Abnormal ESR2 expression in primary tumors is not associated with increased metastatic potential. This suggests that ESR2 expression in primary tumors is not a reliable indicator of metastatic potential. Instead, ESR2 expression in primary tumors may be a marker of tumor aggressiveness. This finding has significant implications for the development of targeted therapies for breast cancer.

This report reevaluates the metastatic potential of ER+ MCF-7 and T47D human breast cancer cells in orthotopic mouse tumors using ZsGreen as a sensitive new tracking method. Cells stably expressing this fluor and grown as xenografts in mammary glands of nude mice were absolutely estrogen dependent. Tumor fluorescence was stable and superficial LN metastases were easily detected microscopically in lymphatics in local and distant LNs and, occasionally, in distant organs. If the primary tumors were ER+ and PR+, then metastases retained receptors, but their expression levels and that of the proliferation marker tumor cells from the primary site, or their deposition and growth at metastatic sites.
5-bromo-2′-deoxyuridine (BrdUrd) were modified by the LN microenvironment. In addition, the hyaluronan receptors, CD44, which control chemotactic behavior of tumor cells (22), were up-regulated in cells transiting the lymphatics and retained in LNs. These data suggest strategies for suppressing LN metastases and indicate that once in the LNs, tumor cells have reduced hormonal sensitivity.

Materials and Methods

Cell lines. MCF-7 cells were originally purchased from American Type Culture Collection (Manassas, VA). Generation of T47D cells that express one PR isoform was previously described (23), and in the present study PR-B expressing T47D cells were used exclusively. The ZsGreen-N1 expression vector, PT-67 packaging cell line, and pLNCX2 retroviral vector were purchased from Clontech/Becton Dickinson (Franklin Lakes, NJ). Fluorescent retroviral vectors were generated by cloning the DNA coding sequence of ZsGreen into the pLNCX2 vector using the restriction enzymes HindIII and NotI and T4 ligase. Plasmids were then transfected into PT-67 packaging cells with a standard calcium phosphate protocol. Stable retrovirus producing cells were selected by 2 weeks of treatment with 500 μg/ml G418. MCF-7 or T47D cells were plated at ~20% confluence and incubated in filtered, virus-containing supernatant. Cells were serially transduced two to three times for 24 hours each round, at which point they exhibited heterogeneous green expression. Cells were then subjected to G418 selection, followed by aseptic fluorescence associated cell sorting (FACS), to isolate a homogeneously bright green subpopulation. These cells were returned to culture and remained bright green through multiple passage generations. Expression of ER and PR in the selected cells was confirmed by immunohistochemistry (see below).

Xenograft tumor growth and metastases. All animal procedures were done under a protocol approved by the University of Colorado Institutional Animal Care and Use Committee. Ovariectomized female athymic nude/nu mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) at 5 to 6 weeks of age. To establish tumors, animals were anesthetized with Avertin and injected into the opening of the lactiferous duct of the abdominal mammary gland (#4) of 5- to 6-week-old, estradiol-supplemented 129/1nu mice supplemented with an estradiol-releasing silastic pellet (24). This injection route led to formation of well-circumscribed tumor nodules distant from the subiliac LN in the gland, as shown by a whole mount 3 days after injection (Fig. 1A, top). In contrast, whole mount of a tumor at 12 weeks shows the subiliac LN, still physically distinct from the primary tumor but already highly involved with tumor cells (Fig. 1A, bottom).

This protocol was used to track the spread of ER+ tumors beyond the #4 mammary gland using whole-body imaging of live mice done weekly. Figure 1B shows an image of tumor tracks photographed through the skin of an intact mouse at 12 weeks. Bright green bilateral tumors are present in mammary gland #4 at the groin, and both subiliac mammary gland LNs contain tumor cells. Also clearly visible are tumor cell tracks in the epigastric collecting lymphatic vessels (LV) draining the tumor cranially on the right, as well as tumor cells in the right axillary LN. Similarly, three distinct green fluorescent clusters of tumor cells are faintly seen in the left lymphatics and axillary LN.

