Morphology, Growth, Chromosomal Pattern, and Fibrinolytic Activity of Two New Human Neuroblastoma Cell Lines


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SUMMARY

Neuroblastoma cell lines LA-N-1 and LA-N-2 were established from neuroblastoma cells in the bone marrow and in the primary tumor, respectively, of two children with metastatic neuroblastoma. Morphology, growth in vitro and in athymic nude mice, chromosomal pattern, and fibrinolytic activity of these cell lines and of previously established human neuroblastoma cell lines IMR-32, SK-N-MC, and SK-N-SH were compared. Most LA-N-1 cells were tear-drop shaped, small cells with processes; they tended to grow in clusters. LA-N-2 was comprised of elongated cells and small round cells, the latter growing in dense clumps on the former. Electron microscopy revealed numerous cytoplasmic dense cores in many LA-N-1 cells but none in LA-N-2 cells. During logarithmic growth in vitro, doubling times for LA-N-1, LA-N-2, SK-N-MC, SK-N-SH, and IMR-32 cells were 32, 56, 23, 36, and 26 hr, respectively. Cells of all lines formed colonies in soft agar, and, after variable latency periods, LA-N-1, LA-N-2, SK-N-MC, and IMR-32 cells formed tumors in athymic nude mice. The marker chromosome(s) characteristic of each cell line was present in more than 90% of cells of a given line. Significant plasminogen-dependent fibrinolytic activity was present in cells of all lines. These studies indicate that LA-N-1 and LA-N-2 cells arose from single but different aberrant progenitor cells and that they have properties of neuroblastoma cells. They also demonstrate that cell lines derived from human neuroblastomas are heterogenous as are the tumors in children.

INTRODUCTION

Neuroblastoma, a malignant neoplasm that arises in the adrenal medulla or sympathetic ganglion, is the most common solid tumor in children (26). The prognosis for approximately 66% of children with this tumor is extremely poor (4), indicating the need for new and improved therapy. One approach to developing new therapy is to use well-characterized, continuously growing human neuroblastoma cell lines as experimental in vitro and in vivo models of the tumor. With this approach, a large number of potentially useful therapies could be evaluated; then the most successful could be used in clinical trials. Ideally, such experimental studies should have available a number of human neuroblastoma cell lines with properties similar to those observed for neuroblastoma in children. However, only 3 established lines, IMR-32 (22), SK-N-SH, and SK-N-MC (2), are generally available.

We have established 2 new human neuroblastoma cell lines, LA-N-1 and LA-N-2. This paper reports that cells of these lines have morphological, chromosomal, fibrinolytic, and growth characteristics of malignant cells and of neuroblastomas; we report elsewhere that these cells retain neurochemical properties of the tumors from which they were derived (24, 25).

MATERIALS AND METHODS

Tumors and Cell Culture. LA-N-1 was derived from neuroblastoma cells in bone marrow of a 2-year-old male with clinical Stage IV neuroblastoma. The child had elevated levels of vanillylmandelic acid and homovanillic acid in his urine, and his tumor did not respond to chemotherapy (cyclophosphamide, dimethyltriazenoimidazolecarboxamide, vincristine, and Adriamycin). The bone marrow specimen that was cultured was obtained 5 months after diagnosis and initiation of chemotherapy. It contained many neuroblastoma cells and was placed directly into 25-sq cm tissue culture flasks (No. 3014; Falcon Plastics, Oxnard, Calif.) in Waymouth’s medium (Microbiological Associates, Los Angeles, Calif.) containing 30% heat-inactivated fetal calf serum (Reheis Chemical Co., Phoenix, Ariz.), glutamine (100 mM), and gentamycin (50 μg/ml). After 4 days, clumps of adherent cells were present; these were washed, and the medium was replaced. Medium was changed every 3 to 4 days until cells became subconfluent, at which time they were removed with trypsin-Versene (1 part 0.25% trypsin in Tris-0.9% NaCl solution and 3 parts 0.02% Versene in Dulbecco’s phosphate-buffered saline, pH 7.3; Microbiological Associates) and seeded into 2 flasks. This procedure was continued with aliquots of cells being viably frozen in liquid nitrogen vapor at various passages.

