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Cell Surface Sialoprotein Alterations in Metastatic Murine Colon Cancer Cell Lines Selected in an Animal Model for Colon Cancer Metastasis[1]

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ABSTRACT

Alterations in cell surface proteins and glycoproteins may play a key role in determining the metastatic behavior of tumor cells. The cell surface proteins of a series of related murine colon cancer cells selected in an animal model for colon cancer metastasis (R. S. Bresalier *et al.*, Cancer Res., *47:* 1398–1406, 1987) were therefore compared by a variety of biochemical methods. Lactoperoxidase-catalyzed iodination of cell surface proteins followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated quantitative and qualitative differences in the cell surface protein profiles of parental cell line 51B (low metastatic potential) and its metastatic derivatives 51B LiM 5 and 51B LiM 6. Labeling of sialic acid-containing proteins suggested that, in the case of at least four of these proteins (M_r 170,000, 120,000, 95,000, and 55,000), this represented an increase in radioactive labeling of sialoglycoproteins from the metastatic lines. Affinity chromatography of solubilized ^{125}I-labeled cell membrane proteins revealed a 2- to 3-fold increase in wheat germ agglutinin and *Sambucus nigra* lectin binding associated with the metastatic lines, compared to the poorly metastatic parent. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of material eluted from these columns demonstrated enhancement of proteins from the metastatic cells corresponding in molecular weight to the previously identified major sialoglycoproteins. Neuraminidase-releasable membrane-associated sialic acid and sialyltransferase activities were 2- to 3-fold higher in the metastatic cell lines compared to the parental line. Liver colonization after intrasplenic injection of the various lines into syngeneic mice was dramatically reduced by prior removal of cell surface sialic acid. Immunohistochemical staining of primary and metastatic tumors formed after cecal injection of parental 51B suggested selective metastasis by wheat germ agglutinin-binding tumor cells. These results further support the concept that cell membrane sialylation is important in determining the metastatic potential of cancer cells.

INTRODUCTION

Alterations in cell surface proteins and glycoproteins are common during carcinogenesis and may play a key role in determining the metastatic behavior of tumor cells (1–4). The cell surface is thought to play a role in several stages of metastasis including cell growth regulation, motility during invasion, homotypic and heterotypic interactions with other tumor cells, platelets, and the immune system, and the adherence of metastatic cells to endothelia and the extracellular matrix. Although a universal "metastasis-related cell surface phenotype" has yet to be identified, differences in the sialylation of cell surface glycoproteins and glycolipids are common when comparing cells of low and high metastatic potential (5–20). Cell surface sialylation has been implicated in affecting such diverse characteristics as invasive potential (15), tumor cell-mediated platelet aggregation (18, 21), resistance to T-cell-mediated immune destruction (11, 22), and alterations in tumor cell adherence to basement membrane components and target cells (2, 9, 10, 12). Increased cell surface sialic acid has been shown to correlate with metastatic potential in some studies (5, 8, 15, 17), while in others the position of sialic acid on terminal carbohydrate structures appears more important in determining the extent of metastasis (9, 13, 16, 19). The majority of cultured cell lines used in these studies have been derived from nonepithelial tumors such as murine melanomas (5–8, 14), T-cell lymphomas (9, 11–13, 15, 16, 19), and leukemia-like tumors (9–11, 13, 23).

Colorectal cancer represents the second most common epithelial cancer in North America and the third most common cancer world-wide. Since colon cancer-related mortality is strongly correlated with extent of tumor cell invasion and metastasis at diagnosis (24), there has been a great deal of interest in identifying cellular determinants of colon cancer metastasis. Alterations in cell surface and secreted glycoconjugates have been described in colon cancer tissues and cell lines (17, 18, 25–36), including differences between primary and metastatic tumors (17, 29, 32–34) and between intratumoral cell subpopulations with different biological properties (28, 31). In the case of at least one murine colon carcinoma, sublines which differ in their metastatic potential also differ in their degree of cell surface sialylation (17, 18).

We have recently described the development of an animal model for colon cancer metastasis, which was used to establish and characterize murine colon cancer cell lines which differ in their liver-metastasizing abilities during cecal growth (37). It was demonstrated that the cells selected for enhanced metastatic potential are more invasive *in vivo* and *in vitro* and that they secrete a basement membrane-degrading protease (type IV collagenase) in proportion to their invasive and metastatic abilities. These tumor cells have also been shown to adhere rapidly to pure cultures of hepatic sinusoidal endothelial cells *in vitro*. The present study was undertaken to compare the cell surface proteins and glycoproteins of these related colon cancer cell lines by a variety of biochemical means. The degree of cell surface sialylation, cell membrane sialoprotein profiles, and sialyltransferase levels are also compared.

MATERIALS AND METHODS

Reagents. Lactoperoxidase, glucose oxidase, galactose oxidase, *N*-acetylneuraminic acid (sialic acid), *N*-acetyl glucosamine, neuraminidase type X from *Clostridium perfringens*, and NP40[3] were purchased from Sigma Chemical Co. (St. Louis, MO); sodium dodecyl sulfate, acrylamide, and bisacrylamide from U.S. Biochemicals (Cleveland, OH); albumin from Calbiochem (La Jolla, CA); sodium [^{3}H]borohydride (52 Ci/mmol) and carrier-free Na^{125}I from ICN (Irvine, CA); Autofluor from National Diagnostics (Manville, NJ); biotinylated WGA from EY Laboratories (San Mateo, CA); streptavidin-peroxidase

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[3] The abbreviations used are: NP40, Nonidet P-40; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HBSS, Hanks' buffered saline solution; IdUR, iododeoxyuridine; Man, mannose; OSM, ovine submaxillary mucin; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SNA, *Sambucus nigra* lectin; WGA, wheat germ agglutinin.

conjugate from Zymed Laboratories (South San Francisco, CA); agarose-bound WGA and SNA from Vector Laboratories (Burlingame, CA); and UDP-[U-^{3}H]galactose (specific activity, 289 mCi/mmol) and CMP-[4,5,6,7,8,9-^{14}C]sialic acid (173 mCi/mmol) from New England Nuclear (Boston, MA). OSM was purified from ovine submaxillary glands as described by Tettamanti and Pigman (38), and transferrin was obtained from Sigma. Sialic acid was removed from OSM and transferrin by heating with 0.1 N HCl at 80°C for 90 min. The samples were neutralized with 0.5 N NaOH and dialyzed against 5 to 6 volumes of distilled water for 48 h. The dialyzed samples were lyophilized and used as acceptors for the sialyltransferase assays.

