In Vitro Growth and Drug Sensitivity of Tumor Colony-forming Units from Human Tumor Xenografts

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ABSTRACT

To investigate the feasibility of using tissue obtained from human tumor xenografts for in vitro screening of new antineoplastic agents, we grew human tumor colony-forming units (CFU) in semisolid agar from xenografts serially passaged in nude mice. Growth of human tumor CFU was accomplished from nine xenografts representing five different histological tumor types (ovarian carcinoma, adenocarcinoma of the colon, malignant melanoma, epidermoid carcinoma of the lung, and malignant astrocytoma). Cloning efficiency ranged from 0.04 to 0.1% and showed significant variability both between tumor types and between individual animals bearing the same type of xenograft. A high percentage of tumor CFU was in S phase (47 ± 20% (S.D.)) as determined by the thymidine "suicide" technique. The number of tumor CFU observed increased linearly with increasing numbers of cells plated. In vitro drug sensitivity of the tumor CFU was assessed to Adriamycin, cis-platinum, and melphalan. The patterns of drug sensitivity were found to be reproducible and stable over a period of 9 months. Drug sensitivity curves to Adriamycin for five xenografts representing four tumor types showed complex patterns with plateau portions similar to those described for tumor CFU from primary tumors. The rank order of sensitivity of the tumors was compared to that of normal granulocyte-macrophage progenitors and, with the exception of the melanomas, was found to correlate well with clinical experience (order of sensitivity = colon < ovary < bone marrow). Growth of human tumor CFU from xenografts represents a reproducible and stable means for the study of the biology of tumor CFU and has potential applications as a means for screening new anticancer agents.

INTRODUCTION

Recent development of techniques for growing colony-forming cells from primary human cancers in semisolid media has allowed new approaches to the study of these tumors (7, 12) and to prediction of in vivo response to chemotherapeutic agents (1, 17, 22). Preliminary studies suggest that assessment of the fraction of tumor CFU killed by in vitro exposure to established drugs can reliably predict in vivo responses (1, 17, 22). It has been proposed that such systems may be useful for the screening of new chemotherapeutic agents prior to clinical trials.

Many types of human cancers will grow as xenografts in athymic (nude) mice (13, 16). Tumors propagated in nude mice have been used in the investigation of new chemotherapeutic agents (10, 13), and screening of new chemotherapeutic agents using nude mouse xenografts has been incorporated into the preclinical screening procedures of the National Cancer Institute (5).

We have investigated the feasibility of using serially transplantable human tumor xenografts as a constantly available source of tissue for screening new chemotherapeutic agents using the tumor colony-forming-unit assay. In these studies, we have successfully grown human tumor CFU from s.c. human tumor xenografts in nude mice using the semisolid system developed by Hamburger and Salmon (8) and Hamburger et al. (9). We have assessed the in vitro response of these CFU to established chemotherapeutic agents and demonstrated that this system can be used as a reproducible, adjunctive screening procedure for new chemotherapeutic agents and as a model for the study of human tumor CFU.

MATERIALS AND METHODS

Human Tumor Xenografts. Human tumor xenograft tissue was obtained from tumors serially passaged in nude mice. Nu/nu mice on a BALB/c background were obtained from the Nude Mouse Research Center of the University of California's San Diego Cancer Center. Tumors were established from primary human cancers and passaged by s.c. injection as described elsewhere (16). Xenograft tumors were excised for study when they reached a diameter of approximately 1.5 cm. Xenografts used in these studies included: adenocarcinomas of the ovary (T17, T385); adenocarcinomas of the colon (T183, T219); malignant melanomas (T242, T354, T355); an epidermoid carcinoma of the lung (T222); and a malignant astrocytoma (T24).

Processing of Xenograft Tissues. Tumor xenografts were aseptically removed from mice killed by cervical dislocation. The tumor was freed from surrounding connective tissue and overlying skin and placed in HBSS (Grand Island Biological Co., Grand Island, N. Y.). The tumor was then gently minced with dissecting scissors, and the tissue mince was gently pressed through a sterile, 40 mesh wire screen. A single-cell suspension was formed mechanically by passing the tumor cell suspension through serially smaller needles and by gentle pipeting with a Pasteur pipet. Total nucleated cells were counted in a hemocytometer.

