Characterization of Tumor Cell Dissemination Patterns in Preclinical Models of Cancer Metastasis Using Flow Cytometry and Laser Scanning Cytometry

David Goodale,1 Carolina Phay,2 Carl O. Postenka,1 Michael Keeney,2,3 Alison L. Allan1,3,4,5*

Abstract

The inability to sensitively detect metastatic cells in preclinical models of cancer has created challenges for studying metastasis in experimental systems. We previously developed a flow cytometry (FCM) method for quantifying circulating tumor cells (CTCs) in mouse models of breast cancer. We have adapted this methodology for analysis of tumor dissemination to bone marrow (BM) and lymph node (LN), and for analysis of these samples by laser scanning cytometry (LSC). Our objective was to implement these methodologies for characterization of tumor cell dissemination in preclinical models of cancer metastasis. Human cancer cells were injected into mice via mammary fat pad (MFP; spontaneous metastasis), tail vein (TV; targets lung), or intracardiac (IC; targets bone) routes. At several time points postinjection (4 h to 8 weeks), mice were sacrificed and blood, LNs, and BM were collected. Samples were immunomagnetically enriched and labeled with human leukocytic antigen–fluorescein isothiocyanate and CD45-PE antibodies (FCM/LSC), and propidium iodide (FCM) prior to quantitative analysis. Following MFP injection, CTCs increased over time, as did disseminated cells to the LN. Interestingly, tumor cells also spontaneously disseminated to BM, peaking at 2 weeks postinjection. Following TV injection, CTCs were initially high but decreased rapidly by 1 week before increasing to peak at endpoint. Combined with an observed concurrent increase in disseminated cells to LN and BM, this suggests that tumor cells may shed into the circulation from lung metastases that establish following initial cell delivery. Following IC injection, CTCs increased over time, peaking at 4 weeks. Tumor cells in the BM (most prevalent site of metastasis after IC injection) remained at moderate levels until peaking at endpoint. Combined use of FCM and LSC allows sensitive quantification of disseminated tumor cells in preclinical models of metastasis. These methods will be valuable for future studies aimed at testing new therapeutics in the metastatic setting.

Key terms
breast cancer; metastasis; preclinical animal models; tumor cell dissemination; flow cytometry; laser scanning cytometry

*Correspondence to: Alison L. Allan, London Regional Cancer Program, 790 Commissioners Road East, London, Ontario, Canada N6A 4L6. Email: alison.allan@lhsc.on.ca

© 2008 International Society for Advancement of Cytometry
Given the multistep nature of the metastatic cascade, there should be several opportunities for early identification and therapeutic targeting of metastatic cells before they become a clinical problem. Indeed, the presence of circulating tumor cells (CTCs) in the bloodstream of cancer patients has been recognized for over a century (9), although a lack of sensitive technology precluded the detailed study of these cells until recently. However, technological advances have now facilitated the identification, enumeration, and characterization of individual disseminated cells in breast cancer patients using methods such as PCR (10–12), flow cytometry (FCM) (13–15), image-based immunologic approaches (16–19), immunomagnetic techniques (20,21), and microchip technology (22). In breast cancer patients with either metastatic or apparently localized disease, there is growing evidence that the presence of individual CTCs in the blood or disseminated tumor cells (DTCs) in the bone marrow (BM) may be an important indicator of the potential for metastatic disease and poor prognosis [reviewed in (23–27)]. Furthermore, the histopathological identification of very small metastatic tumor deposits in the axillary LN have recently been reported to be prognostically significant (28). However, the biological implications of individual occult tumor cells in regional and distant sites remains poorly understood, particularly with regards to the functional and mechanistic details of their progression to clinically relevant metastases. Therefore, preclinical modeling of the pattern and kinetics of CTC and DTC dissemination in breast cancer and the relationship to endpoint metastatic disease would be extremely valuable.