Cell Lines. Parental murine colon cancer cell line 51B and its metastatic derivatives 51B LiM 5 and 51B LiM 6 were established as previously described (37). 51B consists of a heterogeneous population of cells and forms poorly to moderately differentiated carcinomas with limited metastatic ability when grown in the cecal walls of syngeneic BALB/c mice. 51B LiM 5 and 51B LiM 6, cell lines with high liver-metastasizing ability during cecal growth, were established by serially selecting cells which metastasized from cecum to liver in the previously described model. These cell lines metastasize to the liver in 100% of animals after cecal injection and have enhanced liver-metastasizing potential compared to parental 51B when injected s.c. or i.v. (37). Cell lines were grown and maintained in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), in a 7% CO_2 environment. Early passage cell lines (passaged fewer than 15 times) were used for all studies.

Histological Examination and Histochemistry. Specimens for histological examination were fixed in 10% formalin for 24 h and embedded in paraffin. Serial 5-μm sections were then cut and stained with hematoxylin and eosin or processed for histochemistry. WGA binding in fixed tissues was determined using the streptavidin-peroxidase technique, as previously described (39). Biotinylated WGA was used at a working concentration of 2 μg/ml. The lectin specificity was confirmed by incubating 50 μl of biotinylated lectin with 50 μl of 0.5 M hapten sugar (*N*-acetylglucosamine) before application to duplicate tissue sections.

Labeling of Cell Surface Proteins and Glycoconjugates. Lactoperoxidase-catalyzed iodination of cell surface proteins was performed by a modification of a previously described method (40). Cells were grown to confluency in 75-cm^2 tissue culture flasks and washed 4 times with PBS (pH 7.4) and then once with 3 mM glucose in PBS. The tyrosine-containing residues of cell surface proteins were labeled *in situ* by the addition of 2 ml of 3 mM glucose in PBS, 10 μl (5 units) of lactoperoxidase, and 250 μCi of carrier-free Na^{125}I. The reaction was initiated by the addition of 10 μl (15 units) of glucose oxidase and continued for 30 min at 25°C. Cells were washed 4 times in PBS containing 1 mM NaI and 5 mM PMSF and scraped into 4 ml PBS-PMSF. Labeled cells were then centrifuged at 600 × *g* for 10 min, resuspended in 500 μl PBS-PMSF, sonicated twice for 20 s, and then ultracentrifuged at 100,000 × *g* for 1 h. The membrane-enriched pellet thus obtained was resuspended in 500 μl PBS-PMSF, and aliquots were taken for protein determination (41) and SDS-polyacrylamide gel electrophoresis.

Labeling of cell surface proteins with galactose oxidase and tritiated sodium borohydride was performed as previously described (40), with modifications. Cells cultured in 75-cm^2 tissue culture flasks were labeled *in situ* after being washed 4 times with isotonic PBS (pH 7.4). Washed cells were treated with 25 units (45 μg) of galactose oxidase in 2.5 ml Hanks' balanced salt solution (pH 7.0) for 60 min at 37°C. Cells were then washed with PBS (pH 8.3) and incubated with 2.5 mCi of tritiated sodium borohydride for 30 min at 25°C in 2 ml HBSS (pH 8.0). The labeled cells were then washed 4 times in PBS-PMSF and membrane-enriched fractions were prepared for SDS-PAGE as above. Parallel flasks were incubated with 0.2 units of neuraminidase type X (*C. perfringens*) in 2.5 ml HBSS (pH 6.0), for 30 min at 37°C, prior to labeling.

Cell surface sialoproteins were labeled according to a modification of the method of Gahmberg and Andersson (42). Cells grown in 75-cm^2 flasks were washed 4 times with PBS (pH 7.4) and treated for 20 min with 2 ml 2 mM sodium periodate in the dark at 4°C. Glycerol (0.2 ml of 0.1 M solution in PBS) was added for 10 min. The cells were washed with PBS (pH 8.0) and then incubated for 30 min with 2.5 mCi tritiated sodium borohydride in 2 ml HBSS (pH 8.0). Labeled cells were washed with PBS-PMSF and membrane-enriched fractions were prepared for SDS-PAGE as above.

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (43), using a discontinuous buffer system and 3% stacking and 7% running gels. Equal amounts of protein or equal amounts of radioactivity were applied to parallel lanes for each cell line. Gels containing ^{3}H-labeled proteins were fixed and stained with Coomassie brilliant blue, soaked in Autofluor (National Diagnostics), dried, and used to expose Kodak X-O-Mat AR film. Gels containing ^{125}I-labeled proteins were dried and X-ray film was similarly exposed.

Lectin Affinity Chromatography. Cell surface proteins were labeled by lactoperoxidase-catalyzed iodination, and membrane-enriched fractions were prepared as described above. Proteins were solubilized by sonicating briefly in PBS-PMSF containing 1% NP40 and extracting for 2 h at 4°C. After centrifugation at 100,000 × *g* for 1 h, the supernatant was applied to a column of agarose-bound WGA (Vector Laboratories), succinylated WGA or SNA. The WGA and succinylated WGA columns were washed with 3 volumes of PBS (pH 7.1) containing 0.1% NP40 and eluted in a stepwise fashion with 0.2 and 0.5 M *N*-acetylglucosamine. The SNA column was eluted with 0.5 M lactose in PBS (pH 7.1) containing 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate. Material from the eluted peaks was dialyzed against 4 liters of distilled water, concentrated by lyophilization, and submitted for SDS-PAGE.

Sialic Acid Determination. Neuraminidase-releasable membrane-associated sialic acid was determined as follows. Cell membrane-enriched fractions were prepared from approximately 10^7 cells, as described above, and resuspended by sonicating in 500 μl HBSS, pH 6.0. A 50-μl aliquot was removed for protein determination, and the remainder was incubated at 37°C for 1 h, in a shaking water bath, with 0.2 units (100 μl) affinity-purified neuraminidase type X from *C. perfringens*. The mixture was centrifuged at 100,000 × *g* for 1 h and the supernatant was assayed for free sialic acid by the thiobarbituric method (44). Standard curves were constructed for each experiment with known concentrations of sialic acid, and determinations were made in the linear portion of the curve.

Glycosyltransferase Assays. Cultured cells (75% confluent monolayers) were washed 3 times with PBS and harvested by scraping into 8 ml of PBS with a cell scraper. Cells were centrifuged, and the cell pellet was suspended and sonicated in 1 ml of PBS (pH 7.1). Aliquots were used directly for glycosyltransferase assays or frozen at −70°C.

