Abstract

It has been established that the invasive behavior of cancer cells can be regulated by alterations in their extracellular environment. We investigated whether extracellular matrix isolated from nulliparous and postlactating (involuting) rat mammary glands differentially modulated the metastatic behavior of human breast cancer cells. Using modified Boyden chamber and three-dimensional culture assays, nulliparous mammary matrix was found to suppress motility and invasion in highly metastatic MDA-MB-435 cells, whereas involution mammary matrix supported motility and invasion in highly metastatic MDA-MB-435 cells, but not in cells with low metastatic potential. Biochemical characterization of the matrices revealed intact fibronectin (FN) and low matrix metalloproteinase activity in nulliparous mammary matrix and fragmented FN and high matrix metalloproteinase activity in the matrix isolated from involuting glands. Purified intact FN was found to inhibit cell invasiveness, whereas FN fragments enhanced cell invasiveness in a matrix metalloproteinase-dependent manner. These data suggest that physiological changes that occur in the mammary extracellular matrix as a result of reproductive status alter the in vitro parameters of metastatic potential.

Introduction

Over 100 years ago, Paget put forth the seed and soil hypothesis, suggesting that a cancer cell (the seed) will not metastasize unless the extracellular environment (the soil) is permissive, a concept that subsequently has been verified (1). The seed and soil hypothesis is also applicable to the ability of a cancer cell to disseminate from the primary tumor. Recent in vitro studies have shown that the ability of a cancer cell to migrate and invade, which is a prerequisite to dissemination, can be regulated by the composition of the extracellular environment (2–4). The mechanisms by which cell-ECM interactions influence the metastatic behavior of tumor cells are varied and include: (a) changes in ECM glycoprotein composition, which can alter cell adhesion and motility; (b) increased ECM-degrading protease activities within the stroma, which presumably facilitate the movement of tumorigenic cells by disrupting basement membrane and stromal barriers; and (c) release of bioactive ECM fragments and/or growth factors that promote tumor cell progression (for review, see Ref. 4). Whereas sufficient data exist that demonstrate that changes in pathogenic tumor-stromal interactions can play critical roles in determining whether a tumor cell will remain dormant or become invasive, it is not known whether physiological changes in the ECM can also contribute to tumor cell dormancy or dissemination in vivo.

Materials and Methods

Animals. Female Sprague Dawley rats (six rats/reproductive stage) were obtained from Taconic Farms (maurue adult nulliparous rats [70 ± 3 days of age], age-matched rats in mid-pregnancy [day 8], and age-matched rats 2–4 days after weaning). For fully regressed, postlactational mammary tissue, MGs were isolated from rats 2 months after weaning. Rats were euthanized by CO2 inhalation, and the inguinal MGs (pairs 4–6) were isolated. Lymph node-free glands were quickly frozen in liquid nitrogen and stored at −70°C until use. For each MG, tissue was also processed for routine histology to verify that the gland was inflammation free. Vaginal cytology and cervical histology were evaluated to confirm reproductive status.

Isolation of Stroma. Isolation of stroma was conducted as described by Hahn and Ip (6), based on the original procedure described by Kleinman et al. (7). Briefly, frozen MG pairs 4–6 (with lymph nodes removed) were pulverized and extracted with a high-salt/N-ethylmaleimide solution [3.4 mM NaCl, 50 mM Tris-HCl (pH 7.4), 4 mM EDTA-Na2, and 2 mM N-ethylmaleimide] containing a proteinase inhibitor mixture (100 μg/ml phenylmethylsulfonyl fluoride and 50 μg/ml aprotonin, leupeptin, and pepstatin) at 4°C. Homogenates were pelleted and washed three times at RCFmax 110,000 × g (30 min, 4°C), and supernatants containing soluble proteins were discarded after each wash. The ECM-enriched pellets were resuspended in a mid-level salt/urea solution [2 M urea, 0.2 M NaCl, 50 mM Tris-HCl (pH 7.4), 4 mM EDTA-Na2, and 2 mM N-ethylmaleimide] with proteinase inhibitor mixture and extracted overnight at 4°C. Samples were pelleted at RCFmax 110,000 × g, and the ECM-enriched supernatants were removed and extensively dialyzed (Mw 12,000–14,000 molecular weight cutoff; Spectrum) against a low-salt buffer [0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), and 4 mM EDTA-Na2], followed by dialysis against serum-free DMEM:Ham’s F-12 (Sigma) supplemented with 1 μg/ml gentamicin at 4°C. Matrices were stored on ice at 4°C and used within 2 weeks of isolation. Six animals were used per stage. For the Boyden chamber invasion assay, two separate preparations (batches) of mammary matrix were evaluated.

