Characterization of a Melanoma Antigen with a Mouse-specific Epitope Recognized by a Monoclonal Antibody with Antimetastatic Ability

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INTRODUCTION

Metastasis of tumor cells is known to be a complex phenomenon affected by a multitude of factors. These include loss of adhesiveness to extracellular matrix and changes of cell surface phenotypes that influence immune responses and allow tumor cells to escape from host defense mechanisms. Cell surface components are thought to play a major role also in the interaction of tumor cells with their environment. In fact, several investigators have suggested that changes in glycoproteins on the cell surface are important in cancer metastasis. In addition to the above findings, recent studies on inhibition of tumor metastasis by monoclonal antibodies against cell surface molecules also provide clear evidence for the importance of cell surface properties. Nicolson has shown that the expression of the corresponding antigen on tumor cells which was defined by monoclonal antibodies correlates with enhanced colonization of tumor cells. Moreover, Vollmers, Birchmeier, and co-workers have described monoclonal antibodies that interfere with adhesion of tumor cells to the extracellular matrix, such as fibronectin and laminin, and inhibit lung colonization of B16 melanoma.

Gunji and Taniguchi have also reported that a single injection of a syngeneic monoclonal antibody (M562) recognizing a mouse-specific melanoma antigenic epitope significantly reduced the numbers of lung metastatic colonies of B16 melanoma cells in vivo. Therefore, it is quite likely that the antigen recognized by the antibody might associate with some metastatic potential of B16 melanoma cells. Furthermore, Nozue et al. have demonstrated that B16 melanoma variant clones losing expression of the M562 and M622 antigens largely showed a reduced metastatic ability, correlating with a loss of adhesiveness to extracellular matrix.

Here, we describe the biochemical properties of a mouse melanoma membrane glycoprotein recognized by a monoclonal antibody, which might play an important role in lung metastasis of B16 melanoma cells.

MATERIALS AND METHODS

Monoclonal Antibodies. Three antimalanoma monoclonal antibodies (M562, M622, and M2590) were established from fusion of P3U1 cells with C57BL/6 spleen cells hyperimmunized with syngeneic B16 melanoma cells, and their properties have been described elsewhere (10). The isotype of these antibodies was IgM. M2590 was shown to recognize hematoxin (O6)-R-1, a monoclonal antibody (IgM) raised against a protease of hamster fibroblasts, and anti-Thy-1 (IgM) were used as controls for anti-mouse melanoma antibodies (M562 and M622).

Cell Lines and Their Culture. B16 melanoma cells were maintained in RPMI-1640 medium supplemented with 4% fetal calf serum. MEA-1, a subclone of B16, expresses melanoma antigen recognized by M562, M622, and M2590. A mutant clone, MEB-4, which does not express the antigen detected by either M562 or M622, was established by mutating B16 melanoma cells with N-nitro-N-nitrosoguanidine as described elsewhere.

Immunoprecipitation. Subcellular cell monolayers were cultured in the presence of [3H]glucosamine (10 ^Ci/ml) or [14C]glucosamine (3 ^Ci/ml) for 3 days or [35S]cysteine (50 ^Ci/ml) overnight. The radio-labeled cells were washed, harvested with EDTA, and solubilized by sonication in 50 mM Tris-HCl (pH 7.5) buffer containing 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 150 mM NaCl. Insoluble materials were removed by centrifugation at 105,000 x g for 30 min, and the lysate was used for immunoprecipitation. The conditioned medium of the labeled cells was precipitated with 70% ammonium sulfate. The precipitates were dissolved with water and dialyzed against 50 mM Tris-HCl (pH 7.4):150 mM NaCl buffer. NP-40 and deoxycholate were then added to make a final concentration of 0.5%. The cell lysate and the conditioned medium were incubated with purified antibodies (50 ^g/ml) overnight at 4°C. The antigen-antibody complexes were precipitated with anti-mouse IgG coupled to Sepharose (Zymed Lab., Inc.) and washed with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.5% NP-40. The precipitates were analyzed by SDS-PAGE under reducing conditions. The gel was processed for fluorography using Enlightening (New England Nuclear, Boston, MA).

Cell Surface Labeling and Affinity Purification. Cells were harvested with EDTA at subconfluence and iodinated using lactoperoxidase (0.2 mg/ml), [125I]iodide (200 ^Ci/10^7 cells; Na125I, New England Nuclear), and 0.06% H2O2 as described. Iodinated cells were washed 3 times with 10 mM KI in PBS. The cell lysates were incubated with antibody-coupled Sepharose overnight at 4°C. The antigen was eluted with 3 M NaSCN in PBS (pH 7.4) followed by SDS-PAGE.

