Isolation and Characterization of Spontaneous Wheat Germ Agglutinin-resistant Human Melanoma Mutants Displaying Remarkably Different Metastatic Profiles in Nude Mice

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ABSTRACT

Spontaneous wheat germ agglutinin (WGA)-resistant mutants of the MeWo human malignant melanoma line were isolated after sequential selection in increasingly toxic concentrations of WGA, without prior mutagenesis. They were isolated in an attempt to obtain "membrane glycosylation mutants" having significantly altered metastatic properties when grown in nude mice, and to characterize the biochemical (oligosaccharide) changes associated with altered metastatic behavior. The lines were assessed for their sensitivity to other lectins, membrane glycoprotein profiles, ploidy levels, and their ability to produce "artificial" metastases in nude mice by i.v. inoculation. One mutant, called 70-W, manifested a 3- to 4-fold resistance to WGA compared with wild-type cells. When inoculated into NIH Swiss nude mice, 70-W cells not only produced extensive lung colony formation but also showed an extraordinary ability to disseminate widely and extensively in a clinical fashion to many extrapulmonary sites such as the subcutis, mesentery, muscle, and brain. Moreover the majority of these metastases were deeply pigmented facilitating visual identification of very small visceral metastases. A second mutant called 3S5 was isolated and found to be highly resistant to WGA (>20-fold resistance). This line was virtually devoid of metastatic ability and was found by biochemical analysis to be phenotypically similar to the class I WGA resistant non-metastatic mutants previously isolated from the highly metastatic murine tumor MDAY-D2 which are known to be deficient in sialic acid and galactose. The similarity between these and earlier results using lectin resistant mutant rodent cell lines strongly suggests that sialylated glycoconjugates contribute to the metastasis of both animal and human tumors of different tissue origin. These new spontaneously derived WGA resistant MeWo mutants should be valuable new tools for the study of human tumor progression in vivo and factors involved in metastasis, especially the contribution of oligosaccharide moieties of cell surface glycoconjugates.

INTRODUCTION

Metastasis is a rather complicated biological process involving a series of steps, many of which are thought to be affected by plasma membrane structures. To help define these structures and their functions, we and others have attempted to isolate "membrane glycosylation mutants" manifesting either gross attenuation or amplification of wild-type metastatic ability. Such mutants have been obtained from a variety of mouse tumors (or Chinese hamster ovary) cell lines by exposing them to toxic concentrations of membrane-binding lectins such as WGA* and selecting for rare lectin-resistant mutants (1–5). The rationale for this type of selection protocol is that cell surface glycosylation mutants can be obtained in this manner (5, 6) and that certain membrane-associated glycosylation changes should affect tumorigenicity (7) and/or metastatic behavior. Indeed, WGA*-mutants in particular have been successfully used in a number of instances to study aspects of the biology of metastasis. They have been isolated from a wide spectrum of mouse tumors, e.g., the B16 melanoma (1, 2) the RAW 117 lymphosarcoma (3), and MDAY-D2 leukemia-like tumor system (4, 5). Many of these WGA* mutants were shown to have a greatly reduced or complete loss of metastatic capability compared to the wild-type cells. Since WGA exerts its toxic effects by binding to sialylated glycoconjugates (8) it is not surprising that WGA* mutants are often undersialylated (9, 10). Recently, the loss of metastatic potential in a series of lectin-resistant variants of the highly metastatic murine MDAY-D2 tumor was found to be related to the type of glycosylation defect (11, 12). Thus, loss of sialylated lactosamine antennae (i.e., so-called "class I") lectin-resistant mutants) or decreased branching of the trinamnosyl core for Asn-linked oligosaccharides (i.e., so-called "class III" mutants) resulted in loss of metastatic potential (11, 12).

