Rapid Growth of Human Cancer Cells in a Mouse Model with Fibrin Clot Subrenal Capsule Assay

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ABSTRACT

Rapid in vivo growth of cultured human cancer or leukemia cells was achieved by implantation into the subrenal capsule of mice. A solid structure, necessary for accurate implantation and measurement of tumor growth in this model, was provided by stepwise addition of fibrinogen and thrombin to the tumor cells, leading to rapid enzymatic formation of a solid tumor-fibrin matrix. Human leukemia and epithelial cancers increased in volume between 6- and 40-fold when measured 6-10 days after implantation into normal or immunosuppressed mice. Immunosuppression of host CD-1 mice was achieved by cyclosporine given daily after tumor implantation, cyclophosphamide given preimplantation combined with cyclosporine, or whole-body irradiation given preimplantation. Confirming the validity of tumor measurements, tumor histology in the immunosuppressed mice revealed cell proliferation, invasion, and neovascularization. Similarly, no artifactual measurement of tumor growth was observed by nonviable cancer cells, implanted after in vitro exposure to a known cytotoxic concentration of thiotepa. This model provides an economical, short-term technique for the in vivo study of human tumor growth, for the evaluation of new cancer therapies, and for in vitro - in vivo drug activity correlations in specific types of human cancer or leukemia cell lines.

INTRODUCTION

The examination of cultured tumor cells, derived from mice or other rodents, has provided insights into the biology and treatment of cancer. Rodent cancers have been utilized widely because of their consistent growth capacity, both in cell culture and in animal models. Unfortunately, the biological behavior and therapeutic responses of commonly used rodent tumor systems do not reflect those of common human malignancies (1).

Stimulated by the need for experimental models that more closely resemble human neoplasia, numerous cell lines have been established in serial passage from human cancer specimens. These cultured cells are used for investigations of new cancer therapies, based on the hypothesis that such results will correspond to the sensitivity or resistance of human cancers from which the tumor cells were derived (2). Although human tumor cell lines are useful for such in vitro experiments, they do not generally provide practical models for experiments in vivo. Preclinical development of cancer therapy requires the evaluation of drug combinations, pharmacokinetics, and the therapeutic activities of metabolites that are generated in vivo (1). Furthermore, many new therapeutic strategies are targeted against biological interactions of cancer cells with the host (i.e., tumor angiogenesis, invasion, and metastases), and these cannot be examined by using only cells in culture.

Congenitally athymic (nude) immunodeficient mice can be used as hosts for the propagation of human tumor cell lines, passaged as solid tumor grafts in the s.c. site. However, tumor growth in these animals often requires many weeks or months. Furthermore, many cultured tumor cell lines fail to produce tumors in these mice, or do so with such low frequency as to make this system impractical.

A short-term method for the growth of human tumors in mice was developed by Castro and Cass (3) and later refined by Bogden et al. (4), using solid tumor fragments implanted under the kidney capsule. SRC assay allows precise measurement (accurate to 0.1 mm) of changes in tumor size, as judged by a stereomicroscope with an ocular micrometer. A 7-fold greater blood flow has been demonstrated for SRC-implanted as compared to s.c. tumors, suggesting that the SRC is advantageous for delivery of nutrients and systemic antitumor agents (5). Some human tumors can be studied 4-6 days after implantation into normal mice before immune rejection, providing economic advantages over use of nude mice (4). Immunosuppression of normal mice further extends the duration of tumor growth and improves the potential of the assays for discriminating active treatments (6).

The SRCA has not been well defined with direct implantation of cultured cell lines or human leukemias, since this technique requires a solid matrix for accurate implantation and serial measurement. In this report, we describe a simple, rapid technique for direct implantation and quantitative analysis of cultured human cancer or leukemia cells in the SRCA. Our studies demonstrate that (a) stepwise additions of fibrinogen and thrombin provide a solid FC as a tumor cell matrix; (b) the FC matrix maintains homogeneity and viability of implanted tumor cells; (c) rapid growth in vivo is observed by diverse types of human epithelial or leukemia cell lines; and (d) these tumors display histological features that represent malignant growth in vivo, including frequent mitoses, neovascularization, and invasion into normal tissues.

