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RAPID COMMUNICATION

Separation of High and Low Metastatic Subpopulations from Solid Tumors by Centrifugal Elutriation

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Abstract. We have isolated from murine solid tumors (B16a) subpopulations of cells possessing high and low metastatic potential. Tumors were dispersed by collagenase treatment. The resulting heterogeneous population of cells (i.e., viable and non-viable tumor cells and host cells) were separated by centrifugal elutriation. Four of the fractions (100, 180, 260, 340) contained tumor cells of high viability (> 95%) and high purity (< 1% host cell contamination). The four fractions were characterized by flow cytometry and found to differ in distribution of cells in G_1, S and G_2. The cell populations were also found to differ in metastatic potential as determined by their ability to form lung colonies following intravenous injection. The 340 fraction was approximately 5-fold more metastatic than the 100 fraction. We also observed that cells from the 100 fraction failed to induce platelet aggregation whereas cells from the 340 fraction induced significant platelet aggregation. These observations demonstrate that cells of B16a tumors are heterogeneous for phenotypic characteristics (i.e., metastatic potential; platelet aggregation, etc.) and that their ability to induce platelet aggregation is positively correlated with metastatic potential.

Introduction. Malignant solid tumors contain subpopulations of cells that differ in phenotypic characteristics such as metastatic potential, growth rate, etc. (1,2,3). In order to identify only those characteristics that regulate and/or characterize metastatic potential, homogeneous subpopulations of tumor cells must be isolated from the heterogeneous cell population of a solid tumor. Thus, comparison of the phenotypic properties of subpopulations with high and low metastatic potential can be performed without using a separate tumor line as the "normal" control. Previously, two methods have been used to isolate populations of cells with different metastatic potential. One selected cells in vitro for an enhanced characteristic thought to be important to the metastatic process. These have included adhesive (4) and invasive properties (5) and resistance to natural killer cell lytic activity (6). The second method selected metastatic variants in vivo. In this method, metastatic lesions are isolated and reinjected (subcutaneously or intravenously) to form secondary metastases. These metastases in turn, are isolated and reinjected into host mice. After 8 - 12 isolations and reinjections, this selection process usually yields a tumor variant of high metastatic potential (4,7). In both methods, the "high" and "low" metastatic properties are characterized by comparing selected clones derived from the same tumor line (8,9), but maintained as separate and distinct tumor lines (e.g. B16F1 and B16F10).

We have developed a third method to isolate subpopulations of cells with high and low metastatic potential. This method utilizes centrifugal elutriation to fractionate heterogeneous populations of cells derived from a single tumor or from a pool of several syngeneic tumors.

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Materials and Methods. The B16 amelanotic melanoma (B16a) was obtained from the DCT-Animal and Human Tumor Bank. Routine tumor transplantation was performed every two weeks into male, syngeneic host mice (C57BL/6J; Jackson Laboratory), of between 17 and 22 g (approximately 49 days old). All animals were quarantined for at least two weeks before use in experimental studies and were housed under identical conditions of photoperiod, feeding regime, temperature, etc. Tumor brei (implanted into the left axillary region) was used for routine transplantations as recommended in the DCT Tumor Bank (NCI, Bethesda, Md.), Inventory of Transplantable Animal and Human Tumors (November 1978). In order to maintain their metastatic phenotype, tumor lines were routinely restarted from liquid N_2 stocks after eight to ten isotransplant generations in vivo. In addition, each line was recharacterized, which included histology, transplantability, screening for murine tumor viruses, growth rate (in vitro doubling time), and metastatic potential (lung colonies formed in the experimental metastasis and spontaneous metastasis assays).

