Optimization and Characterization of the Capillary Human Tumor Clonogenic Cell Assay

Francis Ali-Osman and Patricia A. Beltz

Northwest Neuro-Oncology Research Laboratory, Department of Neurological Surgery, University of Washington, Seattle, Washington 98195 [F. A. O.] and Hipple Cancer Research Center, Wright State University School of Medicine, Dayton, Ohio 45460 [P. A. B.]

ABSTRACT

The capillary human tumor clonogenic cell assay (HTCA) has been shown to have important advantages over conventional HTCAs. In the present report, this promising novel HTCA was further optimized and characterized using 46 primary human tumor specimens, 6 human tumor cell lines (1 astrocytoma, 2 colon carcinomas, 1 melanoma), and 2 murine leukemias. Hydrocortisone, epidermal growth factor, heat-inactivated fetal calf serum, and horse serum were investigated for their ability to modulate tumor colony formation in the assay. Critical assay parameters that can affect tumor colony formation, namely, cell seeding density, agarose concentration, culture volume, capillary tube geometry, and capillary tube sealing, were also investigated. The results showed that serum (optimum concentration, 20%) was obligatory for tumor colony formation, and that both epidermal growth factor (50 ng/ml) and hydrocortisone (2.5 ng/ml), although supportive of colony growth, were not absolute requirements. Plating at 2.5-3 x 10^5 cells/ml in a culture volume of 50 μl/capillary tube and an agarose concentration of 0.2% optimized colony formation (number, size, and distribution of colonies along the capillary tube) by primary human tumor cells. The cell lines generally formed colonies best at lower seeding densities and in lower culture volumes (30 μl/tube). Colony formation was significantly better in unsealed than in sealed capillary tubes and growth was just as good, and in some cases, better in round capillary tubes than in square ones. Using ovarian carcinoma cells, the Cellscan prototype system was demonstrated as feasible for automated counting and evaluation of tumor colony growth in capillary tubes. A comparison of the capillary HTCA and the agar double-layer assay in Petri dishes produced a median plating efficiency of 0.18 for the capillary HTCA and 0.036 for the Petri dish method. The overall success rate was 77% for the former and 53% for the latter assay.

INTRODUCTION

The in vitro clonogenic growth of human tumor cells obtained from fresh tumor biopsies, effusions, and aspirates has been reported by several investigators using different HTCA methods (1-4). Of these, the technique originally described by Hamburger and Salmon (1) utilizing an agar double layer in Petri dishes has been, by far, the most widely used. Although advances have been made that have improved its performance, overall, low PEs and low rates of successful cloning still constitute major problems investigators using this assay technique have to contend with (5-7).

In initial attempts to improve upon the performance of HTCAs, Ali-Osman and Maurer (8, 9) described a clonogenic cell assay in glass microcapillary tubes for tumor cell lines and further modified it to successfully clone cells obtained from fresh human tumor specimens (4, 10). Prior to these studies, the only reported attempts (11) to clone tumor (rat hepatoma) cells in agar contained in capillary tubes were not very successful. Capillary tubes had, however, been used successfully to isolate and grow in liquid medium, single cells of the Earle's strain L mouse sarcoma, and the EL 4 mouse lymphoma cell lines (12), and to clone hematopoietic cells in methylcellulose (13) and soft agar (14-18). However, since the first reports of the successful cloning of tumor cells in agar contained in capillary tubes, the technique has been utilized by other investigators to successfully clone cells derived from many histological types of human tumors (19-22) and cell lines. Drug sensitivity studies including both prospective and retrospective in vivo-in vitro correlative trials have also been performed with the assay (23-26).

Although the studies mentioned above demonstrated unique advantages of the capillary HTCA over conventional assays (4, 10, 20, 21) none of them had examined in detail the many critical parameters that can affect its optimum performance. In this report we describe, using primary human tumor specimens and established cell lines, the further characterization, optimization, and standardization of the capillary HTCA. The need for and the optimum concentrations of colony growth-supporting culture supplements, namely, HC, EGF, HS, and FCS, were investigated. Critical assay parameters, namely, cell seeding density, agarose concentration, capillary tube geometry, culture volume per capillary tube, and capillary tube sealing, were also investigated. The overall performance of the capillary HTCA was compared, head-to-head, with that of the double-layer agar HTCA. A prototype automated system for counting and analyzing colony growth in capillary tubes was also evaluated.

