[CANCER RESEARCH 41, 2262–2266, June 1981]
0008-5472/81/0041-0000$02.00

A Correlation between Cell Surface Sialyltransferase, Sialic Acid, and Glycosidase Activities and the Implantability of B16 Murine Melanoma[1]

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ABSTRACT

A murine melanoma variant (B16-F10^{lr6}), resistant to lymphocytic cytolysis, has been shown previously to produce lower numbers of tumor nodules in the lung of C57BL/6J mice following i.v. inoculations. These differences found in tumor implantation and lymphocyte recognition may be due to changes in surface properties of this cell line. Therefore, membrane-bound sialic acid (released by *Vibrio cholerae* neuraminidase treatment), ectosialyltransferase activity, and total cellular glycosidase levels were measured in this cell line and compared with levels in its parent melanoma tumor cell line, B16-F10, which was selected for its enhanced ability to form tumor nodules. The results of these studies indicate a correlation between the degree of lung implantation and the amount of tumor cell sialic acid accessible to neuraminidase cleavage, tumor cell surface sialyltransferase activity, and several cellular glycosidase activities. These results are consistent with the idea that membrane structural changes in the glycocalyx may account for the ability of a tumor cell to implant and metastasize.

INTRODUCTION

Many of the properties of malignant tumor cells which enable them to be released from a primary tumor and implant at a secondary site are thought to be "surface-membrane directed." These properties include changes in the biochemical composition of membrane glycoproteins and their carbohydrate moieties, as well as changes in surface enzyme activities. It is conceivable that these cell surface alterations are important for cellular implantation, one of the steps in the metastatic process (5, 8, 22).

Recently, it has been shown that fusion of parent B16-F1 murine melanoma cells with membrane vesicles obtained from the highly metastatic variant B16-F10 caused an increase in the number of tumor nodules appearing in the lungs of animals receiving these i.v. implants (20, 21). These experiments show that differences in surface membrane composition between the F1 and F10 variants may serve as the basis for the observed variations in lung implantability. Yet, in another recent article, the wide differences in metastatic behavior of B16 melanoma variants (F1, F10, F10^{lr6}) were not correlated with any major qualitative changes in several surface parameters (23). But, in the same study, quantitative differences were found in some surface components between these cell lines, and, perhaps, one should expect differences to be only quantitative in nature since all the variants do implant in the lung but in varying degrees only.

In this report, we investigated several cell surface glycoconjugate-related parameters in B16-F10 and B16-F10^{lr6} melanoma. The B16-F10 line was selected from the B16-F1 tumor for its increased lung implantation properties (9). The B16-F10^{lr6}, a low-lung-implanting variant comparable to the B16-F1, was selected for its resistance to cytolysis by immune syngeneic lymphocytes (12). Since both implantation and resistance to lymphocyte cytolysis (11) are presumed to be membrane-directed phenomena, experiments were designed to study several cell surface-related parameters of these 2 melanoma variants. The amount of cellular sialic acid accessible to neuraminidase, ectosialyltransferase activity, and exoglycosidase activities was quantitated. These parameters may be involved in the mechanisms of cell to cell recognition, adhesion, communication, and metastasis.

MATERIALS AND METHODS

Animals. Eight- to 10-week old inbred C57BL/6J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Tumor Cells. B16 murine variant melanoma lines with different abilities to form lung nodules after i.v. administration (designated as B16-F10 and B16-F10^{lr6}) were initially developed by Dr. I. Fidler and obtained from Dr. G. Poste. These tumor cell lines were maintained *in vitro* under culture conditions recommended by Fidler (9). In all cases, cells were removed from monolayer culture by scraping with a rubber policeman.

***In Vivo* Studies.** B16 melanoma cells were harvested from nonconfluent monolayers. The cells were washed and resuspended in RPMI-1640-HM.[4] The percentage of viable tumor cells was determined by the trypan blue exclusion test. The cell suspension was diluted so that cell count adjusted to 5×10^4 viable cells in 0.2 ml and was injected into the tail vein of each mouse. Mice were killed 21 days later, and the number of pulmonary nodules was counted under a dissecting microscope. In one experiment, mice were given injections i.v. of 10^4 viable B16-F10 cells and pretreated *in vitro* with 100 units of protease-free VCN per ml of RPMI-1640-HM (pH 7.0) at 37° for 30 min. Cell viability remained higher than 95% as determined by trypan blue dye exclusion. VCN, a hydrolase having broad substrate specificity, was obtained from Calbiochem-Behring, La Jolla, Calif. One unit of activity is defined as that

[1] This work was supported in part by Grants CA-19814 and CA-13038 from the National Cancer Institute, USPHS, Department of Health and Human Services.

