Development and Characterization of a Human Sarcoma Cell Line, MES-SA, Sensitive to Multiple Drugs1

W. Graydon Harker, F. Roy MacKintosh,2 and Branimir Ivan Silic3

Oncology Division, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

A cell line designated MES-SA has been developed from a uterine sarcoma. Cells from the surgical tumor specimen were grown in a soft-agar clonogenic assay, with a relatively high plating efficiency of 0.5% and sensitivity to multiple drugs. Histologically, the surgical specimen and tumors developing after MES-SA inoculation into nude mice were identical, consisting of sheets of anaplastic sarcoma cells amid scant hyalinized stroma. The nonepithelial origin of this line was supported by ultrastructural analysis and negative mucin staining. Growth in monolayer was established by seeding colonies from soft agar into liquid media and has been maintained for over 21 months (greater than 100 passages), with a population-doubling time for the cell line of 22 hr. The MES-SA line readily forms colonies in soft agar with plating efficiencies ranging from 10 to 20%. Tumor cell inoculation s.c. into nude mice produces tumors within 2 to 3 weeks and subsequent tumor volume-doubling times of 7 to 10 days. MES-SA has a modal chromosome number of 45. Karyotypic abnormalities include: monosomic forms of chromosomes 5, 6, and 7; a 5q, 6p translocation; and one marker chromosome. In vitro sensitivities to doxorubicin, dactinomycin, mitomycin C, and bleomycin have been demonstrated by clonogenic assay. These drug sensitivities remain stable over long periods of monolayer growth and after passage in nude mice.

INTRODUCTION

The establishment of cell lines from human cancers provides important tools for the investigation of cancer etiology, biology, and therapy. Lines from several human tumors have been reported, but most have been derived from tumors of epithelial origin. Cell lines generated from human sarcomas to date include those from osteogenic sarcoma (1, 4, 13, 16, 21, 29, 30, 38), liposarcoma (1, 3, 13, 16, 24-27), giant-cell sarcoma (1, 29), synovial cell sarcoma (29), neurogenic sarcoma (37), fibrosarcoma (1, 20, 29, 31), rhabdomyosarcoma (6, 20, 22, 40), chondrosarcoma (7, 9, 14, 39, 42), Ewing’s sarcoma (8), Kaposi’s sarcoma (12), and leiomyosarcoma (10) sources. The extent of these characterizations varies considerably. Details regarding tumorigenicity in nude mice and drug sensitivities are seldom mentioned in these reports.

Sarcomas of uterine origin are uncommon; approximately 3000 are diagnosed annually in the United States, representing 5 to 10% of all malignant uterine tumors (2). Cell lines originating from these uterine sarcomas are also rare (17). We report here the establishment of a human sarcoma cell line of uterine origin (malignant mixed müllerian tumor) designated MES-SA, which grows readily in monolayer and soft agar, is tumorigenic in nude mice, and retains sensitivity to several anticancer agents. Characterization of the cell line and xenografted sarcomas is described.

MATERIALS AND METHODS

Source of Material. The patient, a 56-year-old female, underwent uterine curettage in May 1980 to evaluate postmenopausal bleeding. The curettage specimens were positive for undifferentiated tumor. A total abdominal hysterectomy was performed which revealed malignant mixed müllerian tumor. Recurrence of tumor was then documented in October 1980 with the development of a pelvic mass. The histology of this recurrence was documented to be a poorly differentiated sarcoma, consistent with elements of the previously diagnosed mixed müllerian tumor. Tumor samples were obtained at the time of surgery for culture and in vitro drug sensitivity testing.

Soft Agar Clonogenic Assays and Initial Cultivation In Vitro. Sections of the solid tumor sample from the patient or nude mice were disaggregated by fine mincing followed by exposure to enzymes. The enzyme solution consisted of a mixture of 0.02% collagenase II (Sigma Chemical Co., St. Louis, Mo.), 0.02% DNase I (Boehringer Mannheim, Mannheim, West Germany), and 0.05% Pronase (Sigma) in HBSS4 at pH 7.4. Following incubation in enzyme solution at 37° for 30 min, the cell suspension was filtered through gauze and a 200 mesh (90-μm) stainless steel screen. The cells were centrifuged at 200 x g for 10 min and then resuspended in W:M media (1:1) (Grand Island Biological Co., Grand Island, N. Y.), supplemented with gentamicin (10 μg/ml), cefoxitin (100 μg/ml), insulin (5 μg/ml), and 15% NBCS, and counted by hemocytometer. Drug sensitivity testing was then performed as described below, and cells were plated in the upper layer of the 2-layer soft-agar system.

