A Melanosomal Membrane Protein Is a Cell Surface Target for Melanoma Therapy

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ABSTRACT

Differentiation antigens on cancer cells are recognized by the immune system. A prototype set of these autoantigens in melanoma cells are the melanosomal glycoproteins, expressed in both melanomas and normal melanocytes. These are intracellular proteins that can be recognized by both antibodies and T lymphocytes. While one can understand how T cells can respond to intracellular proteins, based on cellular requirements for antigen processing and presentation, it is more difficult to understand how antibody responses to melanosomal proteins could lead to tumor rejection. We demonstrate that gp75 is expressed on the cell surface as well as intracellularly in human and mouse melanomas. The surface expression of gp75 can be augmented by IFN-γ and during tumor growth in vivo. Surface expression of gp75 on mouse melanoma cells correlates with the ability of a monoclonal antibody (mAb) against gp75 to reject melanomas in syngeneic mice. Antibody-mediated rejection seems to require the Fc portion of the antibody.

INTRODUCTION

Until recently, little was known about the structure of antigens recognized on cancer cells by the immune system. The identification of a handful of potentially immunogenic tumor antigens has shown that these antigens are not typically foreign. Most of the central work in human cancer immunology has been done in melanoma. Surprisingly, studies of melanoma have shown that self molecules are typically recognized (1). A majority of melanoma antigens recognized by the immune system are expressed both on malignant cells and their normal cell counterparts (e.g., melanocytes), defining them as differentiation antigens (2). Furthermore, autoantibodies against differentiation antigens predict a favorable outcome in patients with metastatic melanoma (3). Thus, studies point to differentiation antigens as one set of dominant antigens recognized on human cancer cells and suggest that immunity to differentiation antigens may alter the progression of melanoma.

Melanosomes are specialized cellular organelles. Melanosomal glycoproteins expressed specifically in melanocytes are among the best-characterized melanoma antigens (4–6). Three of these antigens are melanosomal membrane glycoproteins (tyrosinase, gp75/TRP-1, and the gp100 protein) that are recognized by T cells, and two of these antigens are recognized by autoantibodies (tyrosinase and gp75; Refs. 1, 4, 7–10). Because T cells recognize peptides that are processed and presented from intracellular compartments, it was predicted that melanosomal proteins could be recognized by T cells (4). However, it is unclear how melanosomal proteins might be recognized by antibodies in intact cells. Antibodies against the brown locus protein gp75 or gp75/TRP-1 have been shown to efficiently localize to human melanoma xenografts (11). Furthermore, a mouse mAb against gp75 can be used to treat mouse melanomas (12). One explanation has been that tumor necrosis can spill intracellular contents, allowing access to antibodies. However, tumors without any necrosis (e.g., <1-2 mm in diameter) are rejected by antibodies against gp75. Here, we show that gp75 is expressed on the cell surface, allowing antibody-mediated tumor rejection. Cell-surface gp75 can be up-regulated by the host inflammatory cytokines, specifically IFN-γ. Finally, we characterize the antitumor effects and manifestations of autoimmunity mediated by antibodies against gp75.

MATERIALS AND METHODS

Mice and Tumors. C57BL/6 (6–8-week-old females) were obtained from The Jackson Laboratory (Bar Harbor, ME). CB-17 mice with either scid/scid or scid/scid;bg/bg traits were purchased from Taconic Farms, Inc. (Germantown, NY). B16F10 is a mouse melanoma cell line of C57BL/6 origin kindly provided by Dr. Isaiah Fidler (M. D. Anderson Cancer Research Institute).
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Center, Houston, TX). B78H.1 is a variant of B16 melanoma that does not express the gp75 antigen (12). B78H.1 cells were transfected with syngeneic gp75 cDNA expressed in the plasmid pcEXV-3 (13). Cell lines were tested routinely for mycoplasma contamination. Cell lines were grown in Eagle’s MEM + 1% nonessential amino acids, 100 µg/ml each of penicillin and streptomycin, and 2 mM glutamine (Life Technologies, Inc., Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO). B16F10 melanoma cells were detached with 0.02% EDTA in PBS and were washed twice in PBS. Then 1 × 10^5 cells were injected s.c. into the flanks of mice. Two weeks later, mice that had s.c. B16F10 tumors were sacrificed. Tumor tissue was removed and minced between two frosted microscope slides (Fisher Scientific, Pittsburgh, PA) to disaggregate mechanically. A single-cell suspension from the tumor was obtained by passing tumor tissue through a cell strainer (Falcon, Lincoln Park, NJ), and cells were cultured short-term at 37°C CO2 in a 37°C incubator. For B16F10 melanoma lung metastases, C57BL/6 mice were inplanted B16F10 lung metastases and primary ex- plated B16F10 tumors (B16F10 Tumor; B) were stained on ice with mAb TA99. Cells were evaluated by flow cytometry as described in “Material and Methods.” Abscissa, fluorescence intensity; ordinate, relative cell number. The lighter line is a control representing the staining of B16F10 cells that were incubated with control mAb. C, induction of cell surface expression of gp75. B16F10 cells were cultured for 3 days with different amounts of supernatant from conA-stimulated syngeneic splenocytes (0, 1, or 10% v/v conA/medium). Cells were stained with mAb TA99 or control mAb and analyzed as described. The lighter line is a control representing the staining of B16F10 cells that were incubated with control mAb. D, induction of cell surface expression of gp75 analyzed on B16F10 cells by the MHA assay. Reactivity was scored according to the proportion of target melanoma cells covered by RBC rosettes. A test was read as negative when wells showed <10% rosetted target cells.