MATERIALS AND METHODS

Primary Tumors

Tumor specimens were obtained incidental to surgery on approved protocols and in accordance with federal and institutional guidelines. Specimens were placed in McCoy's 5A medium containing 5% FCS and preservative-free heparin and transported to our laboratories, usually within 2-24 h after obtainment. No specimens were excluded because of the size or type of tumor. However, a minimal viability of 30% was required of a cell suspension for inclusion in the study. For each tumor, the histological diagnosis of malignancy was established independently by a pathologist as part of the routine diagnostic workup of the patient. Table 1 summarizes the number and types of primary human tumors used in this study.

Tumor Cell Lines

Two human adenocarcinomas of the colon (HT 29 and Colo 205), a human malignant melanoma (HT 144), a human malignant astrocytoma (HTB 17), and two mouse leukemic cell lines (P 388 and L 1210) were used in various parts of this study. All six cell lines were obtained from the American Type Culture Collection, Rockville, MD, and were cryopreserved in RPMI 1640 containing 20% FCS and 10% dimethyl sulfoxide. Prior to use, thawed cells were washed twice, cultured in RPMI 1640 or MEM containing 15% FCS, and passaged once.
Capillary Tubes

Round 100-µl capillary tubes (Clay Adams, Parsippany, NJ) were cut to 9.75 cm in length, washed for 2 h in an ultrasonic bath with deionized water containing a nontoxic detergent, and finally rinsed with five exchanges of double-distilled water. The cleaned capillary tubes were dried heat sterilized for 2 h before use. Square capillary tubes were obtained from Glass Company of America, New York, NY, and were also cleaned as described above before being used to clone cells.

Growth Factors and Enzymes

EGF was purchased from Collaborative Research Inc., Bedford, MA, and hydrocortisone, DNase, collagenase, and neutral protease were obtained from Sigma, St. Louis, MO.

Preparation of Single Cell Suspensions

After freeing tumor specimens of macroscopically visible fat and other nontumor material and/or gross necrosis, each specimen was weighed and finely minced with crossed scalpels. An enzyme mixture consisting of 0.02% deoxyribonuclease, 0.04% collagenase, and 0.2% neutral protease was added and the mixture stirred for 30–90 min at 37°C. The cell suspension thus obtained was diluted with McCoy’s 5A medium and passed through a 60-µm sterile nylon mesh. The cells were pelleted at 300 × g for 10 min, washed twice with fresh McCoy’s 5A medium, and resuspended in plating medium. A viable cell count was performed by the trypan blue exclusion method and the stock cell suspension was adjusted with fresh plating medium to the desired cell concentration.

Capillary Tumor Cell Cloning

The basic method used was a modification of that described by Ali-Osman and Maurer (4, 9, 10). The standard plating medium consisted of CMRL 1066 (GIBCO, Grand Island, NY) supplemented with 20% heat-inactivated FCS, 5 mM ascorbic acid, 200 µM L-glutamine, 1.5 units/ml preservative-free insulin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 2.5 µg/ml human transferrin, 1:2 nonessential amino acids, 3 µg/ml catalase, 44 mg/ml L-asparagine, 30 µg/ml L-serine, 150 µg/ml sodium pyruvate, and 1 µg/ml gentamycin. Tumor cells were suspended in plating medium in a 1.5-ml sterile reaction tube and, if required, test solutions were added to the desired final concentration. Agarose (1%) (SeaPlaque; Marine Colloids, Rockland, ME) was added to a final concentration of 0.2%. Total culture volume was 300 µl. After thorough mixing, 50 µl (or other specified volume) of the plating mixture was drawn into each triplicate capillary tube by means of an automatic pipetter (Gilson, France). The cultures in the tubes were then allowed to gel on a cold surface and the tubes placed in specially made racks (Stafford Gage and Tool, Vandalia, OH). The racks were placed in 15-cm-diameter Petri dishes and incubated at 37°C in a fully humidified 5% CO2-air atmosphere. Representative capillary tubes were monitored weekly to determine colony growth.

The cell lines were set up similarly, except that the plating medium used was RPMI 1640 (Dulbecco’s minimal essential medium for HTB 17) supplemented with 15% heat-inactivated FCS. In the standard assays, cells were seeded at 1 × 104 cells/ml (HT 29, Colo 205, and P388), 5 × 104 cells/ml (HT 144), 2.5 × 105 (HTB 17), and 2.5 × 103 cells/ml (L 1210). The capillary tubes were unsealed throughout these studies, except in the studies comparing tumor colony formation in sealed and unsealed tubes.