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LA-N-2 was derived from the primary tumor of a 3-year-old female with clinical Stage IV neuroblastoma. Diagnosis was confirmed pathologically; this child did not have elevated levels of catecholamines, their precursors, or their metabolites in her urine but did have cystathioninuria. A portion of the biopsy specimen obtained for diagnostic purposes before initiation of therapy was cut into 1-mm fragments and seeded into 25-sq cm culture flasks containing Waymouth fetal calf serum medium as above; the remainder was freeze-stored in the vapor phase of liquid nitrogen. After the 1st 5 days of culture, medium was changed every 3 to 4 days. After approximately 2 weeks, clumps of adherent cells were observed; these were allowed to become subconfluent, at which time they were removed with trypsin-Versene and seeded into new flasks. As with LA-N-1, aliquots were viable frozen in the vapor phase of liquid nitrogen at various passages.

Other neuroblastoma cell lines studied included SK-N-SH, SK-N-MC (2), and IMR-32 (22). These cells all were grown in Eagle’s minimal essential medium (Microbiological Associates), containing 15% heat-inactivated fetal calf serum, glucose (100 mM), and gentamycin (50 µg/ml). When cells became confluent, they were removed with trypsin-Versene and seeded into new flasks.

All cell lines were examined for contaminating microorganisms. All tests, including culture, fluorescence (14), and electron microscopy for Mycoplasma and for viruses, were negative.

**Light and Electron Microscopy.** Phase-contrast microscopy of living cells attached to culture flasks was carried out with a Nikon inverted microscope. For electron microscopy, cells attached to culture flasks were rinsed in 0.1 M cacodylate buffer, pH 7.3, and then were fixed overnight in 2% glutaraldehyde with 0.1 M cacodylate buffer. The cells were then washed with buffer, scraped from the surface of tissue culture flasks, and pelleted by centrifugation. Pellets were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, washed in buffer, dehydrated in alcohol and propylene oxide, and embedded in Epon 812. Thin sections were mounted on carbon-formvar-coated, 1-hole grids and stained with uranyl acetate and lead citrate. Sections were examined and photographed with a Siemens IA electron microscope.

**Isoenzyme Analysis.** Lysates of cultured neuroblastoma cells were analyzed electrophoretically by standard techniques for the enzymes: adenylate kinase (6), 6-phosphogluconate dehydrogenase (6), adenosine deaminase (19), phosphoglucomutase I (6), acid phosphatase (17), glucose 6-phosphate dehydrogenase (18), and esterase D (8).

**Growth in Vitro.** Doubling time and saturation density were determined by seeding 5 to 50 x 10⁶ cells into 2-sq cm wells of tissue culture plates (No. FB-16-24-TC; Linbro of the Pacific, Van Nuys, Calif.), followed by counting adherent cells at 24-hr intervals in 3 replicate wells. Cell doubling times were calculated from the best-fit line for logarithmic growth, and saturation density was calculated once growth stopped. Medium was changed every 4 days to ensure adequate growth conditions. Plating efficiency of cells on a plastic surface was determined by seeding 10⁵ cells into 60-mm culture dishes and counting colonies after 2 weeks. The ability of cells to form colonies in soft agar was determined by seeding 10⁶ or 10⁷ cells into 60-mm culture dishes and then examining dishes weekly with a stereo microscop for colonies of 8 or more cells (10).

**Growth in Nude Mice.** Four-week-old female homozygous nude mice (NIH Swiss background; mice obtained from the Frederick Cancer Research Center, Breeding Section, Frederick, Md.) were given s.c. injections of 5, 10, or 30 x 10⁶ cultured cells suspended in 0.5 ml of complete tissue culture medium. Both time to detectable tumor (3-mm diameter) and subsequent growth of tumor were determined.

**Chromosomal Analysis.** Cells were exposed to Colcemid at a concentration of 1 µg/ml for 2 to 3 hr. After hypotonic treatment with 0.075 M potassium chloride:0.97% sodium citrate (1:1), the cells were fixed in methanol:acetic acid (3:1). Chromosome preparations were made according to the air-dry technique (1). The following day, chromosomes were banded by immersing slides once for 15 to 30 sec in trypsin-EDTA solution at room temperature and then by staining with 10% phosphate-buffered Giemsa at pH 6.8 (Gurss Giemsa; Biomedical Specialties, Santa Monica, Calif.) (16).

