Overexpression of Fibroblast Growth Factor 1 in MCF-7 Breast Cancer Cells Facilitates Tumor Cell Dissemination but Does Not Support the Development of Macrometastases in the Lungs or Lymph Nodes

Lurong Zhang, Samir Kharbanda, Sandra W. McLeskey, and Francis G. Kern

ABSTRACT

Mice bearing primary tumors produced by LacZ-tagged MCF-7 human breast carcinoma cells transfected with fibroblast growth factor (FGF) 1 have frequent micrometastases, but macrometastases are not observed. i.v. injection of FGF-1-transfected tumor cells produced no pulmonary micrometastases, and removal of primary tumors resulted in the disappearance of spontaneous micrometastases. Thus, failure of micrometastases to proliferate was not due to inhibitory factors released from the primary tumor, and the presence of the primary tumor is required for maintenance of the micrometastases. This indicates that the micrometastases result from continued seeding from the primary tumor balanced by clearance from the metastatic site. Tumor emboli trapped in the vessels of lungs and lymph nodes and single tumor cells observed in the pulmonary vein implied that FGF-1-overexpressing MCF-7 cells are deficient in their ability to extravasate. The frequency of tumor cells incorporating bro- mooxyuridine was consistently lower in lung tissues when compared with primary tumors, indicating that disseminated tumor cells were unable to maintain a high rate of proliferation. Increased angiogenesis resulting from FGF-1 production by the transfected cells with a concomitant increased rate of intravasation into developing blood vessels may be the underlying determinant of spontaneous micrometastases produced by these cells when compared with parental MCF-7 cells.

INTRODUCTION

Metastasis is the product of complicated interactions between tumor cells and stroma. Tumor cells must detach from the primary tumor, enter the circulation, extravasate at distant sites, and proliferate in the parenchyma of distant organs (1–3). Although a number of factors have been shown to be involved in this process, including proteases (4, 5), adhesion molecules (4, 6, 7), motility factors (3), and angiogenic factors (1, 8), the precise mechanism of the metastatic process remains unclear. However, a sufficient blood supply via tumor microvessels is critical for both primary tumor growth and secondary metastasis (1, 8, 9). Therefore, increased angiogenesis may increase opportunities for intravasation of tumor cells into the circulation (10, 11).

A recent report (12) suggests that intravasation mediated by activation of a plasmin-metalloproteinase cascade may be a rate-limiting step in the metastatic process. However, circulating tumor cells can commonly be found in patients with many types of solid tumors [Refs. 10 and 13–21 (reviewed in Ref. 13)]. Thus, the importance of intravasation as a rate-limiting step of metastasis is currently under debate. Likewise, recent studies seem to indicate that extravasation is a relatively common event and therefore perhaps not rate-limiting (5, 22). However, others have proposed that all sequential steps of metastasis are rate-limiting (1) and selective (23). Although the reasons for these controversies may have to do with the validity of the particular models of metastasis used, it is also possible that for different tumors, different steps of the metastatic process are either more or less important as rate-limiting steps.

In previous studies, we have demonstrated that transfection of FGFRs can confer an antiproliferative and hormone-independent in vivo growth phenotype to MCF-7 breast carcinoma cells. In addition, FGF-1 or FGF-4 overexpression confers a metastatic phenotype on these nonmetastatic and noninvasive cells (24–26). The results of the current study indicate that overexpression of FGF-1 by MCF-7 cells can induce transient microdeposits of tumor cells in vessels of distant organs that do not seem to extravasate efficiently into the parenchyma and may fail to proliferate at the distant site. We submit that the increased dissemination of tumor cells is a result of enhanced intravasation via the large number of FGF-1-stimulated blood vessels within the primary tumors. Thus, this xenograft tumor model provides evidence that the processes of intravasation and extravasation can be distinct, requiring different attributes, and, together with control parental MCF-7 cells, provides the opportunity to study these processes separately.