MATERIALS AND METHODS

Cell Cultures. A431 vulvar cancer (American Type Culture Collection), CX1 colon and LOX melanoma (NCI-Frederick Cancer Research Center), and T24 bladder cancer (Dr. J. Fogh Sloan Kettering Institute for Cancer Research) were grown as monolayer cultures in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified, 10% CO2 incubator. HL60 leukemia cells were grown as a suspension culture in the same supplemented medium plus nonessential amino acids (Gibco) and L-pyruvate (Gibco), and these cells were obtained after l.p. passage in athymic nude mice (A. Bogden and W. Cobb, Bogden Laboratories). Human karyotypes of HL60 and T24 cells were confirmed in our laboratory, and the human karyotype and non-Hela isoenzyme pattern of other cell lines were established by the source laboratories. All cells were determined to be Mycoplasma free by culture techniques.

Nonmalignant WI38 fetal lung fibroblasts (American Type Culture Collection) and FS2 foreskin fibroblasts (Dr. R. Sager, Dana-Farber Cancer Institute; Ref. 7) were maintained in early-passage monolayers with the same medium and culture conditions.

The abbreviations used are: SRC, subrenal capsule; SRCA, subrenal capsule assay; FC, fibrin clot; CSA, cyclosporine; CVT, cyclophosphamide.

Received 10/6/86; revised 3/17/87; accepted 4/15/87.

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1 Supported by USPHS Grants CA01157 and CA42802 and American Chemical Society Grant PDT-221C.
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[CANCER RESEARCH 47, 3824-3829, July 15, 1987]
Fibrin Clot Formation. Fibrinogen (bovine; Sigma Chemical Co., Lot 55F9305) was stored at -70°C in aliquots after dissolving in phosphate-buffered saline (20 mg/ml). Optimal activity was achieved by slow dissolving over 1-2 h on a rotor, with care to avoid bubbling or clumping. Thrombin (bovine; Sigma, Lot 114 F-9461) was stored at -70°C in aliquots (20 units/ml) after dissolving in double-strength Dulbecco’s modification of Eagle’s medium (Gibco).

To provide a fibrin matrix, logarithmically proliferating cells were first obtained as a single cell suspension. Monolayer cultures were suspended by treatment with trypsin-EDTA solution (Gibco) and washed 3 times in complete medium to remove trypsin-EDTA and cellular debris. The cells were then counted and centrifuged at 100 x g for 5 min to form a loosely packed cell pellet, and the supernatant was discarded. To facilitate subsequent handling of the cell pellet and reagents, this last centrifugation was performed after transferring the desired cell number with 1.2 ml complete medium into a 1.5-ml microcentrifuge tube (VWR Scientific). In a typical experiment, 1 x 10^7 centrifuged cells produced a pellet with a volume of about 250 μl; 15 μl fibrinogen (20 mg/ml) were added and dispersed throughout the cell pellet by gentle tapping; 8 μl thrombin (20 units/ml) were then added and dispersed in a similar manner. The added volumes did not exceed 10% of the total volume to ensure over 90% cellularity of the implanted grafts.

The cell pellet was then incubated at 37°C for 5-10 min. The thrombin produced enzymatic cleavage of the fibrinogen, resulting in a solid FC matrix surrounding the tumor cells. Using a sterilized, triangular metal spatula wetted with cold medium, the pellet was rimmed onto a Petri dish with RPMI 1640 supplemented with penicillin, streptomycin, and 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer (Sigma), then immediately cut with a no. 10 scalpel into about 100 1- to 1.4-mm fragments for implantation. Thus, each implant contained about 1-2 x 10^6 cells, and the cell number per implant could be monitored and modified by altering the number and size of sectioned fragments.

