Jpn. J. Cancer Res. 86, 1060–1067, November 1995

Expression of Sulfomucins in Normal Mucosae, Colorectal Adenocarcinomas, and Metastases

Yoshifumi Matsushita,[1,5] Noriko Yamamoto,[1] Hiroshi Shirahama,[1] Sadao Tanaka,[2] Suguru Yonezawa,[1] Takao Yamori,[3] Tatsuro Irimura[4] and Eiichi Sato[1]

[1]*Department of Pathology II, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890,* [2]*Pathology Division, Kagoshima-shi Ishikai Hospital, 7-1 Kamoike-shinmachi, Kagoshima 890,* [3]*Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170 and* [4]*Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113*

We have examined the expression of specific mucin antigens in tissue sections from 92 cases of colorectal carcinoma, using sulfomucin-specific monoclonal antibody (MAb) 91.9H. The expression of sulfomucins was high in normal mucosae and much lower in primary colorectal carcinoma, in metastatic lesions in lymph nodes or in liver. The intracellular localization of sulfomucins was also different among these tissues. In normal mucosae, MAb 91.9H binding was seen in the supranuclear area, presumably Golgi complexes, the luminal surface, and secretory products. In primary colorectal carcinomas and in their metastatic lesions, MAb 91.9H was preferentially localized in the cell surface and substances attached to the luminal surface of glandular structures. Analysis of the lysates of normal and tumor tissues showed that very-high-molecular-weight components contained the antigenic epitopes. The intensity of MAb 91.9H binding was lower in tumors at advanced stages than in tumors at early stages. These high-molecular-weight components were apparently reactive with MAb FH6 specific for sialyl-Le^X (s-Le^X) structures. Histological specimens with low levels of MAb 91.9H reactivity often exhibited relatively high levels of MAb FH6 reactivity. These two mucins may have reversed expression during carcinogenesis and carcinoma progression, and this change may be related to metastatic potential.

Key words: Colorectal cancer — Mucin antigen — Metastasis

Cancer metastasis is one of the most critical prognostic factors for patients with malignant tumors. The mechanism of metastasis involves a variety of tumor cell-host interactions.[1–8] In the metastatic cascade, cell surface molecules are believed to play crucial roles in recognition and in the interaction between tumor cells and host cells. Various molecules have been proposed to be involved in these cellular events, based on studies with experimental systems,[9–12] but little is known about the significance of these molecules as determinants of metastasis in human cancers. We have previously reported that the levels of several high-molecular-weight mucin-like glycoproteins in colorectal carcinoma at advanced stages were different from those at early stages.[13–21] Significant differences in mucin expression were observed between primary sites and metastases. For example, the contents of *Ulex europaeus agglutinin-I*-binding high-molecular-weight glycoproteins (type-II blood group-H antigens and/or Lewis-Y antigen) or monoclonal antibody (MAb) 91.9H-binding sulfomucins were lower in metastatic lesions than in primary tumors.[13, 19, 21] Conversely, MAb FH6 binding was higher in metastatic lesions.[17, 20] These results suggested that blood-group related antigens and/or high-molecular-weight mucins may influence the metastatic behavior of tumor cells. In experimental animals, it was reported that a combination of various biological, biochemical or molecular properties contribute to the metastatic potential.[22–27] Among these properties, carbohydrate antigens may affect metastatic potentials by influencing tumor cell-induced platelet aggregation, tumor cell adhesion to extracellular matrices, and sensitivity to the host immune systems.[7, 22, 26–33] Furthermore, it was recently reported that sialyl-Lewis^X (s-Le^X) functions as an adhesion molecule.[9, 10]

In this study, we investigated the expression of sulfomucins recognized by MAbs 91.9H to see how the expression of high-molecular-weight sulfomucin changes during carcinoma progression and/or metastatic formation. MAb 91.9H binding was often observed in cells which were not reactive with MAb FH6. This unique pattern of sulfomucin expression may provide new information on the role of altered glycosylation during colorectal carcinoma progression to metastatic phenotypes.