Sialyltransferase activities were determined using asialotransferrin [(Galβ1–4GlcNAc)$_2$Man$_3$GalNAc$_2$] and asialo-OSM (GalNAcα1–OSer/Thr) as acceptors. Assay mixtures consisted of cacodylate buffer (pH 6.8), 20 mM $MnCl_2$, 200 μM ATP, 400 μg of appropriate acceptor, 1.75–2.0 μM (70,000–80,000 cpm) CMP-sialic acid, 50–100 μg of cellular protein, and 0.1% Triton X-100, in a total volume of 100 μl (45). All assays were performed within the linear range of both enzyme concentration and incubation time. The amount of CMP-sialic acid (unlabeled and labeled) used yielded optimum transfer of labeled sialic acid to the acceptors. Reaction mixtures were incubated at 37°C for 90 min, and the reaction was terminated by the addition of 2 ml ice-cold 1% phosphotungstic acid in 0.5 M HCl. Precipitates were washed 3 times with cold phosphotungstic acid and dissolved in 500 μl of NCS tissue solubilizer (Amersham/Searle, Arlington Heights, IL). The solubilized precipitates were mixed with Omnifluor (New England Nuclear) toluene cocktail and counted in a liquid scintillation counter. Net radioactivity transferred to exogenously added acceptor was determined after subtracting the amount of radioactivity transferred to endogenous acceptors.

Galactosyltransferase activity (β1–4-galactosyltransferase) was determined using a reaction mixture consisting of 0.1 M PBS (pH 7.1), 20 mM $MnCl_2$, 200 μM ATP, 10 mM GlcNAc, 17–20 nM UDP-[^{3}H]-galactose (50,000–60,000 cpm), and 0.1% Triton X-100, in a total volume of 100 μl (45). The mixture was incubated at 37°C for 90 min, and the reaction was stopped by placing it on ice. The reaction mixture

was then applied to a 0.5 × 2 cm Dowex 1 × 8 formate (100–200 mesh) column (Bio-Rad, Richmond, CA), and the products were separated by washing each column with 2 ml distilled water (46). An aliquot of each eluate was mixed with ACS II scintillation fluid (Amersham, Arlington Heights, IL) and counted in a liquid scintillation counter. Values obtained without added acceptor were taken as radioactivity transferred to endogenous acceptors and were subtracted to yield net radioactivity transferred to the exogenously added acceptor.

Liver Colonization after Neuraminidase Treatment. Tumor cells were grown to confluency in 75-cm^2 tissue culture flasks, washed 3 times with PBS, and incubated *in situ* for 30 min with 0.2 units neuraminidase type X and *C. perfringens* in 2 ml PBS (pH 7.0). These conditions were found to remove greater than 95% of cell membrane-associated sialic acid while maintaining cell viability. Control cells were treated identically except that neuraminidase was omitted during incubation. Greater than 90% of cells remained viable during the procedure, as determined by trypan blue exclusion. Cells were then washed 5 times with PBS, harvested, resuspended in serum-free Dulbecco's modified Eagle's minimal essential medium at a concentration of 10^7 cells/ml, and used in a modification of a splenic injection model for liver colonization (47). BALB/c mice (15-g females; Simonsen Laboratories, Gilroy, CA) were anesthetized with methoxyfluorane by inhalation and prepared sterilely and the spleen was exteriorized through a flank incision. One million cells in 100 μl were slowly injected into the splenic pulp through a 27-gauge needle over 1 min, followed by splenectomy. Animals were sacrificed 2 weeks later, the livers were removed and weighed, and tumor nodules were counted.

The fate of untreated and desialylated tumor cells after splenic injection was determined by injection of IdUR-labeled tumor cells. Metastatic tumor cell line 51B LiM 5 was grown as above and 0.5 μCi/ml [^{125}I]IdUR was added to the culture medium 24 h prior to harvesting. This concentration of isotope was previously shown to label greater than 95% of tumor cells without affecting viability (37). Neuraminidase-treated and control cells were prepared as described above, and 10^6 viable cells in 100 μl were injected into the splenic pulp over 1 min, followed by splenectomy 1 min later. Animals were sacrificed at various intervals after injection by cervical dislocation, and livers were removed (n = at least 6 animals/time point/group). The radioactivity present in the spleen (removed after injection), liver, and remaining organs of each animal was determined by gamma-counting in a Beckman 7000 counter.

RESULTS

Electrophoretic Profiles of Radiolabeled Cell Surface Proteins. Coomassie blue staining of electrophoretically separated cell surface proteins from parental cell line 51B and its metastatic derivatives 51B LiM 5 and 51B LiM 6 did not show substantial differences in protein profiles between these cell lines. Lactoperoxidase-catalyzed iodination of cell surface proteins followed by SDS-polyacrylamide electrophoresis, however, demonstrated quantitative and qualitative differences in the cell surface protein profiles of 51B (low metastatic potential) and its metastatic variants (Fig. 1*A*). The highly metastatic cell lines had similar protein profiles, which differed from that of the poorly metastatic parental line. Quantitative differences were present in proteins of approximate molecular weights 170,000, 120,000, 95,000, 66,000, 55,000, and 40,000 (designated bands 3, 5, 6, 10, 11, and 14) with a slight shift in molecular weight of the 95,000 protein.

Labeling of sialic acid-containing glycoproteins by mild periodate oxidation and sodium borotritide reduction at 4°C suggested that increases in the M_r 170,000, 120,000, 95,000, and 55,000 proteins (bands 3, 5, 6, and 11) from the metastatic cell lines correspond to quantitative alterations in the sialoproteins from these cell lines (Fig. 1*B*). This was further suggested by galactose oxidase-sodium borotritide labeling of galactose- and *N*-acetylgalactosamine-containing glycoproteins in the presence or absence of prior neuraminidase treatment (Fig. 2). Labeling of bands 3, 5, 6, and 11 was enhanced in all cell lines by prior neuraminidase treatment, indicating that these glycoproteins are sialylated. A proportionately greater enhancement with neuraminidase treatment was suggested for some bands (principally 3 and 5) in the metastatic cell lines.

Lectin Affinity Chromatography. WGA is a lectin which binds specifically to oligosaccharides containing exposed *N*-acetylglucosamine or sialic acid. Affinity chromatography of solubilized ^{125}I-labeled cell surface proteins revealed a 2- to 3-fold increase in WGA-binding proteins, which were sequentially eluted from the column with 0.2 and 0.5 M hapten sugar (*N*-acetylglucosamine), from the highly metastatic cells compared to those from parental 51B (Fig. 3*A*). Electrophoresis of the peak fractions of WGA-binding material eluted from the column again suggested that glycoproteins corresponding to sialoproteins are altered on the metastatic cells (Fig. 3*B*). When solubilized cell surface proteins were applied to a succinylated WGA column (which binds *N*-acetylglucosamine but not sialic acid), the quantitative differences previously seen between the cell lines on the WGA column were abolished, suggesting that those differences were due to differences in the sialic acid content of the cell membrane fractions.