[2] Recipient of an American Cancer Society, Eleanor Roosevelt International Fellowship awarded by the International Union against Cancer.

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Received February 19, 1980; accepted March 9, 1981.

[4] The abbreviations used are: RPMI-1640-HM, Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 10 mM 3-(*N*-morpholino)propanesulfonic acid; VCN, *Vibrio cholerae* neuraminidase.

amount of enzyme capable of removing 1 μg of *N* acetylneuraminic acid from human α_1-acid glycoprotein in 15 min under optimal conditions.

Sialic Acid Determination. The amount of free sialic acid released from whole cells by VCN (100 units/ml for 1 hr (pH 5.5) at 37°) was quantitated according to the modified thiobarbituric acid assay (1). *N*-Acetylneuraminic acid (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. Control cells were incubated and treated in an identical manner but without neuraminidase treatment; control values were subtracted from those values obtained with enzyme-treated cells.

Cellular protein was determined by the method described by Lowry *et al.* (16). Bovine serum albumin was used as a standard.

Ectosialyltransferase. The activity of cell surface sialyltransferase was determined as described by Bernacki (2). The monolayer cells growing in semiconfluent culture were harvested and pretreated with neuraminidase (100 units/ml for 30 min at 37°, pH 7.0) to desialylate cell surface glycoconjugates. These cells were washed, and 2 ml of RPMI-1640-HM (Grand Island Biological Co., Grand Island, N. Y.), pH 7.0, were added along with 1 μCi of cytidine 5′-monophosphate-N-[*acetyl*-^{14}C]-neuraminic acid (specific activity, 227 mCi/mmol; Amersham/Searle Corp., Arlington Heights, Ill.); the final incubation volume was 2040 μl. After an incubation (1 hr, 37°), the reaction was terminated with the addition of 1% phosphotungstic acid; the acid precipitates were washed twice with 2 ml of 10% trichloroacetic acid. The remaining insoluble material was then dissolved in 0.2 ml N NaOH, neutralized with 0.2 ml N HCl, and added to 10 ml of ACS cocktail (Amersham). The incorporated radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. The activity of the enzyme was expressed as cpm of *N*-[*acetyl*-^{14}C]neuraminic acid incorporated per mg of cellular protein per hr. Control cells were treated in an identical manner with the exception of the neuraminidase treatment. Ectosialyltransferase activity is linear for up to 2 hr after which it begins to level off. This probably is due to a limitation of suitable cell surface acceptors for the enzymatic addition of sialic acid.

Colorimetric Assays for Glycosidase and Acid Phosphatase. Enzyme activities were determined with 0.1% Triton X-100 homogenates of the subconfluent cultures of the 2 variant cell lines. The enzyme assay was carried out at pH 4.3 in 0.2 M citrate buffer, using 6 μmol of *p*-nitrophenyl derivatives as substrate; after 1 hr of incubation at 37°, the reactions were terminated with 0.4 M glycine-NaOH buffer, pH 10.5, and the absorbance of the released *p*-nitrophenol was measured at 420 nm. To express the specific activity of the enzymes, nmol of hydrolyzed *p*-nitrophenol per hr per mg cellular protein were calculated. *p*-Nitrophenol was used as standard (4). Cellular protein was estimated by the method of Lowry *et al.*

Neuraminidase Assay. Enzyme activity was determined with 0.1% Triton X-100 homogenates of the subconfluent cultures of the 2 variant cell lines. The enzyme assay was carried out at pH 5.5 in 0.1 M sodium acetate buffer using a tritiated sialic acid-containing derivative of fetuin. Fetuin-[^{3}H]sialic acid was prepared by reduction of periodate-treated fetuin with [^{3}H]-$NaBH_4$ as described previously (3). The amount of acid-soluble radioactivity produced during the enzyme assay was quantitated by scintillation counting methods. Enzyme activity was expressed as equivalent units of VCN which was assayed under identical conditions. One unit of VCN liberated 55,400 cpm of [^{3}H]sialic acid per hr.

Statistical Analysis. Data were analyzed using Student's *t* test.

