Mouse Monoclonal Antibody (FKH1) Detecting Human Melanoma-associated Antigens

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ABSTRACT

A mouse monoclonal antibody, FKH1, was produced to detect cytoplasmic melanoma-associated antigen. FKH1 was raised using cultured human melanoma cell line KHm-6 as an immunogen. Reactivity of this antibody was assessed by immunohistochemical techniques against cell lines and normal and neoplastic tissues. Positive reactions were seen against 5 human melanoma cell lines. It stained cytoplasm of melanoma cells in a diffuse and granular pattern in indirect immunofluorescence. Immunoelectron microscopy showed diffuse distribution of immunoreactive in the cytoplasm of KHm-1 cells excluding melanosomes and other organelles. Reactivity against frozen and alcohol-fixed, paraform-embedded melanocytic tumors was also tested with IIF or indirect or avidin biotinylated horseradish peroxidase complex immunoperoxidase techniques. All cases of frozen sections from benign and malignant melanocytic tumors showed positive staining with FKH1. In fixed tissues, however, reactivity was 11 of 14 (79%) in malignant melanoma and 28 of 42 (67%) in other melanocytic tumors. FKH1 did not react against normal melanocytes and nonmelanocytic tumors except APUDoma and 2 glioblastoma cell lines. It failed to stain the B-16 mouse melanoma cell line, neuroblastoma cell line, breast carcinoma cell line, and T-cell lymphoma cell line. Normal human peripheral nerves were nonreactive with FKH1.

In immunoelectroblot study, FKH1 bound with proteins having molecular weight of 71,000 and 55,000 extracted from KHm-6 cells. It was suggested that FKH1 is a useful monoclonal antibody in diagnostic study of human malignant melanoma specimens.

INTRODUCTION

Monoclonal antibodies have been used to identify and characterize tumor-associated antigens. A malignant melanoma is a tumor in which extensive work has been done with monoclonal antibodies. A number of monoclonal antibodies against cell surface MAA, with varying degrees of specificity, have been produced (1-18) but only a few monoclonal antibodies detecting cytoplasmic MAA have been reported (19-22). Most of those membrane MAA are denatured by conventional histological fixation and an embedding procedure, a major disadvantage when used in immunopathological diagnosis. We describe the production and characterization of a monoclonal antibody which can detect cytoplasmic MAA apparently preserved in fixed melanoma cells.

MATERIALS AND METHODS

Cell Lines. Cultured human melanoma cell lines KHm-1, KHm-3, and KHm-6 were established as described previously (23). Human melanoma cell lines A375 and A101D, mouse melanoma cell line B-16, glioblastoma cell lines A-382 and A-172, neuroblastoma cell line IMR-32, breast carcinoma cell lines MCF-7 and BT-20, and T-cell lymphoma cell line MOLT3 were obtained from the Cell Culture Laboratory, Children’s Hospital of Michigan, Detroit, MI (Table 1). Cell lines were grown in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. Mouse SP2/0-Ag-14 (SP2/0) myeloma cells were grown in RPMI 1640 medium containing 10% fetal bovine serum.

Establishment of Hybridomas. SP2/0 cells were fused with spleen cells from a BALB/c mouse that had been immunized with two inoculations of homogenized KHm-6 melanoma cells according to the method of Köhler and Milstein (24). Hybrid cells were grown in hypoxanthine aminopterine thymidine medium and the supernatant was screened for reactivity against KHm-6 cells by indirect enzyme-linked immunosorbent assay using the BRL kit (Bethesda Research Laboratories Inc., Gaithersburg, MD). These hybridomas were then cloned by the limiting dilution technique to obtain a single-cell clone (25). Selected clones were expanded in tissue culture and peritoneal cavities of mice primed with 0.5 ml pristane (Sigma Chemical Co., St. Louis, MO). Spent culture media or ascitic fluid were used as the source of antibody.

