Growth of Cell Colonies in Soft Agar from Biopsies of Different Human Solid Tumors

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ABSTRACT

Colony growth in soft agar of cells disaggregated from 87 solid tumor specimens was evaluated. Tumors were disaggregated by an enzymatic method consisting of microtome slicing of tissues and incubation at 37° for 2 hr in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% fetal bovine serum, 0.8% collagenase II, and 0.002% DNase I. Tumor cells were cultured in a two-layer agar system (0.5% agar feeder, 0.3% agar plating layer) in enriched medium with or without addition of different factors. Fifty-eight of 87 malignant tumors (16 of 22 melanomas, 11 of 16 sarcomas, 8 of 16 pulmonary carcinomas, 8 of 11 colon carcinomas, 5 of 8 breast carcinomas, 2 of 3 carcinomas of unknown site, 2 of 2 ovarian carcinomas, 1 of 1 urinary bladder carcinoma, 0 of 1 kidney carcinoma, 1 of 1 epiglottic carcinoma, 1 of 1 pancreatic carcinoma, 1 of 1 pleural mesothelioma, 1 of 1 lung hemangiopericytoma, 0 of 1 malignant schwannoma, 0 of 1 malignant giant cell tumor, and 1 of 1 neuroblastoma) grew in the soft-agar system yielding an overall cloning success rate of 67%. The number of colonies (30 or more cells) after plating 5 x 10⁶ cells ranged from 50 to 3774 per plate, yielding plating efficiency from 0.01 to 0.8%. Considerable variations in plating efficiency were noticed among tumors of the same type. A linear relationship was obtained between the number of cells plated and the number of colonies formed. Tumor cells washed after enzymatic disaggregation yielded more colonies per plate than did tumor cells plated without washing. Refrigeration of tumor tissue for 24 hr in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% fetal bovine serum did not decrease the number of colonies. The number of colonies formed was not increased with two different cell-conditioned media. Murine and rat red blood cells did not affect tumor colony growth. The cells plucked from individual colonies and stained by the Wright-Giemsa and Papanicolaou methods had the same morphological characteristics as did tumor cells in the original cell suspension. The histological pattern of the original tumors was maintained after passage from agar methyl cellulose into the nude mouse.

INTRODUCTION

The formation of colonies of tumor cells in semisolid culture medium has many potential applications such as: (a) cloning of individual cells from the tumor population; (b) detection and characterization of regulatory factors controlling cell growth and differentiation; (c) characterization of biophysical properties of tumor stem cells; (d) development of individualized predictive trials of anticancer drugs in a manner analogous to techniques used for selection of antibacterial agents; and (e) measurement of sensitivity of tumor stem cells to anticancer drugs, hormones, immunological agents, and radiation.

It has been shown that cells derived from hamster tumors induced by certain adenoviruses formed colonies in soft agar; cells from normal mouse tissues did not (10). These colonies had histological characteristics similar to those of the tumor from which they were derived, and they induced progressively growing tumors when transplanted into mice. These findings suggested that: (a) human tumor cells might form colonies in agar which would be morphologically similar to the parent tissue; and (b) the agar culture method might differentiate neoplastic from nonneoplastic tissues.

Several investigators have evaluated colony growth in soft agar of cells from human tumors (2, 3, 5–8, 11, 18, 19, 22). We have also recently demonstrated the ability to clone different solid human tumor stem cells in soft agar (13–15, 20). Initial results have been very encouraging, but success in obtaining colony growth is not achieved in every case; cloning efficiencies are often low.

In this report, we described the growth of different human solid tumor cells in soft agar.

MATERIALS AND METHODS

Tumors. Histologically proven primary, recurrent, and/or metastatic malignant melanomas, sarcomas, carcinomas (of several origins including lung, colon, breast, unknown site, ovary, urinary bladder, kidney, pancreas, and epiglottis), pleural mesothelioma, hemangiopericytoma, malignant schwannoma, malignant giant cell tumor, and neuroblastoma were studied.

