ABSTRACT

Of 33 surgical specimens of osteosarcoma obtained from 24 patients, eight were established as transplantable tumor lines in immune-deprived CBA/CaJ inbred mice. Each line retained the histological characteristics of the corresponding primary tumor and produced human lactate dehydrogenase isozymes. Volume doubling times, which ranged from a mean of 12.3 ± 5.6 to 39.3 ± 9.8 days, were stable for individual lines over multiple passages. Flow cytometric analysis indicated similar cellular DNA content values in the primary human tumors and established xenograft lines; the presence of two separate stem lines, as in the original tumors, was observed in the laboratory models. Comparison of two methods of immune deprivation indicated that thymectomy, whole-body irradiation, and bone marrow reconstitution was associated with a higher rate of successful engraftment than was thymectomy, 1-β-D-arabinofuranosylcytosine treatment, and whole-body irradiation. Bone marrow-reconstituted mice also showed less variability in tumor volume doubling time.

We conclude that osteosarcoma can be heterotransplanted into bone marrow-reconstituted mice with a relatively high success rate and that the xenografts retain features characteristic of the tumors of origin. The availability of these models should prove useful in the development of new therapeutic regimens and in understanding the biology of osteosarcoma.

INTRODUCTION

Osteosarcoma, the most common primary bone tumor in children (1), can now be added to the list of malignant solid tumors that are curable in a majority of patients (2, 3). Although use of intensive adjuvant chemotherapy has produced 5-year survival rates >60% in children presenting without metastases, refinements in treatment are urgently needed. More than a third of patients will relapse after receiving currently available therapy, and treatment-related morbidity is considerable. Variations in therapeutic protocols, a lack of active new agents, and poor understanding of the biological factors underlying the chemosensitivity of osteosarcoma have been major obstacles to the development of uniformly effective treatment strategies. Accordingly, a reliable laboratory model of osteosarcoma would permit controlled evaluation of new therapeutic regimens and manipulation of factors that might affect treatment outcome.

Xenografts of various human cancers retain features of the original primary tumors, including chemosensitivity and growth characteristics, and therefore offer useful alternatives to tissue culture systems and experimental animal tumor models. Previous research has demonstrated the utility of human rhabdomyosarcoma xenografts in the study of the biochemical determinants of drug sensitivity and in selection of agents that may offer useful alternatives to tissue culture systems and experimental animal tumor models. Previous research has demonstrated the utility of human rhabdomyosarcoma xenografts in the study of the biochemical determinants of drug sensitivity and in selection of agents that may have clinical application (4-7). In this article, we report successful establishment of human osteosarcoma xenografts in immune-deprived mice and demonstrate retention of biological features characterizing the tumors of origin.

MATERIALS AND METHODS

Immune Deprivation of Mice. Four-week-old female CBA/CaJ inbred mice (The Jackson Laboratories, Bar Harbor, ME) were caged in an air-conditioned room (23.3 ± 1.1°C) lighted for 12 h each day. Cages, litter, and food (Purina No. 5010, autoclavable) were sterilized, and mice were placed in fresh cages twice weekly. Two different types of immune deprivation were investigated.

In technique 1, mice underwent thymectomy at 4 weeks of age; 3 weeks later they received a single injection of 1-β-D-arabinofuranosylcytosine (200 mg/kg i.p.) followed in 48 h by whole-body irradiation (950 cGy from a 137Cs source at a dose rate of 170 cGy/min) (8). This method of immune deprivation was termed 8AraCt.

In technique 2, mice received whole-body irradiation 3 weeks after thymectomy; this was followed within 6 h by bone marrow reconstitution with 3 × 10^6 cells from thymectomized syngeneic mice. This procedure was designated 8tBM (7, 9).

For certain experiments, C57Bl/6J athymic nude mice (Charles Rivers, Wilmington, MA) were also used.

Source of Tumors and Histology. All but one of the tumors were obtained from primary human osteosarcomas at the time of definitive surgery; the exception, HxOs29, was obtained from a pulmonary metastasis. None of the patients had received chemotherapy. Tumor specimens (2 to 4 mm^3) were implanted surgically in the s.c. dorsal flanks of anesthetized mice 1–2 weeks after irradiation. The incision wound was irrigated with a solution containing penicillin (200,000 units/ml) and streptomycin (20 μg/ml). All procedures were performed in a type B laminar flow hood. For histological evaluation, tumors were fixed in 10% neutral buffered formalin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by one of the authors (B. L. W.).

Lactate Dehydrogenase Isozymes. LDH^3 isozymes were separated by flat-bed electrophoresis (200 V, 95 min, 2°C) on Cellogel analytical strips (Kalex Scientific Co., Manhasset, NY), as described previously (7).

