Highly Pigmented Human Melanoma Variant Which Metastasizes Widely in Nude Mice, Including to Skin and Brain

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ABSTRACT

The properties of a highly malignant human melanoma variant cell line which metastasizes in nude mice in a tissue-specific pattern are described. The variant, called 70-W, was isolated from the MeWo malignant melanoma by exposure of the latter to stepwise increasing concentrations of the toxic lectin, wheat germ agglutinin. After nine cycles of treatment a population of wheat germ agglutinin-resistant cells was obtained that manifested a 4-fold resistance to wheat germ agglutinin, a property which was found to be stable in culture for over 6 months in the absence of the lectin. Intravenous inoculation of 70-W cells into 4-6-week-old nude mice revealed remarkable differences in metastatic (organ colonization) behavior. Whereas the parent MeWo cells gave rise only to lung metastases, most of which were amelanotic, injection of the 70-W cells resulted in multiple skin (s.c.) and brain metastases, as well as dissemination to bone marrow, ovarian, mesenteric (gut-associated), muscle, and abdominal metastases all of which were highly melanotic. This is the first report of a melanoma tumor in nude mice. They were found to be bilateral and confined to the deeper layers of the cerebral cortex. The unique malignant behavior of 70-W cells in nude mice should facilitate studies of host and tumor cell factors involved in human melanoma metastasis, melanogenesis, and development of new treatment strategies for disseminated human malignant melanoma.

INTRODUCTION

In 1977 Tao and Burger (1) reported that stepwise exposure of metastatic B16 melanoma cells to toxic concentrations of WGA could be used to isolate WGA mutants which were severely deficient in metastatic capacity. Subsequent somatic cell genetic and biochemical studies revealed an oligosaccharide alteration (2, 3) present in the mutant cells which appeared to account for their attenuation in malignant behavior. During the ensuing decade several laboratories have successfully used this approach to select phenotypically stable, homogeneous populations of lectin-resistant glycosylation mouse tumor mutants which manifest grossly altered metastatic properties (reviewed in Refs. 4 and 5). The most detailed studies are those of Dennis et al. (5-8) using the highly metastatic MDAY-D2 mouse tumor; these investigators isolated several classes of genetic (WGA) mutants having defined biochemical changes which correlated with partial or complete loss of metastatic capacity (5-8). Loss of sialylated polyolactosamine structures correlated with loss of metastatic behavior (5-8).

These studies indicated a direct role of cell surface oligosaccharides in the process of metastasis (9); they also revealed a powerful method of obtaining phenotypically stable, homogeneous, mutant tumor cell subpopulations manifesting very large quantitative differences in metastatic capacity with which to undertake basic biochemical and genetic studies into the complex nature of metastasis.

Our laboratory decided to adopt a similar approach using human tumors with the objective of selecting mutants which would manifest grossly altered metastatic behavior in athymic nude mice. We initiated our studies with a human malignant melanoma cell line called MeWo which metastasizes to the lungs of nude mice after i.v. or s.c. inoculation of the cells (10). We initially succeeded in isolating WGA clones which actually manifested a greater, not lesser, metastatic capacity after selecting cloned WGA cells from a mutagenized population of MeWo cells (11).

More recently we isolated spontaneous WGA MeWo variants (i.e., with no prior mutagenesis involved) by using a multistep selection protocol involving stepwise increasing concentrations of WGA (12). Two remarkable sublines were obtained. One (called 355) was virtually completely deficient in pulmonary metastatic capacity, whereas the other (called 70-W) manifested widespread visceral, deeply pigmented nodular metastases as well as lung metastases after i.v. inoculation of the cells. The purpose of this paper is to provide a detailed summary of the biological, histopathological, and metastatic characteristics of 70-W variant cells inoculated i.v. into NIH nude mice. We show its remarkable propensity to colonize many different organs and tissues characteristic of clinical melanoma, including most notably the brain and skin (s.c.) tissue.

