74% of the normal colon tissue-derived molecules carried the epitope. The tumor-derived mucins bound, on the average, 34 ± 15 (SD) antibodies/1000 nm of the protein core while the mucin from normal colon tissue carried 12 ± 11 antibodies/1000 nm of the protein core. These data indicate that the increased expression of AM-3 epitopes during malignant transformation of the human colon is due to accumulation of AM-3-positive mucin as well as a higher number of accessible AM-3 epitopes on this mucin.

INTRODUCTION

Malignant transformation of colonic mucosa is accompanied by numerous morphological, functional, and immunological alterations. Aberrant expression of carbohydrate structures present on colon cell-derived mucins and glycolipids is frequently associated with cancer. Most of these structures with altered expression in cancer are related to ABO and Lewis blood group antigens (for review see Refs. 1–3).

The transformation of the human colon is accompanied by ectopic expression of blood group antigens (4), deletion of antigens usually expressed in normal mucosa (4, 5), or modification of an existing structure (6). Of particular interest are the antigens the increased expression of which parallels the gradual transformation of the colonic mucosa (adenoma-carcinoma sequence). These include the carbohydrate core antigen T (7) as well as a number of peripheral antigens with a common carbohydrate type 2 chain structure (8–10). These antigens are also consistently detected on mucins present in serum of cancer patients. The data obtained by Kim et al. (9) and Kannagi et al. (11) led to the unifying concept that type 2 chain elongation and fucosylation are biosynthetic functions characteristic of malignant cells, resulting in accumulation of the modified Le" and Le' blood group antigens in cancer tissue. The question whether the increased amounts of tumor-associated carbohydrate antigens in tissue and in serum are due to accumulation of certain mucin subpopulations or rather to the actual increase of mucin glycosylation has not been addressed in detail. Kannagi et al. (11) deduced from the solubility of serum mucins in perchloric acid that malignant transformation is associated with the increased number of type 2 chains and the decrease of type 1 chains on the mucins.

The antigen detectable with the AM-3 antibody belongs to the family of carbohydrate epitopes the expression of which in tumor tissue increases in the course of the adenoma-carcinoma sequence (12). Immunohistochemical studies have shown that the antibody reacts preferentially with carcinomas of the colon (98%), esophagus (83%), stomach (75%), breast (78%), and pancreas (60%). The corresponding normal tissue was not stained at all (colon, stomach) or was stained with a lower intensity and with a different pattern (esophagus, breast, pancreas) (12). The neuraminidase-sensitive carbohydrate epitope is present on a large mucin with a molecular weight of > 440,000. In the present study, the AM-3 antibody has been used for monitoring the purification of the AM-3-positive mucin from normal and cancerous colonic tissue and for the comparison of the extent of their glycosylation. The data obtained by immunoblot and rotary shadowing indicate that the increased number of the available epitopes is due to the increased fraction of AM-3-positive mucins as well as to the increased density of the epitope on these molecules.

MATERIALS AND METHODS

Purification of the Mucin. Fresh, nonneoplastic tissue and tissue from colorectal carcinomas used for mucin extraction were obtained at the time of surgery. The tumor was dissected free of necrotic tissue and fat. Colon segments located 10 cm or further from the tumor were considered normal. Tissue was washed with saline, dried with paper towel, and weighed. Tissue pooled from 5 patients (total, 5 g) was minced with a scalpel and boiled for 10 min either in 15 ml of extraction buffer (10 mM NaH$_2$PO$_4$-10 mM Na$_2$HPO$_4$-1 mM MgCl$_2$-30 mM NaCl-1 mM dithiothreitol-5 mM phenylmethylsulfonyl fluoride-0.02% NaN$_2$-0.01% DNase) or in the guanidine hydrochloride buffer used for column elution (4 mM guanidine hydrochloride-1 mM NaCl-5 mM EDTA-0.1 mM Tris, pH 8.0). The denatured sample was homogenized and centrifuged at 100,000 × g for 1 h as described previously (12). The 100,000 × g supernatant was fractionated on a Sepharose CL-6B column (2.6 × 35 cm). The 3.5-ml fractions were collected and their content of AM-3-positive molecules was determined by ELISA. Fractions containing the mucin were pooled, CSCI was added to a final density of 1.39 g/ml.

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3 The abbreviation used is: ELISA, enzyme-linked immunosorbent assay.
guanidine hydrochloride was added to a final concentration of 4 M, and the mixture was centrifuged for 65 h at 140,000 x g in a Kontron ultracentrifuge at 20°C. The gradient was harvested in 1-mL fractions, in which the density and the content of AM-3 mucin were determined. AM-3-positive fractions were pooled and dialyzed against 1 mM Tris-HCl buffer, pH 8.0, for protein determination and slot-blotting. Rotary shadowing was carried out with nondenatured samples.