Tumor Colony-forming Assay. The assay for tumor colony-forming cells in semisolid agar was adapted from that described by Hamburger and Salmon (9) for human ovarian carcinoma cells. The underlayer contained 1 ml of enriched McCoy's Medium 5A (Grand Island Biological Co.) with 10% heat-inactivated FCS (Flow Laboratories, Rockville, Md.), 5% horse...
serum (Grand Island Biological Co.), and 25% medium conditioned by BALB/c adherent spleen cells (see below) in 0.5% agar. The overlayer contained 1 to 7.5 x 10^5 tumor cells/ml in Connaught Medical Research Laboratories medium (Grand Island Biological Co.) as modified by Hamburger and Salmon (8), + 15% horse serum (Flow Laboratories) in 0.3% agar in a final volume of 1 ml. Assays were performed in triplicate 35-mm tissue culture dishes (Lux Scientific Corp., Newbury Park, Calif.) and incubated in 7.5% CO_2 at 37°C for 7 to 14 days. With an inverted microscope, aggregates of greater than 30 tumor cells were scored as colonies.

The morphology of the colonies obtained in these cultures was similar to that of tumor CFU described previously for primary human tumors (7, 8). The composition of colonies growing in the plates was assessed by removing single colonies with fine needles and placing them on slides. The intact or dispersed colonies were then stained with Wright-Giemsa or Papanicolaou’s stain. Tumor colonies were composed of large, distinctive cells under the inverted microscope, and stains of cells contained in the colonies showed them to be identical in morphology to cells from the original tumor. On no occasion were colonies of cells seen which appeared to be composed of murine hemopoietic cells.

Preparation of Adherent Spleen Cell-conditioned Medium. Medium conditioned by mineral oil-primed, BALB/c adherent spleen cells was prepared according to the method of Hamburger et al. (9). Six- to 8-week-old female BALB/c mice were given i.p. injections of 0.3 ml of mineral oil 4 to 12 weeks prior to sacrifice. The mice were then sacrificed by cervical dislocation, and the spleens were removed aseptically. The spleens were teased apart with needles, and a single-cell suspension was created by pipeting; 1 x 10^6 cells/ml were allowed to adhere to tissue culture dishes in Roswell Park Memorial Institute media with 10% FCS. After a 2-hr incubation at 37°C in 7.5% CO_2, the nonadherent cells were removed by washing 3 times with HBSS. The adherent cells were then overlaid with Roswell Park Memorial Institute medium + 10% FCS and incubated for 3 days at 37°C in 7.5% CO_2. Conditioned medium was harvested, centrifuged, Millipore filter sterilized, and stored at -20°C until use.

Assay for Normal Granulocyte-Macrophage Progenitors (CFU-GM). Assay for normal human granulocyte-macrophage progenitors was performed as described previously (21). Normal bone marrow, from patients undergoing routine orthopedic procedures or normal peripheral blood was collected in heparin. The cells were subject to Ficoll-Hypaque sedimentation (Pharmacia Fine Chemicals, Piscataway, N. J.) at 1200 x g for 20 min. The interface cells were collected and washed 3 times in α-medium with nucleosides (Grand Island Biological Co.). Peripheral blood cells were subject to overnight incubation with 2-aminoethylisothiouronium bromide-treated SRBC’s (14). SRBC rosette-negative cells were then depleted from the peripheral blood by a second Ficoll-Hypaque sedimentation in order to concentrate peripheral blood colony-forming unit cells. The final plating mixture contained α-medium with nucleosides + 15% FCS in either 0.8% methylcellulose or 0.3% agar.

Bone marrow or SRBC rosette-depleted normal peripheral blood cells were plated on WBC feeders after incubation with Adriamycin (see below). WBC feeders were prepared from normal peripheral blood cells as described by Pike and Robinson (15) and contained 1 x 10^6 normal, peripheral blood mononuclear cells in 1 ml of McCoy’s Medium 5A + 15% FCS in 0.5% agar. Bone marrow cells were plated in overlayers at 1 to 2 x 10^5 cells/ml, and SRBC rosette-depleted peripheral blood cells were plated at 5 x 10^5 cells/ml in triplicate 35-mm plates. Cultures were incubated in 7.5% CO_2 at 37°C for 10 days, and aggregates of greater than 20 cells were scored as colonies. Cultures for normal human colony forming unit cells grew 154 ± 103 colonies/plate (S.D. for 5 experiments).