**Fibrinolytic Activity.** Intact cell fibrinolytic activity was determined as previously described (11). Briefly, 5 x 10⁶ cells in 5 ml Eagle’s minimal essential medium or Waymouth’s medium containing 10% human serum were added to 60-mm culture dishes that had been coated with 125I-labeled fibrin (2 µg/sq cm; 500 cpm/µg). The 125I-labeled fibrin dishes were incubated for 24 hr at 37°, and then 0.5 ml of the supernatant medium was counted in a gamma counter. The fibrinolytic activity was expressed as the percentage of the total 125I-labeled fibrin that was solubilized in 24 hr. Plasminogen dependence of fibrinolytic activity was tested using lysates of cells (9).

**RESULTS**

**Morphology.** Light and electron microscopic characteristics of LA-N-1 and LA-N-2 cells were determined (Fig. 1). The predominant cell in LA-N-1 was a tear-drop-shaped cell with processes; these cells tended to grow in clusters. In general, the appearance of LA-N-1 cells was similar to that of IMR-32 and SK-N-SH cells. LA-N-2 was composed mostly of large, elongated cells with processes, but some were small and round. The latter tended to grow in dense clusters on the former and to detach readily.

Electron microscopy revealed that many LA-N-1 but not LA-N-2 cells had cytoplasmic dense cores of approximately 100-nm diameter. Some SK-N-SH cells also had a single cytoplasmic dense core; however, the frequency of such cells was much less than that observed for LA-N-1. Rarely, an IMR-32 cell had dense cores; SK-N-MC cells did not have dense cores. None of the cells had cytoplasmic or nuclear viral particles.

**Isoenzymes.** Analysis of 7 isoenzymes for each cell line showed that all were of human origin and that all had distinct isoenzyme patterns (Table 1). None had isoenzyme patterns of HeLa cells.
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Table 1
Isoenzymes of human neuroblastoma cell lines

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>LA-N-1</th>
<th>LA-N-2</th>
<th>SK-N-MC</th>
<th>SK-N-SH</th>
<th>IMR-32</th>
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<tbody>
<tr>
<td>Adenylate kinase</td>
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<td>1-1</td>
<td>1-1</td>
<td>1-1</td>
<td>1-1</td>
</tr>
<tr>
<td>6-Phosphate-glucuronate dehydrogenase</td>
<td>AA</td>
<td>AA</td>
<td>AA</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>1-1</td>
<td>1-1</td>
<td>1-1</td>
<td>1-1</td>
<td>1-1</td>
</tr>
<tr>
<td>Phosphoglucomutase I</td>
<td>1-1</td>
<td>2-1</td>
<td>1-1</td>
<td>1-1</td>
<td>2-2</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>A</td>
<td>BA</td>
<td>BA</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Esterase D</td>
<td>2-1</td>
<td>1-1</td>
<td>2-2</td>
<td>1-1</td>
<td>1-1</td>
</tr>
</tbody>
</table>

**Growth in Vitro.** Colony formation on plastic surfaces and in soft agar, doubling time, and saturation density were determined (Chart 1; Table 2). Single cells of all lines formed colonies on plastic and in agar. Seeding 10^6 cells onto plastic and 10^5 cells into agar resulted in 1 to 8% and 1 to 43% plating efficiency, respectively. LA-N-2 cells formed the lowest number of colonies in each test system. Doubling times were 23 to 36 hr except for LA-N-2 which was 56 hr. Saturation densities for LA-N-1 and LA-N-2 cells were 3.8 and 3.5 x 10^5/sq cm, respectively, whereas they were 5.0 to 5.6 x 10^5/sq cm for cells of the other lines.

**Growth in Nude Mice.** Cultured neuroblastoma cells (5 to 30 x 10^6) were injected s.c. into 4-week-old female homozygous mice to determine the latent period and incidence of tumors. In a representative experiment (Chart 2), tumors appeared after injection of 5 x 10^6 LA-N-1, LA-N-2, SK-N-MC, and IMR-32 cells in 7 of 12, 1 of 6, 11 of 12, and 5 of 12 mice, respectively. Median times from injection to development of 3-mm-diameter tumors were 43 days for LA-N-1, 33 days for LA-N-2, 8 days for SK-N-MC, and 59 days for IMR-32. Injection of 10^7 or 3 x 10^7 LA-N-1 cells increased the incidence of tumors to 83 and 100% and decreased the latent period to 26 and 19 days, respectively. However, increased numbers of cells did not significantly influence these parameters for LA-N-2 or IMR-32. Chromosomal pattern and pathology of these tumors confirmed that they were human neuroblastomas. Although SK-N-SH cells have been reported to grow in nude mice (7), they did not do so in 3 experiments when 5 to 30 x 10^6 cells were injected.