SRC Assay. The SRC assay followed the technique described by Bogden et al. (4). Female CD-1 mice (24-28 g; Charles River Laboratories, virus and pathogen free) were anesthetized with chloral hydrate, placed on a sterile surface, and swabbed with ethanol-Zephiran chloride solution. A 1-cm incision was made through the skin and body wall in the region of the left kidney. The kidney was partially exteriorized and a small slit was made in the renal capsule. The implanted fragment was loaded onto the tip of a 19-gauge trocar and inserted through the slit to deposit under the capsule. Immediately after implantation, the longest and shortest diameters of the implant were measured in situ with a dissecting microscope, equipped with an ocular micrometer calibrated so that 10 ocular micrometer units equaled 1 mm. The kidney was then replaced into the body cavity, and the incision was closed with sterile wound clips. The implantation procedure required less than 2 h for a typical experiment involving 30-40 mice.

Mice were randomly assigned to treatment groups (5-7 mice/group) after implantation. At the termination of assay, each animal was weighed and sacrificed by cervical dislocation under anesthesia. The abdominal cavity was exposed, and the left kidney was removed and weighed and sacrificed by cervical dislocation under anesthesia. The kidney was then placed under the dissecting microscope for measurement of final tumor size. Tumor sizes were expressed as the average of longest and shortest diameters, as described by Bogden et al. (4), or as tumor volume by the formula (length x width x width x 1/3).

Dugs and Irradiation. CSA was purchased from Sandoz Pharmaceuticals as a liquid suspension (100 mg/ml) and diluted in oil (Miglyol 812; Dynnimat Nobel) prior to injection. CSA injections (80-120 mg/kg of body weight) were done s.c. on a daily basis starting up to 2 days after tumor implantation. Cyclophosphamide (Cytoxan; Mead Johnson) was dissolved in saline immediately before use and injected i.p. (150 mg/kg of body weight) 24 h before tumor implantation. Whole-body irradiation was achieved with a General Electric Maxitron X-ray at 60 rads/min, administered 4-16 h prior to implantation.

Histological Staining. Initial FC pellets and tumor-bearing kidneys were fixed in buffered 10% formalin, embedded in paraffin, and serial sections were prepared and stained with hematoxylin and eosin. In addition, trypan blue studies were also used to monitor the quality (initial cell viability) of implanted grafts between replicate experiments.

RESULTS

Histological and Gross Appearance of FC Cell Pellets. The FC protocol was effective in providing a solid matrix for a variety of human cancer or normal cells, and no adverse changes were observed in cell morphology (Fig. 1). After fibrin formation, the cell pellets were transferred rapidly into buffered medium and sectioned into 1-mm implants, and cell viability was well preserved throughout the 1- to 2-h period needed for the SRC implantation (less than 10% decrease in the number of viable cells by trypan blue exclusion). As shown in Fig. 1, the cells maintained a discrete, solid structure under the kidney capsule, and 3-dimensional growth was observed macroscopically and quantified directly by changes in average diameter, or by the computed tumor volume.

To confirm the validity of tumor measurements, we investigated histological features in multiple cross-sections of formalin-fixed tissues. As shown in Fig. 2, these sections demon-
Growth of FC Implants versus Passaged Solid Tumors. After passage in nude mice, human cancers commonly reveal marked heterogeneity in terms of viability and cellularity (9). This heterogeneity can lead to unreliable growth and passage into other nude mice, posing a problem for studies that optimally require uniform growth and passage. We studied implants from the CX1 human colon tumor line, taken from monolayer cell culture and implanted by the FC technique, or from solid CX1 tumor that grew after s.c. injection of the same cells into athymic nude mice. Growth of the FC implants exceeded the s.c. passaged tumors, even on day 12 after implantation into CSA-immunosuppressed mice (Fig. 3). Histological studies, performed on representative samples of s.c. passaged tumor grafts (day 0 implants), revealed numerous areas of microscopic necrosis, despite our preselection of “viable” tumor material by gross appearance; the overall viability of the s.c. passaged grafts was also demonstrated by growth of new tumors after s.c. implantation of representative material into nude mice. In addition, the initial growth rates of A431, LOX, and HL60 cells (Fig. 4) exceeded those in published reports with the same tumor cells or related sublines, implanted s.c. in nude mice (10-12). Taken together, these results suggest that the FC technique can improve the homogeneity of implanted tumor cells and sustain their growth capacity in vivo, especially when compared to s.c. passaged tumors in nude mice.