At sacrifice, intravital fluorescence microscopy was used to examine tumor spread to visceral tissues as shown in Fig. 1C and D. An axillary LN micrometastasis is seen in one mouse at 12 weeks (Fig. 1C, left) and a fully involved LN metastasis in another mouse at 12 weeks (Fig. 1C, right). Figure 1C also shows the afferent LV full of tumor cells, with the parallel epigastric vein devoid of such cells. Tumor cells also spread medially to the abdomen. Shown in Fig. 1D is early invasion into a periaortic LN investigator that counted cells from random fields of stained sections using Image Pro software version 4.50.29 (Media Cybernetics, Inc., Silver Spring, MD). Each field was quantified either as a percentage of positively stained cells or an Allred score (ref. 25% percent of labeled cells added to the intensity of immunolabeling). Fields from the same tissue section were averaged and SPSS software version 12.0.2 (SPSS, Inc., Chicago, IL) was used to analyze the data. A Mann-Whitney U test was used to compare BrdUrd-positive HRP-stained cells in independent, nonparametric samples of estradiol- and placebo-treated mouse tumors. Paired t test was used to compare fluorescent-stained cells positive for CK18/BrdUrd, ER, or PR of tumor, LN, and associated lymphatic embolus triad from one mouse to those of other triads from other mice. A marginal homogeneity test was used to compare ER+ and PR+ HRP expression on Allred scores in nonparametric mouse trial samples. For all tests, P < 0.05 was considered significant.

Results

An in vivo model of estrogen-dependent human breast cancer LN metastasis. The ER+ human breast cancer cell lines MCF-7 and T47D are commonly used to study the biology of estrogen action in vitro. These cells also grow as estrogen-dependent xenografts in nude mice (8, 24, 26), but most published reports state that orthotopic tumors fail to metastasize. Because, clinically, ER+ tumors metastasize frequently (4–6), we decided to reevaluate the metastatic potential of ER+ human breast cancer cells in xenografts using sensitive new tracking methods. To this end, MCF-7 and T47D cells were retrovirally transduced with ZsGreen, G418 selected, and the brightest green subpopulation was isolated by FACS sorting. Confocal imaging showed that ZsGreen was highly expressed in both cell types, in the cytoplasm and nuclei (not shown). Intense fluorescence is maintained through multiple passages, the fluor is nontoxic, and photobleaching studies show that ZsGreen, a coral reef protein, is much brighter and more photostable than green fluorescent protein, a jellyfish protein (not shown).

Visualizing lymphovascular tumor cell spread. One million fluorescent tumor cells in Matrigel were injected into the lactiferous duct of the abdominal (#4) mammary gland of 5- to 6-week-old, ovariectomized nude/nu mice supplemented with an estradiol-releasing silastic pellet (24). This injection route led to formation of well-circumscribed tumor cell nodules distant from the subiliac LN in the gland, as shown by a whole mount 3 days after injection (Fig. 1A, top). In contrast, whole mount of a tumor at 12 weeks shows the subiliac LN, still physically distinct from the primary tumor but already highly involved with tumor cells (Fig. 1A, bottom).

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(left) and also much more extensive metastases to periaortic and renal LNs (right).

Bilateral tumor-filled lymphatic tracks exiting a primary tumor are clearly seen in Fig. 2A. LV full of tumor cells extend both cranially and medially from the tumor. It is possible that the tortuously branching network of lymphatics exiting the tumor cranially represents lymphangiogenesis. A higher-power view of the laterally branching iliac LV shows that the tumor cells within it coalesced into emboli rather than moving as discrete cells. In response to mechanical pressure applied either to the tumor or the efferent lymphatic root during dissections, fluorescent emboli in collecting lymphatics can often be seen moving into their draining LN (not shown). This has implications for the order in which breast surgical procedures and sentinel node biopsies are done. In general,

