Quantitative Relationships of Intravascular Tumor Cells, Tumor Vessels, and Pulmonary Metastases following Tumor Implantation

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SUMMARY

An experimental model has been developed to quantify some of the major processes initiated by tumor transplantation and culminating in pulmonary metastases. The T241 fibrosarcoma, chosen because of its high hematogenous metastatic propensity and reproducible biological behavior, is transplanted into the femoral region of the C57BL mouse. Experiments are performed at specified times after transplantation to determine the dynamics of tumor growth, density, and size distribution of perfused tumor vessels, entry rate of tumor cells into the circulation, and the number of pulmonary metastases. Estimates of the entry rate of tumor cells into the circulation are obtained by perfusing tumors with an oxygenated, cell-free medium to allow the counting of single tumor cells and tumor cell clumps collected in the venous effluent. The tumor vascular network appears at approximately Day 4, while tumor cells are first observed in the perfusate approximately 5 days after transplantation. The concentration of effluent tumor cells, singly and in clumps, increases rapidly until Day 10, after which the rate of cell entry into the perfusion is diminished. A linear relation is found between the density of perfused vessels and the concentration of effluent tumor cells. Metastases, first observed on Day 10, increase rapidly with time, at a rate similar to that of the concentration of effluent tumor cell clumps containing 4 or more cells. These studies suggest that dynamics of hematogenously initiated metastases depend strongly on the entry rate of tumor cell clumps into the circulation, which in turn is intimately linked to tumor vascularization.

INTRODUCTION

In numerous studies, one or more of the parameters involved in the hematogenous metastatic process have been considered. None to date has evaluated quantitatively the dynamics of the entire process starting with tumor transplantation and ending in the development of metastases. Previous studies of circulating tumor cells have been limited to identifying tumor cells in a blood sample by microscopic or bioassay methods (3, 8, 9) or to demonstrating tumor cells in venous effluent samples following tumor manipulation (11, 19, 26). The importance of vascularization of the tumor has been established by Warren et al. (30, 31), Folkman et al. (7), Eddy and Casarett (4), Tannock (23), and Tannock and Hayashi (24). Quantitative studies of the changes in the rate of tumor cell entry into the circulation during the process of tumor growth and vascularization have not been reported. The time course of development of pulmonary metastases has been evaluated by Wexler et al. (32, 33, 34) and Romsdahl et al. (18, 19). However, the quantitative relationships of tumor cell liberation from the primary tumor to the temporal development of metastases require further study. In the present studies, a model system utilizing a transplantable murine tumor and standardized quantitative techniques has been developed to monitor the time course of the change in primary tumor volume, tumor vascularization, the number of single tumor cells and tumor cell clumps in the tumor venous effluent, and the number of pulmonary metastases. A major objective of this study is the evaluation of the quantitative interrelationships of these variables during the metastatic process in this system.

MATERIALS AND METHODS

Tumor-Host System. The 9- to 10-week-old C57Bl/6J male mouse (The Jackson Laboratory, Bar Harbor, Maine) is used. This inbred strain minimizes the general biological variability between animals and, in particular, variations in the immunological and vascular response to a transplanted tumor. Furthermore, this murine strain has a low incidence of spontaneous pulmonary tumors. The transplantable T241 fibrosarcoma (National Cancer Institute, Bethesda, Md.) was chosen because it not only exhibits rapid hematogenous spread, but also has very large cells and a reproducible pattern of growth and development (33, 34).