Flow Cytometry Analysis. Cells were stained for 30 min on ice with saturating concentrations of mAbs and FITC-conjugated rabbit anti-mouse IgG (Accurate Chemicals, Westbury, NY). Cells were washed twice in PBS containing 1% BSA and 0.1% sodium azide after each incubation. The stained cells were analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA). Routinely, 1 × 10^4 events were collected on a live gate.

MHA Rosetting Assay. Rabbit anti-mouse immuno- globulin MHA assay was used for the detection of cell surface antigens (14). B16F10 cells (2 × 10^5 cells/well) were seeded in Terasaki plates (Nunc, Naperville, IL) and primary ex- planted B16F10 tumours were stained on ice with mAb TA99. Cells were evaluated by flow cytometry as described in “Material and Methods.” Abscissa, fluorescence intensity; ordinate, relative cell number. The lighter line is a control representing the staining of B16F10 cells that were incubated with control mAb. C, induction of cell surface expression of gp75. B16F10 cells were cultured for 3 days with different amounts of supernatant from conA-stimulated syngeneic splenocytes (0, 1, or 10% v/v conA/medium). Cells were stained with mAb TA99 or control mAb and analyzed as described. The lighter line is a control representing the staining of B16F10 cells that were incubated with control mAb. D, induction of cell surface expression of gp75 analyzed on B16F10 cells by the MHA assay. Reactivity was scored according to the proportion of target melanoma cells covered by RBC rosettes. A test was read as negative when wells showed <10% rosetted target cells.

Fig. 1  The cell surface expression of gp75 was assessed in B16F10 melanoma cells by the MHA assay. Reactivity was scored according to the proportion of target melanoma cells covered by RBC rosettes. A test was read as negative when wells showed <10% rosetted target cells.

Fig. 2  A and B, cell surface expression of gp75 analyzed on B16F10 cells by flow cytometry. Cultured B16F10 cells (A) and primary ex- planted B16F10 tumor (B) were stained on ice with mAb TA99. Cells were evaluated by flow cytometry as described in “Material and Methods.” Abscissa, fluorescence intensity; ordinate, relative cell number. The lighter line is a control representing the staining of B16F10 cells that were incubated with control mAb. C, induction of cell surface expression of gp75. B16F10 cells were cultured for 3 days with different amounts of supernatant from conA-stimulated syngeneic splenocytes (0, 1, or 10% v/v conA/medium). Cells were stained with mAb TA99 or control mAb and analyzed as described. The lighter line is a control representing the staining of B16F10 cells that were incubated with control mAb. D, induction of cell surface expression of gp75 analyzed on B16F10 cells by the MHA assay. Reactivity was scored according to the proportion of target melanoma cells covered by RBC rosettes. A test was read as negative when wells showed <10% rosetted target cells.