Colony Counting and Evaluation

After incubations of 2–3 wk (primary tumors), 7 days (L 1210 and P 388), and 10 days (HT 29, Colo 205, HT 144, and HTB 17), the content of each capillary tube was flushed out gently onto a clean microscope slide such that the agarose gel was in one straight piece. Colonies of 50 µm or greater in diameter were then counted under inverted phase microscopy at 100× magnification. Alternatively, colonies were allowed to air dry on the slides, fixed in 95% ethanol, and stained with hematoxylin before counting. For cytological evaluation of the colonies, “squash” preparations of the cultures were made by placing a second glass slide onto the first one with the culture and pressing gently on it. After taking the slides apart, both were air dried, fixed in 95% ethanol, and stained using the Papanicolaou technique or with hematoxylin-eosin.

Quality Control

Quality control for preexisting cell clumps and preformed cell aggregates was performed in two stages. First, during the preparation of single-cell suspensions, aggregates of 40 µm or greater in diameter were counted and if there were more than 20 such aggregates per 106 cells, the cell suspension was reaspirated and filtered through a 30-µm mesh sieve and recounted. Secondly, after the cultures had been allowed to set, the contents of 3 representative capillary tubes were flushed onto clean glass slides, and aggregates equal to or greater than 40 µm in diameter were again counted. The assay was invalidated if more than four such aggregates per capillary tube were observed.

Growth Optimization

Culture Supplements. The culture supplements tested were heat-inactivated FCS and HS (0–30%), EGF (0–100 ng/ml), and hydrocortisone (0–10 ng/ml). In addition to the dose-response studies, we examined the effect of 50 ng/ml EGF and 2.5 ng/ml HC on colony formation of 24 primary human tumor cells. These concentrations were selected based in part on published (27–29) ranges of EGF and HC concentrations at which in vitro growth of tumor cells was enhanced, and partly on our own pilot studies. Stock test solutions were added to triplicate cultures containing 2.5 × 103 primary tumor cells/ml to achieve the above concentrations. For the cell lines, cultures were set up as described earlier and, from the only culture supplement tested, was added to a final concentration of 0–30%.

Agarose Concentration. To determine the effect of agarose concentration on colony formation, cultures were set up as described above and 60 µl of stock agarose solutions of varying concentrations were added such that final agarose concentrations of 0.1–0.5% were achieved in culture. Fifty µl (primary tumors) and 30 µl (cell lines) of the culture mixtures were then drawn into triplicate capillary tubes and the tubes incubated as described above.

Cell Density. The standard cloning procedure used was as described earlier except that the stock cell suspensions were prepared such that addition of 100 µl of each to the culture mixture resulted in final cell densities ranging from 1 × 104 to 1 × 106 cells/ml (primary tumors) and 1 × 104–1 × 105 cells/ml (cell lines).

Culture Volume. The effect of the culture volume contained in each capillary tube on tumor colony formation was examined using cells of primary breast, ovarian, and renal cell carcinomas, and all 6 cell lines. Cultures were set up containing 0.2% agarose and tumor cells at the final densities described earlier. Volumes ranging from 15–75 µl of the culture mixtures were drawn into triplicate capillary tubes and allowed to gel and the tubes were incubated as previously described.

Capillary Tube Geometry. To study the effect of capillary tube geometry on colony formation, cultures of P 388, L 1210, and Colo 205 were set up as described earlier. Thirty µl of the culture mixture were

### Table 1 Types and numbers of primary tumor specimens used in the capillary HTCA studies in this report

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Breast carcinoma</td>
<td>15</td>
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<tr>
<td>Ovarian carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Melanoma</td>
<td>3</td>
</tr>
<tr>
<td>Renal carcinoma</td>
<td>0</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0</td>
</tr>
<tr>
<td>Wilms’ Tumor</td>
<td>1</td>
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<tr>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
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</tr>
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</table>

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**Quality Control**

**Growth Optimization**

**Culture Supplements.** The culture supplements tested were heat-inactivated FCS and HS (0–30%), EGF (0–100 ng/ml), and hydrocortisone (0–10 ng/ml). In addition to the dose-response studies, we examined the effect of 50 ng/ml EGF and 2.5 ng/ml HC on colony formation of 24 primary human tumor cells. These concentrations were selected based in part on published (27–29) ranges of EGF and HC concentrations at which in vitro growth of tumor cells was enhanced, and partly on our own pilot studies. Stock test solutions were added to triplicate cultures containing 2.5 × 103 primary tumor cells/ml to achieve the above concentrations. For the cell lines, cultures were set up as described earlier and, from the only culture supplement tested, was added to a final concentration of 0–30%.

