Expression of Melanoma-associated Antigens by Normal and Neurofibroma Schwann Cells

Alonzo H. Ross,2 David Pleasure, Kenneth Sonnenfeld, Barbara Atkinson, Barbara Kreider,3 Donna M. Jackson, Ingrid Taff, Elio Scarpini, Robert P. Lisak, and Hilary Kопrowski

The Wistar Institute of Anatomy and Biology [A. H. R., D. M. J., H. K.]; The Children's Hospital of Philadelphia, [D. P., B. K.]; Hospital of the University of Pennsylvania [B. A., R. P. J.]; Philadelphia, Pennsylvania 19104; Mt. Sinai School of Medicine, New York, New York 10029 [K. S., I. T.]; and University of Milan School of Medicine, Milan, Italy [E. S.]

ABSTRACT

The cell surface antigen distribution on traumatic neuromega Schwann cells and neurofibroma Schwann-like cells was characterized using monoclonal antibodies that define melanoma-associated antigens. Immunofluorescence staining of cultured cells, immunoprecipitation of radioiodinated antigens from cells placed in short-term cultures, and immunoperoxidase staining of frozen tissue sections revealed most of the melanoma-associated antigens tested on traumatic neuromega and neurofibroma Schwann cells and on fetal and adult femoral nerve. The cross-reactivity of the antibodies with neural cells may reflect the common neural crest embryological origin of Schwann cells and melanocytes. Cell sorter analysis of neurofibroma cells using a monoclonal antibody directed against the melanoma nerve growth factor receptor resulted in cell cultures highly enriched for Schwann-like cells which may bear the genetic defect responsible for neurofibromatosis. The antigen detected by this monoclonal antibody is the neurofibroma nerve growth factor receptor and the antibody was a potent inhibitor of nerve growth factor binding to neurofibroma cells.

INTRODUCTION

Schwann cells are a glial element in the peripheral nervous system and are embryologically derived from the neural crest (1). In adults, these cells surround all peripheral nerve fibers and, except in the case of injury or disease, have a very low rate of division (2). Neurofibroma is a relatively common autosomal dominant disorder most often involving benign dermal lesions containing Schwann-like cells, fibroblasts, mast cells, and neuronal processes (3). The primary proliferating cell is thought to be the Schwann-like cell (4).

Little is known of the molecular or cellular properties of the Schwann-like cells, as compared with those of melanoma cells (5). A large number of anti-melanoma MAbs are available that have been used to characterize the distribution of melanoma-associated antigens among normal and transformed tissues (6, 7). This information is important for applications of MAbs to therapy and diagnosis as well as to the determination of the melanoma-associated antigen function and role in the transformed phenotype. Many MAbs do not bind to normal tissue melanocytes but do bind to nevi, the benign melanocytic lesions, and to neural-related tumors such as astrocytomas and neuroblastomas. This binding pattern may be related to the neural crest embryological origin of melanoma (1). We previously reported that the staining pattern of human peripheral nerves and neurofibromas with anti-NGF MAbs suggested that normal Schwann cells and Schwann-like cells in neurofibromas expressed the NGF receptor (8) and that Schwann-like cells specifically bind NGF (9).

To better understand the growth and immunological properties of normal and neurofibroma Schwannian cells and their correlation, if any, with tumor progression, we have analyzed the antigenic profiles of these cells using anti-melanoma MAbs. We report here that traumatic neuromega Schwann cells and neurofibroma Schwann-like cells express many of the antigens detected with anti-melanoma MAbs. These anti-melanoma MAbs also exhibited reactivity in femoral nerve frozen tissue sections. The cross-reacting antigens detected on the Schwann cells are similar to the corresponding melanoma-associated antigens as judged by SDS-PAGE. These results define new cross-reactivities of the anti-melanoma MAbs with peripheral nerves which are relevant to future therapeutic and diagnostic applications of the MAbs, identify tumor-associated antigens present on neurofibroma cells, provide a means by cell sorting of preparing pure cultures of neurofibroma Schwann-like cells, and demonstrate that two tumors of similar embryological origin, neurofibroma and melanoma, express a similar antigenic profile.