MATERIALS AND METHODS

Cell Lines. FGF-1 clone 18 is a clonal cell line of LacZ-transfected MCF-7 breast carcinoma cells retransfected with the cDNA for human FGF-1 (26). To isolate the clone 18-lung cell line, 10 million clone 18 tumor cells were injected into the mammary fat pads of ovariectomized nude mice treated with 17β-estradiol (0.72 mg/pellet; 60-day release). After 40 days, 100 mg of lung tissue of each mouse was sterilily harvested, minced, and digested in 1 ml of a mixture of 800 µg/ml collagenase (C-9891; Sigma, St. Louis, MO), 100 mg/ml bovine pancreatic protease (P-4630; Sigma), and 0.5 mg/ml bovine pancreatic DNase (DN-25; Sigma) in IMEM (Biofluids Inc., Rockville, MD) at 37°C for 1 h. After washing with IMEM, the disassociated cells were cultured in 10% fetal bovine serum-IMEM containing 1000 units/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml gentamycin. The approximate number of foci of blue-stained metastatic foci was scored microscopically (27). The following scoring system was used to score the degree of metastasis, based on the estimated counts: (a) –, no blue spots; (b) +, about 1–50 blue spots; (d) ++, about 100–200 blue spots; and (e) ++++, >200 blue spots.
Experimental Lung Metastasis Assay. One million clone 18 or clone 18-lung tumor cells were injected into the tail veins of ovariec-tomized nude mice supplemented with 17β-estradiol (0.72 mg s.c. pellet; 60-day release). Representative mice were sacrificed at 2, 24, 48, and 72 h and 1 week and 2 weeks after injection. Lung metastases were evaluated as described above.

Analysis of Extent of Metastases after Primary Tumor Removal. Five million tumor clone 18 or clone 18 lung cells were injected each of two mammary fat pads located on opposite sides of 4–6-week-old ovariec-tomized nude mice (athyemic NCR nude; National Cancer Institute, Frederick, MD) supplemented with 17β-estradiol (0.72 mg s.c. pellet; 60-day release) as described previously (26). Tumors sizes were measured twice weekly with calipers. On days 19 and 25 after inoculation, one mouse from each cell line was checked for the extent of lung metastases as described above. On day 26 after inoculation, tumor-bearing mice were randomly divided into three groups (5–10 mice/group, depending on the number of tumor-bearing mice). The mice in one of the groups were sacrificed to determine the incidence (the number of mice with metastases/total mice examined) and the extent of the metastases, using the scoring system above, in both the lungs and lymph nodes. The mice in another group (the remain group) were sacrificed 32 days after tumor cell injection, when the tumors reached a size that necessitated sacrifice of the mouse. Mice in the third group (the removal group) had the tumors resected. At 14 and 44 days after tumor resection, one mouse from each removal group was sacrificed to assess metastases. Fifty days after tumor removal, all of the remaining mice were sacrificed, and lung and lymph node metastases were assessed. Two mice in the removal groups, one for each cell line, had tumors reappear at the site of the tumor excision. They were sacrificed at the same time as the rest of the mice (50 days after excision) and comprise the regrowth group.

PECAM-1 and BrdUrd Immunohistochemistry. PECAM-1 staining of tumor-associated mouse endothelial cells was accomplished with a rat mono-clonal antibody against murine PECAM-1 (Mec 13.3; 01951D; PharMingen, San Diego, CA), as described previously (27). Primary tumors and lung tissues from mice receiving i.p. injections of 1 mg of BrdUrd (B5002; Sigma) in PBS 8 h before sacrifice were processed for immunohistochemical assessment of tumor cell proliferation as described previously (27). Briefly, tumors and tissues were fixed in 10% buffered formalin, and 5-μm-thick paraffin sections of lungs and primary tumors were deparaffinized and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 30 min. The slides were then incubated in a blocking solution of 5% BSA + 2% normal rabbit serum in PBS for 1 h. Rat anti-BrdUrd primary antibody (MAS 250; Accurate Antibodies, Westbury, NY) was added at a 1:320 dilution in blocking solution, and the slides were incubated for 1 h at room temperature. Biotinylated rabbit antirat IgG (5 μg/ml) was applied for 1 h, and, after washing with PBS, a streptavidin-conjugated peroxidase solution (BA 4000; Vector Laboratories, Burlingame, CA) was applied for 1 h at room temperature. The BrdUrd incorporation in tissues was then visualized by incubation with 0.05% diaminobenzidine and 0.01% H2O2 for 15 min. The slides were counterstained with hematoxylin, dehydrated, coverslipped, and examined using a Olympus AH-2 microscope. For primary tumors, the percentage of BrdUrd-positive cells was determined by counting 100 cells in three to five random fields (×200) from each of five different tumors. For sections from lung tissues, the number of BrdUrd-positive cells/number of total X-gal-stained cells was determined for six different tumor-bearing animals by counting multiple fields and sections. Over 1600 X-gal-stained cells were counted.