Disaggregation of Tumors and Nonneoplastic Tissues. Single-tumor-cell suspension was prepared as described by Slocum et al. (21). Briefly, surgical specimens collected into sterile wells on ice were trimmed free of normal and necrotic elements and sliced (0.5 mm) with a Stadie-Riggs microtome (Arthur H. Thomas Co., Philadelphia, Pa.). The slices were briefly minced with scalpels in RPMI 1640³ containing 10% heat-inactivated fetal bovine serum. The tissue mince was poured over a 100-mesh filter, and the retained tissue was further washed with
medium. The remaining tissue was incubated in a mixture containing 0.8% collagenase II (Worthington Biochemical Corp., Freehold, N. J.) and 0.002% DNase I (Sigma Chemical Co., St. Louis, Mo.) in RPMI 1640 with 10% fetal bovine serum at 37° in a humidified atmosphere of 5% CO₂ in air for 2 hr. In studies on washing, digested tissue was then washed with fresh medium as above.

**Culture Assays for Tumor Colony-forming Cells.** Two different soft agar assays and agar-methyl cellulose assay were used to culture tumor cells. Briefly, the soft agar assay of Pluznik and Sachs (16) and Bradley and Metcalf (1), consisting of a 2.5-ml lower layer of 0.5% agar in double-strength Eagle’s minimal essential medium containing 10% fetal bovine serum, was placed in a 35- x 10-mm plastic Petri dish and permitted to solidify at room temperature. Cells to be tested for colony formation were suspended in a plating layer (0.85 ml) of 0.3% agar in double-strength Eagle’s minimal essential medium containing 10% fetal bovine serum. The method of Hamburger and Salmon (7) without conditioned medium was also used to culture several tumor samples. Briefly, an underlayer of 0.5% agar in enriched McCoy’s Medium 5A containing 10% fetal bovine serum was prepared (1 ml in a 35-mm Falcon plastic Petri dish). Cells to be tested for colony formation were suspended in a plating layer of 0.3% agar in enriched CMRL Medium 1066 containing 15% horse serum (Flow Laboratories, Rockville, Md.).

A methyl cellulose plating layer over an agar underlayer assay, developed by Buick et al. (3), was also used for plucking of colonies and their transplantation into nude mice. Subsequent cellular manipulations are much more easily achieved after growth in methyl cellulose than they are after growth in agar. Briefly, an underlayer of 0.5% agar in enriched McCoy’s Medium 5A containing 10% fetal bovine serum was prepared (1 ml in a 35-mm Falcon plastic Petri dish). The plating layer consisted of 1 ml of McCoy’s Medium 5A containing the same enrichments and 0.8% methyl cellulose (Methocel; Dow Chemical Co., Midland, Mich.). Cells were routinely plated at a concentration of 5 x 10⁶ cells/ml in the 1-ml plating layer.

After the plating layer was poured, under the conditions of either method, the agar and agar-methyl cellulose were permitted to solidify at room temperature and were inspected for cell clumps (none was noted in the experiments reported in this study). Cultures were set up in quintuplicate and incubated at 37° in a humidified incubator in an atmosphere of 5% CO₂ and 95% air.

**Counting of Colonies.** Cultures were examined with an inverted-phase microscope at 100 x and 200 x. Final colony counts were made between 2 and 3 weeks after plating. Groups of 30 or more cells were considered as colonies, whereas groups consisting of fewer cells were counted as clusters (8).

**Factors Affecting Colony Growth.** The effect of conditioned medium from human and mouse cells and the effect of mouse and rat RBC on tumor colony growth in soft agar were studied. These materials were put into the lower layers of 0.5% agar. Ten and 20% conditioned medium from a human sarcoma cell line was kindly supplied by Dr. H. Preisler of this Institute.