In order to propagate a cell line from the original clonogenic assay, colonies growing in the upper agar layers of untreated control wells were disaggregated into fresh W:M media. Cells from these colonies were then seeded into 24-well Linbro plates (Flow Laboratories, Inc., McLean, Va.). Those wells developing monolayer tumor cell growth were subsequently harvested with 0.06 M EDTA and passaged into Comin culture flasks (Coming Glassworks, Comin, N. Y.).

In Vitro Growth Characteristics. Growth curves were obtained by seeding 5.0 x 104 viable tumor cells into multiple 15 x 60 mm tissue culture dishes (Falcon Plastics, Division of Becton Dickinson and Co., Oxnard, Calif.) containing W:M media, insulin, 15% NBCS, gentamicin, and cefoxitin. Dishes were incubated for 7 days at 37° in a 5% CO2 humidified atmosphere. At 24-hr intervals after plating, duplicate dishes were harvested with EDTA. Cell number and viability were determined by hemocytometer and trypan blue dye exclusion. Essential and optimal conditions for MES-SA cell growth were determined by incubating cells in serum-free medium containing a "balanced salt solution" and "W:M" media, respectively.

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2 Present address: VA Medical Center, 1000 Locust Street, Reno, Nev. 89502.
3 Recipient of a faculty development award in clinical pharmacology from the Pharmaceutical Manufacturers Association Foundation. To whom requests for reprints should be addressed, at Oncology Division, Room M-011, Department of Medicine, Stanford University Medical Center, Stanford, Calif. 94305.
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in various media: (a) Waymouth's; (b) McCoy's 5A; (c) W:M media; and (d) Dulbecco's modified Eagle's medium (Grand Island Biological Co.); and various concentrations of NBCS and insulin.

In Vitro Drug Sensitivity Testing. Cells in the logarithmic phase of monolayer growth were harvested using 0.06 M EDTA and then washed with HBSS:10% NBCS prior to centrifugation at 200 x g for 10 min. These cells, and those from disaggregated solid tumor specimens from the patient or nude mouse, were then exposed to various drugs, as follows.

Stock solutions of standard anticancer agents were diluted in sterile 0.9% NaCl solution or HBSS and stored at -70° in aliquots adequate for one assay. Tumor cell suspensions were adjusted to a concentration of 1.0 x 10⁶/ml and incubated with or without drug for 1 hr at 37° in HBSS:10% NBCS. Drug concentrations ranged from 0.001 up to 10.0 μg/ml with 3 to 5 concentrations studied for each drug. Following incubation, the cells were centrifuged at 200 x g for 10 min, washed twice with HBSS:10% NBCS, and resuspended in 0.3% agar in W:M media supplemented with 15% NBCS, gentamicin (10 μg/ml), cefoxitin (100 μg/ml), and insulin (5 μg/ml). One-half ml of this mixture containing 250,000 viable cells from the original surgical specimen, or 10,000 to 25,000 cultured cells, was then pipetted onto 0.5-ml lower layers containing W:M media, 15% NBCS, and 0.5% agar. Control and drug-treated cells were plated in triplicate in 16.6-mm-diameter wells of 24-well Linbro plates (Flow Laboratories, Inc., McLean, Va.). After preparation of both the bottom and top layers, the plates were examined under an inverted microscope to confirm the presence of a good single-cell suspension. The plates were then incubated at 37° in a 5% CO₂ humidified atmosphere.

