Monoclonal Antibody against Human Colonic Sulfomucin: Immunochemical Detection of Its Binding Sites in Colonic Mucosa, Colorectal Primary Carcinoma, and Metastases

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ABSTRACT

Previous studies using metabolic labeling of fresh colonic mucosa and colorectal carcinoma with [35S]sulfate followed by biochemical analysis demonstrated that the amount of a sulfated high-molecular-weight glycoprotein expressed in primary colorectal carcinoma was lower than that in normal mucosa, and that the amount further decreased in liver metastases. This suggested that this sulfated molecule represented a sulfomucin previously defined by histochemical reactivity with a cationic dye. We have extracted and partially purified this high-molecular-weight sulfated glycoprotein from normal human colonic mucosa. We immunized mice with the partially purified sulfomucin and generated hybridomas. One cloned hybridoma, designated as 91.9H, produced a monoclonal antibody strongly reactive with a component which migrated at an identical position as the metabolically [35S]sulfate-labeled high-molecular-weight glycoprotein after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The reactive molecules appeared to have a polydisperse nature with a molecular weight ranging between 400,000 and 900,000. The [35S]sulfate-labeled high-molecular-weight glycoprotein was bound to Staphylococcus Protein A-agarose coated with this monoclonal antibody but did not bind to unconjugated Protein A-agarose. The immunoprecipitated substance also migrated at an apparent molecular weight range of 400,000 to 900,000. The reactivity of monoclonal antibody 91.9H with the extracts of normal mucosa, colorectal primary carcinoma, and metastasis was compared by dot blot assay on a nitrocellulose membrane. This antibody was more reactive with the extracts of mucosa adjacent to carcinoma tissues than with the carcinoma extracts. Primary tumors showed higher reactivity than metastases in most of the cases. These results strongly suggest that this antibody is specific to colonic sulfomucins or at least to mucins closely related to colonic mucins previously identified by metabolic labeling with [35S]sulfate.

INTRODUCTION

Carcinoma cells of a variety of origins are known to produce very high molecular weight glycoproteins containing a large number of serine- and threonine-linked carbohydrate chains. These glycoproteins often serve as antigenic determinants when human tumor cells or tumor cell membranes are used as immunogens in mice (1–9). Although the diagnostic value of these antigens and the therapeutic use of these monoclonal antibodies have been studied in many laboratories, the biological importance of various different types of mucins in determining malignant behavior of human carcinoma cells is not clear. Regulation of the expression of different immunochemical determinants has not been elucidated. We recently studied changes in human colorectal carcinoma tissues during tumor progression and acquisition of metastatic phenotypes by using tissue specimens available from surgical resections of tumors. We reported specific changes in high-molecular-weight glycoproteins with three different carbohydrate determinants: glycoproteins expressing ABO/Lewis blood group antigens (10), high-molecular-weight sialoglycoproteins (11, 12), and sulfated glycoproteins (13). In contrast, some other phenotypes, such as collagenolytic enzyme activity, did not show any correlation with metastatic potential (14).

The high-molecular-weight sulfated glycoproteins were found in smaller amounts in primary carcinoma than adjacent normal mucosa and almost disappeared in liver metastases (13). These glycoproteins were expressed in greater amount at the invasive edges of primary carcinomas than the luminal edges (13). The significance of these findings was 2-fold. (a) A family of high-molecular-weight glycoproteins which is histochemically designated as sulfomucin (15, 16) could be biochemically identified and quantitated using metabolic [35S]sulfate labeling of normal or tumor tissues in vitro followed by biochemical analysis. (b) The change of mucin production from sulfated to sialylated is one of the phenotypic alterations during tumor progression in addition to an early event in colon carcinogenesis (16–19). The sulfated high-molecular-weight glycoproteins from normal colonic mucosa and colorectal carcinoma were compared by electrophoresis before and after alkaline treatment and treatment with various proteases, and we have tentatively concluded that mucosa and carcinoma tissue produced very similar sulfomucin molecules in different quantity. Our next investigation focused on: (a) examination of the biological roles of the sulfomucin molecule as a possible determinant of tumor invasion and metastasis; (b) assessment of whether the expression of this molecule could be a useful prognostic indicator for colorectal carcinoma; (c) detailed studies on the intratumoral heterogeneity of the expression of this molecule; and (d) structural characterization and analysis of molecular diversity, focusing particularly on differences from sialomucins. For these purposes, purification and direct determination of sulfomucin were necessary in place of indirect methods relying upon metabolic labeling of tissue samples. Thus, we have attempted to develop a MAb3 specific to sulfomucin produced by normal colonic mucosa and colorectal carcinoma.