Cell Line Propagation. The human breast cancer cell lines used were MCF-7 and derivatives of MDA-MB-435. The breast tumor cell line MCF-7 (obtained from American Type Culture Collection) was passaged as described by the supplier in Eagle’s MEM with nonessential amino acids, 1 mM sodium pyruvate, 10 μM insulin, and 10% FBS. The highly metastatic MDA-MB-435 empty vector control (C-100 cells) and MDA-MB-435 cells transfected with the breast metastasis suppressor gene nm23-H1 (H1-177 cells) were obtained from Dr. Pat Steeg (National Cancer Institute, Bethesda, MD) and cultured in DMEM (Sigma) supplemented with 10% heat-inactivated FBS.

Motility assays were conducted as reported by Kantor et al. (8). Cells were resuspended in serum-free medium and plated at 125,000 cells/filter (24-well
format filters; Falcon). Filters (pore size, 8 μm) were precoated with either 10 μg/ml EHS murine tumor matrix (Matrigel; Collaborative Biomedical) or 10 μg/ml isolated MG matrix. The cells were stimulated to migrate across the filter by providing a chemoattractant (0.5% FBS) in the assay chamber beneath the coated filter to which the cells were added. For FN experiments, filters were coated with 10 μg/ml gelatin control, 10 μg/ml intact FN, or 10 μg/ml FN120, a M 120,000 proteolytic fragment containing the RGD cell binding domain (Life Technologies, Inc.). High motility C-100 cells were assayed 4 h after plating, and low motility cells (MCF-7 and H1-177) were assayed 24 h after plating. Cells were fixed in 10% neutral buffered formalin for 5 min and stained in 0.1% crystal violet. The cells, which crossed through the pores toward the chemoattractant, were quantified photographically, as described previously (8). Assays were performed in triplicate.

**Invasion Assays.** To occlude 8-μm pores, transfilters were coated with 200 μl of 200 μg/ml EHS tumor matrix or isolated MG matrix. Cells were suspended in serum-free medium and plated at 125,000 cells/filter in 24-well plates using 0.5% FBS as chemoattractant in the lower chamber. The number of invasive cells, evaluated 24 h after plating, was quantified as described above. For FN reconstitution experiments, 0, 10, and 20 μg/ml intact FN or FN120 were mixed on ice with 200 μg/ml gelatin, and filter pores were occluded as described above. For experiments designed to investigate the effects of inhibition of MMP activity, the MMP inhibitor GW9471 (British Biotech BB-24) was added to medium at the time of cell plating to a final concentration of 0.5 μm, as described previously (9). Invasion assays were performed in triplicate.

**Three-Dimensional Culture Model.** C-100 cells were overlaid as single cell suspension onto a thick matrix substratum in DMEM supplemented with 2% FBS at a concentration of 100,000 cells/well (24-well plate; Falcon). The matrix substratum was composed of 300 μl of rat MG matrix (isolated based on equal wet weight of mammary tissue) and mixed 1:1 with EHS control matrix. EHS matrix was added to the rat MG matrix to facilitate polymerization. The development of organoids in this three-dimensional culture model is dependent on the migration of single cells into cell aggregates (9). Organoid development was monitored over 120 h using an inverted light microscope (Zeiss Axioscope 25) and photographed with a Polaroid camera at ×100. Matrix substratum and overriding organoids were harvested at 72 and 120 h after plating, fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5-μm sections before staining with H&E. Cytological sections were viewed on a Zeiss Axioscope and photographed.

**Biochemical Analyses.** Zymography and Western analyses were performed as described previously (5). MG stromal preparations were loaded by either wet weight isolation or equal protein concentration, as determined by the BioRad protein assay kit. The rat-specific FN probe was a generous gift from Dr. Pamela Norton (Thomas Jefferson University, Philadelphia, PA). The FN probe was sequenced to verify 100% homology to rat (University of Colorado Health Sciences DNA Sequencing Core Facility). Mammary tissue RNA was isolated using a modified phenol/chloroform procedure (RNAwiz kit; Ambion), with six animals/group. [32P]UTP-labeled antisense FN RNA and rat-specific actin RNA (Ambion) probes were transcribed with the Maxiscript kit (Ambion) and gel-purified. The probes, 2 ng of actin (1.02 × 10^6 cpmp) and 0.5 ng of FN (2.05 × 10^5 cpmp), were combined with RNA samples (2.6–4.75 μg; prenormalized for actin), precipitated, pelleted, and cohybridized overnight at 45°C in RPA III hybridization buffer (Ambion). Hybridized samples were RNase-digested at 37°C for 1 h. Denatured samples were resolved on urea-polyacrylamide gels and exposed to Hyperfilm MP (Amer sham). Semiquantification of the signal was performed on scanned gels (Adobe Photoshop 4.0; Epson Expression 636 scanner) using the UN-SCAN-IT gel automated digitizing system.

