Fate of Melanoma Cells Entering the Microcirculation: Over 80% Survive and Extravasate

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Materials and Methods

Cell Culture and Fluorescent Labeling

B16F10 melanoma cells (5) and a clone, 6-5, of B16F10 cells transfected with a TIMP-1 expression vector were grown and subcultured as described (1, 6, 7). The level of TIMP-1 mRNA in clone 6–5 is 5–7-fold higher than in parental B16F10 cells (6) and was confirmed prior to injections. Cells were fluorescently labeled (green) with the use of Fluoresbrite carbohydrate microspheres of 0.05–0.07 μm in diameter (Polysciences, Inc., Warrington, PA) as described previously (2). Invariably, all cells in the suspension became labeled during this process. Mean relative plating efficiency (1) of labeled cells was 92 ± 9.6% (SE; n = 6) for B16F10 cells and 107 ± 20.7% (n = 5) for clone 6–5. Membrane integrity of labeled cells was assessed before injections by ethidium bromide (0.01 mg/ml) as described (1, 3). Fluorescence microscopy was used to determine the percentage of cells excluding (green fluorescence, intact cell membrane) or taking up (red fluorescence, loss of cell membrane integrity) ethidium bromide. Before the injections, 94% of parental and 96% of TIMP-1 overexpressor cells excluded ethidium bromide.

Intravital Videomicroscopy

In vivo videomicroscopy of the chick embryo CAM model was used to answer two questions: (a) What proportion of the total number of injected cancer cells that reach the CAM microcirculation remains viable within the tissue one day later? and (b) What proportion of the total number of injected cancer cells reaching the CAM microcirculation succeeds in extravasating? Detailed descriptions of our intravital videomicroscopy procedures have been published previously (4, 8). In brief, i.v.-injected cancer cells arrested in the CAM microcirculation are observed in vivo with the use of an inverted microscope with oblique fiber optic transillumination and episcopic illumination at the fluorescence excitation wavelength (450–490 nm). Real-time images of the CAM microcirculation in living embryos are viewed with the use of a video camera and monitor and are recorded on SVHS videotape. Quantification of cell numbers at various locations and times p.i. is performed during the experiment and, subsequently, by analysis of the videotapes.

Assessment of Absolute Numbers of Cancer Cells Surviving in Tissue

We have devised a new technique to accurately quantify the proportion of the injected cancer cells present at any given time. The technique is based on the standard method for measuring distribution of blood flow (9). Polystyrene microspheres 15 μm in diameter are included in the cell suspension before injection, enabling us to compare the numbers of cells per microsphere before injection with the corresponding ratios in the tissue after injection obtained by in vivo videomicroscopy. The microspheres remain trapped by size restriction within the microcirculation and, therefore, provide an absolute standard against which to compare the numbers of cancer cells present.
Fluorescently-labeled parental or TIMP-1 overexpressor cells (10^6 cells/embryo) and 15-μm polystyrene microspheres (~10^5/embryo; Polysciences, Inc., Warrington, PA) were injected together into a 0.1 ml OptiMEM (GIBCO-BRL, Burlington, Ontario) into a CAM vein of 11–13-day-old chick embryos. Mean cell diameters were 20 μm for parental and 19 μm for TIMP-1 overexpressor cells. All embryos were also injected (i.v.) with 0.1–0.2 ml of Tween 20-1% solution in citrate saline. The intact cancer cells:15-μm microspheres ratio in the CAM after injection, divided by the ratio in the syringe, was calculated for each injection (10 fields of view: X20 objective). For calculating the ratios in vivo, an aliquot of the cell and microsphere injection mixture was examined before each injection (10 fields of view: X20 objective). For calculating the ratios after injection, a minimum of 10 fields (X20 objective) were analyzed, and 15–20 polystyrene microspheres per embryo were counted for each observation or experiment.

Continuous Assessment of Cell Survival from Injection to Extravasation. Video images from the monitor were used to define the locations of cancer cells (intravascular, in process of extravasation, or extravasated; Refs. 1, 4, 8), as well as their survival in the CAM, as measured by ability to exclude ethidium bromide. Fluorescently labeled parental or TIMP-1 overexpressor B16F10 cells within 2 or 3 fields of view (X20 objective) were monitored individually and continuously over extended periods of time (0.5–8.0 h) to quantify the dynamics of extravasation and to identify any loss of cell membrane integrity. At the end of each experiment, many fields of view were scanned in search of signs of cell destruction. Photographs were taken during the experiments with a 35-mm camera attached to the microscope, and also directly from the monitor, during subsequent review of the videotapes. Data analysis was based on the number of experiments rather than the number of cells. For statistical analysis, Mann-Whitney U tests were used because the majority of the populations analyzed did not show a normal distribution.