MATERIALS AND METHODS

MAbs. The production and characterization of the anti-melanoma MAbs have been described (6, 8, 10). MAbs P3X63Ag8, ME31.3, ME19-19, ME20.4, ME75-29, ME061, ME13-17, and ME491 are IgGl isotype and MAbs MENu4B and ME37-7 are IgG2a isotype. Table 1 summarizes the antigens detected and cross-reactivities of the MAbs. The proteoglycan antigen identified in this study with Mab ME31.3 has also been extensively studied by other groups using MAbs 9.2.27 (15), 155.8 (16), 225.28S (17), and B5 (18). The M, 97,000 glycoprotein identified in this study with Mab ME061 has been extensively studied using MAbs 96.5 (19, 20) and L235 (18) and has been shown to be structurally related to transferrin (21).

Tissue Samples. Neurofibromas removed for therapeutic purposes and femoral nerves removed postmortem from adults with no apparent neuropathies were quick frozen. Fragments of traumatic neuromega were obtained at the time of delayed secondary nerve repair. Fetal femoral nerves were obtained from traumatic abortuses at 19 or 22 wk gestation. The fetal neural samples were rapidly frozen by immersion in isopentane cooled with liquid nitrogen.

Cell Cultures. The distribution of melanoma cell lines WM-9 and A875 has been described (22, 23). Single cell suspension were prepared from freshly excised neurofibromas and traumatic neuromega. Tissue samples were minced in Hanks' balanced salt solution with 1 mm EDTA and lacking magnesium and calcium, then transferred to RPMI 1640 medium containing 15% fetal calf serum-25 mm 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid, dispase, 1.25 units/ml (crude; Boehringer)-0.05% (w/v) collagenase (Worthington)-0.1% (w/v) hyaluronidase (Sigma), and incubated overnight at 37°C in 5% CO2/95% air. The next day the medium was removed and replaced with RPMI 1640 without serum containing dispase, 1.25 units/ml and 0.05%...
collagenase. After 30 min at 37°C, the suspension was pipetted vigorously, then spun down and the pellet washed three times with RPMI 1640 medium containing 15% (v/v) fetal calf serum. The cells were seeded on poly-L-lysine coated glass coverslips.

Radioiodination, Immunoprecipitation, and Electrophoresis. Cells were labeled by the lactoperoxidase procedure and extracted with 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)] (24). Immunoprecipitations were carried out using 100 μl of hybridoma culture supernatant per sample. Antigen:mouse immunoglobulin complexes were bound with 50 μl goat anti-mouse immunoglobulin (Sigma) and washed three times with solubilizing buffer. The samples were analyzed by SDS-PAGE using a 10% gel (25). Autoradiography was carried out using a Cronex Lightning-Plus intensifying screen (DuPont).

Immunofluorescence and Immunoperoxidase Microscopy. Cells to be analyzed by immunofluorescence microscopy were incubated without prior fixation with undiluted hybridoma culture supernatant, washed with MEM, overlaid with rhodamine-conjugated goat anti-rabbit immunoglobulin (Cappel), washed with MEM, fixed with ice-cold 5% acetone in ethanol, washed five times with MEM, and washed once with water. Preparation of frozen sections and immunoperoxidase staining have been described (14, 26).

Fluorescence-activated Cell Sorting. Cells to be analyzed were released from the substratum with dispase, 1.25 units/ml and 0.05% (w/v) collagenase. The cells were washed with Dulbecco's PBS (lacking Ca²⁺ and Mg²⁺) and resuspended at 5 × 10⁶ cells/ml in hybridoma culture supernatant diluted 1:2 with PBS for 1 h at 4°C. The cells were pelleted, washed three times with PBS, resuspended at 5 × 10⁹ cells/ml in fluorescein-conjugated rabbit anti-mouse immunoglobulin (60 μg/ml) and incubated for 1 h at 4°C. The cells were washed in the same manner and resuspended in PBS at 1 × 10⁷ cells/ml. An aliquot of the cells was analyzed by flow cytometry (Ortho Cytofluorograf 50 HH). The cutoff for positive staining was determined using cells stained in parallel with P3X63Ag8, a nonspecific control MAb. The remainder of the cells were sorted on a Becton Dickinson FACS IV retaining the 50% most fluorescent of the positive cells for cell culture.

