Establishment and Characterization of a Cell Line (Wa-2) Derived from an Extrarenal Rhabdoid Tumor


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ABSTRACT

A new human cell line (Wa-2) derived from an extrarenal rhabdoid tumor has been established. The cell line grows as a monolayer consisting of round- and spindle-shaped cells. Injection of cells into nude mice results in the growth of solid tumors within 2 wk of inoculation. These solid tumors have the microscopic appearance similar to that of the original tumor from which the cell line was derived. Moreover, the tumors have all the features of rhabdoid tumors, including the intracytoplasmic hyaline globules, large prominent nucleoli, and inclusion bodies made up of whorls of fibrillar material. Transplanted tumor cells stain positive for vimentin, cytokeratin, actin, and desmin and negative for myoglobin and neuron-specific enolase. Karyotyping of the cell line at different passages and cells derived from the transplant tumors consistently revealed a diploid number of chromosomes with a reciprocal translocation between chromosomes 18 and 22 (t[18;22] (q21;p11.2)). In fibroblasts derived from the patient, no translocation could be found. Culturing the cells in the presence of 1-/3-D-arabinofuranosylcytosine induces differentiation, characterized by the outgrowth of neuron-like processes and the morphological appearance of cells similar to neuroblasts. Southern blot analysis showed no amplification of the N-myc oncogene. Since no published cell line derived from rhabdoid tumors exists, this cell line should be helpful to further elucidate the histogenesis and histological origin of the malignant rhabdoid tumor.

INTRODUCTION

Rhabdoid sarcoma is a very malignant and rare tumor in infancy and early childhood. Usually occurring in the kidney (1–3), rhabdoid tumors arising from extrarenal sites have been described (4–11). Histologically, the tumors consist of oval to polygonal cells with eosinophilic cytoplasm. The presence of eosinophilic inclusions composed of whorls of intermediate filaments has suggested rhabdomyoblastic differentiation (2), but ultrastructural studies could not demonstrate any signs of cross-striations, and there is no evidence of a myogenic origin (12, 13).

Rhabdoid sarcoma is regarded as an entity separate from Wilms' tumor or rhabdomyosarcoma (2, 3, 12, 14). However, the origin of this tumor is still an open question. There are several hypotheses concerning the histogenesis and etiology. A neural crest origin has been suggested because of similarities with APUD tumors (2). A histiocytic origin has been discussed because of staining for lysozyme and the formation of rosettes in tissue culture with sheep erythrocytes (14). Additionally, a dual epithelial and mesenchymal origin has been hypothesized (15). However, the histological origin of this tumor has not been well defined. This is hampered by the fact that no established cell line from rhabdoid tumor exists.

In this paper we report the establishment of a cell line derived from a paravertebral tumor with features of rhabdoid tumor. This cell line should help to elucidate the histogenesis and biology of this very aggressive malignancy.

MATERIALS AND METHODS

Case Record. A 17-mo-old boy had been referred to our hospital because of obstipation and paralysis of the left lower extremity. A subsequent myelography revealed an obstruction at the T11 level. Computer tomography showed an intraspinal epidural tumor with an extraspinal paravertebral extension through the intervertebral foramina with infiltration of the left parietal pleura and paravertebral muscles. The tumor was removed subtotally, and chemotherapy with actinomycin D, ifosfamide, and vincristine was started. This regimen did not show any effect on the tumor growth, since subsequent computer scans revealed local tumor growth during therapy. Another subtotal tumor resection was performed. A chest X-ray was positive for pleural effusions and for signs of carcinomatous lymphangiosis. A pleural puncture was performed and was positive for tumor cells. The boy died 4 mo after the first diagnosis was made.