Results

Absolute Numbers of Cancer Cells Surviving in Tissue. In the first series of experiments (n = 34), embryos were injected i.v. with fluorescently labeled parental or TIMP-1 overexpressor B16F10 cells plus 15-μm polystyrene microspheres. The cell:microparticle ratio present in the syringe before injection was approximately 10:1 (see Table 1). After injection the cell:microparticle ratios in the CAM were analyzed at 0–15 min or 24–31 h p.i. Cells (a total of 5989) were individually observed within the CAM at these times to assess cell membrane integrity and to establish cell:microparticle ratios. These ratios in vivo, either immediately after injection or 1 day later, did not differ significantly from the ratios in the syringe. Therefore, the CS indices did not differ significantly from unity (Table 1), indicating that any cell loss or destruction over this period was minimal.

Continuous Monitoring of Survival and Extravasation of Individual Cells. In the second series of experiments (n = 14), fluorescently labeled parental or TIMP-1 overexpressor cells were monitored continuously within the CAM for periods of 30 min to 8 h (mean, ~2 h) to quantify any cell loss or disruption of membrane integrity during the processes of arrest and extravasation. Table 2 shows that no loss of membrane integrity was observed in either cell line between 0 and 24 h p.i.; 100% of observed cells remained intact. Confirmation of the ability of ethidium bromide to identify damaged cells within the microcirculation was obtained by i.v. injection of a detergent, which caused trapped cancer cells to change from green to red fluorescence. For both cell lines, initial arrest occurred by size restriction within microvessels, and the extravasation process started after 2 h p.i. Some cells remained completely intravascular up to 6 h p.i. By 12–24 h p.i., 91 and 85% of the observed parental and TIMP-1 overexpressor cells, respectively, had finished the process of extravasation. After extravasating from capillaries, cancer cells from both lines migrated through the mesenchyme toward microvessels, and many of the cells presented extensions wrapped around the vessels, as we observed previously (1, 4, 8).

The fate of the injected cells during the first 24 h p.i. is presented schematically in Fig. 1. The graphs show the time course of extravasation from the capillaries to the underlying mesenchyme. The percentages of cells that were intravascular, in the process of extravasation, or extravasated are shown immediately after injection and at four subsequent time intervals: >0–2 h, 2–6 h, 6–12 h, and 12–24 h. The timing of extravasation of parental versus TIMP-1 overexpressor cells was not significantly different. The values for the percentage of cells observed at each location have been converted to take cell loss into account, yielding values representing the percentage of cells injected (100% = total number of injected cells that arrested in the CAM originally). This was done with the use of the cell survival indexes from Table 1 for the 24-h point, and assuming a linear loss from the time of injection on this basis, 81% and 82%, respectively, of injected parental or TIMP-1 overexpressor cells that originally reached the CAM had extravasated 1 day later. By this time, the percentage of injected cells that were lost was 11% for parental and 4% for TIMP-1 overexpressor B16F10 cells.

Discussion

Metastasis is an inefficient process, and it is widely accepted that very few of the cancer cells shed into the vascular system succeed in forming tumors. A series of steps in the metastatic process, including survival in the circulation, arrest in the microcirculation, extravasation, and growth in the new target organ, have been deduced from many experimental studies (10–12). However, the contribution to metastatic inefficiency of each of these steps remains an important unanswered question.

For this study, we devised a new experimental procedure to quantify and follow the survival or loss of injected cancer cells by intravitreal videomicroscopy, enabling us to determine the contributions to metastatic inefficiency from the early stages of the metastatic process. By injecting cells together with inert microspheres that remain arrested in the microcirculation, and knowing the injected cell:microparticle ratio,
we were able to monitor the fate of injected cells and detect any loss of cells in vivo. Any reduction in the cancer cell:microsphere ratio would imply cell loss. Our results suggested that a small amount of cell loss may have occurred by 24 h p.i., but due to variability between experiments, the mean CS index was not significantly different from unity for either cell line. As a complement to this technique, we made continuous and real-time observations in vivo of a series of 404 individual cells over extended periods of time, covering consecutively most of the period from injection to 24 h later. In addition, we analyzed a total of 2885 cells at the conclusions of these experiments.

For each cell observed, the color of the fluorescence (green or red) indicated whether membrane integrity was maintained. We found that virtually all of the observed cells remained intact during our direct observation.