RESULTS

Detection of Melanoma-associated Antigens on Traumatic Neuroma Schwann and Neurofibroma Cells. Cells from a traumatic neuroma and from a dermal neurofibroma were placed into short-term culture for 1 day and then radioiodinated by the lactoperoxidase method. For comparison, the melanoma cell line WM-9 was also iodinated. The cells were solubilized, and specific antigens were immunoprecipitated with anti-melanoma MAbs and analyzed by SDS-PAGE. Fig. 1 shows the results for MAbs ME31.3, ME37-7, ME75-29, and ME20.4. ME31.3 is specific for the melanoma-associated chondroitin sulfate proteoglycan and its core protein gp260 (27). Both species were detected in the traumatic neuroma, neurofibroma, and melanoma samples (lanes 1, 5, and 9). The gp260 component is marked on the gel, and the proteoglycan component is visible as a broad band in the stacking gel (delineated by arrows). Additional bands in lanes 1 to 4 are due to higher background immunoprecipitation for the traumatic neuroma. The ME37-7 antigen, HLA-DR, was detected only in the melanoma sample (lane 10). The ME75-29 antigen, gp120, was detected in the neuroma, neurofibroma, and melanoma samples (lanes 3, 7, and 11). The ME20.4 antigen, the NGF receptor, was also detected in all three samples (lanes 4, 8, and 12).

Table 1 Properties of anti-melanoma MAbs and corresponding antigens

<table>
<thead>
<tr>
<th>MAb</th>
<th>Antigen</th>
<th>Normal adult tissues</th>
<th>Tumors</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME31.3</td>
<td>gp260 glycoprotein + M, 500,000 proteoglycan</td>
<td>Nevus, sweat gland, basal keratinocytes, connective tissue in colon, capillary endothelium</td>
<td>Astrocytoma</td>
<td>5-7, 11-12</td>
</tr>
<tr>
<td>ME31.3 and ME19-19</td>
<td>M, 260,000 glycoprotein</td>
<td>Nevus, adrenal medulla, peripheral nerves</td>
<td>Astrocytoma</td>
<td>5-6</td>
</tr>
<tr>
<td>ME491</td>
<td>M, 30,000 to 60,000 glycoprotein</td>
<td>Nevus, some epithelial and hematopoietic cells</td>
<td>Astrocytoma, some carcinomas</td>
<td>13</td>
</tr>
<tr>
<td>ME37-7 and ME13-17</td>
<td>HLA-DR</td>
<td>Nevus, B-lymphocytes, macrophages</td>
<td>Lymphoma, some carcinomas</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 2 Immunoprecipitations from Schwannian cells using anti-melanoma MAbs

<table>
<thead>
<tr>
<th>MAb</th>
<th>No. cultures positive/no. cultures tested</th>
<th>Traumatic neuroma Schwann cells</th>
<th>Neurofibroma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME31.3</td>
<td>2/2</td>
<td>2/2</td>
<td>1/1</td>
</tr>
<tr>
<td>MENu4B</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>ME20.4</td>
<td>2/2</td>
<td>2/2</td>
<td>1/1 (weak)</td>
</tr>
<tr>
<td>ME75-29</td>
<td>0/1</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>ME491</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>ME37-7</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

The ME491 antigen present in cultured neurofibroma cells was identified by Western blotting (Fig. 2). The ME491 antigen was not detected by immunoprecipitation (Table 2) probably because most of the antigen is intracellular and, hence, not accessible to cell surface lactoperoxidase iodination (13).

The protein bound by MAb ME20.4 was demonstrated to be the neurofibroma NGF receptor in binding studies with 125I-labeled NGF (Fig. 3). ME20.4 ascites fluid at a 10^-6 dilution strongly inhibited 125I-labeled NGF binding. Two other MAbs, ME19-19 and ME13-17, which bind to other proteins on the neurofibroma cell surface, did not inhibit 125I-labeled NGF binding at these dilutions.