Tumor Transplantation and Volume. A 10-day-old i.m. transplant is harvested, excluding any area of necrosis and connective tissue. The tumor is minced into fragments no larger than 0.2 cu mm on a McIlwain tissue chopper (Brinkman, Westbury, N. Y.). The fragments are moistened in Ringer's solution containing 100 units of penicillin and streptomycin per ml (Grand Island Biological Co., Grand Island, N. Y.), loaded into a 1-ml syringe, and inoculated immediately. Each recipient is given 40 mg of
tumor fragments (75 to 80% viable), which are injected into the right anterior medial thigh region just proximal to the insertions of the vastus medialis and rectus femoris. The needle entry site is below the knee. Fragments rather than single cells are inoculated to minimize the possibility of inadvertent i.v. injection. At intervals of 5, 9, 10, 11, 12, 14, 15, 20, 21, and 25 days following inoculation, the hosts are sacrificed, and tumor volume is determined by displacement with 0.9% NaCl solution.

**Tumor Perfusion.** Tumor vascularity and rate of entry of tumor cells into the local venous drainage are quantified by perfusion of the vascular plexus that supplies the region where the tumor is growing. At various times after tumor inoculation (Days 3, 4, 5, 7, 8, 10, 11, 12, and 15), individual animals are anesthetized i.p. with sodium pentobarbital, 100 mg/kg, and placed on a heating pad. Under a stereoscopic microscope, a midline abdominal incision is made and the vessels in the right iliac region are exposed. Other possible venous return routes, both dorsal and ventral, are ligated. Tumor trauma is minimized by avoiding any direct contact with the tumor.

The venous effluent from the femoral region is collected by cannulating the external iliac vein via the inferior vena cava. The orifice of the cannula is carefully located just distal to the hypogastric branch. The external iliac artery is cannulated, the entry point being just distal to the pudic trunk. The vessel cannulations are performed usually within 2 min after the vessels are exposed. The cannulated vascular plexus is incorporated into the perfusion circuit (Chart 1) in which the constituents of the cell-free perfusate (Grand Island Biological Co.) are those reported by Folkman *et al.* (6). Perfusion is immediately initiated by means of a Harvard constant-flow syringe pump, while oxygenation is performed by a Dow beaker gas permeator (Cordis Corp., Miami, Fla.). Perfusate from a reservoir passes through the oxygenator, fills a 50-ml syringe, and is then pumped through a heating bath to the tumor. Pressure is measured (Statham Instruments Transducer P23A, Oxnard, Calif.; E for M Recorder PR-6, White Plains, N. Y.) proximal to the arterial cannula. Pump flow is adjusted until the tumor perfusion pressure is 90 to 100 mm mercury, after correction for the resistance of the arterial cannula. Thus the pressure is approximately equal to the mean blood pressure of the mouse.

The total venous effluent is collected by gravity in a syringe barrel (Swinnex filter holder, Millipore Corp., Bedford, Mass.) connected to a 47-mm in diameter Nucleopore filter (Nuclepore Co., Pleasanton, Calif.) containing 8-μm pores. The resident blood volume within the tumor at the time of cannulation is included in the sample. After 5 ml of perfusate pass the filter, the collected cells are immediately fixed and mounted on a glass slide.

**Identification of Perfused Vessels.** Tumor perfusions are followed by perfusion with a stain solution, the viscosity of which is adjusted to that of the perfusate. The stain solution is a mixture of 0.04 g crystal violet resorcin (MC&B Manufacturing Chemists, Norwood, Ohio) and 60 ml ethanol acidified with 1 ml concentrated HCl, and is diluted to 100 ml with water. The cannulated vessels are tied off, and the tumor is excised and fixed in formalin for 1 week. The tumor is bisected (25, 29), and unstained histological slides are prepared. Cross-sections of unperfused and perfused tumors at each day after transplantation are microscopically evaluated to determine the time at which tumor vascularization commenced. Vessels are identified by the presence of erythrocytes or injected stain material. Only those vessels present within tumor tissue are counted and their smallest transverse diameter measured by ocular micrometer.

**Pulmonary Metastases Assay.** In a separate study, animals harboring 5-, 7-, 10-, 15-, 21-, and 26-day-old tumors are sacrificed and their lungs are studied for metastases. The excised lung is inflated intratracheally with neutral buffered formalin and fixed in inflation for 5 days. The lung is then submerged in silver nitrate (5%) solution for 30 min, washed in tap water, and examined with a stereomicroscope by transillumination. Metastases appear as dense spots and are categorized for size as greater or smaller than 0.5 mm. Macroscopic observations of metastases are confirmed by histological methods.