Subpopulations of B16a tumor cells were obtained from primary subcutaneous tumors of similar age (± 2 days) and weight (approximately 2 g). Tumors were minced to brei (approx.1-2 mm^3) and aseptically dispersed using sequential collagenase digestion (type CLS III, Worthington Biochem., Freehold, NJ) as previously described (10). Centrifugal elutriations were performed by a modification from our previously published procedure (10,11) using a Beckman J2-21 centrifuge and JE-6 rotor with Sanderson chamber. Briefly, seven fractions (designated 60, 80, 100, 180, 260, 340, and 700) instead of twelve fractions, were isolated. The 60 and 80 fractions consisted primarily of host cells and the 700 fraction consisted of cellular debris and cellular aggregates. Each of the remaining four fractions (100, 180, 260, 340) typically consisted of 100% monodispersed cells (i.e., no cellular aggregates), of which, > 99 % were tumor cells, of > 95 % viability. Tumor cell viability was determined by trypan blue vital exclusion. Host cell contamination was determined by light microscopic examination of cell samples air dried and differentiated with Wright's stain.

In pilot studies, cells from all fractions were tested for lung colony forming potential. Only cells from tumor cell enriched fractions formed lung colonies (data not shown). In these studies, cells (3.7×10^4) from each of the four tumor cell enriched fractions (100, 180, 260, 340) were injected i.v. (tail vein) into host mice. All mice were sacrificed 21 days post injection. Lungs were removed, fixed in Bouin's for at least 24 hrs, and visible tumor colonies were enumerated.

Cells from each fraction were pelleted by centrifugation (200 x g x 7 min) and resuspended in Eagle's Minimal Essential Media (MEM), Gibco, Grand Is. NY) supplemented with 10% fetal bovine serum (Gibco) at 1×10^6 cells/ml. An equal volume of cold (4°C) absolute ethanol was added drop-wise to a final concentration of 50% ethanol. Cells were fixed for a minimum of 1 hr at 4°C, centrifuged (200 x g x 7 min) and the supernatant discarded. Each pellet was resuspended in 0.5 ml of RNase type II-A (1 mg/ml, Sigma, St. L ouis, MO) in phosphate buffered saline (50 mM, pH 7.4, Ca^{++} and Mg^{++} free) and incubated for 30 minutes at 37°C. Propidium iodide, 0.5 ml (100 µg/ml, Sigma) was added to the cell suspension for a final concentration of 50 µg/ml. Cells were then placed on ice until analysis. Approximately 20,000 propidium iodide stained cells from each fraction were analyzed in a FACS 440 dual laser flow cytometer (Becton-Dickinson, Mountain View, CA) with an argon 5 watt laser, operating at 200 mW output at 488 nanometers. Using the Consort 40 Acquisition and Analysis software, DNA histogram curve fitting was done via the G. poly program as developed by Phil Dean (Lawrence Livermore National Laboratory, Livermore, CA).

Mouse platelet rich plasma (PRP) was prepared from blood drawn with a 20-gauge needle into heparin at a 1:9 ratio (v/v). Aggregometry studies were performed using a model DP247E dual-channel aggregometer (Sienco, Morrison, Co.) as previously described (12). Final tumor cell concentration was held constant at 2.5×10^7/ml. Platelet aggregation was initiated by the addition of 20 ul of tumor cells into 250 ul of platelet rich plasma ([platelets] = 2×10^9/ml). All studies were run in triplicate. Platelet aggregation was verified by light microscopy and by transmission and scanning electron microscopy (data not shown).

In vitro data were analyzed for significant differences by one way analysis of variance. Because in vivo data distribution were non-parametrically distributed, we used the

Kruskal-Wallis test to identify significant differences between groups. Studies with groups demonstrating a statistically significant ($p < 0.05$) distribution were further analyzed by the Q test.

Results. In the present studies, we used the B16 amelanotic melanoma (B16a), which is spontaneously metastatic from subcutaneous primary tumors. Tumors were enzymatically dispersed by collagenase (Type III) digestion. The resulting heterogenous population of cells (i.e., viable and non-viable tumor cells and host cells) was separated by centrifugal elutriation into seven fractions (60, 80, 100, 180, 260, 340, 700). The 60 and 80 fractions contained host cells (macrophages, PMN's, etc.), cellular debris, and non-viable tumor cells, and the 700 fraction contained cellular aggregates. The remaining four fractions (100,180,260,340) contained tumor cells of high viability (> 95%) and high purity (< 1% host cell contamination). These fractions were characterized for cell cycle distribution by flow cytometry (using DNA staining). Each fraction was found to differ in the percent of cells in G_1, S and G_2 phases (Figure 1). This distribution was reproducible between experiments. The 100 fraction always had the greatest percentage of cells in G1 phase and the 260 and/or 340 fraction had the smallest percentage of cells in G1 phase.