MATERIALS AND METHODS

Cell Line. The human melanoma cell line MeWo was originally derived and established in culture from a lymph node metastasis (13). It was diagnosed as a nodular malignant melanoma and was heavily pigmented in the patient from which it was derived. A WGA mutant of MeWo, designated 70-W, was isolated by the sequential, stepwise selection in increasing concentrations of WGA, without using prior mutagen treatment. Briefly, 2 x 10^6 cells were plated in 100-mm culture dishes in the presence of WGA and incubated for varying periods of time (with fresh changes of the WGA-containing medium if necessary); this selection procedure was repeated 9 times. The detailed protocol such as concentrations and periods of WGA treatment in each selection was described previously (12). The concentrations used for the stepwise selections ranged from 30 to 70 μg/ml. Initially after each selection, surviving cells were grown to mass culture in the absence of WGA. However, from the 7th to the 9th selections, the cells were grown continuously in the presence of WGA, i.e., the selection pressure (WGA) was always maintained but increased in a stepwise manner. The superscript "a" in a designation of the cell lines signifies that the line was established from an "artificial" metastasis formed by the i.v. inoculation of tumor cells into an NIH Swiss nude mouse. For example, 70-W-μt1, 70-Wmes1, 70-Wova1, and 70-W-μ1 were established from individual lung, mesentry, ovary, and s.c. metastases, respectively.

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4 The abbreviations used are: WGA, wheat germ agglutinin; WGR, wheat germ agglutinin resistant; L-PHA, leukophytohemagglutinin; D₅₀, concentration which reduced the isotope incorporation to 50% of control; i.d., intradermally.

5 C. Urmacher, Sloan Kettering Cancer Center, New York. Personal communication.
tively, in NIH Swiss nude mice which had been given i.v. injections of 5 x 10^6 70-W cells 40 days earlier. All the cell lines were maintained as monolayer cultures in antibiotic-free RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 7% fetal calf serum (GIBCO) at 37°C in a humidified 5% CO2-95% air atmosphere, and they were subcultured at least every 7 days by trypsin-EDTA treatment. With respect to this protocol it is important to note that when 70-W cells become near-confluent, the culture medium becomes rapidly acidic for reasons which are not clear. This does not occur with the MeWo parent line or the 3SS variant. The cell lines were periodically screened for Mycoplasma contamination and only Mycoplasma-free cell lines were used in this study. They were also tested for the presence of 13 different viruses by “MAP” testing, as described previously (12), and found to be virus free.

Lectins. The source of lectins used was as follows: WGA (Boehringer Mannheim, Dorval, Quebec, Canada); lectin from Bandeiraea simplicifolia (Sigma Chemical Co., St. Louis, MO); concanavalin A (Pharmacia Canada, Ltd., Montreal Quebec, Canada), and L-PHA (Pharmacia). Each lectin was dissolved in phosphate-buffered saline, sterilized by filtration, and stored at 4°C. The binding specificities of these lectins have been summarized by Dennis and Laferte (5).

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 (12). Cells (2-3 x 10^6/well) were incubated at 37°C for 2-3 days in flat-bottomed 96-well plates containing serial dilutions of lectins, pulsed with 2 mcI of [methyl-3H]thymidine, and harvested 4 h later onto glass fiber discs using a Titertek harvester (Flow Laboratories). The discs were counted in liquid scintillation counter and the lectin Dso was determined (12).

Chromosome Analysis. Exponential growth phase culture cells were harvested after any mitotic inhibitors and suspended in 0.075 M KCI at 37°C for 15-20 min. The cells were pelleted, fixed with methanol:acetic acid (3:1), dropped onto glass slides, and stained with Giemsa. One hundred metaphase spreads were counted for each line analyzed.

Animals and Experimental Metastasis Assays. Female specific-pathogen-free NIH Swiss nude mice were obtained from Taconic Farms, Inc. (Germantown, NY) and were first used at 6 to 9 weeks of age. The mice were housed in a contained facility within laminar flow filtered air containment cabinets and fed sterilized water and mouse chow. Experimental metastasis assays were performed by the careful injection of 1 x 10^6 or 5 x 10^6 tumor cells in serum-free RPMI 1640 in the form of a single cell suspension into the lateral tail vein of NIH Swiss nude mice. The mice were sacrificed when they became moribund in the case of 1 x 10^6 cell inoculation (approximately 140 days later) and 40 days or 9 weeks later in the case of 5 x 10^6 cell inoculations. The metastases in other organs were examined both microscopically and macroscopically.