Properties of the AM-3 Antibody. The monoclonal antibody AM-3 has been obtained through immunization of mice with mucins extracted from colon carcinomas (12). The antibody belongs to the IgM class in which the density and the content of AM-3 mucin were determined. The expression of which in the normal colon is below the detection limit of immunohistochemistry. The amounts of the epitope increase in parallel with the progress of malignant transformation (adenoma-carcinoma sequence) and were detectable in all investigated colonic carcinomas (12).

Immunoblotting. For determination of the relative content of AM-3 epitope by slot-blotting, serial dilutions of each preparation were applied to a polyvinylidenefluoride membrane (Millipore, Bedford, MA) in a Bio-Rad slot-blot chamber, incubated with AM-3 antibody, and detected with β-galactosidase conjugate as described (12). The intensity of the signal was evaluated with a Hoefer reflectance scanning densitometer (Hoefer, San Francisco, CA) and plotted against the protein content of the sample. The same procedure was applied for determination of the relative amounts of the epitope in homogenates of normal and cancerous tissues from different patients.

Detection of AM-3 Epitopes in ELISA. Samples to be tested were adsorbed to 96-well Immulon microtiter plates (Dynatech, Denkendorf, Germany) for 24 h at 4°C, washed three times with phosphate-buffered saline containing 0.1% Tween, and blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. The bovine serum albumin was removed, and the plate was washed three times and incubated with AM-3 antibody for 2 h at 4°C. The detection of the bound antibody followed by goat anti-mouse IgM-antibody conjugated with β-galactosidase (2 h incubation at 37°C) and, after washing, with 100 µl o-nitrophenyl-β-D-galactopyranoside (0.9 mg/ml; Sigma, Deisenhofen, Germany) as the substrate. After 2 h incubation at 37°C, the reaction was stopped with 50 µl of 1 N Na2CO3 and the absorbance was measured with a Dynatech ELISA reader at 410 nm. For detection of small amounts of the antigen, a more sensitive ELISA with 4-methylumbelliferyl-β-D-galactoside as the substrate was used. The detection of the reaction product followed in a Fluoroskan (Flow Laboratories, Muenchen, Germany) at 365 nm excitation and 450 nm emission wavelength.

Purification and Conjugation of AM-3 Antibody to Peroxidase. Mouse ascites was fractionated on a Sephacryl 300 column (16 x 70 cm). Fractions containing IgM were identified in ELISA, pooled, and dialyzed. The pool contained 90–95% immunoglobulin as tested in sodium dodecyl sulfate electrophoresis. Conjugation to peroxidase was carried out as described by Avrameas and Ternynck (13). Briefly, 10 mg peroxidase (Sigma) were activated in 1.25% glutaraldehyde for 18 h, dialyzed, and coupled to 5 mg IgM dissolved in 1 M Na2CO3/NaHCO3 buffer, pH 9.5. The coupling reaction was continued overnight at 4°C and stopped with 0.2 M lysine solution in H2O. The reaction mixture was dialyzed and the conjugate was separated from the free peroxidase on the Sephacryl 300 column. The fractions of the eluate were tested in ELISA for their IgM content and for mucin detection capacity. Fractions containing the conjugate were pooled, portioned, and kept at 4°C.

Sandwich ELISA with AM-3-Peroxidase Conjugate. Sandwich ELISA for mucins was carried out on Immulon ELISA microtiter plates (Dynatech) under conditions which were previously optimized. In short, the AM-3 antibody (7.5 ng/ml) as catcher was adsorbed for 1 h at room temperature. The mucin solutions were incubated overnight at 4°C. The tracer antibody (AM-3 conjugate) was incubated for 5 h at 4°C. The ELISA signal was carried out with a Dynatech ELISA reader at 410 nm.

Visualization of the Mucins by Rotary Shadowing. The rotary shadowing was carried out as described previously (14). In short, mucin molecules were diluted in 20 mM magnesium acetate, spread on a hypophase of benzyl(dimethyl)alkylammonium chloride, picked up on carbon-coated grids, fixed with ethanol, dried, and rotary shadowed with platinum-tungsten at an angle of 5 degrees.

