Characterization of Cell Lines Derived from Xenografts of Childhood Rhabdomyosarcoma


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ABSTRACT

Three human rhabdomyosarcoma cell lines (Rh10, Rh18, and Rh28) have been established from three independently derived xenografts. These lines have been characterized as mesenchymal in origin (reactivity to desmin and vimentin antibodies) and as expressing a human fetal muscle surface antigen recognized by monoclonal antibody 5H11. Measurable levels of creatine phosphokinase have been detected in the cell lines. Rh10 and Rh28 exhibit the same chromosomal translocation and express an atypical lactate dehydrogenase isoenzyme which may be homologous to those previously reported in other tumor types. The karyotype analysis has confirmed that each cell line was derived from its respective tumor and thus provides a unique model for future investigations.

INTRODUCTION

RMS is the most common soft tissue sarcoma in children (1, 2) and represents 4–8% of all malignant diseases in children under 15 years of age (3, 4). Tumors arise de novo from skeletal muscle, and in many instances demonstrate morphological characteristics of differentiated muscle tissue. Histological fidelity is retained when these tumors are heterografted into immune-deprived mice (5), and such xenografts have been of value in determining biochemical determinants of drug sensitivity, and for identifying agents which may have clinical application in the treatment of RMS (6–8). Morphological characteristics of tumors suggest that embryonic tumors in children may result from a developmental disturbance during organogenesis that causes arrest at an early stage in the normal differentiation process to adult tissue (9). This embryonic tissue continues to proliferate inappropriately and produces a mass of immature tissue termed embryonal tumor. The genetic basis is unknown, but expression of recessive mutant alleles at the WAGR locus on human chromosome 11 band p13 and the Rb-1 locus on human chromosome 13 band q14 are involved in the development of Wilms tumor (10, 11) and retinoblastoma (12), respectively. Recent evidence indicates a chromosomal pathogenic mechanism common to RMS, hepatoblastoma, and Wilms tumor (13). The implication is that a common etiology in these three tumors involves pleiotropic expression of a mutant allele at the Wilms tumor (WAGR) locus on human chromosome 11 band p13. Data obtained by us (14) and others (15, 16) suggests also that in some RMS a nonrandom specific translocation, t(2;13), may occur, the breakpoint being close to the Rb-1 locus on chromosome 13.

In order to further study the relationship between cytogenetic observations and reported loss of heterozygosity at the WAGR locus, and “arrested” differentiation of RMS, we have established and characterized three cell lines of RMS that were established originally as xenografts. Since the LDH isoenzyme profiles of mouse and human are distinctive, these patterns were examined to verify the absence of mouse stromal contamination in the cell cultures. Creatine phosphokinase isoenzymes have been well characterized during myogenic cell differentiation. The RMS have therefore been examined for the presence of the foetal (BB), transitional (MB), and adult (MM) enzyme forms. Antibodies to muscle related or nonmuscle proteins were also used to further characterize the cell lines for comparison to their respective xenografts. Karyotype analyses of the cell lines and the xenograft tissue were used to confirm the human origin and fidelity of the established lines. These parallel in vitro-in vivo models may be of value in subsequent studies on the arrested differentiation of these cells in comparison to myogenesis of nonneoplastic populations of cells.

MATERIALS AND METHODS

Immune Deprivation of Mice. To establish the HxRh28 xenograft (human tumor xenografts have the Hx prefix), CBA/CaJ inbred female mice were immune deprived by the technique of infant thymectomy (4-week-old mice) followed 3 weeks later by whole body irradiation (950 rads; 137Cs source) given 48 h subsequent to a single i.p. administration of 1-ß-D-arabinofuranosycytosine (200 mg/kg) as described (6). Both HxRh10 and HxRh18 were established in mice immune deprived by thymectomy, irradiation, and bone marrow reconstitution. All xenografts were passaged subsequently in mice receiving 1-ß-D-arabinofuranosycytosine priming.