In this paper, we present data on the development and characterization of a MAb, designated as 91.9H (mouse IgG1), prepared against partially purified sulfomucin from human colonic mucosa. Immunochemical studies demonstrated that this MAb bound to normal colonic sulfomucins or at least to mucins closely related to sulfomucins. The same differential expression of sulfomucins among normal colonic mucosa, primary colorectal carcinoma, and liver metastases was observed, as we had previously shown by biochemical analysis of sulfomucin (13).

MATERIALS AND METHODS

Analytical Methods. Protein was estimated as previously described by Lowry et al. (20). Total neutral sugar was measured by the phenol

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3 The abbreviations used are: MAb, monoclonal antibody; BSA, bovine serum albumin; CHAPS, 3-[3-chloroallylpropyl(dimethylammonio)]-1-propanesulfonate; DPBS, Dulbecco's phosphate-buffered saline; SDS, sodium dodecyl sulfate; i.d., intradermal.
Source of Colorctal Mucosa and Carcinoma Tissues and Purification of Sulfated Glycoprotein from Colorctal Mucosa. Tissue specimens were obtained from patients undergoing surgery of colorectal carcinoma. Colorectal carcinoma was routinely staged according to Astler and Coller's modification (24) of Dukes' classification (25). Fresh tissue specimens were immediately processed for metabolic labeling or frozen in liquid nitrogen and stored at -70°C. Two normal colorectal mucosa specimens used for purification of sulfomucin were obtained from the rectum of a 58-yr-old man and the ascending colon of a 63-yr-old woman resected during surgical treatment of cancer. Approximately 100 cm² of mucosa were separated from the muscularis.

The extracts from the normal mucosa from two patients were processed separately, combined at this step, dialyzed against distilled water, and lyophilized. [³⁵S]Sulfate-labeled and nonlabeled specimens were processed separately during the entire purification procedure. The lyophilized extracts were dissolved in 40 ml of 50 mM sodium acetate buffer (pH 6.0) containing 50 mM sodium chloride, 8 mM urea, and 0.2% CHAPS. The material eluted at 0.2 M sodium chloride was pooled (Peak I), dialyzed against distilled water, and lyophilized. This material was dissolved in 17.5 ml of 10 mM Tris-HCl (pH 8.0) and fractionated on a Sepharose CL-4B column (2.5 x 80 cm) in the presence of 8 M urea, 0.2% CHAPS, and 0.2% sodium acetate buffer (pH 6.0) containing 4 M guanidine hydrochloride. The fractions were eluted with 0.1 M glycine-HCl buffer (pH 2.5).

Preparation of Monoclonal Antibodies. Five-wk-old BALB/c mice were immunized by i.d. injection of partially purified colonic sulfomucin emulsified in Freund's complete adjuvant (1:1) and 1.25 µg/ml of amphotericin B and incubated with a 1:1 mixture of Dulbecco's modified minimal essential medium and Ham's F-12 medium containing antibiotics as above and [³⁵S]labeled disodium sulfate at a concentration of 50 µCi/ml. Incubation was performed to a ratio of approximately 10 cm² of mucosa tissue to 10 ml of medium under humidified conditions with 5% carbon dioxide at 37°C for 48 h. At the end of the incubation, the medium was removed, and the tissues were rinsed with chilled DPBS. From the labeled or fresh mucosa, epithelial cells were removed by scraping the surface with a glass slide. The epithelial cells from 50 cm² of mucosa were suspended in 60 ml of calcium and magnesium-free DPBS containing 2 mM phenylmethylsulfonyl fluoride. After ultrasonication on ice for 20 s, the suspension was centrifuged at 10,000 x g for 1 h. The pellets were resuspended in 10 ml of the same buffer and centrifuged again at 100,000 x g for 1 h. The supernatants from the first and second centrifugations were combined.

