In Vitro Properties of Human Melanoma Cells Metastatic in Nude Mice

Dorothee Herlyn,² Dimitrios Iliopoulos, Pamela J. Jensen, Annette Parmiter, Janet Baird, Hak Hotta, Koji Adachi, Alonzo H. Ross, Jadranka Jambrosic, Hilary Koprowski, and Meenhard Herlyn


ABSTRACT

We have developed a human melanoma metastasis model in nude mice. In this model, a human variant cell line (451-LU) was obtained that spontaneously metastasized in nude mice. This variant cell line was selected from the lung of a nude mouse after several in vivo passages of human melanoma WM164 cells previously isolated from a melanoma metastasis of a patient. The WM164 cells were not competent for metastasis in nude mice prior to this selection. We compared the phenotypes of the parental nonmetastatic cell line and the metastatic variant with respect to growth at clonal seeding densities in protein-free medium (growth factor independence), in vitro invasion through reconstructed basement membranes, secretion of proteolytic enzymes, expression of tumor-associated antigens, and chromosomal abnormalities. Metastatic 451-LU cells showed significantly increased growth factor independence when grown at clonal seeding densities as compared to the parental cells. In in vitro chemoinvasion assays, metastatic 451-LU cells were significantly more invasive than the parental cells. The metastatic variant secreted collagenase and tissue type plasminogen activator at levels 10- and 3-fold higher than the parental WM164 cells, respectively. Polyclonal antibodies to tissue type plasminogen activator significantly inhibited invasion through reconstructed basement membranes. In metastatic 451-LU cells, expression of nerve growth factor receptor was elevated, recently been developed in nude mice (1-7). These models have secretion of proteolytic enzymes, and increased chromosome mode as aneuploid with a mode of 97 chromosomes, whereas the parental nonmetastatic cells had a mode of 52 chromosomes.

Our studies suggest that metastatic melanoma cell variants selected in vivo show increased independence of exogenous growth factors when grown at clonal cell densities, enhanced invasiveness in vitro, greater secretion of proteolytic enzymes, and increased chromosome mode as compared to the nonmetastatic parental cells. The data further suggest that melanoma cells isolated from metastatic lesions and maintained in vitro have an unstable invasive phenotype but that metastatic variant cells can readily be selected.

INTRODUCTION

Models for spontaneous metastasis of human melanoma have recently been developed in nude mice (1–7). These models have helped to define the three major steps within the metastatic cascade: cell attachment to basement membranes, membrane degradation, and cell locomotion (8). Cells selected in these animal models have a relatively stable metastatic phenotype. Cultured cells from metastatic melanoma lesions, on the other hand, show instability of their metastatic phenotype. They are noninvasive when injected into nude mice (7) and have antigenic and genetic similarities with primary melanoma cells (9). Instability of the metastatic properties of cells from metastatic lesions occurs either in vitro growth or as a result of variant selection during rapid proliferation in the human host (10).

We have developed a human melanoma metastasis model in nude mice and demonstrated inhibition of metastasis by a MAb³ to GD2/GD3 gangliosides (7). In this model, a human variant cell line (451-LU) was obtained that spontaneously metastasizes in nude mice. This variant cell line was selected from the lung of a nude mouse after several passages in vivo of human melanoma WM164 cells, which were previously isolated from a melanoma metastasis of a patient (11) but are without competence for metastasis in nude mice (7).

The current study characterizes the phenotypes of the metastatic variant (451-LU) cells and nonmetastatic, parental (WM164) cells with respect to growth at clonal cell densities, invasiveness of the cells, proteolytic enzyme activity, expression of melanoma-associated antigens, and karyotype. These studies demonstrate that metastatic cells, derived from a heterogeneous population of nonmetastatic cells, have distinct phenotypic characteristics, suggesting that metastases are produced by the selective growth of specialized aggressive subpopulations of metastatic cells that preexisted in the parent tumor, rather than by random survival of circulating tumor emboli.

MATERIALS AND METHODS

Cell Lines. Human melanoma cell line WM164 and its clone E9, both without competence for metastasis in nude mice, have been described (7, 11). After four sequential passages of WM164 cells in vivo in nude mice, variant cell line 451-LU and its clone G4 were obtained, both of which spontaneously metastasized to the lungs and lymph nodes in 100% of nude mice following s.c. injection of 5 × 10⁶ tumor cells (7). 451-LU cells retained their metastatic capability in nude mice over a >1-year period of in vitro culture of the cells. In vitro selection of WM164 cells in a chemoinvasion assay (see below) resulted in the isolation of the highly invasive (both in vitro and in vivo) variant cell line, WM164-Bch4. Melanoma cell line WM9 derived from a different patient (11) was used in control experiments.