RESULTS

FGF-1-overexpressing MCF-7 Cells Fail to Develop Experimental Lung Metastasis. Although the MCF-7 cell line was established from a pleural effusion of a 69-year patient with metastatic carcinoma (28), the cells are poorly metastatic and poorly invasive (12, 25, 26). We have previously reported that transfection of LacZ-tagged MCF-7 breast carcinoma cells with FGF-1, FGF-4, or VEGF165 produced cell lines with increased spontaneous metastatic potential in comparison with the parental MCF-7 cells (24–26, 29). A clonal MCF-7 derivative that stably expresses a transfected bacterial LacZ gene was used as the recipient in these studies, allowing a sensitive X-gal staining assay to be used to semiquantitatively detect the presence of micrometastases in lungs, lymph nodes, and other organs (25). Overexpression of either VEGF, FGF-4, or FGF-1 allows disseminated tumor cells to be detected in large numbers and with essentially 100% frequency in either the lungs or lymph nodes of tumor-bearing mice. In contrast, the frequency with which disseminated tumor cells can be detected in estrogen-supplemented mice bearing parental cell tumors is essentially zero, even when these tumors are the same size as the tumors composed of either FGF- or VEGF-overexpressing cells.

Despite the large number of tumor cells present within the lungs of either FGF or VEGF tumor-bearing mice, macrometastatic tumor nodules were never observed. This raised the possibility that the injection of X-gal-stained cells in distant organs of tumor-bearing mice was due to the presence of the cells that had been introduced into the circulation at the time of tumor cell injection and, as a result of angiogenic growth factor overexpression, were able to persist in these organs until the time of animal sacrifice.

To address this possibility, one million clone 18 cells, which overexpress FGF-1, were injected into the tail veins of nude mice, and metastases were examined in the lungs after harvesting at 2, 24, 48, and 72 h and 1 week and 2 weeks after the tail vein injection. Two hours after tumor cell injection, there were abundant blue-stained tumor cells in the lung. However, the number of these cells decreased rapidly with increasing time, and at 72 h or later, no blue-stained tumor cells were visible (Fig. 1 and Table 1).

Only a small proportion of circulating tumor cells may have the ability to grow in metastatic sites (2). Therefore, the above-mentioned results might be explained by a lack of lung-colonizing ability of the large majority of the cells we injected into the tail vein. To rule out this explanation, we established the clone 18 lung cell line that was derived by retrieving clone 18 tumor cells from the spontaneous metastases present in the lungs of mice bearing primary clone 18 tumors that grew after mammary fat pad injection. This cell line could form well-vascularized tumors in nude mice with or without estrogen supplementation, indicating that those cells that were present in the lungs continued to produce and respond to FGF. These cells were also injected into the tail veins of recipient mice as described above. The results were similar to those obtained with unselected clone 18 cells (Fig. 1 and Table 1). Thus, the tail vein injections seeded the circulation with many more tumor cells than would be expected from the random accidental intravasation of cells at the time of mammary fat pad inoculation. Nonetheless, neither unselected cells nor cells selected from a metastatic site produced micrometastases. Therefore, we conclude that spontaneous lung micrometastases are not the result of a one-time seeding of cells into the circulation that might result from tissue trauma or vessel injury at the time of mammary fat pad injection.