**Identification of Tumor Cells.** Tumor cells are accurately identified for counting by distinguishing each of the hematogenous cellular components to be differentiated from tumor cells. Lymphocytes, macrophages, and tumor cells are characterized and quantitated in the following manner. Lymphocyte control populations are obtained by mincing the spleens of healthy C57BL/6J mice. The recovered cells are suspended in perfusate and mixed with absolute alcohol, and the suspension is filtered through an 8 μm pore, 47-mm Nucleopore filter. The filter is then mounted, air dried, and stained by the Papanicolaou method. This technique is used for control and tumor cell characterization. A population of macrophages and lymphocytes is harvested from the peritoneum 24 hr following introduction of a standard mineral oil challenge. A lymphoblast control...
population is obtained by incubating lymphocytes (1.5 x 10^6 cells) derived from excised mouse spleen with concanavalin A for 3 days (17). The tumor cell population is characterized with the use of cells obtained by mincing and dispersing cells from a 10-day-old T241 fibrosarcoma.

The cells from each population are examined by means of a semiautomated microscopic image analyzer (IMANCO, Image Analysing Computers, Inc., Monsey, N. Y.). The nuclear area and total cell area of 100 cells from each population are measured. Each unit corresponds to an area of 0.14 sq μm. Quantitative criteria for identifying tumor cells and distinguishing them from other cell types are established by preparing histograms of the frequency distribution of the areas of cell and nucleus (Charts 2 and 3). Those cells that fall in the regions of overlap (1000 to 1750 units) are further evaluated, utilizing the ratio of nuclear to cell area. The following steps are then used in the identification and characterization of tumor cells from each experiment: (a) all cells on each filter are scanned; (b) cells with a total cell area greater than 1750 units (cell diameter, > 14 μm) are classified as tumor cells; (c) cells with a total cell area between 1000 and 1750 units (9 to 14 μm diameter) are classified as tumor cells only if the following criteria are met: (i) nuclear hyperchromaticity, (ii) presence of nucleoli (iii) eccentricity of nucleus, and (iv) nuclear/cell area ratio, <0.7; and (d) both the number of clumps and the number of cells in each clump are recorded.

RESULTS

Tumor Volume. Tumor volume increases rapidly in the 1st 10 days, but at a continuously decreasing rate (Chart 4). The shape of the growth curve is similar to that of other transplantable sarcomas and can be represented by a Gompertz function (22).

Cells in the Effluent. Cells collected from the tumor perfusion include clusters of polymorphonuclear leukocytes, lymphoblasts, erythrocytes, endothelial cells, single tumor cells, and tumor cell clumps. Clusters of leukocytes are frequently found in contact with tumor cells. In the 1st 5 to 10 days, the clusters consist of polymorphonuclear leukocytes and macrophages; after this period, lymphocytes become the predominant cell type. Tumor cells are oval or round, but not spindle shaped. The hyperchromatic tumor cell nuclei are usually eccentric, and nucleoli can often be identified. About 90% of the tumor cells are identified on the basis of cell size alone. Tumor cells in a clump are usually larger and better preserved than single tumor cells. The number of tumor cells in a clump ranges from 2 to 30. A histogram of clump-size distribution is shown in (Chart 5). Approximately 10% of the total number of tumor cells...
in a sample are in clump form. No tumor cells have been identified in the venous effluent before Day 5. The increase in the concentration of tumor cells in the effluent approaches an exponential form initially (Chart 6); however, the rate of increase diminishes between Days 10 and 15. Similarly, the concentration of tumor cell clumps (Chart 7) rises rapidly from Day 5 to Day 12, then levels off between Days 12 and 15. This curve, however, has a slower rate of increase than that for total tumor cells (Chart 6).

