ABSTRACT

Two neuroblastoma cell lines established from tumor tissue taken from one individual are described. The first of these was established from a bone marrow aspirate (RT-BM) and the other from a right axillary lymph node (RT-LN) of a 1-yr-2-mo-old patient with Stage IV disease. The original lines were cloned in soft agar to yield six clones of the bone marrow-derived line (RT-BM 1-6) and 12 of the lymph node line (RT-LN 1-12). Chromosomal analysis of the original lines and clones showed they all have either identical or very similar karyotypes, with a deletion of chromosome 1p. Transmission electron microscopy indicates all contain neurosecretory (dense core) granules and neurotubules. In addition catecholamine metabolites of dopamine and noradrenaline have been identified. Different growth characteristics of the lymph node and bone marrow lines have been identified. RT-LN lines grow in a single cell layer with neurite processes, whereas bone marrow-derived lines form focal aggregates with neurite processes. In addition the colony-plating efficiency of the lymph node-derived lines is higher than those derived from bone marrow.

Comparison of the cell surface antigen profile of the original tumor tissue, parent lines, and clones demonstrates they all bind seven of a panel of nine monoclonal antibodies. The expression of these antigens has remained stable in vitro for 25 passages undertaken over a 2-yr period. The definition of antigens that are expressed on the membranes of neuroblastoma cells in a stable form can aid in the differential diagnosis of neuroblastoma from other “small round cell tumors of childhood” and hopefully contribute to a greater understanding of the biology of this highly malignant tumor.

INTRODUCTION

Neuroblastoma, arising from tissue derived from the neural crest, is one of the most common malignant solid tumors of childhood. Histologically it can be grouped with lymphoblastic leukemia/lymphoma, rhabdomyosarcoma, and Ewing’s sarcoma as one of the small round cell tumors of childhood. Occasionally the similar morphological appearance of these tumors can represent a considerable diagnostic problem to the pathologist (1, 2).

Using human cell lines to define cell characteristics unique to neuroblasts is fraught with difficulties as some can convert between having neuroblastic characteristics and epithelial-like morphology (3, 4). In addition, loss of catecholamine production (5) and changes in karyotypes during culture have been reported (6).

MATERIALS AND METHODS

Clinical History of Patient R. T. R. T. was a 1-yr-2-mo-old Japanese infant who presented with poor appetite and anemia on July 16, 1983. On clinical examination a right upper abdominal mass and axillary lymphadenopathy were observed. A bone survey revealed general bone metastasis, involving the right orbit, ribs, femoral, iliac, and lumbar bones. Pyelography i.v. and computer-aided tomography of the abdomen showed a primary tumor arising from the right adrenal gland. Analysis of bone marrow aspirates revealed that normal hematopoietic progenitor cells had been almost totally replaced by tumor. High levels of the catecholamine metabolites, vanilmandelic acid and homovanillic acid, were identified in the patient’s urine. Biopsy of the right axillary lymph node led to the histological diagnosis of Stage IV neuroblastoma. The patient was treated with chemotherapy consisting of a combination of cyclophosphamide, vincristine, and doxorubicin, followed by external beam irradiation to the bone metastases. Although partial regression of the primary abdominal tumor was observed, the patient died of her disease on October 22, 1983. Autopsy findings showed general metastatic spread of neuroblastoma throughout the body.

Cell Culture. Tumor samples for cell culture were taken prior to chemotherapy. A mononuclear cell fraction of cells from a bone marrow aspirate was prepared by Ficoll-Hypaque centrifugation on July 18, 1983. Cells were cultured in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) at 37°C in 5% CO2. Forty-eight h later a biopsy sample from a right axillary lymph node was disaggregated into small fragments and placed in complete culture medium. The medium was changed every 3 to 4 days. Cells were subcultured into new flasks by trypsinization when cultures were in a subconfluent state.

 Colony Formation in Soft Agar and Cloning. The ability of cells to form colonies in soft agar was determined as previously reported (9). Cells (5 to 20 × 10^3) were suspended in 1.0 ml of 0.3% Bacto-agar (Difco Lab., Detroit, MI) in complete culture medium and seeded into 18 U.S.C. Section 1734 solely to indicate this fact.1This work was supported in part by grants for cancer research from the Ministry of Health and Welfare and from the Ministry of Education (No. 59480236) of Japan. Also supported in part by NMRDC Work Unit MR0001-01.01.1300. The opinions and assertions contained herein are the private opinions of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

2 To whom requests for reprints should be addressed.

Recently several MoAbs3 that bind to cell surface components of neuroblast have been described. These have proved valuable in the differential diagnosis of lymphoblastic leukemia/lymphoma, rhabdomyosarcoma, and Ewing’s sarcoma from neuroblastoma (7, 8). However, few studies have been undertaken to define the homogeneity in antigen expression between tumor cells isolated from the same patient. In addition, the relationship between antigens expressed on fresh tumor and those defined on cell lines established from the same material has never been described.

