A New $M_r$ 55,000 Surface Protein Implicated in Melanoma Progression: Association with a Metastatic Phenotype\(^1\)

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**ABSTRACT**

Emergence of the invasive phenotype is a key event in the progression of human melanoma from benign proliferative lesions to malignant lesions. Recently, we successfully selected in vivo from a poorly metastatic M4Beu human melanoma cell line two variants (7GP and TIP26) that generate a higher frequency of spontaneous metastases to the lungs into immune-suppressed neonatal rats. Both cell lines showed no significant differences in the integrin profile of the subunits analyzed except for $\beta_3$, which was reduced to a background level in metastatic variants. To investigate how these variant sublines of human melanomas manage to sustain growth in the absence of $\alpha\beta_3$, a subtractive immunization approach was used to elicit host antibody responses against cell surface proteins expressed on metastatic variants. In this study, a new monoclonal antibody (MoAb), LY1, that is highly specific for the 7GP and TIP26 variants, was isolated. LY1 identifies a membrane protein of $M_r$ 55,000 on melanoma variants with epitopes that were resistant to sugar-cleaving enzymes. Immunostaining cells from variants by LY1 showed that staining is distributed to the cell periphery with high labeling intensity at the cell-to-cell contact points. This MoAb significantly inhibited invasion of metastatic variants through a reconstituted basement membrane (Matrigel) in vitro. Moreover, tumor growth of metastatic variants was dramatically affected in vivo with this MoAb. In vitro studies indicate that the LY1 MoAb does not inhibit chemotactic migration of the metastatic variants, the adhesion of tumor cells to vitronectin, collagen IV, fibronectin, and laminin, or cell proliferation. Expression of this antigen is high in human striated muscle, heart, liver, and pancreas. Using 59 fixed, paraffin-embedded archival tissues of human melanomas and nevi, LY1-reactive cells were not observed in melanocytes, nevi, or radial growth phase primary melanomas. In sharp contrast, LY1 selectively stained melanocytes derived from the vertical growth phase of many primary melanomas and metastatic melanomas. These results provide evidence that the $M_r$ 55,000 protein expressed by selected variants with increased metastatic properties in vivo plays a functionally important role in determining metastasis. This molecule may represent a new metastatic risk marker in human melanoma and may be of biological importance in the identification of fatal metastatic subpopulations that have acquired competence for metastasis production.

**INTRODUCTION**

Malignant melanoma is a highly invasive and metastatic tumor with incidence and mortality that in recent years have been increasing faster than those of any other cancer (1). Because adjuvant therapy of proven efficacy is not currently available for these patients, the search for a specific marker for those tumor cells most likely to metastasize may lead to the development of a new prognostic indicator (2).

Benign to malignant progression results from the sequential emergence of a series of aggressive subpopulations of cells that preexisted in the parent tumor (3, 4). Such metastatic variants differ from the parental tumor in a number of properties such as transcription factors, cell surface enzymes, motility, signaling, angiogenic factors, and cell adhesion molecules (5–8). Recently, receptors that mediate cell-to-cell and cell-to-substratum adhesion were shown to be key components in the metastatic cascade (9–12). An integrin such as $\alpha\beta_3$ has been shown to be expressed by an increasing proportion of primary melanomas in the transition from RGP\(^3\) to VGP, suggesting that this integrin plays an active role in melanoma progression (13, 14). Likewise, the intercellular adhesion molecule 1, MUC18, HLA-DR, and gangliosides GD\(_2\) and GD\(_3\) exhibit enhanced expression on melanoma cells as they progress to a more metastatic phenotype (15–19). However, the absence of expression of many of these antigens was frequently observed on tissue sections (13, 14, 20), suggesting that additional tumor cell markers that are indicative for metastatic capacity are needed.

Using a metastasis model, which mimics the early events of metastasis in humans, we recently selected from a poorly $\alpha\beta_3$-positive metastatic human melanoma cell line, M4Beu, a whole series of $\alpha\beta_3$-negative variants that generate a higher frequency of spontaneous metastases into immune-suppressed neonatal rats and aggressively proliferate in vivo after s.c. implantation into athymic nude mice (21). These results suggest that expression of such a phenotype can confer metastatic ability to variants that have emerged from the in vivo selection pressure. Such dramatic changes in the expression of a major integrin on the surface of biologically aggressive subpopulations of tumor cells prompted us to search for the nature of the cell membrane molecules that mediated the metastatic ability of tumor cells.

In this study, a chemical immunosuppression approach (22) was used to raise monoclonal antibodies to functional cell surface antigens that showed minimal expression on the parental melanoma cell line M4Beu, but that significantly enhanced on metastatic melanoma variants. Immunosuppression was achieved by the use of the tolerizing cytotoxic drug cyclophosphamide, which reduces the recognition of the common immunodominant cell surface antigens present on the two phenotypically distinct melanoma cell types by selectively killing proliferating clones of B cells (23). This approach has proved to be a powerful technique for the development of function-blocking mouse monoclonal antibodies directed against less immunodominant proteins such as maturation-specific sperm surface molecules, cerebellar neuron, nasal retinal axons, and axonal proteins expressed by premigratory, migrating retinal ganglion cells (22) and to specific-tumor associated antigens (24).