Elderberry bark lectin (SNA) is a lectin which preferentially recognizes oligosaccharides which contain the terminal sequence Neu-5-Ac(α2–6)Gal/GalNAc (48, 49). Affinity chromatography of solubilized cell surface proteins on agarose-bound SNA revealed increased SNA binding by the highly metastatic cells compared to parental 51B (Fig. 4*A*). Electrophoresis of SNA-binding proteins eluted from the column demonstrated quantitative increases in labeling of three of the four sialoglycoproteins (3, 5, 6) previously shown to be increased in the metastatic cell lines (Fig. 4*B*).

Sialic Acid Content of Cell Membranes. Neuraminidase-releasable membrane-associated sialic acid was determined for parental murine colon cancer cell line 51B and its metastatic derivatives 51B LiM 5 and 51B LiM 6. Neuraminidase-releasable sialic acid was approximately 2-fold higher on the metastatic cell lines compared to the parental cell line (Table 1).

Sialyltransferase Activity. Sialyltransferase activities associated with parental cell line 51B and metastatic lines 51B LiM 5 and 51B LiM 6 were determined using asialotransferrin [(Galβ1–4GlcNAc$_2$)Man$_3$] and asialo-OSM (GalNAcα1–Ser/Thr) as acceptors. Sialyltransferase specific activities using either acceptor were higher for the metastatic cell lines and correlated with both the metastatic potential and amount of cell membrane-associated sialic acid of the cell lines (Table 2). In contrast, there was no difference in the β1–4-galactosyltransferase activities of the cell lines.

WGA Binding to Experimental Primary and Metastatic Tumors. Parental cell line 51B is heterogeneous *in vitro* (37) and forms moderately to poorly differentiated tumors when injected into the ceca of syngeneic animals. Examination of hematoxylin- and eosin-stained sections of the primary tumor formed after cecal injection of 51B revealed two major cell populations (Fig. 5*A*). Immunohistochemical analysis using biotinylated wheat germ agglutinin and streptavidin peroxidase staining demonstrated preferential binding of WGA to the cell surface of one population (Fig. 5*B*). Examination of liver metastases from 51B and its more metastatic derivatives revealed the metastasizing tumor cells to avidly bind wheat germ agglutinin

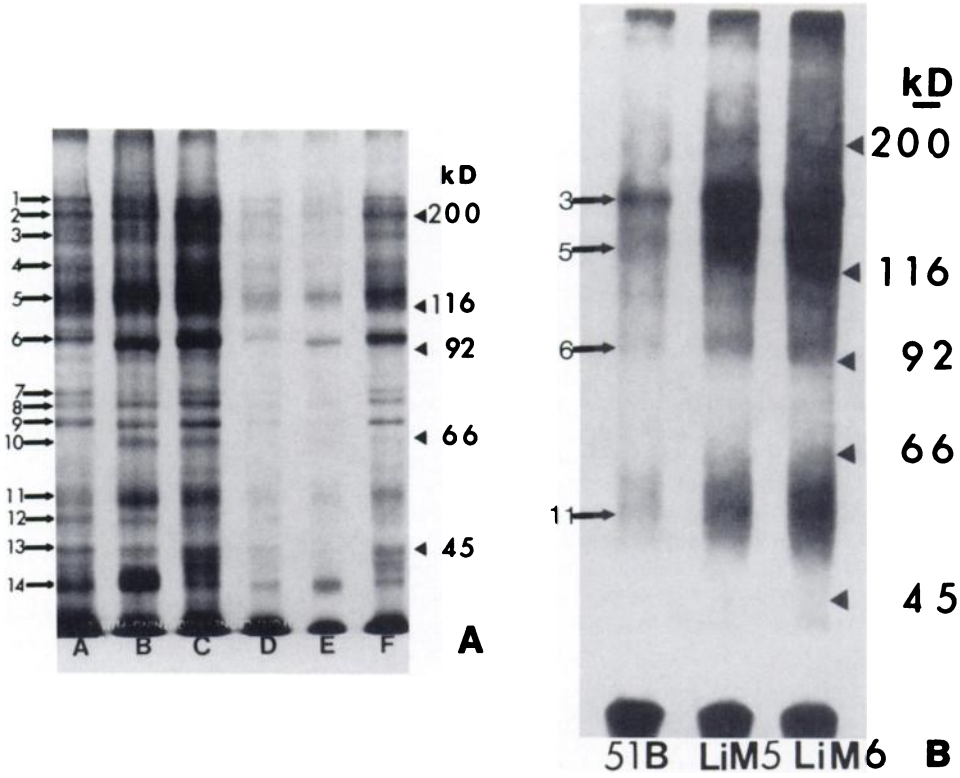


Fig. 1. Cell surface proteins from parental (51B) and metastatic (LiM 5, LiM 6) murine colon cancer cell lines separated by SDS-PAGE. *A*, radioiodinated (lactoperoxidase-catalyzed iodination) proteins. Equal amounts of radioactivity (*lanes A, B,* and *C*) or equal amounts of protein (*lanes D, E,* and *F*) were applied to parallel lanes. *Lanes A* and *D*, 51B parental line; *Lanes B* and *E*, 51B LiM 5; *Lanes C* and *F*, 51B LiM 6. *B*, sialic acid-containing glycoproteins. Equal amounts of protein were applied to parallel lanes. Sialic acid-containing glycoproteins were labeled by mild periodate oxidation and sodium borotritide reduction at 4°C.

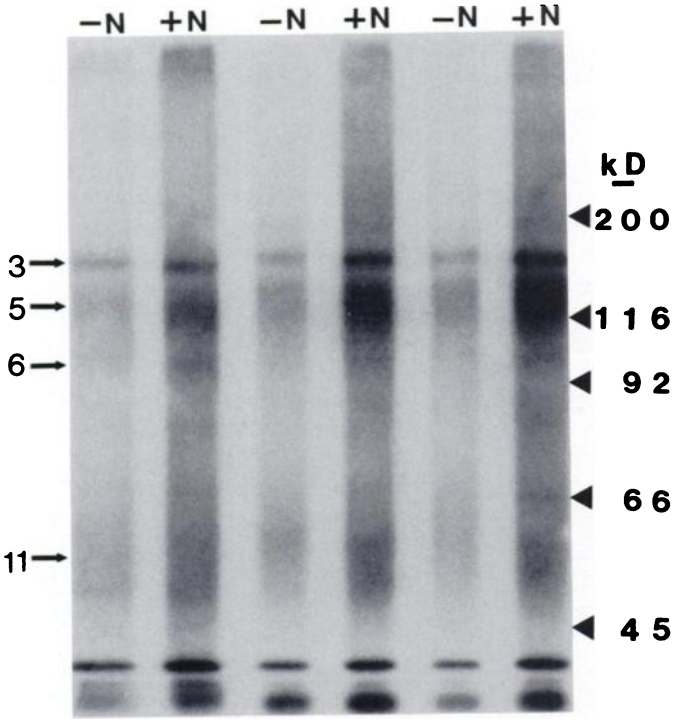


Fig. 2. Galactose- and *N*-acetylgalactosamine-containing glycoproteins from parental (51B) and metastatic (51B LiM 5, 51B LiM 6) murine colon cancer cell lines labeled by the galactose oxidase-sodium borotritide method in the presence (+*N*) or absence (−*N*) of prior neuraminidase treatment to remove cell surface sialic acid. Equal amounts of protein were applied to parallel lanes.