Figure 1. Whole-body and intravital images of ZsGreen-expressing ER+ human breast cancer cells and their LN metastases. A, excised abdominal mammary gland from a 3-day-old MCF-7 tumor, in relation to the subiliac LN (top, dashed circle), and from a 12-week-old MCF-7 tumor with a LN metastasis at a similar site (bottom). B, whole-body image showing bilateral T47D tumors (T), subiliac LN metastases (*), collecting LV (arrows), and axillary LN macrometastases (right) and micrometastases (left; dashed circles). C, intravital image of MCF-7 axillary LN metastasis showing a micrometastasis (left) and a macrometastasis (right) with a collecting LV draining into it. D, intravital image of an MCF-7 metastasis to the left para-aortic LN (left) and more progressive disease showing coalesced left and right para-aortic LNs and further tumor spread caudally to the renal LNs (right).

Figure 2. Identification of cancer cells in LVs. A, top, intravital image of a T47D tumor, showing its metastasis to the subiliac LN, and cells moving through the epigastric and iliac LVs. Bottom, higher magnification of the iliac LV showing clusters of cells moving inwards toward the para-aortic LNs. B, the isolated epigastric LV from (A) showing cancer cells moving through a collecting valve (*) in route to the axillary LN. C, left, an isolated collecting LV full of MCF-7 cancer cells (*) paralleling a milk duct. Right, a serial section confirms the identity of the cancer cells and shows maintenance of ZsGreen fluorescence after tissue processing into paraffin blocks.
tumor cell spread to the superficial LNs is restricted to the side of the body containing the primary tumor, and spread to deeper LNs becomes bilateral with progressive disease (Fig. 1D). Directional valves (asterisk) within the LV do not inhibit tumor cells moving through LVs, as shown in Fig. 2B. Lastly, fluorescence microscopy can be used to analyze clinicopathologic features of tumors and their metastases. Importantly, ZsGreen fluorescence is maintained throughout the histologic fixation process. Figure 2C shows the H&E stain of a collecting LV filled with tumor cells within the mammary gland fat pad. A milk duct runs alongside it. Fluorescence microscopy of a serial section confirms that tumor cells are restricted to the LV.

**Estrogen-dependent tumor growth and rate of LN metastases.** Figure 3 summarizes the primary tumor growth pattern of MCF-7 and T47D cells during 12 weeks of observation and the extent of LN metastasis as assessed by whole-body and intravitral imaging. Growth from both cell types is estradiol dependent, with tumors nearly tripling in size in ~12 weeks (Fig. 3A). Both MCF-7 and T47D tumors produce LN metastases at the same rate, which increases with time and tumor size. At 2 weeks, no metastases occurred. By 4 weeks, ~14% of tumors generated LN metastases. This increased to ~75% at 12 weeks. Development of LN metastases seems to be size dependent (Fig. 3B). A separate time-course study of tumors grown for 1, 6, or 12 weeks showed that metastases to superficial subiliac and axillary LN, or to deeper visceral lateral iliac and aortic lumbar LN surrounding the aorta and kidneys, tend to occur at the same rate.

** Estradiol and LV development.** Because only estradiol-treated tumors yielded LN metastases, we next studied the effects of estradiol on tumor-associated LV development. For these studies, tumors were grown with or without estradiol supplementation for short term (3-10 days) or long term (6-12 weeks) and LV were quantified with lymphatic-specific LYVE-1 and Prox-1 (not shown) antibodies. Peritumoral LVs were identified in both treatment groups and likely represent lymphatics found adjacent to veins and arteries in the normal mouse mammary gland (Fig. 4). Intratumoral LVs were not seen after short-term estradiol treatment or in the estradiol-deprived dormant tumors. Fifty-eight percent of long-term estradiol-treated tumors contained intratumoral LVs (Fig. 4). ZsGreen fluorescence was quenched during the immunolabeling process, allowing for double immunostaining with anti-LYVE and anti-BrdUrd antibodies (Fig. 4) to assess the proliferation state of the LV. This procedure showed the LVs to be BrdUrd positive, suggesting that lymphangiogenesis was occurring. However, double labeling for LYVE and ER expression showed absence of ER in the LVs (not shown). We conclude that LV proliferation, although restricted to the estradiol-dependent growing tumors, is either not itself estradiol dependent or is occurring in response to paracrine signals from surrounding ER+ tumor cells.