In this paper, we describe two new human neuroblastoma cell lines derived from tumor tissue taken from a single patient with Stage IV disease. Subsequently 6 and 12 cloned lines from each parent line have been established. Morphological, biochemical, and cytogenetic characteristics of these lines have been investigated, and the cell surface antigen profiles on these cell lines have been compared with the patient’s fresh tumor cells, isolated from the bone marrow.
35-mm Petri dishes (Lux Scientific, Newbury Park, CA). The plates were incubated at 37°C in a 5% CO₂-humidified atmosphere. Colonies consisting of at least 20 cells were counted after 10 days with an inverted microscope, and the colony plating efficiency was calculated. Colony-forming cells were transferred to 96-well microtiter plates and subsequently expanded to 25-cm² flasks.

Electron Microscopy. For transmission electron microscopy, cells attached to culture flasks were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer. These were subsequently harvested by scraping from the surface of the dishes using a rubber policeman. Cells were fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated through a series of graded ethanol, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Hitachi H-500H electron microscope.

For scanning electron microscopy, cells on coverslips were fixed in 2% glutaraldehyde, dehydrated in graded concentrations of ethanol, and dried. These were coated with platinum and observed with a Hitachi S-520-LB scanning electron microscope.

Catecholamine Estimations. Cells were harvested from culture flasks and washed with phosphate-buffered saline. For qualitative assays of catecholamines, cells were smeared onto glass slides, and formaldehyde-induced catecholamine fluorescence was examined by the method of Falck-Hillarp (10). For quantitative assays of catecholamines, harvested cells were homogenized, and dopamine, adrenaline, and noradrenaline levels were determined by high-performance liquid chromatography (11). Results are expressed as the protein basis.

Chromosome Analysis. The karyotype of the parent and cloned cell lines was analyzed by a modification of Seabright's trypsin-Giemsa banding technique (12).

MoAbs. Four MoAbs raised against human hematopoietic cells (BA-1, BA-2, J-5, and OKIa1), 3 against either human fetal or adult brain (UJ-13A, UJ-127-11, and anti-Thy-1), and 2 against human neuroblastoma cell lines (HSAN1.2 and PI153/3) are listed in Table 1. All MoAbs except HSAN1.2 were in ascites form, the former being a hybridoma culture supernatant.

Indirect Immunofluorescence. A 15-µl aliquot of cell suspension containing 1 to 3 × 10⁶ cells, whose viability was more than 90%, was incubated with 15 µl of the appropriately diluted MoAbs for 30 min at room temperature. MoAbs were used in antibody excess (4 times the antibody concentration needed to saturate antigenic sites on a reference neuroblastoma cell line, SMS-KAN, for all MoAbs except J-5 and OKIa1, and on a reference pre-B-acute lymphocytic leukemia cell line, NALM-6, for J-5 and OKIa1 MoAbs) as previously reported (5, 8, 9). After 2 washes, antibody binding was visualized by incubation with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (Cappel Lab., West Chester, PA). As a control, normal mouse IgG was used. All reagents contained sodium azide (0.1% by volume) to prevent antigenic modulation. After washing, the cells were observed under an Olympus BH2-RHK microscope equipped with a USH 1021 100-W mercury bulb. A minimum of 200 cells was counted, and the percentage of stained cells showing either partial or complete ring staining was determined (5, 8, 9). The results are expressed as the mean of between 3 and 4 experiments.

RESULTS

Establishment of Cell Lines. The tumors biopsied in July 1983 from bone marrow and a right axillary lymph node and explanted into tissue culture flasks grew in the form of adherent cells. Cells were reseeded into new flasks when cultures were in a subconfluent state. Parent cell lines were designated RT-BM and RT-LN as a reference to the tumor site from which they were established. At passage 6 both lines were cloned in soft agar. Six clones of the RT-BM-derived line were obtained and 12 from the lymph node line.

Colony Formation. The number of colonies identified in soft agar was dependent on the concentration of cells seeded into the Petri dishes. At equivalent concentrations the RT-BM line had a cloning efficiency of 1.0% against 1.7% for the RT-LN line. These differences in cloning efficiency were mirrored on analysis of the clones derived from the parent lines (RT-BM-4 and RT-LN-1) (Fig. 1).