The extracts from the normal mucosa from two patients were processed separately, combined at this step, dialyzed against distilled water, and lyophilized. [³⁵S]Sulfate-labeled and nonlabeled specimens were processed separately during the entire purification procedure. The lyophilized extracts were dissolved in 40 ml of 50 mM sodium acetate buffer (pH 6.0) containing 50 mM sodium chloride, 8 mM urea, and 0.2% CHAPS (Buffer A) and applied to a column of DEAE-Sephacel (Pharmacia, Uppsala, Sweden) (2.8 x 8 cm) previously equilibrated with Buffer A. The column was eluted with Buffer A until no materials having absorption at 280 nm or radioactivity were detected, and further eluted first with 0.11 M sodium acetate buffer (pH 6.0) containing 0.2 M sodium chloride, 8 mM urea, and 0.2% CHAPS, then with 0.23 M sodium acetate buffer (pH 6.0) containing 0.5 M sodium chloride, 8 mM urea, and 0.2% CHAPS (13). The material eluted at 0.2 M sodium chloride was pooled (Peak 1), dialyzed against distilled water, and lyophilized. This material was dissolved in 17.5 ml of 10 mM Tris-HCl buffer (pH 8.0) and fractionated on a Sepharose CL-4B column (2.5 x 100 cm) in the presence of the same buffer for the elution. The fractions were assayed for their absorption at 280 nm, for total neutral sugar (21), and for radioactivity in the case of radiolabeled samples. Fractions with high content of neutral sugar and sulfate label were pooled and used as the final preparation of sulfomucin.

Preparation of Monoclonal Antibodies. Five-wk-old BALB/c mice were immunized by i.d. injection of partially purified colonic sulfomucin emulsified in Freund's complete adjuvant (final 10 to 30 µg/ml per 0.1 ml per mouse). Additional antigen (5 to 15 µg) was administered by i.d. injection with Freund's incomplete adjuvant at 2- to 4-wk intervals. The final boosting was given by i.v. injection of 15 µg of antigen 3 days prior to the mice. The booster was repeated 3 times with 0.05% Tween-20 in DPBS. Fifty µl of hybridoma culture supernatant were added to each well, and the plates were incubated at room temperature for 2 h. The plate was then washed 3 times with 0.05% Tween-20 in DPBS. Fifty µl of 1:1000 diluted goat anti-mouse IgG-peroxidase conjugate (BioRad, Richmond, CA) were added to each well. The plates were incubated for 1 h at room temperature and washed 3 times with Tween-20 in DPBS. Then 150 µl of 0.15 mg/ml of 2,2-azino-di(3-ethylbenzthiazolinsulfonate) (Behringer-Mannheim, Indianapolis, IN) with 0.015% hydrogen peroxide in 0.1 mM sodium citrate buffer, pH 4.0, were added to each well. After incubation at room temperature for 20 min, the reaction was stopped by the addition of 35 µl of 1 M sodium fluoride to each well, and absorption at 414 nm was measured by a Titertek (Flow, McLean, VA) multispecimen reader. Immunoglobulin subclasses of the antibodies were determined by a similar enzyme immunoassay using a typing kit with defined anti-immunoglobulin antibodies obtained from Behringer-Mannheim.

Electrophoretic Analysis. Immunoperoxidase staining of colonic sulfomucin with MAb's after electrophoretic separation on polyacylamide gels was performed as follows. Twenty µg of partially purified sulfomucin containing approximately 4000 cpm of [³⁵S]sulfate were dissolved in 2% SDS, 3% mercaptoethanol, 0.5 mM EDTA, 10% glycerol, and 62.5 mM Tris-HCl (pH 6.8) (electrophoresis sample buffer). Electrophoresis was carried out on 5% slab gels as previously described (26). The gels were fixed in 25% isopropanol:10% acetic acid for 2 h at room temperature and neutralized by repeated washing in 25 mM Tris-HCl buffer (pH 7.3) containing 0.14 M sodium chloride. The gels were incubated first with hybridoma culture supernatant (diluted 1:10) and then with goat anti-mouse IgG-peroxidase conjugate diluted 1:200 in 0.05% Tween-20 in DPBS containing 2% BSA. During incubation, the gels were continuously shaken for 4 h at room temperature. After each incubation, they were washed in 25 mM Tris-HCl buffer (pH 7.3) containing 0.14 M sodium chloride for 18 to 24 h with several changes. The bound antibodies were made visible by incubating with 0.4 µg/ml of 4-chloro-1-naphthol in 5 mM Tris-HCl buffer (pH 7.4) containing 0.02% hydrogen peroxide for 15 min. The dried gels were photographed and processed for autoradiography.