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**Culture Volume.** The effect of the culture volume contained in each capillary tube on tumor colony formation was examined using cells of primary breast, ovarian, and renal cell carcinomas, and all 6 cell lines. Cultures were set up containing 0.2% agarose and tumor cells at the final densities described earlier. Volumes ranging from 15–75 µl of the culture mixtures were drawn into triplicate capillary tubes and allowed to gel and the tubes were incubated as previously described.

**Capillary Tube Geometry.** To study the effect of capillary tube geometry on colony formation, cultures of P 388, L 1210, and Colo 205 were set up as described earlier. Thirty µl of the culture mixture were
drawn into each of a set of six round capillary tubes and a set of six square capillary tubes. After the cultures had gelled, the tubes were placed in racks and incubated as described earlier.

Capillary Tube Sealing. Twelve primary human tumors and three cell lines, L 1210, P 388, and HTB 17, were used in this study. Cultures were set up as described above for the volume experiments. Fifty μl (primary tumors) and 30 μl (cell lines) were drawn into the capillary tubes (12 tubes per tumor or cell line). Six replicate tubes were immediately sealed with Critoseal (Monoject Scientific Products, St. Louis, MO) and the other six were left unsealed. Both sets of tubes were placed in the same capillary tube holder and incubated as described earlier. At the end of the incubation period, the two ends of the sealed capillary tubes were broken off with the help of an ampule file and the cultures flushed onto glass slides and counted as described earlier for unsealed tubes.

Automated Capillary Tube Scanning. This was performed using a modification of the Cellscan prototype automated data collection-analysis system (30) (Triton Biosciences, Oakland, CA). The system consists of a microprocessor and HP series 200 PC which interface with an inverted microscope (Olympus; Tokyo, Japan) with an automated x-y stage capable of securely holding an 18-tube rack. Our modification of the prototype involved bypassing the standard light source of the microscope and using a dual fiberoptic cold light source (Nikon; Tokyo, Japan) to indirectly illuminate the capillary tubes. This “dark-field” illumination system darkens the culture background and “lights up” colonies and clusters within the capillary tube. Each tube was scanned at a 100× magnification. During the scanning, light scatter from each colony and the exact position of the colony along the length of the capillary tube were picked up by a photodetector, processed, and stored on a hard disc in the computer. To reduce background signals and improve counting reproducibility, the outside of each capillary tube was cleaned thoroughly with a moist fiber-free paper before each scanning. The tube positions were also maintained by securing the capillary tube to the outer frame of the tube holder. Each tube was scanned 3 times at Day 0 and at the end of the experiment and was also always counted manually. Both the number and peak integrals of the colonies were determined at a threshold setting corresponding to a 50-μm colony diameter.

Head-to-Head Comparison with Double-Layer Agar Technique. For this comparison, the agar double-layer technique as described by hamburger and Salmon (1) was used. Briefly, a 1-ml base layer containing 0.6% agarose in enriched McCoy’s 5A medium (1) was prepared in 35-mm-diameter sterile Petri dishes and after gelling was overlaid with 1 ml of a plating layer consisting of 5 x 10⁶ cells suspended in enriched CMRL 1066 medium (1) containing 0.3% agarose. The plates were allowed to gel at 4°C and incubated at 37°C in a humidified 5% CO₂/air environment. A set of three plates was prepared for each tumor. To control for cell aggregations and preformed cell clumps, 0.1 ml of a 1 mg/ml p-iodonitrotetrazolium violet solution was applied to the surface of the plating layer of two dishes on Day 0, and after a 24-h incubation at 37°C and 5% CO₂, cell aggregates of 40 μm or greater in diameter were counted using an inverted microscope. If the number was less than 15 per plate, the assay was considered valid and the cultures were incubated for a further 21 days. At the end of the incubation period, each plate was treated with p-iodonitrotetrazolium violet solution as described earlier and colonies equal to or greater than 50 μm in diameter were counted. All colony counting was performed with the Omnicorn FAS III image analyzer (Bausch and Lomb, Rochester, NY). The standard capillary HTCA cloning technique as described earlier in this report was used in this comparative study. The final cell seeding density was 2.5 x 10⁴ cells/ml and the final agarose concentration was 0.2%.

