Adrenal Carcinoma Tumor Progression and Penultimate Cell Surface Oligosaccharides

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ABSTRACT

Many previous studies have implicated cell surface saccharides, and sialylglycoconjugates in particular, as important mediators of tumor cell metastasis. In this report, we have used three different specific sialidases and a highly sensitive high-performance liquid chromatographic sialic acid assay to probe the cell surfaces of several murine adrenal carcinoma variants. In contrast to several earlier studies on other metastatic variants, we find no significant differences in the overall levels of cell surface or total cellular sialic acid among three Y1 murine adrenal carcinoma variants with widely different metastatic phenotypes. However, using highly purified, linkage-specific sialyltransferases, in conjunction with F. cholerae sialidase, to probe the cell surface saccharide topography of specific penultimate oligosaccharides, we do find striking differences in oligosaccharide structures underlying the sialic acid moieties. Two tumorigenic and metastatic variants (F2 and F4) contain about 6-fold more penultimate Galβ1→3GalNAc sialylation sites than a related nonmetastatic variant (HSR) when CMP-[3H]-N-acetylneuraminic acid and the Galβ1→3GalNAc α2,6 sialyltransferase are used to probe the adrenal carcinoma cell surfaces. The metastatic variants also are found to contain 4- to 4.5-fold more Galβ1→3GalNAc sialylation sites than the nonmetastatic variant when the Galβ1→3GalNAc α2,3 sialyltransferase is used as a cell surface probe. Earlier work, which used the same sialyltransferase probes on sialidase-treated murine melanoma variants (A. Passaniti and G. W. Hart, J. Biol. Chem., 263: 7591-7603, 1988), also showed similar quantitative differences in penultimate structures between metastatic variants. However, in contrast to the adrenal carcinoma cells, the highly metastatic melanoma cells have severalfold lower levels of sialylatable penultimate Galβ1→3GalNAc and Galβ1→3GalNAc saccharides compared to their nonmetastatic counterparts. Thus, while the precise structural alterations or surface accessibilities of penultimate saccharides appear to be cell type dependent, these results suggest that pronounced changes in penultimate structures do correlate with the ability of the cells to undergo spontaneous metastasis from a subcutaneous tumor.

To further evaluate the hypothesis that cell surface glycoconjugates and, in particular, sialylated saccharides are directly involved in determining the metastatic phenotype, we used a highly sensitive high-performance liquid chromatographic sialic acid assay, in conjunction with a specific sialidase and two STases to probe the cell surfaces of metastatic variant adrenal carcinoma cells. A goal of this study was to determine if the observations from the melanoma model were generally applicable to metastatic tumor cells of widely different origins or cell types (6). Three invasive adrenal carcinoma variants were investigated for cell surface sialic acids, total cellular sialic acids, and specific penultimate oligosaccharides. These cells were clonally derived from a mixed population parental line by limiting dilution (7). The most invasive clone (F2) attaches to immobilized laminin and invades reconstituted basement membrane (matrigel) and forms multiple organ metastasis when injected s.c. into nude mice. The second invasive clone F4 attaches to laminin but does not spread or invade on matrigel and forms multiple organ metastasis when injected s.c. into nude mice. The noninvasive clone (HSR) does not attach to laminin or matrigel and produces no metastasis. Our data show that like the murine melanoma model, there are no differences in cell surface or total cellular sialic acids among these three adrenal carcinoma variants. However, in contrast to our findings in the melanoma system in which sialylatable penultimate structures are substantially reduced on highly metastatic cells, the metastatic F2 and F4 adrenal carcinoma cells contain substantially more penultimate Galβ1→3GalNAc and Galβ1→4GlcNAc sialylation sites than nonmetastatic HSR cells.