Tumors. Both HxRh10 and HxRh18 have been described previously (5). Both are of embryonal histology and were established directly as xenografts from surgical material derived from the primary site. HxRh28 was derived from a metastasis in the axillary node. Both the human tumor and its xenograft have alveolar histology. HxRh10 and HxRh28 are poorly differentiated and HxRh18 shows moderate differentiation.

Development of Cell Cultures. Xenograft tissue was removed aseptically and minced with crossed scalpels in Hanks’ balanced salt solution containing 80 µg/ml gentamicin. The suspension was separated from the particulate matter and centrifuged at 800 x g. The pellet was suspended in RPMI 1640 containing 20% fetal calf serum, 40 µg/ml gentamicin, and plated at high density in T-25 flasks. The monolayers were maintained for a period of 2 weeks in the above medium. At this time, the serum was gradually reduced to 10% and gentamicin replaced by penicillin-streptomycin. Cells were grown fresh medium and/or passed twice weekly.

Cellulose Acetate Gel Electrophoresis. Cells were lifted in trypsin-EDTA, washed twice in PBS pH 7.4, pelleted at 800 x g and resuspended in an equal volume of 0.25 M mannitol 0.1 mg/ml EDTA. After two cycles of freeze thawing the suspension was centrifuged (12,000 x g, 20 min/4°C). The supernatant was applied to cellulose acetate gels, presoaked in 0.04 M barbitone running buffer, pH 8.8, and electrophoresed in the cold at 200 V, for 35 min. Visualization buffers were as previously described for LDH (5) and CPK (17). Formazan development was monitored and at the appropriate time, gels were fixed for 5

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: RMS, rhabdomyosarcoma; LDH, lactate dehydrogenase; CPK, creatine phosphokinase; PBS, phosphate buffered saline.
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min in water: acetic acid: glycerol (69:30:1) and stored in 7% glycerol until photographed.

CPK Enzyme Assay. CPK activity in cell homogenates of the cell lines was determined using the Stat-zyme CPKs-1 kit (Worthington). Enzyme activity was measured by monitoring the rate of conversion of NAD to NADH according to the manufacturer’s protocol.

Rabbit Antimouse Serum Production. Female New Zealand white rabbits were injected s.c. with 10⁷ B-16 mouse melanoma cells in Freund’s complete adjuvant at weekly intervals for 4 weeks. Serum was prepared from 50 ml of blood drawn one week following the final injection from an immunized and a control rabbit. The sera were stored at −20°C in small aliquots. Serum from a nonimmunized rabbit was used as a source of lytic complement.

Indirect Immunofluorescent Staining. Cells were plated at 2.5 × 10⁴ cells/cm² in 35-mm culture dishes containing sterile collagen coated coverslips and incubated for a minimum of 24 h in culture medium. Cells were fixed with ethanol:acetic acid (3:1) and incubated for 45 min at room temperature with 1:100 fold dilution of monoclonal antibody 5.1 H11 in PBS (18). The coverslips were washed five times with PBS and incubated an additional 45 min with rabbit antimouse fluorescein isothiocyanate (1:10 dilution in PBS). After washing five times with PBS the cells were mounted under 50% glycerol, 0.5% azide, and examined for fluorescence (excitation, 450 nm; emission, 495 nm). Monoclonal antibody 5.1 H11 was a gift from Dr. F. S. Walsh, Institute of Neurology, London.

Immunoperoxidase Staining. Cell lines were plated onto a 4-well tissue culture chamber slide (Lab-Tek Tissue Culture, Miles Scientific, Naperville, IL). After confluence was reached, the slides were immersed in prechilled acetone for 10 min, and subsequently washed in PBS at pH 7.6 for 1 min. Tumor tissue from xenografts was fixed for 48 h in absolute ethanol and embedded in paraffin. Sections were deparaffinized and hydrated through xylene and graded series of alcohols, followed by a 5-min rinse in distilled water.

