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A Rapid Method for Establishing Short-Term Primary Cultures of Human Tumor Cells from Fresh Tumor Biopsies (39087)

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In vitro studies of human malignant cells frequently require a rapid method for the establishment of primary, short-term cultures from fresh surgical biopsies of solid tumors. The current methodology to obtain such cultures is frequently unsuccessful (1–3). The adaptation of human tumor cells to continuous *in vitro* culture is complex and seems to depend on multiple factors. The need for estrogen or other hormones in some cases of breast cancer is an appropriate example (4, 5). The reported rate of success in obtaining primary cultures from melanoma varies between complete failure (1) and 77.8 % (6). Low rates of success were also reported in carcinomas in general (3), and those arising in the gastrointestinal tract in particular (1).

Little attention has been paid to the low proportion of viable cells that can be extracted from human solid tumors (7). The need for viable fresh human tumor cells for immunological and other studies has led to attempts at separation of viable from nonviable tumor cells by differential flotation on density gradients both in animal tumor models (8) and in the human (9). We have demonstrated the advantage of separated viable tumor cells over nonviable cells in terms of their immune reactivity *in vitro* (10), and we have hypothesized that the failure to establish primary cultures *in vitro* may be related to an inhibitory effect of the high proportion of nonviable tumor cells present in unseparated solid tumor cell suspensions (11). We examined the potential of the viable tumor cell fractions obtained by hypaque-ficoll specific density solution separation of mechanically dispersed tumor cell suspension to develop rapidly into proliferating primary tumor cell cultures.

Materials and methods. Fresh tumor biopsies were obtained under sterile conditions from 10 patients with the diagnosis of melanoma, carcinoma of the colon, and sarcoma. The surgical tumor specimens were cleaned of debris and fat and then mechanically dispersed into a free cell suspension by mincing, teasing, and sieving through a screen as previously described (10). The only modification was the addition of 5 % fetal calf serum to the tissue culture medium used for the preparation of cell suspension. To remove any residual fat fragments, the cells were spun down at 150*g* for 10 min at room temperature (International Centrifuge, UV Model). The cell pellet was resuspended in 10–20 ml of fresh medium containing 5 % fetal calf serum. After the cells were counted and their viability determined by trypan blue dye exclusion, they were carefully layered on top of a 10 ml mixture of ficoll and hypaque (24 parts of 9 % ficoll and 10 parts of 33.9 = hypaque were mixed thoroughly to give the solution of a final density of 1.080 g/ml), in a 15 × 150 ml screw cap tube. This was centrifuged at 1500*g* for 15 min at room temperature.

The viable cell fraction at the interface between the culture medium and the gradient mixture was aspirated and the cells were washed and resuspended in 5–10 ml of Ham's F-10 medium (Gibco Laboratories, Grand Island, New York) containing 20 % fetal calf serum. The cell count and the viability were redetermined. Five × 10^5 viable tumor cells were plated into each T-30 falcon culture flask containing 7–10 ml of F-10 medium with 20 % fetal calf serum. The seeded flasks were incubated at 37°C in a moist atmosphere of 5 % CO_2 in air. Cultures were examined daily with an inverted phase contrast microscope for evidence of tumor cell growth. Establishment of a primary tumor cell culture was determined by the observation of a doubling of the number of adherent

cells in the flask and their subsequent continued proliferation for a minimum of 2 weeks. Identification of tumor cells was achieved by morphological examination of stained, cytocentrifuge—prepared slides of cultured cells. The latter were detached by striking the culture flask sharply against a laboratory bench or by incubating its contents for 5 min with a mixture (v/v) of 0.25% trypsin (Gibco, Grand Island, New York) and 1% EDTA (versene, Hach Chemical Co., Iowa). Established primary cultures were fed three times a week by discarding and replacing 50% of the culture medium.