INTRODUCTION

Tumor cell surfaces have been extensively analyzed in order to understand the metastatic phenotype (1). According to Dennis (2), terminal carbohydrate moieties may aid tumor cell invasion of blood vessels by increasing their adhesion to the vessel walls. Sialylation of cell surface Galβ3 and GalNAc residues have been correlated with lung colonization potential using a series of murine tumor lines (3). Elevated cell surface sialic acid has been shown to correlate with spontaneous metastatic potential and invasiveness in vitro (4). Membrane structural changes in the glycoalyx may account for the ability of a tumor cell to implant and metastasize (5). In a recent study, two purified sialyltransferases, one α2,6 N-linked and the other α2,3 O-linked oligosaccharide specific, were used as cell surface probes for desialylated Galβ1→4GlcNAc and Galβ1→3GalNAc residues. These studies indicated that the differences in the lung-colonizing abilities of B16 melanoma metastatic variants do not correlate with the numbers or sialylation states of specific penultimate oligosaccharide structures on their surfaces. However, the relative levels of specific types of penultimate saccharide structures do correlate with the ability of the cells to undergo spontaneous metastasis from a subcutaneous tumor (6).

Materials and Methods

Cell Culture

HSR cells were established from a mixed population parental Y1 murine adrenal carcinoma tumor and obtained from Dr. Donna L. George (University of Pennsylvania). Derivative clonal lines F2 and F4 were established from the parental Y1 (American Type Culture Collection CCL 79) cells by limiting dilution. Fresh cells were retrieved from frozen stocks, and cells were grown in minimal essential medium.
Arthrobacter sialidase, Dulbecco's modified Eagle's medium-harvested and acid treated in the same fashion. Samples were dried and stored at -70°C until they were assayed.

Total Sialic Acid Content of Adrenal Carcinoma Cells

CDP-hexanolamine-Sephadex affinity resin (6, 12). Enzyme (5 µl; 2.5 mU) was added to reaction mixtures containing 35 µl deionized water, 12 µl of 5X buffer (50 mM sodium cacodylate, 2.5% Triton CF-54, pH 6.0), 1.25 µl bovine serum albumin (40 mg/ml). To start the reaction, 5 µl of isotopically diluted CMP-[3H]NeuAc was added (9.25 nmol containing 0.013 nmol CMP-[3H]NeuAc) at 4°C, and incubation was continued at 37°C for 0, 2, 5, or 10 min. Reactions were stopped by the addition of 5 µl of 20% SDS and boiling for 5 min. Samples were applied to Sephadex G-50 minicolumns (1-ml bed volume in tubing syringes) and washed through the column with 50 mM ammonium formate (250 µl) followed by 350 µl of the same buffer. Activity was quantitated by liquid scintillation counting.

Galβ1→4GlcNAc α2,6 Sialyltransferase. The method of Weinstein (10) was used to assay enzyme activity. Asialo α1-acid glycoprotein was used as the substrate. Enzyme (5 µl; 2.5 mU) was added to reaction mixtures containing 35 µl deionized water, 12 µl of 5X buffer (50 mM sodium cacodylate, 2.5% Triton CF-54, pH 6.0), and 1.25 µl bovine serum albumin (40 mg/ml). To start the reaction, 5 µl of isotopically diluted CMP-[3H]NeuAc was added (9.25 nmol containing 0.013 nmol CMP-[3H]NeuAc) at 4°C, and incubation was continued at 37°C for 0, 2, 5, or 10 min. Reactions were stopped by the addition of 5 µl of 20% SDS and boiling for 5 min. Samples were applied to Sephadex G-50 minicolumns (1-ml bed volume in tubing syringes) and washed through the column with 50 mM ammonium formate (250 µl) followed by 350 µl of the same buffer. Activity was quantitated by liquid scintillation counting.

Galβ1→3GlcNAc α2,3 Sialyltransferase. The method of Rearick et al. (13) was modified, 10 µl enzyme were added to assay mixtures containing 8.75 µl deionized water, 10 µl of 5X buffer (50 mM sodium cacodylate, 2.5% Triton CF-54, pH 6.0), 1.25 µl bovine serum albumin (40 mg/ml), and 10 µl lactose (288 mg/ml). To start the reaction, 10 µl of isotopically diluted CMP-[3H]NeuAc were added (0.13 nmol containing 0.039 nmol CMP-[3H]NeuAc) at 4°C. Reactions were continued at 37°C for 0, 15, or 30 min. Control samples lacking lactose substrate were also assayed. The reactions were stopped by diluting with 1 ml deionized ice water, and the diluted mixture was applied to a 1-ml QAE-Sephadex column as described before. [3H]NeuAc-lactose was eluted with 50 mM ammonium formate and quantitated by liquid scintillation counting.

