Identification of Melanoma-associated Antigens Using Fixed Tissue Screening of Antibodies

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ABSTRACT

Early culture supernatants from hybridomas that were obtained through fusions of mouse myeloma cells with lymphocytes of melanoma-immunized mice were screened for their reactivity with a paraffin-embedded cell block of a melanoma cell line, using a biotin:avidin immunoperoxidase procedure. Eleven monoclonal antibodies were derived that define several new melanoma-associated antigens. The antigens include a neutral glycolipid, gangliosides, membrane-associated proteins, cytosolic proteins, and strongly secreted proteins. These antibodies, which detect antigens that withstand tissue fixation and embedding procedures, were tested for reactivity in fixed cell lines, as well as in melanoma biopsies. These antibodies may provide powerful tools in diagnostic studies of human malignant melanoma biopsy material.

INTRODUCTION

In this paper, we described the production of hybridomas through fusion of mouse myeloma cells with splenocytes of mice that were immunized with human melanoma cells. Eleven MAbs secreted by these hybridomas were screened for their ability to bind antigen(s) expressed by melanoma cells after fixation and paraffin embedding of tumor tissue. A major goal of such studies is to produce antibodies with diagnostic usefulness. Antigens that bind MAbs in radioimmunonassay or in mixed hemagglutination assay often do not withstand routine tissue fixation and embedding procedures (3, 4, 7, 9, 11-13, 16, 17, 20), or the antibody demonstrates reactivity with normal skin components (6, 8), thereby obviating the advantage of using MAbs in immunohisto-pathological procedures. We describe here the derivation of 11 MAbs against melanoma-associated antigens which can detect antigens retained in fixed melanoma tissue and which delineate melanoma cells from normal skin components.

MATERIALS AND METHODS

Cell Lines and Production of Hybridomas. Human malignant melanoma cell lines were established and grown as described previously (16). Cells of these lines were used for primary i.p. immunization of mice, followed 4 to 11 weeks later by an i.v. booster injection as described previously (9, 12, 16). Details of fusion techniques have been described previously (9, 12). Malignant melanoma cell lines were used for immunization in all cases, except for one case in which a crude melanosome preparation was used (Experiment 149). Melanoma SK Mel 23 cells were lysed by incubation in distilled water, the nuclei were removed by centrifugation at 800 × g, and the supernatant was layered on a 4-step sucrose gradient. This preparation was centrifuged at 20,000 × g, and material from the pigmented layer containing the crude melanosomes was used for immunization of mice.

Screening of Antibodies. Following centrifugation, the supernatant was assayed for binding to cells of either a colon carcinoma line or a normal skin section. If a positive reaction was obtained in this assay (Fig. 2), the antibody binding to melanoma cells. Those supernatants that maintained a strong positive reactivity (+ +) with a melanoma cell line (Fig. 1) were assayed for binding to cells of either a colon carcinoma line or a normal skin section. If a positive reaction was obtained in this assay (Fig. 2), the hybridoma fusion product was cloned by limiting dilution during initial preparation of the hybridoma supernatants. The supernatants were then assayed for binding in an IP assay to a section of a fixed melanoma cell line. Alternatively, the hybridoma fusion product was cloned by limiting dilution during initial growth, and the progeny of the clones were tested for secretion of antibody binding to melanoma cells. Those supernatants that maintained a strong positive reactivity (+ +) with a melanoma cell line (Fig. 1) were assayed for binding to cells of either a colon carcinoma line or a normal skin section. If a positive reaction was obtained in this assay (Fig. 2), the antibody was discarded; if a negative or weakly positive reaction was
obtained, those hybridomas not cloned initially were cloned by limiting dilution. Progeny cultures were restested after further growth, and all supernatants that remained strongly positive after restest of clones were tested on a fixed tissue panel composed of normal skin, 10 fixed melanoma cell lines, 5 cases of SSM of the skin, and 8 cases of melanoma metastatic to lymph nodes, s.c. tissue, or lung.