Results. The primary cultures which were established by using the viable cells from surgical biopsies of solid tumors are shown in Table I. Tumor specimens consisted of seven malignant melanomas, one colon carcinoma, and two sarcomas. The mechanically dispersed single cell suspension derived from these surgical biopsies invariably had low viability ranging from 7–29%. Differential flotation on top of a ficoll-hypaque specific density solution resulted in a significantly higher viability of the cell fractions recovered from the interface between the tissue culture medium and the ficoll-hypaque solution. The viability of the separated cells ranged from 46–97%. Using those viable fractions, primary cultures were established in every case. Tumor cells grew and could be identified as such in all cases within 24 hr after plating. Tumor cells enlarged and formed vacuoles and cytoplasmic processes within 2–3 days. Fig. 1 shows a 4-day-old primary culture established from viable cells derived from a surgical biopsy taken from a patient with malignant melanoma. An electronmicroscopic examination of these cells confirmed the presence of melanoma cells by the demonstration of numerous melanosomes in the cytoplasm (Fig. 2). Primary cultures of malignant melanoma could be subsequently subdivided without the use of trypsin-EDTA for the detachment of cells from the surface of the culture flasks. Ninety percent of the melanoma cells were promptly detached by simply striking the culture flask sharply once or twice against a pad of gauze laid on the laboratory bench. Cell suspensions recovered this way grew as quickly as those which were detached by using trypsin/EDTA. It is noteworthy that primary cultures established from viable cells derived from other malignancies were difficult to detach without the use of trypsin/EDTA. Only a minority of these newly established primary cultures ultimately became predominately fibroblastic, but this did not occur until after the passage of 4–5 weeks. We have further attempted to identify the presence of fibroblasts by electronmicroscopy in some viable floating fractions of tumor cells immediately after separation on the density gradient. We found virtually no fibroblasts present in preparations derived from a lymph node involved with melanoma nor in those derived from a primary comedo carcinoma of the breast.

TABLE I. PRIMARY CULTURES ESTABLISHED WITH VIABLE CELLS EXTRACTED FROM SOLID TUMORS

Experiment number	Source of tumor cells	Diagnosis	Percent viability before f-H separation	Percent viability after f-H separation	Primary cultures established (days)[a]
1	surgical biopsy	melanoma	29	95	2
2	surgical biopsy	melanoma	22	80	2
3	surgical biopsy	melanoma	8	90	2
4	surgical biopsy	melanoma	9	92	2
5	surgical biopsy	melanoma	19	97	2
6	surgical biopsy	melanoma	8	78	2
7	surgical biopsy	melanoma	7	67	2
8	surgical biopsy	colon carcinoma	10	50	3
9	surgical biopsy	sarcoma	21	46	3
10	surgical biopsy	sarcoma	7	81	3

[a] Primary cultures were considered established when a significant number of tumor cells introduced into culture flasks had enlarged and were morphologically recognizable.

Discussion. The results of this study indicate that viable tumor cell fractions recovered after ficoll-hypaque density centrifugation readily grow and develop into a primary

