The Significance of Hematogenous Tumor Cell Clumps in the Metastatic Process

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SUMMARY

The relationship between the size distribution of vessels in an implanted tumor, the size distribution of tumor cell clumps collected in the venous effluent of the tumor, and the development of pulmonary metastases have been studied. The purpose is to evaluate the importance of clumps and their site of formation in the metastatic process. The results demonstrate a negative exponential character for both the size distribution of effluent tumor clumps and the tumor vessel population. Tumor trauma or massage increases total tumor cells and clumps released into the effluent. Serial amputation demonstrates that tumor cells are continuously being released on a day-by-day basis in vivo. A linear relationship exists between the proportion of vessels with diameters large enough to pass a tumor clump of a given size and the proportion of clumps of that size within the venous effluent. Injection of tumor cells in clumps of 6 to 7 cells produces a significantly greater number of metastatic foci than does a similar number of single tumor cells; larger clumps produce significantly more metastatic foci than do smaller clumps matched for the number of cells. These studies verify the significance of tumor clumps in the metastatic process. It is suggested that tumor cell clumps arise locally within the vascular bed of the tumor.

INTRODUCTION

The concentration and size distribution of tumor cell clumps that enter the circulation play a significant role in the hematogenous metastatic process (2, 4, 7, 11). Our previous studies (6) have indicated that tumor cell clumps are a reproducible finding in the venous effluent of a transplanted T241 fibrosarcoma. The present study was undertaken to confirm this observation and analyze it more fully. Previous investigators (2, 11) have recognized that i.v.-injected tumors in clump form have a greater tendency to form metastases than do the same number of single tumor cells. However, no data have been available on the size distribution of tumor cell clumps released spontaneously into the venous effluent of a primary tumor. A better estimate of the importance of clumps in the unperturbed hematogenous metastatic process requires quantitative information on the size distribution of clumps entering the circulation and their relative propensity to form metastases. Consequently, the first objective of this study is to quantify the size distribution of clumps of tumor cells in the effluent blood and to evaluate the effect of factors that may modulate the clump distribution. These factors include time, trauma, and ablation of the primary tumor. An additional objective of this study is the verification of the hypothesis that a given number of tumor cells in clump form are of greater potential in the production of metastases than are a similar number of single tumor cells. This has been accomplished by study of the pulmonary metastases resulting from a single i.v. infusion of tumor cells of known concentration and clump size distribution.

One further objective is the evaluation of the relative importance of local or intratumor factors in the formation of clumps of tumor cells. This has been accomplished by comparison of the vessel size distribution within the tumor and with the size of clumps present in the venous effluent of the primary tumor. One of the possible determinants of clump size may be vessel size within the primary tumor, since a tumor cell clump must pass the vascular plexus in order to enter the systemic circulation. By comparing the distribution of sizes of the tumor clumps and vessels, some indication of the importance of local formation of clumps may be gathered.

MATERIALS AND METHODS

Tumor-Host System. The transplantable T241 fibrosarcoma C57BL mouse system was chosen because it exhibits rapid hematogenous spread and a reproducible pattern of growth and development (6, 12). As described previously (6), the tumor is transplanted in 30-mg aliquots into the murine femoral muscle.

Effluent Tumor Cell Clumps. The rate of entry and size distribution of tumor cell clumps in the local tumor venous effluent is studied as described previously (6). In this method the circulation to the tumor is isolated and perfused at controlled pressure via the iliofemoral vessels. The perfusate is a cell-free oxygenated nutrient medium at 37°C (6). A quantity of 20 ml of venous effluent is collected and passed through a Nuclepore filter (8 μm pore size). Cytological elements retained on the filter are quantitated. Tumor cells are identified by size and nuclear to cytoplasmic ratio (6). The tumor cell clumps are counted and placed in the following size categories: single cells, clumps of 2 to 3 cells, 4 to 5 cells, 6 to 7 cells, and 8 or more cells.