At this point, both cell lines and tissue sections were incubated for 30 min in 0.3% methanolic peroxide. They were subsequently incubated for 20 min with dilute normal horse serum and 15% bovine serum albumin. All incubation steps were interspersed with washes in PBS at pH 7.6. Slides were incubated with monoclonal anticytokeratin AE1-AE3 (Hybritech, San Diego, CA; dilution 1:50) antivimentin for cell lines (Lab Systems, Chicago, IL; dilution 1:50), antivimentin for alcohol-fixed tissue (DAKO, Santa Barbara, CA; dilution 1:50), antineurofilament (dilution 1:100, Lab Systems), and antidesmin (1:100 dilution; Boehringer Mannheim, Indianapolis, IN). Incubation times were 30 min for antivimentin, anticytokeratin, and antineurofilament and 18 h for antidesmin. Subsequently the avidin-biotin-peroxidase method was employed using reagents (Vectastain) and directions supplied by Vector Laboratories, Burlingame, CA. Diaminobenzidine was used as the chromogen, and slides were incubated in copper sulfate solution to intensify the staining reaction and in hematoxylin as a counterstain.

For negative controls, cell lines and sections were incubated with mouse ascites fluid (MOPC 21, Sigma Chemical Co., St. Louis, MO) of the same dilution as the primary antiserum. For positive controls, sections of skin (cytokeratin and vimentin), muscle (desmin), and ganglioneuroma (neurofilament) were employed. All controls yielded the expected staining reactions.

Cytogenetic Analysis. Cell lines were harvested 1 to 2 days after seeding in an attempt to obtain adequate metaphases in the logarithmic growth phase. Colcemid was added to the flasks to a final concentration of 0.05 µg/ml. After 1 h cells were trypsinized and resuspended in KCl (0.075 M) for 20 min. The cells were then fixed several times in methanol:acetic acid (3:1) and slides were prepared by air drying and then stained with Giemsa and trypsin-Giemsa. Five cells per line were photographed and karyotyped.

RESULTS

Xenografts. Characterization of xenografts HxRh10 and HxRh18, their growth and chemosensitivity have been reported previously (5). HxRh28 was derived from a metastasis in the axillary node, prior to the patient receiving therapy. Tumor was transplanted into 28 mice, which resulted in growth of a single tumor. This was transplanted subsequently and routinely grows...
in >80% of recipient mice. Examination of the xenograft derived tumor shows alveolar histology similar to the patient tumor. Of interest is that HxRh28, implanted in the s.c. site, is metastatic to the paraaortic and axillary nodes in approximately 25% of recipient mice. Metastatic spread is predominantly (if not exclusively) to lymph nodes, paraaortic, and axillary nodes sometimes growing to >1 cm diameter. The histology of the xenograft at the s.c. site is similar to that of the metastatic tumor (Fig. 1) and characteristic of poorly differentiated alveolar RMS. When a tumor brei was inoculated into the hind leg muscle, HxRh28 was invasive, again giving metastasis to the paraaortic nodes. We have not examined rigorously other organs by serial section to detect micrometastasis, however, there was never gross evidence of involvement in any mouse necropsied.

Establishing Cell Lines in Vitro. Rhabdomyoblasts were established in culture using the procedure described in “Materials and Methods.” In order to determine whether early cultures had contamination by murine stromal cells, cultures were analyzed for species specific LDH isoenzymes. Lactate dehydrogenase isoenzymes for Rh10, Rh18, and Rh28 cultures and mouse skeletal muscle are shown in Fig. 2. In Rh18 cultures, human specific isoenzymes from H4 to M4 were observed. In both Rh10 and Rh28 cultures an additional band migrating cathodal to human H3M was visualized (Fig. 2, arrow). Migration of this band was coincident with mouse LDH3, and could have signified contamination by murine cells. In the absence of LDH substrate, lithium lactate, none of these bands was visualized, indicating that the aberrant band in Rh10 and Rh28 cultures was an isoenzyme of LDH.

In order to further characterize these cultures numerous metaphase spreads were analyzed for acrocentric (mouse) chromosomes. None of the cell lines (Rh10, Rh18, Rh28) were found to contain mouse cells. However, a fourth line, from xenograft HxRh12, was found to contain exclusively murine fibroblasts (designated 12 Mur) with a transformed phenotype. These studies suggested that the aberrant LDH isozyme in cultures of Rh10 and Rh28 were human, possibly LDH-Z (19), rather than due to murine cell contamination.