(Fig. 5*C*). Sugar specificity was confirmed by abolition of WGA binding in the presence of 0.5 M hapten sugar (*N*-acetylglucosamine).

Cell Surface Sialic Acid Effect on Liver Colonization. In order to determine whether removal of sialic acid from the cell surface would affect the ability of colon cancer cells to form secondary foci in the liver, cells of high and low metastatic ability were treated with neuraminidase *in situ*, and liver colonization was tested after splenic injection. Control cells were handled identically but were not treated with neuraminidase prior to injection. Animals were sacrificed 2 weeks after injection and the livers were examined for tumor. Few tumor nodules were noted in the livers of animals injected with parental cell line 51B (range, 3 to 12), and almost none were present after prior neuraminidase treatment (range, 0 to 1). Animals injected with cell line 51B LiM 5 had extensive tumor throughout the liver (range, 220 to >500 nodules), which was dramatically decreased by prior removal of cell surface sialic acid (range, 6 to 68 nodules) (Table 3, Fig. 6). Injection of [^{125}I]IdUR-labeled tumor cells demonstrated that prior neuraminidase treatment did not alter the number of viable tumor cells leaving the spleen and initially reaching the liver (Table 4). Rather, it appeared that, by 12 h after injection, fewer desialylated cells remained in the liver compared to the untreated cells.

DISCUSSION

Metastasis is a selective process by which certain tumor cells disseminate to form secondary foci at distant sites. Cell membrane glycoproteins and glycolipids play an important role in many biological functions such as cell-cell interactions, growth regulation, differentiation, and malignant transformation, and tumor cell metastasis may require alterations in membrane properties determined by cell surface glycoconjugates (1–4). While an universal alteration in cell membrane structure has not been clearly defined on all metastatic cells, quantitative differences in cell surface sialic acid and qualitative changes in

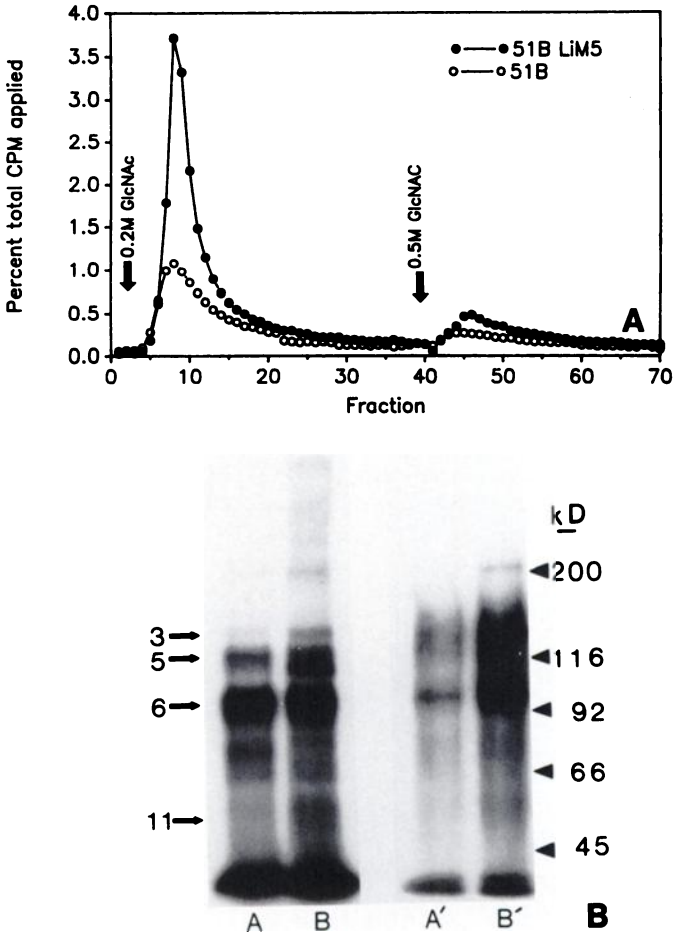
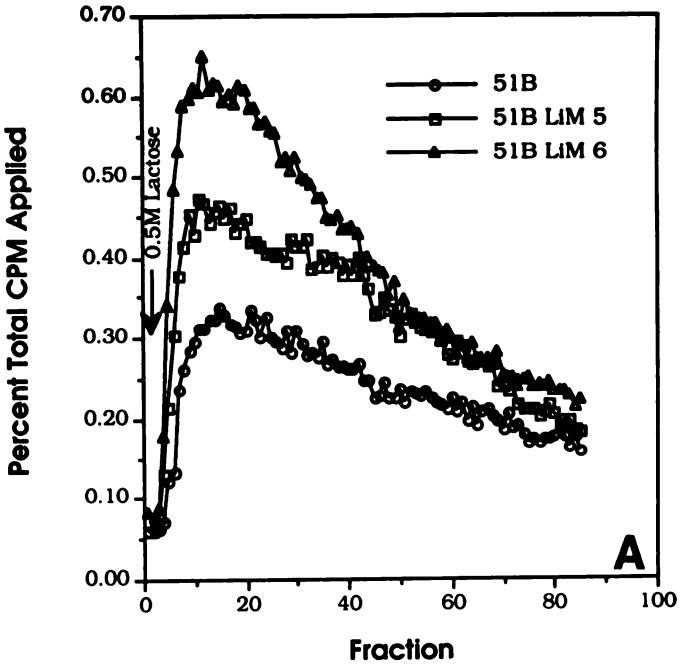


Fig. 3. Affinity chromatography of solubilized ^{125}I-labeled cell surface proteins on an agarose-bound WGA column. *A*, WGA-binding material was sequentially eluted from the column with 0.2 and 0.5 M hapten sugar (*N*-acetylglucosamine). 51B, parental murine colon cancer cell line; 51B LiM 5, metastatic colon cancer cell line. Similar differences were found when comparing 51B to metastatic line 51B LiM 6 (data not shown). *B*, SDS-PAGE of cell membrane proteins sequentially eluted from a wheat germ agglutinin affinity column. *A* and *A'*, proteins from parental line 51B; *B* and *B'*, proteins from metastatic line 51B LiM 5. *A* and *B* represent proteins eluted with 0.25 M *N*-acetylglucosamine; *B* and *B'* represent proteins eluted with 0.5 M *N*-acetylglucosamine.