Table 1 MoAbs for determination of cell surface antigen expressions

<table>
<thead>
<tr>
<th>MoAbs</th>
<th>Reported specificity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA-1</td>
<td>B-cell associated</td>
<td>5, 8, 17</td>
</tr>
<tr>
<td>BA-2</td>
<td>Leukemia associated</td>
<td>5, 8, 18</td>
</tr>
<tr>
<td>J-5</td>
<td>Common ALL⁵ antigen</td>
<td>19</td>
</tr>
<tr>
<td>OKIa1</td>
<td>HLA-DR antigen</td>
<td>20</td>
</tr>
<tr>
<td>UJ-13A</td>
<td>Neuroectodermal associated</td>
<td>5, 8, 21</td>
</tr>
<tr>
<td>UJ-127-11</td>
<td>Neuroectodermal associated</td>
<td>5, 8, 22</td>
</tr>
<tr>
<td>Anti-Thy-1</td>
<td>Thy-1 antigen</td>
<td>7, 8</td>
</tr>
<tr>
<td>HSAN1.2</td>
<td>&quot;Neuroblastoma&quot;</td>
<td>8, 23</td>
</tr>
<tr>
<td>PI153/3</td>
<td>Neural, pre-B ALL, common ALL</td>
<td>8, 24, 25</td>
</tr>
</tbody>
</table>

⁵ ALL, acute lymphocytic leukemia.

FIG. 1. Percentages of colony-plating efficiency in parent cell lines (RT-BM and RT-LN, passage 6) and cloned cell lines (RT-BM-4 and RT-LN-1, passage 6) were 1.0, 1.7, 1.1, and 1.9%, respectively. Bars, SE.

FIG. 2. Transmission electron microscopy showed that RT-BM cells (passage 12) contained neurotubules (large arrows) and a few dense core granules (small arrows) characteristic of neuroblastoma cells. ×23,000.
IDENTICAL NEUROBLASTOMA ANTIGEN EXPRESSION

Fig. 3. Left, RT-BM cells formed focal aggregates of cells with neurite processes. x 520. Right, RT-LN cells grew as a single layer with neurite processes. x 520.

Table 2 Catecholamine fluorescence and levels of catecholamine in bone marrow tumor cells and parent and cloned cell lines

<table>
<thead>
<tr>
<th>Catecholamine fluorescence</th>
<th>Parent cell lines</th>
<th>Cloned cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RT-BM (passage 10)</td>
<td>RT-LN (passage 10)</td>
</tr>
<tr>
<td>Catecholamine (ng/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>NT*</td>
<td>0.42 ± 0.08*</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>NT</td>
<td>ND</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>NT</td>
<td>0.18 ± 0.04</td>
</tr>
</tbody>
</table>

* NT, not tested; ND, not detected.
* Mean ± SE from 3 separate experiments.
* Significant differences from dopamine levels in RT-BM and RT-BM-4 (P < 0.01).

Electron Microscopy. Transmission electron microscopy showed that RT-BM cells (passage 12) contained neurotubules and a few dense core granules characteristic of neuroblastoma cell lines (Fig. 2). Clones from these lines also demonstrated these organelles (data not presented). Scanning electron microscopy demonstrated that RT-BM cells formed focal aggregates of cells with neural processes, whereas RT-LN cells grew as a single layer with neural processes (Fig. 3). These different growth characteristics in the 2 parent cell lines have been retained for 25 passages over a 2-yr culture period. In addition the clones derived from the parent lines also exhibit differences in growth characteristics.

Catecholamines. The results of catecholamine-induced fluorescence and the levels of catecholamines in fresh tumor cells from bone marrow and in cell lines, estimated by high-performance liquid chromatography, are shown in Table 2. Catecholamine-induced fluorescence was not observed in either the fresh tissue or any of the cell lines obtained. However, by quantitative assays, dopamine and noradrenaline were detected in the cell lines. Levels of noradrenaline were similar in parent and cloned cell lines, whereas levels of dopamine were significantly higher in RT-LN and RT-LN-1 than in RT-BM and RT-BM-4 (P < 0.01).

Chromosome Analysis. The karyotype of parent lines RT-BM and RT-LN at passage 6 and 2 clones at the same passage (RT-BM-2 and RT-LN-1) showed 46XX, del(1)(p22) characteristic of neuroblastoma. All lines analyzed also contained double-minute chromatin bodies.4

Determination of Cell Surface Antigen Expression by a Panel of MoAbs. Table 3 shows the binding profiles of fresh bone marrow tumor cells, as well as the parent and cloned cell lines

4 M. Morita et al., manuscript in preparation.
Two catecholamine metabolites (dopamine and noradrenaline) were observed. The plating efficiency of the lymph node of chromosome Ip, which is a characteristic of neuroblastoma, while lymph node-derived cell lines grow in a single cell layer. Although identical expression of surface membrane antigens on all cell lines was observed, variations in the percentage of cells binding antibody in each line were determined by indirect immunofluorescence.