Growth of Osteosarcoma Xenografts. The growth of engrafted tumors was assessed by measuring two perpendicular diameters at 7-day intervals, using electronic calipers (Maxcal digital caliper Fred Fowler, Co., Newton, MA with an NSK 232 interface). Tumor volumes were calculated by the formula (π/6) × d^3, where d is the mean diameter. Calculations were performed on a Suntronics AT Computer (IBM compatible) with Maxcal and Lotus 123, with simultaneous use of programs.

Flow Cytometry. Tumors were minced with scalpels in RPMI 1640 medium, and debris was allowed to sediment for 5 min. Aliquots of tumor cell suspensions were stained with propidium iodide and analyzed by flow cytometry, as previously described (10). The DNA index of each tumor stem line was determined by calculating the ratio of the modal channel number for tumor G0-G1 phase cells versus that for normal human diploid cells. A mixture of equal numbers of normal human blood leukocytes and tumor cells was stained and analyzed separately to identify the modal fluorescence of G0-G1 phase tumor cells relative to that of G0-G1 phase normal cells. Wright-stained smears of aliquots of each tumor cell suspension were analyzed for percentages of tumor cells and contaminating blood leukocytes.

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2 To whom requests for reprints should be addressed, at Department of Hematology-Oncology, St. Jude Children's Research Hospital, P.O. Box 318, Memphis, TN 38101.

3 The abbreviation used is: LDH, lactate dehydrogenase.

William H. Meyer, Janet A. Houghton, Peter J. Houghton, Bruce L. Webber, Edwin C. Douglass, and A. Thomas Look


Development and Characterization of Pediatric Osteosarcoma Xenografts1
normal diploid cells (DNA index = 1.0) if the percentage of cells in the diploid G0-G1 peak of the DNA histogram was at least 20% greater than the percentage of normal blood leukocytes determined from the morphological analysis (10).

Cytogenetic Analysis. Xenograft material was finely minced prior to a 24-h culture in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were treated with colcemid (0.05 μg/ml) for 1 h, followed by trypsin and KCl, and fixed in methanol-acetic acid. Samples were air dried, stained with Giemsa and trypsin-Giemsa, and then photographed (11).

RESULTS

Heterotransplantation into Immune-deprived Mice. Thirty-three tumor specimens, surgically excised from 24 patients, were transplanted into immune-deprived (θAraCr) mice. Eight of these specimens were established as transplantable lines, designated as HxOsl, 2, 6, 8, 9, 17, 29, and 33. HxOs9 and HxOs29 were obtained from the same patient; the former line was derived from the primary tumor and the latter from a pulmonary metastatic lesion that developed several months after resection of the primary tumor. An additional line, HxOs10, which was derived from the same patient as HxOsl8, differentiated into a bony tumor by passage 3 and could not be transplanted further. Only one of eight metastatic tumor specimens was transplantable; 7 of the 25 primary specimens were propagated for at least three passages. By life-table analysis using the Mantel-Cox test for significance (Blossom STAT-PACK, Release 2; Data Management Branch, Division of Computer Research and Technology, NIH, Bethesda, MD), the survival times of patients did not correlate with success in establishing a transplantable line.

Histological Characteristics. The histological features of each of the xenografts were similar to those of the corresponding primary human tumor. Two engrafted tumors (HxOsI and HxOs33) are osteoblastic osteosarcomas with no other differentiation. HxOs1 is a cellular tumor with some calcified osteoid. It has a moderate mitotic rate and appears to lack giant cells. HxOsl3 is a very highly cellular tumor with rare areas of calcified osteoid; its mitotic rate is high and some giant cells are present. It may possess a telangiectatic component, as suggested by the presence of blood-filled pools.

Two tumors (HxOs2 and HxOs2l) demonstrated chondroid features. HxOs2 is a moderately cellular large cell osteosarcoma with a minor chondroid component. Focal necrosis is present, and the mitotic rate is high. HxOs2l is a moderately cellular chondroblastic osteosarcoma with abundant collagen matrix that produces chondroid and rare tumor osteoid. Nuclear pleomorphism is rare.

Three tumors (HxOs8, HxOs9, and HxOs29) showed some fibroblastic differentiation. HxOs8, an osteoblastic osteosarcoma, has abundant extracellular osteoid and fibrous matrix. Occasional giant cells are present. Cellularity is lower in this xenograft than in most of the others examined. HxOs9 and HxOs29 are histologically similar in all respects. The tumors are primarily fibroblastic with moderate cellularity and some tumor osteoid. Occasional calcification and giant cells are present.

