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

Barbara Atkinson, Carolyn S. Ernst, Barbara F. D. Ghrist, Meenhard Herlyn, Magdalena Blaszczyk, Alonzo H. Ross, Dorothy Herlyn, Zenon Steplewski, and Hilary Koprowski


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 defined several new melanoma-associated antigens. The antibodies 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 MAbs3 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 radiimmunoassay 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 (8, 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.

Immunoglobulin isotypes were determined by Ouchterlony immunodiffusion and by indirect radiommunoassay (9).

Specimens and IP Technique. Monolayer cultures were trypsinized, and the cell suspensions were centrifuged and resuspended in Bouin’s fixative. After 1 to 2 hr in fixative, the cells were centrifuged at 3000 rpm for 10 min, and the cell pellet was gently scraped from the tube with a spatula, wrapped in filter paper, and dehydrated and embedded in paraffin by routine procedures. Paraffin blocks from selected specimens of malignant melanoma and normal skin which were fixed in either Bouin’s fixative or 10% neutral buffered formalin were identified from the diagnostic files of surgical pathology or dermatopathology at the Hospital of the University of Pennsylvania, Philadelphia, PA. IP studies were performed on 5-μm sections with a biotin:avidin kit (Vector Laboratories, Burlingame, CA), using modifications of the method of Hsu and Raine (10). Slides were deparaffinized, hydrated, and washed for 5 min in running water. Endogenous peroxidase was inhibited by treatment with 0.3% H2O2 in absolute methanol for 15 min (1). Successive antibody incubations were performed at room temperature using 10% normal horse serum in PBS with 0.10% bovine serum albumin (10 min), tissue culture supernatant containing mouse MAb (30 min), biotin-labeled horse anti-mouse immunoglobulin (1:200, 30 min), and avidin:biotin complex (10 min). Slides were treated for 5 min with a solution of 0.06 M DAB in PBS containing 0.01% H2O2, counterstained with hematoxylin, washed for 2 min, and mounted directly in aqueous medium (18).

Slides were scored as “++” if a strong, dark brownish-black color (DAB) or dark red (AEC) was present, “+” if a dark brown color (DAB) or pinkish red (AEC) was present, and “−” if the reaction was similar to or only slightly greater than the control.

Screening Technique. Cultures of hybridomas were grown in 24-well plates, and colonies were picked and placed in 96-well plates after approximately 2 week of growth. After an additional week of growth, the colony supernatants were harvested, diluted 1:4 in PBS, and tested for binding in 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 $7 \times 10^7$ 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 $\mu$Ci of $[^{35}S]$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 x g, washed with PBS, resuspended (about 0.2 ml) in 1.0 ml of swelling buffer (1 mM MgCl$_2$: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 x 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 x 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 radioimmunoassay was performed with this fraction as described previously.

**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 hydridomas. 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 react detectably 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 immunosorbent assay procedures against a variety of targets, including whole live cells, KCI 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 antimelanoma 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.

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<th>Table 1</th>
<th>IP tissue reactivity of selected MAbs</th>
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<td>Reactivity (antibody-positive cases/total no. of cases tested)</td>
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<td>Anti-body</td>
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<td>ME121</td>
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* Reactivity of all antibodies was weak.
In an effort to select an antibody that would be useful in immuno-histopathologic 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. Although radioimmunoassay is more sensitive in detecting low-level reactivity, only antigen on the surface membrane is detected, whereas reactivity with cytoplasmic components is broader than that usually obtained by screening in live cell radioimmunoassay. Although radioimmunoassay is more sensitive in detecting low-level reactivity, only antigen on the surface membrane is detected, whereas reactivity with cytoplasmic components is also observed in IP. The cross-reactivity of 61% of our antibodies with other tumors or normal tissue probably derives from the detection of ubiquitous cell components released from the cytoplasm.

Despite the selection in IP for a cytoplasmic distribution of antigen, 7 of 11 antibodies still showed cell surface reactivity in live cell radioimmunoassay, and the antigens defined by 5 of the antibodies (3 antibodies to the same antigen) were immunoprecipitated from surface membrane fractions. The fixed tissue IP assay is relatively insensitive to the surface membrane, because the cut surface of the cell membrane is small relative to the internal cytoplasmic compartment. The fixation process itself may also alter some antigens and may mask other antigens so that this assay detects a different variety of antigens. Immunoprecipitation of metabolically labeled cell fractions permitted identification of the tumor-associated antigens and the assignment of the antigen to one or more cell fractions. The ME165 antigen is particularly interesting with regard to the latter, since the M, 200,000 antigen was found in both cytosol and membranes, but a M, 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 MAbs 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.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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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