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β1–6 Branching of Asn-Linked Oligosaccharides Is Directly Associated with Metastasis

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Neoplastic transformation has been associated with a variety of structural changes in cell surface carbohydrates, most notably increased sialylation and β1–6–linked branching of complex-type asparagine (Asn)-linked oligosaccharides (that is, –GlcNAcβ1–6Manα1–6Manβ1–). However, little is known about the relevant glycoproteins or how these transformation-related changes in oligosaccharide biosynthesis may affect the malignant phenotype. Here it is reported that a cell surface glycoprotein, gp130, is a major target of increased β1–6–linked branching and that the expression of these oligosaccharide structures is directly related to the metastatic potential of the cells. Glycosylation mutants of a metastatic tumor cell line were selected that are deficient in both β1–6 GlcNAc transferase V activity and metastatic potential in situ. Moreover, induction of increased β1–6 branching in clones of a nonmetastatic murine mammary carcinoma correlated strongly with acquisition of metastatic potential. The results indicate that increased β1–6–linked branching of complex-type oligosaccharides on gp130 may be an important feature of tumor progression related to increased metastatic potential.

ONE OF THE MORE CONSISTENTLY observed alterations following neoplastic transformation is a shift toward the synthesis and expression of larger Asn-linked oligosaccharides (*1–5*). Such changes have been detected in both rodent (*1–3*) and human tumor cells (*5*) transformed by chemical mutagens (*1*), oncogenic viruses (*2*), or by transfection with DNA obtained from neoplastic cells (*3*). In a number of studies the change in size has been attributed to an increase in sialic (neuraminic) acid content of the structures (*4–6*). More recently, rodent cells transformed with polyoma virus (*7*) or cells transfected with activated H-*ras* oncogenes (*8*) have been shown to be more highly branched at the trimannosyl core of Asn-linked glycans because of the addition of β1–6–linked lactosamine antennae (that is, galβ1–4GlcNAcβ1–6). Since many of the lactosamine antennae are substituted with sialic acid, increased branching may also contribute to the transformation-related increase in sialic acid. Branching of complex Asn-linked oligosaccharides to produce tri-, tri′-, and tetra-antennary structures appears to depend on the action of UDP-GlcNAc:α-D–mannoside β1,4*N*-acetylglucosaminyltransferase (that is, GlcNAc transferase IV) and UDP-GlcNAc:α-D–mannoside β1,6*N*-acetylglucosaminyltransferase (that is, GlcNAc transferase V) (*9*) (Fig. 1). After the action of the GlcNAc transferases, processing is completed by the addition of galactose and sialic acid to produce the common sialyllactosamine antennae. Yamashita *et al.* compared six GlcNAc transferase activities and found only transferase V was elevated two times more than normal in polyoma virus–transformed BHK cells (*10*).

As a first approach to determine whether expression of specific oligosaccharide structures on malignant cells contribute to their tumorigenic or metastatic behavior, we isolated glycosylation mutants of the highly metastatic, murine tumor cell line called MDAY-D2 (*11*). For example, the class 1 genotype was consistently nonmetastatic in both syngeneic and immunosuppressed nude mice. The biochemical basis of this mutation appeared to be a deficiency in the transport of uridine diphosphate (UDP)–galactose into the Golgi apparatus, and, consequently, Asn-linked oligosaccharides lacked the typical sialylated lactosamine antennae (*12*). By taking advantage of the hypersensitivity of the class 1 cells to BSII lectin from *Bandeirea simplicifolia*, we were able to select single-step revertants that simultaneously regained the sialylated lactosamine antennae and the highly metastatic phenotype, thereby showing a direct association between the glycosylation defect and loss of metastatic potential in these cells (*13*).