mAbs and Cytokine. The mouse mAb TA99 (IgG2a), which binds to the brown locus product gp75 or TRP-l, was purified from mouse ascites by protein A affinity column (Phar- macia LKB, Piscataway, NJ). TA99 F(ab')2 fragments were constructed by using pepsin digestion. IgG2a mAb UPC10 (Sigma) was used as a control mAb. Cells were treated i.p. with mAb TA99, TA99 F(ab')2, or control mouse IgG2a mAb UPC10 diluted in 0.3–0.4 ml of PBS three times/week. Recom- binant mouse IFN-γ was from Genzyme Corp. (Cambridge, MA), and recombinant human IL-2 was from Chiron (Em- eryville, CA). To make conA-activated supernatant, mouse spleen cells (1 × 10^6 cells/ml) were cultured for 48 h in RPMI 1640 (Life Technologies, Inc.), 50 µM 2-mercaptoethanol (Sigma), and 10% fetal bovine serum in the presence of 5 µg/ml conA (Boehringer Mannheim, Indianapolis, IN). Residual conA in the supernatant was removed by absorption with 10 mM methyl α-D-mannopyranoside (Sigma).
Fig. 3 TA99 treatment against B78gp75 melanoma. B78gp75 cells express intracellular gp75 but not cell surface gp75. B78gp75 cells \(5 \times 10^5\) were injected s.c. into the flank of C57BL/6 mice. Mice were treated i.p. with control mouse IgG2a mAb UPC10 or mAb TA99 injected at a dose of 330 \(\mu\)g (days 0, 2, 4, 7, and 9). This TA99 treatment inhibited the growth of B16F10 and JB/RH melanomas that express cell surface gp75 (12). Tumor growth was measured in millimeters using calipers. The longest surface length \(a\) and its perpendicular width \(b\) were measured, and tumor size was reported as \(a \times b\). Tumors were checked at least three times/week and allowed to grow to 400 mm\(^2\). Bars, SD of tumor size with 5–7 mice/group.

immunoglobulin conjugated to human type O RBCs were added to the wells. The plates were incubated for 1 h at room temperature. After blotting, the plates were washed six times with PBS, and rosetting was read under the light microscope.

**ADCC Assay.** Target cells were radiolabeled with 200 \(\mu\)Ci (1 Ci = 37 GBq) of \(^{51}\)Cr for 1 h and then plated with the effector cells (50 \(\mu\)l/well) in 96-well flat-bottom microplates (Falcon). The naive spleen cells, which were cultured with 500 units/ml of recombinant human IL-2 for 5 days, were used as effector cells. Purified antibodies (50 \(\mu\)l/well) were added to each well. Plates were incubated at 37°C for 6 h. The supernatants were removed, and radioactivity was measured with a gamma counter.

Percent cytotoxicity

\[
\text{Percent cytotoxicity} = \left( \frac{\text{sample release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \right) \times 100
\]

**ELISA Assay for IFN-\(\gamma\).** NK cells were isolated from splenocytes with biotinylated anti-mouse NK1.1 mAb (PharMingen) and Dynabeads M-280 streptavidin (Dynal, Oslo, Norway) and cultured with 2000 units/ml of recombinant human IL-2 and 50 \(\mu\)M 2-mercaptoethanol for 7 days. Fresh B16F10 cells \((4 \times 10^5\) cells/well; 1 ml/well) were seeded as target cells in 24-well plates (Costar Corp., Cambridge, MA). Splenocytes or isolated NK cells \((4 \times 10^5\) cells/well; 0.5 ml/well) were added as effector cells. Purified antibodies (50 \(\mu\)g/ml; 0.5 ml/well) were added in each well. Supernatants were taken at 4, 6, 14, 24, 46, 73, and 132 h and assayed for IFN-\(\gamma\) by means of a sandwich ELISA (PharMingen).

**RESULTS AND DISCUSSION**

The Melanosomal Membrane Protein gp75 is Expressed on the Cell Surface. The gp75 glycoprotein is a type I membrane molecule that is synthesized in the endoplasmic reticulum, transported through the Golgi complex, and sorted to the endosomal compartment and to melanosomes (15, 16). The gp75 glycoprotein contains an intracellular retention signal that sorts it to the endosomal compartment (16), leading to stable intracellular retention. An unexpected finding has been that mAb to gp75 can localize to melanoma tumors in xenografts and induce tumor rejection in syngeneic hosts, despite the intracellular location of gp75 (11, 12). To assess whether gp75 also reaches the plasma membrane, live unfixed B16F10 melanoma cells were analyzed for expression of gp75. Cell surface expression of gp75 was detected by MHA assays using mAb TA99 (Fig. 1). Plasma membrane expression was confirmed by flow cytometry (Fig. 2A). Similar cell surface expression was shown for the gp75\(^+\) murine melanoma JB/RH and the gp75\(^+\) human melanomas SK-MEL-19 and SK-MEL-23 (data not shown).
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1. **Introduction**