Preparation of Melanoma and Fibroblast Protein Extract. Extracts of KHm-6 cells were prepared by lysing cells in 1% (w/v) deoxycholate/1% (v/v) Nonidet P-40/1 mM phenylmethylsulfonyl fluoride/7 mM NaCl/1 mM MgCl2/100 mM NaF/7 mM Tris buffer, pH 7.4 (26). The lysates were centrifuged at 4°C for 10 min at 7000 x g to sediment nuclei. The supernatants were dialyzed against distilled water and stored at −20°C after lyophilization. Dermal fibroblasts cultured under the identical condition were lysed in a similar fashion and the extracts were prepared for the control studies.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting. The extracts were separated on 10% sodium dodecyl sulfate gel (27) and transferred electrophoretically to nitrocellulose paper (28). The nitrocellulose paper was incubated with diluted culture supernatant of FKH1 and immunoperoxidase staining was done by using the ABC kit (Vector Laboratories Inc., Burlingame, CA). 4-Chlor-1-naphthol (Sigma Chemical Co., St. Louis, MO) was used as a chromogen. In the control studies monoclonal antibodies against keratin (EKH4, AN3) (29, 30), elastic fiber (NKH1), myelin basic proteins (P-2, P-24) (31), and neurofilament (AHNF1) (31) were used instead of FKH1.

Immunoelectron Microscopy. KHm-1 melanoma cells (2 x 10^6) were inoculated s.c. into BALB/c athymic nude mice. After 10 weeks a mouse was killed and tumors were used for immunoelectron microscopic examination. A tumor was fixed in periodate-lysine-parafomaldehyde solution and processed by the method of McLean and Nakane (32). Frozen sections (10-12 μm) were placed on albumin-coated glass slides and stained with FKH1 by indirect immunoperoxidase technique. Sections were postfixed with 1% OsO4 and embedded in Araldite (R. P. Cargille Lab. Inc., Cedar Grove, NJ).

Indirect Immunofluorescence. Fresh specimens of malignant melanoma, other types of benign and malignant tumors, and normal skin (20 white and 50 black skin specimens) were obtained from surgical patients (Tables 2 and 3). A portion of each specimen was snap-frozen in liquid nitrogen, while the remainder was fixed with 70% ethanol, embedded in paraffin, and used for routine histopathological diagnosis and FKH1 testing.

Cryostat sections were cut at 4-5 μm and stained with IIF without fixation.
Cell lines were grown on tissue culture chamber slides (Miles Scientific, Naperville, IL) for 2–3 days and tested. Supernatant medium from cultures of SP2/0 was used as negative control.

Indirect and ABC Immunoperoxidase Staining of Tissue Sections. Indirect or ABC immunoperoxidase technique was used to determine the reactivity of FKHI against frozen tissues and alcohol-fixed paraffin-embedded tissues. 70% ethanol-fixed paraffin-embedded sections were dewaxed in xylene and taken to 80% ethanol through graded alcohols. Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in 80% methanol at 4°C for 30 min. The peroxidase color reaction was developed with either 3,3′-diaminobenzidine tetrahydrochloride (Aldrich Chemical Co., Milwaukee, WI) or 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO), or 3-amino-9-ethylcarbazole. 

Immunoelectron Microscopy. The granular, electron-dense immunoreaction products were shown to distribute diffusely in the cytoplasm of the KHm-1 cell. There was no increase in electron density in the nucleus and mitochondria as well as in stage II and stage III melanosomes (Fig. 3). In other organelles as far as they were intact, no increased density over the control was observed. No cell surface membrane components were labeled. It was concluded that nonstructural cytoplasmic proteins are labeled with FKHI.

Table 1 Cell lines tested for binding with FKHI by IIF

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHm-1</td>
<td>Malignant melanoma</td>
<td>2+</td>
</tr>
<tr>
<td>KHm-3</td>
<td>Malignant melanoma</td>
<td>2+</td>
</tr>
<tr>
<td>KHm-6</td>
<td>Malignant melanoma</td>
<td>2+</td>
</tr>
<tr>
<td>A375</td>
<td>Malignant melanoma</td>
<td>2+</td>
</tr>
<tr>
<td>A101D</td>
<td>Malignant melanoma</td>
<td>2+</td>
</tr>
<tr>
<td>B-16 (malignant melanoma (murine))</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>A382</td>
<td>Glioblastoma</td>
<td>1+</td>
</tr>
<tr>
<td>A172</td>
<td>Glioblastoma</td>
<td>1+</td>
</tr>
<tr>
<td>IMR-32</td>
<td>Neuroblastoma</td>
<td>–</td>
</tr>
<tr>
<td>MCP-7</td>
<td>Breast carcinoma</td>
<td>–</td>
</tr>
<tr>
<td>BT-20</td>
<td>Breast carcinoma</td>
<td>–</td>
</tr>
<tr>
<td>MOLT3</td>
<td>T-cell lymphoma</td>
<td>–</td>
</tr>
</tbody>
</table>

* Intensity score of fluorescence: –, no fluorescence; +, moderate/slight fluorescence; 2+, intense fluorescence.