Statistical Analysis. All colony counts were expressed as the mean ± 1 SD. PEs were determined using the formula

\[
PE = \frac{\text{mean no. of colonies per dish (or tube)}}{\text{no. of cells seeded per dish (or tube)}}
\]

To compare the performance of the capillary HTCA with that of the agar double-layer method, the median PE and the success rates obtained with the two methods were compared. Success rate was defined as

\[
\text{No. of specimens which yielded positive growth} = \frac{\text{total number of specimens assayed}}{\text{no. of specimens which yielded positive growth}}
\]

For this, positive growth was defined arbitrarily as a net increase over Day 0 controls of 5 or more colonies per capillary tube and 15 or more colonies per Petri dish. The Student’s t test (at P = 0.05) was used to determine the significance of the effect of culture modification and culture parameters on colony formation, and of the performance of the capillary HTCA versus that of the agar double-layer assay.

RESULTS

Colony Growth and Morphology

Fig. 1 shows (a) phase contrast and (b) dark-field illumination micrographs of a section of a round capillary tube containing primary human breast carcinoma colonies. At optimal dark-field illumination, there is minimal interference either by the agarose matrix or by light reflection off the curved glass surface. A section of the agarose gel that has been flushed out of a capillary tube onto a glass slide is shown by direct phase contrast and dark-field microscopy in Fig. 1, c and d, respectively. Fig. 2, a–d, shows the native state morphology by inverted phase microscopy of colonies of different tumor types. The “glandular” formation around the ovarian adenocarcinoma colony is a rare observation. Fig. 3, a–c, illustrates the cytomorphology of the cells of a malignant melanoma, a clear cell renal carcinoma, and a transitional bladder carcinoma cloned in the capillary HTCA from primary specimens. The cellular and especially the nuclear pleomorphism of the cells in the colonies is quite distinct.

Optimization of Growth

Culture Supplements. The dose-response curves for the effect of EGF and HC on tumor colony formation by cells of five representative tumors are shown in Fig. 4. A significant increase (135 and 210% of controls) in the number of colonies after EGF supplementation was observed for breast and ovarian tumors, the highest stimulation being at 25–50 ng/ml EGF. Similarly, tumor colony growth was stimulated by culture supplementation with 2.5–5 ng/ml HC. Above 5 ng/ml, HC inhibited colony growth of the majority of tumors, particularly those that had been initially stimulated by low concentrations of the hormone. Table 2 summarizes the results of the effects of EGF (50 ng/ml) and HC (2.5 ng/ml) on colony formation by cells of 24 primary human tumors. Colony formation was stimulated in 54% of the tumors by EGF and 37.5% by HC at these concentrations.

The effects of both FCS and HS on colony growth of five representative tumors are shown in Fig. 5. Three of the 5 tumors showed higher colony formation for FCS over HS at the same concentrations. In 2 cases (ovary and lung), there was no statistically significant difference in tumor colony stimulation by the sera types and in one case of infiltrating ductal carcinoma of the breast, HS was actually better than FCS in supporting colony formation. None of the tumors formed colonies in the absence of serum. The optimal serum concentration was approximately 20% for both serum types. Highest colony formation was at 10% FCS for the murine cell lines and 20% for all of the human tumor cell lines.

Cell Density. Eight primary carcinoma specimens (breast, lung, ovarian, colorectal, and renal cell) were used for this study. Colony formation by all 8 tumors was highest at seeding
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Fig. 1. Section of a round glass capillary tube showing primary human breast carcinoma colonies (a) by phase-contrast microscopy and (b) by indirect illumination. Section of an agarose culture (containing colonies of the same tumor as in a) that has been flushed out onto a glass slide, c, inverted phase contrast microscopy; d, the same culture by indirect illumination.

densities of 2.5–3 × 10³ cells/ml. The optimal cell seeding densities for the cell lines were L 1210, 2.5 × 10³ cells/ml; P388, 1 × 10⁴ cells/ml; HT 29, 1 × 10⁴ cells/ml; Colo 205, 1 × 10⁴ cells/ml; HT 144, 4 × 10⁴ cells/ml; and 2.5 × 10⁴ cells/ml for HTB 17. Cell densities beyond these resulted in a nonlinear relationship between the number of cells seeded and the number of colonies formed, and colony growth was better at the tube ends than at the center. Fig. 6 summarizes the results of the studies on the effect of cell seeding density on colony formation by primary ovarian and lung carcinomas and by the cell lines P388, L 1210, and Colo 205.