Identification of Perfused Tumor Vessels. Tumors are
perfused with a low-viscosity crystal violet stain solution to identify tumor vascular structures (6). The tumor is bisected and unstained histological slides are prepared. This staining procedure colors the vascular wall a deep blue with little or no leakage of stain into surrounding tissue. All vascular structures present in tumor tissue are counted and their smallest transverse diameter is measured by ocular micrometer.

Infusion (i.v.) of Tumor Cell Clumps. For investigation of the possibility that circulating tumor cells incorporated in clumps may have a greater propensity to form pulmonary metastatic foci, an aliquot of tumor cells with a known clump size distribution is infused into the tail vein of recipient mice. The recipient mouse is anesthetized with a sublethal dose of sodium pentobarbital (30 mg/kg). Under a stereomicroscope a longitudinal midline incision, 1 cm, is made in the dorsal connective tissue overlaying the tail vein. The vein is carefully exposed and a cannula is inserted in the lumen. Injection volumes of 0.05 ml are infused over a 10-min period.

Cell Clump Separation. Tumor cell clumps are obtained from the nonnecrotic portions of 10- to 14-day-old tumors. After being minced thoroughly with scissors, the tumor tissue is diluted with an equal weight of Ringer’s solution. The suspension is gently agitated for 0.5 hr. For the preparation of single tumor cells the suspension is passed through Whatman filter paper (Grade Course 4, maximum effective pore size of 25 μm) in a 47-mm Swinnex filter holder. The cell concentration of the filtrate is found using a WBC-counting procedure under a hemocytometer. Clumps are seen in a size range of 2 to 20 cells. Single cells comprise less than 25% of the total cells. In the cell suspensions, 90% of the single cells and 95% of the clumps are judged viable by trypan blue exclusion. In order to demonstrate the transplantability of the cell suspensions (single cells with less than 1% clumps, or clumps with less than 25% single cells), cells or clumps were injected i.m. into a series of mice in adjunct studies. In these experiments, inoculation of 1 × 10^6 cells as single cells or clumps showed equivalent rates of tumor development and indicated the viability and growth potential of these preparations.

Twelve days following i.v. clump infusion, macroscopic pulmonary metastases are assayed in excised lungs by transillumination as described previously (6).

Tumor Trauma. Two types of trauma are administered to groups of tumor-bearing animals, manual massage and intratumor 0.9% NaCl solution injection. These 2 groups are studied and compared to a control group which is not subjected to trauma. Animals are anesthetized by CO₂ narcosis. The femoral tumor-bearing region of animals in the 1st experimental group is vigorously massaged digitally for 30 sec. Animals in the 2nd experimental group are given injections of 0.2 ml 0.9% NaCl solution into the tumor. Both of these forms of trauma are performed 7 days postimplant. Pulmonary metastatic foci are assayed 6 and 14 days posttrauma. In addition, to estimate the effect of tumor trauma on the venous effluent clump size distribution, 12-day-old tumors were traumatized by massage and the size distribution of tumor venous effluent clumps was evaluated concurrently in the collected perfusates.

Tumor Amputation. Before amputation, animals are anesthetized with sodium pentobarbital (30 mg/kg). Since manipulation of the tumor during the amputation procedures may cause release of tumor cells into the venous effluent, the femoral region proximal to the tumor is ligated before excision. A loop of black silk ligature is placed around the proximal femoral region as close as possible to the head of the femur. The loop is gradually tightened and securely fastened to occlude the circulation totally. The femur and associated tissue at the base of the stump are ligated and a skin flap is closed with braided silk suture. The clean wound is covered with Polymyxin B-Bacitracin-Neomycin ointment. Amputations were performed on groups of 10 mice 3, 4, 7, 9, 10, and 12 days following tumor implantation.