Table 2 Benign and malignant melanocytic tumors tested for binding with FKHI by IIF or indirect immunoperoxidase method

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Frozen</th>
<th>Fixed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nevus pigmentosus</td>
<td>8/8</td>
<td>1/1</td>
<td>9/9</td>
</tr>
<tr>
<td>Junctional</td>
<td>9/9</td>
<td>7/9</td>
<td>16/18</td>
</tr>
<tr>
<td>Compound</td>
<td>7/7</td>
<td>8/15</td>
<td>15/22</td>
</tr>
<tr>
<td>Intradermal</td>
<td>1/1</td>
<td>3/4</td>
<td>4/5</td>
</tr>
<tr>
<td>Blue nevus</td>
<td>NT*</td>
<td>6/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Halo nevus</td>
<td>3/3</td>
<td>2/3</td>
<td>5/6</td>
</tr>
<tr>
<td>Dysplastic nevus</td>
<td>NT</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Lentigo nevus</td>
<td>SSM</td>
<td>3/6</td>
<td>7/10</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>LMM</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Neurofibroma</td>
<td>NM</td>
<td>3/3</td>
<td>4/4</td>
</tr>
<tr>
<td>Metastatic melanoma</td>
<td>NM</td>
<td>4/4</td>
<td>11/11</td>
</tr>
</tbody>
</table>

* NT, not tested; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma.

Table 3 Tumors tested for binding with FKHI by IIF

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of tumors tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurofibroma</td>
<td>0/7</td>
</tr>
<tr>
<td>Granular cell schwannoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Basal cell epithelioma</td>
<td>0/6</td>
</tr>
<tr>
<td>Squamous cell carcinoma of the skin</td>
<td>0/4</td>
</tr>
<tr>
<td>Paget’s disease</td>
<td>0/2</td>
</tr>
<tr>
<td>Mycosis fungoides</td>
<td>0/6</td>
</tr>
<tr>
<td>Dermatofibrosarcoma protuberans</td>
<td>0/2</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>0/7</td>
</tr>
<tr>
<td>Kidney carcinoma</td>
<td>0/3</td>
</tr>
<tr>
<td>Lung carcinoma (squamous cell type)</td>
<td>0/3</td>
</tr>
<tr>
<td>Lung carcinoma (small cell type)</td>
<td>1/2</td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>1/1</td>
</tr>
<tr>
<td>Carcinoid tumor</td>
<td>1/1</td>
</tr>
<tr>
<td>Normal skin</td>
<td>0/20</td>
</tr>
</tbody>
</table>

Cell lines were grown on tissue culture chamber slides (Miles Scientific, Naperville, IL) for 2–3 days and tested. 

Indirect and ABC Immunoperoxidase Staining of Tissue Sections. Indirect or ABC immunoperoxidase technique was used to determine the reactivity of FKHI against frozen tissues and alcohol-fixed paraffin-embedded tissues.

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RESULTS

Monoclonal Antibody. Two hybridomas were found to be positive against KHm-6 cell line by enzyme-linked immunosorbent assay and IIF. They were cloned by the limiting dilution technique. One of cloned hybridomas producing IgGsb was designated as FKHI and used for further characterization. Cytoplasm of KHm-6 cells was stained in diffuse and granular pattern in IIF (Fig. 1). No reactivity of FKHI with intact KHm-6 cells was observed in immunoadherence assay, suggesting that no antigenic substances are present on the cell surface.