**The triad of primary tumor, lymphatic emboli, and LN metastases: proliferation.** Our ability to dissect a primary tumor, its draining lymphatic collecting duct filled with tumor cells, and the downstream tumor-filled LN, from the same mouse, allows assessment of the role of the microenvironment in tumor cell proliferation and steroid receptor expression. BrdUrd uptake, measured with BrdUrd primary antibodies coupled to a HRP or a green fluorescent secondary antibody, was used to assess the proliferation rate of cells in these different compartments. In the absence of estradiol, tumor cells at the injection site remain viable and proliferate slowly with a labeling index <7% (Fig. 5A), but at a rate insufficient to elicit an overall increase in tumor size at 12 (Fig. 3) or 16 to 20 weeks (not shown). These dormant tumors yield no LN metastases. In the presence of estradiol, primary tumors exhibit extensive BrdUrd uptake with a labeling index of ~20%.

To accurately quantify dispersed tumor cells and eliminate the contribution of proliferating normal host cells, tumor cells were immunolabeled with CK18 and a red secondary antibody. In this case, proliferating cells were quantified with anti-BrdUrd and a green fluorescent secondary antibody, and some sections were also counterstained with blue 4',6-diamidino-2-phenylindole (DAPI). Figure 5B shows two “triads” of tumor, LV and LN. Each triad came from the same mouse; the two sets were taken from two different mice. In triad 1, although the primary tumor cells have a high labeling index, proliferation in the LV embolus is low and mainly restricted to cells at the periphery, and in the LN, tumor cells are present only in the subcapsular sinus. The remainder consists of normal DAPI-stained LN cells (some of
which are also undergoing cell division). Triad 2 exhibits a different pattern, with extensive proliferation in the primary tumor and throughout the LV emboli, and the LN is highly involved with rapidly proliferating tumor cells.

Figure 5C quantifies all proliferation data. It shows, first, that the two methods of assessing BrdUrd uptake yielded similar results (compare the two +E "tumor" sets—one assessed with HRP and the other with dual BrdUrd/CK18 immunohistochemistry). As expected, compared with dormant controls, estradiol-treated primary tumors exhibit a statistically significant increase in proliferation rate from $\sim 7$ to $\sim 20\%$. And, compared with the primary tumors, metastatic cells in the LN microenvironment have a statistically significant ($P = 0.003$) further increase to $\sim 27\%$. Proliferation rates of embolic cells in the LV were variable and not statistically different from the other two compartments, although they tended to resemble those in the primary tumor more closely.

The triad of primary tumor, lymphatic emboli, and LN metastases: ER and PR. ER and PR immunohistochemistries for two sets of vehicle versus estradiol-treated triads using MCF-7 derived tumors show the heterogeneity observed (Fig. 6). In the absence of estradiol, levels of ER in tumors are always high. Estradiol treatment leads to extensive tumor ER down-regulation. ER tend to remain low in the LV and LN of Fig. 6 (top) but are somewhat restored in the LN of the second triad (bottom). No PR are expressed without estradiol induction. Estradiol treatment leads to heterogeneous PR expression in tumors, which tends to decrease in LV and LN of the same mouse. Quantitation of PR data from several mice is shown in Fig. 6. There is a clear trend of decreased PR expression as cells spread into LV and LN ($P = 0.052$). A second experiment (not shown) using fluorescent immunohistochemistry for ER and PR showed a similar trend of reduced PR between five tumors and their matched LNs ($P = 0.09$). Each immunohistochemical procedure showed a
nonsignificant slight increase in ER expression in the LN metastases compared with the down-regulated ER in the estradiol-treated primary tumor.