**Biochemical Analysis of Antigens.** About 7 × 10⁷ WM 164 melanoma cells in a plastic flask (75 sq cm) were placed in methionine-free medium for 30 min, washed twice with Dulbecco’s PBS, and incubated for 2 days with fresh methionine-free medium containing 100 μCi of [³⁵S]methionine. The media, containing molecules shed by the cells in culture, were removed and used in immunoprecipitation. The cells were then incubated for 10 min in 5 ml of Versene to release them from the flask surface. The cells were pelleted at 200 × g, washed with PBS, resuspended (about 0.2 ml) in 1.0 ml of swelling buffer (1 mM MgCl₂: 1 mM KCl: 10 mM Tris, pH 8.1), and quickly frozen in a dry ice:acetone slurry (19). The cell suspension was thawed and centrifuged at 130,000 × g for 1 hr at 4°C. The supernatant contained the soluble cytosolic fraction, and it was retained. The pellet was then solubilized in 4 ml of ice-cold solubilizing buffer [0.5% Nonidet P-40:140 mM NaCl:10 mM NaF:10 mM Tris:5 mM EDTA: aprotinin (100 Kallikrein IU/ml):1 mM phenylmethylsulfonyl fluoride, pH 7.5]. After 30 min, the solubilized fraction was centrifuged at 130,000 × g or 1 hr at 4°C to remove nuclei and insoluble material. The supernatant was retained and referred to as the membrane fraction. These three fractions, the retained media, the soluble cytosolic constituents, and the membranes were then used in our standard immunoprecipitation procedure (14, 15). Glycolipid extract from WM 9 melanoma cell line was prepared and subjected to Folch partition (5). The upper phase material was concentrated to dryness and dissolved in chloroform:methanol:ether (30:60:8), and 2 fractions, neutral glycolipid and ganglioside, were obtained by chromatography in DEAE-Sephadex A-25 (acetate form) (21). Solid-phase radioimmunossay was performed with this fraction as described previously (2).

**RESULTS**

Twenty-eight fusions between mouse myeloma cells and spleenocytes obtained from mice that had been immunized with melanoma resulted in the production of 2501 hybridomas. Of these, 382 (14.4%) secreted antibodies binding to cells of melanoma culture line which had been fixed and embedded; 222 (61%) of these hybridomas secreted antibody that also bound to normal skin or cells of colorectal carcinoma and were therefore discarded. Some hybridoma colonies were then expanded and restested. Of 92 colonies handled in this fashion, only 28 supernatants retained reactivity, and these were cloned by limiting dilution. Of the 39 colonies cloned immediately, 27 supernatants retained reactivity on fixed tissue sections, and this reactivity was studied using a panel of fixed cell lines and melanoma biopsies of different stages.

The results of the binding of 11 of these MAbs are seen in Table 1. These 11 MAbs were from 5 different fusions. All reacted with cells of many fixed melanoma lines and at least one skin biopsy of SSM. ME425 reacted with 9 of 10 cell lines and 4 of 4 SSM but did not react with any of 6 metastatic melanomas. All of the MAbs demonstrated weak reactivity with normal structures, but the staining of melanomas was always quite strong, and it could be differentiated from the background. Antibodies ME491, ME492, and ME493 gave identical tissue staining patterns, with 491 and 492 showing the strongest IP reaction. These antibodies were positive in all SSM tested, and they define individual invasive and noninvasive malignant melanoma cells in tissue sections. Although these MAbs also stain normal macrophages in the skin and other sites but no other skin component, macrophages are easily distinguishable from melanoma because of their smaller size and their presence as single cells; their presence does not interfere with the diagnostic interpretation. Melanophages did not detectably react with these antibodies.

Antibody ME109 also differentiates melanoma cells in SSM (Fig. 3) and in one case of metastatic melanoma (Fig. 4) from the occasional low background staining of squamous epithelium, lymphocytes, and cells in sweat glands. No reactivity is seen in the metastatic melanoma stained with the P3 control antibody (Fig. 5).

The characteristics of the melanoma-associated antigens defined by the antibodies are presented in Table 2. All but one of the antigens were shed by melanoma cells maintained in serum-free medium, and 7 of the 11 were present on the cell surface. ME491, ME492, and ME493 antibodies each immunoprecipitated a heterogeneous protein with a molecular weight of 30,000 to 60,000 which was found in the membrane fraction and in smaller quantities in the culture supernatant. By contrast, ME165 immunoprecipitates 2 antigens, a M, 200,000 antigen detected in the cytosol and membrane fractions of melanoma cells and a M, 27,000 antigen in the culture supernatant. No antigens were identified for ME425 and ME510. Two of the antigens detected by antibodies ME416 and ME515 represent gangliosides, and ME325 detects a neutral lipid antigen.

**DISCUSSION**

Most investigators have screened supernatants from hybridoma colonies by radioimmunoassay or enzyme-linked immunoabsorbent assay procedures against a variety of targets, including whole live cells, KCl extracts, antigens from cell membrane and subcellular components, and antigens secreted into tissue culture medium (3, 4, 7–9, 11–13, 16, 17, 20). We had previously tested over 100 antibodies with antimeanoma specificity obtained by these methods and had not found an antibody that reacted strongly to delineate melanoma cells from surrounding cells in fixed tissue sections.
In an effort to select an antibody that would be useful in immunohistopathologic diagnosis of melanoma, we screened supernatants of early hybridomas on a fixed cell pellet of a melanoma cell line. This screening plan was successful and has led to the detection of at least 7 new melanoma-associated antigens (Table 2). Three of the MAbs selected from 2 fusions detected the same antigen, and 4 MAbs did not immunoprecipitate extracts of the cells. Two of the MAbs detected gangliosides, and one recognized a carbohydrate structure present on both glycoprotein and neutral lipid. Several of the MAbs obtained are highly promising for their potential diagnostic use in tissue, since they react quite strongly with most SSM and are only weakly reactive with normal components, as detected in the IP assay. The cross-reactivity of 61% of our antibodies is detected, whereas reactivity with cytoplasmic components is only antigen on the surface membrane