**Results**

To determine whether the reproductive state of mammary stroma alters two parameters of the metastatic phenotype, motility and invasion, *in vitro* assays were performed with human breast cancer cells and isolated rat MG matrix. Three tumorigenic cell lines were investigated: (a) low motility MCF-7 cells; (b) highly motile control transfected MDA-MB-435 cells (C-100 cells); and (c) MDA-MB-435 cells transfected with a known suppressor of metastasis, nm23-H1 (H1-177 cells; Ref. 8). ECM isolated from nulliparous and postlactation involuting rat MGs and EHS murine tumor matrix were used as substrata. MCF-7 cells displayed low motility on all matrices tested (Fig. 1). C-100 cells displayed low motility on matrix isolated from nulliparous animals, whereas motility was enhanced approximately 10-fold on matrix isolated from involuting MGs and on EHS tumor matrix. In contrast, motility was suppressed on all matrices in MDA-MB-435 cells transfected with nm23-H1 (H1-177 cells), consistent with previous reports demonstrating the inhibition of migration by nm23-H1 (8).

The effect of reproductive state on the ability of the MG matrix to support cancer cell invasion was also evaluated. Cells were overlaid onto filters in which the 8-μm pores were occluded with matrix so that transit of cells to the lower chamber required matrix digestion (2, 3). As observed in the motility assay, the invasive potential of C-100 cells was highly dependent on the reproductive state of the mammary matrix. C-100 invasion on involution MG matrix was enhanced 3–20-fold over the nulliparous levels (Fig. 1, b and c). MCF-7 cells and MDA-MB-435 cells transfected with nm23-H1 (H1-177 cells) were not observed to be invasive on nulliparous, involution, or EHS tumor substrata (data not shown).
A second in vitro three-dimensional culture assay, which is dependent on both cell motility and invasion (9), was used to further evaluate the effects of MG matrix on cancer cell behavior. In this assay, mammary matrix was evaluated based on wet weight extractability and was not normalized for protein concentration, allowing for the detection of quantitative differences between matrices. The rationale is based on our observation that the concentration of ECM extracted per mg of tissue is consistently higher in involuting MG (≈2 mg matrix/g tissue, for a final concentration of ≈1000 µg/ml) than in nulliparous MG (≈0.4 mg matrix/g tissue, for a final concentration of ≈200 µg/ml). The increase in concentration of ECM proteins in isolated involution matrix may reflect the in vivo extracellular environment because elevated levels of matrix proteins have been reported in involuting mammary tissue (5, 9). Using the modified Boyden chamber assays, it is not possible to test high concentrations of matrix proteins because concentrations of ECM above 50 µg/ml inhibited motility, and concentrations above 250 µg/ml inhibited invasion. However, in the three-dimensional organoid assay, MG matrix can be evaluated as isolated. Single cell suspensions of C-100 cells overlaid onto thick substrate of EHS tumor matrix formed simple sphere-shaped organoids by 72 h after plating (Fig. 2, a and b). More complex organoids resembling mammary ducts developed when C-100 cells were overlaid onto involution MG matrix (Fig. 2g). In contrast, organoids did not develop when C-100 cells were plated on nulliparous MG matrix; instead cells organized into loose two-dimensional sheets (Fig. 2d and e). These data suggest that EHS tumor and involution MG matrices supported the requisite cell migration required for organoid formation, whereas nulliparous MG matrix did not, observations consistent with the modified Boyden motility assay results (Fig. 1a). Evaluation of 5-µm sections of 120 h organoids demonstrated that EHS tumor and involution MG matrices also supported cancer cell dissemination from primary organoids into the surrounding matrices. Apparent “growth cones” of cells disseminating from the primary organoid were evident at 120 h in organoids that formed on EHS tumor and involution MG matrices (Fig. 2, c and i), but not on nulliparous MG matrix (Fig. 2f), corroborating the modified Boyden chamber results in which invasion was observed maximally on involution MG and EHS matrices and was absent or reduced on nulliparous MG matrix (Fig. 1, b and c).