Tumor trauma and changes in the rate of perfusion produce marked alterations in the concentration of tumor cells collected in the venous effluent from tumors perfused on Days 10 and 12. Tumor massage results in at least a 10-fold rise over the initial concentration of tumor cells, as well as a higher proportion of large clumps. An abrupt doubling in perfusion flow rate yields approximately a 2-fold increase (above control levels) in the total concentration of tumor cells.

**Pulmonary Metastases.** No metastases are identified macroscopically prior to Day 10. The number of pulmonary metastases increases from an average of less than 1 on Day 10, to 20.1 ± 2.8 (mean ± S.D.) on Day 25 (Chart 8). Most pulmonary metastases are less than or equal to 0.5 mm in diameter and increase in size as well as number with time.

**Perfused Tumor Vessels.** Considerable variability is observed in the density and size distribution of perfused tumor vessels in 12-day-old tumors. The vessels > 30 µm or greater in diameter are of particular interest when related to the size of individual tumor cells and the diameter of tumor clumps. There is a linear relationship between the density of perfused vessels of diameter > 30 µm and the concentration of both total tumor cells (r = 0.94) and tumor cell clumps (r = 0.97) (Chart 9). A less significant linear relationship is found between the total density of per-
Intravascular Tumor Cells and Metastases

Chart 8. Semilog plot of the number of metastases versus time after tumor implantation. Means ± 1 S.D. are plotted. The large S.D. on Day 10 is caused by the presence of metastatic foci in only 50% of the animals.

Chart 9. The relationship of number of perfused tumor vascular channels greater than 30 μm, concentration of total tumor cells (○), and tumor cell clumps (2 cells or more) (O) observed in the tumor venous effluent. The regression line (0.97) represents the best least squares fit of cell clumps as a function of tumor vessels 30 μm and larger in size. Note the intersection of the regression line near zero.

DISCUSSION

Tumor perfusion has been used for a variety of studies by Griffiths and Salsbury (9), Gullino et al. (10), Folkman et al. (6), and Fisher and Fisher (5). Perfusion is used here to study the rate of release of tumor cells, singly and in clumps, and to monitor changes following implantation. The method has the following major advantages. The collected volume of venous effluent can be as large as needed for an adequate sample. The ability to control variables such as pressure, flow, perfusate constituents, and perfusate viscosity allows for the quantitative analysis of those physiological and biochemical variables that influence tumor cell washout. Use of a cell-free perfusate reduces the number of hematogenous component cells that must be differentiated from tumor cells. The addition of dye or contrast media to the perfusate can be used to study the functional tumor vasculature and its relationship to tumor cell washout.

Tumor cell identification is considered accurate since, in actual practice, 90% of the tumor cells can be distinguished from other cell types on the basis of size alone, without using additional criteria of nuclear appearance or nuclear/cell area ratio. Most of the larger tumor cell clumps (4 or more cells) are in a “molded” configuration, rather than in a loose aggregation. Since perfusate samples are collected and fixed immediately, it is our belief that the tumor cell clumps observed in the venous effluent are actually liberated as such into the circulation during the natural course of the pathological process. The observation of tumor cell clumps in the venous effluent may be of primary importance in the development of pulmonary metastases. Owing to their larger diameter, clumps would have a greater tendency to arrest in the pulmonary precapillary vessels (12, 13, 35). Furthermore, the centrally placed tumor cells in a clump may be protected from immunologically mediated destruction. In this study, trauma of the tumor mass increases the rate at which tumor cells are released from the tumor, and thus confirms the previous observations of Tyzzer (26) and others (11, 19).