RBC were prepared as described by Courtenay and Mills (4). In brief, blood was obtained by cardiac puncture from August rats and BALB/c mice, and the buffy coat was removed after centrifuging. RBC were rinsed 3 times by resuspending in phosphate-buffered saline (0.85% sodium chloride-0.01 M phosphate buffer, pH 7.4) and centrifuging, and they were finally made up to the original volume with culture medium. RBC were stored at 4° for a period of 15 days, and 0.25 ml of ½ dilution was put into the lower layer.

Conditioned medium from the adherent spleen cells of BALB/c mice was prepared as described by Hamburger and Salmon (7). Mice were given i.p. injections of 0.2 ml of mineral oil. Four weeks later, single-cell suspensions were made from the spleens, and 5 x 10⁶ cells were placed into a 60-mm Falcon Petri dish for 2 hr to permit the cells to adhere. The dishes were then rinsed 3 times in cold phosphate-buffered saline. Cells were incubated for 3 days at 37° in RPMI 1640 with 15% heat-inactivated fetal bovine serum. The conditioned medium was decanted and centrifuged at 400 x g for 15 min, and the supernatant was then passed through a Millipore filter and stored at 20°. This conditioned medium (0.25 ml) was put into a lower layer of 0.5% agar.

**Refrigeration of Tumors.** Surgical specimens collected into sterile plastic tubes containing 50 ml of RPMI 1640 with 10% fetal bovine serum were refrigerated. Disaggregation of tumors as described above was done 24 hr later.

**Preparation of Slides for Morphology Studies.** For morphology studies, slides of the original tumor cell suspension, intact agar-methyl cellulose cultures, and individual colonies were prepared. Individual colonies were removed from the Petri dishes with a fine capillary pipet and, after being suspended in 1 drop of heat-inactivated fetal bovine serum, the cells were deposited on slides. Air-dried slides of the original cell suspension and individual colonies were stained with Wright-Giemsa and Papanicolaou stains (9, 12). Permanent slides of intact cultures of tumor cells were prepared as described by Salmon and Buick (17). Cells plucked from individual colonies, and cells from permanent slides were compared with the original cell suspension. Histological slides of each tumor specimen were stained with hematoxylin and eosin and then analyzed. The slides were read by Z. P. Pavelic, who is a board-certified pathologist.

**Transplantation of Tumor Cells Growing in Agar Methyl Cellulose into the Nude Mouse.** Eight- to 10-week-old homozygous nude female mice of BALB/c background were used. They were obtained from Harlan/Sprague Dawley Animal Resources, Madison, Wis. The animals were housed in a laminar air-flow room at 25–28° under aseptic conditions. Filtered air and sterilized bedding, cages, food, and water were used. Single-tumor-cell suspensions produced from a single colony (10⁵ cells) and from a pool of largest tumor colonies (3.5 x 10⁵ to 10⁶ cells) growing in agar methyl cellulose were rinsed in antibiotics (streptomycin and penicillin) and injected s.c. into the groin and axilla with an 18-gauge needle into 1 to 4 sites/mouse. The mice were observed every weekday.

**RESULTS**

**Relationship between Number of Cells Plated and Colony Formation.** There was a linear relationship between the number of malignant melanoma (Chart 1A), Ewing’s sarcoma (Chart 1B), lung adenocarcinoma (Chart 1C), and ovarian adenocarcinoma (Chart 1D) cells plated per dish and the number of colonies formed from 10⁴ to 50 x 10⁴ cells plated.