MATERIALS AND METHODS

Colorectal cancer specimens Ninety-two cases of colorectal carcinoma specimens were examined. Eight adeno-

[5] To whom correspondence should be addressed.

carcinomas of the colon and rectum in Dukes stage A, forty-nine Dukes stage B, twenty-seven Dukes stage C, and eight colon carcinomas in Dukes stage D were obtained from patients who had undergone surgery and pathologically examined at Kagoshima-shi Ishikai Hospital and Kuroki Hospital in Kagoshima city. These specimens were processed for routine pathological examinations. Fresh frozen specimens were prepared for biochemical studies from some materials immediately after surgery. All cases were clinically and pathologically confirmed as adenocarcinoma of the colon or rectum, and were classified according to Dukes' classification as modified by Astler and Coller.[34, 35]

MAbs MAb FH6 was a gift from Prof. Hakomori (The Biomembrane Institute and University of Washington, Seattle).[36–38] MAb FH6 recognizes terminal carbohydrate s-Le^X antigen, which is a sialylated form of stage-specific embryonic antigen SSEA-1 and also a ligand for selectins expressed on endothelial cells. MAb 91.9H was raised against purified sulfomucin from normal colonic mucosae and this antigen is expressed on the MUC2 gene products. The characteristics of this MAb were described previously.[19, 21] In gastric cancer, the epitope detected by MAb 91.9H may be expressed on other proteins than MUC1 or MUC2 core protein.[39]

Immunohistochemistry Immunohistochemical staining methods were described previously.[20] Briefly, serial sections of surgical specimens fixed in buffered formaldehyde and embedded in paraffin were deparaffinized and treated with 0.03% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. The sections were rehydrated and washed with Dulbecco's phosphate-buffered saline (DPBS), then incubated with 1% bovine serum albumin (BSA, RIA grade, Sigma, St. Louis, MO) dissolved in DPBS for 2 h at room temperature or overnight at 4°C. MAb FH6, specific for s-Le^X carbohydrate antigen, was diluted to suitable concentrations (1:5–1:20 of culture supernatant of hybridoma cells and 1:200–1:1,000 of ascites fluid produced by intraperitoneally injected hybridoma cells) in 1% BSA in DPBS. After incubation for 2 h at room temperature or overnight at 4°C, the sections were washed several times with DPBS and incubated with biotinylated anti-mouse IgM for 60 min at room temperature. After several washes, the sections were incubated for 60 min with solutions of avidin-biotin-horseradish peroxidase complex (ABC staining kit including anti-mouse IgG or IgM was purchased from Vector Laboratories, Burlingame, CA) for 60 min at room temperature. After several washes with DPBS, the sections were incubated with a peroxidase substrate solution prepared by mixing 1.5 ml of 30 mg/ml diaminobenzidine (DAB from Dojin Pharmaceuticals, Kumamoto), 150 ml of 100 m*M* PBS, and 100 ml of 30% hydrogen peroxide. The sections were then counterstained with Meyer's hematoxylin and mounted with Eukitt mounting reagent (O. Kindler, Germany).

Classification of degree of monoclonal antibody reactivity with tumor cells Because of the heterogeneity of the cancer, the degree of MAb staining of individual tissue sections was classified according to the percentage of stained carcinoma cells among all carcinoma cells in the entire section as described previously.[20] In the immunohistochemical examination of colorectal carcinoma tissues with MAb 91.9H, the positivity was estimated, using the classification described below, by at least two pathologists independently. When the two pathologists assigned different classifications, the results were discussed until agreement was reached. To compare the distribution of carcinoma-related mucin, some freshly obtained and frozen tissues were stained with MAb FH6 and MAb 91.9H in serial sections. We examined the cytoplasm, cell membrane (cell surface or luminal surface), and associated secretory products independently and assigned a positive score when at least one of these components was positive. The grading of the staining was as follows: 0, totally negative to less than 5% of at least one of the three areas, described above, of carcinoma cells stained; +1, 5–30% of at least one area of carcinoma cells stained; +2, 30–60% of at least one area of carcinoma cells stained; and +3, >60% of at least one area of carcinoma cells stained. Adenomas in the examined cases were excluded from the evaluation. In the primary carcinomas and metastatic lesions from the same individuals, MAb reactivity was compared.