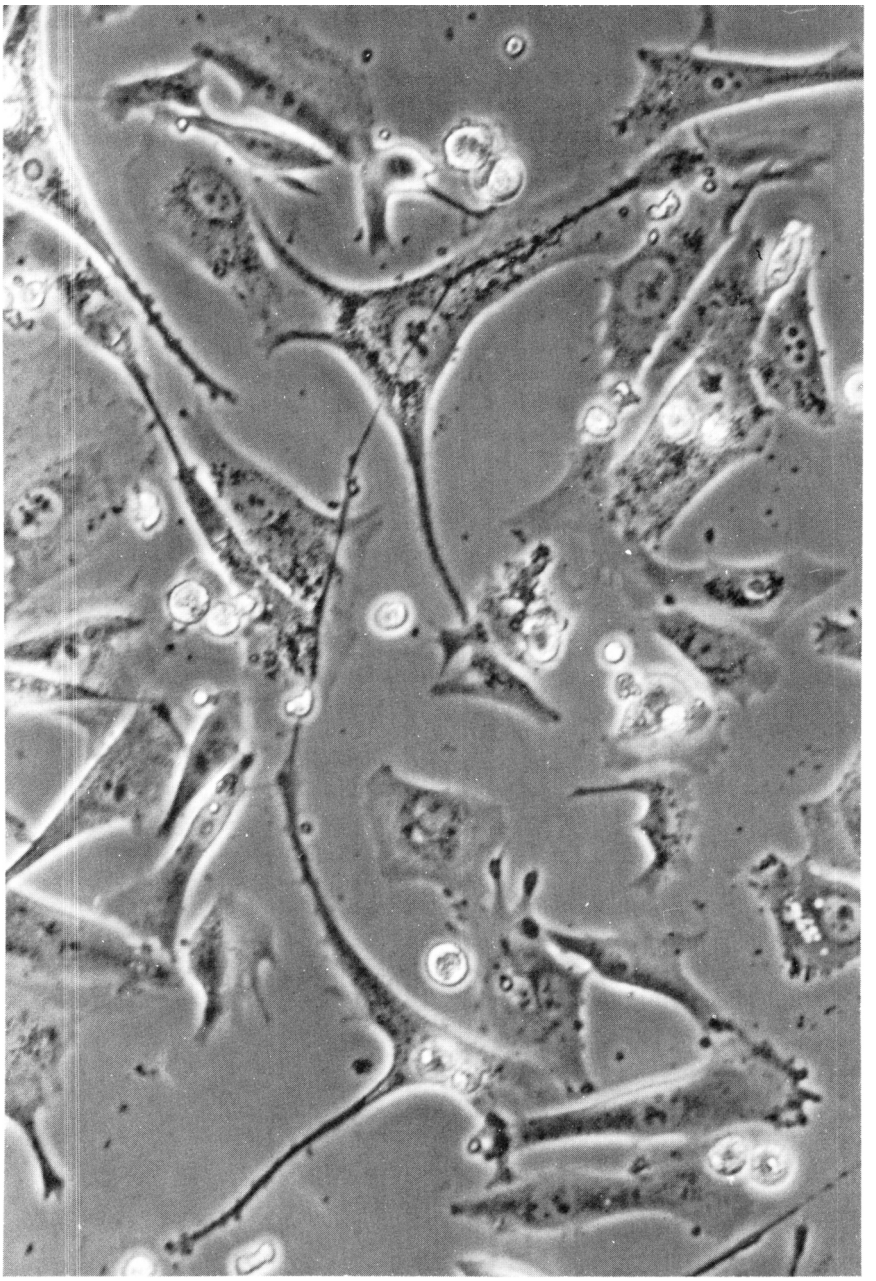

FIG. 1. A 4-day-old primary tumor cell culture established with viable cells obtained from a fresh surgical biopsy of metastatic melanoma. × 235.

culture. The elimination by density centrifugation of nonviable tumor cells, red blood cells, granulocytes, and cellular debris from the viable tumor cell fraction appears to be crucial to our success in the rapid establishment of primary tumor cell cultures derived from various solid tumors. The possible ill effect of nonviable tumor cells on the immunobiological reactivity of the viable cells has been proposed (11). It is also possible that the frequent failure to establish primary cultures (1, 2) and the common delays in