Treatment of gp80 with Various Glycosidases. The antigen was treated with sialidase (0.1 unit/ml; Nakarai Chem., Ltd., Tokyo, Japan) in 50 mM sodium acetate buffer (pH 5.2), fucosidase (65 million units/ml) in 100 mM sodium citrate phosphate buffer (pH 6.5), or Endo H (200 million units/ml) in 150 mM citrate phosphate buffer (pH 5.0) containing 100 mM NaCl and 0.1% bovine serum albumin. To treat the antigen with Endo D (200 million units/ml) in 50 mM citrate phosphate buffer

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(pH 6.5), it was first incubated with endogalactosidase (200 milliunits/ml) in 5 mM sodium acetate buffer (pH 5.8). The glycosidases (Endo H and Endo D) were obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan, and Endo F was purchased from Boehringer Mannheim Yamanouchi Co., Tokyo, Japan.

**Immunofluorescent Staining.** Cells were inoculated on glass coverslips. At various time intervals after inoculation, cells were fixed with 10% formalin and stained with either M562 or M622 as described previously (13).

**Antibody-binding Inhibition Assay.** Melanoma cells (2 × 10^5) were incubated with various doses of nonlabeled monoclonal antibodies at 4°C for 1 h, washed 3 times with PBS, and then incubated with 50 μl of ^[35]S-labeled monoclonal antibodies (100 μg/ml; specific activities of M562, M622, and M2590 were 1146, 606, and 957 cpm/ng, respectively) at 4°C for 1 h. The radioactivity was counted by an autogamma counter (ARC500; Aloca Co., Tokyo, Japan).

**RESULTS**

**Immunoprecipitation**

A B16 melanoma subclone (MEA-1) was labeled with glucosamine and immunoprecipitated by monoclonal antibodies. As shown in Fig. 1A, both M562 and M622 specifically precipitated a glycoprotein with a molecular weight of 80,000 (gp80) from both cell lysates (Fig. 1A, Lanes 2 and 3, respectively) and conditioned medium (Fig. 1A, Lanes 5 and 6). The control antibody did not precipitate gp80 (Fig. 1A, Lanes 4 and 7). The same antigen was also immunoprecipitated from the parent B16 melanoma cells labeled with either [H]glucosamine or [35S]cysteine (data not shown). Moreover, both M562 and M622 also precipitated gp80 from the surface of iodinated MEA-1 cells (Fig. 1B, Lanes 1 and 2, respectively). This suggests that the gp80 is indeed expressed on the outer surface of the membrane.

As shown in Fig. 2, gp80 was not precipitated from MEB-4, a mutant subclone, which was negative for reactivity toward both M562 and M622 antibodies. When NIH 3T3 or L-cells, negative for M562 or M622 reactivity, were labeled with [3H]glucosamine under the same conditions as described above, gp80 was not precipitated by these antibodies (data not shown).

**Effect of Tunicamycin on Antigen Expression**

Study with Immunoprecipitation. When the cells were labeled with [14C]glucosamine in the presence of TM (2 μg/ml), no radioactive molecule was specifically precipitated by the antibodies (Fig. 3A, Lanes 2 and 3). The same result was obtained when the immunoprecipitation was carried out using externally labeled cells that had been treated with TM (Fig. 3B, Lane 2). Meanwhile, when cells were labeled with [35S]cysteine in the presence of TM, a M, 69,000 molecule was precipitated by M562 and M622 (Fig. 3C, Lanes 4 and 5), but not by the control antibody (Lane 6) instead of gp80 precipitated from TM-nontreated control cells (Fig. 3C, Lanes 1 and 2).

Study with Immunostaining. The antigen recognized by M622 and M562 antibodies was found to be resistant to trypsin treatment (data not shown). We therefore treated cells with 0.02% Pronase for 2 min at room temperature to remove the antigenic determinant from the cell surface. Cells were then cultivated in the presence or absence of TM (2 μg/ml). Cells cultured in the absence of TM became stainable with both M562 and M622 within 1.5 h after the treatment. The reactivity of the cells with the antibodies increased thereafter and reached a normal level by 4 h after Pronase digestion (Fig. 4, A to D). On the other hand, when cells cultured in the presence of TM did not spread out their cytoplasm even at 24 h after inoculation and under these conditions, no cells became significantly reactive with M562 and M622 by this time, if TM-treated cells were made permeable by treatment with 0.2% Triton X-100, a slight increase in reactivity of the cells with M562 was observed (data not shown).