RESULTS

The Incidence of Artificial Lung Metastases Produced by the i.v. Injections of Tumor Cells from B16 Variant Lines. In these experiments, the mice were killed 21 days after the i.v. inoculation of 5×10^4 viable cells of either the B16-F10 or B16-F10^{lr6} variant tumor lines maintained in tissue culture. All of the mice given injections of B16-F10 cells developed pulmonary tumor nodules, whereas only 23 of 28 developed these nodules following the inoculation of B16-F10^{lr6} cells. The average number of resultant pulmonary tumors differed among the groups; tumor cells from the B16-F10 line yielded 5 times more pulmonary nodules than did cells from the B16-B10^{lr6} line (Table 1). The differences between the incidence of pulmonary metastases were significant at a $p < 0.001$ level. No decrease in the number of lung nodules was found in mice given i.v. injections of *in vitro* neuraminidase-treated B16-F10 tumor cells as compared to untreated controls.

Sialic Acid Content of B16 Variant Melanoma Lines. The results of the quantitative determination of neuraminidase-releasable sialic acid of the variant melanoma cell lines are presented in Table 2. The treatment of suspended cells *in vitro* with neuraminidase released 2.2 ± 0.4 μg of *N*-acetylneuraminic acid per mg of cellular protein from the B16-F10 cell line but only 1.0 ± 0.2 μg from the B16-F10^{lr6} cells. The difference in the amount of released sialic acid was statistically significant at $p < 0.05$ level. That means that the highly metastasizing B16-F10 cells contained more neuraminidase-sensitive sialic acid as compared to the low-lung-implanting B16-F10^{lr6} cells.

Activity of B16 Ectosialyltransferase. The B16 melanoma variant lines were found to contain ectosialyltransferase enzyme activity. These cells incorporated significant quantiites of *N*-[*acetyl*-^{14}C]neuraminic acid from cytidine 5′-monophos-

Table 1

Incidence of pulmonary metastatic nodules in C57Bl/6J mice inoculated i.v. with 5×10^4 viable B16 melanoma cells

B16 variant lines	No. of pulmonary nodules[a]
F10	54.8 ± 28.2[b]
F10^{lr6}	11.6 ± 8.4

[a] Twenty mice/group. Lung nodules were counted 21 days after i.v. inoculation.
[b] Mean ± S.D. Differences between the incidence of pulmonary metastases were statistically significant at $p < 0.001$ level.

Table 2

Amount of neuraminidase-releasable sialic acid of B16 melanoma variant lines in culture

B16 variant lines	*N*-acetylneuraminic acid (μg/mg protein)
F10	2.2 ± 0.4[a]
F10^{lr6}	1.0 ± 0.2
% of decrease[b]	54.8
Significance	$p < 0.05$

[a] Mean ± S.D. Results are an average of 3 separate assays performed in triplicate.
[b] Percentage of decease $= \frac{F10 - F10^{lr6}}{F10} \times 100.$

Table 3

Sialic acid: ectosialyltransferase activity of intact B16 melanoma variant lines in culture

B16 variant lines	Specific activity (cpm/mg protein/hr)		
	VCN-treated	Untreated	Treated − control
F10	2693 ± 502[a]	1282 ± 198	1411
$F10^{lr6}$	1804 ± 393	1140 ± 213	664
% of decrease[b]			53

[a] Mean ± S.D. of 3 separate assays performed in triplicate.

[b] Percentage of decrease $= \frac{F10 - F10^{lr6}}{F10} \times 100$.

Table 4

Glycosidase and acid phosphatase activities of B16 melanoma variant lines in culture

Enzyme	B16 variant lines		
	F10	$F10^{lr6}$	F10/$F10^{lr6}$
β-Galactosidase (EC 3.2.1.23)	18.2 ± 7.0[a]	12.2 ± 4.1	1.5
β-L-Fucosidase (EC 3.2.1.38)	3.2 ± 0.2	2.6 ± 0.3	1.2
α-D-Mannosidase (EC 3.2.1.24)	22.1 ± 2.0	11.3 ± 1.4[b]	2.7
N-Acetyl-β-D-galactosaminidase (EC 3.2.1.53)	30.3 ± 3.2	11.2 ± 0.2[b]	2.7
N-Acetyl-β-D-glucosaminidase (EC 3.2.1.30)	113 ± 13	62.1 ± 3.1[b]	1.8
Acid phosphatase (EC 3.2.3.2)	68.1 ± 6.2	65.2 ± 6.1	1.0
Neuraminidase[c] (EC 3.2.1.18)	0.29 ± 0.13	0.46 ± 0.11	0.6

[a] Mean ± S.D. of 2 separate assays performed in triplicate. Enzyme activity is expressed as nmol per hr per mg protein.