Since the class 1 mutation also inhibited ganglioside biosynthesis (*14*), these studies did not indicate which classes of glycoconjugates were required for expression of the metastatic phenotype. We therefore selected glycosylation mutants with defects limited to Asn-linked oligosaccharide biosynthesis. The class 3 mutants of MDAY-D2 cells were selected in medium containing leukoagglutinin (L-PHA) and BSII lectin; the latter lectin was added to eliminate class 1 mutants (*13*). The choice of L-PHA for mutant selection was based on the known binding specificity of the lectin for tri′- and tetra-antennary complex-type oligosaccharides (*15*). L-PHA binding requires the β1–6–linked lactosamine antennae and has recently been used to detect these structures in transformed cells (*8*). Two clones, KBL1 and KBL2, with identical lectin sensitivity profiles were isolated, and although the mutants were highly tumorigenic, their metastatic potential was dramatically reduced (Table 1). Compared to MDAY-D2, the mutants were poorly metastatic when injected by either intravenous or subcutaneous routes.

The structural changes in the class 3 lectin resistant mutants could be deduced from the lectin staining of glycoproteins separated by

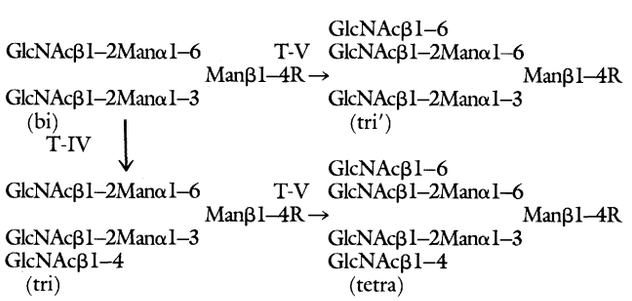

Fig. 1. GlcNAc transferases IV and V initiate the peripheral antennae in tri-, tri′- and tetra-antennary complex-type oligosaccharides. Only structures with the β1–6–linked antennae and further substitutions of galactose bind L-PHA (*15*). R = GlcNAcβ1–4GlcNAc1-Asn.

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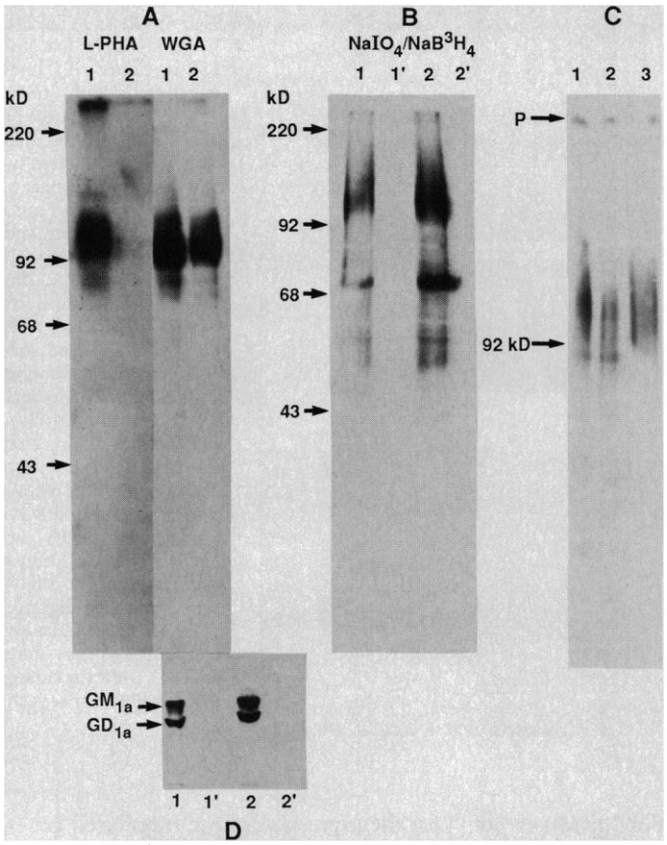