RESULTS

Lectin-resistant Phenotype and Its Stability of 70-W Cells. We assessed the sensitivity of 70-W cells to a panel of different lectins as well as the relative stability to these lectins when grown in the absence of WGA for up to 9 months in culture. 70-W cells, which were obtained after 9 successive cycles of selection in increasing concentrations of WGA and whose isolation process took more than 1 year to complete (12), showed a 3-4-fold resistance to the sialic acid-binding lectin, WGA, when compared to wild-type MeWo cells (12), but no significant difference in the sensitivity to an N-acetylgalactosamine-binding lectin, B. simplicifolia lectin, or a mannoside-binding lectin, concanavalin A, as previously published (12). As for the sensitivity to L-PHA, which binds to galactose in tri- and tetraantennary (branching) complexes, 70-W cells were slightly more sensitive than the MeWo cells, although both cell lines were quite resistant to the high concentration of the lectin (i.e., D50 > 125 µg/ml). There was only a minimal difference of these lines in their population-doubling times in vitro when grown in 7% fetal calf serum (41.4 and 44.5 h in MeWo and 70-W, respectively). The lectin-resistant phenotypes were found to be stable in tissue culture for at least 6 months.

Organ Colonization Pattern of 70-W Cells in Nude Mice. Table 1 summarizes the metastatic profile results obtained following the i.v. inoculation of MeWo parent (wild-type) cells or the 70-W variant. We injected 5 x 10^6 cells i.v. into NIH Swiss nude mice and sacrificed the animals 40 days later; inoculation of MeWo cells produced predominantly amelanotic lung nodules with a median number of 55.5; there was no evidence of metastases to extrapulmonary sites. This is consistent with our previous observations (10-12). In marked contrast, however, injection of 70-W cells produced a larger number of lung nodules (median, >200), and the majority of them were intensely pigmented (12). In this respect it should be noted that the MeWo melanoma was highly pigmented in the patient from which it was derived (see “Materials and Methods”). Moreover, the majority of mice in this group manifested extensive extrapulmonary nodular metastases in a clinically relevant or “tissue-specific” fashion. For example, multiple, pinhead to 1-mm s.c. metastases (Fig. 1A), multiple metastatic deposits in the brain (Fig. 1B), gastrointestinal tract (Fig. 1C), rib cage (Fig. 1D), and other sites (see Table 2, Experiment A) were observed; all were deeply pigmented. Extrapulmonary, deeply pigmented metastases were also obtained when the mice were given i.v. injections of 1 x 10^6 tumor cells although the frequencies were not always as high (data not shown). Mice which were given injections of 70-W cells had a somewhat larger number of lung nodules than those of MeWo control group, and most mice had metastases at other sites, whereas extrapulmonary metastases were never observed in mice that were given i.v. injection of the parental MeWo cells (Table 1). Thus uniquely qualitative as well as quantitative changes were apparent between MeWo and 70-W cells in terms of their metastatic phenotype after i.v. injection. To our knowledge, there have been no previous reports of brain metastases in nude mice following injection or implantation of human tumors, including melanomas.

Histological Examination of Primary and Metastatic s.c. Tumor of 70-W in Nude Mice. The histological characteristics of the primary tumor at the site of s.c. injection was studied on hematoxylin and eosin-stained sections of tissue fixed in formalin and embedded in paraffin (Fig. 2). The microscopic appearances were similar for both the MeWo and 70-W cell lines. The tumor was established in the s.c. fat where it had formed a large mass. Superficially it was abutting on the underlying surface squamous epithelium and had penetrated the epithelium to form an ulcer. The margins of the mass were relatively rounded and circumscribed with some compression of the adjacent mesenchymal tissue but no true capsule. There was no evidence of irregularity of the margins of the mass and no infiltration extension into the surrounding tissue was seen (Fig. 2).