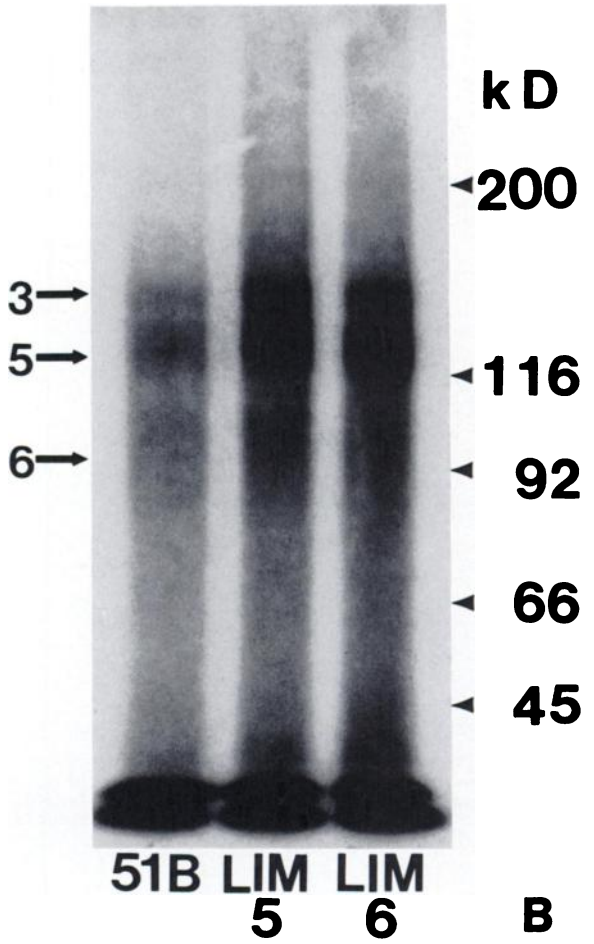


Fig. 4. Affinity chromatography of solubilized ^{125}I-labeled cell surface proteins on an agarose-bound SNA column. *A*, material bound to the column eluted with 0.5 M lactose. *B*, SDS-PAGE of cell membrane proteins eluted from the column.

sialylated oligosaccharides are commonly associated with metastasis (5–20, 23, 34, 50). The current study compares the cell surface proteins and glycoproteins of murine colon cancer cell lines which differ in their spontaneous liver-metastasizing ability during cecal growth. Metastatic lines were derived by serial selection from a heterogeneous parental cell line in an animal model for colon cancer metastasis (37). Thus, all cell lines compared are genetically related but were selected for alterations in their metastatic phenotype.

SDS-polyacrylamide gel electrophoresis of ^{125}I-labeled cell surface proteins revealed alterations in several proteins from the metastatic lines compared to the less metastatic parent. In the case of at least four of these proteins (approximate M_r, 170,000, 120,000, 95,000, and 55,000), this represented an increase in the radioactive labeling of sialoglycoproteins from the metastatic lines (mild periodate oxidation-sodium borotritide reduction). This was confirmed by galactose oxidase labeling of galactose- and *N*-acetylgalactosamine-containing glycoproteins. All cell lines were poorly labeled by this method prior to neuraminidase treatment, suggesting that the nonreducing termini of the oligosaccharide side chains of these glycoproteins are highly sialylated. Neuraminidase pretreatment enhanced the labeling of glycoproteins similar in molecular weight to those labeled by mild periodate oxidation, and a proportionally greater enhancement was seen for some proteins in the metastatic colon cancer cell lines.

An increase in cell surface sialoglycoproteins on the metastatic variants was further supported by lectin affinity chromatography of solubilized cell membrane proteins. WGA binds to the oligosaccharides of glycoconjugates containing exposed *N*-acetylglucosamine or sialic acid. A 2- to 3-fold increase in wheat germ binding was observed in the metastatic lines when compared to the poorly metastatic parental line. This difference was not seen after application of membrane proteins to a succinylated WGA column (binds *N*-acetylglucosamine but not sialic acid), suggesting that the differences seen in WGA binding were due mainly to different degrees of sialylation of membrane glycoproteins. Application of solubilized membrane proteins to agarose-bound *Sambucus nigra* lectin, a lectin which preferentially binds α2–6-linked sialic acid, again confirmed increased sialylation of the metastatic cell lines. SDS-PAGE of material eluted from the WGA and SNA columns demonstrated enhancement of proteins from the metastatic cells corresponding in molecular weight to the previously identified major sialoglycoproteins. The above data are in keeping with the approximately 2-fold higher neuraminidase-releasable sialic acid and sialyltransferase activities associated with the metastatic cell lines.

A functional relationship between cell surface sialic acid and liver-colonizing ability of the murine colon cancer cell lines used in this study was suggested by comparing their ability to form liver nodules after their introduction into the portal system (splenic injection). This experiment confirmed the greatly increased ability of 51B LiM 5 to colonize the liver, compared to parental line 51B, and demonstrated a dramatic reduction in liver tumor foci after removal of sialic acid from the surface of these cells by neuraminidase treatment. Tracking of iododeoxyuridine-labeled tumor cells demonstrated that the decrease in tumor burden by neuraminidase treatment was due to decreased liver colonization and not due to a difference in the number of cells leaving the spleen and reaching the liver after splenic portal injection.

Immunohistochemical staining with WGA of primary tumors formed after cecal injection of parental cell line 51B revealed

Table 1 *Neuraminidase-releasable membrane-associated sialic acid in primary and metastatic colon cancer cell lines*

Cell line	Metastatic ability	Sialic acid (μg/mg protein)
51B	Low	1.49 ± 0.13[a]
51BLiM5	High	2.83 ± 0.04[b]
51BLiM6	High	2.96 ± 0.41[c]

[a] Numbers represent mean ± SD of four separate experiments.
[b] $P < 0.001$ compared to 51B.
[c] $P < 0.01$ compared to 51B.