**Colony Growth in Soft Agar of Cells Disaggregated from Different Human Solid Tumors.** Cell doublings and appear-
Colony Growth of Human Tumors in Soft Agar

The colony growth of human tumors in soft agar was monitored in most trials. Cell doublings were observed between 24 and 48 hr after plating; clusters (4 to 30 cells) appeared within 2 to 10 days; colonies (30 or more cells) appeared 6 to 12 days after plating. Cultures were not refed, and the life of cultures ranged from 3 to 4 weeks. At that time, degenerative changes and necrosis of cells were noted. Table 1 summarizes the number and percentage of tumors producing 50 or more colonies; the colony range; and the plating efficiency of cells disaggregated from human malignant melanoma, sarcoma, carcinoma (including those of the lung, colon, breast, unknown site, ovary, urinary bladder, kidney, pancreas, and epiglottis), pleural mesothelioma, hemangiopericytoma, malignant schwannoma, malignant giant cell tumor, and neuroblastoma. The number of tumor specimens producing colonies ranged from 40% for anaplastic lung carcinoma to 100% for ovarian carcinoma. The data indicate that, in 58 of 87 cases, there was growth of tumor cells in soft agar yielding an overall success in cloning of 67%. The data also indicated that the plating efficiency ranged from 0.01 to 0.8%.

Relationship between Washed and Unwashed Tumor Cells and Colony Growth. The results of the colony growth of malignant melanoma, sarcoma, and ovarian carcinoma cells disaggregated by collagenase type II and then either washed or not washed with RPMI 1640 containing 10% fetal bovine serum just prior to plating are shown in Table 2. The results indicate that, in 7 of 8 cases, washing of tumor cells after enzymatic disaggregation yielded more colonies per plate than did plating of tumor cells without washing. In 3 of 8 cases (Tumors 1, 2, and 6), however, unwashed cells did not grow, washing of tumor cells yielded 138.0 ± 19.7 (S.D.), 125.3 ± 39.5, and 209.0 ± 24.0 colonies, respectively. This represents a 2- to 26-fold increase in the number of colonies formed from cells washed after disaggregation by collagenase II.

Effect of Refrigeration of Tumor Specimen on Colony Growth. Table 3 summarizes the colony growth of freshly disaggregated tumor cells from malignant melanoma, sarcoma, and kidney and ovarian carcinoma and compares it to that of cells disaggregated from a portion of the same tumor tissue kept at 4° for 24 hr prior to plating. The results indicate that, in 4 of 8 cases, there was no colony growth of tumor cells disaggregated either before or after 24 hr refrigeration. In one case (Tumor 8), plating of tumor cells after refrigeration yielded more colonies than plating before refrigeration. In contrast, Tumor 4 yielded the opposite result.

Experiments on Factors Possibly Affecting Colony Growth. The effect of conditioned medium from human sarcoma and mouse cells and that of mouse and of rat RBC on tumor cell colony growth in soft agar was studied. The results

Table 1

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<tr>
<th>Type of tumor</th>
<th>No. of specimens producing colonies/total no. of specimens tested</th>
<th>% of specimens producing 50 or more colonies</th>
<th>No. of colonies</th>
<th>Plating efficiency (%)</th>
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<tr>
<td>Malignant melanoma</td>
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<td>Sarcoma</td>
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<td>Pulmonary carcinoma</td>
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<td>50–399</td>
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<td>40</td>
<td>50–3774</td>
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<tr>
<td>Adenocarcinoma</td>
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<td>60</td>
<td>50–327</td>
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<td>Colon carcinoma</td>
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<td>Carcinoma of unknown site</td>
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<td>Ovarian carcinoma</td>
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<td>Other</td>
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*Pleural mesothelioma, 1 of 1; epiglottis carcinoma, 1 of 1; lung hemangiopericytoma, 1 of 1; urinary bladder carcinoma, 0 of 1; kidney carcinoma, 0 of 1; malignant schwannoma of a forearm, 0 of 1; islet cell carcinoma, 1 of 1; malignant giant cell tumor of a femur, 1 of 1; retroperitoneal neuroblastoma, 1 of 1.
of the colony growth of malignant melanoma, lung carcinoma, and pleural mesothelioma cells after putting these materials into a lower layer of 0.5% agar are shown in Table 4. Conditioned medium enhanced colony growth in 2 trials; in 4 cases, conditioned medium did not support colony growth; in one trial, the number of colonies was decreased. The data also indicate that conditioned medium from the adherent spleen cells of BALB/c mice and from murine and rat RBC did not have an effect on tumor cell colony growth. These are the results of a typical experiment. Fourteen other trials on different patients have given similar results.