The cytological appearances of the tumor cells were essentially similar for both the MeWo and 70-W primary tumors. The tumor masses were composed of a mixture of epithelioid and spindle-shaped cells displaying a high degree of pleomorphism. The cells had large nuclei with prominent eosinophilic nucleoli. Many cells had multiple nuclei. There was more necrosis in the 70-W tumor (50-60% of the tumor area) in comparison to the MeWo tumor (10-15% of the tumor area). In the 70-W tumor the necrosis consisted of smaller scattered foci. There was significantly more melanin pigment in the 70-W tumors which was present in 70-80% of the tumor cells. However, there was considerable heterogeneity in the distribu-
tion of melanin within the tumor. Only about 5% of individual cells contained pigment in the parental MeWo tumor. The mitotic activity was similar for the tumors derived from both cell lines.

In both tumors the adjacent host tissue was largely unaffected. In some areas there were occasional dilated blood vessels. Intravascular tumor was not seen beyond the tumor mass. Scattered mast cells and macrophages were present, many of which contained melanin pigment. Occasional neutrophils and mononucleus cells were also seen.

Skin-associated metastases formed after i.v. inoculation of 70-W cells, usually consisting of small, discrete, circumscribed lesions present in the s.c. tissue adjacent to the s.c. muscle with no or minimal epidermal involvement (Fig. 3). This was similar to what was observed in the patient from whom the tumor was derived. The cells displayed a lesser degree of pleomorphism compared to the primary tumor. Individual cell pigmentation was seen in the majority of cells (approximately 70% of all cells) similar to the primary tumor. There was no significant host cell response and no intravascular tumor was noted.

Metastatic tumor deposits were also noted in other sites, especially the brain; the latter were bilateral and confined to the cerebral cortex; no involvement of the meninges was noted (Fig. 3).

**Lectin Sensitivity and Ploidy of Cell Lines Established from 70-W Metastases.** In the MeWo tumor system, cell lines established from spontaneous lung metastases always had different ploidy levels from the parental MeWo cells (10, 11). Thus, to determine whether metastases of 70-W following i.v. inoculation retained cellular characteristics of 70-W cells, we established 7 cell lines from artificial metastases (3 lines from individual lung nodules, 2 from mesentery metastases, 1 from an ovarian metastasis, and 1 from a s.c. metastasis) and determined their lectin sensitivity and ploidy levels. As we reported previously (10, 11), the MeWo parental line was predominantly hypodiploid (mode, 44 chromosomes), whereas 70-W displayed a broad spectrum of chromosomes with a modal number of 76 (data not shown). All the 7 cell lines established from 70-W metastases had near-triploid chromosome modes (64–72 chromosomes) (data not shown), and distribution patterns of the chromosome numbers were very similar to those of the parental 70-W line. These cell lines also showed similar resistance to 70-W cells against WGA (3–5-fold resistance over MeWo parental lines). These cell lines also showed similar resistance to 70-W line. These cell lines also showed similar resistance to 70-W mes2 and 70-W sc1 manifested different organ-colonizing properties from the "parental" 70-W cells. 70-W mes2 seemed to have a higher lung-colonizing ability than 70-W, and the lung nodules were predominantly melanotic but also contained some amelanotic nodules. The frequency of extrapulmonary metastases of 70-W mes2 was lower than 70-W. 70-W sc1 produced melanotic but a smaller number of lung nodules and unexpectedly did not metastasize to extrapulmonary sites (Table 2). The reasons for this are unclear and are currently under investigation.

Thus it would appear that some of the malignant and differentiated properties of the melanoma cells observed in situ in the patient from whom the tumor was derived were lost upon its establishment in culture but would be "resurrected" by selection of certain WGA variant cell lines, such as 70-W.