Comparison of early passage tumors with the primary tumors from which they were derived disclosed essentially identical histological features (Fig. 1). In general, the histological features of xenografts were preserved after multiple passages in mice, although some tumors showed increased cellularity and increased numbers of giant cells in later passages (Fig. 2). There was also variation in the amount and type (osteoid, fibrous, or chondroid) of stroma. HxOs2, for example, had primarily chondroid with some osteoid matrix after the initial passage, whereas after passage 2 it lacked chondroid but had some osteoid matrix. At passage 8 to 10, abundant chondroid and minimal osteoid matrix were noted. By passage 18, the tumor showed both chondroid and osteoid matrix and resembled the primary tumor more closely than did tumors in several of the intermediate passages. These observations may reflect histological variability in different areas of the same tumor or differences in the microenvironment that modulates the proliferative activity of the cells.

LDH Isozyme Profile. Each tumor line growing in mice produced human-specific LDH isozymes (Fig. 3), distinguishing these xenografts from murine origin. Small amounts of mouse isozyme were sometimes present, owing to infiltration of the xenografts by murine stromal cells (7).

Growth Characteristics. The growth rates of each of the xenografts are presented in Table 1. Mean (±1SD) volume doubling times ranged from 2.3 ± 5.6 days (HxOs2) to 29.3 ± 9.8 days (HxOs2l) and were stable over many passages in individual tumor lines. The growth pattern approximated logarithmic rather than Gompertzian growth, indicating a constant growth rate regardless of tumor size. Logarithmic growth properties have also been observed for xenografts established from rhabdomyosarcomas and human colorectal adenocarcinomas (7, 8).

The doubling times of the human osteosarcoma xenografts were similar to mean values reported for pulmonary metastases of osteosarcomas [22 and 50 days (12-14)] but were longer than those for rhabdomyosarcomas, which range from 7.2 ± 1.5 days to 19.8 ± 2.3 days (7).

Effect of Immune Deprivation Technique on Growth of Osteosarcoma Xenografts in Mice. In xenografts established as heterotransplantable lines, the rate of successful engraftment of serial passages, expressed as (number of implants/number of progressively growing tumors) × 100, ranged from 14 to 60%. In most instances, either both tumor fragments engrafted or neither grew. In rare instances (<10%), a tumor grew in one flank but not the other. These findings suggested that the failure of established xenografts to engraft resulted from host factors rather than tumor-related factors. We tested this impression with the use of the xenograft HxOs9 and mice that were immune deprived by either θAraCr or θBM; HxOs9 was also heterotransplanted into CD athymic nude mice. Representative growth curves from HxOs9 tumors (passage 19) are shown in Fig. 4. The engraftment rate was 100% for θBM mice, significantly higher than take rates for θAraCr-prepared mice and for athymic nude mice (Table 2; χ² = 24.47; P < 0.01). In addition, the tumor doubling times showed less variability in the θBM model, compared with the θAraCr and nude mouse models.

Flow Cytometric and Cytogenetic Analysis. Two separate tumor stem lines were found by flow cytometric analysis of each of the five osteosarcoma xenografts studied; cellular DNA content values were similar to those found in the original tumors (Table 3). Fig. 5 compares the tumor stem lines detected by flow cytometry in HxOs8, passage 11 (Fig. 5A), with the original tumor (Fig. 5B). In the xenograft, stem lines with DNA indices of 1.11 and 2.19 were detected, together with a minor population of mouse blood cells with a DNA index of 1.0. Mouse blood cells have a DNA index identical to human blood cells in these analyses, despite the very different cytogenetic appearance of human compared to murine chromosomes.

Tumor cells obtained from the original tumor showed both
Fig. 1. Photomicrographs of representative sections of the primary human tumor (A) and the second passage HxOs9 xenograft (B), demonstrating identical histological and cytological features.

Fig. 2. Photomicrographs of representative sections of HxOs2. A, human tumor; B, passage 18 showing increased cellularity through serial passage with maintenance of histological and cytological features, including presence of chondroid matrix.

Table 1 Tumor volume doubling times for osteosarcoma xenografts
See "Results" for comparison with doubling times for metastatic osteosarcoma from patients.

