Abstract

In this study, we report that needles containing chemoattractants can be used to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor in a live rat as a pure population suitable for further analysis. The most efficient cell collection requires the presence of chemoattractant cytokines, such as epidermal growth factor and serum components, and occurs with 15-fold higher efficiency in metastatic tumors compared with nonmetastatic tumors. Although tumor cells of the nonmetastatic tumors show a motility response to serum, they were not collected with high efficiency into needles in vivo in response to serum, indicating that additional factors besides motility are required to explain differences in cell collection efficiencies between metastatic and nonmetastatic tumors. The results reported here indicate that needles filled with growth factors and matrigel, when inserted into the primary tumor, can faithfully mimic the environment that supports invasion and intravasation in vivo. Furthermore, the results indicate that the same cell behaviors that contribute to chemotaxis in vitro also contribute to invasion in vivo.

Introduction

Metastasis involves the escape of cells from the primary tumor either via lymphatics or blood vessels, transport to and arrest in a target organ, and growth of metastasis in the target organ (1). Each of these steps is a multicomponent process, with potentially different tumor cell properties and molecules playing critical roles at different steps (2). Recently, emphasis has been on the development of molecular arrays to identify new genes and proteins that contribute to specific steps in metastasis. Such approaches are crucial in the analysis of cancer as a genetic disease and in the identification of key genes that might be used in diagnosis and therapy. However, array-based approaches treat the tumor as a black box. Ideally, high-resolution methods for the analysis of metastasis at the cellular level, such as imaging of cells within tumors, when combined with array-based approaches, could be used to accurately evaluate the roles of specific gene products in the individual steps of metastasis at the cellular level. The use of Laser Capture Microdissection as a front end for array-based gene discovery is such a fusion approach (3). However, some of the cell behaviors that are believed to be essential for metastasis, such as adhesion and motility (4, 5), cannot be used as criteria in the selection of cells for analysis from fixed material because the behavior and history of individual cells cannot be inferred from fixed material. Methods for the collection of cells from living tumors in which key cell behaviors can be observed and used as the criteria for cell collection need to be developed. One such cell behavior is the chemotaxis of tumor cells. Metastatic tumor cells are believed to chemotax to cytokines that are normally found in association with blood vessels (6–8). We developed a cell graft breast tumor metastasis model in rats that is syngeneic and orthotopic that permits the imaging and tracking of cell behavior in live tumors (8, 9). Using this model, we have observed, in metastatic primary tumors, the highly persistent linear locomotion of a subpopulation of tumor cells toward blood vessels in vivo using intravital imaging. This locomotion resembles the chemotaxis of cells observed in culture (9, 10) and is correlated with metastatic potential (10, 11). Tumor cell chemotaxis is also correlated with the accumulation of metastatic tumor cells around, and their polarization toward, blood vessels in the primary tumor. Furthermore, chemotaxis is correlated with the efficient intravasation into, and survival of tumor cells in, the systemic circulation (8). Because these properties are not observed in nonmetastatic tumors prepared from cells in the same way (7, 8), polarization and chemotaxis toward blood vessels are believed to be important in intravasation and metastasis (8). In this study, we report that chemotaxis can be used to advantage to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor in vivo as a pure population suitable for further analysis.

Materials and Methods

Serum Upshift of Cells in Vitro. MTLn3-GFP and MTC-GFP cells were plated in 35-mm dishes at a density of 50,000 cells per dish 18 h before the experiment. On the day of the experiment, cells were starved for 3 h in 2 ml of MEM containing HEPES and 0.69% BSA, which is the isotonic equivalent of 10% FBS. The upshift was performed as described before (12), with the exception that the cells were stimulated with 10% FBS. Briefly, the dishes were covered with a thin layer of heavy mineral oil (Sigma #400-5) and placed in an enclosed microscope preheated to 37°C. Using a CCD camera, single-frame images were collected using NIH Image every minute. After 4 min, 2 ml of MEM with HEPES and 20% FBS were added to the dish, and image frames were collected for an additional 16 min.

In Vitro Cell Collection. MTLn3 cells were plated in a 35-mm dish 18 h before the experiment to be 60–80% confluent at the time of the experiment. On the day of the experiment, cells were starved using MEM-BSA, the isotonic equivalent of 5% FBS, for 2 h. During this time, 26-gauge syringe needles were prepared by filling them with 10 μl of Matrigel mixed 1:1 with MEM-BSA or MEM-BSA containing EGF for a final concentration of 0.5 nm, 2.5 nm, 5 nm, 25 nm, 50 nm, or 250 nm EGF. After starvation, the needles were attached to the side of the plate using paraffin to hold them in place with the bevel of the needle facing the bottom of the plate so that the matrigel was in contact with the surface of the plate. Dishes were placed into a 37°C/5.0% CO2 incubator for up to 6 h. After this time, the contents of each needle was extruded into a new 35-mm dish containing MEM with 5% FBS (growth medium). Cells that had entered the needle were allowed to grow into clones for 6 days to determine cell count and viability. Positive clones, checked by GFP fluorescence and cell morphology, were then counted.