**DISCUSSION**

The major findings in this study are the remarkable metastatic and pigmentation properties of the variant 70-W human malignant melanoma cells; whereas i.v. inoculation of the MeWo parent led to the exclusive formation of predominantly amelanotic pulmonary nodules, 70-W cells manifested deeply pigmented lesions in multiple organ sites including the skin (s.c. tissue), brain, intestines, ovaries, bone marrow, and abdomen, all sites commonly associated with melanoma metastases in humans (14).

Of particular interest is the observation of brain and skin metastases. Cerebral metastases are associated with a very poor prognosis in melanoma patients (15–17); about 5–20% of melanoma patients develop clinical signs of central nervous system involvement (15, 16), and autopsy results have shown frequencies of central nervous system involvement as high as 50–75% (16, 17). Similarly skin metastases are seen in over 50% of advanced melanoma patients (14). Despite these "trophisms," neither skin nor brain metastases have been detected following direct i.v. inoculation of human malignant cells into mice. Mouse melanoma brain metastases can be obtained but require the use of specialized preselected variants obtained by intracardiac injection of tumor cells into mice and removal of brain metastases, followed by resection (18) or by direct intraarterial (intracarotid artery) injection of the cells (19, 20). When this is done, certain mouse melanoma cell lines (e.g., B16) give meningeal metastasis, whereas others (e.g., K1735) do not involve the meninges but are instead found to colonize the deeper parenchymal tissues of the brain (20). In the case of the 70-W cells, we noted brain metastases in both lobes of the cerebral cortex with no meningeal involvement. The presence
of 70-W brain metastases also presumably reflects the ability of these cells to pass through the lung capillary bed and eventually enter the arterial circulation. It may be that the parental MeWo cells are unable to achieve this. As stated earlier, this is the first report of brain metastases of a human tumor in nude mice. Moreover, they are easily obtained after i.v. injection, and the pattern of involvement is ideal as a general tumor model of brain metastasis since, according to Conley (19), metastatic brain tumor deposits should initially develop and lie within neural parenchyma with little or no involvement of the leptomeninges, skull, or extracranial structures of the head (19).

With respect to skin or s.c. metastases, this is the second report of their occurrence in nude mice following injection of human melanoma cells, the first being our prior results (11) with mutagenized single-step selected WGA* sublines of MeWo (Fostad, Phil and colleagues have unpublished evidence of skin metastases following injection of a newly selected human malignant melanoma variant called FMXI).* 70-W skin metastases were confined to s.c. tissues and manifested a deeply pigmented, nodular appearance. They did not involve the epidermis. It is of interest to note that nodular-type melanoma tends to give more local skin and bone metastases, whereas the superficial spreading type is more frequently associated with general lymph node involvement (21). Lymph node metastases were not commonly observed in these experiments (whereas skin metastases were); moreover, the MeWo cell line was diagnosed as a nodular

* Fostad et al., personal communication.

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Table 2 Experimental metastatic properties of MeWo, 70-W, and cell lines established from artificial metastases of 70-W in NIH Swiss nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of mice used</th>
<th>Lung nodules</th>
<th>Extrapulmonary metastases</th>
<th>Pigmentation characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeWo</td>
<td>6</td>
<td>115.5</td>
<td>None</td>
<td>Mixture of melanotic and amelanotic (predominantly amelanotic)</td>
</tr>
<tr>
<td>70-W</td>
<td>6</td>
<td>&gt;200</td>
<td>4/6 s.c., 4/6 brain, 1/6 mesentery, 3/6 muscle, 3/6 bone, 2/6 ovary, 1/6 diaphragm, 1/6 abdominal wall, 1/6 heart</td>
<td>Melanotic</td>
</tr>
<tr>
<td>70-W mes*2</td>
<td>6</td>
<td>&gt;200</td>
<td>1/6 s.c., 2/6 brain, 1/6 mesentery, 1/6 bone, 1/6 muscle, 1/6 abdominal wall</td>
<td>Mixture of melanotic and amelanotic (predominantly melanotic)</td>
</tr>
<tr>
<td>70-W sc*1</td>
<td>6</td>
<td>&gt;74</td>
<td>None</td>
<td>Melanotic</td>
</tr>
</tbody>
</table>

NIH Swiss nude mice were given i.v. injections of 5 x 10^6 tumor cells and sacrificed 9 weeks later. 70-W cells which were injected had been kept in culture in lectin-free medium for 7 months after the isolation protocol.