Sialidase Digestion of Intact Cells

For the determination of cell surface sialic acids released from adrenal carcinoma cells by V. cholerae sialidase or Arthrobacter sialidase, Dulbecco's modified Eagle's medium-harvested cells were washed twice with wash buffer [10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.3, 24 mM sodium bicarbonate, 0.14 M NaCl, 5 mM pyruvate, and 1 mM CaCl₂]. Intact cells (10⁶) were incubated for 2 h at 4°C in 0.2 ml of the same buffer containing 0–20 mU of V. cholerae sialidase, 0–1.5 mU of NDV sialidase, or 0–20 mU of Arthrobacter sialidase. Cell supernatants were stored at -70°C until assayed for released sialic acid by a HPLC micro-TBA method (14).

Total Sialic Acid Content of Adrenal Carcinoma Cells

Total cellular sialic acid was determined by hydrolysis of washed cell pellets with 200 µl 0.1 N HCl for 60 min at 80°C, in screw-capped polypropylene microtubes under nitrogen gas. Samples were neutralized by adding 20 µl of 1 N NaOH plus 20 µl of 0.2 M sodium phosphate, pH 7.0. As a control, known amounts of N-acetylneuraminic acid were added to equivalent amounts of buffer and acid treated in the same fashion. Samples were dried and stored at -70°C until they were assayed for total cellular sialic acid by a HPLC micro-TBA method (14).

Sialyltransferase Radiolabeling of Intact Cells

This procedure has been described in detail previously (6, 12). Briefly, sialidase-treated or mock-treated adrenal carcinoma cells were washed in RPMI 1640 (Gibco) and resuspended in STase buffer. The cells were then treated with the indicated concentrations of STase at 4°C. To initiate the labeling reaction, 5 µl (2 µCi) of CMP-[3H]NeuAc (26.2 Ci/mmol) was added to 175 µl of cells and enzyme. After 6 h, the reaction was stopped by centrifugation through 1 ml 30% sucrose. The cell pellets were resuspended in 900 µl lysis buffer (containing 1% aprotinin and 1 mM phenylmethylsulfonyl fluoride) and incubated for 45 min at 4°C. The postnuclear supernatants were added to 100 µl of 20% SDS, boiled for 10 min, and stored at -70°C until they were used.

Preparation of Radiolabeled Macromolecules from Postnuclear Supernatants

Postnuclear supernatants which had been stored at -70°C were thawed quickly at 37°C and fractionated on Sephadex G-50 fine (1 x 30 cm) columns in 50 mM ammonium formate, 0.1% SDS, and 0.02% sodium azide. Fractions were collected, and radiolabeled macromolecular materials eluting in the void volume (V₀) were pooled, concentrated by lyophilization, and precipitated in acetone:water (8:1) at -20°C overnight.

Sds-PAGE and Fluorography

Equal amounts of cell protein precipitates prepared from postnuclear supernatants were separated on 10% SDS-PAGE gels according to the method of Laemmli (15). Gels were fixed in 50% methanol, treated with EnHance (NEN Research Products) for 1 h, incubated in deionized water for 30 min, and dried under vacuum. Kodak X-Omat AR film (Eastman Kodak) was used for fluorography.

β-Elimination of Radiolabeled Macromolecules

Acetone-precipitated [3H]NeuAc-labeled proteins were resuspended with 0.1 N sodium hydroxide, 1 M sodium borohydride at 37°C for 24 h (16) and stopped with 4 M acetic acid on ice until neutralized. The reaction mixtures were fractionated on Sephadex G-50 fine (1 x 30 cm) columns in 50 mM ammonium formate, 0.1% SDS, and 0.02% sodium azide. Fractions (1.0 ml) were collected with macromolecular materials eluting in the void volume (V₀) and oligosaccharides in the included volume (Vᵢ).