[b] Differences are statistically significant at the $p < 0.02$ level.

[c] Activity is expressed as equivalent units of VCN per mg cellular protein.

phate-*N*-[*acetyl*-^{14}C]neuraminic acid, its nucleotide sugar precursor, following pretreatment of these cells with neuraminidase. A comparison of the activity levels of ectosialyltransferase of B16-F10 and $B16\text{-}F10^{lr6}$ lines is shown in Table 3. It was found that the activity level in the highly metastatic B16-F10 cells was 2-fold higher than the level on the low-implanting $B16\text{-}F10^{lr6}$ cells following neuraminidase treatment. This finding is consistent with data in Table 2, indicating that more sialic acid was removed from B16-F10 cells by VCN treatment, thereby exposing more available acceptor sites for resialylation.

Activity Levels of Glycosidases and Acid Phosphatase. Exoglycosidase activities were generally found to be elevated in highly metastatic B16-F10 lines as compared with the low-lung-implanting $B16\text{-}F10^{lr6}$ line with the exception of neuraminidase, which was lower in the B16-F10 line. The relative activity levels of fucosidase and neuraminidase were very low in both cell lines, while the activity levels of galactosidase, mannosidase, and hexosaminidase were much higher. The latter 2 enzyme types were significantly elevated in the B16-F10 cell line. Acid phosphatase activity was similar in both cell lines (Table 4).

DISCUSSION

It is generally believed that metastatic behavior of tumor cells may be an expression of unique membrane alterations. Thus, differences in metastatic properties of various tumors might be related to differences in their cell surface components (28). The different implanting variants of the very same tumor are considered particularly useful for studying cellular properties that impart an increased propensity to implant and form tumors following an i.v. inoculation. Therefore, we studied several membrane properties of 2 variants of B16 melanoma which differ in their lung implantability.

Fidler and Bucana (11) and Fidler *et al.* (12) developed a useful model for studying experimental metastases by selecting out a B16 melanoma tumor cell variant (F10) which produced a high number of lung tumor nodules following i.v. inoculation. Bosmann *et al.* (5) and Yogeeswaran *et al.* (30) demonstrated that this F10 variant had significantly more neuraminidase-accessible sialic acid than the parent F1 line. Studies by Raz *et al.* (23) have also shown differences in sialylation of membrane glycoproteins of B16 variants. Their findings indicate an inverse relationship between sialylation of a major membrane sialoglycoprotein and lung implantability. The apparent discrepancies between these findings and the others may be due to major contributions of neuraminidase-susceptible sialic acid by gangliosides (30). Also, it is assumed that only cell surface sialic acid is removed by neuraminidase; however, it is possible that some intracellular sialic acid is also released by neuraminidase treatment which contributes to the total amount of sialic acid measured, and comparisons made between the amount of sialic acid removed by VCN and that labeled either metabolically or with enzyme treatments also may not be identical. Nevertheless, Burger *et al.* (8) have shown that a wheat germ agglutinin-resistant B16 melanoma line (26), having decreased surface sialic acid, also loses its ability to metastasize to the lung. These findings are similar to those reporting a correlation between cell surface sialylation and metastatic properties of a high- and low-metastatic line derived from polyoma-induced Wistar-Furth renal sarcoma or RNA virus-transformed BALB/c3T3 cell lines (18, 29). All of these studies are consistent with our own. In the experiments presented here, the amount of free sialic acid released from intact B16-F10 and $B16\text{-}F10^{lr6}$ cells by *in vitro* treatment with neuraminidase was measured, compared, and found to correlate with the ability of the tumor cells to implant and grow in lung tissue. The low-implanting $B16\text{-}F10^{lr6}$ cells had less than one-half the neuraminidase-susceptible sialic acid as compared with the high-implanting B16-F10 tumor cells. These positive correlations point to the potential biological role of sialic acid in the process of tumor cell implantation and cellular adhesiveness.