Removal of Primary Tumors Results in the Disappearance of Lung Metastases. Long-lived secreted angiogenesis inhibitors that are produced by the primary tumor have been described which inhibit the growth of disseminated tumor cells; the best characterized of these are angiotatin and endostatin (30, 31). To determine whether the FGF-1-transfected MCF-7 primary tumors were producing a similar substance that could overcome the growth-stimulatory effect that would be expected from the production of FGF-1, an experiment similar to the one used by others to demonstrate the dependence of the metastasis-inhibiting activity of angiotatin on the continued presence of the primary tumor was performed. Mice were injected with either FGF-1 clone 18 or clone 18 lung cells. Tumors were allowed to develop to an approximate size of 1 cm3. All of the animals from a group that were sacrificed at this point had numerous micrometastatic deposits of blue-stained cells in either the lungs or lymph nodes (Fig. 2).

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the resulting increased density of intratumoral blood vessels provide
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duced by the parental
-LacZ
-transfected MCF-7 cells (24–26, 29), micrometastases
produced by the FGF-1 transfectants could be due to increased in-
travasation of the tumor cells into the more abundant blood vessels
stimulated by FGF in the primary tumors (26). Immunohistochemistry
using an antibody specific for murine PECAM-1, a marker for mouse
endothelial cells, shows increased blood vessel density in the tumors
produced by FGF-1-transfected cells compared with those produced
by the parental
-LacZ
-expressing MCF-7 cell line ML-20 (Fig. 3). We
hypothesize that the production of an angiogenic growth factor and
the resulting increased density of intratumoral blood vessels provide
avenues for the intravasation of tumor cells and thereby facilitate
micrometastasis formation.

Tumor Cells in Micrometastases Fail to Extravasate. Because
we never observed pulmonary macrometastases in mice bearing tu-
mors produced by FGF-transfected cells, and because it seems that
abundant tumor cells gained entry to the circulation, we examined
lung and lymph node specimens closely to see whether tumor cells
were contained within vessels or had invaded the parenchyma of the
lung or lymph node. Careful examination revealed that whereas a
small number of tumor cells appeared in the parenchyma of the lungs
and lymph nodes, many multiple-cell tubular aggregates of tumor
cells could be seen within the microvasculature of the lungs (Fig. 4A
or in the lymphatic vessels (Fig. 4B). These results imply that the large
majority of tumor cells contained in “micrometastases” are actually
confin ed to the vessels of distant organs. Furthermore, individual
X-gal-positive cells were visible in large pulmonary veins (Fig. 4C),
implying that they were in the process of being cleared from the lung.

Table 1 i.v. injection of FGF-1-transfected MCF-7 breast cancer cells does not
produce permanent lung metastasis

<table>
<thead>
<tr>
<th>Time after</th>
<th>Injection</th>
<th>Cell Line</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
<td>Clone 18</td>
<td>5/5 (+++++)</td>
<td>5/5 (+++++)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>5/5 (+++++)</td>
<td>5/5 (+++++)</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>Clone 18</td>
<td>0/4</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>3/5 (1+; 1++; 1++)</td>
<td>3/5 (1+; 1++; 1++)</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>Clone 18</td>
<td>1/5 (+)</td>
<td>1/5 (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>4/5 (2++; 2++)</td>
<td>4/5 (2++; 2++)</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>Clone 18</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>Clone 18</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>Clone 18</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

a Clone 18 cells are FGF-1-transfected MCF-7 cells (26).
b Number of mice with blue cells in the lung/number injected.
c Extent of metastases, according to scoring system (above).
d Clone 18 lung cells are clone 18 cells harvested from the lungs of a tumor-bearing
mouse (see “Materials and Methods”).
We also sought to determine whether cells comprising the micrometastases had a lower proportion of proliferating cells when compared with cells within the primary tumor by determining the percentage of tumor cells that had incorporated BrdUrd over an 8-h period before animal sacrifice. We consistently observed that primary tumors were composed of 25–35% BrdUrd-positive cells, whereas the frequency of BrdUrd-positive tumor cells present in lung tissues was consistently between 5% and 15% (Fig. 4, D and E). These results suggest that most of the cells comprising the micrometastases formed by the FGF-1 transfectants lack the ability to extravasate or proliferate. In addition, they imply that the deposits of cells within the vasculature of the lung are dynamic collections of cells that result from a balance between continual seeding from the primary tumor and continual clearance.