At Days 10 to 14 in culture, the number of colonies formed in control and drug-treated wells was counted using an Olympus inverted-phase microscope (Olympus Optical Co., Tokyo, Japan) at x40. Colonies were defined as cell aggregates measuring greater than 50 μm in diameter and containing 30 or more cells. PEs are calculated by dividing the mean colony count from triplicate wells by the number of cells plated per well. Drug effects are expressed in terms of percentage of control survival, which is calculated by dividing the PE of drug-treated wells by the control PE and multiplying by 100.

Chromosomal Analysis. Cultures of MES-SA cells in exponential growth were harvested for chromosomal preparation using standard cytogenetic techniques (19, 33). Twenty metaphase spreads were selected for analysis after Giemsa banding.

Tumorigenicity Studies. Ten to 12-week-old NIH Swiss nude mice were used for s.c. inoculation of MES-SA cell suspensions. Monolayer cells in logarithmic phase of growth were harvested with EDTA, washed with HBSS:10% NBCS, and resuspended in W:M media at cell concentrations of 0.2 x 10⁶ or 0.6 x 10⁶ cells/ml. A dorsal interscapular injection of 0.1 ml of either cell suspension (containing 2.0 x 10⁶ and 4.0 x 10⁶ cells, respectively) was then administered to mice in triplicate. Weekly bidimensional (length (L) x width (W)) measurements of developing tumors were recorded. Tumor volumes (V) were estimated by using the formula:

\[ V = \frac{1}{2} L \times W^2 \]

Animals were sacrificed after 3 to 4 weeks of tumor growth for clonogenic drug sensitivity and histopathological studies.

Enzyme Analysis. LDH (EC 1.1.1.27), GOT (EC 2.6.1.1), and GPT (EC 2.6.1.2) activities were assayed in cell homogenates and serum-free media from MES-SA cultures. MES-SA cells (2 x 10⁶) were seeded into tissue culture flasks containing W:M media and 15% NBCS. After 10 days of growth, this medium was discarded, and the monolayer cells were washed twice with 0.9% NaCl solution to remove the remaining NBCS. Serum-free HBSS containing 0.1% glucose and antibiotics was then added. After incubation for 16 hr, the spent HBSS was decanted, centrifuged at 1,500 x g for 10 min, and stored for enzyme analysis. The cells in monolayer were harvested with EDTA, centrifuged at 12,000 x g for 5 min, and resuspended in serum-free HBSS. The cell suspension was then sonicated for 45 sec at 20° with the microprobe of a sonicator-cell disruptor (Heat-Systems Ultrasonics, Inc., Plainville, N. Y.), and the resultant cell homogenate were centrifuged at 12,000 x g for 15 min in an Eppendorf No. 5412 microfuge. Total LDH, GOT, and GPT activities of the cell homogenates and supernatants were assayed using a Technicon SMAC (sequential multiple analyzer with computer) instrument (Technicon Instruments Corp., Tarrytown, N. Y.). Electrophoretic patterns of LDH isoenzymes were determined by methods described previously (36).

Protein concentrations in cell homogenates and serum-free supernatants were measured by a dye-binding assay (5) (Bio-Rad Laboratories, Richmond, Calif.). Enzyme activities are expressed as milliunits/mg protein in the supernatants and milliunits/10⁶ cells in cell homogenates. A milliunit is defined as that amount of enzyme that converts 1 nmol of substrate to product per min.

Histopathology. For light microscopy, sections of the original and recurrent tumors in the patient and MES-SA-induced tumors in nude mice were fixed in 10% formalin, embedded in paraffin, sectioned at 4 to 6 μm, and stained with hematoxylin and eosin. Sections of selected blocks were also stained with reticulin and periodic acid-Schiff stains.

Drug-treated and untreated control wells from Days 10 to 14 MES-SA soft agar cultures were selected for histologic section and staining. Agar bilayers were fixed in B-5 fixative (prepared by mixing 10 ml of a 37 to 40% formaldehyde solution with 90 ml of a water base containing 0.067% mercuric chloride and 0.021% sodium acetate) for 2 hr and then washed 3 to 4 times with tap water. Colony containing upper agar layers were removed from the 0.5% agar underlayers and stored in 70% alcohol. After paraffin embedding, the agar layers were serially sectioned at 4 to 6 μm and stained with hematoxylin and eosin.