There are several in vivo preclinical mouse models available for studying breast cancer metastasis, including “spontaneous” metastasis models, “experimental” metastasis models, and transgenic models (29,30). Spontaneous metastasis models allow for investigation of all steps of the metastatic cascade and involve injection of cancer cells into the correct orthotopic site [i.e., the mammary fat pad (MFP) for breast cancer cells], growth of a primary tumor, and eventual development of spontaneous metastases in distant organs (most commonly in LN and lung following MFP injection) (29,31–34). In contrast, experimental metastasis models involve direct injection in the blood circulation, and thus circumvent the initial steps of primary tumor growth and intravasation (29). Depending on the route of injection, cells can be targeted for metastatic growth in different organs using this assay. For example, one of the most commonly used injection routes, the tail vein (TV), targets cells for delivery and growth in the lung (29,35). Other routes of injection include the mesenteric vein to target liver, or an intracardiac (IC) injection route which theoretically targets the entire circulation but is usually aimed toward development of metastatic growth in the bone (29,35–38). The occurrence and extent of metastasis in preclinical models has typically been measured by macroscopic or histological examination at endpoint (29). However, these approaches are limited in their sensitivity and are often performed on a small number of organ sections which may not be representative of the true extent of metastasis. Therefore, although these preclinical models have been valuable for providing scientists with the ability to test the overall effect of specific molecules or drugs on endpoint metastasis, they are ultimately “black box” assays that do not allow detection and tracking of individual DTCs during metastatic progression.

To address this problem, we previously reported the development of a novel and sensitive multiparameter FCM assay to quantify CTCs in preclinical mouse models of breast cancer metastasis (39). In the present study, we have adapted this methodology for analysis of tumor cell dissemination to BM and LN and for analysis of these samples by laser scanning cytometry (LSC). The overall goal of the study was to implement these cytometry methods for characterization of tumor cell dissemination patterns and kinetics in spontaneous and experimental models of breast cancer metastasis. Our novel findings suggest that the combined use of FCM and LSC is highly complimentary and allows for sensitive quantification of CTCs and DTCs in different preclinical models of breast cancer metastasis. These methods will allow us to gain a greater understanding of the biology of metastasis and will be extremely valuable for future studies aimed at preclinical testing of new therapeutics in the metastatic setting.

**Materials and Methods**

**Cell Culture**

The human cancer cell line MDA-MB-435HAL (a kind gift from Dr. David Griggs; Pfizer, St. Louis, MO) is a green fluorescent protein-expressing, metastasis-derived variant of the MDA-MB-435 cell line. This subcloned variant was isolated after multiple in vivo passages and was selected for its enhanced MFP tumor growth rate and increased metastasis to lung (40). Cells were grown in minimal essential medium with Earle’s salts and L-glutamine (MEM) supplemented with 25 mmol/L HEPES buffer, 1 mmol/L sodium pyruvate, 1× MEM vitamin solution, and 10% fetal bovine serum (FBS). Media, supplements, and PBS were obtained from Invitrogen (Carlsbad, CA). FBS was obtained from Sigma Chemical (St. Louis, MO). It should be noted that the MDA-MB-435 cell line was originally isolated from the pleural effusion of a woman with metastatic breast adenocarcinoma (41). Recently, a debate has arisen over the origins of this cell line, whether it was derived from the M14 melanoma cell line (42,43). However, its expression of milk proteins (44) and propensity to metastasize from MFP but not from subcutaneous sites (29) are consistent with it being a breast carcinoma.

**Sample Collection**

Fresh whole blood, LNs, and BM were collected from female athymic NCR nude mice (nu/nu) (aged 6–15 weeks; Harlan Sprague–Dawley, Indianapolis, IN). Blood (300 μL/mouse) was collected via terminal cardiac puncture of the right ventricle using a 22-G needle attached to a 1-mL syringe precoated with heparin (10,000 IU/mL; Leo Pharma, Thornhill, ON). Six LNs per mouse (two brachial, two axillary, two inguinal) were harvested into cold PBS + 10% FBS, minced cross-wise with scissors, passaged three times through a 16-G
neuclide and five times through an 18-G needle, filtered through 70 μM mesh, and resuspended in cold PBS + 10% FBS. For BM collection, one hind tibia and femur per mouse were flushed with cold PBS + 10% FBS, washed twice, and resuspended in PBS + 10% FBS. For FCM and LSC protocol development, samples consisting of MDA-MB-435HAL human cancer cells "spiked" into mouse blood, LN homogenates, and BM at various concentrations (10–0.001%) were prepared by serial dilution as described previously (39). All samples were prepared within 2 h of collection.