We first immunized mice with a poorly metastatic M4Beu melanoma cell line and generated a series of immune responses, which were selected on the basis of their abilities to produce antibodies that block cell-to-cell and cell-to-substratum adhesion.

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\(^4\) The abbreviations used are: RGP, radial growth phase; VGP, vertical growth phase; MoAb, monoclonal antibody; IAP, integrin-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AEC, 3-amin0-9-ethylcarbazole.

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noma cell line. The immunosuppressive drug cyclophosphamide was then used to tolerize mice to M1B.eu, followed by multiple immunizations with a highly metastatic melanoma variant, TIP26. These immunization procedures enabled us to produce several potential MoAbs that have a selective ability to bind to highly metastatic tumor variants. Here, we report the characterization of one MoAb (LY1) that recognizes a M$_r$ 55,000 surface antigen (p55), which plays a functional role in the process of tumor progression and metastasis. Expression of p55 was preferentially detected in primary melanomas in the VGP as well as metastatic lesions but not on melanocytes, nevi, or primary melanomas in the RGP. This cell surface protein of M$_r$ 55,000 may represent a new tumor progression and metastatic risk marker in human melanoma.

**MATERIALS AND METHODS**

**Antibodies, Chemicals, and Reagents**

Monoclonal antibodies against the human a$_1$ (clone HP2B6), a$_2$ (G9), a$_3$ (clone M-KID 2), a$_5$ (HP2/1), a$_6$ (SAM1), a$_{GoH3}$, a$_1$ (clone AMF7), and b$_3$ (clone SZ21) integrins were purchased from Immunotech (Marseille, France). Other reagents were obtained as follows: NuSerum, Matrigel basement membrane matrix, and Bicocat cell culture inserts were from Becton Dickinson Labware (Bedford, MA); cyclophosphamide was from Sigma (St. Louis, MO), prestrained high molecular weight protein marker and horseradish peroxidase color development reagent were from Bio-Rad (Hercules, CA); protease inhibitor mixture was from ICN (Costa Mesa, CA); Hybond-C and [${}^{3}$H]thymidine were from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom); a cell-proliferating kit, N-glycosedase F; recombiant O- glycoidasite (from *Diplocoocus pneumoniae*), and neuraminidase (from *Arthrobacter ureafaciens*) were from Boehringer Mannheim (Indianapolis, IN); and a RAL S55 staining kit was from Reactif RAL (Paris, France).

**Cell Culture and Selection of α,β, deficient Cells**

The human M$_1$B.eu cell line was derived from a lymph node metastasis of a patient with malignant melanoma (25). M$_1$B.eu cells expressing α,β, (α,β,$^+$ positive) are unable to give lung metastases after orthotopic injection of the cultured cells into immunosuppressed newborn rats (low incidence of spontaneous lung colonization; Ref. 21). The α,β, negative variant, designated TIP26 (high incidence of spontaneous lung colonization), was established in culture after two direct successive orthotopic transplantations of M$_1$B.eu tumors into immunosuppressed newborn rats (21). The α,β, negative 7GP (high incidence of spontaneous lung colonization) metastatic variant was selected in *vivo* through eight serial transplantations of lymph node metastases of the M$_1$B.eu cell line into immunosuppressed newborn rats (21). Human melanoma cell lines TIP26L and TIP26R (high incidence of spontaneous lung colonization) are clones that were sorted from TIP26 cells on the basis of their ability to bind the peanut agglutinin lectin using a fluorescence-activated cell sorter (26). These melanoma cell line variants have a doubling time $(30 \pm 0.5 \text{ h})$ similar to that of the parent M$_1$B.eu.

**Cell Line**

The cells were cultured as monolayers in RPMI 1640 or McCoy’s 5A medium supplemented with 10% fetal bovine serum as described previously (21). Cultures were routinely checked and found free of *Mycoplasma* contamination using the Hoechst 33258 fluorescence staining procedure. To minimize the possibility of phenotypic drift, variants were maintained in culture for 4–6 weeks after which they were replaced with new frozen stock. Passages 26–40, counted from the first passage of the original variant, were used in these studies. SK-MEL-2 and SK-MEL-24 human melanomas, MG-63 human osteosarcoma, and MCF-7 human breast carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The DEV human medulloblastoma cell line was a gift from Dr. B. Jacquetmont (INSERM U433, Lyon, France; Ref. 27). IAP-negative, vector-transfected OVO10 and IAP-transfected OVO10* ovarian carcinoma cell lines were kindly supplied by Dr. F. P. Lindberg (Washington University School of Medicine, St. Louis, MO; Ref. 28).