Immunoelectrophoretic Study. Two species of KHm-6 protein extract having molecular weights of 71,000 and 55,000 were revealed to react with FKHI under reducing condition (Fig. 2D). Extracts from normal human fibroblasts (control) showed no reactivity with FKHI (Fig. 2E). EKH4, AN3, NKH1, AHMY1, and AHNF1 did not label any one of these extracts as transferred electrophoretically to nitrocellulose paper.

Immunoelectron Microscopy. The granular, electron-dense immunoreaction products were shown to distribute diffusely in the cytoplasm of the KHm-1 cell. There was no increase in electron density in the nucleus and mitochondria as well as in stage II and stage III melanosomes (Fig. 3). In other organelles as far as they were intact, no increased density over the control was observed. No cell surface membrane components were labeled. It was concluded that nonstructural cytoplasmic proteins are labeled with FKHI.

Reactivity against Benign and Malignant Melanocytic Tumors. In fresh-frozen tissues, all melanocytic tumors were positive with FKHI whereas, in alcohol-fixed paraffin-embedded tissues, reactivity was 11 of 14 (79%) in melanoma and 28 of 42 (67%) in other melanocytic tumors (Table 2). Three cases of amelanotic melanoma included in melanoma series were all positive. There was no difference in histological features between positive and negative cases. Individual tumor cells within the one lesion were stained with almost the same intensity (Figs. 4 and 5). Positive stainings of nevus cells was not associated with the presence of melanin (Fig. 6). In addition, nevus cells in the upper dermis, which are cuboidal and have varying amounts of melanin (A-type nevus cells) and nevus cells in the middermis, which are smaller than A-type nevus cells and synthesize less melanin (B-type nevus cells), were also positive with FKHI regardless of the presence of melanin (Fig. 6). Neural nevi, C-type nevus cells (no melanin synthesis), and nevus corpuscles were FKHI negative.

FKHI reacted with 4 other human melanoma cell lines in IIF but failed to react with the B-16 mouse melanoma cell line. FKHI stained two glioblastoma cell lines only weakly. None of the other cell lines tested was positive with FKHI (Table 1).

Reactivity against Other Neoplasms. Neurofibroma and granular-cell myoblastoma showed no reactivity with FKHI. Malignancies involving the skin such as basal-cell epithelioma, squamous cell carcinoma, Paget’s disease, mycosis fungoides, and dermatofibrosarcoma protuberans were negative. Three out of 4 cases of small cell carcinoma of the lung, pheochromocytoma, and carcinoid tumor, termed APUDoma, were stained with FKHI (Table 3).

Reactivity in Normal Skin. When normal skin was tested with FKHI, normal melanocytes and other neuroectodermal components (nerves and nerve endings) of the skin were negative (Fig. 7). Perifollicular and periglandular networks of sensory and secretory nerves and their endings were entirely negative. Keratinocytes of the epidermis, hair follicle, and eccrine and apocrine glands (secretory as well as ductal segments) were all...
Fig. 1. a. FKH1 staining of KHm-6 cells in IIF. Cytoplasms are stained in diffuse and granular pattern. b. phase contrast view of KHm-6 cells as used in a; there are many nonmelanosomal granules (*), which apparently correspond to FKH1-positive granules in a (a and b, × 400).

Fig. 2. Electrophoretic transfer of cytoplasmic extracts to a nitrocellulose paper. Lane A: Molecular weight standards stained with Coomassie blue: Phosphorylase B (M, 92,500), albumin (M, 66,200), ovalbumin (M, 45,000), carbonic anhydrase (M, 31,000), trypsin inhibitor (M, 21,500), and lysozyme (M, 14,400). Lane B: cytoplasmic extracts of KHm-6 cells stained with Coomassie blue. Lane C: cytoplasmic extracts of normal human fibroblasts stained with Coomassie blue. Lane D: Immunoblot detection of cytoplasmic MAA. Two bands having molecular weight of 71,000 and 55,000 are revealed to react with FKH1 under reducing condition. Lane E: Immunoblotting of fibroblast protein extracts. FKH1 failed to stain any of those bands shown in Lane C. kd, kilodalton.

Fig. 3. Immunoelectron micrograph of KHm-1 melanoma cell. a, control; b, electron-dense reaction products(*) for FKH1 distributed diffusely in the cytoplasm. Cristae of stage II melanosomes (arrowheads) and mitochondria (m) are clearly identified because there is no deposition of dense immunoreaction product. (a and b, × 25,000).

cells were the only component of the normal skin which reacted with FKH1.