Electrophoretic analysis Electrophoretic analysis of high-molecular-weight mucin from colorectal carcinoma was performed as previously described[20] with slight modifications. Briefly, frozen tissues were thawed and homogenized with 10 volumes of 0.5% NP-40 in 0.25 *M* sucrose, 10 m*M* Tris-HCl, 50 m*M* $CaCl_2$, and 10 m*M* phenylmethylsulfonyl fluoride pH 7.2. Supernatants collected after centrifugation at 13,000 rpm for 5 min were assayed for protein content by Lowry's method.[40] This supernatant was mixed with 1/2 volume of 6% sodium lauryl sulfate, 3% 2-mercaptoethanol, 30% glycerin, and 5 m*M* EDTA in 18.75 m*M* Tris-HCl buffer pH 6.7 and heated at 100°C for 5 min. From these treated extracts, samples corresponding to 100 μg of protein from each were loaded on gels for electrophoretic analysis. Polyacrylamide gel electrophoresis was performed in 1.5 mm thick 3.3% polyacrylamide running gels with 3% stacking gels supported by Gel Bond (FMC BioProducts, Rockland, ME) as described previously.[20] The gels were fixed in 25% isopropanol and 10% acetic acid solution for 4 h and neutralized by repeatedly rinsing them in 10 m*M* Tris-HCl buffer pH 7.4. After 24–48 h, the gels were reacted with each MAb at a suitable dilution at 4°C for 4

h. After having been washed with PBS several times for 30 min each, the gels were reacted with biotinylated anti-mouse immunoglobulin (Sigma) and with horseradish peroxidase-conjugated avidin (Bio-Rad, Richmond, CA). 4-Chloro-1-naphthol solution (Nacalai Tesque, Kyoto) (3 ml of 0.3% 4-chloro-1-naphthol in ethanol, 21 ml of H_2O, 120 ml of 1 *M* Tris-HCl buffer pH 7.4, and 16 ml of 30% hydrogen peroxide) was used as a peroxidase substrate.

RESULTS

Immunohistochemistry Representative profiles of the binding of MAbs 91.9H and FH6 to colorectal carcinoma, adjacent normal mucosae and liver or lymph node metastasis are shown in Fig. 1. Fig. 1a demonstrates the distribution of sulfomucins (MAb 91.9H binding) in cytoplasm, cell surface, and secretory products in the glands of colonic mucosae. In contrast, MAb FH6