**Labeling and Immunomagnetic Enrichment Procedure**

Sample labeling and immunomagnetic enrichment was carried out essentially as described previously (39). Briefly, blood, LN homogenates, and BM samples were subjected to red blood cell lysis in 1× NH₄Cl and washed with PBS prior to labeling with 10 μL of mouse antihuman leukocytic antigen (HLA) antibody (clone W6/32) conjugated to fluorescein isothiocyanate (FITC) (Sigma) and 10 μL of rat antimouse pan-leukocytic CD45 antibody (clone 30-F11) conjugated to phycoerythrin (PE) (Caltag Laboratories, Burlingame, CA). Samples were then immunomagnetically enriched using the EasySep™ PE Selection Kit (Stem Cell Technologies, Vancouver, BC) as per the manufacturer’s instructions, using 2 × 5 min incubations in the EasySep magnet. This immunomagnetic enrichment procedure has previously been established to provide ~10-fold enrichment of tumor cell detection by FCM (39). After incubation, the fraction containing the tumor cells (supernatant) was fixed and permeabilized using the Intra-Prep™ Fix/Perm Kit (Beckman Coulter, Fullerton, CA). At this stage, ~1/10th of the concentrated sample (10 μL; equivalent to a starting volume of ~30 μL of whole blood) was aliquoted for LSC analysis. The remainder of each sample was resuspended in 500 μL of PI (50 μg/mL) (Beckman Coulter) and incubated for 15 min at room temperature following by 45 min at 4°C in preparation for FCM analysis.

**FCM Analysis**

A four color XL-MCL flow cytometer (Beckman Coulter) was configured to detect the HLA-FITC signal in FL1 (525-nm bandpass filter), CD45-PE in FL2 (575-nm bandpass filter), and PI in FL3 (625-nm bandpass filter). Setup and compensation was adjusted on a ~10% mixture of MDA-MB-435HAL cells and mouse leukocytes. A threshold region was set on DNA content based on PI fluorescence equivalent to a diploid mouse leukocyte (cells with lowest DNA content). By plotting integral PI signal versus the ratio of the PI peak/PI integral signal, it is possible to identify debris, cell doublets and clumps which can be excluded from further analysis (39). A minimum of 100,000 PI+ events were collected per sample. Gated events which were HLA+CD45− were considered to be positive tumor events.

**LSC Analysis**

Aliquots (10 μL) of concentrated samples of blood, LN homogenate, or BM labeled with HLA-FITC and CD45-PE were placed onto a glass slide and covered with a glass cover slip (18 × 18 mm²; VWR International, Mississauga, ON) just prior to analysis with an iCys LSC (CompuCyte, Cambridge, MA). Setup and compensation was adjusted on a ~10% mixture of MDA-MB-435HAL cells and mouse leukocytes. The LSC acquisition protocol was configured with primary contour set on light scatter. Green fluorescence (HLA-FITC) and orange fluorescence (CD45-PE) were excited with a 488-nm argon ion laser and measured using standard filter settings. The intensities of maximal pixel (pixel size: 0.5 × 0.5 μm²) and integrated fluorescence were measured and recorded for each event. One scan field (40 × 0.5 μm) per sample was analyzed. Cell morphology and fluorescence parameters were confirmed by visualizing microscopy images through the gallery function in the iCys software. Events which were confirmed to be HLA−CD45− were considered to be positive tumor events.

**In Vivo Metastasis Assays**

All animal procedures were conducted under a protocol approved by the University of Western Ontario Council on Animal Care. For all experiments, 6–7-week-old female athymic nude (nu/nu) mice (Harlan Sprague–Dawley) were used. MDA-MB-435HAL cells were prepared in sterile PBS and injected in a 100 μL volume into the second thoracic MFP (2 × 10⁶ cells/mouse), lateral TV (1 × 10⁶ cells/mouse), or IC via the left ventricle (2 × 10⁵ cells/mouse) as described elsewhere (32,34,37,39). At various time points postinjection (MFP = 1, 2, 4, and 8 weeks; TV = 4, 24, 48 h, 1, 4, 6, and 7 weeks; IC = 4, 24, 48 h, 1, 4, and 5 weeks), five mice/time point were sacrificed and blood, LNs, and BM were harvested, processed, and analyzed by FCM or LSC as described earlier. Endpoints were chosen based on previous experience with these model systems (our unpublished data), morbidity due to primary tumor (mean MFP tumor size at endpoint = 1725 mm²), and/or metastatic burden. Time points were preselected based on the predicted endpoint and the hypothesized behavior of the MDA-MB-435HAL cells following implantation via the various injection routes.

Additional tissues (lung, liver, spleen, ovary, adrenal gland, brain, bone) were also collected at necropsy and fixed in 10% neutral-buffered formalin before processing. It should be noted that we were unable to carry out histological analysis of LN micrometastases because all LN material was processed for the cytometry studies. Tissues were embedded in paraffin wax, sectioned (4 μm thick), and subjected to standard hematoxylin and eosin (H&E) staining. Stained slides were evaluated by light microscopy in a blinded fashion by an experienced pathologist in order to observe histopathological characteristics and identify incidence and regions of metastatic involvement.