Electron microscopy was performed with a JEOL 100 CX transmission instrument at 100 kV. Contour lengths were measured on photographically enlarged electron micrographs (final enlargement, 60,000) with a Planix digital planimeter (Hall and Watts, Links Trading Estate, Yiovill, Summerset, United Kingdom). The precision of the instrument was approximately 2% and the overall accuracy of the contour-length measurements was estimated to be better than 10%.

The mucin-antibody reaction was carried out by incubating the grids, on which mucin may be fixed, on a droplet of 5 mM magnesium acetate containing 20 µg/ml antibody and 0.1% Tween for 0.5 h. The grids were then washed three times with magnesium acetate-Tween and twice with magnesium acetate solution to remove nonspecifically bound antibodies. The rotary shadowing followed as described for pure mucas.

Neuraminidase Digestion of the Mucin. Neuraminidase from Arthrobacter ureafaciens (Boehringer, Marburg, Germany) was used to study the effects of gradual removal of the AM-3 epitope. Mucin preparation obtained after column chromatography was incubated for 1 h in acetate buffer (0.05 M sodium acetate-0.1 M CaCl2, pH 5.5) and different amounts of neuraminidase. The reaction was stopped by boiling (20 min) and serial dilutions were applied to a slot-blot apparatus and detected by immunoblotting.

Protein Determination. The determination of protein concentration was performed with Bio-Rad reagent, according to the recommendations of the producer (Pierce, Heidelberg, Germany).

RESULTS

Purification of the AM-3-positive Mucin from Normal and Cancerous Colonic Tissue. Denaturation of the tissue enzymes by boiling, homogenization in the extraction buffer, and 100,000 x g ultracentrifugation yielded a 14-fold enrichment of the antigen in the supernatant from tumor tissue and a 5-fold enrichment in the normal tissue preparation. The supernatants were then fractionated on Sepharose CL-6B, on which mucins from both sources eluted in the void volume of the column (Fig. 1), increasing the purification factor to 191-fold for tumor-derived and 25-fold for normal tissue-derived mucins. Finally, both mucin preparations banded as a single peak at a density of 1.40 g/ml (Fig. 2) after isopycnic gradient centrifugation. The overall purification, determined in the slot-blot procedure, was about 300-fold as related to the homogenate and the recovery was about 34% of tumor mucin and about 5% of the normal mucosa-derived mucin (Fig. 3; Table 1). The slopes of the curves obtained after evaluation of the slot-blot data remained constant after each step of the procedure, indicating that the molecules did not alter in the course of the purification. The tumor-derived mucin preparations always gave steeper curves than the preparations obtained from normal colonic mucosa (Fig. 3).

Detection of Qualitative Differences in Mucins by Immunoblotting and Sandwich ELISA. The results obtained in ELISA after purification of the mucin from pooled tissue of 5 patients were verified on the material from 3 patients investigated separately. The content of AM-3-positive mucin in the 100,000 x g supernatants obtained after extraction of normal colonic mucosa or colon carcinoma of three different patients was compared in a slot-blot assay using hybridoma supernatant as

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from colonic carcinoma and 133 of 143 measured molecules (93%) isolated from normal colonic mucosa were 100–600 nm long (Fig. 6).

The incubation of the mucins with AM-3 antibody yielded immune complexes which could be visualized by electron microscopy. Of 105 measured molecules 103 molecules (97%) of tumor-derived mucins bound the antibody, at a density between 3 and 25 IgMs/molecule. By contrast, 46 of 62 molecules (74%) from the normal mucosa-derived mucins carried the antibody at the density between 1 and 24 IgMs/molecule. The number of bound immunoglobulins was dependent on the length of the protein core and the density of IgMs on the mucin (Fig. 5, B, D, E).

The distribution of the antibody along the molecules was random with no noticeable clustering. The density of the bound immunoglobulins per protein chain length showed a different distribution in normal tissue and carcinoma-derived mucins (Fig. 7) and indicated that in the tumor the most frequent density was 30–40 antibodies/1000 nm while in the normal tissue the mucins carrying less than 10 immunoglobulins represented the most frequent population. On the average, 34 ± 15 (SD) antibodies/1000 nm of the protein chain were present on mucins isolated from carcinomatous colonie tissue. On normal mucosa-derived mucins the average density was 12 ± 11 antibodies/1000 nm of protein chain.

**DISCUSSION**

In the present work, the molecular basis of different expression of the AM-3 epitope in normal and cancerous colonie tissue was investigated. For that purpose, the mucins carrying the epitope were purified from both tissues pooled from 5 patients and the distribution of the epitopes on the protein core was assessed by electron microscopy. The purification procedure was designed to prevent enzymatic digestion of the protein core or the carbohydrate moiety. The AM-3-positive mucins from both normal and carcinoma tissues eluted in the void volume of Sepharose CL-6B column and banded in a cesium chloride gradient at the density of 1.40 g/ml, suggesting no substantial difference in molecular mass between the molecules.