For analysis of colony-forming efficiency, invasiveness, and enzyme activity, cells were maintained in protein-free WM449 medium (12). For all other assays, cells were grown in Leibovitz L-15 medium supplemented with 10% fetal bovine serum.

Mice. Female nude mice (nu/nu, BALB/c background) were obtained from Harlan Sprague-Dawley, Indianapolis, IN. Mice were housed in a germ-free environment; tumor cell inoculations were performed under a laminar flow cabinet.

Growth Assays. Two types of growth assays were performed with melanoma cells adapted to continuous growth in protein-free medium (12). Growth at high cell density was determined by seeding cells at 2 × 10⁶ cells/cm² in 2-cm² wells precoated with 1% gelatin, and counting cells with a Coulter cell counter on days 1, 3, 5, 7, and 9 after seeding. Growth at clonal cell densities was determined by seeding cells at 30, 60, 90, 180, and 360 cells/cm² in 10-cm² wells coated with 1% gelatin. Seven days later, cells were fixed with 10% formaldehyde in Dulbecco’s modified phosphate-buffered saline, stained with eosin solution, and PD time (h) was calculated by using the following formula:

$$PD (h) = \frac{h \text{ of incubation}}{\log (\text{mean no. of cells/colony})}$$

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² To whom requests for reprints should be addressed.
³ The abbreviations used are: MAb, monoclonal antibody; EGF, epidermal growth factor; NGF, nerve growth factor; PA, plasminogen activator; PD, population doubling; pPA, tissue type plasminogen activator; pUPA, urokinase type plasminogen activator; cDNA, complementary DNA; SSC, standard saline solution; GD3, GD2, GD1, abbreviations for gangliosides follow the Svensson nomenclature. J. Neurol. 106: 613, 1983.
Colony-forming efficiencies (%) were determined as described (13). All determinations were done in duplicate, and values presented in Table 1 are the means of three independently performed experiments.

Invasion and Motility. Invasion of melanoma cells through a reconstituted basement membrane (Matrigel, Collaborative Research, Inc., Bedford, MA) was tested as described (14). The highly invasive variant cell line WM164-Bch4 was obtained from WM164 cells by four serial treatments with specific or normal goat IgG at a final concentration of 100 ng/ml before passaging of these cells in the Boyden chamber assay. Inhibition was quantified as the difference between the number of invaded cells with and without specific antibody treatment. Motility of cells was determined essentially as described for the invasion assay except that filters were left uncoated. All determinations were done in duplicate, and values presented in Table 2 are the means of two to three independently performed experiments.

PA Activity Assay. PA activity in cell lysates and conditioned media was measured with the 125I-fibrin plate assay as described by us previously (15). To determine the type of PA activity present, aliquots of conditioned media were preincubated with anti-uPA IgG (20 µg/ml), anti-tPA IgG (4 µg/ml; gift of Dr. Paul Horan, Smith Kline & French Laboratories), or normal rabbit IgG (20 µg/ml) for 30 min at room temperature prior to addition to the fibrin plate assay (16, 17). For radioimmunodiffusion assay a double antibody procedure was used, similar to that described by others (18). Briefly, wells of microtiter plates were coated with goat antibodies to either human tPA or uPA (American Diagnostica, New York, NY). Samples of conditioned media or lysates were then added to the wells followed by the addition of rabbit anti-tPA or anti-uPA IgG (16, 17). Antibody binding was detected by 125I-labeled goat F(ab')2 against rabbit IgG (Organon Teknika, West Chester, PA). In both assays, single chain tPA (American Diagnostica) and single chain uPA (gift of D. Moir, Collaborative Research) were used as standards.

Data were standardized to 1 mg of cell protein in the lysates. Protein concentrations in the lysates were determined by the Bio-Rad (Bio-Rad, Richmond, CA) assay using bovine serum albumin as a standard.

Immunocytochemical staining was performed directly in the 6-well tissue culture plates with anti-tPA IgG (17) or affinity-purified anti-uPA IgG (16), as described previously (17).