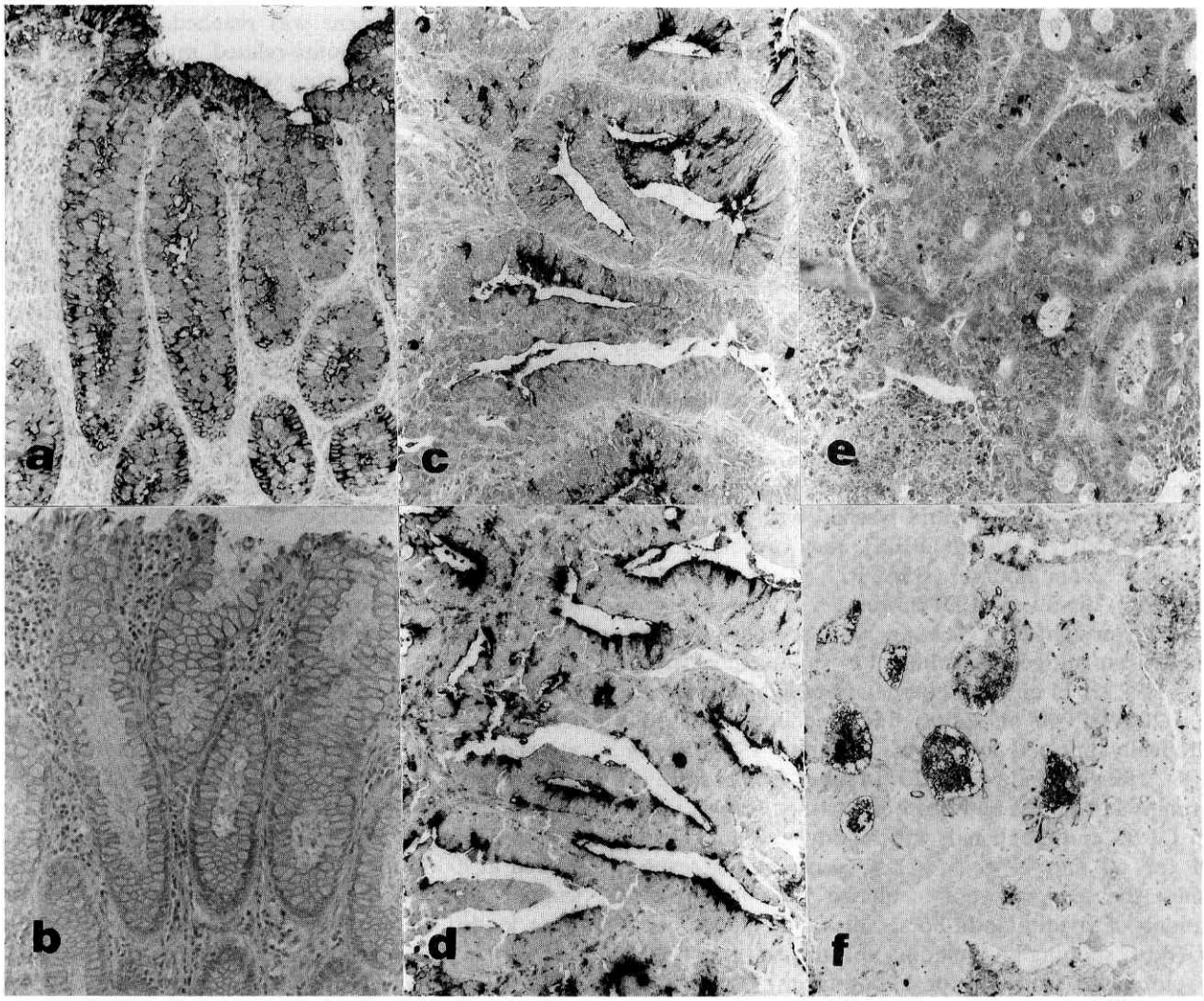

Fig. 1. Staining of the normal rectal mucosae and primary and/or metastatic carcinomas of a patient at stage D by MAbs 91.9H or FH6. Photos a and b show normal mucosae, c and d show primary lesions, and e and f show liver metastasis. Sections in a, c, and e were stained with MAb 91.9H and those in b, d, and f with MAb FH6, and all were counterstained with hematoxylin. Photo a shows the presence of sulfomucins in normal colonic mucosae. In contrast, MAb FH6 did not show any reactivity to the normal colonic mucosae. In photos c–f, in the primary carcinoma and metastatic foci, both MAbs show reactivity to cytoplasm, luminal surface and/or secretory products and their distributions were different. ($\times$60)

reveals no reactivity to normal colorectal mucosae (Fig. 1b). In Fig. 1c, MAb 91.9H is focally positive in primary colon carcinomas. MAb FH6 also demonstrates focally positive staining (Fig. 1d). A comparison of serial sections indicates that some cells were reactive with both antibodies. Fig. 1e and f reveal positive staining of MAb 91.9H and FH6, respectively, in a metastatic lesion from the same patient as in Fig. 1a–d. There are some differences in the expression and localization of the mucin antigens detected by MAb 91.9H and FH6. MAb FH6 apparently binds secretory products, whereas MAb 91.9H does not. In Fig. 2a–d, these two MAbs demonstrate a clear contrast in normal Lieberkuehn's glands. In these colon carcinoma specimens, patterns of MAb 91.9H staining are similar to those of MAb FH6 staining, though differing in detail.