The source of AM-3 antibody. Serial dilutions of the antigens from each source yielded straight lines of different slopes, indicating a qualitative difference in the interaction with the antibody. The slot-blot lines of mucin preparations from different colon carcinomas had similar slopes (Fig. 4A). The mucin preparations obtained from normal colonie mucosa of different patients had different slopes and yielded signals consistently lower than tumor mucins (Fig. 4A). Treatment of carcinoma extracts with neuraminidase to give partial removal of AM-3 epitopes yielded mucins with ELISA curves overlapping with those of the normal mucin (Fig. 4B).

Both experiments (Fig. 4) gave the same results by using the slot-blot technique or ELISA with purified AM-3 antibody as catcher and peroxidase-labeled antibody for detection (not shown). These data suggested that the tumor-derived mucin might carry a higher number of epitopes.

Evaluation of the Mucins by Rotary Shadowing and Electron Microscopy. The mucins purified from pooled normal or cancerous colorectal tissues were further investigated by electron microscopy. Electron microscopy of colonie mucins shows that these macromolecules are linear flexible threads of variable length (Fig. 5, A and B). The appearance and length distribution were similar for normal tissue- and tumor tissue-derived mucins; 231 molecules of 266 measured molecules (87%) isolated

![Fig. 1](#) Purification of the AM-3 antigen from colon carcinoma (•) and normal colonie mucosa (○). A 100,000 x g supernatant was fractionated on a Sepharose CL-6B column and the fractions were analyzed for AM-3 content in ELISA with o-nitrophenyl-β-galactopyranoside or 4-methylumbelliferyl-β-D-galactoside as the substrate.

![Fig. 2](#) CsCl density gradient ultracentrifugation of AM-3-positive mucins: •, from colon carcinoma; ○, from normal colonie mucosa. Fractions were monitored for the presence of AM-3 epitopes in ELISA and density was determined by weighing.

![Fig. 3](#) Quantitation of AM-3 antigen in preparations from (•) colon carcinoma or (○) normal colonie tissue. Aliquots of the material obtained after each preparation step were serially diluted and 100 µl of each dilution were applied onto a polyvinylidene difluoride membrane. After immunodetection with AM-3 antibody and scanning, the signals were plotted against the applied protein amount. The obtained ELISA curves were parallel in each group, the curves for tumor mucins being steeper than for normal mucins. For clarity only the last curve for tumor-derived mucin is shown.
A signal corresponding to 10.25 ng protein in the eluate of the tumor preparation was defined as 1 unit. Protein was quantitated with the Bio-Rad reagent.

Table 1. Purification of AM-3-positive mucins from colon cancer (C) or normal colon mucosa (N)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total units</th>
<th>Units/mg protein</th>
<th>% of recovery of the antigen</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>C 288 N 337</td>
<td>C 148 N 3.8</td>
<td>0.51 N 0.011</td>
<td>100 C 100 N 1</td>
<td>1 C 1 N 1</td>
</tr>
<tr>
<td>100,000 x g</td>
<td>C 23 N 27</td>
<td>C 164 N 1.4</td>
<td>7.1 N 0.052</td>
<td>111 C 37 N 14</td>
<td>14 C 5 N 5</td>
</tr>
<tr>
<td>Eluate</td>
<td>C 1.2 N 1.1</td>
<td>C 117 N 0.3</td>
<td>97.5 C 0.27</td>
<td>79 C 8 N 191</td>
<td>25 C 10 N 10</td>
</tr>
<tr>
<td>CsCl pool</td>
<td>C 0.323 N 0.052</td>
<td>C 51 N 0.17</td>
<td>157.9 C 3.3</td>
<td>34 C 4.5 N 310</td>
<td>300 C 150 N 150</td>
</tr>
</tbody>
</table>

from both sources. The total recovery of the epitope was 35% from the tumor tissue and 20% from the normal tissue. The final enrichment, calculated as an increase of the slot-blot signal per protein weight, was about 300–310 for each preparation. This result of a rather simple three step procedure compares well with previously described data on mucin purification. Johnson et al. (15) achieved a 47-fold enrichment and 15% yield by TAG-72 mucin purification, and Prat et al. (16) obtained a 400-fold enrichment and a 36% yield of CAR-3 mucin. The DEAE ion exchange chromatography applied by Sheer et al. (17) resulted in a 1000-fold enrichment of TAG-72 molecules. In the case of AM-3-positive mucins there was a considerable loss of material on ion exchange column (not shown); therefore it was not routinely used in the present work.