Table 1. Population doubling time of nonmetastatic WM164 cells and metastatic 451-LU cells maintained in protein-free medium

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>WM164</th>
<th>451-LU</th>
<th>WM164-Bch4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonal seeding density</td>
<td>221 ± 34</td>
<td>94 ± 16</td>
<td>166 ± 2*</td>
</tr>
<tr>
<td>High seeding density</td>
<td>71 ± 18</td>
<td>59 ± 15</td>
<td></td>
</tr>
</tbody>
</table>

PD (b) was calculated as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Without preincubation with antibody</th>
<th>Preincubation with cells with anti-tPA antibody* (% of invasion inhibition)</th>
<th>Motility*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM164</td>
<td>9.2 ± 3.1</td>
<td>5.3 ± 2.7 (42.3)</td>
<td>69.8 ± 6.3*</td>
</tr>
<tr>
<td>451-LU</td>
<td>25.6 ± 6.6*</td>
<td>9.2 ± 3.4 (64.6)</td>
<td>49.8 ± 8.8*</td>
</tr>
<tr>
<td>WM164-Bch4</td>
<td>20.4 ± 0.9*</td>
<td>5.0 ± 0.7 (75.5)</td>
<td>163.1 ± 2.3</td>
</tr>
</tbody>
</table>

* Assays were performed in Boyden chambers with Matrigel-covered filters (a) or uncovered filters (b).

b Assays were performed in Boyden chambers with Matrigel-covered filters (a) or uncovered filters (b).

c Cells were preincubated with goat anti-tPA antibodies at 100 µg/ml for 90 min before testing their invasiveness in chemoinvasion assays.

Colagensase Activity. Serum-free culture supernatants of cells were tested for collagenolytic activities. Type IV collagen, radiiodinated with 125I, using the Bolton Hunter reagent (New England Nuclear, Boston, MA), was absorbed to polyvinyl chloride microtiter wells, and collagenolytic activity of protein-free supernatants of 3-day-old confluent melanoma cultures was measured by the release of radioactivity from the wells after a 3-h incubation at 36°C. Values are expressed as ng collagenase activity/ml, using bacterial collagenase type IV (Sigma, St. Louis, MO) as standard.

Expression of Melanoma-associated Antigens. Binding of MAbs to melanoma cells was tested in mixed hemadsorption assay (19). Briefly, sheep erythrocytes preincubated with mouse anti-sheep erythrocyte antibodies and then reacted with goat anti-mouse immunoglobulin antibodies were used as indicator cells. Melanoma cells were incubated with MAbs present in tissue culture supernatants of hybridoma cultures and binding was tested by adding indicator cells to cultures. MAbs used for studies were ME31.3 [anti-chondroitin sulfate proteoglycan (20)]; MEDA3 [anti-130/105 kDa (21)]; ME77.1 [anti-120 kDa (21)]; PL17 [anti-190/94 kDa (13)]; ME77.36 [anti-125/104/99 kDa (22)]; ME491 [anti-highly glycosylated 30/60 kDa (23)]; 425 [anti-EGF receptor (24)]; ME20.4 [anti-NF-1 receptor (25)]; ME96-1 [anti-p97 or melanotransferrin (22)]; 13-17 [anti-HLA-DR (26, 27)]; and 452 [anti-170 kDa]. Binding of anti-G0/G0, G0 ABE361 (28) to melanoma cells was tested by indirect flow cytometry (29).

Southern, Northern, and Western Blotting Analyses. For Southern blotting analysis, high-molecular-weight DNA was extracted from melanoma cells with 50 µg/ml proteinase K, 0.5% sodium dodecyl sulfate, 50 mM Tris, 10 mM EDTA, pH 8.0, followed by phenol and phenol-chloroform extractions, and ethanol precipitations (30). Ten µg DNA was digested with the appropriate restriction enzyme and fractionated by 1% agarose gel electrophoresis. The DNA was transferred to nitrocellulose filters and hybridized with the appropriate overnight at 42°C with 32P-labeled NGF receptor cDNA probe pH1-3 (31) in 50% formamide, 5 x SSC (1 x SSC = 0.15 M NaCl and 15 mM sodium citrate, pH 7.0). 5 x Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. The blots were washed at 65°C with a final wash of 0.2 x SSC containing 0.1% sodium dodecyl sulfate.