Selection of cells for lymphatic transit: CD44. Hyaluronan regulates various aspects of cell behavior, particularly cell migration and invasiveness. It does so by binding to specific receptors on the cell surface, the best characterized of which is CD44 (22). Human breast cancer cells migrate directionally in response to a hyaluronan gradient depending on CD44 expression levels (22). Breast cancers secrete factors that up-regulate hyaluronan expression in osteoblasts (27), which may promote bone metastasis. Figure 7A shows CD44 expression levels in two primary tumors and in their LV and/or LN metastases. Set A (top) clearly shows extensive overexpression of CD44 in tumor cells occupying the LN microenvironment, compared with the same tumor cells in the mammary gland, where CD44 expression is sparse and heterogeneous. Set B includes a matched LV embolus and again shows sparse and heterogeneous CD44 in the tumor, but homogenous and elevated CD44 in both the LV embolic tumor cells, and the LN metastases.

Distant metastases. Metastases to distant organs were occasionally observed with these models. Figure 7B (left) shows H&E staining of tumor cells metastatic to the lung. Their identity is confirmed by intense ZsGreen fluorescence in a serial section (Fig. 7B, right). These lung metastases could have arisen by direct peritumoral venous invasion and spread (the most direct route to the lungs from distant body sites) or they may reflect indirect spread from an overwhelmed lymphatic system via lymphatic-venous anastomoses. Both mechanisms may contribute to distant organ metastasis in humans (28). It should be stressed that these metastases were rare and they occurred only in animals that also had extensive LN spread.

Perineurial invasion. Perineurial invasion was also noted in tumors arising from both cell lines. Perineurial invasion is observed in a variety of human carcinomas and may contribute to local-regional tumor spread, although it has not been shown to be an independent prognostic indicator in human mammary carcinomas (29). Although not as frequent as lymphatic invasion, perineurial invasion was occasionally found at tumor margins and along the peripheral nerves that parallel the iliac veins. Figure 7C shows an H&E stain (left) and CK18 immunohistochemistry (right) of T47D tumor cells (arrow) tracking along a peripheral nerve (asterisk).

Discussion

LN metastasis in association with tumor size is the single most powerful indicator of poor prognosis in mammary carcinoma (30, 31). Pathologic data sets of patients with breast cancer indicate that, at diagnosis, presence of tumor-infiltrated LNs is common, with estimates ranging from 30% to 50% of cases (32–34), depending on tumor size. The dominant feature of the present model is rapid and reliable LN metastasis of ER+ cells
from orthotopic tumor sites. Two different cell lines, MCF-7 and T47D, generated estrogen-dependent solid tumors and reliably produced metastases to local and distant LNs. Under conditions of the studies, which were terminated at 12 weeks, tumor cells in LV and LN metastases were observed in 75% of all mice and in essentially 100% of mice, the primary tumors of which exceeded ~75 mm² in area (Fig. 3). Clinically, the association between tumor size and LN involvement is well known (35). Metastases to other sites, like the lungs (Fig. 7B), were occasionally observed, as were cells migrating along peripheral nerve tracks (Fig. 7C). The ability of the latter routes to reliably colonize distant organs undoubtedly will require longer observation times. This is suggested by the studies of others. Half a million ER− MDA-231 breast cancer cells, injected directly into the circulation, nevertheless required 10 to 12 weeks before exhibiting limb bone metastases in 30% of mice (36). ER+ ZR75 cells injected into the circulation of mice required 3 to 6 months to yield similar bone metastases (37).

Use of modified breast cancer cells overexpressing hormones (38), growth factors (14, 39), and oncogenes (12) shows that the metastatic potential, route of dispersal, time to metastasis, and preferred organ arrest site of any cell line are dependent on unique factors elaborated by that cell, and that much remains to be learned about the role of estrogens and progestins on these processes. For example, it has been reported that clinically, ER+ tumors are more likely to spread to bones whereas ER− tumors more commonly metastasize to the viscera (40, 41). ER+ metastasis models, reflecting the clinical situation, could help define molecular mechanisms involved in such tissue specificity.