Comparison of Colony Growth in 2 Soft-Agar Systems. Table 5 summarizes the colony growth of malignant melanoma; colon, breast, and ovarian carcinoma; and neuroblastoma cells in 2 different soft agar systems. The results indicate that, in 5 of 9 cases (tumors 1, 2, 3, 7, and 8), cells plated in the assay media of Pluznik and Sach (16) and Bradley and Metcalf (1) formed slightly more colonies than did cells plated in the assay of Hamburger and Salmon (7). In contrast, in 3 of 9 cases (tumors 4, 6, and 9), tumor cells growing in the assay media of Pluznik and Sach (16) and Bradley and Metcalf (1) formed more colonies than did cells plated in the original cell suspension (Fig. 1C). The cytological characteristics of the cell colonies of human malignant melanomas, sarcomas, and lung and colon carcinomas. More than 64% of these gave colonies in agar, but plating efficiency was low (<1%). This could be because the proportion of clonogenic cells in the tumor was small or because of some special metabolic requirements of these cells. There was no correlation between the growth pattern and morphology of the colonies and the histological type of the tumor.

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DISCUSSION

These studies have demonstrated that 67% of the investigated human solid tumor cells have the ability to grow in semisolid medium. This observation agrees with those of other investigators (3–5, 7, 8). Considerable differences in plating efficiency were observed for the same tumor type and among different tumor types. The majority of tumors tested were malignant melanomas, sarcomas, and lung and colon carcinomas. More than 64% of these gave colonies in agar, but plating efficiency was low (<1%). This could be because the proportion of clonogenic cells in the tumor was small or because of some special metabolic requirements of these cells. There was no correlation between the growth pattern and morphology of the colonies and the histological type of the tumor.

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The linear relationship between the number of tumor cells seeded and the number of colonies per plate, a finding which is in agreement with that of other investigators (3, 8), indicates that a colony can be initiated by a single-colony-forming unit. In most cases, washing of tumor cells after enzymatic disaggregation yielded more colonies per plate than did plating of tumor cells without washing. This could be due to cellular damage occurring when the cells are exposed for too long to enzymes. Refrigeration of tumor tissue in RPMI 1640 containing 10% fetal bovine serum did not decrease the number of colonies. This observation should permit long distance transportation of tumor specimens as well as optimal laboratory experimental scheduling.

Neither mouse nor rat RBC provided additional support for the colony growth of tumor cells investigated. Courtenay and Mills (4) have reported that rat RBC added to the agar improved the plating efficiency of cells from a number of different tumor xenografts. They found a labile growth factor released by RBC that might be capable of producing diffuse colonies similar to those seen in marrow cultures. The successful growth of colonies from most tumors taken directly from patients has demonstrated the applicability of this assay in clinical oncology.

Improvements in the disaggregation techniques of solid tumors, advances in semisolid culture techniques, and discovery of factors affecting colony growth should increase the plating efficiency and the success rate in this culture system.

REFERENCES

6. Hamburger, A. W., and Salmon, S. E. Primary bioassay of human myeloma
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Fig. 1. Morphological examination of enzymatically disaggregated tumor cells. A, typical 21-day-old colony grown directly from a metastatic malignant melanoma and viewed in agar. × 150. B, cell from a metastatic malignant melanoma taken from original suspension. Wright-Giemsa, × 1400. C, cell of a metastatic malignant melanoma grown for 19 days in agar and plucked from individual colony. Wright-Giemsa, × 1400.
Fig. 2. Photomicrographs of the original surgical specimens and of the tumor following growth in soft agar and transplantation into the nude mouse. A. original ovarian carcinoma. H & E, x 370. B, the same tumor after growth in agar and in the nude mouse. H & E, x 370.
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