Antiserum raised in rabbits against murine B-16 melanoma cells was shown to be capable of specifically lysing the mouse cells from a mixed 12 Mur:RD rhabdomyosarcoma population. After five treatments with antiserum the mixed cultures did not exhibit mouse LDH isoenzymes, whereas cultures treated only with normal serum showed an intense band of mouse LDH5. After 3 days of growth following the fifth treatment, these cultures remained completely free of detectable mouse LDH (Fig. 3).

Continued treatment with this antiserum, however, did not diminish the expression of the atypical LDH isozyme in either the Rh10 or Rh28 cell line. When the LDH isoenzyme profiles from the xenografts were reexamined, both HxRh10 (see Fig. 2 and Ref. 5) and HxRh28 (Fig. 2B, lane 1), but not HxRh18 xenografts, exhibit a band migrating cathodal to H3M.
The characteristic pattern of the Rh28 cultures, only H4 and the atypical band, was apparent in the xenograft. More convincingly, a portion of the patient biopsy from which HxRh28 was derived, had been cryopreserved and was thus available for LDH determination. The full complement of human LDH isoenzymes (H4 to M4) was detected with the extra atypical band migrating cathodal to HAL identical to that shown in cultured cells (Fig. 2A, lane 4). The additional normal bands could be attributed to a second population within the tumor (e.g., normal stroma). Although the original biopsy material was not available for comparison with the Rh10 line, the above evidence suggests that the atypical isozyme (LDH-Z) is of human origin.

Characterization of Rhabdomyosarcoma Cell Lines. Population doubling times for Rh10, Rh18, and Rh28 during logarithmic growth were 66.7, 57.8, and 58.8 h, respectively. As shown in Fig. 4, both Rh18 and Rh28 exhibit an initial delay in growth subsequent to plating. Rh10 was found to consistently plateau after 3–4 days in culture, the final density being dependent upon the initial plating density.

Rh28 cell cultures exhibit morphological characteristics which are similar to skeletal muscle cells in culture. Fig. 5 shows typical fields of spindle shaped rhabdomyoblasts. Occasional irregular or spindle shaped syncitia appear in cultures of Rh28, but rarely in Rh10 or Rh18 cultures. In addition, directional polarity has been observed in dense cultures of Rh10 and Rh28.

Creatine Phosphokinase (CPK). In normal myogenesis transition from the BB homodimer (fetal) through the MB heterodimer to the MM (adult) form of enzyme, occurs during myoblast fusion. Profiles for CPK isozymes were examined in each of the RMS xenografts and cell lines, Fig. 6. Each cell line demonstrated BB and MM homodimers. CPK activity in soluble fractions of these cell lines was determined enzymatically, and compared with activity in the nonmuscle line, EPP (human fibroblast). The total CPK activity in Rh10, Rh18, and Rh28 was 157.2, 246.8, and 474.3 milliunits/mg protein, respectively, as compared to 10.3 milliunits/mg in the nonmuscle EPP line. These activities compare well with reported values for chick myoblast cultures prior to fusion.

Reactivity of Cell Lines with Antibodies. Each of the cell lines demonstrated a surface antigen recognized by monoclonal antibody 5.1 H11, Fig. 7. The antibody recognizes an antigen expressed on the surface of myoblasts, myocytes, and myotubes, but not fibroblasts (18). Two human colon adenocarcinoma cell lines (WiDr and DLD-1) as well as 12 Mur fibroblasts showed no reactivity using the same indirect immunofluorescence assay (data not shown).

The reactivity of cell lines and their respective xenografts using antibodies specific for desmin, neurofilament, vimentin, and cytokeratin was also examined. These antibodies were tested in this laboratory against a variety of normal and neoplastic tissues and were found to be highly specific. Anticyto-
Fig. 7. Phase contrast bright field (A) and fluorescent (B) photomicrographs of cell lines Rh10 (1), Rh18 (2), and Rh28 (3) subsequent to indirect immunofluorescence staining with monoclonal antibody 5.1 H11. 430 X.