Growth of Human Cancer or Leukemia Cell Lines. In normal mice, human cancer and leukemia cells of diverse histological types exhibited distinct growth rates on days 0–6 (Figs. 3 and 4), followed by immune rejection. In contrast to this common pattern of tumor regression on days 6–9 in normal mice, a dramatic increase in tumor size was observed after day 6 in immunosuppressed mice, demonstrated by growth in 3 dimensions (Fig. 1) and by the computation of tumor volume as shown in Fig. 4. Studies with A431 cancer cells demonstrated improved growth on days 9–10 in mice immunosuppressed with CYT plus CSA versus CSA alone (Fig. 4A). As shown in Table 1, no statistical difference was observed between tumor sizes in mice immunosuppressed by whole-body irradiation (650 rads) versus CYT plus CSA (P > 0.2; Student’s t test). Similar results were obtained with HL60 cells in whole-body-irradiated mice (data not shown).

In independent experiments, consistent growth was observed by the same cancer cell lines, implanted after multiple (1–10)
RAPID GROWTH OF HUMAN CANCER CELLS IN SRCA

Fig. 4. Growth of human tumor cells in the SRCA. Tumor volumes were computed by the formula (length × width × width × 1/3) (31). Cell culture, FC and SRC implantation techniques were done as described in text. Points, tumor volumes on various days after implantation relative to average initial volume on day 0; bars, SE. A, A431 vulvar cancer cells; B, LOX melanoma cells; C, HL60 leukemia cells. Tumor regression after day 6 in control (immuno-competent) mice corresponded to histological evidence for immune rejection (6, 8).

Table 1 Growth of human tumor or normal cells in the SRCA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells</th>
<th>Change in average tumor diameter (OMU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td></td>
<td>Change in tumor size (average diameters, by ocular micrometer units) ± SE, measured by change from base line (size on day 0). Cells were passaged in culture up to 10 times between individual experiments. T24 cells + thiotepa were exposed in culture to 40 μg/ml thiotepa for 1 h before implantation, a treatment producing over 95% cell kill by clonogenic survival. Immunosuppression was achieved by CYT plus CSA for day 9–10 groups, as described in Figs. 1 and 4, or by 650 rads whole-body irradiation administered 4–16 h before tumor implantation (groups designated +RT, NT, not tested; T24, bladder cancer; A431, vulvar cancer; FS2, foreskin fibroblasts; WI38, fetal lung fibroblasts.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 6</td>
</tr>
<tr>
<td>Cancer</td>
<td></td>
<td>+21.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>T24</td>
<td>+20.7 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>T24 + thiotepa</td>
<td>-0.5 ± 1.4</td>
</tr>
<tr>
<td>1</td>
<td>A431</td>
<td>+7.3 ± 1.1</td>
</tr>
<tr>
<td>2</td>
<td>A431</td>
<td>+7.2 ± 1.4</td>
</tr>
<tr>
<td>3</td>
<td>A431</td>
<td>+9.1 ± 1.1</td>
</tr>
<tr>
<td>4 (+RT)</td>
<td>A431</td>
<td>NT</td>
</tr>
<tr>
<td>5 (+RT)</td>
<td>A431</td>
<td>NT</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>+2.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>FS2</td>
<td>-0.1 ± 2.0</td>
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<tr>
<td>2</td>
<td>FS2</td>
<td>+0.5 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>FS2</td>
<td>NT</td>
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<tr>
<td>1</td>
<td>WI38</td>
<td>+2.1 ± 1.6</td>
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</tbody>
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* OMU, ocular micrometer units.