Although the use of lectin-resistant mutants to study the biology of metastasis of rodent tumors has been informative, this type of experimental approach, with one exception, has yet to be applied to tumors of human origin. The exception is a preliminary study from our laboratory in which the metastatic properties of a number of cloned WGA* variants isolated from a human melanoma line (called MeWo) were evaluated in nude mice (13). Some of these mutants were actually found to be more aggressively metastatic than the wild-type parental cells (13). None was found to be less metastatic (biochemical analysis of the mutants was not undertaken). Unfortunately the mutants in this study were obtained in a single-step selection after the melanoma cells were subjected to treatment with a strong alkylating agent mutagen, namely methylmethane sulfonate to increase the frequency of WGA* mutants before the actual selection was applied. There was thus the distinct possibility that the mutagen treatment itself was responsible for the phenotypic changes detected or greatly influenced the extent of these changes. In this regard there have been a number of reports, many from our own laboratory, documenting heritable high frequency changes in the tumorigenic or metastatic properties of tumors as a result of prior exposure to mutagenic agents in vitro (14, 15).

We therefore decided to attempt to select WGA* mutants from the MeWo cell line spontaneously, that is, without prior mutagenesis, and to biochemically analyze any mutants found to deviate significantly in malignant behavior from the parental cells. The purpose of this report is to summarize our initial findings in this endeavor and to describe the fact that WGA* mutants encompassing a broad spectrum of metastatic properties can indeed be spontaneously isolated using multistep selection protocols.

MATERIALS AND METHODS

Cell Lines. The human melanoma cell line MeWo was isolated in 1974 from a lymph node metastasis in a 78-year-old Caucasian male
with blood type A+ (16), and was sent to us by Dr. J. Fogh, through the Sloan Kettering Institute Human Tumor Cell Laboratory and Cell Bank, as described previously in detail (16). The cells were maintained as a monolayer culture in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 7% fetal calf serum (GIBCO) at 37°C in humidified 5% CO₂-95% air atmosphere and were subcultured approximately every 7 days by trypsin-EDTA treatment. The cell lines were periodically checked for Mycoplasma contamination and only Myco-

plasma-free cell lines were used in this study. The cell lines were also checked for virus contamination (by Microbiological Associates) and were found to be free of the following murine viruses: Sendai; MHV; PVM; Reo3; GDV1; K; ectromelia; MVM; polyoma; lactate dehydro-
genase; M.ad.; and lymphatic choriomeningitis.

Lectins. The source of lectins used was as follows: WGA (Boehringer Mannheim, Dorval, Quebec, Canada), BSII (Sigma Chemical Co., St. Louis, MO), concanavalin A (Pharmacia Canada, Ltd., Montreal, Que-

bec, Canada), and L-PHA (Pharmacia). Each lectin was dissolved in phosphate buffered saline, sterilized by filtration, and stored at 4°C, as described previously (17).

Selection of WGA-resistant Variants. To isolate spontaneous WGA' MeWo mutants, the cells were subjected to stepwise increases in concent-

rations of WGA sequentially, without prior mutagen treatment. Briefly, 2 x 10⁶ cells were plated into 100-mm culture dishes (Falcon) in the presence of WGA and incubated for varying periods of time (with fresh changes of the WGA-containing medium if necessary), and this selection procedure was repeated from 3-9 times, as described in “Results.” The WGA' cells were not cloned prior to injection, although the nature of the selection protocols and results would suggest that the lines were clonal or comprised a very limited number of clones. Concentrations and periods of WGA treatment in each selection are summarized in Fig. 1. Derivation of the mutagen-induced WGA-resistant mutant (MeWo-M-50), which was described previously (13), is also shown.

Lectin Sensitivity. The ability of tumor cells to proliferate in the presence of increasing concentrations of lectin was assayed by measuring [methyl-³H]thymidine incorporation into DNA. Cells (2 x 10⁶/well) were incubated at 37°C for 3-5 days in flat-bottomed 96-well plates containing serial dilutions of lectins, pulsed with 2 μCi of [methyl-³H] thymidine, and harvested 4 h later onto glass fiber discs using a Titertek scintillation counter and the lectin concentration which reduced the presence of increasing concentrations of lectin was assayed by measuring [methyl-³H]thymidine incorporation into DNA. Cells (2 x 10⁶/well) were incubated at 37°C for 3-5 days in flat-bottomed 96-well plates containing serial dilutions of lectins, pulsed with 2 μCi of [methyl-³H] thymidine, and harvested 4 h later onto glass fiber discs using a Titertek harvester (Flow Laboratories). The discs were counted in a liquid scintillation counter and the lectin concentration which reduced the isotope incorporation to 50% of the control value was determined.