Two interpretations can be considered concerning the concentrations of tumor cells collected in the perfusate. The 1st is that the rate of tumor cell release during perfusion is the natural release rate. The natural release rate is defined as the rate that occurs under in vivo circumstances without trauma, perfusion, or other events that may influence the release of tumor cells. This observation is supported by the following analysis. If a blood flow of 1 ml/min is assumed, which is in the range of the present flow values, then the calculated total number of tumor cells released over 24 hr would be $1.4 \times 10^3$ cells on postimplant Day 5, $3.8 \times 10^4$ on Day 10, and $1.5 \times 10^5$ on Day 15. Koike (13) estimated the number of i.v.-injected tumor cells necessary to establish pulmonary metastases in approximately 50% of animals and found a value of $1 \times 10^5$ cells for Sarcoma 37, which has a cell-size distribution very similar to that of the present in the periphery. After Day 12, the total vascular density decreases, although the ratio of vessels $\geq 30 \mu m$ to vessels $<30 \mu m$ increases.
T241 fibrosarcoma. Summation of the total number of tumor cells released into the circulation during the 1st 9 days after tumor transplantation yields a value of approximately $1 \times 10^5$ tumor cells. This value agrees with the range reported by Koike (13). Romsdahl et al. (18) studied metastasis formation in mice bearing the T241 fibrosarcoma following serial amputation of the extremity bearing the tumor. He found that amputation of tumor-bearing limbs 9 days after transplantation resulted in a 50% incidence of pulmonary metastases. These data suggest that the concentration of tumor cells observed in our studies is in a range similar to the "natural" release rate.

The 2nd possibility is that the rate of tumor cell release during the perfusion may be greater than the "natural" release rate, but proportional to the available number of tumor cells subject to dislodgement. This alternative is based on the observation in individual perfusions that the concentration of tumor cells in the 1st 2 ml of perfusate is greater than the concentration in the next 3 ml. In subsequent collections the concentration gradually diminishes. This phenomenon may be the result of pressure and flow changes during tumor vessel cannulation and initiation of perfusion, which initially may dislodge tumor cells at a greater than natural rate. However, even if the concentration of effluent tumor cells in these studies is unnaturally high, the standardized perfusion and collection methods assure that the effluent concentration is proportional to the natural release rate for similar postimplant times.

In these studies, the concentration of effluent tumor cells and tumor cell clumps is time dependent under otherwise identical perfusion conditions. It is, however, sensitive to tumor trauma and perfusion flow alterations. These factors could affect the dislodgment of tumor cells at the mural-lumenal interface, where tumor cells may exist in various stages of vascular penetration and endothelial adherence.

The tumor doubles in volume at least 3 times before tumor cells are first identified in the perfusion. From Day 5 to Day 10, the concentration of cells entering the circulation increases more rapidly than does the tumor volume. Beyond 10 days when tumor necrosis is first developing, both the tumor volume and the concentration of tumor cells increase at slower rates. At this time the rate of increase in tumor volume is more comparable to the increase in concentration of tumor cells in the effluent.

**Tumor Vessels.** In attempting to evaluate the quantitative relationship between the number of perfused tumor vessels and the concentration of tumor cells washed out, one must account for 3 potential sources of error. (a) The tumor vessels identified histologically may not be the same vessels that were perfused during the collection of tumor cells. (b) There are age-dependent changes in tumor vessel density and size distribution when tumor vascularization is occurring (4, 21) and in tumors with an established vascular supply (1, 29). (c) Vessels measured on a bisected tumor surface may not be a valid sampling of the vessels throughout the entire tumor.

The 1st source of error was minimized by adjusting the viscosity of the stain solution to that of the perfusate. There was no significant interruption of flow when the stain solution was introduced into the perfusion circuit, and the stain solution was administered at the standard pressure. Still, there is no absolute assurance that the vessels identified and counted histologically are those that were perfused by the nutrient solution. The 2nd source of error is minimized by comparing tumors matched in age and volume. The 3rd possible source of error is negligible, since vascular densities were observed to be similar in different areas of the same tumor. Vogel (29) and Tannock and Steel (25) also found a similar vascular density in different areas of single mammary adenocarcinomas. Measurement of every vessel in any bisected transection of the tumor is, therefore, considered to be a valid sampling of tumor vessel population.