Drug Sensitivity Testing. Tumor cell suspensions or suspensions of normal hemopoietic cells were exposed in vitro to the following cytotoxic agents: Adriamycin (provided through the courtesy of Farmitalia Carlo Erba, Milan, Italy); cis-dichlorodiamine-platinum (cis-platinum) (provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.); and melphalan (Burroughs-Wellcome Co., Research Triangle Park, N. C.). Adriamycin was dissolved in distilled water, and the drug concentration was confirmed by UV absorption on a Gilford 250 spectrophotometer (ε_480 = 17.2 A/mg/ml-cm). Cis-platinum was dissolved in distilled water. Injectable melphalan was dissolved in a propylene glycol diluent and diluted with HBSS. All solutions were stored in stock form at -20°C until use and were not subjected to repeated freezing and thawing.

Tumor cells or normal hemopoietic cells were incubated in Connaught Medical Research Laboratories medium with various drug concentrations for 1 hr at 37°C. The drug concentrations selected for in vitro testing were those used by Salmon et al. (17) in studies of primary ovarian carcinomas. These were: Adriamycin (0.05, 0.1, 0.15, and 0.3 µg/ml); cis-platinum (0.02, 0.04, 0.06, and 0.6 µg/ml); and melphalan (0.05, 0.10, 0.15, and 0.3 µg/ml). After 1 h of incubation with the drug, the tumor cells were washed 2 times with HBSS and plated in triplicate 35-mm plates. Drug effects were expressed as percentage of CFU survival as a function of drug concentration. Only experiments growing greater than 20 tumor CFU per 5 x 10^5 cells were analyzed for drug assays.

Assessment of Tumor CFU in S Phase. The percentage of tumor CFU synthesizing DNA was assessed by the thymidine suicide technique (11). Tumor cell suspensions (10^7 cells/ml) were incubated in HBSS with high-specific-activity [3H]thymidine (100 µCi/ml) (New England Nuclear, Boston, Mass.) for 20 min at 37°C. The cell suspension was then washed 2 times in α-medium containing 10^-5 M cold thymidine. The percentage of CFU in S phase was expressed as the percentage of reduction in tumor CFU relative to controls incubated with cold thymidine.

RESULTS

Tumor CFU have been grown successfully from 9 different human tumor xenografts representing 5 different tissues of origin (Table 1). Certain xenografts presented special technical problems which have limited the number of successful (>20 colonies/5 x 10^5 cells) cultures (see “Discussion”). Considerable variability was observed in the cloning efficiency (colonies/number of cells plated) between both tumor types and individual xenograft tumors (Table 1). The cloning efficiency in these experiments did not exceed 0.1% (range, 0.04 to 0.1%).

The number of human tumor CFU grown from these xenografts increased with increasing numbers of cells plated. Chart 1 demonstrates this relationship for 3 experiments using the ovarian tumor xenograft T17. The absence of nonlinear por-
tions of the curve argues strongly for a lack of significant cell-
cell interactions in the growth of the tumor CPU, and extrapo-
lation of the curve through the origin suggests that these
colonies originate from single cells. Identical results were ob-
tained in studies of the colon carcinoma xenografts T183 and
T219 and the epidermoid carcinoma T222 (data not shown).

Thymidine suicide experiments were performed to assess the
percentage of tumor CPU in S phase at the time of plating.
In 10 experiments on the ovarian (T17), colon (T219, T183),
melanoma (T242, T353), and astrocytoma (T24) xenografts,
the mean reduction in tumor CPU which resulted from a 20-min
exposure to high-specific-activity tritiated thymidine was 47 ±
20% (range, 0 to 93%).

Xenografts carried in nude mice are a potential source of
continuously available CPU for repetition of the assay. In a
highly selective environment such as a nude mouse, however,
it is likely that ongoing selection pressures may be exerted on
the tumor implant. This possibility was examined by a series of
tests of the sensitivity of tumor CPU from one xenograft (T17)
to a panel of 3 cytotoxic drugs. If selection pressures occurred,
a drift in the pattern of drug sensitivity of the tumor CPU might
be observed over time. In fact, no such drift was observed.