The percentages of MAb 91.9H-reactive cells in the histological sections are summarized in Fig. 3a–d. As can be seen in these figures, almost all the normal mucosae contain MAb 91.9H-binding sulfomucins. The

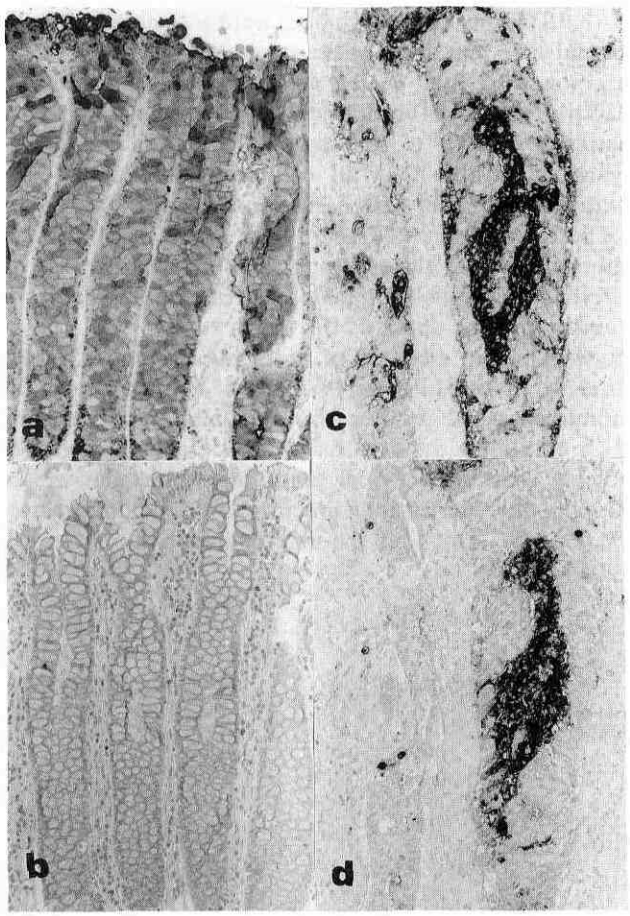

Fig. 2. Staining of the normal rectal mucosae and primary carcinomas of a patient at stage B by MAbs 91.9H or FH6. Photos a and b show normal mucosae, and c and d show primary lesions. The sections in a and c were stained with MAb 91.9H and those in b and d with MAb FH6, and all were counterstained with hematoxylin. Photo a shows the presence of sulfomucins in normal colonic mucosae. In contrast, MAb FH6 did not exhibit any reactivity to the normal colonic mucosae, as seen in Fig. 1. In photos c and d, in the primary carcinoma, both MAbs show reactivity to cytoplasm, luminal surface and/or secretory products, though their distributions were not identical. (×60)