Chromosome Analysis. Exponential growth phase culture cells were harvested without mitotic inhibitors and each sample was electrophoresed on a 0.2 M NaCl-1 mM CaCl₂-1 mM MgCl₂-0.02 mM NaN₃. 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 h. 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. For L-PHA staining, proteins were electrophoresed into nitrocellulose and the transfer was incubated with 1 μg/ml L-PHA for 45 min followed by 3 washes in phosphate-buffered saline-0.1% bovine serum albumin. The blot was then incubated with rabbit anti-L-PHA antisera for 45 min and allowing 3 washes, goat anti-rabbit IgG conjugated with alkaline phosphatase was added at 1:3000. The blot was washed 5× and developed using the Bio-Rad Western blot kit.

Identification of Sialic Acid. Tumor cells (2 x 10⁶/10 ml) were labeled with 330 μCi of [α-D-[³H]glucosamine for 3 days. The cells were washed, lyophilized, and resuspended in 0.2 mM formic acid (pH 2). The samples were heated to 70°C for 60 min, and then released sialic acid was dialyzed into 100 ml water. The residue in the dialysis bag were made 0.1 M HCl and heated to 80°C for 60 min. The dialysates were pooled, concentrated to 2 ml, and passed over an AG50W-X12 column equilibrated in water. The flow-through was concentrated and passed over an AG3-4X column equilibrated in 10 mM sodium formate. The column was washed with 10 mM formic acid, and the sialic acids were eluted with 1 mM formic acid. Samples were dried to remove the formic acid, suspended in 0.05 ml water, and applied to an Alltech NH₄ high pressure liquid chromatography column equilibrated in 72% ace-

tate-28% acetonitrile (pH 5.2). The column was run in the isocratic mode at 2 ml/min and radioactivity was monitored with a Flow One beta counter (Radiomatic Instruments).

Animals and Experimental Metastasis Assays. Female specific-pathogen-free NIH Swiss nude mice obtained from Taconic Farms, Inc. (Germantown, NY) were used at 8 weeks of age. They were kept in a segregated room served by a vertical laminar flow air supply located in the Animal Colony of the Ontario Cancer Institute, Toronto, Ontario, Canada, which is a closed or “contained” facility.

Artificial metastasis assays were performed by injecting 5 x 10⁶ tumor cells in 0.5 ml RPMI 1640 medium without serum into the lateral tail vein of NIH Swiss nude mice. The mice were sacrificed 40 or 63 days later, and the metastatic nodules on the lung surface were counted. Extrapulmonary metastases in other organs were also examined macroscopically.

RESULTS

Lectin Sensitivity and Membrane Glycoprotein Analysis of WGA' Human Melanoma Cells. As outlined in Fig. 1, we isolated 2 new spontaneous uncloned WGA' mutants. Previous studies had utilized mutagenic treatment and a single-step selection protocol (13). Since mutagen treatment itself can cause alterations in the malignant potential of tumor cells (14, 15), we attempted to isolate mutants without prior mutagenesis. We found that such mutants could not be obtained unless...
JV-acetylneuraminic acid is 2-3 times more resistant to neuraminidases (25) which could increase the sialic acid content of cell surface oligosaccharides by decreasing its turnover rate. Based on the association between increased sialylation of tumor cell glycoconjugates and increased metastatic potential (26), the latter seemed to be an intriguing possibility. To examine this, the cells were grown in [3H]glucosamine, and then sialic acids were isolated and separated by high pressure liquid chromatography. However, the MeWo parent, 70-W, and M-50-17 cells had more than 99% N-acetylenuraminic acid (Fig. 3) and no significant difference was seen among these lines.