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Passage</th>
<th>Doubling time (days)</th>
<th>No. of tumors studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>HxOs1</td>
<td>4</td>
<td>29 ± 11.6±</td>
<td>12</td>
</tr>
<tr>
<td>HxOs2</td>
<td>3, 4</td>
<td>14.0 ± 5.2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>19, 20</td>
<td>12.3 ± 5.6</td>
<td>33</td>
</tr>
<tr>
<td>HxOs8</td>
<td>2</td>
<td>33.9 ± 12.5</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>11, 12</td>
<td>28.6 ± 8.4</td>
<td>26</td>
</tr>
<tr>
<td>HxOs9</td>
<td>9, 10</td>
<td>17.6 ± 7.8</td>
<td>44</td>
</tr>
<tr>
<td>HxOs21</td>
<td>2</td>
<td>39.3 ± 9.8</td>
<td>20</td>
</tr>
<tr>
<td>HxOs29</td>
<td>10, 11</td>
<td>18.0 ± 4.0</td>
<td>25</td>
</tr>
<tr>
<td>HxOs33</td>
<td>3</td>
<td>23.5 ± 7.7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>24.0 ± 11.8</td>
<td>9</td>
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* Mean ± SD.

Fig. 3. Separation of LDH isozymes present in osteosarcoma xenografts. Mouse isozymes are designated LDH 1–5 and human isozymes H4–M4. Each line of xenograft demonstrated human-specific LDH isozymes.

near-diploid and near-tetraploid stem lines, with DNA index values similar to those found in the xenograft. The sensitivity of DNA measurements did not permit the near-diploid stem line to be distinguished from blood cells that were present in the primary tumor. Morphological analysis of the cell suspension indicated that 90% of the cells were tumor cells and that 49% of the cells had a DNA index of 1.0; hence a stem line with near-diploid cellular DNA content must have been present. Similar DNA index values were observed for the other four xenografts, compared to measurements obtained from the original tumors, with differences that reflect the limits of detection.

Fig. 4. Representative growth curves for HxOs9 passage 19, by the method of immune deprivation. A, 8AraC; B, 8-BrdU; C, CD/nu,nu athymic nude.
of relative cellular DNA content by this method.

Metaphases were obtained from HxOs2, HxOs8, and HxOs33 (Fig. 6). In all three cases the chromosomal pattern was very complicated. Although human chromosomes could easily be identified, numerous and complex structural abnormalities made complete and accurate karyotyping impossible. In each case, however, the hyperdiploid chromosome numbers were consistent with the elevated DNA index detected by flow cytometry.

DISCUSSION

Xenograft models of human osteosarcoma afford a valuable tool for basic and clinically applied research, especially when one considers the difficulty of establishing osteosarcoma cell lines in vitro. Such xenograft models have relied almost exclusively on athymic nude mice (15–21) and have been used for in vitro drug testing (17, 21) and for investigation of monoclonal antibody localization (15, 20), effects of interferon (16), and manipulation of irradiation (18). Xenografts that can be serially manipulated of irradiation (18). Xenografts that can be serially

As shown in Table 3, the range of growth rates of osteosarcomas is similar for xenografts growing in the s.c. space of mice and pulmonary metastases in patients. Bauer et al. (19) reported tumor volume doubling times of 5.8 to 14.9 days for early passage osteosarcoma xenografts in nude mice. However, Nakaamura and Kitagawa (17) reported volume tumor doubling times in nude mice of 10 days to 6 weeks, a finding similar to our data. The rate of growth can be variable, since xenografts are derived from different patients and reflect the unique properties of the tumor rather than the host environment.

LDH isoenzyme analysis was done to distinguish these tumors as being of human rather than mouse origin (7). Although minor contamination with mouse stroma was noted, the LDH isozyme patterns conclusively demonstrate the human origin of these tumors. This analysis is not sufficiently sensitive to exclude the possibility of genetic alteration and is not relied upon for that purpose. Careful histological review of each passage in mice is needed to ensure fidelity with the primary human tumor.

Our observation of two separate tumor stem lines by DNA flow cytometry in each of the xenograft lines parallels findings in the original tumors. Bauer et al. (19) have also reported that osteosarcoma xenografts may have multiple stem lines, includ-
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ost often, growth rates in two lines examined over serial passage concurrently with an increase in growth rate. We have not assumed particular significance in light of observations published by Look et al. (23). We distinguished a marked difference in treatment outcome between patients with near-diploid tumor stem lines and those with hyperdiploid lines only. This indicates that future animal model studies of the chemore sponsiveness of osteosarcoma will need to consider tumor cell ploidy as an influential treatment variable.

Chromosomal analysis in three xenografts was consistent with the hyperdiploidy noted on flow cytometry. The highly complex chromosomal pattern with numerous numeric and complex structural abnormalities has been previously noted in osteosarcoma (24, 25).

Human osteosarcoma xenografts can be established and maintained in immune-deprived mice. Well characterized xenograft models of osteosarcoma should provide unique opportunities for investigations of the biochemical pharmacology of antineoplastic agents in this relatively rare childhood tumor.

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