**Subtractive Immunization**

The technique and schedule used to immunize mice were as described previously (22). Briefly, subconfluent monolayer cultures of poorly metastatic melanoma cells were detached by the addition of nonenzymatic cell dissociation solution and washed twice in sterile PBS. The washed cells were resuspended in the same buffer and injected i.p. $(2 \times 10^6$ cells per animal$)$ into 8-week-old female BALB/c mice. Twenty-four and 48 h later, these mice were injected i.p. with 200 mg/kg cyclophosphamide diluted in sterile PBS. Two weeks later, blood was collected, and the serum was screened for the presence of anti-poorly metastatic melanoma antibodies using an ELISA technique (29). Three days after the last injection, cells from the metastatic variant were detached from the culture plate as described above, and $2 \times 10^6$ cells were inoculated i.p. into mice. Boosting was performed 3 weeks later by injecting these animals i.p. with $3 \times 10^6$ metastatic variant cells. Three days after the last injection, sera were collected and screened for differential binding toward the poorly metastatic and metastatic cultures by FACScan (Becton Dickinson).

The next day, the mouse with a selective differential immune response to highly metastatic versus poorly metastatic melanoma cells was killed by cervical dislocation, and the spleens from the hosts were taken for fusion with the BALB/c myeloma SP2/0Ag14. A control study was performed with animals inoculated with the poorly metastatic parental cell line and metastatic variants in the absence of cyclophosphamide.

**Production of Monoclonal Antibodies**

Details of the fusion procedure were as described previously (30) using standard procedures (31). Supernatants from the wells containing growing hybridomas were screened for their reactivity to highly metastatic variants and poorly metastatic parental cell line using ELISA and FACScan techniques. The positive clones were cloned by limited dilution, and monoclonal antibodies were purified from ascitic fluid by gel filtration on Sephacryl S-300. Classes and subclasses of the monoclonal antibodies were determined by the Amer- sham isotyping kit (Amersham International, les Ullis, France) following the instructions given by the manufacturer.

**Screening of Monoclonal Antibodies**

In the first stage of screening, an ELISA was used to evaluate antibody binding to poorly metastatic and highly metastatic melanoma cells (29). Briefly, melanoma cells were harvested with nonenzymatic cell dissociation buffer and added $(2 \times 10^6$; 0.1 ml) to each microtiter well containing 50 µg/ml polyclonal. Fifty to 100 µl of hybridoma culture supernatant were added to wells and incubated for 1 h at 37°C. Bound antibodies were detected after sequential addition of peroxidase-conjugated rabbit anti-mouse IgG and IgM (heavy and light chain specific; Dakopatts, Copenhagen, Denmark) and the substrate 1,2-phenylenediamine. Nonspecific binding was performed with pre-immune sera and subtracted from the total binding. In the second stage of screening, positive hybridoma culture supernatants were analyzed by flow cytometry as described previously (21) using either culture medium or preimmune sera as a control. Of 66 culture supernatants tested, 8 (LY1–LY8) hybridomas were found by FACScan containing antibodies directed against the metastatic variant and not to the poorly metastatic parental cell line. One of these monoclonal antibodies, designated LY1, was chosen for its strong reactivity and was used for further studies. For immunocytochemical staining, cells grown on 12-well multistest slides for 48 h were fixed for 1 h in cold methanol before the addition of the first MoAb or normal mouse serum as described previously (10). Thereafter, the cells were washed extensively with Ca$_{2+}$- and Mg$_{2+}$-free PBS followed by sequential incubation with the biotinylated antimuscle immunoglobulin and streptavidin conjugated to alkaline phosphatase using the DAKO (Glostrup, Denmark) LSAB "kit. Staining was completed after incubation with the naphth-fuchsin solution.

**Western Blot Analysis**

Cells were lysed in Lubrol lysis buffer (20 mM Tris (pH 7.4), 0.15 mM NaCl, and 1% Lubrol) containing a protease mixture cocktail according to the instructions of the manufacturer (ICN). The lysates were centrifuged at 12,000 × g at 4°C for 15 min and separated by electrophoresis in a 10% polyacrylamide gel containing SDS according to the method of Laemmli (32).
The proteins were then electrophoresed and transferred to nitrocellulose paper (Hybond-C). The nonspecific binding was blocked by soaking the electrophoretic blots for 1 h in Tris-buffered saline [0.02 m Tris-HCl (pH 7.6), and 0.15 m NaCl] containing 3% BSA. The membranes were incubated with purified MoAb for 1 h. Bound antibodies were detected after sequential addition of a goat antimouse IgM antibody conjugated to peroxidase (1:2000 dilution, Dakopatts) and a horseradish peroxidase color development reagent obtained from Bio-Rad. Control experiments were performed with a nonimmune mouse serum or an irrelevant mouse IgM isotype antibody.

Enzyme Treatment

The cell lysates were subjected to enzyme digestion according to the instructions of the manufacturer (Boehringer Mannheim). Briefly, 20 µg of the lysates were heat denatured in the presence of 1% SDS and diluted to 0.1% SDS. Denatured samples were then treated with 4 milliunits of neuraminidase, 5 milliunits of O-glycosidase F alone, 4 units of N-glycosidase alone, or N-glycosidase in combination with O-glycosidase F for 17 h at 37°C before the Western blot analysis. Control samples were incubated without enzymes.