Culture Volume. Five primary carcinomas (breast, ovarian, and renal cell) and all six cell lines were investigated for this parameter. The range of culture volume per capillary tube examined was between 15 and 75 µl. Although, as shown in Fig. 7, the number of colonies formed increased for culture volumes of up to 75 µl, beyond 50 µl (30 µl for cell lines) colony number did not relate linearly to culture volume and colonies were often largest and most numerous at the end than in the middle portions of the capillary tube. The PEs also dropped significantly at volumes greater than 30 or 50 µl per capillary tube.

Agarose Concentration. The effect of agarose concentration on colony formation was investigated using cells of primary breast, ovarian, and renal cell carcinomas as well as the cell lines. The results (Fig. 8) shows optimal colony formation at a range of 0.2–0.3% agarose for all of the primary tumors. Below 0.15% agarose, colonies were generally loose and the gel matrix was very fluid, allowing the colonies to settle down and drift along the tube upon slight movement. Above 0.4% agarose, colony formation was poor and clusters predominated. There was greater heterogeneity in the optimal agarose concentration for the cell lines. P 388, HT 144, L 1210, HT 29, and HTB 17 cells demonstrated maximal PEs at 0.2–0.3% agarose, similar to cells of primary tumors. For Colo 205, however, the range was much wider (0.15–0.5%).

Comparison of Colony Formation in Square versus Round Capillary Tubes. The bar graphs in Fig. 9 summarize the results of this comparison using P 388, L 1210, and Colo 205 cells and show that for all three cell lines colony growth was significantly higher in round capillary tubes than in square ones.

Effect of Capillary Tube Sealing. Table 3 shows the results of this study. For nine of twelve primary tumors and all three cell lines, sealing of the capillary tubes resulted in decreased colony formation. Under the conditions of this study, there was no contamination or significant shrinking of the gels in the unsealed tubes, as is also evident in the Day 0 and 16 scan profiles in Fig. 10.

Automated Colony Scanning. Fig. 10 shows the scan of a capillary tube containing ovarian carcinoma colonies. Scanning was performed with the Cellscan prototype fitting with indirect illumination. At a threshold setting corresponding to 50-µm
colony size, a colony count of \(23 \pm 0.3\) was obtained. Visual human counting of the same capillary tube section yielded \(21 \pm 2.3\) colonies. The variance of six consecutive Cellscan colony counts of the capillary tube under the same indirect illumination intensity and at the same threshold setting was 0.87.

**Head-to-Head Comparison between Capillary and Agar Double-Layer Assays.** The results of this comparison using 46 different primary tumor specimens are summarized in Table 4. The proportion of tumors that successfully formed colonies was 11% for the capillary technique and 53% for the Petri dish method. Although the range of PEs was similar for both methods, the median PE was more than 5 times higher in the capillary technique (0.18) than in the Petri dish method (0.036). Table 4 also compares the results obtained in this study with those of similar studies by Von Hoff et al. (20, 21).

**DISCUSSION**

Tumor clonogenic cell assays have been shown in several retrospective and prospective clinical trials (25, 26, 31–36) to have potential value in the individualized clinical management of the cancer patient (25–27, 30–36). Furthermore, these assays continued to play increasingly significant roles in new drug development and screening (37–39), in studies of tumor drug resistance (40–42), in the development and evaluation of new clinical cancer treatment protocols (42, 43), and in basic tumor biology and pathology (44, 45). The continued successful exploitation of HTCAs in these studies is, however, dependent in part upon the resolution of some of the major technical and performance problems and limitations of current technologies of tumor cell cloning (5–7). Initial experience (4, 19–21) with the capillary HTCA have shown that this assay successfully addresses some current HTCA problems and that it provides an assay applicable to small tumor specimens. In the present study, we set out to further develop and optimize this novel, promising HTCA and to investigate critical parameters necessary for its successful exploitation. Two tumor colony growth-supporting factors, namely, HC and EGF, were studied. HC at 2.5 ng/ml increased colony formation for most tumors, and concentrations above 5 ng/ml were generally growth inhibitory, particularly for tumors for which colony growth was stimulated at lower HC concentrations. Other investigators (46) have also reported glucocorticoid stimulation of tumor colony formation.