Fig. 2. Characterization of the oligosaccharide defect in class 3 cells. (**A**) Plasma membrane fractions were purified from MDAY-D2 (lane 1) and KBL1 (lane 2) cells and glycoproteins were separated by SDS–polyacrylamide gel electrophoresis. The gels were incubated with ^{125}I-labeled WGA or L-PHA and washed as described (*26*). (**B**) Sialylation of cell surface gp130. MDAY-D2 (lane 1) and KBL1 (lane 2) cells were labeled by the $NaIO_4/NaB^3H_4$ method (*27*). In lanes 1′ and 2′ $NaIO_4$ was omitted. A portion of the labeled cells were solubilized in PBS, 1% Triton X-100, and 2 m*M* PMSF, and proteins from equal cell numbers were separated by SDS-gel electrophoresis. The gels were soaked in Enhance (New England Nuclear), dried, and exposed to x-ray film for 3 weeks. (**C**) Detection of gp130 by Western blotting in MDAY-D2 (lane 1) cells; KBL1 (lane 2); and SP1 mammary carcinoma cells (lane 3). Cell lysate proteins were separated by SDS–gel electrophoresis, electroeluted onto nitrocellulose, and stained with rabbit antiserum to gp130 and an antiserum to rabbit immunoglobulin G coupled with peroxidase. The rabbit antiserum to gp130 was raised against gp130 purified from MDAY-D2 cells (*20*). P indicates the position of the pyronin dye used to mark the lanes. (**D**) Gangliosides were extracted from $NaIO_4/NaB^3H_4$ labeled cells by the method of Folch and separated by HPTLC as described (*14*).

SDS–polyacrylamide gel electrophoresis. The class 3 mutant KBL1 showed a loss of L-PHA binding to gp130 but no change in wheat germ agglutinin (WGA) binding (Fig. 2). The lack of change in the latter suggests the presence of sialic acid, since removal of this residue with neuraminidase eliminates WGA binding (*12*). Cell-surface sialic acid residues were also labeled by exposure to sodium periodate and tritiated sodium borohydride, which confirmed that gp130 was sialylated to a similar degree in the class 3 mutant and wild-type cells (Fig. 2). Since the mutant cells retained sialylated complex-type oligosaccharides but no longer bound L-PHA, it appeared that the defect might be restricted to the addition of the β1–6 antennae. Therefore, GlcNAc transferase V activity, the enzyme that initiates the β1–6–linked antennae, as well as GlcNAc transferases IV and I were compared in cell lysates of mutant and wild-type cells (see Fig. 1). The class 3 mutant KBL1 showed 20% and 60% of the wild-type levels of transferase V and IV activities, respectively, and a twofold increase in transferase I activity (Table 2). This is consistent with the loss of L-PHA–binding oligosaccharides; however, the coordinate change in the activity of GlcNAc transferases IV and I suggests that the mutation may affect a regulatory mechanism for a number of the GlcNAc transferases. The decreased levels of transferases V and IV would be expected to increase the proportion of biantennary complex-type oligosaccharides and this could explain the increased Con A sensitivity of the class 3 cells (Table 1). The biosynthetic defect in our class 3 cells appeared to be similar to that of the lymphoma mutant BW5147-PHA^{R}2.1, which was found to be completely deficient in GlcNAc transferase V activity (*16*). BW5147-PHA^{R}2.1 may also have had decreased GlcNAc transferase IV activity since the cells had reduced levels of triantennary oligosaccharides compared to the parental cell line. As expected, the class 3 mutation did not affect the ganglioside structures found in the cells (Fig. 3).

We have recently shown that swainsonine, a potent inhibitor of Golgi α-mannosidase II that blocks processing prior to the addition of the β1–6 antennae, also inhibits experimental metastasis of both the B16F10 melanoma and MDAY-D2 tumor cells (*17*). Therefore, in both the class 3 mutants and swainsonine treated tumor cells, the β1–6 branched complex-type structures are effectively reduced coincident with loss of metastatic potential. To determine whether enhanced expression of the L-PHA binding oligosaccharides in nonmetastatic or immortalized cell lines may be associated with acquisition of metastatic potential, we attempted to induce the metastatic phenotype in rat 1 fibroblasts and the nonmetastatic mouse mammary carcinoma called SP1 by