Immunoprecipitation and Immunofluorescence Chromatography. MAb 91.9H, ascitic fluid was diluted 1:10 in 0.5 M sodium acetate, 0.015% hydrogen peroxide, and 62.5 mM Tris-HCl (pH 6.8) containing 0.15 mg/ml of 2,2-azino-di(3-ethylbenzthiazolinsulfonate) (Behringer-Mannheim) by eluting 0.5 µl of hybridoma ascitic fluid diluted 1 to 10 with DPBS. The MW of Protein A-agarose was determined by eluting 0.5 µl of Protein A-agarose with 50 µl of 100 mM Tris-HCl buffer (pH 7.3) containing 0.14 M sodium chloride for 18 h at room temperature. After each incubation, they were washed in 25 mM Tris-HCl buffer (pH 7.3) containing 0.14 M sodium chloride for 24 h with several changes. The bound antibodies were made visible by incubating with 0.4 µg/ml of 4-chloro-1-naphthol in 5 mM Tris-HCl buffer (pH 7.4) containing 0.02% hydrogen peroxide for 15 min. The dried gels were photographed and processed for autoradiography.

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Dot-Blot Analysis. To quantitate the sulfomucins content of colonic mucosa and colorectal carcinoma tissues, dot-blot analysis was performed as follows. Minced tissues (approximately 100 mg) were mixed with 0.1 M sodium acetate buffer containing 4 M guanidine hydrochloride, 4% 2,2-azino-di-(3-ethylbenzthiazolinsulfonate) (Behringer-Mannheim, Indianapolis, IN) with 0.015% hydrogen peroxide in 0.1 mM sodium citrate buffer, pH 2.5. The protein content of the extracts was determined by a BCA assay (Pierce, Rockford, IL).

as previously described (20), using BSA as a standard, and diluted to 250 μg of protein/ml with 0.1 M sodium acetate buffer (pH 6.0) containing 4 M guanidine hydrochloride. Two-fold dilutions of the extracts were prepared with the same buffer in 96-well microtiter plates, and these samples were blotted onto nitrocellulose membranes as described previously (27). The staining procedures were the same as the staining of polyacrylamide gels as described above, and hybridoma culture supernatant diluted 1:10 was used. The maximum dilutions of the tissue extracts that produced visible staining were recorded and designated as titration scores.

RESULTS

Purification of Sulfomucin from Normal Colonic Mucosa. In a previous study, we demonstrated that primary colorectal carcinoma tissues produced sulfated glycoproteins very similar to those produced by normal colonic epithelial cells (13). Therefore, we decided to purify and characterize sulfated glycoproteins from normal colorectal mucosa which is available in relatively larger quantities than carcinoma tissue. As previously described (13), the major sulfated macromolecules produced by colorectal mucosa tissues or colorectal carcinoma tissues that were eluted from DEAE-cellulose columns with 0.2 M sodium chloride contained alkaline-labile sugar chains to which sulfate groups were attached (13). Thus, we tentatively called this molecule sulfomucin.

[35S]Sulfate-labeled or nonlabeled sulfomucin was isolated from the normal mucosa of two individuals as described in "Materials and Methods." Fig. 1 is the elution profile of [35S]-sulfate-labeled macromolecules on DEAE-cellulose in the presence of 8 M urea. Fractions eluted when the concentration of sodium chloride was elevated to 0.2 M were pooled, dialyzed against water, and lyophilized. This material was then dissolved in 25 mM Tris-HCl buffer (pH 8.0) and fractionated by gel filtration on Sepharose CL-4B as shown in Fig. 2. A sharp peak of neutral sugars appeared immediately after the void volume. The major peak of [35S]sulfate-labeled material also was seen at the same position. This peak was distinct from the major peak of absorption at 280 nm, which corresponded to proteins. The fractions consisting of the major portions of neutral sugar and sulfate-label were pooled, dialyzed against water, and lyophilized. The yield was approximately 45 mg from 300 cm² of colonic mucosa tissues. The lyophilized material contained sulfate at 1:4.5 weight ratio to neutral sugar and 1:4.5 to amino acid. Although 20 μg of partially purified sulfomucin separated by polyacrylamide gel electrophoresis were not visibly stained by Coomassie brilliant blue, a single component with a polydisperse nature was seen by autoradiography (see Fig. 3, Lane 1). An apparent range (Mₙ 400,000 to 900,000) was estimated from the migration position of intact laminin (Mₙ 880,000), large subunit of laminin (Mₙ 440,000), and small subunit of laminin (Mₙ 220,000) on the same gels.