To image the cells moving toward the needle, a dish was plated for 40–50% confluency before the experiment. Cells were starved, and a needle was prepared as above containing matrigel mixed 1:1 with MEM-BSA containing 25 nm EGF. Images as single frames were taken using the heated microscope and NIH Image every 30 min, as described above. The dish was kept in a 37°C/5% CO2 incubator between images.

In Vivo Cell Collection. MTLn3-GFP and MTC-GFP cells were injected into female Fischer 344 rats, as described before (8, 9), and tumors were

Jeffrey B. Wyckoff, Jeffrey E. Segall, and John S. Condeelis

Departments of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Departments of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300, Morris Park Avenue, Bronx, NY 10461. Phone: (718) 430-4068; Fax: (718) 430-8996; E-mail: condeelis@ aecom.yu.edu.

2 The abbreviations used are: FBS, fetal bovine serum; MEM-BSA, MEM with 0.35% BSA; EGF, epidermal growth factor; i.d., inside diameter; GFP, green fluorescent protein.
allowed to grow for 2.5 weeks. On the day of the experiment, 33-gauge needles were prepared as above by filling them with matrigel and MEM-BSA, MEM-BSA with a final EGF concentration of 25 nM, or MEM-BSA with a final FBS concentration of 10%. All needles included 0.01 mM EDTA (pH 7.4) to sequester heavy metals that might be released by the needle. A rat was anesthetized using 5% isoflurane and laid on its back. The isoflurane was reduced to 2%, and a small patch of skin over the tumor was removed. Three 25-gauge needles (guide needles) with blocking wires were inserted to a depth of 2 mm. The blocking wire was removed, and one of the matrigel-filled needles was inserted into each guide needle (as shown in Fig. 3). The needle was then left in the tumor for 6 h. The isoflurane concentration was slowly lowered to 0.5% during the course of the experiment to keep the rat’s breathing even and unlabored. After 6 h, the needles were withdrawn, extruded into 35-mm dishes containing growth medium, and all cells were counted immediately. The percentage of cells with GFP fluorescence was determined.

As a control for the effects of matrigel, a 33-gauge needle was filled as above with MEM-BSA and agarose, for a final concentration of 1%, and the in vivo experiment was performed as above.

Results

In Vitro Cell Collection. As has been shown previously, MTLn3 cells are chemotactic to EGF with an optimum concentration at 5 nM EGF (12). Also, it has been shown that MTLn3 cells, when placed in a gradient generated using a pipette filled with 50 μM EGF, will orient toward and locomote in the direction of the pipette exhibiting true amoeboid chemotaxis (10). MTLn3 cells are metastatic when reinserted into the mammary fat pad of a Fischer 344 rat. We prepared an artificial microenvironment using microneedles filled with matrigel and either EGF or serum as the chemoattractant to simulate invasion and intravasation into a container that could be withdrawn to collect the chemotactic/invasive subpopulation of cells.

To establish the concentration necessary to attract MTLn3 cells into the needle, needles were filled with a range of EGF concentrations from 0.5–250 nM and inserted into a cell culture. At times up to 6 h of collection, the needles were withdrawn from the culture and the contents were extruded into a new dish with growth medium, and the cells were allowed to grow for 6–7 days to determine cell counts and test viability. The number of cells entering the needle was determined by the number of GFP fluorescent clones that grew during this time. At the peak concentration of 25 nM EGF, an 8-fold increase in the number of cells entering the needle was seen, when compared with buffer alone (Fig. 1). The number of cells collected decreased at 50 nM EGF, and by 250 nM EGF the number of cells collected returned to near background.

The differences in EGF concentration optimum for cell response between the upshift (5 nM; Ref. 12), the pipette experiment (50 μM; Ref. 10), and the collection experiment reported here (25 nM) can be explained by the differences in diffusion of EGF in the different experimental designs. In the upshift, there is no gradient involved and the cells see an equal and constant concentration of EGF. For the pipette experiment the gradient is created by a pipette with an i.d. of <1 μm, and the concentration outside of the pipette is only a fraction of the concentration in the pipette. For the in vitro cell collection experiments reported here the i.d. of a 26-gauge needle is 250 μm; hence, a larger percentage of EGF is delivered per unit time so that a much lower EGF concentration is necessary than in the pipette experiment (10).