Adhesion to Extracellular Matrix Proteins

The cell adhesion assay was performed as described previously (33). Briefly, 1–2 µg of fibronectin, laminin, collagen IV, or vitronectin diluted in PBS (pH 7.2) were adsorbed on each well of the microtiter plate for 1 h at 37°C. Cells (10⁵) resuspended in serum-free RPMI 1640 containing 0.35% BSA were then added to coated wells after 20 min of incubation with purified MoAb (10 µg/10⁶ cells) or with an irrelevant mouse IgM isotype antibody. After a further incubation at 37°C for 60–90 min, nonadherent cells were removed by gently washing the wells. One hundred µl of medium and 10 µl of MTT labeling reagent (0.5 mg/ml) were added to each well and incubated for 4 h at 37°C following the manufacturer’s instructions (Boehringer Mannheim). Then 100 µl of 10% SDS in 0.01 M HCl were added for 12 h. The solubilized formazan product in the wells was spectrophotometrically quantified using an ELISA reader.

Migration (Chemotaxis) Assay

Chemotaxis was assayed using uncoated 24-well BioCoat cell culture inserts (Becton Dickinson Labware, MA) with an 8-µm-porosity polyethylene-terephylate membrane. Briefly, cells were removed with nonenzymatic cell dissociation buffer and resuspended at 5 × 10⁵/ml in serum-free RPMI 1640 containing 0.35% BSA. Cells (5 × 10⁴) pretreated for 20 min with 5–10 µg/ml MoAb or an irrelevant mouse IgM isotype antibody were then added to the upper compartments of the BioCoat chambers (6.25-mm membrane size). The wells of the lower chamber were filled with RPMI 1640 containing 10% NuSerum, and the chambers were each assembled by placing the uncoated membrane between the lower and upper compartments according to the manufacturer’s instructions (Becton Dickinson). The migration assay was carried out at 37°C for 15 h, after which the filters were removed, fixed, and stained with the RAL 555 staining kit. Cells on the upper surface of the filters were removed by wiping with a cotton swab, and migration was determined by counting of the cells that had migrated to the lower side of the filter with a microscope at ×100 magnification. Experiments were assayed in triplicate, and at least eight fields were counted in each experiment.

Invasion Assay

Invasive ability of the melanoma cells in vitro was carried out as described previously (34) with modifications. Invasion was measured by using 24-well BioCoat cell culture inserts with an 8-µm-porosity polyethylene-terephylate membrane coated with Matrigel basement membrane matrix (100 µg/cm²). Briefly, the Matrigel was allowed to rehydrate for 2 h at room temperature by adding warm, serum-free RPMI 1640. The wells of the lower chamber were filled with RPMI 1640 containing 10% NuSerum, and the chambers were each assembled similarly to the method described above for migration assay. Cells (10⁶) were seeded in the upper compartment (6.25-mm membrane size) in serum-free RPMI 1640. Cells were treated with 0.5–1 µg of either MoAb or an irrelevant mouse IgM isotype antibody for 20 min before the invasiveness assay. The invasion assay was carried out at 37°C in a 5% CO₂ humidified incubator for 48–72 h. Fresh antibody was added each day. At the end of the invasion assay, filters were processed and quantitated for the migration assay. Experiments were assayed in triplicate, and at least 10 fields were counted in each experiment.

Tumorigenicity in Nude Mice

Swiss nude mice (nu/nu) were purchased from IFFA-CREDO (Arbrsele, France). Subconfluent cultures of melanoma cells were harvested with non-enzymatic cell dissociation buffer, washed three times with serum-containing medium, and then resuspended in PBS (pH 7.2). Two hundred µl of cells (2 × 10⁶) were inoculated s.c. on the belly of nude mice as described previously (10), after preincubation for 20 min with either MoAb (16 µg/10⁶ cells) or an isotype control mouse IgM. Tumor growth rate was determined by weekly measure of two perpendicular diameters of the tumor. All experimental groups contained five mice. The effect of the MoAb on cell growth, viability, and cytotoxicity was measured by using [3H]thymidine uptake or the MTT cell proliferation kit following the manufacturer’s instructions (Boehringer Mannheim, IN) as described previously (33).

Immunohistochemical Analysis of Normal and Melanoma Tissues

Tissues Studied. Histopathological sections of the following normal human adult tissues were investigated: heart, brain, kidney, liver, lung, pancreas, spleen, and muscle. Serial 5-µm sections of paraffin-embedded tissue were obtained from Novagen (Madison, WI). Surgical biopsy material of malignant lesions of melanocyte origin was obtained from the Departments of Pathology and Dermatology at Hôpital de l’Antiquaille. The lesions were classified by histopathological examination of paraffin sections. Tumor thickness, Clark’s level of invasion, and the growth phase of each lesion were determined on hematoxylin-stained sections as described previously (35). The depth of invasion of lesions used in this study ranged from levels I to V, and the vertical tumor thickness varied between 0 (in situ) and 10 mm. Primary cutaneous melanomas were considered to be in RGP if the epidermal component was larger than the dermal component and if atypical melanocytic cells lay beyond the basement membrane and extended into the papillary dermis. Lesions that did not fulfill these criteria were considered to be in VGP wherein tumor cells grow downward into the underlying mesenchymally derived dermis. The tumor lesions were classified as belonging to one of the following groups: benign skin lesions including 16 nevi (8 dermal, 4 compound, 3 congenital, and 1 junctional; n = 16) and 2 dermatofibromas (n = 2); 10 cutaneous melanomas in RGP (tumor thickness ≥ 0.75 mm; n = 10); 23 cutaneous melanomas that had entered VGP (tumor thickness ≥ 1.0 mm; n = 23); and 8 metastatic melanomas to lymph nodes (n = 8). Samples that show both the RGP and VGP compartments were analyzed separately.