For electron microscopy, 1-mm-cube tissue fragments and single-cell suspensions harvested from monolayer growth were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The material was then rinsed 3 times in buffer, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and infiltrated and embedded in Spurr's resin. Thin sections were stained with 6% uranyl acetate and lead citrate and examined in a Philips 201 electron microscope.

Statistical Methods. The statistical significance of differences in growth related to selection of nutrient medium type was determined by single-classification analysis of variance followed by an a posteriori multiple-comparison (Student-Newman-Keuls) test (35).

RESULTS

Establishment of Cell Lines. Monolayer growth was established in 4 of the wells to which cells obtained from untreated control tumor colonies had been seeded. The growth characteristics and drug sensitivities of the cell lines derived from these 4 wells were quite similar so that one line, designated MES-SA, was selected for more extensive characterization. Fibroblast contamination, frequently observed in lines grown from tumor explants, was not observed in these wells, possibly due to the inhibitory effect of soft agar on fibroblast growth (23). The cell line has been examined for and found free from Mycoplasma contamination by agar culture (Mycotrim-TC; Hana Media, Inc., Berkeley, Calif.).

In Vitro Growth Properties. MES-SA monolayer growth is distinctive. A diverse cell morphology is seen with the majority of cells being large and polygonal (Fig. 1a). Distinct nuclear membranes and multiple, large nucleoli are seen. Cell colonies growing on culture surfaces maintain well-defined boundaries and seldom reach confluency because of rapid nutrient depletion related to high cell density (Fig. 1b). Large floating colonies or spheroids are occasionally produced which appear viable by trypan blue exclusion. Similar spheroid type of growth is seen when MES-SA cells are plated in liquid media over a soft-agar

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layer rather than directly on plastic (Fig. 1c).

Serum supplementation of nutrient media is essential for MES-SA growth. Chart 1 displays the results of experiments designed to determine the effect of varying concentrations of NBCS on growth in monolayer. Whereas MES-SA cells cultured without serum did not proliferate, identical growth curves were seen with cells grown in serum concentrations ranging from 5 to 15%, thus indicating that growth was supported by as little as 5% NBCS (lowest concentration tested). The addition of porcine insulin (5 μg/ml) to W:M media containing 15% NBCS did not appear to enhance growth. Growth in Waymouth’s media supplemented with 15% NBCS, insulin, and antibiotics was inferior to growth in similarly supplemented Dulbecco’s modified Eagle’s medium and McCoy’s medium 5A after 144 hr of incubation (Table 1). Differences between Waymouth’s and W:M media were not statistically significant. Similarly, differences in cell counts obtained from cultures all 4 medium types at earlier time points were not significantly different.

Cells from the patient's recurrent tumor grew well in soft agar, with a PE of 0.5%. The PE of MES-SA in agar has ranged from 5 to 21%. Because of these high PEs compared to the original spontaneous human tumor, growth and drug exposure experiments with the cell line have been seeded at 1,000 to 25,000 viable cells/ml. Colony growth in agar is rapid, and colony counting can be performed after 8 to 10 days of incubation. Colonies forming in agar appear as loosely bound aggregates of large cells (Fig. 2).

**Table 1**

<table>
<thead>
<tr>
<th>Nutrient Media</th>
<th>Cell no. (x10⁶)</th>
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<tbody>
<tr>
<td>Medium type</td>
<td>48 hr</td>
</tr>
<tr>
<td>Dulbecco’s modified Eagle’s</td>
<td>0.91 ± 0.23</td>
</tr>
<tr>
<td>McCoy’s SA</td>
<td>1.08 ± 0.07</td>
</tr>
<tr>
<td>Waymouth’s</td>
<td>1.10 ± 0.12</td>
</tr>
<tr>
<td>W:M(1:1)</td>
<td>1.06 ± 0.14</td>
</tr>
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* Cells (5.0 x 10⁶) were seeded into multiple flasks containing the various types of nutrient media. Media were replaced in remaining flasks at 48 and 96 hr.
* Mean ± S.D. of triplicate cultures.
* p < 0.05, Dulbecco’s modified Eagle’s medium versus Waymouth’s medium.
* p < 0.05, McCoy’s medium 5A versus Waymouth’s medium.