For Northern blotting analysis, melanoma cells were suspended for 5 min at 4°C in 10 ml of buffer solution containing 10 mM NaCl, 10 mM Tris, 1.5 mM MgCl2, pH 7.5; 0.5 ml Nonidet P-40 (10%) was added and after 10 s the nuclei were pelleted, and the cytoplasmic RNA was extracted from the supernatant with phenol and chloroform-phenol and precipitated with ethanol (30). Polyadenylated RNA was selected by oligodeoxynucleotide cellulose chromatography and fractionated by 1% agarose/formaldehyde gel electrophoresis (3 µg/lane). The RNA was transferred to a nitrocellulose filter and hybridized with nick-translated NGF receptor cDNA by using the same conditions described for the Southern blot.

For Western blotting analysis, crude membrane fractions were prepared from melanoma cells and proteins were extracted with solubilizing buffer (0.5% Nonidet P-40, 140 mM NaCl, 10 mM NaF, 10 mM Tris, 5 mM EDTA, aprotinin (100 kallikrein inhibitory units/ml), 1 mM phenylmethylsulfonyl fluoride (pH 7.5) (31). Reactivities of the antitumor MAbs with proteins in this extract were analyzed by Western blotting under nonreducing conditions with an 11% gel (32). The blots were incubated with anti-NGF receptor MAb ME20.4 in ascites diluted 1:500 with Dulbecco's modified phosphate-buffered saline containing 2% γ-globulin-free horse serum and 0.02% NaN3, or purified ME491 MAb at 5 µg/ml in the same buffer and then incubated with 125I-goat anti-mouse IgG at 200,000 cpm/ml.

Cytogenetic Studies. Each cell line was treated with colchicine for 45 min at 37°C. After treatment with a KCl-sodium citrate hypotonic solution and standard fixation, air-dried slide preparations were made for Giemsa banding (9). Karyotypes were determined from a minimum of 50 counts and two banded karyotypes for each of the three cell lines.

Statistical Analysis. Data were analyzed by Student's t test. Statistically significant differences were determined at P < 0.05 level.

RESULTS

In efforts to define properties characteristic of the metastatic phenotype, the following cell lines were examined for their growth in growth factor-depleted medium, motility, invasiveness, proteolytic enzyme activity, antigenic phenotype and karyotype: (a) melanoma cell line WM164, which does not spontaneously metastasize in nude mice; (b) variant line 451-LU which was selected from WM164 cells in nude mice for the capacity to metastasize spontaneously to the lung from the s.c. injection point (7); and (c) variant line WM164-Bch4, which was selected in vitro in the chemoinvasion assay and which metastasizes spontaneously in vivo in nude mice (not shown). Results are presented for the mass cultures of each cell line. Nearly identical results were obtained for three clones, one each derived from the various lines (not shown).

Growth Characteristics. At clonal seeding densities in medium without exogenous growth factors or other proteins (protein-free medium), 451-LU and WM164-Bch4 cells had significantly ($P < 0.001$, $P < 0.05$, respectively) shorter PD times than WM164 cells, clearly indicating a growth advantage of the metastatic and invasive variants as compared to the parental nonmetastatic and noninvasive cells (Table 1). Furthermore, both 451-LU and WM164-Bch4 cells showed significantly ($P < 0.005$) higher colony-forming efficiencies as compared to WM164 cells (Fig. 1). However, at high seeding density in protein-free medium, PD times of WM164 cells and WM164-Bch4 cells did not differ significantly (Table 1). Thus, under more favorable growth conditions, the metastatic and invasive variant cells have no growth advantage over the nonmetastatic and noninvasive parental cells ($P > 0.05$).

Invasion and Motility. When tested for invasion through a reconstructed basement membrane (Matrigel), 451-LU and WM164-Bch4 cells were 2- to 3-fold more invasive than WM164 cells (Table 2). These differences were significant ($P < 0.001$, $P < 0.005$ for 451-LU and WM164-Bch4 cells, respectively). The in vitro invasion of WM164, 451-LU, and WM164-Bch4 cells was inhibited by 42, 65, and 75%, respectively, by preincubating cells with goat anti-tPA antibodies before the assay (Table 2). Inhibition was significant both for 451-LU ($P < 0.01$) and WM164-Bch4 ($P < 0.005$) cells. Motility through uncoated filters, on the other hand, was significantly higher for WM164 cells than for either 451-LU or WM164-Bch4 cells (Table 2), probably reflecting the smaller size (approximately 40%) of WM164 cells as compared to 451-LU and WM164-Bch4 cells (not shown).