Establishment of the Cell Line. Tumor cells from the pleural effusion were isolated by layering on a Lymphoprep gradient (Nyegaard, Oslo, Norway) and centrifugation for 20 min at 400 × g. The cells were harvested and resuspended in RPMI 1640 culture medium (Biochrm, Berlin, FRG) supplemented with 10% fetal calf serum (Biochrm), 2 mmol of l-glutamine (Biochrm), 50 units/ml of penicillin, 50 μg/ml of streptomycin (Biochrm), and 25 mmol of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Biochrm). The tumor cells were then transferred to 24-well culture plates (Costar). After several days, the cells became adherent and started to grow as a monolayer. When they reached confluency, they were trypsinized and transferred to larger culture dishes. Only a few fibroblasts were present at the initiation of the culture, and they disappeared after four weeks. After stable growth in culture, cells were split every 3 to 4 days by trypsinization.

Histological and Ultrastructural Investigations. Tumor tissue from the primary tumor obtained during surgery and from tumors transplanted into nude mice was fixed in 10% formaldehyde or in absolute alcohol and embedded in paraffin. Sections were cut at 3 μm and stained with hematoxylin:eosin and periodic acid-Schiff.

For ultrastructural studies, tumor tissue from the primary tumor or single tumor cell suspension obtained from the cell line by short trypsinization was fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer, pH 7.4. The cell suspension or tissue was then postfixed in 1% cacodylate-buffered osmium tetroxide, washed in buffer, dehydrated in ethanol, and subsequently stained overnight in 70% ethanol saturated with uranyl acetate. Dehydration was continued using absolute ethanol and propylene oxide. Tissue or cells were then embedded in Araldite (Ciba, Basel, Switzerland) and sectioned on a LKB Nova ultramicrotome. Sections were stained with lead citrate. Electron micrographs were taken with a Zeiss EM 10 electron microscope.

Immunohistological Studies. For immunohistological studies, paraffin-embedded tumor tissue sections from the primary tumor or from
transplanted tumors were cut at 3 μm, placed on poly-L-lysine-coated glass slides, deparaffinized in xylene, rehydrated in a graded series of alcohol, and stained using the peroxidase-antiperoxidase method. The antibodies used for the immunohistological studies, their reactivity, their source, and working dilutions have been described previously (16).

Chromosomal Analysis. Colcemid (4 μg/ml; Sigma) was added to the cell culture or to cells derived from transplanted tumors 2 h prior to harvest. The harvest and chromosomal preparation were standard. Hypotonic treatment was done with 0.075 M potassium chloride solution (Sigma) for 20 min. Freshly made fixative (methanol:glacial acetic acid, 3:1) was used for the washes.

Xenograft Procedures. Cells derived from the cell culture at different passages were injected into the flanks of nude mice (BALB/c-nu/nu). Within 2 wk of inoculation, solid tumor nodules at the site of the inoculation occurred and were explanted for histological studies as well as for chromosomal analysis.

RESULTS

Histopathological and Immunohistochemical Findings in the Primary and Transplanted Tumors. Histological examination of the primary tumor revealed a highly cellular tumor tissue consisting of closely packed, medium-sized to large round or polygonal cells with ample, eosinophilic, occasionally clear cytoplasmatic organelles including few round or oval mitochondria, short strands of endoplasmatic reticulum, free ribosomes, rare lysosomes, and groups of large, pale staining vacuoles. Persistent findings included large numbers of intermediate filaments which were either arranged randomly or occurred in concentric whorls. Cross-striations were not detected (Fig. 1B).

The immunohistochemical examination of the primary tumor showed a positive staining pattern for vimentin, S-100, actin, and desmin. A few cells could be stained with antibodies against epithelial membrane antigen. Single cells contained cytokeratin. Stains for NSE and myoglobin were negative. Tumor cells derived from the transplanted tumors were strongly positive for vimentin, actin, and desmin. A number of cells were positive for protein S-100, and single cells contained cytokeratin. Stains for epithelial membrane antigen, neuron-specific enolase, and myoglobin were negative. Results of the immunohistochemical examination of the primary and transplanted tumors are summarized in Table 1.

Ultrastructural Studies. Ultrastructural examination of the primary tumor showed round cells with short cytoplasmic processes. Nuclei were round to oval, sometimes elongated, and usually with smooth contours. Nucleoli were prominent and usually centrally located. Cytoplasmatic organelles included few round or oval mitochondria, short strands of endoplasmatic reticulum, free ribosomes, rare lysosomes, and groups of large, pale staining vacuoles. Persistent findings included large numbers of intermediate filaments which were either arranged randomly or occurred in concentric whorls. Myosin filaments were not present, and cross-striations were not seen. Electronmicroscopic studies of cultured cells at different passages (tenth and 52nd passage, respectively) also showed concentric whorls of intermediate filaments (Fig. 2, B and C). Again, cross-striations or myosin filaments were not found.