DISCUSSION

Two parent cell lines were established from bone marrow and right axillary lymph node metastases of a 1-yr 2-mo-old infant with Stage IV neuroblastoma. In addition 6 cloned cell lines were obtained from a bone marrow-derived parent cell line and 12 from a lymph node-derived cell line. Several observations prove these cell lines to be derived from neuroblastoma tumors. In all the cell lines dense core granules and neurotubules were demonstrated by transmission electron microscopy (Fig. 2). Two catecholamine metabolites (dopamine and noradrenaline) were obtained from high-performance liquid chromatography (Table 2). Chromosome analysis demonstrated identical karyotypes in all lines, with double-minute chromosomes and a deletion of chromosome 1p, which is a characteristic of neuroblastoma.

Several differences in the cell lines, derived from the 2 metastatic sites, were observed. The plating efficiency of the lymph node-derived cell lines (RT-LN and RT-LN-1) was higher than marrow-derived cell lines (RT-BM and RT-BM-4) (Fig. 1). Different growth characteristics of the 2 lines were observed as the bone marrow-derived cell lines form focal aggregates of cells, while lymph node-derived cell lines grow in a single cell layer (Fig. 3). The levels of catecholamine metabolites (dopamine) in RT-LN and RT-LN-1 lines were significantly higher than in RT-BM and RT-BM-4 cell lines (Table 2).

Analysis of cell surface membrane antigen expression using a panel of 9 MoAbs showed identical expression of surface membrane antigens on the 2 parent cell lines and 6 cloned cell lines derived from bone marrow and 12 cloned cell lines derived from lymph node tumors. The binding profiles of the 2 parent and total 18 cloned cell lines were identical, although variations in the number of stained cells in each line were observed (Table 3).

**Table 3 Determination of cell surface antigen expressions on bone marrow tumor cells and parent and cloned cell lines by a panel of MoAbs**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Bone marrow tumor cells*</th>
<th>Parent cell lines</th>
<th>Cloned cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA-1</td>
<td>64</td>
<td>93 ± 7*</td>
<td>97 ± 5*</td>
</tr>
<tr>
<td>BA-2</td>
<td>48</td>
<td>87 ± 3</td>
<td>93 ± 6</td>
</tr>
<tr>
<td>J-5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OKIa1</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UJ-13A</td>
<td>94</td>
<td>98 ± 3</td>
<td>99 ± 4</td>
</tr>
<tr>
<td>UJ-127-11</td>
<td>89</td>
<td>83 ± 3</td>
<td>88 ± 7</td>
</tr>
<tr>
<td>Anti-Thy-1</td>
<td>75</td>
<td>99 ± 4</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>HSAN1.2</td>
<td>55</td>
<td>90 ± 4</td>
<td>90 ± 6</td>
</tr>
<tr>
<td>PI153/3</td>
<td>63</td>
<td>90 ± 6</td>
<td>93 ± 7</td>
</tr>
</tbody>
</table>

* Ninety-nine % of bone marrow cells was morphologically determined as tumor cells with the May-Giemsa stain.

MoAbs enables us the differential diagnosis of neuroblastoma cell lines from leukemia/lymphoma, rhabdomyosarcoma, and Ewing's sarcoma, as previously reported by us (8). Few studies of surface membrane antigens on neuroblastoma cells using MoAbs have been reported. In human solid tumors, most of the studies have been undertaken on melanoma cells, which are derived from the same embryological origin as neuroblastoma. All of the reports on melanoma cells demonstrate heterogeneous surface membrane antigen profiles of primary and metastatic tumors in the same patient (13). In addition cell lines derived from different metastases of the same patient (14) and parent and cloned cell lines (15) show antigen heterogeneity.

With regard to the study of surface membrane antigens on neuroblastoma cells, different antigen profiles of UJ-127-11 and PI153/3 have been reported for tumors from 2 metastatic sites (liver and bone marrow) (16), and changes in antigen expression of UJ-13A and UJ-127-11 during the clinical course in a patient also have been reported (16). In contrast to these results, our present study suggests antigen expression in neuroblastoma cell lines, derived from different sites, parallels that upon the original bone marrow tumor cells and remains stable in vitro for at least 2 yr. This suggests that the use of cell lines for determining reactivity of antibodies to fresh neuroblastoma cells is legitimate, particularly as it can be very difficult to obtain fresh tumor tissue. Ultimately it should prove possible to obtain panels of MoAbs to aid in the differential diagnosis of all the small round cell tumors of childhood. This will undoubtedly prove a considerable benefit to the pathologist.

ACKNOWLEDGMENTS

Thanks are due to Dr. Y. Takeuchi for examining catecholamine fluorescence of these cell lines.

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Identical Expression of Cell Surface Membrane Antigens on Two Parent and Eighteen Cloned Cell Lines Derived from Two Different Neuroblastoma Metastases of the Same Patient

Tohru Sugimoto, Tadashi Sawada, Takaiofumi Matsumura, et al.


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