**Statistical Analysis**

Statistical analysis was performed with GraphPad Prism 4.0© (San Diego, CA) and SigmaStat 3.5© (SYSTAT, Chicago, IL) using ANOVA with Krukal–Wallis test (for comparison between more than two groups). Differences between means were determined using the Student’s t test when groups passed both a normality test and an equal variance test. When this
was not the case, the Mann–Whitney Rank-Sum test was used. Correlation between time and the level of DTCs was assessed using the Pearson product moment correlation. Correlation between methods and correlation between primary tumor size and disseminated cells was assessed using multiple linear regression. Unless otherwise noted, data are presented as the mean ± SEM. In all cases, P values of ≤0.05 were regarded as being statistically significant.

RESULTS

FCM Analysis of DTCs

We previously developed a multiparameter FCM assay to quantify CTCs in the blood of preclinical mouse models of breast cancer metastasis. This method was demonstrated to have high specificity and a detection sensitivity of $10^{-5}$, or one tumor cell in 100,000 mouse leukocytes (39). In the present study, we have adapted this methodology for analysis of tumor cell dissemination to LN and BM. The FCM acquisition setup and representative analysis is illustrated in Figure 1A threshold region (R1) was set on DNA content based on PI fluorescence equivalent to a diploid leukocyte (cells with lowest DNA content) (Fig. 1, left panels), and subsequent analyses of HLA+CD45+ tumor cells were gated based on this threshold (Fig. 1, right panels). Representative gated analysis is shown of ~0.01% MDA-MB-435HAL cells in mouse blood (Fig. 1A), mouse LN homogenate (Fig. 1B), or mouse BM (Fig. 1C). Gated PI− events which fell within region R2 were counted as meeting the criteria for mouse leukocytes (CD45−HLA−), and those which fell within region R3 were counted as meeting the criteria for human tumor cells (HLA+CD45+).

LSC Analysis of DTCs

LSC uniquely combines the advantages of FCM, image analysis, and automated fluorescence microscopy such that a large amount of multiparameter data can be simultaneously gathered and quantified for individual events in a heterogeneous population of cells, including visualization of fluorescence and morphology (45). Therefore, although our FCM method is specific and sensitive, we were interested in using LSC as a complimentary method to confirm our findings, particularly because it gave us the power to analyze each HLA+CD45+ tumor event for its morphological properties. The LSC acquisition protocol was configured as shown in Figure 2A, with primary contour set on light scatter. Figures 2B–2D show representative analysis of green integral (HLA–FITC) versus orange integral (CD45–PE) of ~0.01% MDA-MB-435HAL cells in mouse blood (Fig. 2B), mouse LN homogenate (Fig. 2C), or mouse BM (Fig. 2D). Events which fell within region R1 were counted as meeting the criteria for mouse leukocytes if they were CD45−HLA−, and events which fell within region R3 were counted as meeting the criteria for human tumor cells if they were HLA+CD45+. Cell morphology and fluorescence parameters of both populations were confirmed by visualization (Figs. 2B–2D, top insets). The combined use of this LSC assay and the FCM assay described in Figure 1 allowed us to characterize in vivo tumor cell dissemination patterns and kinetics in the blood, LN, and BM of mice injected with MDA-MB-435HAL cells via MFP, TV, or IC routes (Figs. 3–6).

Tumor Cell Dissemination Patterns and Kinetics Following MFP Injection of Human Cancer Cells