**Chart 1.** Effect of serum concentration on MES-SA monolayer growth. MES-SA cells (5.0 x 10⁶) were grown in cultures containing W:M media supplemented with NBCS concentrations ranging from 0 to 15% (v/v). At 24, 60, 120, and 168 hr of incubation, triplicate cultures were harvested and counted. Points, mean cell count from triplicate cultures.

**Chart 2.** MES-SA tumor growth in nude mice. Bidimensional measurements (L x W) were taken of tumors developing in nude mice following s.c. injection of either 2.0 x 10⁶ or 4.0 x 10⁶ MES-SA cells. Tumor volumes were estimated by the formula $V = \frac{1}{2} L \times W^2$. Points, mean tumor volume from at least 3 mice.

**Chart 3.** Drug sensitivities of uterine sarcoma cells from the surgical specimen. Cells from the recurrent pelvic tumor were disaggregated, exposed to standard anticancer agents, and plated in a 2-layer agar culture as described in “Materials and Methods.” Colonies were counted after 14 days of incubation, and drug effects were expressed as a percentage of control colony survival. Sensitivity is defined as a more than 70% reduction in colony survival compared to control. cis-DDP, cisplatin; DTIC, dacarbazine; L-PAM, melphalan; VCR, vincristine; MTX, methotrexate; BCNU, carmustine; points, mean of triplicate assays.
inocula were identical, with a tumor-doubling time of 7 days calculated after the initial lag period. The animals were sacrificed when tumor weights ranged from 3.0 to 5.4 g. Limited autopsies on 2 animals (lung, liver, and kidney), revealed no evidence of metastatic spread. Cells harvested from the tumors of these animals grew well in monolayer and soft agar cultures.

In Vitro Drug Sensitivity Testing. Chart 3 displays the results of the in vitro drug sensitivity testing performed on the cells from the patient’s recurrent sarcoma specimen. As can be seen, the original tumor was most sensitive to mitomycin C and dactinomycin, and a greater than 70% reduction in PE was also seen with doxorubicin and vinblastine at drug concentrations of 0.2 \( \mu g/mL \). Intermediate sensitivity (50 to 70% reduction in drug-treated PE) was seen at a bleomycin concentration of 0.2 \( \mu g/mL \). Sensitivity to mitomycin and dactinomycin is retained by the MES-SA line. The cell line displays greater in vitro sensitivity to bleomycin and doxorubicin than did the original tumor cells but appears to be less sensitive to vinblastine (Chart 4a). The sensitivity shown by MES-SA to doxorubicin, mitomycin, dactinomycin, and bleomycin remains remarkably stable over a period of 1.5 years despite greater than 100 cell passages. MES-SA cells propagated in nude mice retain sensitivity to those 4 agents, as well as resistance to vinblastine (Chart 4b). Both the original tumor cells and the cell line are resistant to several agents, including dacarbazine, cisplatin, and melphalan; MES-SA is also resistant to vincristine, methotrexate, and etoposide (data not shown).

Enzyme Analysis. Aminotransferase levels (GOT, GPT) were very low in both MES-SA cell lysates and spent media from monolayer cultures. LDH activity, however, which had been increased in the patient’s serum during therapy (630 IU, normal less than 200) was readily detectable in both cell lysates and supernatants (Table 2). LDH isoenzyme separation by electrophoresis in agarose revealed a pattern most representative of uterine origin when compared to standards of percentage of distribution of LDH isoenzymes in normal human tissues (Table 3).