Table 3 *Effect of neuraminidase treatment on hepatic tumor burden after intrasplenic injection of primary and metastatic cell lines*

Cell line	Condition	Liver weight (g)	Number of tumor nodules (range)
51B	–	0.96 ± 0.15[a]	3–12[b]
51B	+Neuraminidase[c]	1.03 ± 0.16	0–1
51B LiM 5	—	2.22 ± 0.75	220–500
51B LiM 5	+Neuraminidase	1.12 ± 0.17[d]	6–68[e]

[a] Mean ± SD. $n = 6$ animals/group.
[b] Macroscopic nodules visible to the naked eye.
[c] Neuraminidase type X from *C. perfringens*.
[d] $P < 0.05$ *versus* untreated cells.
[e] $P < 0.01$ *versus* untreated cells.

Table 2 *Sialyltransferase and galactosyltransferase activities in primary and metastatic colon cancer cell lines*

Acceptor	Donor	Reaction	Cell line	Specific activity (pmol/mg protein/h)
Asialo-transferrin	CMP-[^{14}C]NeuAc	NeuAc → Gal-	51B	12.20 ± 1.18[a,b]
			51BLiM5	23.60 ± 1.38[b,c]
			51BLiM6	31.20 ± 3.26[b,c]
Asialo-OSM	CMP-[^{14}C]NeuAc	NeuAc → GalNAc-	51B	4.44 ± 0.71[b]
			51BLiM5	7.24 ± 0.15[b,c]
			51BLiM6	8.62 ± 1.20[b,d]
GlcNAc	UDP-[^{3}H]Gal	Gal → GlcNAc-	51B	8.22 ± 0.21[e]
			51BLiM5	10.65 ± 1.22[e]
			51BLiM6	9.45 ± 0.12[e]

[a] Mean ± SD.
[b] $n = 6$.
[c] $P < 0.005$ compared to 51B.
[d] $P < 0.01$ compared to 51B.
[e] $n = 3$.

Table 4 *Distribution of [^{125}I]IdUR-labeled tumor cells after splenic injection*

[^{125}I]IdUR-labeled tumor cells (cell line 51B LiM 5; 10^6 viable tumor cells/animal = 81,000 to 84,000 cpm) were injected into the spleens of athymic nude mice followed by immediate splenectomy. Animals were sacrificed at various intervals and livers were removed. The percentage of radioactivity remaining in the spleen or present after various intervals in the liver and other organs was determined by gamma-counting.

	% of input radioactivity					
	Spleen		Liver		Other	
Time	−N[a]	+N	−N	+N	−N	+N
0	43.2 ± 11.7[b]	41.7 ± 13.8	51.6 ± 12.0	53.6 ± 10.8	5.2 ± 1.4	4.6 ± 3.4
10 min	41.2 ± 1.1	44.6 ± 1.6	51.0 ± 3.2	50.0 ± 1.6	7.8 ± 4.3	5.9 ± 2.1
1 h	46.0 ± 13.3	42.7 ± 13.1	48.6 ± 13.7	44.2 ± 12.9	5.5 ± 0.9	13.1 ± 1.4
4 h	43.2 ± 10.2	42.6 ± 9.0	49.6 ± 9.9	42.3 ± 8.8	7.2 ± 0.3	15.1 ± 1.1
8 h	40.2 ± 9.8	40.6 ± 7.9	51.0 ± 7.5	46.8 ± 7.0	8.8 ± 3.8	16.2 ± 6.9
12 h	44.0 ± 2.3	42.1 ± 3.0	22.5 ± 7.4[c]	3.6 ± 1.6	7.4 ± 0.9	5.8 ± 2.7
24 h	43.0 ± 4.0	41.9 ± 5.6	8.1 ± 3.8[c]	1.9 ± 0.9	2.3 ± 1.1	2.4 ± 1.2

[a] −N, untreated cells; +N, cells desialylated by prior neuraminidase treatment.
[b] Mean ± SD. $n = 6$ animals/group (except for 24 h, where $n = 9$ animals/group).
[c] $P < 0.05$ untreated *versus* neuraminidase-treated cells.