DISCUSSION

In this report we describe a rapid, simple, and quantifiable technique for direct implantation and short-term growth of human cancer or leukemia cells in mice. The ability of tumor cells to survive and grow from a fibrin matrix is consistent with other investigations in culture and in vivo. Dvorak et al. (14) described a "fibrin cocoon," formed from plasma proteins, that envelops some tumor metastases, and suggested that it may provide a partial barrier to immune rejection. Fibrin has been observed in the earliest stages of many experimental tumor metastases (15), although other studies are not consistent with these (16). Several investigators have utilized tumor transplantation models that contain high-fibrin clots, formed from peritoneal or plasma constituents (17). Using the 6-day SRCA in immunocompetent mice, Stratton et al. (18) reported consistent growth with malignant ascites implanted after the tumor cells had been centrifuged and allowed to clot, with or without added fibrinogen. Furthermore, some studies suggest that fibronectin and related fibrinopeptides, which are components of commercial fibrinogen preparations, can promote angiogenesis (19) and tumor cell proliferation (15, 20).

The SRCA has gained wide popularity as a short-term model for evaluation of cancer therapy in vivo. Using highly cellular human cancers, obtained from s.c. passage in nude mice, reproducible growth can be achieved during the 6-day period after transplantation into immunocompetent mice (4, 21). With such passaged tumors, several studies have demonstrated excellent correlations between the 6-day SRCA in normal mice and results with the same tumors in the s.c. position of athymic nude mice (4, 11, 21). Major advantages of the 6-day SRCA (Figs. 1 and 2) that showed mitoses and other properties of in vivo tumor proliferation, these results provide evidence that the measured growth of FC implants represents the in vivo proliferative capacity of the tumor cells, in contrast to some artificial changes in graft sizes.
include (a) the economy of broad-scale experiments with normal mice versus athymic nude mice (in most laboratories, the cost can be over 10-fold lower than comparable studies with athymic nude mice), and (b) the rapid end point of 6 days, compared to 30+ days typical for s.c. tumors in nude mice.

While the SRCA does provide several advantages with passaged human tumors, a major drawback for some laboratories has been the continued requirement for athymic nude mice bearing s.c. passaged tumors, used as a source of human tumor material for initial implantation. The stringent requirements and high expense to maintain even small numbers of athymic nude mice have limited such in vivo experimentation to the few laboratories or institutions that can support such specialized facilities. In contrast, the FC-SRCA requires only standard facilities for tissue culture and normal mice and, thus, should allow broader experimentation with human cancer or leukemia cells in vivo.

The dramatic improvement in tumor growth, observed in the immunosuppressed mice on days 9–12, is consistent with other studies from our laboratory with solid human tumor grafts (6). Using monoclonal antibodies to various subsets of mouse lymphocytes, our immunoperoxidase studies suggested that CSA-sensitive T-cells (L3T4 positive) are a major component of graft rejection in the 6-day SRC model (6). However, the results with A431 cells (Fig. 4A) indicate that CSA-treated CD-1 mice retain some degree of immunocompetence, which can be blocked further by CYT (22). CYT alone allowed tumor growth for about 6 days, but graft rejection occurred by days 8–10 in mice not given CSA.

Our previous experiments also suggested that the longer (10-day) assay period could improve the capacity of the SRC assay to discriminate active treatments, since differences between treated versus controls are more readily assessed with the larger control tumors (6). Such differences also become important for discrimination of combination therapies versus single agents; our preliminary studies suggest that the evaluation of therapeutic synergism (13) is more readily achieved in the period on days 9–12 after tumor implantation. Similarly, some investigators have reported definite histological evidence for drug response but minimal or no changes in tumor size after treatment with known active antitumor agents (8), and these studies have led to some difficulty in the interpretation of what constitutes a positive therapeutic response, specifically in understanding how decreased tumor volume correlates with tumor cell death (23). Our experiments with chemotherapy-pretreated tumor cells suggest a feasible approach to investigate such changes in tumor sizes on various days after treatment in vivo, related to cell death measured by parallel studies in culture.