Table 1  Cytogenetic abnormalities of RMS lines

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<tr>
<th>Line</th>
<th>Xenograft (in vivo)</th>
<th>Cell line (in vitro)</th>
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<tr>
<td>Rh10</td>
<td>t(2;13)(q35;q14), +del(12)(12;7)(q23;7), +der(16)(1;16)(p22;q24), +mars</td>
<td>t(2;13)(q35;q14), +del(12)(12;7)(q23;7), +der(16)(1;16)(p22;q24), +mars</td>
</tr>
<tr>
<td>Rh18</td>
<td>+del(1)(p22), +del(3)(3;7)(p14;7), +del(6)(q24), +der(11)(1;11;122)(q14;7), +del(16)(q22), +der(22)(1;22)(q31;q11), +mars</td>
<td>+del(1)(p22), +del(3)(3;7)(p14;7), +del(6)(q24), +del(16)(q22), +der(22)(1;22)(q31;q11), +mars</td>
</tr>
<tr>
<td>Rh28</td>
<td>t(2;13)(q35;q14), +der(12)(3;12)(q11;q24), HSR (14q), +mars</td>
<td>t(2;13)(q35;q14), +del(9)(q22) +del(9)(q11), +der(12)(3;12)(q11;q24), HSR (14q), +mars</td>
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keratin decorated sweat gland and duct epithelium of skin tissue while antivimentin stained the fibroblasts and epithelial cells of the same tissue. Antidesmin and antineurofilament stained skeletal muscle tissue and neural elements (i.e., axons and neuronal perikarya), respectively. A cell line derived from a human colon adenocarcinoma (GCx/M) and the xenograft were
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used as control tissues of nonmuscle origin. The colon line was positive for cytokeratin, but did not react with other antibodies. Each cell line and respective xenograft was positive for desmin and vimentin. The Rh18 cell line and xenograft were positive for neurofilament; however, it must be noted that frozen skeletal muscle reacts strongly with the monoclonal antineurofilament which we used.

Karyotype Analysis. Karyotypes for each cell line and the xenograft from which each was derived are presented in Table 1. The tumors from which Rh10 and Rh28 were derived were classified as monomorphous round cell according to the criteria of Palmer (20).

Cell Line Rh10. A karyotype of Rh10 is shown in Fig. 8 (top) illustrating the hyperdiploidy and chromosome markers found in this line. The chromosome markers in this cell line, t(2;13)(q35;q14), del(12)(q12q24), and del(16)(p16q22), are the same as those seen in the xenograft, HxRh10.

Cell Line Rh18. A karyotype of Rh18 is shown in Fig. 8 (middle) illustrating the hyperdiploidy and chromosome markers typical of this line. The numbers of individual chromosomes were variable from cell to cell, chromosome count being in the 70 to 80 range. The presence of several chromosome markers in this line confirmed its derivation from xenograft HxRh18 (21). These included: del(1p12), der(3)(3q21;?p14q24), del(6)(q24), and der(22)(1q22)(3q11q1); all of which were found in both the cell line and the xenograft. An 11p marker found in the xenograft was not seen in the cell line, and the del(16)(q22) of the cell line was variably present in cells analyzed directly from the xenograft.

Cell Line Rh28. A karyotype of Rh28 is shown in Fig. 8 (bottom). Like the karyotype of the xenograft that of Rh28 is near tetraploid (with approximately 25% of the metaphases near octoploid). The t(2;13)(q35;q14) and der(12)(3;12)(q21; q24) translocation markers, and the HSR(14q) are found in both the cell line and the xenograft, HxRh28, indicating their common clonal origin. Better chromosomal resolution in studies of the cell line allowed identification of other abnormalities including del(9)(q22), del(9)(q11), and i(17q), which were discernible only as uncharacterized markers in studies of the xenograft.