Proteolytic Enzymes. Metastatic 451-LU and WM164-Bch4 cells showed 2- to 3-fold higher total PA enzymatic activity than nonmetastatic WM164 cells, both in culture supernatants and in cell lysates (Table 3). These differences were statistically significant ($P < 0.05$; Table 3) except for the PA activity in lysates of 451-LU cells versus WM164 cells. All activities were inhibited by greater than 90% with anti-tPA IgG, indicating that most, if not all, of the PA activity in the cells was due to tPA. In solid-phase radioimmunoassays, the concentration of tPA was significantly ($P < 0.05$ or less; Table 3) higher in WM164-Bch4 cells and 451-LU cells as compared to WM164 cells, both for culture supernatants and cell lysates. All cell lines showed undetectable levels of uPA protein, both in culture supernatants and cell lysates (<2.0 and <0.6 ng/ml cell protein, respectively; results not shown).

Supernatants of 451-LU cells grown in protein-free medium showed ~10-fold higher collagenolytic activity than the supernatants obtained from the same number of WM164 cells (Table 3). These differences were significant ($P < 0.001$).

By immunocytochemical staining, tPA was concentrated along the cytoplasmic membrane in all 3 cell lines. There was no specific staining of cells of any of the 3 lines with antibodies to uPA (not shown).

Melanoma-associated Antigens. WM164, 451-LU, and WM164-Bch4 cells were compared in MHA for expression of the melanoma-associated antigens chondroitin sulfate proteoglycan, various glycoproteins, EGF and NGF receptor, melatontransferrin, HLA-DR, and $G_{D3}$ gangliosides (see “Materials and Methods”). Of all antigens, only NGF receptor and $G_{D3}$ ganglioside expression consistently showed differences between metastatic 451-LU and nonmetastatic WM164 cells. NGF receptor was expressed in ~100% of the metastatic 451-LU cells, ~80% of the invasive WM164-Bch4 cells, and only ~20% of the nonmetastatic WM164 cells (Fig. 2). These results were confirmed by Western blotting analysis (Fig. 3). Extracts of metastatic cell line 451-LU reacted much more strongly than extracts from WM164 cells or from melanoma cell line WM9. These results were not due to a difference in the quality of the protein extracts since antimelanoma MAb ME491 (23) reacted strongly with all three extracts. The results are consistent with Northern blotting analyses of nonmetastatic and metastatic cells with the NGF receptor cDNA probe (Fig. 4). RNA from cell line 451-LU had an intense 3.8-kilobase NGF receptor mRNA, but WM9 cells had a barely detectable band, and no band was visible for WM164 cells. These differ-

![Fig. 1. Colony-forming efficiency of WM164 (△), WM164-Bch4 (●), and 451-LU cells (□) cultured in protein-free medium. Bars, SD of duplicate determinations. Values for 451-LU and WM164-Bch4 cells differ significantly ($P < 0.05$) from those for WM164 cells.](image-url)
Table 3 Plasminogen activator and collagenase activity in WM164, WM164-Bch4, and 451-LU cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PA enzymatic activity (mPU/mg cell protein)</th>
<th>Amount of tPA (ng/mg cell protein)</th>
<th>Collagenase activity in culture supernatants (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture supernatant</td>
<td>Cell lysates</td>
<td>Culture supernatant</td>
</tr>
<tr>
<td>WM164</td>
<td>1151 ± 206</td>
<td>101 ± 111</td>
<td>8.5 ± 2.7</td>
</tr>
<tr>
<td>WM164-Bch4</td>
<td>115 ± 38</td>
<td>384 ± 32</td>
<td>25.1 ± 9.5</td>
</tr>
<tr>
<td>451-LU</td>
<td>381 ± 38</td>
<td></td>
<td>16.4 ± 0.3</td>
</tr>
</tbody>
</table>

* Fibrin plate assay. All results are the mean ± SD of 2-4 experiments; mPU = milliploug unit.
* Solid phase radioimmunoassay (mean ± SD of 2-4 experiments).
* Substrate was 125I-labeled collagen IV.
* For each parameter, values with the same symbols differ significantly; d, e = P < 0.05; f = P < 0.01; g = P < 0.005; h = P < 0.001.