Expression profiling of matched primary and metastatic LN tumor pairs in clinical samples has failed to identify genes that distinguish one from the other (42) because metastases are genetically more closely related to the primary tumor from which they arose than to any other metastasis. However, within matched pairs, select genes are differentially expressed between the tumor and LN metastasis. Common among these are extracellular matrix and cell-matrix interacting genes, like the hyaluronan receptor CD44, which mediates rolling, attachment, and migration of cells on a hyaluronan substratum (43, 44). We show here that CD44 is sparsely and heterogeneously expressed in primary tumors but homogeneously overexpressed in LV tumor cell emboli and LN metastases (Fig. 7). Because directional migration of breast cancer cells towards hyaluronan is dependent on CD44 (22), we propose that the CD44+ subpopulation of primary tumor cells is preferentially propelled into tumoral lymphatics, and from there to LNs. If so, therapeutic targeting of the CD44+ subpopulation in a primary tumor could prevent LN metastases.

There is a high concordance rate (~80-90%) in ER expression between primary tumors and matched LN metastases (5). However, in the same patient, considerable disparities can exist in the concentrations of ER at the two sites, with ER levels often (6, 45) but not always (45) higher in the LN than in the local primary tumor. We observe a pattern (Fig. 6) in which ER levels are higher in the LN than in the primary tumor of the same mouse, an indicator of ER down-regulation failure in the LN microenvironment. It has been reported that there is an association between estrogen-dependent ER down-regulation and improved transcriptional efficacy of the receptors (46). Although it might be counterintuitive to think that in the presence of estrogen, lower ER levels signal a stronger hormone response, this seems to be the case. Thus, the inefficient ER down-regulation in the LN could be associated with poorer hormone responsiveness at that site. In the mouse tumors, PR expression levels (Fig. 6), which are markers of estrogen action and ER function, support this hypothesis. Assessment of PR levels in LNs and matched primary tumors shows a trend towards a decrease in LN PR (Fig. 6). This would be predicted by inefficient ER down-regulation. Clinically, both up-regulation and down-regulation of PR have been reported in the LN compared with primary tumors (45), but this has not been correlated to ER in the same samples.

We also observe a statistically significant increase in the proliferation rate of LN metastases compared with matched primary tumors (Fig. 5B and C). Clinically, increased mitoses in nodal metastases compared with primary tumors have been reported (47), possibly due to release of cytokines and growth factors, such as insulin-like growth factor-I and epidermal growth factor, by the LN (48). The implications of this growth promotion within LNs for failure of hormone treatments in metastatic disease could be important. If, in the LN microenvironment, cytokines and growth factors are the dominant mitogens, then LN metastases would be less sensitive than primary tumors to estradiol-suppressive therapies. Indeed, in metastatic disease,
tamoxifen, which targets ER signaling pathways, is rarely associated with long-term remission, exhibiting decreased mean time to disease progression and decreased duration of response associated with long-term remission, exhibiting decreased mean time to disease progression and decreased duration of response associated with local disease (41, 49, 50).

In a summary, we have developed models of ER+ human breast cancers that rely spread to LNs from solid tumors. Through the use of a new generation fluorescent protein, ZsGreen, we are able to identify cancer cells in tumors, LV emboli, LNs, and, occasionally, more distant sites for further molecular analysis. These studies show that the tumor cells found in LNs express metastatic markers with tumor aggressiveness. At the same time, the LN microenvironment may suppress estrogen sensitivity of tumor cells, which would make them more resistant to primary tumors ER-targeted treatments.

Acknowledgments

Received 5/16/2006; revised 7/6/2006; accepted 7/25/2006.

Grant support: Department of Defense Predoctoral Breast Cancer Training Grant BC050889 (J.C. Harrell); Susan G. Komen Breast Cancer Foundation BCTR0402682 (C.A. Sartorius); and NIH grant CA26869. National Foundation for Cancer Research, the Avon Foundation, and the Breast Cancer Research Foundation grants (K.B. Horvitz). Costs for publication 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.

We thank the University of Colorado Cancer Center Flow Cytometry and Sequencing Core Laboratories.

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