Table 1. Lectin sensitivity and malignant potential of the class 3 lectin resistant mutants. The origin of the highly metastatic tumor DBA/2 called MDAY-D2 has been described (*11*). The class 3 mutants were selected from 10^7 MDAY-D2 cells plated in alpha minimal essential medium plus 7% fetal calf serum (FCS) with 50 μg/ml of L-PHA and 20 μg per milliliter of BSII. The two surviving colonies were subcloned and one clone of each, termed KBL1 and KBL2, were tested for lectin sensitivity. Tumor cell proliferation in the presence of increasing concentrations of lectin was determined by measuring [^{3}H]thymidine incorporation into DNA. Cells were cultured at 3×10^4 per well in 96-well micro test plates containing serial dilutions of lectins. After 20 hours the cells were exposed to 1 μCi of [^{3}H]thymidine for 4 hours and the lectin concentration that reduced the isotope incorporation to 50% of the control was determined (D50). The ratio of mutant to wild-type D50 values for L-PHA ranged between 4 and 5 in five independent experiments. Tumorigenicity or the number of cells required for 50% tumor take was assessed by injecting 10, 50, 10^2, and 10^3 cells subcutaneously into syngeneic DBA/2J mice (five mice per group). Spontaneous liver metastases were counted by visual inspection of the liver of mice that had been injected subcutaneously with 10^5 tumor cells. The mice were examined on days 38 and organs were minced and placed in tissue culture. Only three liver nodules were found in mice injected with class 3 cells and no tumor cells grew out from the organ cultures of livers that had no visible nodules.

Cell line	Lectin sensitivity (D50, μg/ml)				Tumorigenicity (cell number)	Spontaneous liver metastases (nodules/liver)
	L-PHA	WGA	Con A	BSII		
MDAY-D2	5	6	35	>50	50 to 100	15, >100, >100, >100, >100, >100
KBL1	25	6	25	>50	10 to 50	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
KBL2	25	6	25	>50	50 to 100	0, 0, 0, 0, 0, 1, 1, 1

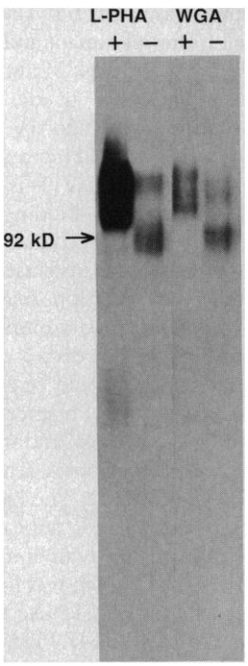

Fig. 3. Enhanced expression L-PHA–binding oligosaccharides on gp130 in v-K-*ras* transfected rat 1 fibroblasts. Plasma membranes were prepared from rat 1 cells (−) and from rat 1 cells transfected with the entire 7.0-kb v-K-*ras* oncogene ligated into the Eco RI site of pBR322 (+) (*19*). The plasma membrane glycoproteins were separated by SDS–gel electrophoresis and stained by direct overlay with ^{125}I-labeled WGA and L-PHA.