We first used our FCM and LSC assays to assess tumor cell dissemination patterns and kinetics in an in vivo model of spontaneous metastasis (29,31–34) (Fig. 3). Following MFP injection of MDA-MB-435HAL cells, FCM analysis demonstrated that CTCs in the blood increased over time [30 ± 6 (1 week) to 190 ± 72 (endpoint) tumor cells/10⁵ leukocytes] (Fig. 3A, solid lines), as did DTCs in the LN [3 ± 1 (1 week) to 223 ± 90 (endpoint) tumor cells/10⁵ leukocytes] (Fig. 3B, solid lines). Interestingly, tumor cells also spontaneously disseminated to the BM (unusual for this model), peaking at 268 ± 125 tumor cells/10⁵ leukocytes (2 weeks) (Fig. 3C, solid lines). Using LSC as a complementary tool for quantitative and morphologic analysis, we observed that CTCs in the blood and DTCs in LN increased over time [blood: 10 ± 5 (1 week) to 101 ± 26 (endpoint) tumor cells/scan field; LN: 3 ± 1 (1 week) to 52 ± 9 (endpoint) tumor cells/scan field] (Figs. 3A and 3B, dashed lines), and DTCs were again detected in the BM with a peak of 235 ± 60 cells/scan field at 2 weeks postinjection (Fig. 3C, dashed lines). Table 1 shows CTC/DTC counts obtained by FCM and LSC in individual mice at endpoint (8 weeks postinjection). For all time points, the pattern and kinetics of tumor dissemination observed was consistent between methods, and the number of events detected by FCM was found to be significantly correlated with those detected using LSC analysis of blood ($R^2 = 0.92; P = 0.04$), LN ($R^2 = 0.92; P = 0.04$), and BM ($R^2 = 0.95; P = 0.03$). For both methods, there was a significant correlation between the time postinjection and the number of disseminated cells detected in the blood ($R^2 > 0.91; P < 0.05$) and LN ($R^2 > 0.97; P < 0.02$), although no such correlation was observed in the BM. Consistent with previous clinical studies (22,46), no significant correlation was observed between the endpoint primary tumor size of individual mice and the level of disseminated cells in blood, LN, or BM.

Tumor Cell Dissemination Patterns and Kinetics Following TV Injection of Human Cancer Cells

We next assessed tumor cell dissemination patterns and kinetics in an in vivo model of experimental metastasis targeting the lung (29,35) (Fig. 4). Following TV injection, FCM analysis demonstrated that CTCs were initially high at 4 h postinjection (336 ± 112 tumor cells/10⁵ leukocytes) but decreased rapidly by 1 week (19 ± 5 tumor cells/10⁵ leukocytes) before increasing to peak at 598 ± 429 tumor cells/10⁵ leukocytes by endpoint (Fig. 4A, solid lines). There was a smaller but somewhat concurrent increase in DTCs observed in the LN (peaking at endpoint; 56 ± 14 tumor cells/10⁵ leukocytes) (Fig. 4B, solid lines) and BM (peaking at 6 weeks postinjection; 37 ± 5 tumor cells/10⁵ leukocytes) (Fig. 4C, solid lines). LSC analysis resulted in the detection of a steady increase in levels of CTCs over time (4 ± 1 tumor cells/scan field at 4 h to 177 ± 58 tumor cells/scan field at endpoint).
DTCs in the LN showed a fluctuating increase over time, peaking at endpoint (47 ± 24 tumor cells/scan field) (Fig. 4B, dashed lines). In the BM, DTC levels peaked at 1 week postinjection (70 ± 13 tumor cells/scan field) and dropped off slightly by endpoint (49 ± 15 tumor cells/scan field) (Fig. 4C, dashed lines). Table 1 shows CTC/DTC counts obtained by FCM and LSC in individual mice at endpoint (7 weeks postinjection). For all time points, the observed pattern and kinetics of tumor dissemination in the blood was consistent between methods, and the number of CTCs detected by FCM was found to be significantly correlated with those detected using LSC analysis ($R^2 = 0.96; P = 0.01$). There was also a significant correlation between the time postinjection and the number of CTCs detected in the
blood by both methods ($R^2 > 0.77; P < 0.03$). However, there was neither a significant correlation between methods for analysis of LN and BM, nor a correlation between time postinjection and the number of DTCs detected in these samples.

**Tumor Cell Dissemination Patterns and Kinetics Following IC Injection of Human Cancer Cells**

We also assessed tumor cell dissemination patterns and kinetics in another in vivo model of experimental metastasis that targets the entire circulation, but it is usually aimed toward development of metastatic growth in the bone (29,35–38) (Fig. 5). Following IC injection and assessment by FCM, CTC levels in the blood were observed to increase over time, peaking at 4297 ± 1705 tumor cells/10^5 leukocytes (4 weeks postinjection) before decreasing to 281 ± 71 tumor cells/10^5 leukocytes by endpoint (Fig. 5A, solid lines). DTCs in the LN increased by 24 h postinjection (98 ± 33 tumor cells/10^5 leukocytes) and maintained this level until endpoint (101 ± 11 tumor cells/10^5 leukocytes) (Fig. 5B, solid lines). Levels of DTCs in the BM (the most prevalent site of metastasis after IC
injection) remained at moderate levels until endpoint, where they peaked at 730 ± 251 tumor cells/10^6 leukocytes (Fig. 5C, solid lines). Using LSC, CTCs in the blood also showed a peak at 4 weeks postinjection (1248 ± 81 tumor cells/scan field) (Figs. 5A, dashed lines). In both the LN and BM, levels of DTCs were observed to peak at endpoint (LN: 63 ± 20 tumor cells/scan field; BM: 256 ± 81 tumor cells/scan field) (Figs. 5B and 5C, dashed lines). Table 1 shows CTC/DTC counts...
obtained by FCM and LSC in individual mice at endpoint (5 weeks postinjection). For all time points, LSC analysis demonstrated patterns and kinetics of dissemination in blood and BM consistent with that observed by FCM ($R^2 > 0.97$; $P < 0.001$), although no such correlation was observed for LN. In addition, there was no significant correlation between the time postinjection and the number of disseminated cells detected by either method in any of the three sample types.