Fig. 2. Disappearance of spontaneous metastases after the removal of primary tumors. Mice were injected in the mammary fat pads at two sites with 5 million clone 18 or clone 18 lung cells. On day 28, mice were randomized into control and removal groups. Control group mice were sacrificed and evaluated for metastases. Removal group mice were subjected to surgical excision of the tumors. A, spontaneous lung metastases in a control mouse 26 days after tumor cell injection. B, lungs of a mouse sacrificed 14 days after removal of the primary tumors. C, lungs of one of the two mice originally in the removal group whose tumors were incompletely excised and regrew (the regrowth group) 50 days after excision of the original tumors. D, lymph node metastases in a control mouse 26 days after tumor cell injection. E, lymph nodes of a mouse sacrificed 14 days after the removal of primary tumors.

We also sought to determine whether cells comprising the micrometastases had a lower proportion of proliferating cells when compared with cells within the primary tumor by determining the percentage of tumor cells that had incorporated BrdUrd over an 8-h period before animal sacrifice. We consistently observed that primary tumors were composed of 25–35% BrdUrd-positive cells, whereas the frequency of BrdUrd-positive tumor cells present in lung tissues was consistently between 5% and 15% (Fig. 4, D and E). These results suggest that most of the cells comprising the micrometastases formed by the FGF-1 transfectants lack the ability to extravasate or proliferate. In addition, they imply that the deposits of cells within the vasculature of the lung are dynamic collections of cells that result from a balance between continual seeding from the primary tumor and continual clearance.

Table 2. Spontaneous metastasis produced by FGF-1-transfected MCF-7 breast cancer cells are cleared after tumor removal

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell line</th>
<th>Lung metastases</th>
<th>Lymphatic metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incidence</td>
<td>Extent[^a]</td>
</tr>
<tr>
<td>Control</td>
<td>Clone 18</td>
<td>7/7[^c]</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>9/9</td>
<td>++++</td>
</tr>
<tr>
<td>Remain</td>
<td>Clone 18</td>
<td>5/5</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>3/3</td>
<td>++++</td>
</tr>
<tr>
<td>Removal</td>
<td>Clone 18</td>
<td>0/5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>0/3</td>
<td>–</td>
</tr>
<tr>
<td>Regrowth</td>
<td>Clone 18</td>
<td>1/1</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Clone 18 lung</td>
<td>1/1</td>
<td>+</td>
</tr>
</tbody>
</table>

\[^a\] Extent of metastases as described in the text and in Table 1.
\[^b\] Number of mice with metastases/number of mice injected.
\[^c\] Number of lymph nodes examined with a particular metastasis score.
DISCUSSION

Although our previous studies have shown that overexpression of angiogenic factors could increase the incidence of micrometastases of MCF-7 breast carcinoma cells (24–26, 29), macrometastases were never found, and none of the tumor-bearing mice would eventually die of lung metastasis. In the present study, we demonstrate that micrometastases do not originate from the seeding of tumor cells into the circulation as a result of the trauma of inoculation but probably result from an increased incidence of intravasation, possibly as a result of increased tumor-associated vessels. Moreover, it would seem that most of the disseminated cells are unable to extravasate and remain trapped in the pulmonary and lymphatic vessels. There seems to be a dynamic equilibrium between cells arriving from the primary tumor and those leaving or being cleared from the metastatic site. Because the ability of tumor cells to form macrometastases depends on their completion of the steps of intravasation into the local circulation, dissemination throughout the circulatory system, arrest in the microvasculature of metastatic sites, extravasation, and growth in the parenchyma of the metastatic site (1, 2), FGF-1-transfected MCF-7 cells may be unable complete the final two steps in the process of metastasis.