Histopathology. The presence of malignant epithelial as well as mesenchymal features led to the diagnosis of a uterine malignant mixed müllerian tumor in the original hysterectomy specimen. At the time of the pelvic recurrence, however, only sarcoma elements were identified. The histological features of the recurrent tumor and those of tumors developing in nude mice are identical (Fig. 4, a and b), consisting of sheets of anaplastic sarcoma cells amid scant hyalized stroma. Prominent round to oval nuclei are seen which contain finely granular chromat in and large nucleoli. Indistinct cell boundaries encompass scant am-

| Chart 4. Drug sensitivities of cultured MES-SA cells (a) or MES-SA cells grown in nude mice (b). Tumor cells harvested from either monolayer or nude mouse sources were exposed for 1 hr to various concentrations (CONC) of bleomycin (BLC), dacitomycin (DCT), doxorubicin (DOX), mitomycin C (MMC), or vinblastine (VBL) After 10 to 14 days of growth in a 2-layer agar culture, colonies were counted in control and drug-treated wells, and drug effects were expressed as percentage of colony survival compared to controls. Remarkable similarities can be seen in the drug sensitivity patterns for all 5 drugs for the cells from the 2 different sources. Points, means of triplicate assays. |
phagolystic cytoplasm.

Hematoxylin-and-eosin-stained sections of MES-SA colonies on Day 14 in soft agar bore striking resemblance to sections of both the patient’s recurrent tumor and tumors developing in nude mice after MES-SA cell inoculation (Fig. 4c).

Ultrastructurally, MES-SA cells display features mentioned above with large centrally located nuclei and prominent nucleoli (Fig. 5a). The cytoplasm contains a few thin profiles of rough endoplasmic reticulum and many free ribosomes. Small mitochondria are present in groups. No epithelial features (i.e., secretory granules, desmosomes, tight junctions, or microvilli) are identified. A prominent feature of these cells is the presence of lipid vacuoles (Fig. 5b). Complex phagolysosomes are often seen.

**DISCUSSION**

The human sarcoma line of uterine origin (MES-SA) described herein was established in tissue culture by propagating tumor colonies developing in soft agar. Others have also reported propagation of human tumor cells in this manner (11, 18, 43). Individual tumor cells growing in monolayer and tumors produced by MES-SA inoculation of nude mice maintain histological features nearly identical to the primary human tumor. These tumors are undifferentiated with large anaplastic cells, prominent nuclei and nucleoli, and scant cytoplasm. The epithelial features seen on sections of the patient’s original tumor are absent on both the recurrent pelvic tumor and xenografted tumors in nude mice, indicating that the recurrence represented only the sarcomatous element of the mixed müllerian tumor.

The karyotype of MES-SA is abnormal, with 3 deletions, one translocation, and one marker chromosome (45,XX,-5,-6,-7, +t(5q, 6p),+ Mar 1). It remains to be seen whether any of these abnormalities are characteristic of human sarcomas, since extensive cytogenetic studies of those tumors have not been reported (32).

Although the development of cell lines from other human sarcomas has been reported, lines from malignant tumors of uterine origin are distinctly unusual. Ishiwata et al. (17) have described a cell line designated SKN, established from a uterine leiomyosarcoma. This line produced tumors in hydrocortisone-treated Syrian golden hamsters following s.c. cheek pouch inoculation. Tumorigenicity in nude mice and in vitro drug sensitivities of this line have not been described. Fogh et al. (10) have reported the establishment of a cell line from a mixed müllerian tumor of uterine origin which is tumorigenic in nude mice, but details of the characterization of that line are not available.

The following features displayed by the MES-SA cell line make it potentially useful for oncological and biological research.

**Multiple In Vitro Drug Sensitivities.** Prior studies of mechanisms of drug resistance and cross-resistance to anthracyclines have used non-human cell lines (15, 28, 34). Possible cross-species differences in those mechanisms of resistance have not been investigated. The degree of sensitivity to anthracyclines, mitomycin, daunomycin, and bleomycin displayed by MES-SA is unusual, and will provide the opportunity to study the development of resistant mutants under various conditions of selection. Differences in drug uptake, efflux, and metabolism may be readily studied in both the sensitive MES-SA line and resistant strains. Preliminary investigations of mechanisms of resistance to anthracyclines and bleomycin have begun in this laboratory using the MES-SA cell line.

**Tumorigenicity in Nude Mice.** The production in nude mice of tumors which retain sensitivities to various chemotherapeutic agents provides an excellent model for evaluating the efficacy and toxicity of new drugs in vivo, after initial screening or evaluation using the same cells in simpler in vitro assays. This line and other lines like it might someday provide a battery of tumors of known sensitivity which can be used for screening of new and potentially useful antitumor agents.

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