Chromosome Analysis. In the MeWo tumor system, lectin-resistant mutants obtained after mutagenesis or cell lines established from spontaneous lung metastases which showed increased malignant properties, always had different ploidy levels from parent cells (13, 16). They were consistently near-tetraploid or triploid in contrast to the predominantly hypodiploid parental MeWo cells. Thus we decided to ascertain whether the newly obtained WGA\(^\text{\textsuperscript{R}}\), 3S5, and 70-W mutants also have similarly altered ploidy levels. The results are summarized in Table 1. As we reported previously (13, 16), the MeWo parent line was predominantly hypodiploid (mode: 44 chromosomes) with a minor population of near-tetraploid cells. Conversely, as shown here, the nonmetastatic 3S5 cells had a near-tetraploid chromosome mode (80–85) with a minor population of near-diploid cells. 70-W cells also had a broad spectrum of chromosome modes (76–121) with a minor population of near-tetraploid cells. Thus deviation of ploidy levels towards tetraploidy in MeWo cells is not always associated with an increase in metastatic aggressiveness.

“Artificial” Metastasis Properties of MeWo WGA\(^\text{\textsuperscript{R}}\) Mutants. Since spontaneous or experimental metastasis assays after the injection of 0.5–1.0 × 10\(^6\) MeWo cells usually take a very long period of time to complete (3–9 months), we injected a larger number of cells (5 × 10\(^6\)) i.v. into NIH Swiss nude mice in our initial experimental analyses and sacrificed the animals 40 or 63 days later. As shown in Table 2 (Experiment 1) and Fig. 4, mice given injections of MeWo cells had predominantly amelanotic lung nodules without any evidence of extrapulmonary metastases. Remarkably, and in marked contrast, no lung metastases were observed in 4 of 6 nude mice given injections of 3S5 cells, despite the huge number of cell injected. The other 2 mice had only one or 2 amelanotic lung nodules, respectively, and we did not detect any other extrapulmonary metastases. Conversely, in the case of 70-W, all lungs but one contained melanotic metastases in the subcutis in 5 of 6 mice, as well as multiple metastatic deposits in the rib cage, mesentery, and brain were detected (see Table 2). In this experiment, the M-50-17 cells did not show such an aggressive metastatic behavior, but as reported previously (13), they produced melanotic lung nodules.

### Table 1: Lectin sensitivity profiles and chromosome numbers of wild-type and WGA-resistant mutants of the MeWo human melanoma line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>WGA sensitivity(^\text{\textsuperscript{a}})</th>
<th>BSII</th>
<th>Concanavalin A</th>
<th>L-PHA</th>
<th>No. of chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeWo parent</td>
<td>5</td>
<td>&gt;125</td>
<td>43</td>
<td>&gt;125</td>
<td>44 (37–87)</td>
</tr>
<tr>
<td>3S5</td>
<td>&gt;125</td>
<td>20</td>
<td>20</td>
<td>&gt;125</td>
<td>84 (40–121)</td>
</tr>
<tr>
<td>70-W</td>
<td>18</td>
<td>&gt;125</td>
<td>29</td>
<td>&gt;125</td>
<td>76 (34–107)</td>
</tr>
<tr>
<td>M-50-17</td>
<td>13</td>
<td>&gt;125</td>
<td>44</td>
<td>&gt;125</td>
<td>69, 70 (57–80)</td>
</tr>
</tbody>
</table>

\(^{a}\) Lectin sensitivity was determined as described in "Materials and Methods."

\(^{\text{D50}}, \) lectin concentration that reduced the isotope incorporation to 50% of the control.

Several cycles of selection were undertaken. The sensitivity of these mutants to a panel of different lectins is shown in Table 1. MeWo parental cells were sensitive to WGA and resistant to the N-acetylgalactosamine-binding lectin, BSII (19), as well as to L-PHA which binds to galactose in tri- or tetraantennary complexes (20). This information as well as details of the binding specificities of other lectins such as WGA has been summarized by Dennis and Laferte (5). In contrast, the 3S5 WGA\(^\text{\textsuperscript{R}}\) MeWo mutant, which was obtained after 3 cycles of selection, was highly resistant to WGA and sensitive to BSII. As to the sensitivity to L-PHA, all of the 4 lines examined manifested the lectin concentration that reduced the isotope incorporation to 50% of the control value at concentrations higher than 125 \(\mu\)g/ml (Table 1). 70-W cells, which were obtained after 9 cycles of selection, a selection process which took more than a year to complete, showed a 3- to 4-fold increase in WGA resistance compared to the parent cells and no differences in BSII sensitivity. The M-50-17 WGA\(^\text{\textsuperscript{R}}\) cells manifested a similar sensitivity pattern as 70-W when tested with WGA, BSII, and L-PHA. Some differences in concanavalin A sensitivity were seen among these cell lines.