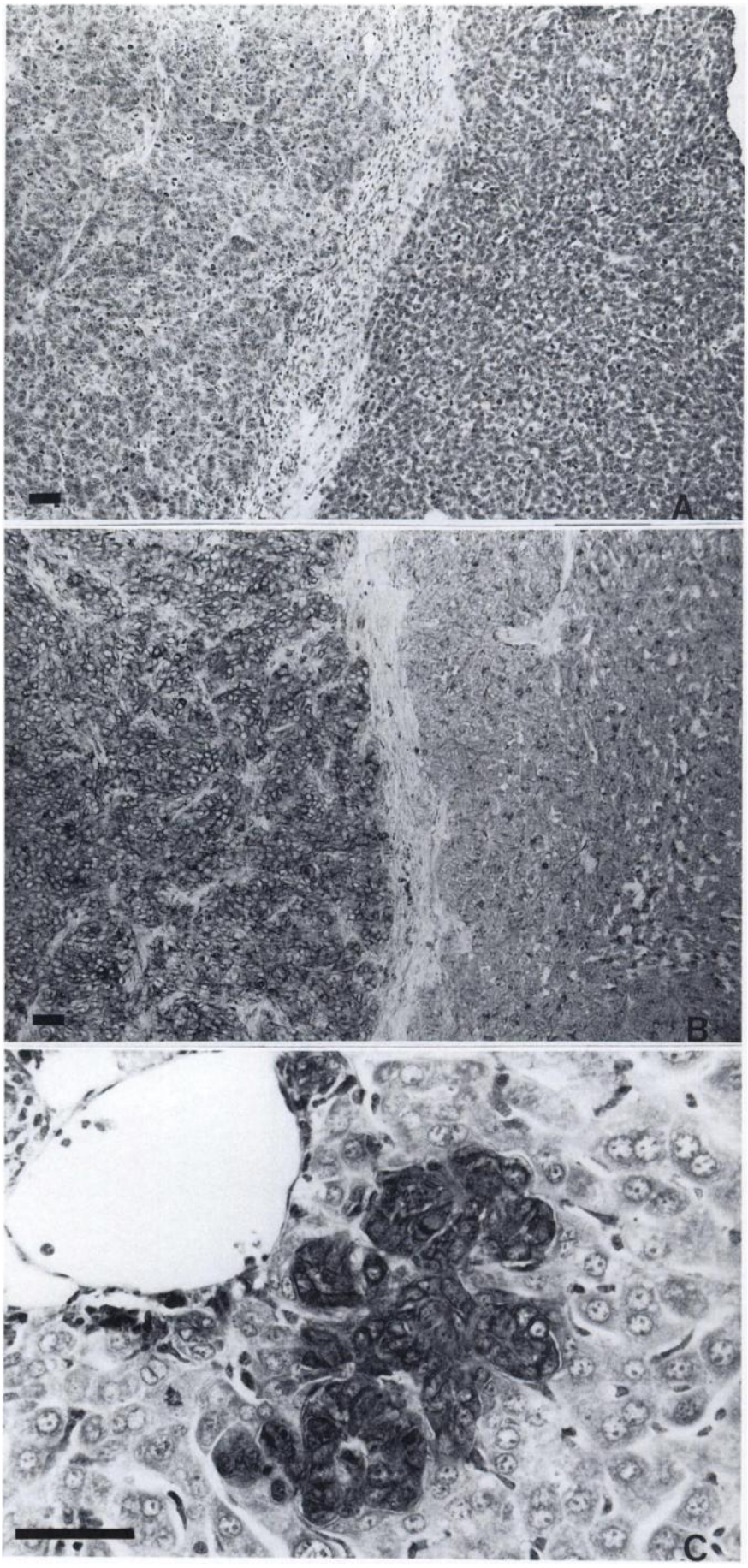


Fig. 5. WGA binding to experimental primary and metastatic tumors. ***Bars***, 50 μm. ***A***, hematoxylin- and eosin-stained tissue section from experimental cecal primary tumor formed after injection of murine colon cancer cell line 51B. Note two cell populations separated by stroma. ***B***, WGA immunoperoxidase staining of cecal primary tumor. Note staining of cell population on ***left***. ***C***, WGA immunoperoxidase staining of liver metastasis from cecal primary tumor.

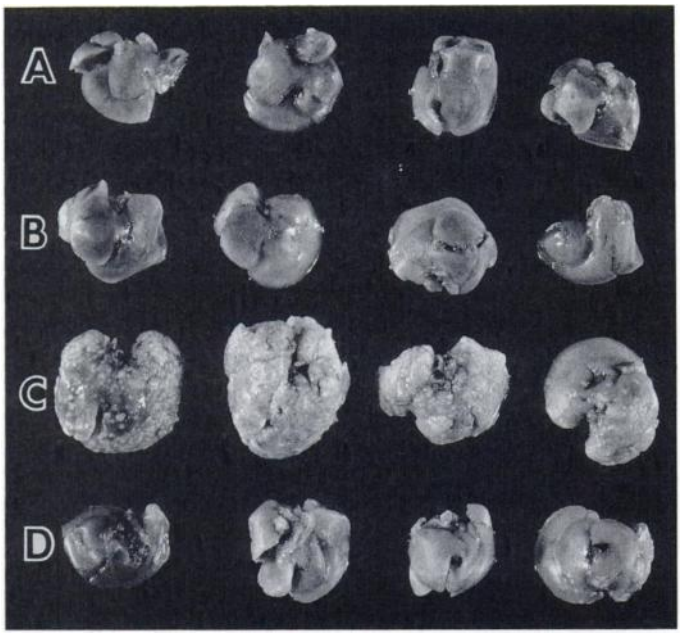


Fig. 6. Livers from BALB/c mice 2 weeks after intrasplenic injection of murine colon cancer cell lines. *A*, livers injected with 51B; *B*, 51B pretreated with neuraminidase prior to injection to remove cell surface sialic acid; *C*, 51B LiM 5; *D*, 51B LiM 5 pretreated with neuraminidase.

binding of this lectin to only a subpopulation of cells in the primary cecal tumor. Spontaneous liver metastases in the same animals, however, demonstrated strong homogeneous binding of WGA, again suggesting selective metastasis by WGA-binding tumor cells. Although it is possible that the difference in WGA binding between the cells of the primary cecal tumor and the liver metastases was a result of environmental factors (34), this is unlikely since cells selected for liver-metastasizing potential strongly bound WGA when grown in other sites (s.c., cecum; data not shown) and *in vitro*.

We found an approximately 2-fold increase in neuraminidase-releasable membrane-associated sialic acid and sialyltransferase activity associated with the metastatic colon cancer cell lines, compared to the poorly metastatic parent. While no significant difference in β1–4-galactosyltransferase (lactose synthetase) was found between the cell lines, we cannot exclude differences in other galactosyltransferases. Sialic acid is known to be present in various linkages on a variety of *N*-linked and *O*-linked oligosaccharides of glycoproteins. Glycoproteins with *N*-linked oligosaccharides containing the nonreducing terminal sequence Galβ1–4GlcNAc may serve as acceptors for the addition of sialic acid catalyzed by β-galactoside α2–6-sialyltransferase, and nearly stoichiometric amounts of sialic acid can be incorporated into asialotransferrin by this enzyme (51). While it is likely then that such a sialyltransferase is elevated in the cells studied (as suggested by SNA affinity chromatography), other sialyltransferases may also be responsible for increased incorporation into asialotransferrin. The sequence NeuAcα2–3Galβ1–4GlcNAc may be found in the *N*-linked chains of certain glycoproteins (52). Although asialotransferrin has not been shown to be an efficient acceptor for such an enzyme (51, 53), our data do not exclude an elevation in a β-galactoside α2–3-sialotransferase. Since sialic acid was also transferred to asialo-OSM by the metastatic colon cancer cells, *O*-linked glycoproteins in these cells may also contain greater amounts of terminal sialic acid, compared to the parental cell line. Sialylated oligosaccharides may be prominent in mucin-type glycoproteins from human colorectal cancers (54–56) and in some cases are responsible for tumor-associated antigenicity (57). The gastrointestinal cancer-associated antigen recognized by monoclonal antibody 19-9, for example, is a sialyl Le^a blood group antigen expressed as a ganglioside in tissues but found in mucin-type glycoproteins in serum (58). Sialylated mucin-type glycoproteins have been associated with metastatic potential in at least one murine colon cancer cell line (17), and alkaline-labile carbohydrate chains on high molecular weight sialoglycoproteins have been described on metastatic human colon cancer cells (34).

Our findings are consistent with observations from other laboratories, which have demonstrated increases in total or neuraminidase-releasable sialic acid and increases in the sialylation of penultimate galactose residues on metastatic murine tumor cells (5, 8, 15, 17). Wheat germ agglutinin binding by metastatic tumor cell variants is also, in at least some cases, due to the presence of sialic acid on terminal *N*-acetyllactosamine structures, the loss of which leads to decreased metastatic potential (20, 50). In the case of some tumor cells, overall levels of membrane-associated sialic acid are not elevated, but the position of sialic acid on terminal carbohydrate structures on which sialic acid appears may be most important in determining the extent of metastasis (9, 13, 16, 19). The present study further emphasizes the importance of cell membrane sialylation in determining the metastatic potential of cancer cells.

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