Cytogenetics. Karyotyping of the cell line at different passages increased number of reticulin fibrils. As in the primary tumor, cross-striations were not detected (Fig. 1B).

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Table 1 Antibody panel used in antigenic characterization of Wa-2 cells

<table>
<thead>
<tr>
<th>Antibody against</th>
<th>Reactivity</th>
<th>Primary tumor</th>
<th>Transplanted tumor</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>Vimentin</td>
<td>+</td>
<td>+</td>
<td>Mesenchymal cells</td>
<td></td>
</tr>
<tr>
<td>Desmin</td>
<td>+</td>
<td>+</td>
<td>Muscle cells</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
<td>Contractile proteins</td>
<td></td>
</tr>
<tr>
<td>Protein S-100</td>
<td>+</td>
<td>±</td>
<td>Neural cells and several other cell types</td>
<td></td>
</tr>
<tr>
<td>Epithelial membrane antigen</td>
<td>-(+)⁴</td>
<td>-</td>
<td>Various epithelial and plasma cells</td>
<td></td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>-(+)</td>
<td>-(+)</td>
<td>Epithelial cells</td>
<td></td>
</tr>
<tr>
<td>Neuron-specific enolase</td>
<td>-</td>
<td>-</td>
<td>Neural cells and several other cell types</td>
<td></td>
</tr>
<tr>
<td>Myoglobin</td>
<td>-</td>
<td>-</td>
<td>Skeletal muscle cells</td>
<td></td>
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* All cells were positively stained.
* Only a few cells were positive.

Fig. 2. Electron micrograph from the paravertebral malignant rhabdoid tumor from the patient with perinuclear intermediate filaments in concentric whorls (A) (× 16,500) and from Wa-2 cells derived from the tenth (B) (× 10,000) and the 50th (C) (× 13,000) cell culture passages. Concentric whorls of intermediate filaments are also present in cultured cells.

EXTRANAL RHABDOID TUMOR

(deptied in detail. The translocation between chromosomes 18 and 22 proved to be a tumor-specific translocation, since karyotyping of fibroblasts derived from the patient revealed diploid chromosomes without any structural aberration (not shown).

Establishment of Xenografts in Nude Mice. To further prove the malignant character of the cell line, single cell suspensions derived from the cell line at different passages were injected s.c. into the flanks of BALB/c-nu/nu mice. Within 2 to 4 wk after injection, growth of solid tumors could be observed in each animal given injections (Table 2). Histological, immunohistochemical, and ultrastructural features of these transplanted tumors were very similar to those of the primary tumor.

In Vitro Growth Kinetics. The doubling time of the cell line at the 50th passage was determined to be 53 h.

Soft Agar Cloning. To determine the cloning efficiency in soft agar, cells from the 50th passage were plated at 1 × 10³ cells/ml. Plates were scored for colonies after 12 days. The cell line has a high cloning efficiency, since about 50% of the plated tumor cells were able to form colonies (>40 cells) in soft agar.

Differentiation Studies. In order to get some hints about the histogenetic origin of MRT, we investigated the possibility of inducing differentiation in the cell culture. In screening experiments it was found that ara-C induces a morphological differentiation of the Wa-2 cells. Therefore, cells between the 50th and the 60th cell passages were incubated with various amounts of ara-C for different time periods. In the absence of ara-C, the cell culture consists of round and polygonal cells (Fig. 4A). Occasionally, some spindle-shaped cells can be seen. In the presence of 200 ng/ml of ara-C for 6 to 10 days, however, a change in the cell morphology of most cells was noted. This included the protrusions of neuron-like processes and the appearance of the cells similar to neuroblasts (Fig. 4B). Additionally, intensive contact formation between the neurite-like structures could be observed (Fig. 4C).