The present investigation was designed to determine the survival and ability to extravasate (by 24–31 h) of injected cells (metastatic B16F10 versus poorly metastatic TIMP-1 overexpressor cells) in the chick embryo CAM. We found that the vast majority (>83%) of cells from both cell lines was intact 24–31 h after injection. The finding that cell destruction was minimal is consistent with our previously reported findings that no significant destruction of B16F10 melanoma cells or D2A1 mammary carcinoma cells was observed over the 2-h period after arrest in the microcirculation of mouse liver or muscle (3). These results are contrary to the prevailing idea that a key contributor to metastatic inefficiency is the early and rapid destruction of cancer cells in the circulation (13). Studies using radiolabeled cancer cells have, in some cases, reported significant cell loss within the first 24 h after injection (with variation between cell lines) based on values derived from radioactive counts remaining in target tissue (for examples, see Refs. 14, 15). The apparent discrepancies between these studies and ours may be due to a variety of factors, including immunogenicity or other attributes of the cancer cells, the immune status of the experimental host (immune-deficient chick embryos or immune-deficient mice), the target organ assessed (e.g., chick CAM versus mouse lung), and the use of radioactivity values to derive a calculated cell loss. In the study reported here, we were able to directly observe and quantify intact cells, the vast majority of which had extravasated by 24 h, and found only minimal cell loss over this time period.

We provided previously (1) a detailed analysis of the timing of extravasation of B16F10 parental and TIMP-1 overexpressor cells and showed that they extravasated with similar kinetics. The results presented here are consistent with those findings. Here we extended that study by showing that the reduced metastatic potential of TIMP-1 overexpressor cells is not due to selective loss of these cells but rather to their reduced ability to grow after extravasation.

Our results indicate that the majority of cancer cells may survive in the circulation and succeed in extravasating, if findings from the cells

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**Table 2 Lack of cell destruction**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time interval (h:min p.i.)</th>
<th>Continuous observation of arrested cells</th>
<th>Single observation of arrested cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of intact cells</td>
<td>%</td>
</tr>
<tr>
<td>Parental</td>
<td>0:00-1:30</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0:00-2:40</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3:49-5:40</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6:00-8:30</td>
<td>29</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6:30-7:00</td>
<td>26</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>7:30-8:30</td>
<td>39</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>18:00-19:30</td>
<td>38</td>
<td>100</td>
</tr>
<tr>
<td>TIMP</td>
<td>0:00-2:00</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3:30-5:45</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5:20-7:20</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6:00-14:00</td>
<td>32</td>
<td>100</td>
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<td></td>
<td>9:00-11:00</td>
<td>26</td>
<td>100</td>
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<td></td>
<td>15:00-17:45</td>
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<td>100</td>
</tr>
<tr>
<td></td>
<td>21:00-24:00</td>
<td>45</td>
<td>100</td>
</tr>
</tbody>
</table>

* a Cells from single fields of view monitored continuously throughout the stated time interval. Seven of the 404 cells showed "clasmatosis" (pinching off cytoplasmic fragments), the cells and fragments exhibiting intact membranes. Hundreds of small fragments were observed circulating freely throughout the experiments, but only 13 fragments showed lack of membrane integrity.

* b Cells observed from multiple fields of view at the end of the stated time interval.

* c Cell membrane integrity assessed in vivo using ethidium bromide. Intact, exclusion (green fluorescence); Non-intact, uptake (red fluorescence). A control experiment was performed in vivo to show trapped cells changing from green to red fluorescence after exposure to a detergent.
tested here can be extrapolated to other cells and tissues in both experimental and clinical situations. The work presented here suggests that a major contributor to metastatic inefficiency may be the ability of individual cells to survive and grow in the target tissue after extravasation because the majority of cells will not go on to form metastases. This view is consistent with our finding of individual cells that were intact but “dormant” in liver tissue 3 weeks after injection of mammary carcinoma cells into mouse liver (2). This suggests that tissue-specific growth regulators (16–19) and heterogeneity of cell responses to them may play a significant role in metastatic inefficiency. Studies on tumor metastasis in patients with peritoneovenous shunts have shown that, in some subjects, cancer cells extravasated into the interstitial tissue but did not grow, despite the large numbers of these cells released into the bloodstream (20). Clearly, metastasis is a complex process with specific tumor cells, experimental assays, and hosts influencing the results (10–12). Our work suggests that regulation of postextravasation growth may be a key stage at which to achieve therapeutic control of the metastatic process.

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References

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