RESULTS

Effluent Clump Size Distribution. The size distribution of tumor cell clumps in the venous effluent of animals studied on Days 7, 10, 12, and 15 following tumor implantation are compared in Chart 1. Clumps are placed in 5 classes: single cells, clumps of 2 to 3 cells, 4 to 5 cells, 6 to 7 cells, and 8 or more cells. At the time periods studied, the distribution is similar for all clump classes other than those containing 8 or more cells. The proportion of clumps of 8+ cells increases significantly with time and is associated with a decrease in the proportion of smaller clumps up to Day 15 of

![Chart 1. Effluent clump size distribution compared over 4 postimplant periods.](image-url)
the study. Since the clump size distribution for all groups other than the largest are similar, the overall distribution is characterized for the total of 22 perfusions in Chart 2. This graph demonstrates that approximately 66% of the effluent tumor cells are in the single cell class while the proportion of cells in larger clump classes decrease exponentially with clump size. However, while the clump size distribution is relatively stationary during this time interval, the total concentration of tumor cells in the effluent is rapidly rising. As reported previously using this system (6) tumor cells are first seen in the venous effluent on Day 5. The estimated total number of tumor cells released over 24 hr rises steeply from $3.8 \times 10^6$ cells on Day 10 to $1.5 \times 10^6$ cells on Day 15.

Metastases following Tumor Amputation. All mice harboring the tumor for 3, 4, 7, or 9 days survived the amputation procedure while only 5 of the 10 from the 12-day group survived the operation. The poor surgical survival in older tumors may be attributable to shock from the loss of residual blood within the excised tumor.

Table 1 shows the steady increase in the number of metastatic foci with time and the rapid decline in the probability that no metastatic foci are present.

Metastases following Tumor Trauma. We have previously reported (6) that tumor trauma in the form of massage results in a 10-fold increase in the concentration of tumor cells in the tumor venous effluent. This increase is associated with a greater proportion of large size clumps. Table 2 shows the effects of tumor trauma administered 7 days after tumor implant.

The effects of tumor trauma are evident as early as 6 days after massage. At this time, tumor cells liberated during trauma will have grown to sufficient size to be identified macroscopically. However, the difference in metastases observed at this time is not statistically different from control values. Both forms of trauma produce a statistically significant ($p < 0.05$) increase in the number of metastatic foci by 12 and 14 days posttrauma.

While the clump size distribution is relatively predictable

![Graph](chart2.png)

**Chart 2.** Clump size distribution combined for all time periods. Data at the 4 time intervals studied are combined from Chart 1. $\bar{S} = 2$ S.E. The negative exponential relationship with increasing clump size reflects that seen at each of the individual time periods.

with time in the unperturbed state (Chart 2), trauma produces a prominent alteration in the distribution as shown in Chart 3. Following trauma, the total number of effluent cells is increased by a factor of about 10 while the clump size distribution shifts noticeably in the direction of larger-sized clumps.

**Metastatic Foci following Single Injection of Clumps.** The injection of single tumor cells and tumor cells in clumps into the mouse tail vein resulted in many metastatic foci that were assayed 12 days later. As shown in Table 3, tumor cells in clump form produced a significantly ($p < 0.05$) greater number of metastatic foci than did the same number of cells in single-cell form. Furthermore, larger-sized clumps produced significantly ($p < 0.05$) more metastatic foci than smaller-sized clumps. Comparison of a similar number of cells injected in the 3 size classes shows an approximate linear relationship between average clump size and the number of metastases formed.

**Tumor Vessel Size Distribution.** The tumor vessel size distributions measured on postimplant Days 7, 10, 12, and 15 are compared in Chart 4. The mean vessel diameter increases with time, but the increase is not statistically significant ($p < 0.05$) during these time periods. The vessel size distribution changed significantly with time only for the vessels larger than $100 \mu m$ in diameter. These $100-\mu m$ vessels are almost nonexistent on Day 7, but comprise 2.2% of the population on Day 15. Since the distribution for different postimplant times are similar, an overall distribution for this time period (7 to 15 days) is characterized in Chart 5. The proportion of tumor vascular structures greater than $5 \mu m$ in diameter decreases exponentially with vessel size.