Southern Blot Analysis. Since the differentiation studies suggested a neuroectodermal origin of the tumor cells, we looked for amplification of the N-myc oncogene. However, Southern analysis of the DNA derived from the cell culture at the 50th passage showed only a single copy of the N-myc oncogene (Fig. 5).

DISCUSSION

In this paper, we describe the establishment of a cell line derived from an extrarenal MRT. The cell line has been in continuous culture for almost 3 yr. Further evidence for the malignant nature of this cell line is provided by the high cloning efficiency in soft agar and by the ability to form solid tumors in nude mice. The transplanted tumors retained the characteristic features which were seen in the primary tumor.

There are several characteristics which favor the diagnosis of MRT in this case. Histological examination of the primary tumor showed, in some areas, round cells with eosinophilic cytoplasm, eosinophilic inclusions, and large prominent nucleoli. In the ultrastructural examination of the primary tumor and of cultured cells, no cross-striation or other signs of myogenic origin were found. The eosinophilic inclusions appeared to consist of whorled aggregates of intermediate filaments of 5 to 10 nm in diameter. These intermediate filaments were very consistent finding, since they could be found in the primary tumor and after almost 3 yr of cell culture. Although these ultrastructural features are commonly found in MRT, they are not specific for MRT, since they may be observed in other malignancies, such as in APUD tumors (2) and in undifferen-
EXTRARENAL RHABDOID TUMOR

Fig. 3. Karyotype of Wa-2 cells derived from the 30th cell culture passage showing 46 chromosomes with an 18;22 translocation. The translocated segment on chromosome 22 is marked with an arrow (A). In Fig. 3B, the translocation t(18;22)(q21;p11.2) is depicted in more detail.

Table 2 Tumorigenicity of Wa-2 cells in nude mice
Wa-2 cells derived from different cell culture passages were injected s.c. into the flanks of nude mice.

<table>
<thead>
<tr>
<th>Cell culture passage</th>
<th>No. of cells injected</th>
<th>No. of mice with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>$5 \times 10^6$</td>
<td>3/3</td>
</tr>
<tr>
<td>30</td>
<td>$5 \times 10^6$</td>
<td>5/5</td>
</tr>
<tr>
<td>50</td>
<td>$5 \times 10^6$</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Timated blastemic cells typical of Wilms' tumor (19). Since MRT most often occurs in infants and small children, the age of our patient and the malignant course of the disease in addition to the histological features further support the diagnosis of MRT in this case.

Immunohistochemical studies have been performed by a number of authors (5, 9, 12–15, 20–25). Positive staining for vimentin intermediate filaments was a consistent finding in most of these studies. Expression of cytokeratin, S-100 protein, NSE, myoglobin, epithelial membrane antigen and desmin shows some degree of variability. In the case reported here, all tumor cells stained positive for vimentin and desmin, faintly positive for S-100 protein, and negative for myoglobin and NSE. The expression of desmin does not prove a myogenous origin of the tumor cell line, since expression of desmin is not restricted to tumors of striated and smooth muscles. It has been observed in other malignancies, such as malignant fibrous histiocytoma (26) and epithelioid sarcoma (27). Positive staining for desmin has already been described in MRT (16, 23).

The histogenesis of MRT is still a matter of debate. Several hypotheses concerning the histological origin exist, including a neuroectodermal, histiocytic, and dual mesenchymal-epithelial origin. From our differentiation studies with ara-C, we suggest a neuroectodermal origin of MRT. Our suggestion is based on the observation that incubation of the cells with ara-C induces the outgrowth of neuron-like processes. These processes come into contact with neighboring cell bodies or other neuron-like processes. Moreover, the cells acquire the morphological appearance of neuroblasts with the protrusion of several processes. Such neuroblast-like cells are typical for cells of neuroectodermal origin, such as neuroblastoma. Additional immunohistochemical studies with differentiated cells are under way to further prove the neuroectodermal origin of this cell line. ara-C, an inhibitor of DNA synthesis, is known to induce differentiation in myeloid cell lines (28) and in human neuroblastoma cells (29). The mechanisms by which differentiation is induced are not well understood. However, ara-C is a highly effective drug in the treatment of myeloid leukemias. It has also been used in the therapy of myelodysplastic syndromes. One can speculate that the clinical efficacy of ara-C may be at least partially due to its differentiation-inducing capacity. Our find-
hxtrarenal rhabdoid tumor