The fraction of neurofibroma cells expressing melanoma-associated antigens was determined by flow cytometry. In an initial study, we analyzed 11 cultures of dermal and plexiform neurofibroma cultures for NGF receptor expression (MAb ME20.4). Between 16 and 66% of the cells were positive for NGF receptor [39.1 ± 16.4% (SD)]. We also analyzed four dermal neurofibroma cultures with MAbs ME20.4, ME491, ME31.3, and ME13-17 (Table 3). ME20.4 and ME491 consistently stained a large percentage of neurofibroma cells, whereas ME31.3 stained a smaller fraction. ME13-17 reactivity was variable, with staining of some cultures but not others. In controls with WM-9 melanoma cells, the ME20.4, ME491, and ME13-17 antigens were unaffected by the protease treatment used to prepare the cells for flow cytometry, but the ME31.3 antigen was greatly reduced.

The identity of the melanoma-associated antigen-expressing cells in the neurofibroma cultures was determined by immunofluorescence microscopy. Fig. 4, A to C shows that the cells stained with MAbs ME37-7 and ME20.4 are the long spindle-shaped cells with oval nuclei (Schwann-like cells). The fibroblastic cells did not fluoresce detectably. The Schwann-like cells but not the fibroblasts were positive for MAbs ME31.3 (5 of 6 cultures), ME37-7 (6 of 6 cultures), ME20.4 (5 of 6 cultures), ME75-29 (3 of 6 cultures), MENu4B (3 of 6 cultures), and

Fig. 2. Detection of ME491 antigen by Western blotting. Membrane extracts from either a neurofibroma (lanes 1 and 3) or melanoma cell line A87S (lanes 2 and 4) were applied to an SDS-polyacrylamide gel and then electroblotted onto nitrocellulose, and incubated with either control MAb P3X63Ag8 (lanes 1 and 2) or MAb ME491 (lanes 3 and 4). The antigens were then visualized with an 125I-labeled second antibody and autoradiography.

Fig. 3. Inhibition of MAbs of 125I-labeled NGF binding to dissociated neurofibroma cells. Dissociated neurofibroma cells were incubated for 90 min at 37°C in binding buffer (RPMI 1640 containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and BSA, 5 mg/ml) containing 125I-labeled NGF (5 ng/ml) and several dilutions of MAb-containing ascites [ME20.4 (O), ME19-19 (©), and ME37-7 (A)]. Triplicate samples were centrifuged for 30 s to separate bound from free 125I-labeled NGF, and bound 125I-labeled NGF was measured in a gamma counter. Nonspecific binding (approximately 30% of total binding) was determined in parallel samples to which unlabeled NGF (10 µg/ml) was added and has already been subtracted from the data shown. Binding in the absence of MAb was 1600 cpm/1.4 x 10^5 cells.

Table 3 Flow cytometry analysis of neurofibroma cells

<table>
<thead>
<tr>
<th>MAb</th>
<th>% of cells positive for melanoma-associated antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME31.3</td>
<td>2 ± 2 (0-5)</td>
</tr>
<tr>
<td>ME20.4</td>
<td>50 ± 22 (19-72)</td>
</tr>
<tr>
<td>ME491</td>
<td>56 ± 9 (47-69)</td>
</tr>
<tr>
<td>ME13-17</td>
<td>13 ± 13 (2-31)</td>
</tr>
</tbody>
</table>

5889
Fig. 4. Phase contrast and indirect immunofluorescence microscopy. Schwann-like cells as visualized in cells cultured from a traumatic neuroma (A and B), a dermal neurofibroma (C and D), or Schwann-like cells enriched from a dermal neurofibroma by cell sorting using anti-NGF receptor MAb (E and F) were analyzed for melanoma-associated antigen expression. Cells were stained with MAb ME37-7 (A to D) or MAb ME20.4 (E and F). Bars, 20 μm (A to D) or 70 μm (E and F).
The antigens detected by MAbs ME31.3, MENu4B, ME20.4, ME75-29, and ME061 by immunoprecipitation were apparently identical from melanoma and neurofibroma as judged by SDS-PAGE. The neurofibroma ME49I antigen detected by Western blotting was less heterogeneous than the corresponding melanoma antigen. Since this antigen is heavily glycosylated (13, 28), the difference may be the result of more homogeneous glycosylation in the neurofibroma. The ME20.4 antigen is indeed the NGF receptor in neurofibroma cells since MAb ME20.4 completely blocked 125I-labeled NGF binding (Fig. 3).