**Histological Incidence of Micrometastasis Following MFP, TV, or IC Injection of Human Cancer Cells**

Finally, we were interested in determining how endpoint micrometastases in distant organs (Fig. 6) related to the kinetics of tumor cell dissemination observed in the different breast cancer models (Figs. 3–5). Tissues collected at necropsy for each time point were processed, sectioned, and stained with H&E before evaluation by an experienced pathologist in order to observe histopathological characteristics (Fig. 6, right panels) and identify incidence of metastatic involvement (Fig. 6, left panels). Interestingly, for MFP (Fig. 6A), TV (Fig. 6B), and IC (Fig. 6C) injection routes, the kinetics of micrometastatic incidence (% of mice with metastases) was consistent but slightly delayed relative to the kinetics of tumor dissemination observed in Figures 3–5 (i.e., DTCs were detected at 1 week postinjection or earlier in all models (Figs. 3–5), while micrometastases were only observed starting at 4 weeks postinjection (Fig. 6). Micrometastases were only observed in the lung of mice injected via the MFP (Fig. 6A) or TV (Fig. 6B), but were observed in multiple organs (ovary, adrenal gland, lung, and bone) following IC injection (Fig. 6C). Table 1 shows a comparison between the levels of CTC/DTCs detected by FCM and LSC and the development of micrometastases in individual animals at endpoint. Although the number of animals in each group ($n = 5$) does not provide appropriate power to draw any statistical conclusions, the data do suggest some interesting trends. For example, following MFP injection, mice that had the highest levels of CTC/DTCs in the blood, LN and BM were also the mice that developed micrometastases. Similarly, following TV injection, mice that had the highest levels of CTCs in the blood were again the mice that developed micrometastases. Finally, although all mice injected via the IC route developed micrometastases in the bone and lung, the two mice with the highest levels of CTCs in the blood also developed micrometastases in the adrenal gland and the ovary.

**DISCUSSION**

There is growing evidence that the presence of individual disseminated cells in the blood, LN, or BM of breast cancer patients may be an important indicator of the potential for metastatic disease and poor prognosis (23–28). In the clinical setting, the immunomagnetic-based CellSearch™ assay (21,47) has resulted in a number of promising studies relating to clinical outcome and prognostic value of CTCs in breast cancer (20,46,48–54). In addition, a well-established immunocytochemical method has provided valuable data regarding the prognostic significance of DTCs in the BM (16,55). However, current American Society of Clinical Oncology guidelines do not yet support the use of CTC or DTC assays for clinical management decisions in breast cancer, mainly because of the wide range of methodologies being used and the need for fur-
Furthermore, although the presence of CTCs and DTCs in the blood of breast cancer patients is hypothesized to reflect the presence of micrometastases and/or aggressive disease, a causative biological link between these cells and metastasis has not yet been demonstrated (25).
Experimental challenges in detecting and quantifying rare metastatic tumor cells in mouse models of human breast cancer has hindered the ability to use preclinical animal models to their full capacity for understanding the metastatic process, particularly with regards to determining the timing and location of CTC and DTC dissemination, quantifying early steps in metastasis, and determining how disseminated cells contribute to endpoint metastases. In the present study, we describe the development and implementation of novel FCM and LSC assays for characterizing tumor cell dissemination patterns and kinetics in three different in vivo models of breast cancer metastasis, including evidence of CTC/DTC contribution to the development of metastatic disease.