Preparation of MAbs and Immunochemical Characterization of MAb 91.9H Reactive Molecules. Splenic lymphocytes from an immunized mouse were fused with SP2/0 myeloma cells and plated into 107 wells. We obtained 10 hybridoma cultures secreting antibodies reactive with partially purified sulfomucin and cloned these hybridomas by limited dilution techniques. Culture supernatants of the cloned hybridoma cells were further characterized for their reactivity with partially purified sulfomucin using an enzyme immunoassay (binding assay), staining of electrophoretically separated [35S]sulfate-labeled purified sulfomucin, and immunohistochemical staining of normal colonic mucosa and colon carcinoma. A hybridoma cell line designated as 91.9H was the only hybridoma producing an antibody reactive to a component containing [35S]sulfate-labeled purified sulfomucin (Fig. 3). A similar result was obtained after agarose
Staining of the membrane was performed by an identical method as stainings of the gels except that 0.5% normal goat serum was included in the blocking solution in addition to 2% BSA (data not shown). Purified sulfomucins metabolically labeled with $[^{35}\text{S}]$sulfate were precipitated with MAb 91.9H-linked Protein A-agarose (Fig. 4). There was only little nonspecific binding to Protein A-agarose. The precipitated $[^{35}\text{S}]$sulfate-labeled sulfomucins showed the same electrophoretic mobility as the starting material as revealed by staining with MAb 91.9H or radioactivity (Fig. 5). These results strongly suggested that MAb 91.9H bound to human colon sulfomucin. $[^{35}\text{S}]$Sulfate-labeled sulfomucins bound to a column of Protein A-agarose coated with MAb 91.9H, but did not bind to normal mouse serum-coated Protein A-agarose (Fig. 6).

Dot Blot Analysis of Tissue Extracts for Sulfomucin Contents by MAb 91.9H. Tissue extracts prepared from normal colonic mucosa, primary colorectal carcinoma, and liver metastases were examined for the reactivity with MAb 91.9H by the dot blot method. Partially purified sulfomucin at levels as low as 10 to 20 ng was detectable by this assay. Typical results are shown in Fig. 7. In these three sets of extracts obtained from three different individuals, the titration score of 91.9H reactivity was the highest with normal mucosa, intermediate with primary carcinoma, and lowest with liver metastases. One of these sets of extracts was also tested for reactivity with normal mouse serum, and another with MAb 115D8 which was generated against a sialylated mucin from mammary epithelial cells (29, 30). Fig. 7 shows that none of these extracts had reactivity with normal mouse serum and that MAB 115D8 had greater reactivity with metastasis rather than primary colon carcinoma or normal mucosa. As shown in Fig. 8, our examination of tissue extracts obtained from a total of 16 Dukes’ Stage D patients revealed that normal mucosa had the greatest reactivity with MAb 91.9H, followed by primary carcinoma and then liver metastases, in 12 of 16 cases ($P = 0.038$). Wilcoxon's signed rank analysis indicated significant differences between normal mucosa and primary carcinoma ($P < 0.05$), and between primary carcinomas and metastases ($P < 0.01$). Fig. 9 shows the categorized data. Kruskal-Wallis tests also revealed significant differences among these three categories ($P < 0.01$). These results strongly suggest that MAb 91.9H is specific for the colorectal carcinoma sulfomucins identified by metabolic labeling with $[^{35}\text{S}]$sulfate and anion exchange chromatography (13).