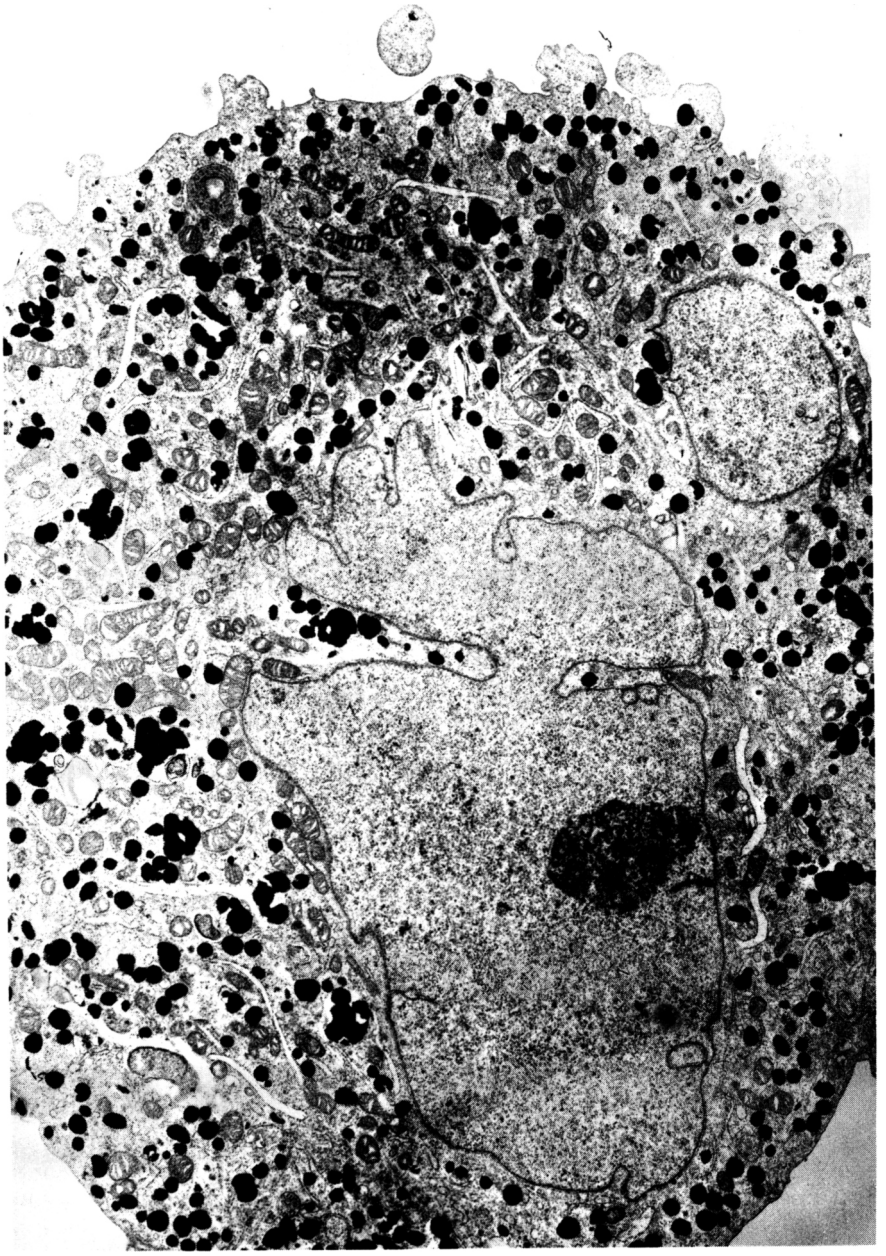

FIG. 2. Electronmicroscopic picture of a single melanoma cell showing numerous melanosomes in the cytoplasm. × 6000.

their growth (3) can be accounted for by such interactions between nonviable and viable tumor cells. It is also possible that ficoll-hypaque has a general enhancing effect on establishment of cell growth in cultures (12). This method does not absolutely prevent, but only reduces the incidence and perhaps delays the appearance of the frequently encountered overgrowth of the primary culture with fibroblasts which becomes obvious within 4–5 weeks.

The use of short-term primary cell cultures

for the demonstration of immunologic specificity is apparently advantageous in performing *in vitro* studies of tumor immunity in man, because of the greater distinction between the reactivity of cancer patients' and normal donors' lymphocytes against short-term tumor cell cultures compared to established long-term tumor cell lines (13). Further studies of cloning procedure intended to separate fibroblasts from tumor cells (14, 15) will be needed to ensure the establishment of long-term cell lines. The rapid method for establishment of short-term primary cultures would be a potential tool with wide application to metabolic, genetic, cytokinetic immunological, and therapeutic studies of human cancer.

Summary. A rapid method for the establishment of short-term primary cultures of human tumor cells obtained from fresh surgical biopsies is described. The method consists of the separation of the viable fraction of tumor cells by differential flotation on ficoll-hypaque density solution and its subsequent seeding into culture flasks. Tumor cell growth is established within 2–3 days. The incidence of overgrowth with fibroblasts is apparently reduced and usually delayed for 4–5 weeks, but cannot be prevented by this method.

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