Fig. 2. (A) Low power photomicrograph of MeWo tumor at site of injection in s.c. tissue. Note rounded tumor margins and minimal host response. H & E, x 290. (B) High power detail of A. Note cellular pleomorphism and sparse intracellular granules of melanin fragment. H & E, x 2210. (C) Low power photomicrograph of MeWo 70-W at site of injection in s.c. tissue. Note well defined margins and pale areas of confluent necrosis in tumor. H & E, x 290. (D) High power detail of C. Note cellular pleomorphism and abundant melanin pigmentation. H & E, x 2210.
malignant melanoma. Taken together, our results show that many of the clinical manifestations of cutaneous nodular malignant melanoma metastases have been stably “resurrected” in the 70-W WGA’ variant of the MeWo cell line. Hence comparison of the 70-W cells with the parental MeWo cells should reveal biological, biochemical, and genetic factors involved in nodular melanoma metastasis that have a high probability of being relevant to the clinical behavior of human malignant melanoma.

Despite the remarkable change in malignant behavior of 70-W cells we still do not have unequivocal proof that this is due to the selection of a glycosylation mutant or variant as a result of the long-term exposure of WGA. Thus it may be argued that cloning alone may have revealed the presence of MeWo cell subpopulations having the malignant properties of 70-W cells. We consider this most unlikely for several reasons: (a) the 70-W cells do manifest a change in WGA binding, namely, they manifest an increase in WGA-binding structures as assessed by lectin blotting of plasma membrane glycoproteins (12) and Scatchard analysis using radiolabeled WGA; (b) we, and Rod-er’s laboratory, have tested over a dozen clones from the MeWo line and none was found to behave like 70W cells; (c) selection of single-step WGA’ variants after mutagenesis resulted in highly melanotic skin-metastasizing cells (11) whereas muta-genesis exposure alone was found to be ineffective.*

The next phase of study in this system must involve a genetic and biochemical analysis of the 70-W cells and establishing a cause and effect relationship between the WGA resistance and enhanced malignant properties of 70-W cells. For example, is the nature of the “mutation” in 70-W cells expressed dominantly or recessively in somatic cell hybrids? Can the WGA’ phenotype be transferred by genomic transfection procedures to WGA-sensitive (and nonmetastatic) mouse melanoma cells? If so, would the recipient cells acquire the metastatic (and/or pigmentation) properties of the 70-W variant cells? With respect to these questions it is encouraging to note (as reported here) that the WGA’ phenotype remains relatively stable for prolonged periods, e.g., 6 months in culture in the absence of WGA.

The unique malignant behavior of the 70-W cells in nude mice we have described in this paper was observed after i.v. inoculation of the cells, and as such is a manifestation of so-called “artificial” metastases. We are now in the process of examining the “spontaneous” metastatic behavior of 70-W cells after their s.c. or i.d. inoculation (i.e., after ‘orthotopic’ transplantation of the cells), with and without tumor excision. These experiments are quite long term in nature (5–10 months, if not longer), which is one of the reasons we utilized the i.v. colonization assay for our initial analyses of the variants. By injecting $5 \times 10^6$ cells, experiments can be completed within 40–50 days; $10^6$ cells can require up to 150 days.

The availability of relatively stable, homogeneous cell populations of human melanoma cell lines manifesting clinically

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* Cornil et al., unpublished observations.

* R. S. Kerbel and M. S. Man, unpublished observations.
relevant patterns of metastatic spread in nude mice should be of great value in helping to define both tumor cell and environmental host factors relevant to the spread and treatment of melanoma in humans. Such studies will be aided by the use of control cell lines and variants having markedly deficient metastatic and melanogenic properties which we have also isolated from MeWo using the WGA selection method described here (12).

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