Comparison among Primary Tumors at Different Stages. The extracts from 54 colorectal primary carcinoma specimens were tested for their reactivity with MAb 91.9H using dot-blot assays. The titration score did not correlate with the stages according to Dukes’ staging with Astler and Coller’s modification (24, 25), or with histological grading (31) (data not shown).

**DISCUSSION**

Colorectal sulfomucin has been a focus of attention for clinical pathologists, since Filipe discovered its presence in colonic mucosa and disappearance in colorectal carcinoma with concomitant increase of sialomucin (15, 30). These discoveries were made using histochemical techniques with cationic dyes. Later Filipe and Branfoot and others reported that these changes were not directly associated with malignant transformation of colonic epithelial cells but rather one of the physiological changes representing a premalignant stage of colonic...
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Fig. 6. Immunoaffinity chromatography of purified [35S]sulfate/[3H]serine double-labeled sulfomucin on Protein A-agarose coated with MAb 91.9H (mouse ascitic fluid) (a), or Protein A-agarose coated with normal mouse serum (b). Two hundred µl each of Protein A-agarose column were used, and sulfomucin corresponding to 12.5 µg of protein as determined by the method of Lowry et al. (20) was applied. Elutions were performed with DPBS containing 1% BSA and 0.05% Tween-20, and 50-µl fractions were collected at 5- to 10-min intervals. Eluting solution was changed to 100 mM glycine-HCl buffer (pH 2.5) as indicated by arrows. The counts from [35S]sulfate (A) and [3H]serine (O) were determined separately.

Fig. 7. Dot-blot assays for the comparison of antibody reactivity of tissue extracts from primary colorectal carcinoma, its liver metastasis, and mucosa adjacent to the primary carcinoma. The extracts of tissues were prepared with 0.1 M sodium acetate buffer containing 4 M guanidine hydrochloride, 4% Zwittergent 3-12, and protease inhibitors as described previously (13) at a ratio of 100 mg of wet tissue/ml. After being diluted to adjust the protein concentration to 250 µg/ml, the samples were serially diluted (2-fold) and blotted to nitrocellulose membrane by gentle aspiration. The membranes were reacted with normal mouse serum, anti-mammary sialomucin antibody 115D8, or MAb 91.9H. After repeated washing, the membranes were reacted with rabbit anti-mouse IgG antiserum and then incubated with 4-chloro-1-naphthol. N, P, and LM represent extracts of normal mucosa adjacent to the primary carcinoma, primary colon carcinoma, and liver metastasis, respectively. We visually examined the stained membrane and define titration score based on the relative intensity. For example, the sixth dot of primary carcinoma stained with MAb 91.9H shows the highest dilution, and thus, the score of this specimen at 250 µg/ml was 6.

Fig. 8. Comparison of MAb 91.9H reactivity with tissue extracts from 16 Stage D colorectal carcinoma patients. The scores of the extracts from normal mucosa (O), primary colorectal carcinoma (P), and liver metastasis (A) from the same individual were connected (---). Abscissa indicates individual patients. As described in the text, the scores of primaries were significantly lower than those of normal mucosa (P < 0.05, Wilcoxon's signed rank test), and the scores of liver metastases were significantly lower than those of primaries (P < 0.01, Wilcoxon's signed rank test).

Fig. 9. Titration scores of MAb 91.9H with normal mucosa, colorectal primary carcinoma, and liver metastases. These plots are based on the same data presented in Fig. 8, but grouped according to normal mucosa (N), primaries (P), or liver metastases (M). Bars indicate medial scores of these three groups. The difference among these three groups was significant as judged by the Kruskal-Wallis test (P < 0.01).

epithelial cells (16, 17). Although the use of these changes seen in colorectal carcinoma and near the tumor margins as markers for diagnosis and prognosis is still debatable (17–19), there was no alternative method to identify these molecules other than by staining with cationic dyes. In a previous report, we showed that sulfated glycoproteins that were eluted from DEAE-cellulose at 0.2 M sodium chloride were present in smaller amounts in colorectal carcinoma than normal colonic mucosa, and further decreased in liver metastases (13). Sulfated glycoproteins produced by colorectal carcinoma cells seemed to be very similar to those produced by normal mucosa (13). We have also suggested that these molecules are sulfomucins previously defined by histochemical methods. To identify and characterize these molecules in more detail, we have developed MABs specific to colonic sulfomucin.