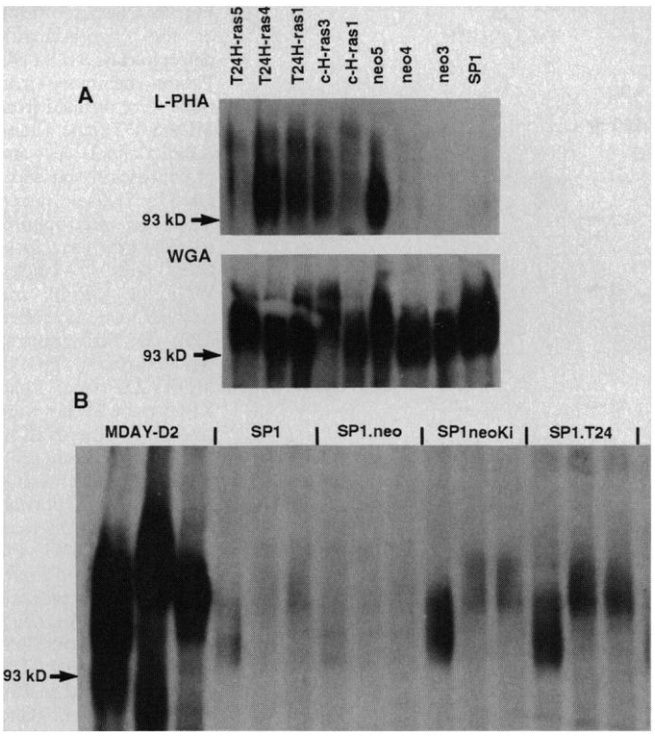

Fig. 4. (**A**) L-PHA and WGA binding to gp130 in cloned SP1 lines transfected with pSV_2neo, pSV_2neo·c-H-ras or pSV_2neo·T24H-ras. Cell lysates were separated by SDS–gel electrophoresis, electroeluted onto nitrocellulose paper, and blotted with ^{125}I-labeled WGA and L-PHA. (**B**) SP1 cells were transfected with pSV_2neo or with pSV_2T24H-ras and surviving colonies were pooled (SP1·neo and SP1·T24, respectively). The cells were injected subcutaneously into CBA/J mice and a metastasis was recovered from the kidney of an SP1·neo–injected mouse (SP1·neoKi). The cell lysates were first digested with: nil, neuraminidase, or neuraminidase plus endo β-galactosidase, left to right, respectively (*28*).

transfecting the lines with oncogenes (*18*). v-K-*ras* transfected rat 1 cells (*19*) injected subcutaneously into BALB/c nude mice produced tumors, and metastases were found in the kidneys and lungs of the tumor-bearing mice. The v-K-*ras* transformed cells showed a large increase in L-PHA binding to a 130-kD glycoprotein but no change in the intensity of WGA staining in this region of the gel (Fig. 3). Therefore it appears that the previously observed increase in β1–6 branching of complex-type oligosaccharides in v-K-*ras* transfected cells (*8*) is heavily represented on gp130. We have recently purified the L-PHA–binding gp130 to homogeneity from MDAY-D2 cells and have raised a polyclonal antiserum in rabbits (*20, 21*). Analysis by Western blotting indicated that immunologically cross-reactive gp130 is present in SP1 mammary carcinoma cells (Fig. 2) as well as a range of normal mouse tissues (*20*).

To segregate the tumorigenic and metastatic phenotypes, we conducted experiments in the tumorigenic but nonmetastatic mammary adenocarcinoma line called SP1. The cells were transfected with either pSV_2neo (neomycin resistance) alone or with pSV_2neo linked to activated T24H-*ras* oncogene or nonactivated c-H-*ras*. G418 resistant clones were selected and then tested for the expression of the transfected genes, spontaneous metastasis in syngeneic CBA/J mice, and expression of the L-PHA–binding gp130 (Figs. 4 and 5). Although some metastatic clones were obtained after transfection with pSV_2neo alone and pSV_2neo·c-H-ras (10 to 20%), there was a significant increase in the proportion of metastatic clones obtained from the pSV_2neo·T24H-ras transfected cells (100%) (*21*). Most significantly, however, the intensity of L-PHA binding to gp130 showed a strong positive correlation with metastatic propensity, regardless of the plasmid constructs that had been introduced into the cells (Fig. 4). In a population of tumor cells heterogeneous for the metastatic phenotype, the subpopulation of cells with high levels of L-PHA binding sites on gp130 would be expected to undergo selective metastasis. To test this hypothesis, we injected a pool of pSV_2neo transfected SP1 cells (50 to 100 clones), which would be expected to have a minor subpopulation of metastatic cells, subcutaneously into syngeneic mice (*21*). Forty days later 100% of the animals had visible metastases in either lungs or kidneys. Tumor cells obtained from metastatic nodules and established in tissue culture showed elevated levels of L-PHA–binding gp130 compared to the injected cells, indicating that the tumor cell with increased β1–6 branching had a selective metastatic advantage over the majority of the cells which expressed low levels of L-PHA–binding gp130 (Fig. 4).