To detect possible changes in Asn-linked oligosaccharide structures, we separated membrane glycoproteins by sodium dodecyl sulfate-gel electrophoresis and stained for lectin-binding glycoproteins. As might be expected from the lectin-sensitivity data (Table 1), glycoproteins from 3S5 cells had lost WGA- and L-PHA-staining oligosaccharides and acquired BSII-binding structures (Fig. 2). The lectin-staining pattern of 3S5 glycoprotein was very similar to that of the class I mutants of the MDAY-D2 mouse tumor line (11) which have been shown to have prematurely truncated glycolipids and Asnlinked oligosaccharides lacking sialic acid and galactose (21, 22). 3S5 may have a mutation in the same gene as that of the class I MDAY-D2 mutants and the Chinese hamster ovary mutant Lec8 which appears to block UDP-galactose transport into the Golgi apparatus (23).

Glycoproteins from the more metastatic mutant 70-W showed small increases in WGA and L-PHA binding. Therefore the basis of the mutant phenotype in these cells would appear to involve a mechanism other than simply loss of WGA binding glycoconjugates. In contrast, the intensity of WGA-staining glycoproteins appeared to be reduced in the M-50-17 mutant. The loss of WGA-staining may indicate a decrease in the level of sialylation. Alternatively, sialic acid may be linked \(\alpha 2-6\) in the mutant rather than \(\alpha 2-3\), the latter being preferred for WGA binding (8). A shift from \(N\)-acetylenuraminic acid to \(N\)-glycolylneuraminic acid in the mutant cells is the basis of the WGA\(^\text{\textsuperscript{R}}\) phenotype in the class II mutants of MDAY-D2 (24). \(N\)-acetylenuraminic acid is 2–3 times more resistant to neuraminidases (25) which could increase the sialic acid content of cell surface oligosaccharides by decreasing its turnover rate.
HUMAN MELANOMA WGA' MUTANTS AND METASTASIS

Fig. 2. Lectin staining of tumor cell membrane glycoproteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Glycoproteins stained with WGA, BSII, or L-PHA as described in "Materials and Methods." Note the absence of WGA (left) and L-PHA-binding (right) proteins in the 3S5 mutant, whereas this line binds BSII in contrast to the MeWo parent (middle). S, standard molecular weight markers; K, molecular weight in thousands.

Fig. 3. Identification of sialic acids in MeWo parent, 70-W, and M-50-17 cells. Sialic acids residues were biosynthetically labeled with [3H]glucosamine, purified as described in "Materials and Methods," and then separated by high pressure liquid chromatography. Integration of the peaks indicated that in MeWo parent (left), 70-W (middle), and M-50-17 (right) cells, more than 99% of the sialic acid was N-acetylneuraminic acid (NeuNaC), and less than 1% N-glycolylneuraminic acid (NeuNGc).

nODULES AS WELL AS ONE EXTRAPULMONARY METASTASIS. AS SHOWN IN TABLE 2 (EXPERIMENT 2), THESE ORGAN-COLONIZING AND PIGMENTATION PROPERTIES OF 3S5 AND 70-W WERE QUITE REPRODUCIBLE AS A SECOND EXPERIMENT PERFORMED SEVERAL MONTHS LATER GAVE VERY SIMILAR RESULTS. THIS WAS ALSO THE CASE WHEN A SMALLER DOSE OF 1X 10^6 CELLS WAS INJECTED I.V. INTO NUE MICE (8 MICE/GROUP), AND THE MICE WERE OBSERVED FOR OVER 100 DAYS. BRIEFLY, 3S5 DID NOT COLONIZE TO LUNGS (MEDIAN NUMBER OF NODULES: 0), WHILE 70-W PRODUCED NOT ONLY A LARGER NUMBER OF LUNG NODULES (MEDIAN: 115) THAN THAT OF MEWO (MEDIAN: 11) BUT ALSO METASTASIZED IN 5 OF 8 MICE TO EXTRAPULMONARY SITES SUCH AS SUBCUTIS AND MESENTERY. M-50-17 PRODUCED A SMALL NUMBER OF LUNG NODULES (MEDIAN: 2), BUT EXTRAPULMONARY METASTASES WERE SEEN IN 7 OF 8 MICE. IN CONTRAST, EXTRAPULMONARY METASTASES WERE NEVER OBSERVED IN NIH SWISS NUE MICE WHICH WERE GIVEN IN I.V. INJECTION OF THE MEWO PARENTAL CELLS. A MORE DETAILLED VISUAL AND HISTOPATHOLOGICAL SUMMARY OF THESE RESULTS WILL BE PROVIDED ELSEWHERE.