We used morphologically normal colonic mucosa from colorectal carcinoma patients as a source of sulfomucin. From extracts of colonic mucosa, we obtained slightly acidic macromolecules and removed nonsulfated protein constituents by gel filtration. Although it was obvious that the large-molecular weight glycoprotein fraction seen in Fig. 2 contained glycoproteins bearing sulfate groups, this fraction might contain high-molecular-weight glycoproteins other than sulfomucins. Assuming that sulfomucin represented a portion of normal colonic acidic mucin, we attempted to separate sulfated high-molecular-weight glycoproteins from nonsulfated high-molecular-weight glycoproteins. We applied a variety of fractionation methods including wheat germ agglutinin-agarose, hydroxyapatite, anion and cation exchange (under various pH), and gel permeation chromatography to fractionate the [35S]sulfate- and [3H]glucosamine double-labeled sulfomucin preparation. However, we could not separate sulfated glycoproteins from nonsulfated
acidic high-molecular-weight glycoproteins that contained only $[^3H]$glucosamine. An exception to this was $[^3H]$glucosamine-label hyaluronic acid occasionally seen in varying amounts in a crude sulfomucin preparation, and it was removed by chondroitinase ABC treatment. Therefore, we concluded that all of the high-molecular-weight component of the Peak I fractions (Fig. 1) which eluted at 0.2 M sodium chloride from DEAE-cellulose contains sulfate groups, and we decided to use this preparation for immunization and screening. Later, we found that Peak I contained predominant portions of MAb 91.9H-reactive substances in colonic mucosa.

The results of immunoblotting, immunoprecipitation, immunoaffinity chromatography, dot blot assays, and immuno-histochemical staining of tissue sections strongly confirmed that MAb 91.9H is specific to sulfomucin. The differential expression between normal mucosa and carcinoma tissues was the only characteristic previously known to define colorectal sulfomucin. The differences between normal and carcinomatous tissues in the titration scores with this MAb coincided with the differences in the relative amounts of sulfomucin determined by metabolic $[^35S]$sulfate labeling of fresh tissues followed by analysis using DEAE-cellulose chromatography (13). Furthermore, the cells stained with the high-iron diamine method (32) were also reactive with MAb 91.9H. However, these results do not necessarily mean that the sulfate group forms an important portion of the antigenic epitope for MAb 91.9H. Removal of sialic acid by neuraminidase treatment did not significantly influence reactivity, indicating that sialic acid is not involved in the antigenic epitope in a negative or positive fashion. It has been suggested that there is heterogeneity in the polypeptide backbones of different types of human intestinal mucins (33, 34). Whether the polypeptide portion is involved in the formation of the antigenic epitope of 91.9H is unknown. The detailed experimental results of our antibody specificity study will be published separately. The results of our immunoprecipitation experiments and immunoaffinity chromatography strongly suggested that only a portion of purified sulfomucin was reactive with MAb 91.9H. The bound and unbound fractions had the same electrophoretic mobility, indicating that sulfomucin with polydisperse nature contained heterogeneous subpopulations having different affinity with MAb 91.9H regardless of their molecular weight. This hypothesis was supported by the fact that immunohistochemical subcellular localization of MAb 91.9H binding sites and high iron diamine staining were slightly different. These results will also be published elsewhere.

Studies of a variety of antigens associated with human colonic epithelium have already been reported. Goldenberg et al. (35) have described "colon specific antigen" representing a family of three antigens associated with normal and malignant gastrointestinal tissues. Burtin et al. (36) have shown the loss of "colon membrane antigen" in colon carcinomas. Gold and Miller (37) have described "colon mucoprotein antigen" which is present in both normal and malignant tissues. They suggested that this antigen in carcinomas had a lower density of carbohydrate moieties on the peptide core than in normal colon tissues, and that the tumor antigen was different from the normal one in its core polypeptide structure (37). A colonic antigen called 3MM was detected by a MAb prepared against isolated colonic glands, but it was absent from colon carcinoma (38). Recently, Muraro et al. (39) have shown a decrease of an antigen called "colon associated antigen" in colon carcinoma from the level of normal mucosa as revealed by a MAb. This antigen has a molecular weight greater than 200,000, and it is distributed in the cytoplasm of goblet cells. The expression of this antigen seemed to be closely associated with morphological differentiation of colon carcinoma, and poorly differentiated carcinoma produced a lower level of this antigen than well-differentiated carcinoma. It appears that the antigenic moiety recognized by MAb 91.9H was similar to this "colon associated antigen." However, MAb 91.9H did not seem to distinguish well and poorly differentiated colorectal carcinomas. Further biochemical studies will be necessary to clarify the differences between the epitopes of these two MAbs. Although MAb 7E12H12 reported by Das and coworkers has shown a similar histochemical distribution in colonic epithelial cells, the antigen for this MAb was a $M_r$ 40,000 protein (40).