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Antigen type</th>
<th>Molecular weight</th>
<th>Fraction present in:</th>
<th>Present on surface</th>
<th>Present in serum-free medium</th>
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</thead>
<tbody>
<tr>
<td>ME109</td>
<td>IgG2a</td>
<td>Protein</td>
<td>92,000, 70,000</td>
<td>Membranes</td>
<td>±</td>
<td>+</td>
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<tr>
<td>ME121</td>
<td>IgG2a</td>
<td>Protein</td>
<td>80,000, 60,000</td>
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<td>+</td>
</tr>
<tr>
<td>ME165</td>
<td>IgG3</td>
<td>Protein</td>
<td>200,000, 160,000</td>
<td>Membranes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ME325</td>
<td>IgM</td>
<td>Protein and neutral lipid</td>
<td>110,000</td>
<td>Membranes and supernatant</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>ME416</td>
<td>IgM</td>
<td>Ganglioside</td>
<td>80,000, 60,000</td>
<td>Membranes and supernatant</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ME425</td>
<td>IgM</td>
<td>Unknown</td>
<td>80,000, 60,000</td>
<td>Membranes and supernatant</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ME491</td>
<td>IgM</td>
<td>Protein</td>
<td>Broad band: 30,000-60,000</td>
<td>Membranes and supernatant</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ME492</td>
<td>IgG</td>
<td>Protein</td>
<td>Broad band: 30,000-60,000</td>
<td>Membranes and supernatant</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ME493</td>
<td>IgM</td>
<td>Protein</td>
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<td>Membranes and supernatant</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ME510</td>
<td>IgM</td>
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<tr>
<td>ME515</td>
<td>IgM</td>
<td>Ganglioside</td>
<td>80,000, 60,000</td>
<td>Membranes and supernatant</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

*<sup>1</sup> Identified using [35Se]selenomethionine-labeled cells.
*<sup>2</sup> Identified using [125I]lactoperoxidase-labeled cells.

The antibodies detected by this technique appear to recognize antigenic determinants which are not similar to melanoma-associated antigens that have been described previously. The molecular weights of these antigens are different, and the pattern of strong antibody reactivity with fixed tissue in IP assay has not been seen with other MAbs to antigens of melanoma (8). None of these MAbs appear to detect HLA determinants either, since the ME165 antigen is particularly interesting with regard to the latter, since the M<sub>2</sub>, 200,000 antigen was found in both cytosol and membranes, but a M<sub>2</sub>, 27,000 antigen was found only in MAb supernatant. A nuclear fraction of melanoma cells was not tested.

Although antibodies that reacted with normal tissue or colon carcinoma cells were eliminated between the cloning stage and development of the final panel, the cross-reactivity with normal tissue was not as apparent then as it was after cloning and selection of the antibody.

The entire panel of MAb obtained by this screening procedure will now be extended, and studies will be conducted comparing antigen expression in different types of melanoma and during stages of melanoma progression from early invasion to deep invasion to metastasis. This method of screening could be applied to hybridomas secreting MAb to other tumors and might result in the selection of MAbs with definitive diagnostic potential.

**REFERENCES**

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11. Johnson, J., Dammer-Deckmann, M., Meo, T., Hadam, M. R., and Ruthmüller, G. Surface antigens of human melanoma cells defined by monoclonal antibod-
Fig. 1. Section of a fixed cell block of the human malignant amelanotic melanoma cell line WM 9 stained with MAb ME491. This reaction was used as the initial criterion to select hybridoma colonies for retesting. The pattern of strong reactivity of some cells and either weak or no reactivity of other cells was most often seen with antibodies that showed the least cross-reactivity with normal cells (IP reaction counterstained with hematoxylin, x 640).

Fig. 2. Intense staining of normal skin squamous epithelium by MAb, which was therefore excluded from further study (IP reaction counterstained with hematoxylin, x 640).

Fig. 3. Section of SSM with strong reactivity using antibody ME109 in IP assay. Individual malignant cells (dark staining) can be seen infiltrating within the squamous epithelium at the top and in the dermis below. The very dark cells at the bottom left contain melanin (IP reaction counterstained with hematoxylin, x 256).

Fig. 4. Section of metastatic melanoma stained with ME109 to demonstrate some malignant cells with strong reactivity and others (bottom of field) with no reactivity (IP reaction counterstained with hematoxylin, x 256).

Fig. 5. Same section as that seen in Fig. 4, stained with control antibody P3 to demonstrate absence of melanin in this field (IP reaction counterstained with hematoxylin, x 256).
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