We believe that the findings of the current study have a number of important biological and technical implications. From a biological perspective, for the first time we were able to quantify individual CTCs and DTCs in our preclinical animal models at very early time points following tumor cell injection and during progression to metastatic disease. In our spontaneous metastasis model, the steady increase in CTCs in the blood and DTCs in the LN over time was perhaps not unexpected and is reflective of the clinical situation in which the lymphatic system is considered to be a primary route for early dissemination of breast cancer (57–59). Furthermore, we observed that the timing of micrometastatic incidence to distant organs such as lung was consistent but slightly delayed relative to CTC and DTC kinetics. For example, DTCs were detected in the blood, LN, and BM as early as 1 week postinjection, while lung micrometastases were only observed starting at 4 weeks postinjection. Interestingly, this timing corresponded to detection of increasing levels of CTCs in the blood and DTCs in the LN, suggesting that, in addition to shedding from the primary tumor, CTCs and DTCs may also be disseminating from secondary metastatic tumors in the lung and possibly the LN. We also observed DTCs in the BM, an unexpected finding given that bone metastases have not been reported to develop spontaneously following orthotopic injection of human breast cell lines (29,60). However, since there was no corresponding histological evidence of bone metastases in this model, these findings suggest [as other studies have (16,61–64)] that the BM may act as a tumor cell reservoir and/or a passive filter interacting with the blood circulation.

Following TV injection, we observed a similar increase in CTCs and DTCs over time. Although the blood seemed to be the major route of tumor dissemination in this experimental metastasis model, the observed concurrent increase in DTCs in the LN and BM and the confirmed histological presence of micrometastases in the lung supports the idea that tumor cells may be shed into the circulation from metastases that establish following initial cell delivery. Following IC injection, we observed the highest levels of CTCs in the blood of any of the three metastasis models. High CTC levels were observed even as late as 2–4 weeks postinjection, when it might be expected that most tumor cells would have been eliminated from the circula-

### Table 1. Comparison of CTC/DTC levels obtained via flow cytometry (FCM) or laser scanning cytometry (LSC) and the development of micrometastases in individual mice at endpoint

<table>
<thead>
<tr>
<th>MOUSE ID</th>
<th>NO. OF CTCs IN BLOOD (FCM&lt;sup&gt;a&lt;/sup&gt;/LSC&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>NO. OF DTCs IN LN (FCM&lt;sup&gt;a&lt;/sup&gt;/LSC&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>NO. OF DTCs IN BM (FCM&lt;sup&gt;a&lt;/sup&gt;/LSC&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>MICROMETASTASES AT ENDPOINT (YES OR NO; SITE)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse 1</td>
<td>180/130</td>
<td>187/51</td>
<td>149/161</td>
<td>Yes; lung</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>100/60</td>
<td>104/44</td>
<td>54/118</td>
<td>No</td>
</tr>
<tr>
<td>Mouse 3</td>
<td>39/33</td>
<td>85/26</td>
<td>34/80</td>
<td>No</td>
</tr>
<tr>
<td>Mouse 4</td>
<td>169/100</td>
<td>166/60</td>
<td>102/139</td>
<td>Yes; lung</td>
</tr>
<tr>
<td>Mouse 5</td>
<td>461/180</td>
<td>578/80</td>
<td>75/124</td>
<td>Yes; lung</td>
</tr>
</tbody>
</table>

**MOUSE ID**

- **MFP injection (8 weeks<sup>d</sup>):**
  - Mouse 1: 180/130, 187/51, 149/161, Yes; lung
  - Mouse 2: 100/60, 104/44, 54/118, No
  - Mouse 3: 39/33, 85/26, 34/80, No
  - Mouse 4: 169/100, 166/60, 102/139, Yes; lung
  - Mouse 5: 461/180, 578/80, 75/124, Yes; lung

- **TV injection (7 weeks<sup>d</sup>):**
  - Mouse 1: 163/113, 59/20, 17/6, No
  - Mouse 2: 264/180, 32/22, 38/72, Yes; lung
  - Mouse 3: 191/122, 91/11, 6/18, Yes; lung
  - Mouse 4: 2313/400, 16/140, 6/78, Yes; lung
  - Mouse 5: 62/70, 80/40, 16/73, No

- **IC injection (5 weeks<sup>d</sup>):**
  - Mouse 1: 108/30, 120/134, 299/100, Yes; bone, lung
  - Mouse 2: 257/50, 126/19, 965/240, Yes; bone, lung
  - Mouse 3: 168/20, 100/77, 58/100, Yes; bone, lung
  - Mouse 4: 358/70, 94/40, 1474/540, Yes; bone, lung, adrenal
  - Mouse 5: 514/170, 63/46, 855/300, Yes; bone, lung, adrenal, ovary

---

<sup>a</sup> Flow cytometry analysis was carried out as described in the “Materials and Methods” section and Figure 1. CTC/DTC numbers obtained by FCM are presented as number of tumor cells/10<sup>5</sup> leukocytes.