The level of gp130 appeared to be similar

Table 2. GlcNAc-transferase activities in cell lysates of MDAY-D2 and class 3 mutant cells grown in tissue culture. Approximately 50×10^6 cells were suspended in 0.2*M* NaCl and lysed by freeze-thawing. The particulate fraction was washed three times with saline. After centrifugation the membrane pellet was solubilized in 200 μl of 1% Triton X-100, 40 m*M* cacodylate, *p*H 7.0, and centrifuged at 2000*g* to remove nuclei and debris. The reaction mixture contained 12 nmol of UDP-(3H)GlcNAc (60,000 cpm/nmol), 0.125 m*M* GlcNAc, 20 nmol of glycopeptide substrate, and 2 m*M* $MnCl_2$ for transferase I and IV+V or 20 m*M* Na_2EDTA for transferase V in a final reaction volume of 40 μl. The ovalbumin glycopeptides $GlcNAc_2Man_3GlcNAc_2$–Asn (transferases IV and V) and $Man_5GlcNAc_2$–Asn (transferase I). Half the reaction volume was cell lysate containing 30 to 100 μg of protein, and after 1 hour of incubation at 37°C, the reaction mixture was passed over a Dowex AGl-X8 column and the excluded material was spotted on paper and washed in 80% ethanol by descending paper chromatography. Product was eluted from the paper and counted to produce the data below. To test for triantennary product, transferase IV+V and V products were applied to Con A Sepharose and found to be 70 to 80% and 100% excluded, respectively. The data show the mean of duplicates ± SEM.

Cell line	GlcNAc transferase activity (nmol/mg per hour)		
	IV+V	V	I
MDAY-D2	0.40 ± 0.02	0.23 ± 0.002	2.70 ± 0.1
KBL1	0.14 ± 0.01	0.048 ± 0.003	5.77 ± 0.02

in all the cloned SP1 transfectants as detected by WGA binding, suggesting that the observed increases in L-PHA binding to gp130 was due to increased β1–6 branching of complex-type oligosaccharides in the metastatic clones. L-PHA–binding gp130 in MDAY-D2 and pSV_2neo·T24H-ras transfected SP1 cells was sensitive to digestion by neuraminidase, resulting in a higher apparent molecular weight on SDS gels (Fig. 4). However, unlike MDAY-D2 cells, L-PHA–binding gp130 in pSV_2neo·T24H-ras transfected SP1 cells was insensitive to endo β-galactosidase digestion indicating the absence of polylactosamine sequences. In some types of cells these repeating sequences of Galβ1–4GlcNAcβ1–3 appear to be more prevalent on the β1–6–linked antennae (*22*); they have also been associated with increased malignancy in human thyroid carcinomas (*23*). Therefore, it is possible that additional substitutions to the β1–6–linked antennae, such as polylactosamine sequences, could enhance the metastatic phenotype of SP1 even further.

Spatial conformation analysis of Asn-linked oligosaccharides suggests that, unlike the other linkages for lactosamine antennae, the β1–6 linkage may allow this antenna to fold back toward the protein backbone where it could affect protein conformation and activity (*24*). It is clear that for certain glycoproteins, such as the peptide hormones, the Asn-linked carbohydrates are required for biological activity, presumably because of their effect on protein conformation (*25*). Alternatively, β1–6 branched oligosaccharides may be specific ligands for mammalian lectin molecules that influences organ colonization or some aspect of tumor cell proliferation.

In conclusion, we have demonstrated a direct association between expression of L-PHA–binding β1–6 branched complex-type oligosaccharides and increased metastatic potential in three independent tumor systems. The loss of L-PHA binding sites on gp130 and GlcNAc transferase V activity in the class 3 glycosylation mutants of MDAY-D2 was associated with a dramatic loss of metastatic potential. Moreover, the enhanced expression of L-PHA binding gp130 in clones of the nonmetastatic tumor line SP1 correlated closely with acquisition of metastatic potential by these clones. These observations, taken together, suggest that acquisition of the L-PHA–binding oligosaccharides are not required for tumor formation per se but are associated with increased metastatic potential. The expression of L-PHA–binding oligosaccharides may depend on a number of factors including cell lineage or the expression of a certain subset of oncogenes, and could account in part for differences in malignant potential of tumors arising in various organs. Although these results show that increased oligosaccharide branching on gp130 is closely associated with enhanced metastatic potential, it remains to be determined whether gp130 or another less prevalent glycoprotein with similar carbohydrate changes is functionally related to metastatic potential.