**Biochemical Characterization of the Antigen**

From the results that no specific molecule was immunoprecipitated from the cells labeled with radioactive glucosamine in the presence of TM and also that TM-treated cells showed very weak reactivity with either M562 or M622, we expect that the gp80 contains asparagine-linked oligosaccharides. To investigate what kinds of oligosaccharides were linked to the peptide, various glycosidases were tested for their abilities to remove the carbohydrate chains. As shown in Fig. 5, treatment with Endo H and Endo D did not change the mobility of the molecule on SDS-PAGE. By the treatment with sialidase, the antigen lost approximately 8,000 daltons. The experiments using [3H]fucose-labeled materials demonstrate that the antigen has fucose in the carbohydrate moiety (data not shown). However, fucosidase did not cause the detectable decrease of the molecular weight of the antigen on the SDS-PAGE.

**Fig. 1. Immunoprecipitation of melanoma antigen from radiolabeled B16 melanoma (MEA-1) cells. In A, extracts of ^[14C]GlcnAC-labeled MEA-1 cells (Lanes 1 to 4) or conditioned medium of MEA-1 cells (Lanes 5 to 8) were immunoprecipitated with M622 (Lanes 2 and 5), M562 (Lanes 3 and 6), or control IgM antibody (Lanes 4 and 7) as described in "Materials and Methods." Immune complexes were collected by centrifugation, solubilized with the gel sample buffer (15), and subjected to SDS-polyacrylamide gel (10%) electrophoresis. Lane 1, cell lysate; Lane 8, concentrated conditioned medium. The ^[14C]-labeled proteins were detected by fluorography. In B, solubilized ^[35]S-labeled MEA-1 cells were incubated with M622 (Lane 1), M562 (Lane 2), or control IgM antibody (Lane 3) without antibody (Lane 4). Immune complexes (Lanes 1 to 3) and cell lysate (Lane 4) were analyzed on SDS-polyacrylamide gel (10%) electrophoresis. ^[35]S-labeled proteins were made visible by autoradiography."
Since the antigen contains sialic acid and glucosamine in the oligosaccharide chains, we tested if the molecule binds to WGA. The antigen was first purified with a M622 antibody-conjugated Sepharose column and then applied to a WGA column (Honen Oil Co., Tokyo, Japan). The gp80 was detected in the eluate from the lectin column with 0.2 M N-acetyl-glucosamine (Fig. 5B). The mobility of the molecule on SDS-PAGE under reducing and nonreducing conditions is shown in Fig. 5C. The molecular weight of the antigen did not significantly change before and after reduction.

Inhibition of Antibody Binding with Monoclonal Antibodies

In order to investigate topographical localization of the mouse melanoma epitopes recognized by two antibodies (M562 and M622), melanoma cells were first incubated with one of the unlabeled monoclonal antibodies to block one epitope, followed by an incubation with radiolabeled antibodies which are either identical to or different from the first antibody. As shown in Fig. 6, the binding of the antibodies was mutually inhibited to the same extent as is caused by the same antibody. This binding inhibition assay was specific, because M2590 recognizing the carbohydrate moiety of GM1 did not affect any binding of M562 or M622 antibody.

DISCUSSION

We have identified and characterized a mouse melanoma-specific antigen recognized by syngeneic monoclonal antibodies, M562 and M622, obtained by fusion of P3U1 and C57BL/6 spleen cells primed with syngeneic B16 mouse melanoma (10). The melanoma specificity of these antibodies has been characterized and was found to be specific for mouse melanoma cells (10). The antibodies react only with mouse melanomas, but not with other tumors, including neuroblastoma, fibrosarcomas, T-cell and B-cell lymphomas, etc., of mouse and human origin. Moreover, the antibody activities of M562 and M622 were not absorbed with high doses of various normal tissues, including brain, skin, and eye, with normal melanocytes.
MOUSE MELANOMA ANTIGEN

With respect to the functional activity of the monoclonal antibody, M562 possesses the ability to block experimental lung metastasis of melanoma (8) and partially but significantly to inhibit anti-melanoma cytotoxic T-lymphocyte activity in the effector phase (14). These results indicate that the M562 melanoma epitope is important in tumor immune responses.