Immunohistological Labeling. Immunohistochemistry on paraffin-embedded tissue sections (5–6 µm) was performed using a catalyzed amplification system (Dako) following the manufacturer’s instructions. Briefly, sections were incubated for 2 h with purified MoAb (1:500) or an isotype control mouse IgM. Sections were then incubated serially with link antibody (biotinylated rabbit antimouse immunoglobulin), streptavidin-biotin complex, amplification reagent (biotinyl-tyramide and hydrogen peroxide), and streptavidin-peroxidase. Bound MoAb was detected with AEC, which gives rise to a red chromogen. These sections were counterstained with Harris’s hematoxylin and finally mounted in aqueous mounting medium. The percentage of stained cells was visually estimated by three independent observers (B. B., E. T., and H. B.). Tumors were scored positive if 10% or more reactive tumor cells showed staining with MoAb, and nevi were scored positive if any reactive areas were seen.

RESULTS

Chemical Immunosuppression for the Poorly Metastatic Parental Melanoma Cell Line with Cyclophosphamide. Suppression of the immune response of mice to poorly metastatic parental melanoma cell line M₄Beu was determined using different cyclophosphamide treatment protocols. An ELISA was used to study the binding of mice sera to the M₄Beu parental cell line. s.c. administration of cyclophos-
phosphamide, added to reduce the immunological response to the M4Beu. cell line at 24, 24, and 48 h after antigen exposure, resulted in increased chemical immunosuppression (from 71 to 98%) compared with control mice not receiving cyclophosphamide. Because two rounds of cyclophosphamide treatments (24 and 48 h) reduced the immunological response to the M4Beu. melanoma cell line, mice were then inoculated with the highly metastatic melanoma variant T1P26, and their splenocytes were fused with myeloma cells. MoAb LY1 was chosen for its strong reactivity against metastatic variants and was identified as an IgMκ antibody by using antimouse subclass-specific antisera. Flow cytometric analysis showed that LY1 showed 2-fold higher levels of binding to the T1P26 metastatic variant than to the poorly metastatic parental cell line M4Beu. (Fig. 1A). This MoAb was observed by immunohistochemistry to bind to the surface of melanoma cell variants (Fig. 1B). This staining appeared localized to the cell periphery with high labeling intensity at the cell-cell contact sites (Fig. 1B).

Flow Cytometry Analysis of LY1 Binding to the Parental Melanoma Cell Line and in Vivo-derived Variants. We next investigated the reactivity of the LY1 MoAb on a panel of variants derived, after in vivo and in vitro selections, from a common progenitor M4Beu. cell line. As shown in Table 1, LY1 binds selectively to highly metastatic melanoma variants but failed to bind to the poorly metastatic parental cell line M4Beu. Moreover, this MoAb also stained SK-MEL-2 and SK-MEL-24, two human melanoma cell lines derived, respectively, from lymph node or cutaneous metastases (Table 1). In contrast, no staining was observed with this MoAb on human breast carcinoma, osteosarcoma, and medulloblastoma cell lines and IAP-negative (OVO10) and IAP-positive (OVO10+IAP) ovarian carcinoma cell lines. In addition, no significant difference in expression of α₁, α₂, α₅, α₆, α₈, and α₁ was observed between the melanoma variants (T1P26 and 7GP) and the parental cell line M4Beu. (data not shown), except for the integrin subunit β₇, which was previously shown to be expressed at a high level on parental M4Beu. cells but was dramatically reduced to a background level on the metastatic T1P26 and 7GP melanoma variants (21).

Biochemical Characterization by Western Blot Analysis of LY1 Melanoma Antigen. To identify the human melanoma-associated cell surface antigen recognized by the LY1 MoAb, Western blot analysis was performed. As shown in Fig. 2A, LY1 immunoblotted a Mr 55,000 major protein from lysates of metastatic variant T1P26 under nonreducing or reducing conditions compared with an irrelevant mouse IgM isotype antibody of the same class as LY1 (Lanes 1 and 2). Other faint immunoreactive protein bands migrating at Mr 55,000, Mr 110,000, and Mr 150,000 were variably detected in the sample of cell lysates (results not shown). In contrast, no identical set of bands could be detected from lysates of the poorly metastatic parental cell line M4Beu, providing further evidence that LY1 is selectively directed against a metastasis-associated antigen (Lane 3). Treatment of the cell lysate with neuraminidase, with N-glycosidase, alone, or with N-glycosidase in combination with O-glycosidase did not affect antibody binding (Fig. 2B).