The present histological studies also demonstrate that certain properties of neoplastic growth in vivo (e.g., angiogenesis and invasion) are more readily assessed during the period on days 6–12 after implantation into the immunosuppressed mice. These results are consistent with the studies by Rybak et al. (24), who described consistent angiogenesis on day 11 after implantation of solid human tumors into the SRC of athymic nude mice. Using syngeneic or allogeneic rodent tumor grafts, Distelmanns et al. (25) reported reproducible invasion on day 8, and proposed a scoring system to quantify effects of drugs against tumor invasion. Using total tumor growth and/or histological changes as therapeutic end points, the FC-SRCA in immunosuppressed mice may provide a more economical technique for in vivo evaluation of antitumor agents that are targeted against such biological properties of human tumor cells.

The problem of drug-drug interactions between some chemotherapy and cyclosporine has been suggested by recent investigations (26, 27). While the mechanism for this interaction (and its relevance for various types of anticancer drugs) is presently unclear, concurrent administration of both CSA and the chemotherapy appears to be necessary for such effects. To avoid such potential interactions, we have routinely administered cancer therapies on day 0 or 1 after tumor implantation, using short half-life agents which are effectively gone by the time we start CSA on day 2. In some experiments, we utilized biological therapies (i.e., tumor necrosis factor) on the same day with CSA, in order to study therapeutic response at a time of early neovascularization; these latter studies demonstrated no increased toxicity to normal or tumor tissues compared to controls. Furthermore, our studies with whole-body irradiation, given before tumor implantation, support the studies by Basler et al. (28) that demonstrate effectiveness of this technique, providing short-term immunosuppression without drug-drug interactions. Other protocols for either short- or long-term immunosuppression of mice have been described (2, 22, 29), and these may offer certain advantages under various experimental conditions.

Bennett et al. (26) reported significant toxicity by CSA alone, administered daily at doses of 60 mg/kg/day. In contrast, we routinely used CSA in doses of 80–120 mg/kg/day with no significant toxicity compared to controls, measured by weight loss or abnormal renal histopathology. However, we utilized an oil-based solvent for CSA, a different weight and strain of mice, and a shorter period for CSA treatments (beginning after animals recovered from anesthesia and resumed water consumption), and several of these factors may account for the differences in toxicity. Preliminary studies with our oil-based CSA in 20- to 22-g BALB/c mice also revealed poor tolerance to doses above 60 mg/kg/day, suggesting that CSA regimens must be optimized for the weight and strain of mice used.

The advantage of in vivo models to develop new cancer treatments has been stressed by both clinical and laboratory investigators (1, 3, 11, 23). While many studies utilize human cancer or leukemia cells in culture, the number of in vivo studies that evaluate the same therapies and human cancer cells are limited by the expense of athymic nude mice, erratic growth and “take” rates, altered genetic properties of passaged tumors, etc. The studies presented here suggest an economical, short-term technique to evaluate in vivo potency, metabolism, and optimal combinations of new anticancer agents, in conjunction with investigations of their activity in specific human cancer or leukemia cells in culture. The CX1, LOX, and HL60 cell lines (Figs. 3 and 4) are currently utilized for similar preclinical investigations by research institutions (11, 30, 31). Our positive results with A431 and HL60 cells also suggest uses for in vivo studies related to growth factors or differentiation, i.e., to extend experiments that commonly use these or related tumor cell lines in culture (10, 12). In addition, these studies may provide a practical experimental model to investigate host-tumor interactions, effects of the tumor cell microenvironment, and other biological properties of human cancer cells in vivo.

ACKNOWLEDGMENTS

We thank A. B. Pardee, A. E. Bogden, J. A. Stratton, and J. A. Bennett for helpful comments, and Patricia Donahoe and Don Kufe for independent and critical reviews of histologies.

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