Results of repeated experiments assessing the effects of cis-
platinum, Adriamycin, and melphalan on T17 tumor CPU are
shown in Chart 2. Good agreement in the patterns of sensitivity
was observed for all 3 drugs, with the exception of one exper-
iment performed near the midpoint in time of these studies
using Adriamycin. The experiments using Adriamycin with T17
and 6 other xenografts now span a period of greater than 9
months without evidence of changes in patterns of tumor
coloncy-forming-unit drug sensitivity for any xenograft studied.

The standard error for triplicate plates from single experimen-
tials was 9.7 ± 6 colonies (96 data points from 21 experi-
ments; range, 2 to 20). The variability of colony counts differed
from tumor to tumor. The standard error of data points for the
ovarian carcinoma xenograft T385 was only 2 ± 0.8 colonies,
while that for the colon tumor T183 was 20 ± 1.5. The large
standard errors encountered in the use of the T183 xenograft
have led us to discontinue experiments using this tumor. De-
spite the large standard errors encountered in experiments
using T183, the pattern of sensitivity to Adriamycin of CPU
from this tumor was congruent with clinical experience; the
the tumor was highly resistant to Adriamycin in vitro (Chart 3B).

The complex patterns of drug sensitivity demonstrated in the
experiments discussed above were confirmed in studies of the
in vitro sensitivity to Adriamycin of tumor CPU from 6 xenografts
representing 4 different histological types (Chart 3). Not all
tumors studied were tested for Adriamycin sensitivity. These
curves are bimodal, composed of discrete linear portions
which level off into plateaus at high drug doses. These data are
similar to those obtained in studies of tumor CPU from primary
tumors (17) and suggest the presence of cell populations in
the specimen which are resistant to Adriamycin under these
conditions of drug exposure.

To improve perspective on relative sensitivity of the tumor
CPU to that of normal human tissues, the sensitivity of tumor
CPU was compared to that of normal granulocyte-macrophage
progenitors (CPU-GM) from peripheral blood and bone marrow
(Chart 3A). Normal hemopoietic progenitors were universally
more sensitive to Adriamycin than were the tumor CPU. A
comparison of results obtained using the CPU-GM assay with
those obtained on tumor CPU suggests that Adriamycin would
have a low therapeutic ratio in the treatment of these tumors,
an observation borne out by clinical trials. The use of the CPU-
GM assay as a contrast to that for tumor CPU, however, does
provide insight into the relative toxicity of a drug for tumor and
normal tissues.

**DISCUSSION**

It is difficult to overemphasize the potential importance of the
development of in vitro clonagenic assays for human solid
tumors (3). Such assays offer prospects for prediction of sen-
sitivity to anticancer drugs and for investigation into the basic
biology of tumor cell growth. Although not formally proven to
be tumor “stem” cells, it is possible that tumor CPU represents
a subpopulation of cells with extensive proliferative potential,
responsible for the maintenance of the tumor mass and there-
fore analogous in some respects to hemopoietic stem cells
(18). As such, tumor CPU provide a logical means for the study
of the effects of chemotherapeutic agents on tumor growth.

In these studies, we have attempted to assess whether
human tumor xenografts in nude mice could provide a readily

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**Table 1**

*Culture of human tumor xenografts for tumor CPU*

<table>
<thead>
<tr>
<th>Tumor of origin</th>
<th>Cultures attempted/successful&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Colonies/5 x 10&lt;sup&gt;4&lt;/sup&gt; cells</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma-ovary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T17</td>
<td>25/16 (64)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>113 ± 103&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25–338</td>
</tr>
<tr>
<td>T385</td>
<td>3/3 (100)</td>
<td>23 ± 1.0</td>
<td>22–24</td>
</tr>
<tr>
<td>Adenocarcinoma-colon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T183</td>
<td>9/5 (56)</td>
<td>202 ± 165</td>
<td>78–433</td>
</tr>
<tr>
<td>T219</td>
<td>4/3 (75)</td>
<td>131 ± 38</td>
<td>106–175</td>
</tr>
<tr>
<td>Melanoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T242</td>
<td>5/3 (60)</td>
<td>53 ± 30</td>
<td>28–87</td>
</tr>
<tr>
<td>T354</td>
<td>4/4 (100)</td>
<td>198 ± 86</td>
<td>98–331</td>
</tr>
<tr>
<td>T355</td>
<td>5/5 (100)</td>
<td>280 ± 239</td>
<td>70–664</td>
</tr>
<tr>
<td>Epidermoid carcinoma-lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T222</td>
<td>6/4 (67)</td>
<td>168 ± 194</td>
<td>78–391</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T24</td>
<td>3/3 (100)</td>
<td>176 ± 121</td>
<td>58–172</td>
</tr>
</tbody>
</table>

<sup>a</sup> Studies growing >20 tumor colonies per 5 x 10<sup>5</sup> cells.
<sup>b</sup> Numbers in parentheses, percentage successful.
<sup>c</sup> Mean ± S.D.
Adriamycin, and cis-platinum on the growth of tumor CPU from the ovarian tumor colony-forming unit kill and the lack of variability in tumor colony-forming unit sensitivity for all 3 drugs over this period of time.