Thus, both cells have similar numbers of NGF receptor genes. Also, there were no obvious gene rearrangements as judged by Southern blotting of melanoma DNA digested with either BamHI or EcoRI (results not shown).

The results obtained in indirect flow cytometry with MAb ME361 against Gm/Gm gangliosides indicate a 2.2-fold increase in the percentage of cells binding the MAb and a 1.8-fold increase in the mean fluorescence intensity per cell in 451-LU cells as compared to WM164 cells (results not shown).

Cytogenetic Studies. The parental cell line WM164 was first karyotyped in 1981 at passage 17 (33). The cells had a modal number of 52 chromosomes, including an isochromosome for the long arm of chromosome 21 (iso21q) and a characteristic marker chromosome (M1) whose origin could not be determined. This cell line was studied on four additional occasions throughout a period of 5.5 years and each time contained the same marker chromosomes (M1, iso21q) and a similar modal number. A few cells in each of these preparations were approximately tetraploid (Table 4). The metastatic variant cell line 451-LU also contained the M1 and iso21q markers present in the parental WM164 line but had a modal number of 97 chromosomes including 5 copies of chromosome 7 and 9 of chromosome 20 (Table 4). WM164-Bch4 cells similarly contained the M1 and iso21q markers with a modal number of 90 chromosomes including 5 and 7 copies of chromosomes 7 and 20, respectively. In addition, a del1(p22) was now present in every cell along with six new marker chromosomes.

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DISCUSSION

Melanoma cells, isolated from a metastatic lesion of a patient and maintained in culture, were shown to represent a heterogeneous population of cells with respect to metastatic capabilities in nude mice and invasiveness through a reconstructed membrane in vitro. Although heterogeneity of the metastatic and invasive phenotype in the parental melanoma cells, described here, may be related to prolonged in vitro culture of the cells, other studies have shown heterogeneity of the metastatic phenotype in murine tumors at an early stage of their development (34). Less than 1% of the parental WM164 cells spontaneously metastasized to the lungs of nude mice (7) or invaded through a reconstructed membrane in vitro (Table 2). Given the origin of WM164 from a melanoma metastasis of a patient, the fraction of metastatic/invasive cells seems rather low and is in the range of that reported for murine tumors (35). Reversion of the metastatic/invasive phenotype, as suggested for WM164 cells, is a common phenomenon (36) and was as high as 10^{-7} to 10^{-1} per cell generation in murine tumor systems (37). However, it is difficult to ascertain whether this phenomenon reflects the situation in vivo or rather may be related to prolonged in vitro culture of tumor cells. Successful isolation from nonmetastatic WM164 cells of invasive variants in vitro, with metastatic properties in vivo (WM164-Bch4 cells), suggests that a subpopulation of invasive cells present a priori in the parental nonmetastatic cells gave rise to the metastatic 451-LU cell line that was isolated under selective pressure in vivo. This is further supported by the similarities in phenotypic differences observed in the in vitro and in vivo selected variants when compared to the nonmetastatic parent line. Phenotypic differences between metastatic/invasive and the parental cells were found with respect to invasiveness, proteolytic enzymes, expression of melanoma-associated antigens, and karyotype. The demonstration of distinct phenotypic differences between metastatic/invasive and nonmetastatic cells seems to indicate that metastases are produced by the selective growth of specialized aggressive subpopulations of cells that preexisted in the parent tumor rather than by random survival of circulating tumor emboli (38, 39).

A striking difference between nonmetastatic and metastatic/invasive melanoma cells is the increased growth factor independence at low cell density of the latter cells. When cultured in protein-free medium at clonal seeding densities, invasive cells had a significantly shorter PD time and higher colony-forming efficiency than the parental cells. The increased growth autonomy of invasive and metastatic cells compared to nonmetastatic cells has recently been demonstrated for human melanoma cells (11, 40) and murine tumor cells (41–43). Independence from exogenous growth factors and higher survival at clonal (nonoptimal) cell densities may be due to the production of growth factors for autocrine growth stimulation, several of which have recently been demonstrated in melanoma cells, including transforming growth factor α (44), platelet-derived growth factor (45), and fibroblast growth factor (46).