We have also detected many of the melanoma-associated antigens on frozen sections of adult and fetal peripheral nerves. Although the exact assignment of the melanoma-associated antigen-bearing cells awaits more detailed histological analysis, possibly including electron microscopy, it is clear that normal elements of the peripheral nervous system express many of the melanoma-associated antigens. Preliminary studies using anti-NGF receptor MAb indicate that cells of the perineurium which are probably of neural crest embryological origin, consistently show strong staining. Schwann cells appeared to stain in some cases but not in others, perhaps reflecting heterogeneity of Schwann cells in different nerves.

The cross-reactivity of these MAbs with normal tissue does not rule out their use for melanoma immunotherapy. The anti-carcinoma MAb CO17-1A, for example, has been used in clinical trials for patients with colon and pancreatic carcinomas and has resulted in remissions for some patients (29). Radiomaging studies have demonstrated that CO17-1A MAb localizes at the tumor in patients even though some epithelial cells in normal colon express the antigen (30, 31). This paradoxical result is apparently due to greater accessibility of the MAb to antigen in tumor than in normal colon.

The melanoma-associated antigen for which we have the most information is the NGF receptor which is not detected in frozen sections of normal melanocytes but is easily detected in sections of nevi and melanomas (8). Human neonatal melanocytes in short-term culture under conditions inducing cell division express low levels of the receptor (5, 6). Both Schwann cells and nerve soma in neonatal monkey dorsal root ganglia also show strong staining.6 In the present study, fetal and adult peripheral nerves were strongly stained with anti-NGF receptor MAb, perhaps reflecting the related neural crest embryological origin of melanocytes, Schwann cells, and sympathetic and sensory neurons. Schwann cells and melanocytes may derive from a common bipotent progenitor cell (32). It has been shown that the expression of other tissue-specific genes is the result of a complex interaction between multiple trans-acting factors (33).

We suggest that the expression of the NGF receptor requires at least two factors, the first of which is normally expressed in neural crest-derived cells and is necessary but not sufficient for expression of NGF receptor. The proposed second factor required for NGF receptor expression is induced in stimulated melanocytic cells capable of undergoing cell division in culture or in nevic or melanoma lesions but not in normal tissue melanocytes, which rarely divide. In sensory nerves and some Schwann cells, this second factor or a substitute factor is expressed. This model suggests many experiments using the recently cloned NGF receptor gene (34) to identify factors

**Table 4 Detection of melanoma-associated antigens by immunoperoxidase staining of neurofibroma frozen sections**

<table>
<thead>
<tr>
<th>MAb</th>
<th>Immunoperoxidase staining of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult femoral nerve</td>
</tr>
<tr>
<td>ME31.3</td>
<td>+</td>
</tr>
<tr>
<td>MENu4B</td>
<td>+</td>
</tr>
<tr>
<td>ME20.4</td>
<td>+</td>
</tr>
<tr>
<td>ME75-29</td>
<td>±</td>
</tr>
<tr>
<td>ME061</td>
<td>±</td>
</tr>
<tr>
<td>ME49I</td>
<td>±</td>
</tr>
<tr>
<td>ME37-7</td>
<td>±</td>
</tr>
</tbody>
</table>

* *+, strong brown color; +, brown color; ±, slight brown color; −, no staining.

M. A. Bothwell and E. Scarpini, unpublished observations.
responsible for the tissue-specific expression of the NGF receptor and its normal expression in melanomas.

ACKNOWLEDGMENTS

We are very grateful to Drs. Allan Rubinstein (Department of Neurology, Mt. Sinai Medical School) and A. Lee Osterman (Department of Orthopedic Surgery, University of Pennsylvania) for their help in obtaining the tissue samples for this study. Marina Aquino provided excellent technical assistance. We thank Jeffrey Faust for running the cell sorter, Marina Hoffman for editorial assistance, and Meenhard Herlyn for helpful discussions and preparation of hybridoma culture supernatants.

REFERENCES

Expression of Melanoma-associated Antigens by Normal and Neurofibroma Schwann Cells

Alonzo H. Ross, David Pleasure, Kenneth Sonnenfeld, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/11/5887

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.