It is not clear whether observed animal-to-animal and tumor-to-tumor variability in cloning efficiency seen in these studies represents differences in xenograft composition, tumor preparation, or culture technique. Because of differences in tumor-to-tumor cloning efficiency for the same xenograft, comparisons of responses obtained by in vivo drug administration to nude mice and subsequent assessment of tumor colony-forming unit content of a xenograft as a measure of cell kill, as performed by others (2), would be difficult to interpret in this system. It appears that in vitro patterns of drug sensitivity over time are relatively stable (Chart 2) and that reliable assessment of drug sensitivity in vitro can be accomplished.

There is acceptable variability within experiments using this system but greater variability between experiments. The test-retest reliability of the tumor colony-forming unit assay system in primary tumors has not been rigorously established, and it is possible that the variation seen in our studies may be a common factor of all such systems in their present form. Automation of colony counting and better tumor dispersal techniques may improve the technical aspects of the assay system in the future. For the present, the use of xenografts allows one to concentrate efforts on tumors which are technically suited for use in this assay system until methodology improves.

Several characteristics of the assay for primary tumor CPU which are important to its potential utility in screening of antineoplastic agents in vitro have been stressed in previous publications. The linearity of the relationship between number of cells plated and CFU formed (9) and the high fraction of cells in S phase, as determined by the thymidine suicide technique (8), are observations in primary tumors which are also obtained in our studies of CFU from human tumor xenografts passaged in nude mice. The cloning efficiencies of the xenografts are similar to those reported by Hamburger and Salmon (8) for primary ovarian tumor CPU and to our own experience in the culture of human primary tumor CPU (plating efficiency, 0.005 to 0.07%).

We addressed the question of whether selection pressures on patterns of drug sensitivity in serially passaged xenografts exist, and we found that CFU from xenografts responded in a similar fashion to exposure to antineoplastic agents over a period of 9 months. However, the establishment of a nude mouse xenograft results in changes in morphological and functional characteristics of some tumors (4) and in increased mitotic activity in others (19). Preliminary evaluation of the response of newly established xenografts to chemotherapeutic agents correlates closely with results obtained in patients (20), but effects of establishment and passage of a xenograft on the in vitro pattern of drug sensitivity of tumor CPU are not known, and, in the absence of such data, one cannot be sure how closely xenografts will continue to reflect the sensitivity of the primary tumors. Further studies of primary tumors in vitro and tumor xenografts established from them as a source of tumor CPU may in the future impart knowledge of the selection pressure exerted by the process of establishing the xenograft.

Even if selection pressure in establishing xenografts does exist, our data suggest that tumor CPU grown from xenografts can be used effectively to answer important questions in cancer biology. The complex nature of drug sensitivity curves obtained in studies of primary tumors (17) and these xenograft CPU suggest the existence of subpopulations of cells which appear
to be resistant to cytotoxic agents in vitro and offer the opportunity to study this phenomenon in detail. The ready availability of xenograft CFU for repetitive study makes them particularly advantageous for this purpose.

This system is well suited to the screening of new antitumor agents in vitro prior to clinical trials. A bank of human xenografts passaged in nude mice, representing both treated and untreated tumors, can be established. Large quantities of tumor material would be available for the screening of new drugs, and extensive and repetitive investigation of agents showing antitumor activity would be possible. Although subject to the limitations of all in vitro systems for the screening of new agents (6), some perspective on the therapeutic ratio of new agents may be gained by comparative studies performed on clonogenic cells grown from normal human tissues. Such studies may provide useful information as to initial doses of drugs to be used in clinical trials, as well as a measure of relative selectivity of the new drug. Comparison studies with drugs of known activity at attainable and well-tolerated in vivo concentrations may also be made.

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