DISCUSSION

Relatively few cell lines have been established from rhabdomyosarcoma of childhood (22–25), possibly reflecting the difficulty in establishing in vitro these cell types. Clearly, however, rhabdomyosarcomas can be propagated in immune deprived mice with high frequency (5). In our studies approximately 40% of surgical specimens from untreated or treated patients have developed into serially transplanted xenografts. In this article we have established three cell lines from three independently derived xenografts. Two of these (Rh18 and Rh28) were from previously untreated specimens. Each cell line demonstrates both vimentin and desmin, thus confirming mesenchymal origin and muscle origin of these cells (26, 27). Antidesmin antibody has proven useful in diagnosis of rhabdomyosarcoma (28–30). Further, each line was positive for human fetal muscle antigen, 5.1 H11, and each had creatine phosphokinase activity. In all stages of human skeletal muscle development the MM homodimer of CPK is dominant. During progressive development of normal muscle cells, however, there is a continued decrease in the BB isoform. Electrophoresis demonstrated that the MM homodimer was a major component of CPK in each cell line. The associated BB isoenzymes in each may suggest an arrested developmental state. Each rhabdomyosarcoma cell line exhibited 15- to 47-fold the total activity determined in the human fibroblast line EFP.

It is of interest that the Rh10 and Rh28 cell lines and the biopsy specimen of Rh28 demonstrate an atypical lactate dehydrogenase isoenzyme. In the Rh10 cell line, all five human isoenzymes are present in addition to the unique form. In contrast the Rh28 cell line has only the atypical migrating form and the H tetramer. The absence of murine metaphase spreads, the inability of the antiserum (lytic for 12 Mur cells) to reduce the intensity of this LDH isoenzyme and, more importantly,
the presence of the atypical isoenzyme in the original Rh28 biopsy specimen, indicate that this isoenzyme is of human origin. Unique LDH isoenzymes have been reported in placenta (31, 32), hydatiform mole (33), and in five of five independently derived choriocarcinomas (34). This new enzyme, which migrates cathodal to H3M has been designated LDH-2' (35) or origin. Unique LDH isoenzymes have been reported in placenta biopsy specimen, indicate that this isoenzyme is of human (31, 32), hydatiform mole (33), and in five of five independently derived cell lines also migrates electrophoretically cathodal to LDH H3M. We suggest that this isozyme may be homologous to that reported in the tissues of trophoblastic origin. Egami et al. (35) reported the expression of two LDH subbands cathodal to H3M and H2M. These subbands were observed in 16 of 31 of the gliomas investigated and suggest that an oncofetal enzyme is expressed in poorly differentiated tumors. Although further enzyme characterization is required, the presence of isoenzymes which migrate similarly to the LDH-Z in both the gliomas and our rhabdomyosarcomas implies that these isoenzymes may be the same and are not limited to cells of trophoblastic origin.

Another similarity between the rhabdomyosarcoma LDH band and LDH-Z from choriocarcinomas is its apparent independence from the M subunit. The profile of the Rh28 cell line reveals only two isoenzymes, H3 and the variant isoenzyme. The JEG-3 choriocarcinoma cell line was cloned and two of these clones, JEG-1 and JEG-7, expressed only the LDH-H3 and LDH-Z isoenzymes (21).

Karyotype analysis confirmed that each line in culture was human, and derived from its respective xenograft. It is of note that both Rh10 and Rh28 cell lines have a similar rearrangement (t(2;13)(q35;q14), which occurs frequently in the alveolar type rhabdomyosarcoma and may be more closely correlated with the monomorphic round-cell cytology described by Palmer (20). The t(2;13) translocation has been reported in advanced stage alveolar rhabdomyosarcoma by Seidal et al. (16) and Turc-Carel (15), and has been demonstrated in four independently derived xenografts (14). The breakpoints of this translocation appear to be at 2q35 and 13q14. 13q14 contains the Rb-1 (retinoblastoma) gene, and homozygous deletions at this locus are postulated to be a primary event in the development of retinoblastoma. Investigation of chromosome 13 has implicated also the 13q14 region in development of osteosarcoma (13). We are aware of one further case with this translocation (36), whereas the RD rhabdomyosarcoma cell line (22) does not demonstrate this abnormality (37). The karyotype analyses presented have confirmed the common origin of the cell lines and their respective xenografts, however, a more detailed cytogenetic analysis is currently in press (14).

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