Sialic acid is attached to endogenous membrane acceptors by sialyltransferases located in the Golgi and the plasma membrane of a variety of cell types. Bernacki (2) and Porter Bernacki (19), using biochemical methods and electron microscope autoradiography, demonstrated the presence of an ectosialyltransferase system on the surface of L1210 leukemic cells. Using the same biochemical criteria, sialyltransferase activity was detected on the surface of these B16 melanoma cell lines. Again, the highly metastatic B16-F10 line had twice the activity of the low-metastatic $B16\text{-}F10^{lr6}$ cells. This demonstrated elevation of sialyltransferase activity on the surface of highly metastatic B16-F10 cells may perform a function of increased synthesis or repair of sialylated glycoprotein constituents of cells in which a higher amount of surface sialic acid has also been detected. Another possible function for the

changes in ectosialyltransferase and their substrates is increased intercellular adhesion. Such a role for cell surface glycosyltransferases in forming specific enzyme-substrate attachments between the surface glycosyltransferases of one cell and the surface glycoconjugates of another has been proposed by Roseman (24) and Roth and White (25).

The B16-F10^{lr6} melanoma line was selected by Fidler and Bucana (11) and Fidler *et al.* (12) for its resistance to cytotoxicity mediated by syngeneic lymphocytes. It was found to form smaller clumps of cells with lymphocytes, and this may be another factor lessening its ability to be arrested and implanted in the lung. Earlier, Fidler (10) had found that, the larger the circulating multicellular emboli, the greater the rate of arrest in the lung leading to the formation of more tumors. Using the same tumor, Nicolson and Winkelhake (17, 28) reported that the highly implanting B16 melanoma variant cells adhered more rapidly to each other in monolayer attachment assay; similarly, the heterotypic rates of adhesion of the more metastatic melanoma cells to host organ cells *in vitro* and to cultured endothelial cells were also higher. Using the same melanoma system, Gasic and Gasic (13) found highly metastatic variant cells heterotypically aggregated with platelets at faster rates as compared to low-metastatic variants. Pearlstein *et al.* (18) also found a correlation between the degree of cell surface sialylation of rat renal sarcoma cells, their ability to aggregate platelets, and their propensity or ability to metastasize. These studies indicate that increased attachment of blood-borne tumor cells to each other, to blood components, and/or to endothelial cells can promote interactions resulting in enhanced tumor cell implantation and metastasis. All of these observations again implicate a role for cell surface glycoconjugate in cell-to-cell adhesion and metastasis.

In our experiments, however, no decrease in the number of lung nodules was found when *in vitro* enzymatically desialylated B16-F10 cells were inoculated i.v., as compared to control. This finding is consistent with observations made by others (7, 27). The lack of *in vivo* response to *in vitro* neuraminidase treatment, however, does not argue against the suggested role of sialic acid in the implantation and metastatic process but may be explained by the rapid regeneration of surface sialic acid on enzymatically "uncoated" cells observed *in vitro* (13, 15). It is very likely that the same regeneration process takes place *in vivo*, explaining why previous removal of sialic acids is without effect on metastatic behavior. This regeneration of surface sialic acid may be facilitated by ectosialyltransferase which was found to be elevated in the highly metastatic B16-F10 variant. This suggests the possibility that the B16-F10 variant is capable of maintaining a higher surface sialic acid content due to increases in the rate of cell surface repair. This capability may impart some sort of selective advantage to this variant for implantation or for further growth of this cell type in the lung (14).

Finally, using the B16 melanoma variant lines, Bosmann *et al.* (5) reported that the more metastatic B16 variants produce higher levels of glycosidases. In our own studies, we also found elevated levels of glycosidases (such as β-L-fucosidase, α-D-mannosidase, *N*-acetyl-β-D-glucosaminidase, and *N*-acetyl-β-D-galactosaminidase) in the highly metastatic F10 line as compared to the low-lung-implanting F10^{lr6} line, with the exception of neuraminidase, which was higher in the B16-F10^{lr6} line. Increased levels of neuraminidase and decreased levels of ectosialyltransferase activity in the B16-F10^{lr6} line might account for the lower levels of sialic acid removed from these cells by VCN treatment. Levels of acid phosphatase activity were similar between the 2 variants, and this result is consistent with the findings of others (23). The increased levels of the certain glycosidases in the B16-F10 cells may enhance the invasive capacity of this variant, altering certain membrane components by sublethal autolysis (6). These differences in glycosidase levels together with the observed increases in the ectosialytransferase activity and surface sialic acid content may alter cell surface-regulated events and account for the increased metastatic behavior of the B16-F10 melanoma variant.

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