Inhibition of Tumor Invasion in Vitro. To determine whether LY1 can affect an important biological property of metastatic melanoma variants, we used an in vitro Matrigel assay. As shown in Fig. 3, the invasive ability of the metastatic variant T1P26 cell line was significantly affected (75% decrease in the invasive potential of the cells) by the LY1 MoAb compared with an irrelevant mouse IgM isotype antibody. In contrast, the invasive ability of the poorly metastatic parental cell line M4Beu. was not significantly affected in the presence of the LY1 MoAb (Fig. 3).

Table 1 Reactivity of LY1 on human tumor cell lines by flow cytometry

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cell origin</th>
<th>LY1</th>
</tr>
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<tbody>
<tr>
<td>M4Beu.</td>
<td>Parental cell line</td>
<td>–</td>
</tr>
<tr>
<td>T1P26</td>
<td>Variant</td>
<td>+</td>
</tr>
<tr>
<td>T1P26L</td>
<td>Variant</td>
<td>+</td>
</tr>
<tr>
<td>Other melanoma cell lines derived from metastatic lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>Lymph node</td>
<td>+</td>
</tr>
<tr>
<td>SK-MEL-24</td>
<td>Cutaneous</td>
<td>+</td>
</tr>
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**Fig. 1.** A, flow cytometric analysis of LY1 MoAb binding to the nonmetastatic parental melanoma cell line and derived metastatic cell variant. A melanoma variant (T1P26) and the parental melanoma cell line (M4Beu.) were first incubated for 1 h at 4°C with the LY1 MoAb followed by a FITC-conjugated rabbit antihuman IgG antibody. Histograms are based on the analysis of 5000 controls. Controls profiles of M4Beu. or T1P26 melanoma cells treated with an isotype control mouse IgM antibody were identical. B, immunohistochemical staining of melanoma variants with MoAb LY1. Cells were grown on 12-well multistest slides, fixed 48 h later, and then incubated with MoAb LY1 (right) or isotype control mouse IgM antibody (left). Signals were detected by the sequential addition of a biotinylated antihuman immunoglobulin and streptavidin conjugated to alkaline phosphatase as described in “Materials and Methods.” Staining is completed by the addition of the naphtol-fuchsin solution.
Inhibition of Tumor Growth in Vivo. To investigate whether LY1 affects melanoma cell growth in vivo, the metastatic variant T1P26 was inoculated s.c. in nude mice, and tumor growth was followed over 4 weeks. As shown in Fig. 4, A and B, melanoma variant T1P26 yielded readily visible tumors as early as 15 days that gave rise to large tumors at 30 days. In contrast, with LY1 added, melanoma variant T1P26 gave rise to small tumors growing at a lower rate than in the controls. Controls included an irrelevant mouse IgM isotype antibody used at the same concentration as that of LY1. To exclude the possibility that LY1 might have a direct cytotoxic effect, cell variants were preincubated with excess LY1 (10 μg/ml) and monitored for cell viability and cell growth. No loss of melanoma cell viability was shown by trypan blue exclusion. Moreover, control and LY1-treated cells showed a similar level of MTT formazan production and cell proliferation in vitro (results not shown). Furthermore, in the presence of fresh rabbit or nude mice sera containing complement, LY1 did not support lysis of melanoma cells, suggesting that the observed inhibition by LY1 in vivo was not due to complement-dependent cytotoxicity or opsonization (data not shown).

Effect of LY1 MoAb on Cell Adhesion to Various Matrix Proteins and Migration (Chemotaxis). We next investigated whether the LY1 MoAb could affect cell adhesion to major matrix substrates such as fibronectin, vitronectin, laminin, and collagen IV. As shown in Fig. 5A, LY1 had no effect on the attachment of the metastatic variant T1P26 to various purified matrix proteins compared with an irrelevant mouse IgM isotype antibody. To examine further the mechanism allowing LY1 to affect tumor growth or invasion, we looked at the ability of LY1 to affect migration of T1P26 cells in the chemotaxis assay. Fig. 5B shows that migration of T1P26 cells toward lower chambers was not affected in the presence of LY1.

Expression of LY1-reactive Antigen in Benign and Malignant Lesions of Melanocyte Origin. The expression of the LY1-reactive antigen was studied by immunoperoxidase staining of paraffin-embedded tissue sections from different lesions representative of different stages of melanoma progression, including benign skin lesions and RGP primary, VGP primary, and metastatic melanomas. MoAb LY1 stained none of the normal melanocytes present in dermal fibroblast (n = 2) and activated melanocyte nevi (n = 16), which included dermal (n = 8), junctional (n = 1), compound (n = 4), and congenital (n = 3) types (Table 2). A typical example of the staining pattern of skin containing a benign melanocytic nevus or RGP of a superficial spreading malignant melanoma is shown in Fig. 6. Cells from nevi (Fig. 6A) and RGP (Fig. 6B) lesions were not stained with LY1 compared with a control section stained with an IgM isotype antibody. In all lesions, staining of keratinocytes was observed. In marked contrast, VGP cells and metastatic cells stained strongly with this antibody (Fig. 6, C and D). There appears to be significant heterogeneity on the staining pattern of VGP and metastatic lesions. Approximately 30–90% of the melanoma cells were stained with LY1.