**Chromosomes.** Chromosomal analyses were performed on the 5 neuroblastoma cell lines (Table 3; Fig. 2). SK-N-SH cells had a mode of 47 chromosomes, and each metaphase contained a marker chromosome (M) that was homologous to the No. 7 chromosome but with 3 extra bands on the long arm. No other marker chromosome was found in this cell line. IMR-32 cells had a modal number of 48 chromosomes with a range of 41 to 51. Marker chromosome M was present in every metaphase, and a few metaphases were found to have 2 or 3 M chromosomes. Giemsa banding showed that M was homologous to the No. 1 chromosome with an elongated, homogeneously stained region on the distal end of the short arm as reported by Biedler and Spengler (3). SK-N-MC cells had a modal number of 45 chromosomes with a range of 43 to 50; however, 4% of cells were polypoid. Two abnormal marker chromosomes, M1 and M4, were found in each metaphase. M1 was a large acrocentric chromosome of undetermined origin, and M4 was homologous to the No. 3 chromosome with an addition of 2 bands on the long arm. No other abnormal chromosomes were seen in these 3 cell lines.

LA-N-1 cells had a mode of 87 chromosomes with a range of 47 to 87; about 4% of cells had 47 chromosomes. An abnormal, large, acrocentric marker chromosome, M, was found in 92% of the cells analyzed. The entire long arm of

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this chromosome was homogeneously stained.

The LA-N-2 line was comprised of cells having 46 to 78 chromosomes with a mode of 73. No metaphase cells had markers M₁ to M₇; however, additional marker chromosomes M₈ and M₉ were discovered. M₈ was a No. 1 chromosome with an additional band on the long arm, whereas M₉ was a dicentric, which appeared to involve rearrangements of 2 No. 1 chromosomes.

Fibrinolytic Activity. Intact cell fibrinolytic activity was readily demonstrated in all cell lines (Table 4). SK-N-SH cells had the most fibrinolytic activity, followed by LA-N-2 and LA-N-1 cells with SK-N-MC cells having the least amount of activity. This fibrinolytic activity was plasminogen dependent (data not shown). Little fibrinolytic activity was found in supernatant media of any of these cell lines.

DISCUSSION

Two new cell lines, LA-N-1 and LA-N-2, have been established from neuroblastomas of 2 children with metastatic disease. LA-N-1 was derived from bone marrow metastases of a catecholamine-producing neuroblastoma, and LA-N-2 was derived from the primary site of a noncatecholamine-producing neuroblastoma. Investigations reported here and elsewhere demonstrate that cells of these lines have properties associated with cancer and also have neurochemical characteristics of the tumors from which they were derived (24, 25).

Anchorage-independent growth often is associated with malignant transformation (10). In vitro, LA-N-1 and LA-N-2 cells form colonies in soft agar, grow in clumps, and reach high saturation densities. All of these observations indicate that replication of these cells is not anchorage dependent. The chromosomal patterns of LA-N-1 and LA-N-2 cells are abnormal, supporting the concept that they are malignant cells (13). Both lines are hypotetraploid and have their own unique marker chromosomes. These markers are present in virtually all cells of a given line, indicating that the 2 tumors were derived from a single but different aberrant cell. This is in agreement with other studies which have shown that...
human tumors most often are derived from 1 malignant progenitor cell (13). Marker chromosomes in LA-N-1 and IMR-32 cells had large, homogeneously stained regions. This confirms previous findings with IMR-32 (3) and demonstrates another human neuroblastoma cell line with this unusual type of chromosome.

Fibrinolytic activity usually is higher in tumor cells than in their normal counterparts (11). In addition, increased fibrinolytic activity has been associated with anchorage-independent growth of tumor cells in vitro (12). Our study of 5 human neuroblastoma cell lines demonstrated that they all have significant plasminogen-dependent fibrinolytic activity. Only 1 human neuroblastoma cell line, SK-N-SH, has been studied previously, and, as in our experiments, it was found to have plasminogen-dependent fibrinolytic activity (23).