The enhancement of colony formation by EGF in the capillary HTCA was highest for breast and ovarian carcinoma cells and least for cells of lung and colorectal tumors. These findings are in general agreement with those for breast carcinoma cell lines (27) and primary human tumors (28, 29, 47). Hamburger et al. (28) showed that not only was colony growth of a wide range of human epithelial-derived tumors stimulated by EGF but that this stimulation was heterogeneous and for many tumors was suppressed by HC. The optimal stimulatory EGF concentration in our studies was 50 ng/ml, the same as that found by Pathak et al. (29) and Hamburger et al. (28) using the agar double-layer assay. These independent but identical findings suggest that response of tumor clonogenic cells to EGF is a true

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**Fig. 2.** Gross morphologies, by inverted phase microscopy, of colonies grown in capillary tubes from cells of primary specimens of (A) infiltrating ductal carcinoma of the breast; (B) colorectal carcinoma; (C) ovarian adenocarcinoma, and (D) malignant melanoma. Note the somewhat glandular formation by the cells at the periphery of the ovarian adenocarcinoma colony.
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Fig. 3. Papanicolaou stain of colonies of (A) malignant melanoma, (B) clear cell renal carcinoma, and (C) a transitional bladder carcinoma in glass capillaries. Cellular and nuclear pleomorphism is very prominent.

Fig. 4. Effect of EGF (upper) and hydrocortisone (lower) on colony formation by primary human tumor cells in agarose-containing glass capillary tubes. Cells were seeded at 2.5 x 10^5 cells/ml. The effect of each culture supplement was expressed relative to controls without supplement.

Table 2 Effect of supplementation of cultures with 50 ng/ml EGF and 2.5 ng/ml HC on colony formation of primary human tumor cells in the capillary HTCA

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No. of specimens</th>
<th>No. of responders</th>
<th>EGF range</th>
<th>No. of responders</th>
<th>HC range</th>
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<td>1.28-5.20</td>
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<tr>
<td>Total</td>
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<td>9 (37.5%)</td>
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</table>

*GEF, growth enhancement factor, obtained by the following equation:

\[
GEF = \frac{\text{no. of colonies in treated cultures}}{\text{no. of colonies in untreated controls}}
\]

biological characteristic of the cells and is independent of the HTCA system. It is likely that EGF response is a function of the EGF receptor status of the tumor cells, as has been previously suggested (48).

The ability of serum to modulate colony formation in the HTCA has also been demonstrated in previous studies (49). We have found the effects of serum to be very dependent upon the type, concentration, and batch, suggesting the need to screen different serum batches to select one with high colony-growth-supporting activity. Cells of both primary tumors and cell lines of human origin varied significantly from the murine leukemias in their serum requirement and there were significant differences in colony-stimulating activity between FCS and HS. The basis of this difference is not completely understood although it may be accounted for in part by differences in content of growth factors, e.g., EGF, insulin, and trace elements in the

5 F.Ali-Osman, unpublished data.
two serum types. We have previously shown that polyamine oxidase present in FCS could oxidize polyamines produced by proliferating tumor cells to intermediates that can affect clonogenic growth of the cells (50).

The culture volume contained in the capillary tube and the plating cell density both affect the size, number, and distribution of colonies along the tube. We found that under optimized conditions 30 μl (cell lines) and 50 μl (primary tumors) per tube provided for most uniform colony growth. These volumes are, however, a function of the length and diameter of the capillary tubes. In contrast to the agar double-layer technique in which cells are seeded at 5 × 10^5 cells/ml (1), we found 2.5–3 × 10^5 cells/ml to provide for the most uniform distribution of colonies along the tube and the highest PEs. It is important to note that evidence by Thomson et al. (51) suggest that not only is the PE and colony size affected by the plating density, but that drug dose-survival curves can also be affected by the number of cells plated. We also observed that colony formation was consistently better in unsealed than in sealed capillary tubes, as used by other investigators (20–22). There was no significant shrinkage of the cultures in the tubes if incubation was in a highly humid environment.