Using tumor cells placed atop a wounded CAM, where they have ready access to a highly vascularized capillary bed, others have shown that the breaching of the vascular wall is a rate-limiting step in intravasation and may be a rate-limiting step for metastasis (12). In our model system, the tumor cells present within highly angiogenic tumors may be able to intravasate and be disseminated to distant sites without their having acquired a mechanism allowing them to breach an intact vessel wall. The high rate of ongoing neoangiogenesis within the FGF-1-overexpressing breast tumors may increase the probability of tumor cells having direct access to immature and incompletely formed vessels. However, if this same vessel-breaching mechanism is also required for extravasation, the metastatic process would be aborted at this stage.

Although we postulate that the discovery of increased spontaneous micrometastases in mice bearing tumors produced by the transfected cell lines is the result of an increased rate of intravasation due to increased angiogenesis in the primary tumor, other factors could be responsible for this phenomenon. Intravascular arrest of circulating tumor cells could be increased with the FGF-1 transfectants in comparison to that of wild-type MCF-7 cells. However, our preliminary analysis of expression of integrins and attachment factors has failed to detect evidence of changed expression in the FGF-1 transfectants compared with the parental MCF-7 cells (data not shown), arguing against increased vascular arrest as an explanation for the increased rate of micrometastases in our transfectants.

Proteases are thought to be essential for tumor cells to degrade the...
basement membrane (4). Therefore, the failure of the tumor cells to extravasate from the circulation could be due to insufficient protease expression or expression of an ineffective or inactive protease. Others have found that transfection of FGF-2 into renal carcinoma cells increased the metastatic potential of these cells and also increased the expression of MMP-2 (32). We have assessed protease activity using gelatin zymography, and we see no distinct differences in MMP-2 or MMP-9 activity in FGF transfectants compared with the nonmetastatic parental MCF-7 cells (data not shown). However, many other proteases, including receptor-localized plasminogen activators, could be important and may be needed in concert with MMP expression for an invasive phenotype (12, 33, 34). FGF-2 induces the expression of uPA in endothelial cells and in L6 myoblast cells or Chinese hamster ovary cells transfected with FGF receptors (35–37). Our preliminary studies suggest that FGF-1 or FGF-4 overexpression also induces the expression of uPA mRNA in MCF-7 cells (38). However, overexpression of either FGF has no effect on uPA receptor mRNA. Thus a FGF-induced increase in protease expression may not result in increased invasiveness if the protease is not subsequently localized properly via an interaction with membrane-bound receptors on the tumor cell, because this process appears to be necessary for activation of a protease cascade (34). Moreover, increased expression of protease inhibitors might also play a role in the failure to extravasate. Studies examining these issues are ongoing in our laboratories.

The view of extravasation as being a rate-limiting step for metastasis has recently been challenged. Nonmetastatic and metastatic cells do equally well in extravasation assays in chick CAM or mouse liver, regardless of their expression levels of adhesion molecules, proteases, or oncoproteins (5, 22, 39–43). Our result showing that FGF-1-transfected cells disappeared from the lung by 24 h after i.v. injection is in apparent conflict with reports that the majority of i.v. injected cells have extravasated by 24–72 h (22, 39–41, 43). Explanations may include differences between the metastatic sites examined. CAM is an avian embryonic tissue that might not present such a formidable barrier to extravasation or might express different adhesion molecules when compared with the vessels of the lung or lymph node in an adult mouse. Liver vessels might also express different adhesion molecules. Moreover, liver contains fenestrated endothelium in the sinusoids, which could possibly facilitate extravasation. Extravasated cells have been observed as single cells in the parenchyma of the liver up to 3 weeks after extravasation (39). Even if it is assumed that the large number of transfected cells that we injected i.v. do extravasate, their disappearance from lung within 24 h is again at odds with these reports. The presence of many tumor cells in large pulmonary veins supports our belief that the majority of our transfected cells do fail to extravasate in the lung and are flushed away by hemodynamic forces. These results are also in conflict with those of others, because they find no cell loss associated with i.v. injected cells (22, 40). Of course, we cannot exclude the possibility that some of our cells can extravasate, and certainly postextravasation events are important determinants in the formation of macrometastasis (22).