We previously reported that B16a tumor cells induce platelet aggregation (12), possibly via a plasma membrane protein with procoagulant (i.e., thrombin-generation) activity (14). Because tumor cell phenotypic characteristics (e.g., appearance of surface antigens, susceptibility to cytotoxic agents) may be dependent on cell cycle (13), we examined samples (5×10^5 cells) from each of the four fractions for differences in the level of expression of procoagulant activity and for ability to induce platelet aggregation. We found significant differences among the four fractions for ability to induce platelet aggregation and expression of procoagulant activity. The greatest difference was between the 100 fraction which failed to induce platelet aggregation, and the 340 fraction which induced significant platelet aggregation (Figure 2). The 180 and 260 fractions demonstrated platelet aggregating activity intermediate to the activity of the 100 and 340 fractions (data not shown). We also observed an identical relationship between procoagulant activity and fraction number - i.e., cells from the 100 fraction had the lowest levels of procoagulant activity whereas cells from the 340 fraction had the highest levels of procoagulant activity (data not shown).

We previously demonstrated the importance of platelets and tumor cell induced platelet aggregation in the metastatic process (12,15). Therefore, because of the significant differences in ability to induce platelet aggregation observed among the four elutriation fractions, we assayed samples of cells from the four fractions for lung colonizing potential (i.e., metastatic potential). Equal numbers (3.75×10^4) of cells from each fraction were injected intravenously (tail vein) into syngeneic mice. We found that cells from the 100 fraction were the least metastatic (formed the smallest number of tumor colonies) and cells from the 340 fraction were the most metastatic (formed the greatest number of tumor colonies; Figure 3). We observed an approximate 5-fold increase in metastatic potential of the 340 fraction over the 100 fraction.

Discussion. Previous studies (16,17,18) have demonstrated that cells isolated from murine solid tumors can be separated by centrifugal elutriation into discrete subpopulations. Those investigators obtained monodispersed tumor cell suspensions by trypsin treatment of murine fibrosarcomas. In contrast, our procedure utilizes collagenase treatment to disperse a murine (amelanotic) melanoma. In general, however, all groups observed that centrifugal elutriation fractionated the heterogeneous cell suspensions by phase of cell cycle. Cells predominantly in G1 phase were eluted first (at the lower flow rates) and cells predominantly in S and/or G2 phase eluted later (at the higher flow rates).

Grdina et al. (16) found that murine fibrosarcoma cells from subpopulations predominantly in the G1 phase formed more tumor colonies when injected intravenously into host mice than cells in S and G2 phase, whereas Meistrich et al. (17) found that cells in G1, S, and G2 had approximately the same tumor colony forming ability in vivo. We observed that cells from the subpopulation predominantly in the G1 phase were the least metastatic in the lung colony assay. There are several possible explanations for these contrasting observations (between tumor colony formation and phase of cell cycle) among the (above) studies. First, different tumors (fibrosarcoma vs. melanoma) were examined. Second, different enzymes (trypsin vs. collagenase) were used to disperse the tumors. Third, the clonogenic potential (in vitro soft agar) of the tumor cells used in these studies may be different. Suzuki et. al. (18) found that clonogenicity was constant among subpopulations obtained from fibrosarcomas, whereas Meistrich et. al. (17) found that