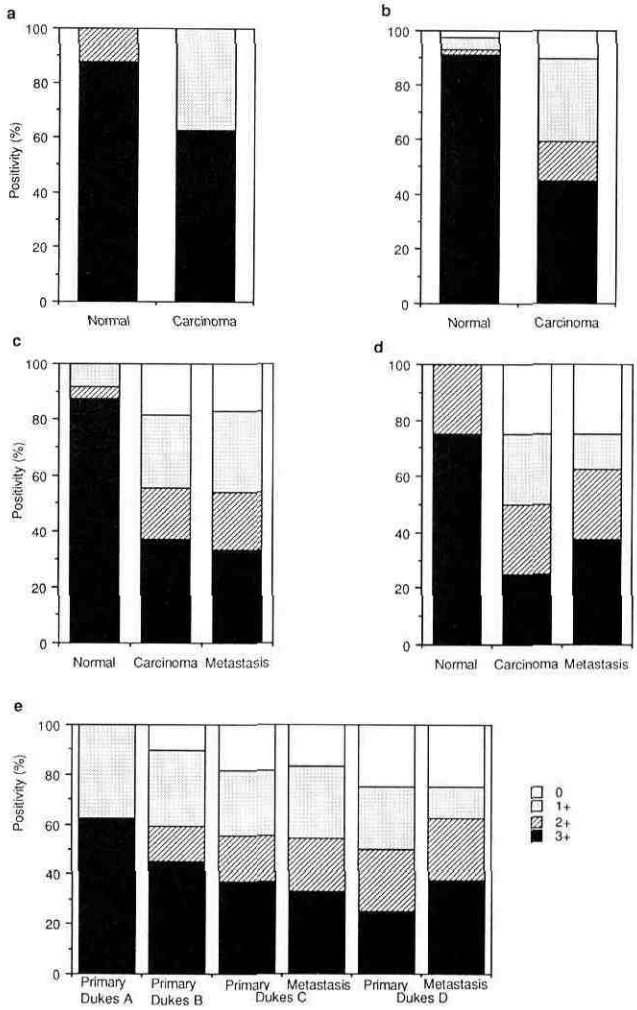

Fig. 3. Expression and intensity of sulfomucins recognized by MAb 91.9H in normal mucosae and carcinomas at each stage classified by Astler and Coller.[35] Charts a–d correspond to stages A–D, respectively. Chart e shows the staining grade of MAb 91.9H-binding sulfomucins in carcinomas at different stages and demonstrates a gradual decrease of expression from stage A to stage D, corresponding to the progression of carcinoma.

expression of sulfomucins was generally lower in carcinoma tissues than in normal mucosae. The intensity of MAb 91.9H reactivity with individual cells was lower in carcinoma tissue than in normal mucosae. For example, nearly 90% of the normal mucosae in the specimens classified as stage C showed very strong diffuse staining. In contrast, less than 40% of the primary carcinoma specimens classified as stage C were strongly reactive with this MAb 91.9H. There was another such difference between primary carcinomas and metastatic lesions. As shown in Fig. 3e, the positivity (degree of positive staining of three different specific areas of carcinoma cells as described in "Materials and Methods") of MAb 91.9H-reactive cells was lower in carcinoma tissue at advanced stages than at early stages.

Fig. 4a–d shows the regional distribution (cytoplasm, cell membrane, and secretory products) of sulfomucins detected by MAb 91.9H among tumors at different stages. In normal mucosae, sulfomucins were observed in the cytoplasm, cell surface and secretory products (Fig. 1c and e, and Fig. 2c). As summarized in Fig. 4a–d, the percentages of MAb 91.9H-reactive cells were lower in carcinoma tissues than in normal mucosae. In particular, there was a striking loss of cytoplasmic sulfomucins when normal and carcinoma tissues were compared. The MAb 91.9H-reactive substances were associated with the luminal side, regardless of the stage or the location of the carcinoma. In Fig. 4e, the percentages of MAb 91.9H-reactive substances in different tissue compartments are plotted according to the stage of the carcinoma. This clearly illustrates that all cell surface-associated sulfomucins decreased with carcinoma progression.

The expression of MAb 91.9H-reactive substances is heterogeneous in the carcinoma tissues, but there is no difference depending on the localization of the tumor, or according to the depth of the tumor.

Electrophoretic analysis of antigenic molecules recognized by MAb 91.9H or MAb FH6 Fig. 5a shows the immunochemical profiles of sulfomucins recognized by MAb 91.9H on 3.3% polyacrylamide gels. The results indicate that MAb 91.9H binds high-molecular-weight components, presumably mucins, of M_r approximately 900,000. The fact that the carcinoma tissue of the pri-