PnGase F Treatment of Radiolabeled Macromolecules

Acetone-precipitated [3H]NeuAc-labeled proteins were resuspended with reaction buffer plus PNgase F and incubated at room temperature for 48 h (16). The reactions were stopped by adding 0.5 ml of 20% SDS and fractionated on Sephadex G-50 fine column as described.

High-Performance Anion-Exchange Chromatography of Resialylated Oligosaccharides

Resialylated oligosaccharides (α2,6 STase labeled) were prepared by PNgase F treatment and desalted by Sephadex G-10 column. Pellicular quaternary amine-bonded (Dionex; Ion-Pac, AS-6) separation at alkaline pH was performed using the following sodium acetate gradient: isocratic at 0.1 M sodium hydroxide, 50 mM sodium acetate for 10 min, then a linear gradient to 0.1 M sodium hydroxide, 300 mM sodium acetate at 70 min. Radioactivity was detected with a Fle-One Radiomatic HPLC detector, and mass was detected by pulsed amperometry.

RESULTS

Cell Surface and Total Cellular Sialic Acid. We used V. cholerae sialidase, NDV sialidase, or Arthrobacter sialidase to release sialic acids from the surfaces of the adrenal carcinoma cell variants. A very sensitive high-performance liquid chromatographic assay was then used to detect released sialic acids. When adrenal carcinoma cells were treated with V. cholerae sialidase, Dulbecco's modified Eagle's medium-harvested and acid treated in the same fashion. Samples were dried and stored at -70°C until they were assayed for total cellular sialic acid by a HPLC micro-TBA method (14).
sialidase at 4°C for 2 h, the amounts of sialic acids released by this enzyme from each adrenal carcinoma variant were found to be the same (Fig. 1A). At saturating enzyme levels, the amount of sialic acids released was 2400 ± 150 (SD) pmol/mg protein from each metastatic variant. Arthrobacter sialidase was also used to release cell surface sialic acids, and the amount of sialic acids released at saturating enzyme from the three metastatic variants was again the same: 2500 ± 100 pmol/mg protein (Fig. 1B). When NDV sialidase was used to release cell surface sialic acids, the amounts of sialic acids released at saturating enzyme from the three metastatic variants were again very similar: F2, 870 ± 50 pmol/mg protein; F4, 1000 ± 60 pmol/mg protein; and HSR, 1000 ± 50 pmol/mg protein (Fig. 1C). The release of less sialic acids by NDV compared to the V. cholerae sialidase and Arthrobacter sialidase is expected, since the NDV sialidase preferentially cleaves the α2–3 sialic acid linkage much more efficiently than the α2–8 sialic acid linkage but cannot release α2–6 linkage. Arthrobacter sialidase preferentially cleaves the α2–6 and α2–3 much more than α2–8 sialic acid linkages, and the V. cholerae enzyme exhibits broad specificity but does display linkage preference (α2–3 > α2–6 >> α2–8) (17).

Total cellular sialic acids were determined by acid hydrolysis and the micro-TBA method. The amounts of total cellular sialic acids were: F2, 9000 ± 450 pmol/mg protein; F4, 9300 ± 500 pmol/mg protein; HSR, 8900 ± 400 pmol/mg protein. Therefore, there are no significant differences in total sialic acids among the three adrenal carcinoma metastatic variants.