Reactivity of LY1 MoAb with Normal Tissues. Analysis of the distribution of the LY1-reactive antigen in normal adult tissues showed that kidney, liver, and pancreas were not stained with this MoAb. In contrast, striated muscle, heart, spleen, brain, and lung were stained by the LY1 MoAb (Table 3).
DISCUSSION

A number of studies suggested that invasive and metastatic cancer cells differ from their biologically indolent counterparts in a wide variety of phenotypes (36, 37). By immunizing mice with two closely related but phenotypically distinct melanoma cell variants and reducing the recognition of common immunodominant cell surface antigens through the use of the immunosuppressive drug cyclophosphamide, we generated several potential monoclonal antibodies that show selective binding to highly metastatic melanoma cell variants. One of the monoclonal antibodies (LY1) recognizes by Western blot an antigen predominantly expressed on the surface of highly metastatic melanoma variants and plays a functional role in the process of metastasis. Several lines of evidence support the above statements: (a) LY1 binds to a major cell surface protein of Mr 55,000 (p55) under nonreducing and reducing conditions, respectively, the epitopes of which do not involve sugar moieties; (b) reducing the availability or functional level of the metastasis-associated antigen using the LY1 MoAb resulted in a significant decrease in tumor cell invasion through Matrigel in vitro; (c) tumor proliferation in vivo was also significantly affected by the presence of bound LY1; and (d) LY1 stained in situ none of the benign melanocytic lesions but strongly stained the metastatic lesions from patients with melanoma.

In addition to the above-mentioned results, primary melanoma cells of the RGP consisting of cells with no demonstrable competence for metastasis (usually those with a thickness $<0.76$ mm) failed to express p55 (35). In striking contrast, the expression of this antigen is dramatically increased in the primary melanoma cells of the VGP. These results have clinical relevance, because previous observations have shown that melanomas in the VGP lesions of $>0.76$ mm, as used in this study, often carry a much worse prognosis and behave genotypically and phenotypically similar to the lesions of distant metastasis (35). These data, to-

Table 2 Immunoeroxidase staining of nevus and melanoma paraffin-embedded tissue sections with LY1 monoclonal antibody

<table>
<thead>
<tr>
<th>Lesions</th>
<th>LY1 positive/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td></td>
</tr>
<tr>
<td>Normal skin</td>
<td>0/2</td>
</tr>
<tr>
<td>Dermal nevi</td>
<td>0/8</td>
</tr>
<tr>
<td>Compound nevi</td>
<td>0/4</td>
</tr>
<tr>
<td>Congenital nevi</td>
<td>0/3</td>
</tr>
<tr>
<td>Junctional nevi</td>
<td>0/1</td>
</tr>
<tr>
<td>Dermatofibroma</td>
<td>0/2</td>
</tr>
<tr>
<td>Primary melanoma</td>
<td></td>
</tr>
<tr>
<td>RGP</td>
<td>0/10</td>
</tr>
<tr>
<td>VGP</td>
<td>19/23</td>
</tr>
<tr>
<td>Metastatic melanoma</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>5/8</td>
</tr>
</tbody>
</table>
gether with the fact that the cell surface expression of p55 is associated with the metastatic potential of a panel of in vivo-selected melanoma variants, lend further support for a critical role of this melanoma-associated antigen in the aggressive phenotype of melanoma cells. It was remarkable to note that our experimental selection procedure led to the appearance of a phenotypic marker of primary melanomas undergoing the radial to VGP transition. It must be noted, however, that the $\beta_3$ integrin subunit with a level of expression that was shown to correlate with the aggressiveness of the tumor (10, 11, 38) is not expressed at any detectable level in melanoma variants used during the course of this study. Whether such $\alpha_v\beta_3$-negative metastatic variants expressing p55 recapitulated their behavior in the human patients remains to be determined.

Further characterization of the tissue distribution of the p55 cell surface protein in several normal human tissues indicated that this melanoma-associated antigen is expressed on striated muscle, heart, spleen, brain, and lung but not kidney, liver, and pancreas. These results suggest that the expression of p55 is not melanocytic specific and is not dependent on the histogenetic origin of the tissue. Indeed, epithelial as well as nonepithelial cells were stained with the LY1 MoAb. Furthermore, a number of epithelial cells (particularly glandular cells) as well as nonepithelial cells were also stained with LY1. The LY1 MoAb did not inhibit adhesion of the metastatic variants to vitronectin, collagen IV, fibronectin, or laminin (Fig. 5). The observations that LY1 strongly stained the cell-cell contacts (Fig. 1B) raise the possibility that p55 is not functioning as an extracellular matrix protein receptor but rather as a cell-cell adhesion molecule.
NH2-terminal residue. Further analysis of the p55 antigen sequence by melanoma cell surface protein have been unsuccessful due to blocked body was detected with AEC.