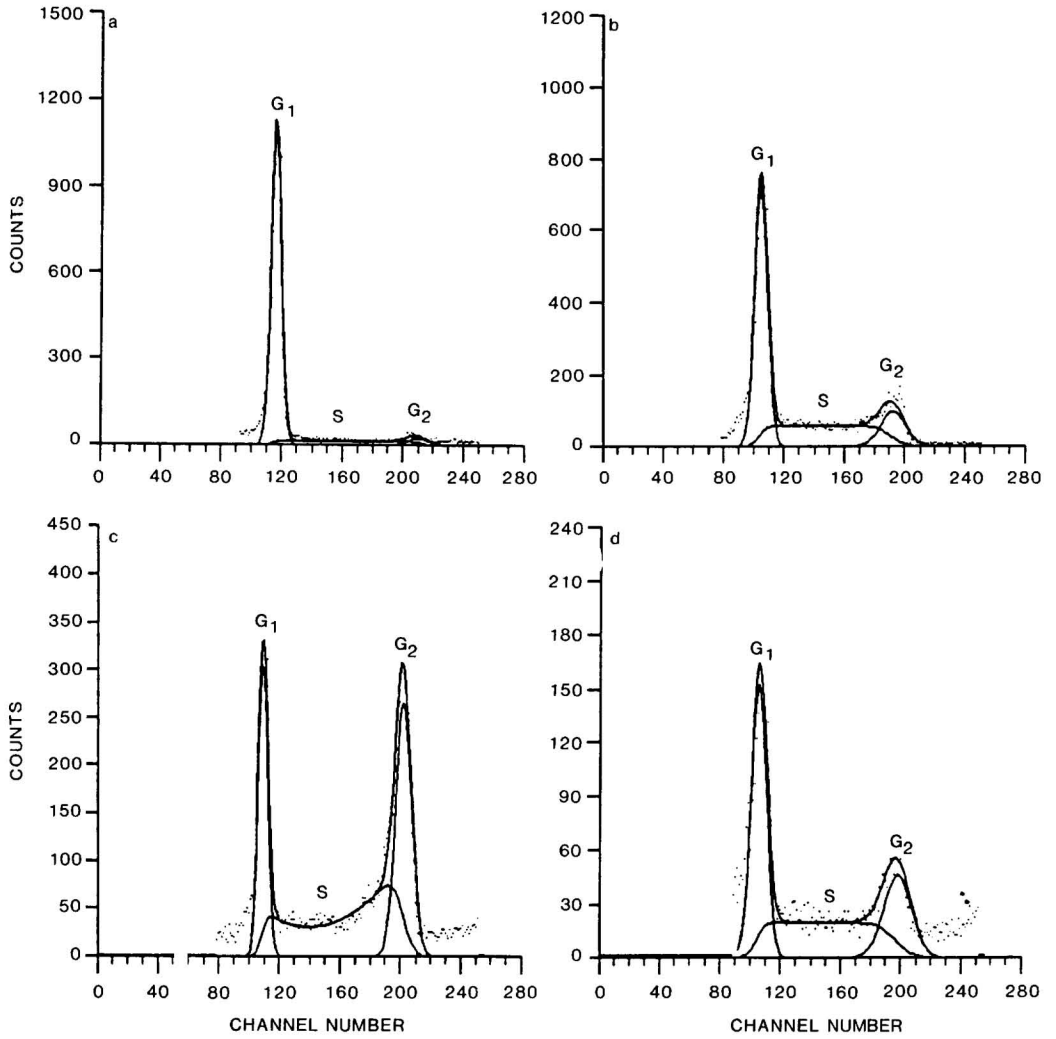
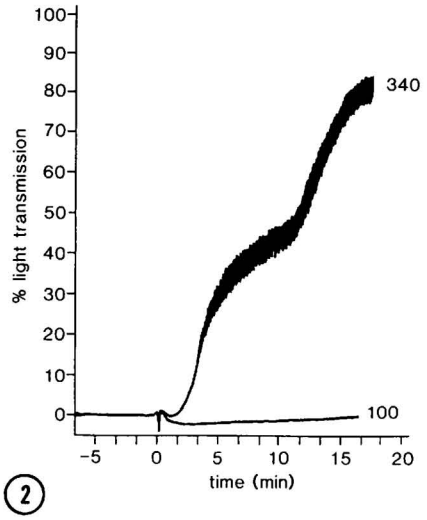