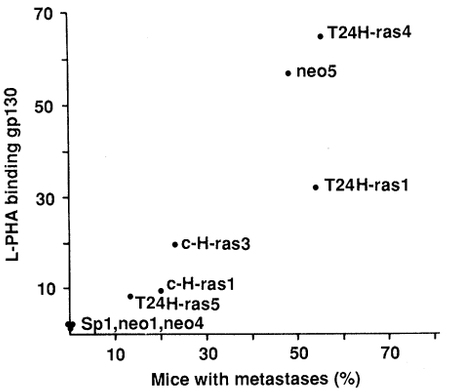

Fig. 5. Correlation of metastatic incidence and expression of L-PHA–binding oligosaccharides on gp130. The intensity of L-PHA–staining gp130 in SP1 cells and cloned transfectant lines shown in Fig. 3 were plotted against the metastatic incidence (that is, number of mice with visible metastases out of numbers of mice injected). The clones were injected subcutaneously into syngeneic CBA/J mice as well as BALB/c nude mice (15 mice per cloned cell line). Metastatic nodules were found in the lungs, and no difference in incidence between CBA/J and nude mice was observed.

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26. SDS–polyacrylamide gel electrophoresis was performed on 12.5% resolving gels under reducing conditions with 40 μg of protein per lane. WGA iodinated with 500 μCi of $Na^{125}I$ and Iodogen beads (Pierce) in 0.5 ml of phosphate-buffered saline (PBS), *p*H 7.0. L-PHA (Pharmacia) was iodinated with 500 μCi of ^{125}I-labeled Bolton-Hunter reagent. The iodinated lectins were passed over a P10 column (1 by 30 cm) equilibrated in sodium phosphate, *p*H 6.8, 0.2*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 0.02 m*M* NaN_3. Gels that had been fixed and stained with Coomassie brilliant blue were equilibrated in the phosphate buffer and incubated with the iodinated lectins for 16 hours. The gels were then washed exhaustively over a 3-day period, dried, and exposed to x-ray film at −70°C for 1.5 days.
27. Tumor cells (5×10^6 per milliliter) in PBS were placed on ice and 25 μl of 0.08*M* $NaIO_4$ was added and left to react for 10 minutes. The cells were washed three times in PBS and 250 μCi of NaB^3H_4 was added. After 20 minutes the cells were washed three times in PBS, then 100 μg of carrier beef brain gangliosides (Supelco, Inc., Oakville, Ontario) was added and the cells were extracted in 50 ml of a mixture of chloroform and methanol (2:1, v/v). Folch partitioning was used to separate glycolipids into neutral and acidic fractions, corresponding to lower and upper phases, respectively. Fractions were analyzed by high-performance thin-layer chromatography (HPTLC) and developed in chloroform, methanol, and 0.02% $CaCl_2$ (60:40:9, v/v), respectively, and then plates were sprayed with En^{3}hance exposed to Kodak XAR x-ray film for 1 week.
28. Aliquots of plasma membrane suspended in 30 μl of 50 m*M* sodium citrate, *p*H 5.1, 1% Triton X-100, 1 m*M* phenylmethylsulfonyl flouride (PMSF), 0.3 μl of Tranylol, and 0.02 m*M* NaN_3 were digested overnight with 0.1 U of neuraminidase (Sigma, type X) or 5 mU of endo β-galactosidase from Escherichia Freundi (Miles). Two times concentrated SDS sample buffer was added and the samples were heated to 95°C for 5 minutes and then separated by SDS–gel electrophoresis.

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