By using a needle loaded with matrigel and 25 nM EGF in MEM-BSA, we were able to capture images of the cells moving toward the pipette, using time-lapse video-microscopy. In Fig. 2, the matrigel surface at the edge of the needle is delineated by the white line and colored gray. At time zero, cells 1 and 2 are seen as nonpolarized cells with no discernable leading edge. After 1.5 h, cells 1 and 2 have oriented themselves toward and moved in the direction of the needle-induced EGF gradient, extending a leading lamellapod toward the needle. Cell 3 has also moved into the field. After 3 h, all three cells can be seen to have moved measurably closer to the needle. The cells move toward the needle at a velocity of 0.32 μm/min, which is comparable with the velocities reported previously (10).

In Vivo Cell Collection. To determine whether cells can be collected from tumors in vivo and, if so, if there is a difference in collection efficiency of cells from nonmetastatic and metastatic tumors, experiments were performed by placing needles into the primary tumors generated by either the nonmetastatic MTC-GFP or the metastatic MTLn3-GFP cell lines. For this, a 33-gauge needle (i.d., 102 μm) was filled as above and inserted into the guide syringe after a blocking wire was removed (as modeled in Fig. 3). The needles were filled with matrigel plus either buffer, 25 nM EGF, or 10% FBS. The 10% FBS was used because the motility of both MTLn3 and MTC cells is stimulated in response to 10% serum (data not shown). After 6 h of collection, needles were withdrawn and the contents of each was extruded into a 35-mm dish containing growth medium, and collected tumor cells were determined by GFP fluorescence. To confirm that only GFP-labeled cells were in the needle, 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) was added to the dish to stain all cells. All DAPI-stained nuclei were in GFP-labeled cells, indicating that only tumor cells were collected.

The number of cells collected for each condition was normalized to the number of cells collected from the MTC-GFP tumors using needles containing matrigel plus buffer (MEM-BSA) only (Fig. 4).
For the needle with 25 nM EGF, 15.3 times more MTLn3 cells were collected from metastatic MTLn3 tumors compared with MTC cells from nonmetastatic MTC tumors under the same conditions of collection. In this case, a maximum of 100 cells was collected. Needles containing 10% FBS showed only a 6.0-fold difference between the two tumor types under the same conditions (Fig. 4). There was a 2-fold increase in the number of MTC cells entering the 10% FBS needle from the MTC tumors compared with the number of cells that entered the needle containing only buffer. This difference was shown to be significant (t test value, 0.027) and is consistent with the increase in motility of MTC cells when stimulated with 10% FBS in vitro (data not shown). We did not attempt to establish long-term cultures of tumor cells collected from the tumors in vivo under any of these conditions.

In addition, in needles containing only buffer, 4.3 times more tumor cells were collected from MTLn3 tumors than from MTC tumors (Fig. 4). To determine whether this was due to a cell response to matrigel, a needle was filled with either 1% agarose containing MEM-BSA or 1% agarose containing 10% FBS in MEM-BSA. MTLn3 cells are able to adhere and grow on agarose. However, agarose was chosen because it is a polysaccharide that cannot be degraded by proteases. The number of cells entering the agarose needles was at background for both the 10% FBS-containing needle (data not shown) and the needle with buffer alone (Fig. 4), indicating that either components within the matrigel or the degradation of matrigel provides a chemotactic signal to the cells.

The collection of cells from the MTLn3 tumors was maximal with needles containing 25 nM EGF, resulting in the collection of about 100 cells in 6 h. Because the diameter of the 33-gauge collecting needle is 100 μm and the average cell diameter is 25 μm, the calculated average velocity of cell motility required to account for the collection of 100 cells in 6 h is 0.3 μm/min. This value is very close to the velocity of cell locomotion observed in vitro during chemotaxis (Fig. 2).

Discussion

In this study, we report that needles containing chemoattractants can be used to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor in vivo as a pure population suitable for further analysis. Our results demonstrate that tumor cells are collected into needles that have been inserted into a primary tumor when they contain either serum, EGF, or matrigel but not agarose, indicating that a tactic signal is required for collection. The most dramatic accumulation of cells in the needles occurs in response to either EGF or serum. EGF is known to be a chemoattractant for MTLn3 cells (10), whereas serum stimulates the motility of both MTLn3 and MTC cells. However, matrigel was sufficient to collect cells above background, indicating that either the matrigel contains cytokines that are chemotactic for these cells or that limited proteolysis resulting from the interaction of the matrigel with the tumor is sufficient to generate a gradient of chemotactic peptides. Either possibility is consistent with the known properties of matrigel (13–15).

Furthermore, MTLn3 cells have a 4-fold greater activity compared with MTC cells (16), which may explain the increase in the number of MTLn3 cells collected into the needles containing matrigel compared with that for MTC cells.