Enzymic Detection of Specific Penultimate Oligosaccharide Structures on Adrenal Carcinoma Cell Surfaces. Adrenal carcinoma cells (F2, F4, and HSR) were harvested and either treated or mock-treated with V. cholerae sialidase (20 mU) to release cell surface sialic acids. The cells were then resialylated by incubating with N-linked specific Galβ1→4GlcNAc α2,6 STase and CMP-[³H]NeuAc. As shown in Fig. 2A, for sialidase-treated cells, at saturating enzyme levels, the STase added 13 ± 2 pmol sialic acids/mg protein (F2) and 12 ± 2 pmol/mg protein (F4) to the metastatic cell surfaces but only 2.0 ± 0.1 pmol/mg protein to the nonmetastatic HSR cells. Mock-treated cells incorporated only 0.5 pmol sialic acid/mg protein for all three metastatic variants (Fig. 2A). These data indicate that metastatic cells (F2 and F4) contain higher levels of exposed Galβ1→4GlcNAc penultimate structures on the cell surfaces than the nonmetastatic cells (HSR). We also used O-linked...
specific Galβ1→3GalNAc α,2,3 STase and CMP-[3H]NeuAc to probe the surfaces of the F2, F4, and HSR cells for accessible enzyme acceptor sites. In the absence of prior sialidase treatment, all three intact metastatic variants incorporated 0.2 pmol sialic acid/mg protein with exogenously added enzyme (Fig. 2B). Sialidase-treated intact nonmetastatic HSR cells incorporated 1 ± 0.1 pmol sialic acid/mg protein, whereas metastatic F2 cells incorporated 4.5 ± 0.1 pmol/mg protein, and F4 cells incorporated 4.0 ± 0.1 pmol/mg protein. From these data it is clear that the metastatic F2 and F4 cells contain substantially more transferase-accessible Galβ1—>3GalNAc structures than the nonmetastatic cells (HSR).

SDS-Polyacrylamide Gel Electrophoresis of Exogenous Sialylated Glycoproteins. Sialidase-treated radiolabeled macromolecules from F2, F4, and HSR cells were separated by 10% SDS-PAGE, and autoradiograms were prepared. Samples were loaded onto gels based upon cell equivalents. As shown in Fig. 3, most of the Galβ1→4GlcNAc α,2,6 STase-labeled macromolecules exhibited an apparent molecular weight greater than 30,000. The metastatic variant (F2, F4) cells were labeled more extensively compared to the nonmetastatic (HSR) cells (Fig. 3). Most of the Galβ1→3GalNAc α,2,3 STase-labeled macromolecules exhibited apparent M, greater than 45,000, and it appeared to label glycoproteins more selectively than the N-linked specific α,2,6 STase.

Protein Linkage of Sialyltransferase Radiolabeled Macromolecules. The amounts of O-linked sialylated oligosaccharides were determined by β-elimination and by sensitivity to PNGase F, an enzyme that cleaves N-linked oligosaccharides. Radioactivity in macromolecules from all three different metastatic variants that were labeled by the N-linked specific α,2,6 STase were completely resistant to β-elimination (Fig. 4A). As expected, the macromolecules shifted to lower molecular weights and produced multiple peaks because the β-elimination reaction results in extensive protein degradation. However, the macromolecules labeled with mucin-type O-linked specific Galβ1→3GalNAc STase were greater than 85% sensitive to β-elimination (Fig. 4B). The Vf fractions contained macromolecules (fractions 1–10), while the Vi fractions contained labeled oligosaccharides (fractions 10–25). Radiolabeled macromolecules were also prepared and treated with PNGase F as described in "Materials and Methods." About 70% of the macromolecules labeled with the N-linked specific Galβ1→4GlcNAc α,2,6 STase were sensitive to PNGase F, while 30% were PNGase F resistant (Fig. 4C). When the Vf fraction was pooled and treated with PNGase F again, very few radiolabeled oligosaccharides were released in the Vi fraction. However, macromolecules labeled with the O-linked specific Galβ1→3GalNAc α,2,3 STase were more than 95% PNGase F resistant (Fig. 4D). Taken together, these data indicate that the α,2,6 STase is almost exclusively labeling N-linked saccharides and the α,2,3 STase is almost exclusively reacting with O-linked saccharides, as expected.

High-Performance, High-pH Anion-Exchange Chromatography of Resialylated N-linked Oligosaccharides. Resialylated oligosaccharides (α,2,6 STase labeled) were prepared from PNGase-treated macromolecules and separated at alkaline pH with a sodium acetate gradient. This chromatographic method is capable of resolving complex mixtures of oligosaccharides, many with very similar structures (18). The pattern of sialylated oligosaccharides is similar in the three adrenal carcinoma cell
lines, each consisting of approximately 90% monosialylated and 10% disialylated oligosaccharides (Fig. 5). The restricted subset of the total oligosaccharide structures that are labeled confirms the high specificity and selectivity of these glycosyltransferase probes.