It has been long suspected that large tumor venules are the site of intravasation (9, 26). For tumors perfused on postimplant Day 12, a linear relationship is found between the average density (number/volume of tissue) of perfused vessels greater than or equal to 30 μm in diameter and the concentration of tumor cells in tumor cell clumps washed out of that tumor (Chart 9). A less significant relationship is found between the total vascular density and the concentration of tumor cells. Salsbury et al. (20) and LeServe (14) recently demonstrated that metastasis from certain transplanted tumors may be prevented by treatment with the drug "ICRF 159." This observation was correlated with a change in the character of the tumor vessels from "poorly defined sinusoids" to "a few well-formed discrete blood vessels." In the perfused T241 fibrosarcoma, for the present experiments, most vessels cannot be distinguished histologically as either arteries or veins by the standard morphological criteria. It is assumed that the identified tumor vessels are newly formed and may present a poor barrier against tumor cell invasion.

Studies on vascularization of tumor transplanted into the hamster cheek pouch show a vascular network is first present 4 to 7 days after implantation (4, 21). In our study, tumor vessels were identified no earlier than 4 days after implantation. Since this is the same time at which tumor cells first appeared in the collected perfusate, these data suggest a dependent relationship between the earliest appearance of tumor cells in the venous effluent and the onset of tumor vascularization.

**Circulating Tumor Cells and Metastases.** The relationship between the number of circulating tumor cells and the subsequent development of metastases has been the subject of extensive study (9, 16). Examination of the rate of development of metastasis in our study reveals that a time delay of about 6 days occurs between the 1st appearance of circulating tumor cells and the earliest demonstration of pulmonary metastases and that the dynamics of these variables are similar (Chart 10). The delay is probably caused by our inability to identify metastatic foci macroscopically until they have grown to at least 0.25 mm in diameter. The similarity in the dynamics can be explained by the supposition that an increase in the rate at which tumor cells arrive at the lung would be associated with a higher probability of initiating metastatic foci. The concentration of total tumor cells, both singly and in clumps, in the effluent does not follow the changes in the number of metastases as
Intravascular Tumor Cells and Metastases

Chart 10. A semilog plot of metastases versus postimplant time compared with the effluent concentration of total tumor cells and tumor cell clumps (4+ cells). The values of tumor cells and clumps are plotted at a time 6 days (t + 6) after their collection. The units on the left ordinate are clumps/ml, and are translated to total cells/ml by multiplying by a factor of 10. The changes in the concentration of cell clumps show a closer correspondence with the development of metastases than do the total tumor cells.

closely as does the concentration of clumps alone (4 cells or more). Compared to single tumor cells, tumor cell clumps are considered to have increased embolization, survival, and growth characteristics (12, 13). A clump of 4 or more cells is calculated to be the threshold for pulmonary precapillary arrest, assuming a mean capillary diameter of 15–20 μm and a minimum tumor cell diameter of 15 μm. Our data suggest that the concentration of tumor cell clumps observed in the venous circulation may provide a better basis for predicting the future development of metastases than the total concentration of tumor cells. An additional comprehensive analysis of these relationships has been performed by the present investigators using a mathematical model and is reported separately (15).

The resulting graphic comparisons (Chart 10) demonstrate 2 phases, a period in which the liberation rate of tumor cells and clumps are linearly related to development of metastases, and a later period in which the correlation is poorer and the number of metastases is overestimated. The 1st phase may be comparable to that observed by investigators (3, 5, 13) who found a linear relationship between the incidence of metastases and the dose of i.v.-injected tumor cells. In their studies, the inoculation of cells was acute and may not have allowed for the natural development of host defenses. The 2nd phase may reflect the development of host defenses which can increase the death rate of circulating tumor cells (2, 27, 28). These predictions must await further experimental verifications.

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REFERENCES


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