A significant finding in this study was that colony formation by cells of both primary tumors and most of the cell lines was best at 0.2% agarose and that concentrations of 0.4% or higher resulted in a rapid drop in both the size and number of colonies formed per capillary tube. This may explain in part why earlier attempts by Braslow and Bowman (11) in which they used 0.4% agar to clone rat hepatoma cells in agar-containing capillary tubes were not very successful.

Our data on the suitability of round tubes for cloning in the capillary HTCA are important because not only are round tubes more readily available but they are much less expensive than squares ones. The main disadvantage of round over square tubes is that of poor colony detectability in the former. This is, however, easily resolved by flushing the tube contents onto glass slides (Fig. 1c) and counting colonies either directly or after appropriate staining. Alternatively, dark-field illumination (Fig. 1, b and d) can be used. In this report, we have shown that the Cellscan automated colony counting system (28) modified with such dark-field illumination can be used to count and evaluate colonies in round tubes.

Fig. 5. Upper, effects of FCS on colony formation by primary human tumor cells and lower, effects of 20% FCS (■) and 20% HS (■) on colony formation by primary human carcinoma cells.

Fig. 6. Relationship between the number of cells seeded per capillary tube and the number of colonies formed for cells of (upper) primary carcinomas, and (lower) cell lines. The volume per capillary tube was 50 μl for the primary tumors and 30 μl for the cell lines.
Currently, a major attraction of the capillary HTCA is its demonstrated ability to improve upon the PEs and success rates achievable with the Petri dish HTCA. However, as the latter technique gets better optimized, higher cloning success rates are also likely to be achieved with it. Consequently, we believe that a more important advantage of the capillary HTCA may be in its making possible clonogenic cell studies not readily performed with other HTCA techniques. In particular, the growth of colonies in a 2-dimensional array in a small volume of low-density agarose allows easy and relatively inexpensive linear scanning of growing tumor colonies to yield quantitative colony growth kinetic data. Abrams et al. (13), Maurer and Henry (52), Ali-Osman and Maurer (8, 9), and Slocum et al. (53) have all used such an approach to study colony growth kinetics in the capillary cloning system.

A major advantage of the capillary HTCA is that the micro amounts of material required for the assay make it the method of choice for assaying small tumor specimens and for testing rare compounds or preparations which may be available only in very small quantities or may be too expensive for large volume testing.

Recently, a perfusion system was described (54) that permits drug exposure in the capillary HTCA to simulate the clinical drug pharmacokinetics. If further developed, this could provide for in vitro drug exposure of tumor cells in a very clinically relevant fashion.

Presently, the reasons why PEs in the capillary HTCA are generally higher than those in the agar double-layer method are only speculative. It is possible that the tube geometry provides for a relatively more hypoxic environment within the culture. Hypoxia has been reported (55), and also observed by us, to increase the PE of most tumors in the standard Hamburger and Salmon HTCA. It is also likely that the small culture volume within the capillary tube may provide for an increased conditioning effect (e.g., higher autocrine growth factor concentrations) in the culture microenvironment that is more supportive of tumor colony growth. However, as discussed by Von Hoff et al. (21), if higher autocrine growth factor concentrations should indeed be involved in the increased PEs observed in the capillary HTCA, this role is likely to be part of a more complex mechanism of stimulation of colony growth in the assay.

The data presented in this paper demonstrate that an opti-
mized capillary HTCA may represent a significant advancement in the technology of tumor cell cloning. It has unique advantages, some of which if appropriately exploited can provide biological data not readily obtainable with other HTCA techniques. The data also clearly show that several technical parameters can limit the performance of the assay and these need to be considered, and in some cases optimized, before using the assay in clinical and other studies. Our results do not define a universal system for the optimized cloning of cells of all tumor types. In view of the extreme heterogeneity, both in the nutritional requirements and in the physiology and biochemistry among different tumor types, such a universal optimized assay is unrealistic. It is our view that further development of the capillary HTCA will best be realized by studying individual tumor types. In addition, more studies are required to further define the place of this assay in the prediction of patients’ response to treatment and in the screening of new anticancer agents.

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Optimization and Characterization of the Capillary Human Tumor Clonogenic Cell Assay

Francis Ali-Osman and Patricia A. Beltz


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