DISCUSSION

A fruitful approach to study the contribution of cell surface complex oligosaccharide structures to the process of metastasis is through the isolation and characterization of lectin-resistant glycosylation mutants (5, 27). Thus, by isolating a series of glycosylation mutants with well-defined defects in oligosaccharide biosynthesis, it has been possible to demonstrate that highly branched and sialylated complex-type structures are required for efficient metastasis of highly metastatic murine leukemia-like MDAY-D2 cells (11, 12). Moreover, these and other studies (1, 3–5) demonstrated that it is possible to derive phenotypically stable genetic mutants manifesting enormous quantitative changes in metastatic behavior when compared to the wild-type lectin-sensitive parental cells. It was also noted that whenever WGA' mutants were isolated and characterized from mouse tumor cell lines they always manifested either a conspicuously reduced (1–5) or no change (11, 17) in metastatic ability; isolation of WGA' mutants having a greater metastatic ability has not been reported.

Several years ago our laboratory undertook the first attempts to adopt this type of experimental approach and analysis to study properties associated with metastasis of human cancer cells (13). It is well known that various types of human tumors, especially melanomas and colon carcinomas, will grow readily in nude athymic mice, although with little evidence of metastatic spread when injected "ectopically" (28). We wished to determine whether the metastatic ability of such cell lines could be significantly altered by putative changes in cell surface glycosylation. To this end, we selected in our initial experiments WGA' mutants from the MeWo human melanoma cell line by using a single-step selection protocol with prior mutagenesis of the cells (13). As summarized above, certain clones of a WGA' isolate were found to be remarkably more aggressive in their metastatic behavior in nude mice. We also noted that these mutants were intensely pigmented, in contrast to the mostly amelanotic parental cells. This facilitated visual detection of
Table 2 Experimental metastases of wild-type and WGA-resistant mutants of MeWo human melanoma in NIH Swiss nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of lung nodules*</th>
<th>Incidence and location of metastases at other sites</th>
<th>Pigmentation characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeWo parent</td>
<td>9, 37, 45, 66, &gt;200, &gt;200</td>
<td>0/6</td>
<td>Amelanotic</td>
</tr>
<tr>
<td>3S5</td>
<td>0, 0, 0, 1, 2</td>
<td>0/6</td>
<td>Amelanotic</td>
</tr>
<tr>
<td>70-W</td>
<td>2, &gt;200, &gt;200, &gt;200</td>
<td>5/6, skin, brain, mesentery, rib cage, muscle, abdominal wall, ovary</td>
<td>Predominantly melanotic</td>
</tr>
<tr>
<td>M-50-17</td>
<td>0, 2, 2, 7, 80, 87</td>
<td>1/6, rib cage</td>
<td>Amelanotic</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of lung nodules*</th>
<th>Incidence and location of metastases at other sites</th>
<th>Pigmentation characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeWo parent</td>
<td>53, 76, 97, 134, &gt;200, &gt;200</td>
<td>0/6</td>
<td>Predominantly amelanotic</td>
</tr>
<tr>
<td>3S5</td>
<td>0, 0, 0, 1, 2, 12</td>
<td>1/6, muscle</td>
<td>Amelanotic</td>
</tr>
<tr>
<td>70-W</td>
<td>25, 145, &gt;200, &gt;200, &gt;200</td>
<td>4/6, skin, brain mesentery, rib cage, muscle, abdominal wall, ovary, diaphragm, heart</td>
<td>Melanotic</td>
</tr>
</tbody>
</table>

* NIH Swiss nude mice were given an i.v. injection of 5 x 10^6 tumor cells and number of surface tumor nodules in lung were counted 40 or 63 days later in Experiments 1 and 2, respectively.