Figure 1a-d. Flow cytometric analysis of fractions 100, 180, 260, and 340. Samples of cells from elutriation fractions 100-340 were analyzed for DNA content (by propdium iodide staining) and location in cell cycle. Plates a-d represent DNA histograms of cell cycle distribution for fractions 100 (plate a), 180 (plate b), 260 (plate c) and 340 (plate d). The proportion of G1 phase cells in the sample populations, as compared to G2 and S phase cells, decreased from the 100 fraction (85%) to the 260 fraction (23%) and then slightly increases in 340 fraction (38%). Concomitantly, the percentage of cells in G2 phase increases progressively from the 100 fraction (3.5%) to the 260 fraction (35%), before slightly decreasing in the 340 fraction (30%).

clonogenicity varied among the subpopulations. Both groups, however, did find that clonogenicity was independent of cell cycle or metastatic potential. We believe a different phenotypic characteristic, i.e., procoagulant and platelet aggregating activity, may account for the heterogeneity of metastatic potential observed in this study .

We observed that tumor cell induced platelet aggregation was positively correlated



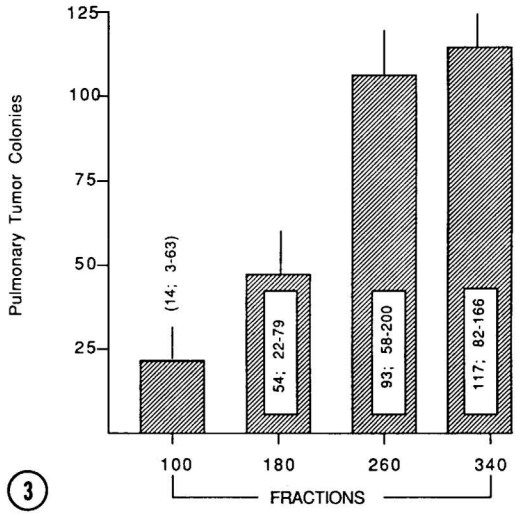


Figure 2. Tumor cell induced platelet aggregation. An equal number (5×10^5) of tumor cell were taken from fractions 100, 180, 260, and 340 and tested for ability to induce platelet aggregation. Addition of tumor cell samples (in 20 ul) to heparinized mouse platelet rich plasma (250 ul) induced significant platelet aggregation and coagulation (fraction 340), intermediate platelet aggregation (fractions 180, 260 - data not shown) or no platelet aggregation (fraction 100).

Figure 3. Pulmonary tumor colony formation. An equal number (3.75×10^4) of cells from each of the fractions (100 - 340) were injected into the tail vein of syngeneic C57BL/6J mice. All mice were sacrificed 21 days post injection and pulmonary colonies were enumerated. Cells from the 100 fraction induced the fewest colonies, whereas cells from the 340 fraction induced the greatest number of tumor colonies. Numbers within bars = median; range, and n=12 for each group.

with metastatic potential. Cells of the 100 fraction failed to induce platelet aggregation and formed few lung colonies in vivo, while cells of the 340 fraction induced significant platelet aggregation and coagulation. This fraction also produced the greatest number of lung colonies in vivo. These discoveries support our previous observations that tumor cell-platelet interactions are an integral part of the metastatic process by B16a tumors. We have shown that platelets facilitate tumor cell adhesion (19), that tumor cells induce platelet aggregation (12,15,20), and that antiplatelet agents (12,15,20) and thrombocytopenia (by Gasic et. al.; 21) inhibit metastasis by B16a and other murine tumors. Thus, one might anticipate that the ability to induce platelet aggregation would be positively correlated with metastatic potential. Additionally, it would appear that the ability to induce platelet aggregation is correlated with B16a cell cycle as cells in G1 phase (i.e., the 100 fraction) lack or have very low levels of procoagulant activity, whereas cells in S and/or G2 phase (i.e, 260/340 fractions) have higher levels of procoagulant activity.

We conclude that centrifugal elutriation of B16a tumor cells isolated from solid tumors provides a powerful tool for the separation of subpopulations with high and low metastatic potential. In this study, we characterized these subpopulations for cell cycle distribution and platelet aggregating activity and found a positive correlation with lung colonizing ability. It seems obvious that this may be a new model system for the preparation of subpopulations of cells from solid tumors for use in the characterization of phenotypic traits (i.e., adhesion receptors, susceptibility to natural killer cell lysis, etc.) regulating the metastatic process.

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