**DISCUSSION**

Effluent Tumor Cells and Perturbations. The size distribution of clumps in the venous effluent of a transplanted T241 fibrosarcoma has been quantified in this study. Previous investigators have noted the presence of circulating tumor cell clumps (3, 7) but have not studied their size distribution. Our present data characterize the size distribution as a negative exponential with largest clumps occurring less frequently. The distribution changes little in the time period studied, although the total concentration of tumor cells rises rapidly. An estimate from our previous studies (6) indicates that up to $1.5 \times 10^6$ tumor cells may...
Table 2
Effect of trauma to primary tumor on number of pulmonary metastases

<table>
<thead>
<tr>
<th>Type of trauma</th>
<th>No.</th>
<th>Day 13</th>
<th>Day 19</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Massage</td>
<td>6</td>
<td>4.0 ± 3.08a</td>
<td>45.2 ± 18.52p</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>2.0 ± 1.41</td>
<td>17.0 ± 6.05</td>
<td>44.5 ± 21.76p</td>
</tr>
<tr>
<td>0.9% NaCl solution injection</td>
<td>6</td>
<td>22.5 ± 10.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a Mean ± S.D.

* p Significantly different from control; p < 0.05.

Trauma performed on Day 7 after implantation of primary tumor.

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Chart 3. Effect of tumor trauma on effluent clumps. Following mechanical trauma to the tumor the distribution favors the release of larger clumps (○ = x; n = 3), compared to nontraumatized effluent ( ). Notice less of an effect on single cells and clumps of 2 to 3 cells.

Table 3
Relationship of number and clump size of injected tumor cells with formation of metastases

<table>
<thead>
<tr>
<th>Total tumor cells injected (× 10⁹)</th>
<th>Av. cells/clump</th>
<th>Metastases 12 days after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.5 Single cells</td>
<td>0.0 ± 0.0*</td>
</tr>
<tr>
<td>8</td>
<td>1.0 Single cells</td>
<td>0.13 ± 0.36</td>
</tr>
<tr>
<td>8</td>
<td>1.0 6-7 cells</td>
<td>1.75 ± 1.22</td>
</tr>
<tr>
<td>8</td>
<td>0.5 10-12 cells</td>
<td>2.8 ± 1.78</td>
</tr>
<tr>
<td>8</td>
<td>1.0 10-12 cells</td>
<td>3.25 ± 2.18</td>
</tr>
</tbody>
</table>

* a Mean ± S.D.

enter the circulation over 24 hr. The studies of Butler and Gullino (1) estimated that 10.53 × 10⁶ tumor cells/day enter the venous effluent from a mammary adenocarcinoma. This difference between their findings and those reported previously by us may reflect variations among tumor-host systems as well as the age of the tumor. We have demonstrated (9) that the rate at which tumor cells enter the circulation is correlated in time with the development of pulmonary metastases and that this relationship can be described mathematically. Trauma to the primary tumor is known to produce an increase in metastases (10). The present studies demonstrate that trauma produces an increase in release of tumor cells characterized by a greater proportion of large clumps (Chart 3). These clumps may be "stripped" from within the lumens of large tumor venules by the trauma. Trauma to the tumor is associated with the release of a greater number of single tumor cells (8) as well as clumps.

In contrast to the effects of trauma, amputation ablates tumor cell entry into the circulation at a defined time. The number of macroscopic metastatic foci observed at times after amputation were initiated by tumor cells that entered the circulation over 24 hr. The studies of Butler and Gullino (1) estimated that 10.53 × 10⁶ tumor cells/day enter the venous effluent from a mammary adenocarcinoma. This difference between their findings and those reported previously by us may reflect variations among tumor-host systems as well as the age of the tumor. We have demonstrated (9) that the rate at which tumor cells enter the circulation is correlated in time with the development of pulmonary metastases and that this relationship can be described mathematically. Trauma to the primary tumor is known to produce an increase in metastases (10). The present studies demonstrate that trauma produces an increase in release of tumor cells characterized by a greater proportion of large clumps (Chart 3). These clumps may be "stripped" from within the lumens of large tumor venules by the trauma. Trauma to the tumor is associated with the release of a greater number of single tumor cells (8) as well as clumps.
Tumor Cell Clumps and Metastases

Static foci formed increases roughly in proportion to the clump size. One \( \times 10^3 \) cells in clumps containing 6 to 7 tumor cells produce 10 times the number of metastases as compared with a similar number of single cells. Doubling the clump size approximately doubles the number of metastases formed for a given number of cells.