We have previously shown that the sulfate groups in sulfomucin are attached to oligosaccharide chains (13). Preliminary analyses of oligosaccharides obtained by alkaline borohydride treatment of sulfomucin suggested that sulfate-bearing oligosaccharides also contained sialic acid. Filipe (14) demonstrated histochemically a decrease of sulfomucins and a simultaneous increase of sialylated mucins in colon carcinomas as well as in transitional mucosa.

Little was known about the changes associated with the metastatic potential of colon carcinoma cells. We have shown that the expression of sulfomucin is significantly lower in liver metastasis than in primaries as determined by biochemical analysis of $[^35S]$sulfate-labeled sulfomucins or by MAb 91.9H reactivity. We have shown that increased wheat germ agglutinin binding to $M_r \sim 900,000$ sialoglycoprotein is associated with increased metastatic potential of colorectal carcinoma (11, 12). Experimental animal studies on metastases indicated that the primary tumor consists of cell populations with different metastatic potentials and that metastases result from the selective growth of those subpopulations having higher metastatic potentials (41, 42). Considerable evidence now exists that the primary colon tumors of mice (43-46) and humans (47, 48) consist of heterogeneous cell populations with different metastatic potentials. The decreased amount of sulfomucin in metastases suggests that a relatively small population of tumor cells in the primary colon tumor that produces less sulfomucin may selectively metastasize to the liver. This possibility may be supported by heterogeneous distribution of sulfomucin in the primary colon tumor. The amounts of sulfomucins in the deep region of primary tumors were smaller than those in the superficial region. Bresalier et al. (49) have also indicated that the amount of the mucin reactive with peanut agglutinin decreases in metastases compared with the mucin produced by corresponding primary colon tumors, suggesting that colon cancer cells with high metastatic potential may produce a mucin lacking an exposed oligosaccharide receptor for this lectin. These phenomena may be explained by alternative mechanisms whereby the production of sulfomucin is halted, modulated, or lost during tumor growth and metastasis due to the influence of surrounding tissues. Nonetheless, it is interesting to test whether the appearance of sialomucins as well as so-called "colon cancer associated" mucin-like antigens and MAb 91.9H reactivity is related. If there is a mechanism that turns off the expression of sulfomucin and simultaneously turns on the expression of sialomucins, then such a mechanism may be related to the fundamental events causing malignant transformation, tumor progression, and metastasis.

MAb 91.9H should have many uses including: (a) examining distribution of sulfomucin in normal and carcinoma tissues; (b) elucidating the molecular structure of sulfomucin and the molecular basis for its differential expression; and (c) proving the
biological significance of sulfomucins. Sulfated glycoproteins may be biologically significant in systems such as cellular differentiation (50), egg-sperm interaction (51), and the processing of glycoprotein hormones (52). Sulfated glycoproteins are important constituents of basement membranes and are involved in cellular adhesion (53, 54). In gastrointestinal systems, the protection of luminal surfaces against protease attack was one of the possible roles of sulfated mucins (55). However, none of these hypotheses was supported by the structural characteristics and biosynthetic regulation of sulfated carbohydrate chains. Furthermore, most of the studies on sulfated glycoproteins were carried out on asparagine-linked carbohydrate chains, and the structures of sulfated carbohydrate chains in serine/threonine-linked carbohydrate chains have not been elucidated. MAb 91.3H may be useful for studying the structural uniqueness of sulfated mucins and the regulatory mechanism of their biosynthesis in a variety of epithelial tissues.

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