Fig. 4. Effect of TM on expression of the melanoma antigen. B16 melanoma (MEA-1) cells were treated with Pronase and incubated on glass overslips as described in “Results.” At the times indicated after incubation in the presence (E to H) or absence (A to D) of TM, cells were fixed and immunostained with M562 as described in “Materials and Methods.”
MOUSE MELANOMA ANTIGEN

Fig. 5. Characterization of the melanoma antigen. A, glycosidase digestion of the antigen. The melanoma antigen labeled with \(^{3}H\)GlcNAc was isolated by M622-conjugated Sepharose and was untreated (Lane 1) or treated with sialidase (Lane 2), fucosidase (Lane 3), Endo H (Lane 4), and Endo D (Lane 5). Electrophoresis on a SDS-polyacrylamide gel (10%) was followed by fluorography. B, binding of the antigen to WGA. \(^{3}H\)GlcNAc-labeled antigen purified by M622 antibody column was applied on a WGA-agarose column. It was eluted with 0.2 M N-acetyl-glucosamine in PBS. The eluate was analyzed on SDS-PAGE (10%). C, effect of 2-mercaptoethanol on the mobility of the antigen. Immunoprecipitates of \(^{3}H\)GlcNAc-labeled antigen were electrophoresed under nonreducing (Lane 1) and reducing conditions (Lane 2).

The molecule recognized by these antibodies is a membrane glycoprotein (gp80), since the same molecule can be labeled with radioactive cysteine, glucosamine, and fucose, and by surface iodination with \(^{125}\)I (Fig. 1). As the antibody precipitated a \(^{35}\)S-cysteine-labeled M, 69,000 molecule from TM-treated B16 melanoma cells (Fig. 3), this represents the size of the core protein. Therefore, about 14% of the molecular weight appears to consist of carbohydrates. In our previous studies, we found that M562 reacted with several proteins with different molecular weights including gp80 (9). In the present data we can clearly demonstrate that the epitope is on gp80.

The results obtained from the TM treatment experiment (Figs. 3 and 4) also show that the sugar moiety is important for transport of the gp80 to the cell surface and for the conformation of the melanoma epitope, because the majority of antibody reactivity to the cells was greatly reduced after TM treatment. Only a faint M, 69,000 band was detected by immunoprecipitation from TM-treated cells (Fig. 3), while no immunostaining was detected (Fig. 4). The discrepancy between immunostaining and immunoprecipitation analyses seems to be due to the sensitivity of these different methods used. Conformational changes of the molecule by removal of the carbohydrate moiety are more likely to explain the decrease of antibody reactivity after TM treatment. Change in molecular conformation of the antigen might also occur after its release from cells, since antibody M562 effectively precipitated antigen from cell lysate but barely precipitated antigen released into the medium.

The metabolic rate of this molecule seems to be rather high, since the results of Pronase treatment demonstrated that the reexpression of the molecule occurred within 1.5 h and the level of the expression became normal 4 h after Pronase treatment.

A similar melanoma antigen recognized by a syngeneic monoclonal antibody with antimitastatic activity has been reported by Vollmers et al. (4–7). They reported that their antibody detected the membrane glycoprotein with a molecular weight of 83,000. However, the molecular weight of their melanoma antigen was estimated to be 105,000 under nonreduced conditions, and the core protein had a molecular weight of 72,000 which was estimated after endoglycosidase F treatment (7). Therefore, the glycoprotein does not seem to be identical to the gp80 described in this report.

Concerning the epitopes recognized by the antibodies, we cannot obtain a definite conclusion from the data shown here of whether both or either one of them recognizes carbohydrate moieties or peptide determinants. Even if the antibodies recognize the peptide epitopes, the carbohydrate seems to be largely responsible for the conformation of the epitope and may build up the tertiary structure of the core protein architecture.

Experiments using these two monoclonal antibodies on inhibition of antibody competition binding suggest that these two determinants may be topographically closely located to each other because one antibody (M562 or M622) competes and interferes with the binding of the other. However, we have previously demonstrated that M562 but not M622 antibody does block experimental lung metastasis of B16 melanoma in vivo (8). This suggests that M562 and M622 epitopes are different from each other and also that only the M562 epitope is a functional site and is responsible for lung metastasis. It is thus quite important to determine the primary sequence of the
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M562 epitope of the melanoma antigen in order to understand the molecular basis of lung metastasis of melanoma cells. This work is now in progress.

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