Both EGF and transforming growth factor α are growth factors found in mammary tissue. MTLn3 cells have around 50,000 EGF receptors/cell, whereas EGF receptors on the MTC cells are not detectable (9). By using EGF as the chemotactrant, we were able to selectively collect 15 times as many metastatic MTLn3 cells from MTLn3-derived metastatic primary tumors as MTC cells from MTC-derived nonmetastatic tumors. Serum, which contains many growth factors with potential chemotactic activity, also stimulated the collection of tumor cells from MTLn3 tumors. Although MTC cells show a motility response to serum, they were not collected with high efficiency into needles in response to serum, indicating that additional factors besides motility are required for the large increase in the number of MTLn3 cells collected in response to serum.

![Fig. 3. Method for using needles for in vivo cell collection. Needles (i.d., 102 μm) filled with matrigel and buffer, 25 nM EGF, or 10% FBS are shown placed in 25-gauge guide needles that are inserted into the primary tumor of an anesthetized rat.](image)

![Fig. 4. Metastatic cells (MTLn3) are more efficient than nonmetastatic cells (MTC) at entering matrigel-filled needles in response to EGF in vivo. Cells were collected from metastatic (MTLn3) and nonmetastatic (MTC) tumors using the in vivo experiment shown in Fig. 3. The maximum response was for cells from the metastatic MTLn3 tumors into EGF- and serum-containing needles. Cells were collected above background from metastatic tumors in response to matrigel in buffer but not agarose. All counts were normalized to MTC cells collected with matrigel in buffer. Bars, the SE of four experiments.](chart)
Morphologically, MTC cells are elongated and polarized both in vivo and in vitro, whereas the MTLn3 cells are generally unpolarized both in culture and in the primary tumor (8, 11). This difference is most dramatically illustrated by using intravital imaging techniques where GFP-expressing tumor cells are imaged directly in the primary tumor (8, 9). In vivo, MTLn3 cells are highly polarized around and oriented toward the blood vessels running through the primary tumor. MTC cells, on the other hand, are polarized throughout the tumor, but the polarity is randomly oriented relative to vessels (8, 11).

Characterization of the cells in vitro confirms the differences between the two cell lines. In cultures that have not been stimulated with a chemoattractant, MTC cells locomote in a linear direction at approximately twice the velocity of MTLn3 cells. MTLn3 cells, under these conditions, are unpolarized and move in random directions or not at all (11). On stimulation with an EGF gradient, the MTLn3 cells become polarized and move linearly at approximately the same speed as the MTC cells, yet have the ability to reorient themselves to follow an EGF gradient with precision (10), a property not seen in MTC cells.

In vivo, in the primary tumor, both cell types move linearly at approximately the same speeds, but the MTLn3 cells tend to move only when they are polarized and in association with a vessel, whereas MTC cells can be seen moving throughout the tumor (8, 9). The ability of the MTLn3 cells to invade into a needle filled with matrigel in response to growth factors is fully consistent with the chemotactic motility exhibited by these cells in vitro, their polarity and locomotion toward vessels in vivo, and with the dramatically increased efficiency of intravasation measured as blood burden of tumor cells in vitro (8). This suggests that chemotaxis may be the key aspect of cell motility that contributes to invasion and intravasation. It also suggests that needles filled with growth factors and matrigel, when inserted into the tumor, may be the key aspect of cell motility that contributes to invasion and intravasation in vivo, and that the same cell behaviors that contribute to chemotaxis in vitro also contribute to invasion in vivo.

An advantage of using the needle collection technique described here for the collection of cells for genomic/proteomic analysis is that the cell behavior can be characterized during the collection process. This can be done by varying the conditions required for cell collection such as the extracellular matrix composition and/or cytokines used as chemoattractants, determining how these changes affect efficiency of cell collection, and then relating these observations to the gene expression and protein composition patterns subsequently obtained from array analysis of the collected cells. Furthermore, cells can also be characterized by intravital imaging during collection to directly visualize the cell-cell and cell-extracellular matrix interactions that contribute to the invasion of the needle under these different conditions. In addition, cells could be cultured and transplanted into other host animals to determine whether they stably retain differential characteristics that contribute to metastatic potential. Finally, by comparing the gene expression patterns of cells collected by invasion into needles with that of cells obtained from the whole primary tumor, the blood, and whole metastatic tumors, genes that contribute to the invasive process uniquely may be identified.

References


Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2000 American Association for Cancer Research.
The Collection of the Motile Population of Cells from a Living Tumor

Jeffrey B. Wyckoff, Jeffrey E. Segall and John S. Condeelis


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/19/5401

Cited articles
This article cites 15 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/19/5401.full.html#ref-list-1

Citing articles
This article has been cited by 28 HighWire-hosted articles. Access the articles at:
/content/60/19/5401.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.