Amplification system as described in "Materials and Methods." Bound monoclonal anti-

Adhesion molecules exhibited increased homotypic adhesion and increased invasiveness through Matrigel-coated filters. These observations, together with previous work showing a direct correlation between the tendency of B16 melanoma variants to undergo homotypic aggregation in vitro and their metastatic potential in vivo (41), strongly suggest a role of p55 in allowing melanoma cells to adhere to each other in various steps of the metastatic process.

Indeed, the human organs that need strong intercellular cohesion (e.g., muscle, heart, spleen, brain, and lung) were those showing high expression of p55 (Table 3). Its function as an adhesive molecule necessary for cell-cell interaction is further supported by our results showing that consistent staining of keratinocytes was noted at the cell membranes but not at the basement membrane-associated basal pole of epithelial cells. Such a role of the p55 cell surface protein in mediating cell-cell interactions is very similar to that of previously described adhesion molecules such as CD146 (also known as melanoma cell adhesion molecule, MUC18, A32 antigen, or S-Endo-1; Ref. 39) or activated leukocyte cell adhesion molecule (40). In these studies, it was shown that transfected melanoma cells with these adhesion molecules exhibited increased homotypic adhesion and increased invasiveness through Matrigel-coated filters. These observations, together with previous work showing a direct correlation between the tendency of B16 melanoma variants to undergo homotypic aggregation in vitro and their metastatic potential in vivo (41), strongly suggest a role of p55 in allowing melanoma cells to adhere to each other in various steps of the metastatic process.

Although it is generally believed that loss of or a reduction in homotypic interactions may be required to facilitate cell detachment from the primary tumor and subsequent migration (9, 19), the increased cell-cell adhesion by p55 seems to contradict the paradigm of tumor progression predicated on the need for tumor cells to reduce cell surface expression of adhesion molecules such as E-cadherin to enable invasion. However, metastasis is a dynamic process characterized by subclonal evolution (3). Up-regulation of p55 in melanomas may provide melanocytic cells with a new phenotype that enhances the ability of tumor cells to reestablish intercellular contact, thereby increasing their ability to evade immune destruction and establish metastatic foci (42).

This metastasis-associated antigen is unrelated to IAP (known also as CD47) in both molecular weight (M, 50,000) and specificity of association with αβ integrin in nucleated cells (43). Moreover, the MoAb LY1 does not bind to the IAP-negative, vector-transfected ovarian carcinoma cell line OVO10 and to IAP-transfected OVO10+ cells, suggesting that it recognizes a different antigen than CD47 (28). Attempts to obtain the NH2-terminal sequence of the M, 55,000 melanoma cell surface protein have been unsuccessful due to blocked NH2-terminal residue. Further analysis of the p55 antigen sequence by gene cloning would provide pertinent information about its biological importance in the malignant progression of human cutaneous melanoma.

The major characteristic of malignant tumor cells is their ability to invade foreign tissues and form metastatic foci at distant locations in the body (44). Such a process requires tumor cell attachment to various matrix proteins followed by migration of the surrounding stroma by tumor cells. As shown in the present report, adhesion of the metastatic variants to major matrix proteins as well as their chemotactic migration were not affected by the LY1 MoAb.

These results suggest that the mechanism by which this antibody affects tumor cell invasion is apparently not related to alterations in the interaction of invasive tumor cells with the basement membrane extracellular matrix or motility of tumor cells. Preliminary results indicate that such variant cell lines produced more of the Mr 92,000 collagenase type IV compared with the parental cell line.5 One possibility is that melanoma-melanoma cell interactions mediated by p55 may regulate (through intracellular signals) the expression of matrix metalloproteinases and that the binding of LY1 to p55 may affect the production of enzymes required for degrading the basement membrane and invasion (39). Indeed, several studies have shown that the invasive potential of tumor cells could be altered via the signal transduction by the cell adhesion molecules (39, 45).

Tumor cell growth is a complex intersection between tumor cells and their surrounding connective tissue components (46). Because the LY1 MoAb significantly inhibited the growth of melanoma cell variants, p55 must play an important role in the proliferation of melanoma in vivo. The finding that the LY1 MoAb did not completely inhibit tumor growth suggests that other adhesion proteins contribute to the full expression of the tumorigenic phenotype of the cells. Maintenance of intercellular contacts by p55 may provide tumor cells with a better chance of survival, as shown previously for E-cadherin-mediated cell-cell adhesion (47, 48). Blocking p55 with LY1 may lead to apoptosis of tumor cells. Alternatively, inhibition of tumor growth may be due to interference of the LY1 MoAb with tumor-host interactions.

In summary, we have identified in this study a Mr 55,000 cell surface protein on αβ-negative metastatic melanoma variants that plays a critical role in the malignant phenotype of human melanoma. Evidence is provided that expression of this cell surface protein in situ is increased dramatically in the melanocytic tumors known to have a relatively high risk of metastasizing. This molecule may represent a new progression marker of biological importance to identify fatal metastatic subpopulations that have acquired competence for metastasis production.

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