The increase in the invasiveness of melanoma cell variants as compared to the parental cells (Table 2) might be at least partially explained by the increased tPA and collagenolytic activity in the variant cells. Invasiveness was significantly inhibited by preincubation of the cells with antibodies to tPA. The preferential expression of tPA and not uPA in all three cell lines is consistent with the reports by other investigators on the prevalency of tPA in human melanoma (47). A correlation between the metastatic phenotype and PA/collagenase activity also has been established in other systems (48, 49).

We have demonstrated here that metastatic 451-LU cells have increased NGF receptor protein and mRNA. However, the role of NGF receptor in metastasis is highly speculative. Current studies on the metastatic properties of a WM164 clone that has been transfected with the NGF receptor gene may clarify this point.

In agreement with our previous results (50) and results reported by Rosenberg et al. (51), we have demonstrated here increased expression of melanoma-associated gangliosides in metastatic versus nonmetastatic lesions of human melanoma. Thus, metastatic 451-LU cells showed higher expression of G_{p2}/G_{p3} gangliosides than the parental nonmetastatic WM164 cells. Since gangliosides have been implicated in the attachment of tumor cells to substrate (52), the increased ganglioside level in 451-LU cells may relate to the invasiveness of these cells.

Repeated cytogenetic studies of WM164 over a period of 5.5 years showed an essentially constant modal number of chromosomes and the same marker chromosomes. These results are indicative of the karyotypic stability of this line in culture. Previous cytogenetic studies have indicated that genetic abnormalities increase in severity with clinical progression of melanoma (53). These abnormalities often include progressive aneuploidy and the appearance of additional marker chromosomes. It is interesting, therefore, that the metastatic 451-LU variant of WM164 had increased aneuploidy as did the WM164 cells that had been passed through a Boyden chamber. In addition to the near tetraploidy of the two selected cell populations, the cells isolated in the Boyden chamber also contained new marker chromosomes and a del1(p22). Deletions of the proximal segment of chromosome 1 are frequently seen in cells derived from advanced melanomas (53). The oncogene N-ras and the β subunit of NGF have been mapped to 1p22 (54). However, the role of these gene deletions in the development of the invasive phenotype in melanoma is unclear. Both variants of WM164 cells showed increased dosages of chromosomes 7 and 20. While the role of chromosome 20 in tumor progression is unclear, interesting associations have been found with respect to chromosome 7. It seems that genes involved in invasion and metastasis are located on human chromosome 7 (55). Increased dosage of chromosome 7 has been detected in advanced metastatic melanomas and has been shown to be associated with high expression of the EGF receptor (56).

The described model of human melanoma metastasis has provided valuable information on the metastatic phenotype of subpopulations of cells derived from a metastasis of a patient. Although studies in animals do not directly bear on the situation in humans, our results obtained in the nude mouse model suggest that metastatic lesions of melanoma in the natural host

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**Table 4: Karyotypic analyses of WM164, WM164-Bch4, and 451-LU cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of karyotypes</th>
<th>No. of chromosome 7</th>
<th>Chromosome 20</th>
<th>Karyotypic markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM164</td>
<td>50</td>
<td>2</td>
<td>52</td>
<td>~14</td>
</tr>
<tr>
<td>451-LU</td>
<td>50</td>
<td>3</td>
<td>97</td>
<td>~100</td>
</tr>
<tr>
<td>WM164-Bch4</td>
<td>50</td>
<td>6</td>
<td>90</td>
<td>~100</td>
</tr>
</tbody>
</table>

- 50 ct chromosome 7
- 4 ct chromosome 20
- M1, iso21q
- M1, iso21q + random markers
- M1, iso21q M2 → M7 random markers + del1(p22)
may contain subpopulations of cells with enhanced capability to further metastasize to additional sites. The demonstration, using in vitro selection methods, of the presence within these lesions of cells (WM164-Bch4) with enhanced invasive capabilities further supports this notion.

We have recently applied the nude mouse model to melanoma cells derived from early nonmetastatic lesions (vertical growth phase) of a patient. A comparison of the metastatic variant cells derived from these lesions with the variants from the metastatic lesion described here will help in our understanding of the evolution of the metastatic phenotype during various stages of tumor progression in melanoma.

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REFERENCES


18. Binnema, D. J., van Nese, J. J. L., and Dooijewaard, G. Quantitation of
HUMAN MELANOMA METASTASIS


In Vitro Properties of Human Melanoma Cells Metastatic in Nude Mice

Dorothee Herlyn, Dimitrios Iliopoulos, Pamela J. Jensen, et al.


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