The failure of our micrometastatic tumor cells to develop into macrometastases could also be due to a failure to grow or an accelerated degree of apoptosis. It has been reported that intravascular proliferation is rare (22). Our efforts to compare labeling indices for BrdUrd incorporation between micrometastases and primary tumors were hampered by the small number of micrometastatic cells and the small number of animals that have been examined thus far. Whereas repeated injections of BrdUrd over time might be required to determine the actual proliferative capability of the disseminated tumor cells, our results at this point suggest that BrdUrd incorporation indices may be lower in micrometastases than in primary tumors. Because our overall results suggest that the micrometastases are transient in nature, much of this incorporation may have occurred while the tumor cells were physically present within the primary tumor. When they are expanded and reinoculated into ovariectomized nude mice, breast tumor cell lines reestablished from the cells present in either the lungs or lymph nodes continue to exhibit in vivo growth phenotypes that we have previously shown to be dependent on the autocrine and paracrine effects of FGF-1 overexpression (44). Thus it would appear that the cells present within these tissues continue to be able to produce and respond to FGF-1. This suggests that whereas the autocrine and paracrine effects of FGF-1 overexpression, in combination with the microenvironment present within the mammary fat pad, provide a strong mitogenic stimulus to tumor cells, the microenvironment within the lungs or lymph nodes does not permit similar effects to be manifested in disseminated tumor cells. Because FGF-1-overexpressing cells demonstrate an enhanced anchorage-independ-ent growth phenotype in vitro (26, 44), the failure of the cells to proliferate once they are disseminated to lungs and lymph nodes is somewhat unexpected. Moreover, the failure to proliferate raises the question of whether these cells actually have enhanced metastatic capabilities beyond their increased ability to gain entry into the circulation. Others have reported that MCF-7 cells can form bone metastases 6–8 weeks after intracardiac injection into the left ventricle (45), a delivery route that bypasses the pulmonary circulation and the subsequent entrapment of tumor cells within lung capillaries. It remains to be determined whether FGF-overexpressing MCF-7 cells have a growth advantage at other sites after intracardiac or i.p. injection.

We also attempted to use a TUNEL assay to determine whether the frequency of apoptotic cells was greater in micrometastases than in tumors. Our preliminary studies did indicate that TUNEL labeling indices were higher in micrometastases than in primary tumors (data not shown). However, this analysis was also hampered by the comparatively small number of micrometastatic cells and the difficulty in identifying labeled cells as tumor cells, especially in lymph nodes, which would be expected to contain apoptotic immune cells. In addition, it was recently discovered that MCF-7 cells do not produce a functional caspase 3, the enzyme largely responsible for the DNA laddering and associated DNA strand breaks detected with the TUNEL assay (46). This recent finding makes it likely that the TUNEL assay may not be an appropriate measure of the extent of ongoing apoptosis in either MCF-7 xenograft tumors or disseminated tumor cells. The current lack of another suitable in situ assay for apoptosis may make it difficult to address this question.

Overall, the results of this study indicate that tumor angiogenesis facilitates intravasation of tumor cells into the circulation from primary tumors. However, we show that this is not sufficient for the formation of macrometastases. Thus, our data imply that the factors that confer upon tumor cells the ability to extravasate and to grow in distant sites may be key determinants in the formation of clinically significant macrometastases. Future studies aimed at discovering determinants that can further enhance the metastatic ability of FGF-1-transfected MCF-7 cells could include investigations into the role of various attachment factors, motility factors, modulators of apoptosis, or activators of proteolytic cascades involved in tumor cell migration through the endothelial cell layer and in basement membrane inva-sion. The increased capacity for autonomous hormone-independent growth and tumor cell dissemination that occurs as a result of overexpression of this angiogenic growth factor should make this a useful model for such studies.

4 S. McLeskey, unpublished data.
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REFERENCES


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