- **Fig. 5** Tumor rejection in mice deficient in T cells and NK cell activity. scid/scid (SCID) or scid/scid:bg/bg (SCID/Bg) CB-17 mice were challenged by injection through the tail vein with 1 × 10^6 B16F10 melanoma cells. Mice (5 mice/group) were treated i.p. with 300 μg of isoate-matched control mAb UPC10 or mAb TA99 three times/week for 2 weeks, starting on the day of tumor challenge. Lung tumor nodules were counted on day 14 after the tumor challenge. Bars, SD.

2. **Expression of gp75**

- **Passive Immunity to gp75 and its Relationship to Surface Expression of gp75.** We have previously shown that treatment with mouse antibody TA99 in C57BL/6 mice bearing B16F10 and JB/RH melanomas leads to tumor rejection (12). As noted above, both B16F10 and JB/RH melanomas express cell surface gp75. B78H.1 melanoma is a gp75-negative variant of B16 melanoma that was selected for its ability to be stably transfected (20). The syngeneic gp75 cDNA expressed in the plasmid pEXV-3 was transfected into B78H.1, and stable clones were selected that expressed gp75. A clone with relatively high expression of cellular gp75 was selected for further experiments (clone B78gp75). There was no detectable cell surface expression of gp75 in B78gp75 transfecants by the sensitive MHA assay even after induction with 100 units/ml IFN-γ, although abundant intracellular gp75 was demonstrated in vesicles inside B78gp75 cells and confirmed by immunoprecipitation with mAb TA99 (data not shown). It was estimated that B78gp75 expressed 62% of the total cellular gp75 level of B16F10 melanoma by ELISA binding to fixed permeabilized cells and by immunoprecipitation and approximately the same level of total cellular gp75 as JB/RH melanoma (data not shown). The lack of cell surface expression of gp75 by B78gp75 cells may reflect certain defects in sorting type I membrane glycoproteins to the cell surface because these cells are markedly deficient in expression of other surface glycoproteins including class I and II MHC molecules. Although treatment with TA99 antibody induced rejection of B16F10 and JB/RH (12), both of which express cell surface gp75, there was no effect on the growth of the B16 variant melanoma B78gp75 (Fig. 3). These results show a relationship between expression of cell surface gp75 and tumor rejection, suggesting that surface expression is a requirement for tumor rejection. Results with the pair of melanomas derived from the B16 line (B16F10 with surface gp75 expression and B78gp75 without surface expression) support this model. Rejection of melanoma did not simply correspond to the total cellular level of gp75 because JB/RH melanoma was rejected by mAb TA99 treatment (12), but B78gp75 melanoma growth was not affected, although both tumors expressed approximately the same levels of gp75.

3. **Up-regulation of gp75 by IFN-γ.** To investigate whether immune factors produced by host mononuclear cells can induce gp75, supernatants from splenocytes stimulated with conA were incubated with B16F10 melanoma cells. Addition of supernatants from 1 and 10% conA-induced syngeneic splenocytes increased the expression of gp75 measured by flow cytometry (Fig. 2C). One component in these supernatants that might be implicated in the induction of gp75 is IFN-γ. IFN-γ was present in these conA supernatants at a concentration of 800 units/ml, as measured by an ELISA assay, and therefore, was present at a final concentration of 8 units/ml in 1% conA medium. Purified recombinant mouse IFN-γ augmented the expression of gp75 at both 10 and 100 units/ml (Fig. 2D). This shows that a host factor, i.e., IFN-γ, can induce increased expression of gp75 in vitro, implicating IFN-γ in the up-regulation of gp75 in vivo. This up-regulation of gp75 is remarkable because we have previously shown that a large number of other melanocyte and melanoma antigens are unaffected by IFN-γ (17).

- It is unclear why a melanosomal protein would be induced by an inflammatory cytokine. The gp75 protein plays a role in melanin synthesis, determining the type of pigment synthesized. The gp75 glycoprotein has 5,6-dihydroxyindole-2-carboxylic acid oxidase activity, which catalyzes an intermediate step in the melanin synthesis pathway (18, 19). The gp75 antigen is encoded by the brown locus and determines coat color in mice (e.g., black, brown, and other colors). One possibility is that the proteins involved in melanin synthesis are coordinately up-regulated during inflammation. The production of epidermal pigmentation is a phenomenon that is commonly observed with cutaneous inflammatory conditions.