Perhaps the most reliable indicator of malignant transformation is tumor formation in vivo (20). Injection of LA-N-1 and LA-N-2 cells into athymic nude mice results in formation of tumors that are similar to neuroblastomas pathologically and that have chromosomal patterns of the cultured cells. Although LA-N-1 cells produce tumors in nearly all mice when 1 to 3 x 10^6 cells are injected, LA-N-2 cells do so infrequently. The low tumorigenicity of LA-N-2 cells may be related to its longer doubling time.

In addition to having characteristics of malignant cells, LA-N-1 and LA-N-2 cells have ultrastructural and neurochemical properties of neuronal tumors. LA-N-1 cells have multiple cytoplasmic dense cores such as those present in sympathetic neurons, pheochromocytomas, and neuroblastomas (21). Dense cores, which are catecholamine storage granules, have been observed in only 1 other human neuroblastoma cell line (15). The action potential Na^+ ionophore, which is a property of neurons (5), is in LA-N-1 and LA-N-2 and also in SK-N-SH and IMR-32 cells (24, 25). This ionophore has been found in some clones of murine C1300 neuroblastoma (5), but it has not been described previously in human neuroblastoma cell lines. Tyrosine hydroxylase, an adrenergic marker, is in LA-N-1 cells, and acetylcholine, a cholinergic marker, is in LA-N-2 cells (24, 25). These neurotransmitter markers indicate that the cell lines retain neuronal properties of the tumors from which they were derived: LA-N-1 was derived from a vanillylmandelic acid- and homovanillic acid-producing tumor, whereas LA-N-2 was from a catecholamine-negative, acetylcholine-positive tumor (data not shown).

Human neuroblastomas and cell lines derived from them are heterogeneous. Neuroblastomas vary in their tumorigenicity (4), and this is maintained in cell lines as reported here. Most neuroblastomas produce catecholamines, their precursors, or their products. LA-N-1 cells, which were derived from a vanillylmandelic acid- and homovanillic acid-producing tumor, have tyrosine hydroxylase and cytoplasmic catecholamine granules and thus probably represent this largest group of neuroblastomas. Both SK-N-SH (2) and IMR-32 (22) cells may represent variants of adrenergic neuroblastomas. SK-N-SH was derived from a vanillylmandelic acid-producing neuroblastoma and has dopamine-ß-hydroxylase (2) but only low levels of tyrosine hydroxylase (24, 25) and occasional cytoplasmic catecholamine granules. It was not reported whether IMR-32 was derived from a catecholamine-producing neuroblastoma; however, it does have tyrosine hydroxylase (24, 25) and rare catecholamine granules. A 2nd small proportion of neuroblastomas does not produce catecholamines. Our studies suggest that these may produce acetylcholine or may have minimal neuronal functions (24, 25). LA-N-2 cells which have the action potential Na^+ ionophore and which produce acetylcholine provide a model for the former; SK-N-MC cells with only choline acetyl transferase (7) may represent the latter. A 3rd group of neuroblastomas may have both adrenergic and cholinergic properties as suggested by recent studies of established cell lines (15).

We conclude from our studies that human neuroblastoma cell lines should provide good experimental models for developing new therapeutic approaches. Our current immunological and chemotherapy investigations support this conclusion. Cultured human neuroblastoma cells have common cell-type-specific cell-surface antigens, and our preliminary results indicate that some of these antigens are on neuroblastoma tissues. In addition, preliminary experiments suggest that LA-N-1 tumors in athymic nude mice do not respond to cyclophosphamide, whereas SK-N-MC tumors do. This response pattern was observed in the patients from whom the cell lines were derived.

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REFERENCES

Characteristics of New Neuroblastoma Cell Lines

Fig. 1. Light and electron microscopy of human neuroblastoma cell lines. A, LA-N-1, Passage 44, × 500; B LA-N-1, Passage 24, × 23,000. Portions of several cells that contain cytoplasmic dense cores are shown. C, LA-N-2, Passage 41, × 500; D, LA-N-2, Passage 40, × 12,000. No cytoplasmic dense cores are present.
Fig. 2. Marker chromosomes of human neuroblastoma cell lines. Trypsin-Giemsa banding patterns of marker chromosomes and of their homologous normal chromosomes are shown for each cell line.
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