The most noteworthy initial finding, reported here, was the relative difficulty in isolating spontaneous, stable WGA' mutants from the parental human melanoma cell line. This observation stands in marked contrast to the relative ease of isolating lectin-resistant mutants from various rodent cell lines which can usually be achieved in a single step, whether or not the cells are mutagenized beforehand (5, 6). An exception was the B16 melanoma which required at least 3 sequential selections in increasing concentrations of WGA to obtain a stable WGA' mutant (1). As shown in Fig. 1, isolation of such WGA' variants of MeWo required a multistep selection procedure not unlike that required for certain types of drug-resistant mutants where the basis of the genetic change leading to the drug-resistant phenotype is gene amplification (29). For one mutant (70-W), it took an exceptionally long period of time to be isolated, and even after 9 cycles of selection performed over more than a year-long period, 70-W showed only 3- to 4-fold resistance to WGA compared to the parent MeWo line.

When we injected 3S5, 70-W, or M-50-17 cells into nude mice, they manifested a wide spectrum of metastatic properties. The 3S5 line, which was highly resistant to WGA, was isolated after 3 cycles of selection in the concentrations of WGA up to 50 μg/ml (Fig. 1), and showed a severe loss in metastatic capacity. This was true even after i.v. injection of as many as 5 x 10^6 cells, a very large dose of cells to be inoculated by this route into a nude mouse. The BSII-resistant phenotype (Table 1), and lectin-staining pattern of membrane glycoproteins of 3S5 cells suggested that they have the same phenotype as the class I nonmetastatic mutants of MDAY-D2 mouse tumor (i.e., the mutants called MDW4, D36W25-1, 210-2A) previously described by us (5), and the "Lee 8" mutant isolated from Chinese hamster ovary cells (6).

Taken together, these results strongly suggest that cell surface glycoconjugate structures contribute to tumor cell metastasis of both murine and human cancers of diverse tissue origins. In contrast to 3S5, the WGA' mutant 70-W showed remarkable metastatic behavior in nude mice after i.v. injection. The cells were able to disseminate widely and extensively to many extrapulmonary sites, including the subcutis. The metastases were so deeply pigmented that even "pinhead"-sized metastases could be easily visualized and quantitated. Mechanisms of acquisition of aggressive metastatic capacity as well as the WGA' phenotype in 70-W and M-50-17 cells are as yet unclear, and since 70-W was obtained after sequential selection, multiple
genetic changes involving phenotypes other than lectin resistance cannot be ruled out. Lectin staining of membrane glycoproteins indicates that sialylated lactosamine antennae on highly branched complex-type oligosaccharides may be marginally increased, consistent with the more malignant phenotype observed in other models. The mutants did not produce N-glycolyneuraminic acid as was previously found for the metastatic WGA class 2 mutants of MDAY-D2 (11).

Our results provide a new approach to successfully induce human tumors to closely recapitulate in nude mice the mode of metastatic spread that they normally manifest in humans. In this respect it should be noted that orthotopic transplant methods have also been successfully used to achieve the same end using human colon and renal cell carcinomas (30–33), but as yet similar results have not been obtained using human melanomas. Thus, there have not been any reports documenting skin or brain metastasis after s.c. (orthotopic) inoculation of human melanomas in nude mice.

In summary, this is the first report documenting the fact that lectin-resistant mutants can be obtained spontaneously from a human tumor and that they can manifest significant changes in metastatic behavior when inoculated into nude mice. The human melanoma WGA’ variants described here, some of which metastasize aggressively in a clinically relevant fashion or which lose their metastatic ability in nude mice and which display a high degree of pigmentation, should be useful new tools for the study of a range of biological problems. These include (a) the relationship between altered expression of specific oligosaccharide structures and malignant potential of human tumor cells, (b) differentiation programs of melanoma cells, (c) testing new experimental anticancer therapy protocols for their effect on disseminated human cancer (melanoma) cells in an in vivo environment, and (d) DNA transfection experimental protocols in relation to melanoma cell biology and metastasis.

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