<sup>b</sup> Laser scanning cytometry analysis was carried out as described in the “Materials and Methods” section and Figure 2. CTC/DTC numbers obtained by LSC are presented as number of tumor cells/scan field.

<sup>c</sup> The presence or absence and location of micrometastases were determined by pathohistological analysis of H&E-stained tissue sections as described in the “Materials and Methods” section.

<sup>d</sup> Respective final time point for each injection route.
tion either by cell death and/or by seeding in distant organs. Indeed, micrometastases were observed in multiple organs including bone, and these metastases may have been contributing to the high CTC levels observed in the blood. At endpoint, the highest level of disseminated cells was observed in the BM, a logical finding considering that bone is the most prevalent site of metastases following IC injection (29,60), and all mice had histologically detectable bone metastases at endpoint.

From a technical perspective, our results demonstrate that the combined use of FCM and LSC allows for sensitive quantification of DTCs in preclinical models of breast cancer metastasis. Although FCM is a proven and reliable high-throughput technology, its major disadvantages for rare event analysis are that high numbers of cells are needed for accurate quantitation, and there is no capacity for morphological analysis (65,66). It has also been suggested that FCM may have limited sensitivity for CTC analysis in human blood samples (67). However, we have found that it has sufficient sensitivity (10^-5) for use in our animal models when combined with immunomagnetic enrichment, perhaps because of the much smaller overall blood volume of a mouse relative to a human (39). Comparatively, LSC has the distinct ability to confirm the true positive nature of identified rare cells by visualization, has been demonstrated to be effective and sensitive for rare event analysis, and can be used for relatively small samples sizes. Disadvantages of LSC include relatively high infrastructure costs, moderate analysis speed, and manual sample handling (17,45,67,68).

Given that both technologies have specific strengths and weaknesses, our goal in the present study was to expand the capacity of our previously validated FCM assay and use LSC as a complimentary (rather than a comparative) approach. As a consequence, we did not perform direct comparisons of absolute CTC/DTC counts between methods. Instead, we were interested in determining whether FCM and LSC demonstrated consistency with regards to the patterns and kinetics of tumor cell dissemination in different sample types and in different metastasis models. In all cases, we observed a significant correlation between methods for analysis of blood samples. Combined with the fact that the LSC assay required only a very small sample size (equivalent to ~30 µL of whole mouse blood), this suggests that LSC may be particularly useful for future studies aimed a longitudinal sampling of mice over time to assess real-time CTC kinetics and the relationship with metastasis and/or response to therapy. Interestingly, there was more variability between FCM and LSC results when analyzing BM and LN samples, and in many cases we detected greater than expected cell numbers by LSC relative to those events detected by FCM in a much larger volume of the same sample. One possible explanation is that both sample types (and BM, in particular) tended to be more “cellular” and contain more cell aggregates than blood, and in the FCM assay these aggregates were lost from analysis based on our gating strategy. In contrast, LSC allowed for visual confirmation of fluorescence and morphological parameters, and thus permitted inclusion of positive events within cell aggregates which may have otherwise been excluded from FCM analysis.

In summary, the ability to quantify CTCs and DTCs following injection of human cancer cells via different routes provides valuable information with regards to the pattern and kinetics of MDA-MB-435HAL tumor cell dissemination in our spontaneous and experimental metastasis models. Furthermore, since our selection and labeling strategy is designed to specifically identify human cancer cells derived from a variety of sources (39), these methods will have broad application for characterizing the tumor cell dissemination patterns and metastatic ability of other human breast cancer cell lines. Although a number of quantitative tools have been previously developed to study in vivo metastasis (69–71), the detection and quantification of rare metastatic events has remained challenging. The novel methods presented in the current study will begin to address this need and have future potential for helping to elucidate the mechanistic details of early steps in metastasis and how these steps relate to the development of life-threatening macrometastases. In addition, these methods will be extremely valuable for the future identification, development, and testing of new therapeutic strategies to combat breast cancer.

ACKNOWLEDGMENTS

The authors thank Dr. Waleed Al-Katib for his assistance with pathohistological analysis. A.L.A. receives salary support from the Imperial Oil Foundation.

LITERATURE CITED

9. Ashworth TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Aust Med J 1869;14:146–149.