DISCUSSION

Five monoclonal antibodies detecting cytoplasmic MAA have been reported. A comparison between these antibodies and
MONOCLONAL ANTIBODY AGAINST MELANOMA

Fig. 4. FKH1 staining of SSM in IIF (a) and in immunoperoxidase method (b). Not only melanoma cells in the lowermost epidermis but also cells migrating into the upper epidermis are stained. (a and b, × 120).

Fig. 5. FKH1 staining of NM with immunoperoxidase method. These are alcohol-fixed paraffin-embedded sections. a, control; in b, nests of melanoma cells are darkly stained. Note that several positive cells are scattered in the middermis (arrow), which may indicate tumor invasion. (a and b, counterstained with hematoxylin, × 100).

Fig. 6. FKH1 staining of compound nevus pigmentosus with immunoperoxidase method. a, Control; b, Both A-type (A) and B-type (B) nevus cells are stained with FKH1 at almost the same intensity: positive staining is not correlated with the presence or amount of melanin. (a and b, counterstained with hematoxylin, × 63).

FH1 is summarized in Table 4. NKI/C-3 has cytoplasmic reactivity as FKH1, but others are different from FKH1 and from each other with respect to molecular nature and staining pattern. The proteins having molecular weights of 71,000 and 55,000 detected by FKH1 are distinct from others such as 465.12S, ME165, and ME325 (Table 4). Moreover, M, 71,000 and M, 55,000 proteins have not been found in cell-surface MAA (2, 7, 8, 10, 12, 16, 18, 19, 23, 25, 35, 36). In immunohistochemical use of monoclonal antibodies, a large area of cytoplasmic staining is certainly an advantage over the thin layer of cell membrane staining to avoid nonspecific retention of chromogens in cell-to-cell or cell-to-ground substance (or collagen) interphase, particularly when tumor cells are packed densely. FKH1 has no affinity to the plasma membrane or cell surface components as demonstrated by immunohistochemical, immunoelectron microscopic and immune adhesive assays. From the therapeutic point of view, a combined use of FKH1 with plasma membrane-specific anti-MAA would be interesting; such antibody could render plasma membrane of the target cells leaky and then FKH1 could attack cytoplasmic vital components. FKH1 is available for such trials.

The smaller number of positive melanocytic tumors after fixation in alcohol and paraffin embedding should be attributed to fixation and/or embedding procedure, since fresh-frozen counterparts of the same tumors showed positive staining with FKH1.

In immunoelectron microscopy, immunoreaction products for FKH1 were distributed diffusely in the cytoplasm of the KHm-1 cell excluding melanosomes and other organelles. As stage III or stage IV melanosomes have high electron density, the possibility that they were labeled by FKH1 cannot be ruled out. The amorphous stage of premelanosomal proteins could
be the antigen of FKH1. However, FKH1 stained various melanocytic tumors, including three cases of amelanotic melanoma, independent of the degree of pigmentation. It is, therefore, concluded that FKH1 does not label melanosomal proteins. The melanosome-independent nature of FKH1 is certainly advantageous for the diagnosis of amelanotic melanoma and leukemias of melanomas.

Among nonmelanocytic tumors tested, only APUDoma showed positive reaction with FKH1. This finding supports the concept that the melanoma is one of the APUDomas (9, 22) and it may be stated that FKH1 is APUDoma-nevus cell specific.

From the clinical and diagnostic pathology point of view, the most useful characteristic of FKH1 is its reactivity with alcohol-fixed, paraffin-embedded tissues. Cell surface antigens are often denatured by conventional histological fixation and embedding procedures, and as a result only fresh-frozen tissues could be used; this is the major limitation to the use of monoclonal antibodies in routine surgical pathology. Therefore, FKH1 is very useful in cases where fresh material is no longer available or when retrospective study is necessary.

In view of its restricted reactivity, FKH1 seems to be one of the most specific monoclonal antibodies available in the detection of melanocytic tumors. Reaction of FKH1 with mast cells can be remedied by using metachromatic dyes such as Giemsa or methylene blue in counterstains.

**REFERENCES**

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