In order to try to estimate the relative importance of clumps in the course of the metastatic process, we must combine the information gained from the metastatic potential of tumor cell clumps. When the number of metastatic foci seen after amputation are compared with those present in the unperturbed tumor-bearing mouse (nonamputated control), the rate of development of metastases appears similar differing only in the time (Chart 6) at which the metastases are observed. The metastatic foci seen after amputation reflect the events of tumor cell release antedating the amputation. Serial tumor amputation is also a means of verifying that tumor cells and clumps are entering the circulation continuously. If tumor cell discharge was a highly sporadic event, then metastases would not accumulate progressively and predictably as is observed in these studies (Table 1; Chart 6).

The Effectiveness of Tumor Cell Clumps Introduced by Injection in Formation of Metastases. Watanabe (11) was one of the first investigators to report that the number of metastases developing was related to circulating clumps rather than single malignant cells. However, Zeidman (13) and Koike (5) recognized the influence of size of the individual tumor cell on the formation of metastases. Recently, Fidler (2) showed that the i.v. injection of clusters of 4 to 5 melanoma cells produced a significantly greater number of metastases than did the same number of cells injected in single cell form. The data presented in this study demonstrate that a given number of tumor cells injected in clump form have a greater propensity for forming metastases than the same number of single tumor cells. Our findings on the relative importance of clumps, therefore, confirms the reports of others in different tumor-host systems (2, 4, 11).

From Table 3, it can be observed that the number of static foci formed increases roughly in proportion to the clump size. One \( \times 10^3 \) cells in clumps containing 6 to 7 tumor cells produce 10 times the number of metastases as compared with a similar number of single cells. Doubling the clump size approximately doubles the number of metastases formed for a given number of cells.

In order to try to estimate the relative importance of clumps in the course of the metastatic process, we must combine the information gained from the metastatic potential of tumor cell clumps.

...
tial of injected clumps with the knowledge of the clump size distribution entering the tumor venous drainage in the intact animal. This analysis is based upon the following assumptions: (a) the clump distribution data obtained in the perfusions are representative of the "natural" distribution occurring in vivo; and (b) metastatic foci are formed from those clumps in accordance with the results observed in our experimental study of injection of prepared clumps. On the basis of the injection studies, we assume a proportional relation between clump size and the number of metastatic foci formed. Consider a population of $1 \times 10^5$ tumor cells entering the circulation from the primary tumor in a clump size distribution similar to that in Chart 3. It can then be estimated that about 7 metastatic foci form from single cells. At least 7 foci originate from clumps composed of 2 to 7 cells, and at least 4 foci originate from clumps of 8 or more cells. Therefore, according to this prediction, a total of 11 metastatic foci would be established from tumor cells in clump form while 7 foci would result from single tumor cells. This analysis supports the contention that a majority of the metastatic foci seen in the hematogenous metastatic process in the present system are a result of tumor cells in clump form.

The finding of clumps in the tumor venous effluent raises the question of how and where they enter the circulation. Does release into the circulation occur only at a few isolated sites in tumor venules or are clumps being released generally throughout the tumor vascular plexus? If the latter condition is more correct, we would expect the size distribution of tumor vessels to be related to and possibly determine the size distribution of effluent tumor cell clumps. This hypothesis assumes that a clump of tumor cells could not pass from a vessel smaller than the dimensions of the clump. Since the minimum diameter of the tumor cells studied here is $15 \mu m$ (6), a vessel of this diameter is chosen as the smallest vessel that can serve as a circulatory entry route for tumor cells. The proportion of effluent tumor cell clumps equal to a given size category (multiples of $15 \mu m$) is plotted in Chart 7 against the proportion of tumor vessels that are larger than that size and can, therefore, serve as an entry site for those clumps. The linear relationship found between these variables lends support to the hypothesis that the tumor vessel size distribution is an important determinant of the size distribution of tumor cell clumps released into the circulation.

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