DISCUSSION

We have found that three metastatic variants of the Y1 mouse adrenocortical tumor which differ in their invasive ability in vitro and their metastatic potential in vivo contain similar levels of total and cell surface sialic acids as determined by a very sensitive HPLC sialic acid assay. However, after pretreatment with sialidase, the metastatic variants contain 6-fold higher levels of Gal\(\beta_1\rightarrow4\)GlcNAc and 4- to 4.5-fold higher levels of the Gal\(\beta_1\rightarrow3\)GalNAc penultimate sialylation sites than the related nonmetastatic variant. These data on sialylatable penultimate structures are the opposite of our findings from identical studies on murine melanoma metastatic variants (6). In these earlier studies sialidase-treated nonmetastatic cells expressed severalfold higher levels of these penultimate structures than their metastatic counterparts. Thus, the quantitative expression of particular penultimate oligosaccharide structures as a function of metastatic phenotype appears to be cell type specific (as might be expected from our knowledge of cell type-specific glycosylation). However, these data together with our earlier work indicate that substantial alterations in the expression of penultimate saccharides on sialylglycoconjugates are likely to be a common feature in the progression of a cell toward a metastatic phenotype.

We previously showed (6) that cellular sialic acid levels do not correlate with the metastasis of melanoma cells in contrast to other studies (3). Schirrmacher et al. (19) have also found that in the Eb/ESb lymphoma tumor system metastatic capacity does not correlate with cell surface sialic acids. Using an independently derived tumor model, we have now confirmed that total cell surface sialic acid content is not a good predictor of tumor metastasis.

By using radiolabeled sialic acids we show that the enzymatic probes used in this study recognized specific penultimate structures on the cell surfaces and that specific differences in macromolecule recognition by these enzymes could be observed on SDS-PAGE. We further show that although the amounts of these penultimate structures differ between these metastatic variants, the distribution of \(\alpha_2,6\) sialic acid oligosaccharide acceptors are similar in all cell metastatic variants.

We have found that cell surface sialic acid levels in the variants as measured by enzymatic release are about 27% of the total cellular sialic acids as measured by acid hydrolysis. These results are similar to those reported previously (14) for lymphoma cells, in which the enzymatic release of sialic acid is less than that expected by acid hydrolysis. Linkage-specific sialylation has also been observed in oligosaccharides isolated from human pancreatic carcinoma (20), where the levels of \(\alpha_2,3\) sialylated Lewis A antigen was found to be 44-fold higher than the levels of \(\alpha_2,6\) sialylated Lewis A antigen in the sera of cancer patients. Normal tissue cells also differ widely in their expression of \(\alpha_2,6\) sialylation structure, as shown recently for isolated murine lymphocytes (21). These studies suggest that although the particular expression of specific sialyl linkages may differ between normal cells and tumor cells from different tissue, the progression to a highly metastatic phenotype may result in phenotypic changes that alter the normal distribution of penultimate or underlying oligosaccharide linkages. These observations are consistent with results from a variety of investigators who have demonstrated changes in oligosaccharide processing as a result of metastatic progression (2).

In conclusion, it now appears that levels of total or cell surface sialic acids do not correlate with the metastatic potential of many murine tumors. Van den Eijnden et al. (22) found that GlcNAc residues may significantly influence the preference of the glycosyltransferases for certain branches, and Dennis et al. (23) showed that \(\beta_1\rightarrow6\) branching of Asn-linked oligosaccharides is directly associated with metastasis. We suggest that specific alterations in the branching or structures of oligosaccharides underlying the terminal sialic acid moieties do correlate with metastasis. These changes in oligosaccharide structures must reflect important underlying genetic or phenotypic
changes in the progression of malignant tumors to the full-blown metastatic phenotype.

ACKNOWLEDGMENTS

We would like to thank Dr. Robert Haltiwanger for his assistance with all LC systems and Dr. Rodney E. Willoughby for preparing the Galβ1→3GalNAc α2,3 porcine submaxillary sialyltransferase. We would also like to thank Drs. R. S. Haltiwanger, M. Takeuchi, R. E. Willoughby, W. G. Kelly, L. Roquemore, L. Blomberg, D. L. Dong, L. Kreppel, and T-Y. Chou for critically reading the manuscript.

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