Fig. 4. Morphology of Wa-2 cells (55th passage) cultured in cell culture medium alone (A) and in the presence of 1-β-D-arabinofuranosylcytosine (200 ng/ml) for 10 days (B and C). ×100.

ings that ara-C induces differentiation of rhabdoid cells may be of clinical interest. The regimen which is successfully used in other pediatric solid tumors is not very effective in MRT. Therefore, ara-C could prove helpful in the therapy of MRT. Moreover, with the help of our continuous cell line, more antineoplastic agents can be screened in vitro.

In view of the aggressive clinical behavior of the primary tumor and the rapid growth kinetics of the cell line as well as of the xenografted cells, the finding of diploid chromosomes even after more than 20 mo in continuous culture is of special interest. Diploid DNA content in MRT has also been described by Schmidt et al. (16). Therefore, DNA content of tumors may not always be a prognostic factor for the clinical outcome of a malignant disease. In other pediatric tumors, such as nephroblastoma and Ewing's sarcoma, diploid DNA content has been reported. In our cell line, a tumor-specific reciprocal translocation between chromosomes 18 and 22 was found in the cell culture as well as in the transplanted tumors. Such a translocation has not previously been described in MRT. This translocation is tumor specific, since fibroblasts derived from the patient showed a normal diploid chromosomal pattern.

The involvement of chromosome 22 in MRT may be of particular interest, because of its involvement in other tumors of neuroectodermal origin, such as the 11;22 translocation in the PNET (30, 31) and in the malignant small cell tumor of the chest wall described by Askin et al. (32). The Askin tumor is now believed to be identical to neuroepithelioma. An 11;22 translocation indistinguishable from that observed in PNETs has also been found in Ewing's sarcoma (33, 34). There is good evidence that Ewing's sarcoma arises in cells of neuroectodermal origin (35, 36). The 18;22 translocation observed in our cell line may therefore be an additional hint to the neuroectodermal origin of MRT. Additionally, loss of regions of chromosome 22 is associated with other tumors of the nervous system, including Schwannomas, neurofibromas, meningomas, and astrocytomas (37). This may suggest a common molecular mechanism in the pathogenesis of these tumors. It is possible that the deleted or translocated regions of chromosome 22 contain a tumor suppressor gene and that deletion or alteration is one of the events leading to tumor growth. However, the translocation in our cell line affects the short arm of chromosome 22, whereas in the above-mentioned tumors the long arm of chromosome 22 is affected.

Another interesting clinical feature of MRT is its association with embryonal tumors of the central nervous system. In one report, renal MRT in six patients was associated with three cerebellar medulloblastomas, one pineoblastoma, one primitive neuroepithelial tumor (probably cerebral neuroblastoma), and one malignant subependymal giant cell astrocytoma (11). In these cases, the brain tumors were unlikely to be caused by metastasis, but rather were second malignancies. The reasons for this biological behavior are not clear. One explanation might be the in vivo production of growth factors by MRT, resulting in the stimulation of growth of susceptible cells preferentially in the central nervous system. Preliminary experiments showed that the Wa-2 cells are able to produce in vitro growth factors such as insulin-like growth factors I and II as well as not yet defined autocrine growth factors. Whether such growth factors are responsible for the secondary malignancies observed in MRT needs further investigation.

In summary, our established cell line derived from MRT offers a good model to study the histogenic origin and the
biology of MRT. Moreover, detailed molecular biological studies can be performed to characterize the genes involved in the 18;22 translocation. The further characterization of the growth factors produced by the cell line could help to better understand the biology of rhabdoid tumors, resulting perhaps in new therapeutic approaches. Additionally, an in vitro drug screening with this cell line is now under way in order to look for more effective drugs for the treatment of this very aggressive malignancy.

REFERENCES

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