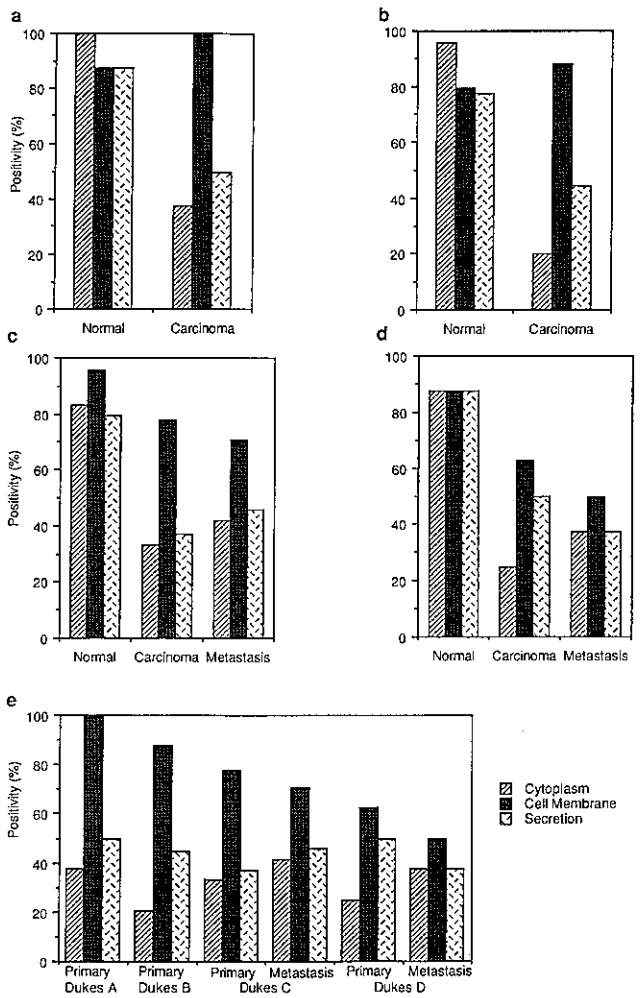

Fig. 4. Distribution of sulfomucins recognized by MAb 91.9H at each Dukes stage. Figs. a–d correspond to stages A–D, respectively. Fig. e demonstrates a gradual decrease of expression of sulfomucins in the cell surface corresponding to the stage of carcinomas. Note the striking loss of cytoplasmic expression of the MAb 91.9H-binding sulfomucins in carcinomas compared with the normal mucosae, too.

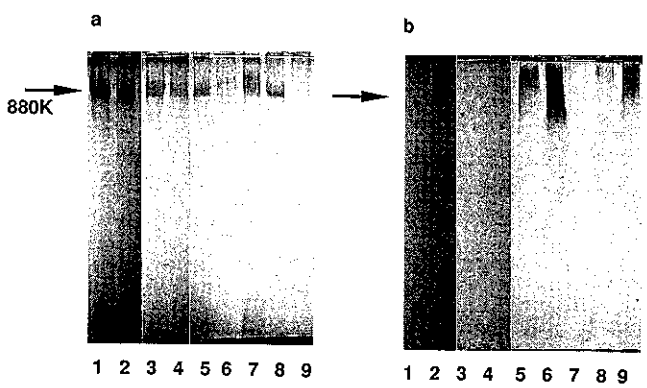

Fig. 5. Figs. a and b show identification of MAb 91.9H binding sulfomucins or s-Le^x antigen detected by MAb FH6, respectively, on 3.3% polyacrylamide gel. Lanes 1 and 2, normal mucosae and stage B2 carcinoma; lanes 3 and 4, normal mucosae and stage C2 carcinoma; lanes 5 and 6, primary carcinoma and liver metastasis at stage D; lanes 7, 8, and 9, normal mucosae, primary carcinoma, and liver metastasis of another case at stage D, respectively. The large arrow indicates 880 kDa marker (laminin).

mary site contains a much smaller amount of sulfomucins than the tissue of normal mucosae, already demonstrated by the immunohistochemical study, was confirmed by this analysis (between lanes 1 and 2, or 3 and 4). The intensity of MAb 91.9H binding to high-molecular-weight components was lower in the extracts of metastatic lesions than in those from primary tumors (between lanes 5 and 6, or 7, 8, and 9). The same extracts were separated on 3.3% polyacrylamide gels and stained with MAb FH6 (Fig. 5b). MAb FH6 binds high-molecular-weight components containing s-Le^X oligosaccharides, in accordance with our previous report.[20] The electrophoretic mobility in the presence of sodium dodecyl sulfate was essentially identical to that of sulfomucins detected by MAb 91.9H (Fig. 5a and b). The intensity of MAb FH6 binding was greater in primary carcinomas than in normal mucosae. Metastatic lesions contained a larger quantity of high-molecular-weight glycoproteins with MAb FH6 reactivity than did the primary sites.