The slot-blot and ELISA signals in the tumor extracts were always higher than in the extracts from normal colonic tissue. These results indicated that the epitope is expressed in larger amounts in tumor tissue and thus corroborated the results obtained previously by immunohistochemistry (12). The analysis of tissue extracts in slot-blot or ELISA indicated further a consistent qualitative difference between the AM-3-positive mucin from tumor and from normal colonic tissue. The slope of the obtained curves was steeper with tumor-derived mucins than with those derived from normal colonic tissue; this result was essentially the same with pooled material (Fig. 3) and with preparations from single patients (Fig. 4A) and was not affected by the interfering proteins in each preparation. This difference in slope was interpreted as a result of different avidity of the AM-3 antibody to the mucin molecules, the avidity to tumor-derived mucins being putatively higher than to their normal counterparts. Since AM-3 is a polyvalent antibody, the higher avidity could be related to the increased number of accessible epitopes present on the mucin core. The diameter of IgM molecules as seen by electron microscopy is about 15 nm (average of 10 measurements). This corresponds to a peptide chain of about 40 amino acids (in pleated sheet conformation), with about ten potential glycosylation sites (18). One IgM molecule may therefore bind several adjacent sugar epitopes, and a higher density of epitopes can be expected to result in a higher avidity of IgM binding to the mucin. Indeed, the partial removal of the epitopes from tumor-associated mucins by neuraminidase treatment yielded a product which behaved in slot-blot and in ELISA in a manner resembling that of the normal mucin.

Electron microscopy showed that the colonic mucins have the form of long flexible threads as they do in the cervical, gastric, and bronchial tissues (19). The length distribution of mucins from normal and tumor tissue was similar, about 90% of molecules isolated from each source being between 100 and 600 nm long. These data indicate that the possible shearing of the native molecule caused by the homogenization step had the same effect on the mucins from both sources. By comparison, the length of the human cervical mucin subunits, which were not homogenized prior to extraction, varied between 100 and 700 nm (14).

Rotary shadowing of mucin complexes with AM-3 antibody showed that 95% of the mucin molecules isolated from the tumor carried the AM-3 epitope, while in the normal mucin preparation about 74% were AM-3 positive. The mucins in normal and cancerous colonic tissue differed also in AM-3 epitope density. The average number of AM-3 antibodies bound to tumor-derived mucins was 34/1000 nm of protein core. To normal colonic tissue-derived mucins 12 molecules were bound per 1000 nm of protein core. This quantitative difference in the epitope density contributed to qualitative difference in the molecules from both sources, demonstrable through the different interaction with the antibody.
The observed increase of the antibody binding to tumor-derived mucins may be due either to a higher number or merely to a better accessibility of the AM-3 epitopes. Further, the large IgM molecule can react with several sugar epitopes; i.e., the number of the bound antibodies can be several times lower than the actual number of accessible epitopes. The resolving power of the procedure is also limited by the dimensions of the IgMs, which permit no more than about 70 molecules/1000 nm of the protein core. Within these limits the obtained data permit the conclusion that the average number of accessible epitopes on mucins from colonic carcinoma is severalfold higher than on mucins from normal colonic tissue.

These results give the first direct experimental evidence for the hypothesis formulated by Kannagi et al. (20), who deduced from solubility in perchloric acid that the tumor-derived serum mucins carry more type 2 chains than their normal counterparts. Preliminary data indicate that the AM-3 antibody indeed detects a type 2 chain-based carbohydrate antigen. Neither the biological effects of the increased density of AM-3 epitope on mucins nor the possible generality of this phenomenon as a carcinoma-associated mucin alteration have been investigated.

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The hypothesis recently formulated by Eggens et al. (21) suggests that surface molecules with multiple sugar epitopes (like Le" determinants) may modulate cell-cell interaction during embryogenesis. Whether the increase of AM-3 epitope density on mucins in the course of the adenoma-carcinoma sequence is related to morphological aberrations and the progress of dysplasia is under study.

In view of the present data, the concept of colon carcinoma-associated antigens deserves reevaluation, since the altered distribution of epitopes may yield a qualitatively different molecule species. These altered molecules may be functionally different and bind with different avidity to antibodies and possibly to physiological receptors. How far this qualitative alteration can be exploited for diagnostic purposes deserves further investigation.

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