4. **A. N. Houghton, unpublished observations.**
A, treated mouse (right) and untreated littermate (left). B, close-up of the coat of a treated mouse.

Characterization of Antitumor Effects in Mice Treated with mAb TA99. In our previous studies, tumor rejection of B16 melanoma cells after treatment with mAb against gp75 was shown to require host NK1.1+ (12). The involvement of NK1.1+ cells suggests that NK cells are involved in tumor rejection mediated by mAb TA99. One possibility is that Fc receptors on NK cells are involved in ADCC mediated by TA99. We showed that the Fc region of TA99 is probably required for tumor rejection by comparing treatment with F(ab')2 fragments of TA99 with whole IgG2a TA99 (Fig. 4). There were no detectable antitumor effects of F(ab')2 fragments. A limitation of this experiment is that the biodistribution and pharmacokinetics of the TA99 fragment are likely different than those of whole IgG. However, even one-tenth of the dose of TA99 used in these experiments gives >70% reduction in melanoma, suggesting that a lower bioavailability of F(ab')2 fragments of TA99 is not necessarily limiting.

In in vitro ADCC assays, no specific lysis (i.e., specific lysis <5%) of B16F10 melanoma cells could be detected in ADCC assays using mouse splenocytes as effector cells (data not shown). No killing was detected with TA99, whether effector cells were fresh splenocytes, IL-2-activated splenocytes (lymphokine-activated killer cells), or adherent IL-2-activated splenocytes, nor whether B16F10 target cells were cultured or explanted from in vivo tumors (data not shown). Likewise, no release of IFN-γ was detected in supernatants when mouse splenocytes + mAb TA99 were incubated with B16F10 target cells (data not shown). Thus, despite the accessibility of cell surface gp75 to mAb TA99 and the potential involvement of NK cells in tumor rejection, there was no detectable ADCC or NK IFN-γ release induced by mAb TA99 bound to target melanoma cells.

These results suggest that a noncytolytic mechanism involving NK1.1+ cells could mediate rejection induced by TA99. To further explore this possibility, we examined the antitumor effects of TA99 in scid and scid + bg/bg mice. The beige mutation causes intracellular vesicle dysfunction and lysosomal malfunction, leading to a loss of NK cell activity (along with marked changes in neutrophil function, coagulation profiles, and other changes). TA99 decreased lung metastases by >70% in both scid/scid and scid/scid:bg/bg mice compared to isotype-matched antibody control treatment (Fig. 5). This experiment further supported a paradigm in which noncytolytic mechanisms of NK1.1+ cells were involved in melanoma rejection mediated by TA99.

At this point, we are unsure how NK1.1+ cells are involved in melanoma rejection mediated by mAb TA99. One possibility is that TA99 binding to gp75 antigen on melanoma cells and to Fc receptors on NK cells leads to NK activation but not to direct tumor lysis (ADCC). Cytokine release by activated NK cells could elicit other effector cells, e.g., macrophages, to kill the tumor. We have not yet found evidence for this scenario because no IFN-γ release was detected when mAb TA99 was incubated with melanoma cells and host mononuclear cells. Another possibility is that traditional NK cells are not involved, but rather another NK1.1+ cell population, e.g., NK1.1+ T cells (21).

Depigmentation in Mice Treated with mAb TA99. We have previously shown that depigmentation occurs after injection of mAb TA99 administered over 2 weeks, but loss of pigmentation in hairs was only observed in regenerating hairs.
within depilated sites. When serum antibody levels against gp75 were maintained over a much longer period (2 months), patchy hair depigmentation appeared in nondepilated areas on four of four mice (Fig. 6). No other changes have been observed in these mice after 6 months of follow-up, including no changes in weight, behavior, or coat texture. These results show that hairs can be affected by TA99 treatment in areas that are not depilated and that there can be surface expression, at least in some circumstances (for instance, activated proliferating melanocytes). This provides a model for autoimmune vitiligo and further supports a relationship between immune recognition of self and tumor rejection. However, substantially less mAb TA99 was required for rejection of transplantable melanoma tumors than for depigmentation, suggesting that hair follicles remain a privileged immunological site relative to tumors.

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