DISCUSSION

Altered expressions of various glycoconjugates are associated with malignant behavior of tumor cells.[1, 3, 8, 17–22, 25–33, 41–46] Blood group type-II H antigen(s) recognized by *Ulex europaeus agglutinin-I* and expressed on high-molecular-weight mucin(s) was decreased during colorectal carcinoma progression to metastatic phenotypes.[13] WGA-binding, mucin-like, high-molecular glycoproteins and mucin containing s-Le^X antigen were increasingly expressed and were associated with progression and metastasis formation of colorectal carcinomas.[15, 17, 18, 20] The present study was designed to examine the distribution of specific sulfomucins in colorectal carcinomas at different stages of progression and to evaluate the feasibility of using a sulfomucin-specific monoclonal antibody (MAb 91.9H) as a clinical marker for recurrence, metastasis, or differential diagnosis. For the latter purpose, additional evaluation with archival samples of various tumor specimens and examination of the relationship between the expression of the sulfomucins and the prognosis of the patients will be necessary.

Our immunohistochemical studies and electrophoretic analysis clearly indicated that the levels of sulfomucins were lower in colorectal primary carcinoma at the advanced stages than at the early stages. In the specimens we analyzed, the electrophoretic mobility of sulfomucin was very similar to that of mucins bearing s-Le^X oligosaccharides detected by MAb FH6 from the same patients. These results and our previous reports on increased expression of s-Le^X antigen in carcinomas at advanced stages or at metastasis strongly suggest that sulfated carbohydrate chains and the s-Le^X epitope are attached to the same polypeptide core.[17, 20] Decreased sulfation may be prerequisite to the formation of the s-Le^X epitope on a single carbohydrate side chain in a mucin molecule. In fact, immunohistochemical localizations of MAb 91.9H-specific sulfomucins and the s-Le^X epitopes revealed by MAb FH6 were not mutually exclusive. Upon electrophoretic separation, these two antigenic epitopes seem to be associated with components having a similar electrophoretic mobility. Comparison of the specimens from normal mucosae, primary colorectal carcinoma, and metastasis indicated that MAb 91.9H-binding sulfomucins were inversely related to tumor progression but MAb FH6 binding was correlated with the progression.

In addition to the differences in the levels of sulfomucins between primary carcinomas at different stages or between primaries and metastatic lesions, the differential localization of sulfomucins within colonic cells should be noted. In normal colonic epithelium, sulfomucins were localized in the cytoplasm, associated with the cell apex, the luminal surface or the secretory products. In carcinomas at primary sites or metastatic lesions, sulfomucins were strikingly lost from the cytoplasm (Fig. 4a–d). We do not know the mechanisms or the biological meaning of the altered localization of MAb 91.9H-binding sulfomucins. The intracellular production and/or transport system for mucins might be modified during malignant transformation and tumor progression. Recently we have reported that the increased expression of s-Le^X antigen correlates with poor survival in patients with colorectal carcinoma.[47] The reversed expression of s-Le^X antigen and sulfomucins between the normal mucosae and carcinoma tissue or between the different stages of the carcinoma tissue indicates that these alterations of the mucins are possibly related to the biological features of colorectal carcinoma.

We think that purification and characterization of these two mucins, and determination of the oligosaccharide structures and core peptides, may cast light on the role of these antigens in the progression and metastasis of colorectal carcinomas, because these mucins carry carbohydrate terminals which have many functions, such as recognition and adhesion.[48]

ACKNOWLEDGMENTS

We wish to thank Prof. S. Hakomori for his generous gift of MAb FH6; Drs. K. Sakoda, K. Kuroki, and H. Yoshii for providing tissue specimens from surgery, and Ms. Y. Arimura and A. Nakanishi for their technical assistance.

(Received June 9, 1995/Accepted August 23, 1995)

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