We have demonstrated previously that mammary tissue from involuting glands has elevated levels of fragmented FN and Ms~97,000 gelatinase activity, consistent with MMP-9 (5, 9). To determine whether FN fragments and MMP activity isolate with involution matrix and contribute to the biological activity of cells plated onto involution matrix, biochemical characterizations of the matrices were performed. Matrices isolated from mid-pregnancy and fully regressed MGs are included for comparison. MMP activity, assayed by a substrate gel analysis, revealed elevated levels of both a Mr~97,000 and a Mr~72,000 gelatinase in involution MG matrix (Fig. 3a). FN integrity was determined by Western analysis. When MG matrix was analyzed based on wet weight extractability (the conditions used in the three-dimensional organoid assay), FN fragmentation was clearly evident in involution matrix (Fig. 3b). When FN was evaluated in matrix samples loaded by equal protein (the conditions used in the modified Boyden chamber assays), the absolute amount of intact FN was low in involution matrix (Fig. 3c and d). The reduced level of intact FN in isolated involution matrix may reflect in vivo MG biology. This argument is supported by FN RNA levels, which were decreased ~70% during postlactational involution (Fig. 3e). Taken together, these data are consistent with our previous results that indicate that mammary FN is targeted for degradation during the period of MG remodeling after weaning in vivo (9) and suggest that FN is a candidate ECM component that may modulate tumor cell motility and invasion.

The role of FN integrity in modulating in vitro C-100 motility and invasion was investigated in reconstitution experiments using the modified Boyden chamber assays. C-100 motility was significantly inhibited by intact FN and enhanced by FN120 fragments in comparison to gelatin controls (data not shown). C-100 invasion was also inhibited by intact FN and stimulated by FN fragments (Fig. 4a). H1-177 cells, which were not invasive when overlaid onto involution mammary matrix (Fig. 1a), were not induced to be invasive when plated onto FN120-containing substrate (data not shown). Evidence that the invasive behavior of C-100 cells is, in part, MMP dependent is demonstrated by suppression of invasion in the presence of the MMP inhibitor GW9471 (Fig. 4b).

Discussion

Tumor-stromal interactions regulate key events in the metastatic cascade in vitro and in vivo (2, 3, 4, 10). Because composition, structure, and function of the MG stroma vary with reproductive state,
involution. In support of this, cells may be resistant to the epithelial cell death signals present during cell survival is a hallmark of cancer cells, suggesting that tumor death (5). In the rodent, death of the alveolar cells appears to be due, involution, normal mammary alveolar cells undergo apoptotic cell organized by active tissue remodeling, mediated in part by MMPs. During the environment of the MG during postlactational involution is characterized by adipose-rich stromal tissue (5, 11). In contrast, the extracellular environment of the MG during postlactational involution is characterized by active tissue remodeling, mediated in part by MMPs. During involution, normal mammary alveolar cells undergo apoptotic cell death (5). In the rodent, death of the alveolar cells appears to be due, in part, to loss of cell adhesion as a result of MMP-mediated degradation of the ECM (5, 12). Importantly, loss of anchorage dependence for cell survival is a hallmark of cancer cells, suggesting that tumor cells may be resistant to the epithelial cell death signals present during involution. In support of this, in vivo data have demonstrated that transformed mammary cells can remain viable during involution (Ref. 5 and the references therein). Because stroma, particularly intact basement membranes, are considered barriers to tumor cell dissemination (2–4, 10), dissolution of these natural barriers, which occurs during postlactational involution of the MG, may be anticipated to facilitate the dissemination of preexisting tumor cells in vivo. In this study, we have demonstrated for the first time that the reproductive state of the rat MG matrix can alter in vitro parameters of metastasis.

Using C-100 cells, derivatives of the highly metastatic human mammary epithelial cell line MDA-MB-435, in vitro motility and invasion were observed to be low on nulliparous MG matrix, which is consistent with C-100 metastatic potential being down-regulated by stromal interactions provided by nulliparous MG matrix. Intact FN was detected in nulliparous matrix, and, in reconstitution experiments, intact FN was identified as a suppressor of motility and invasion. Whether other ECM components, which can actively suppress in vitro tumor cell motility and invasion, are present in nulliparous MG matrix remains to be determined.

In contrast to results obtained with nulliparous MG matrix, C-100 motility and invasion were significantly elevated on matrix isolated from MGs undergoing postlactational involution and on matrix isolated from EHS murine sarcoma. Importantly, neither of these matrices promoted motility and invasion in the less metastatic cell line, MCF-7, or in C-100 cells transfected with the known tumor suppressor gene nm23-H1 (8). Taken together, these observations suggest that EHS tumor and involution MG matrices are permissive, but not instructive, for tumor cell motility and invasion. That is, these matrices do not appear to induce motility or invasion in cells that do not already have the ability to express these phenotypes.

MMP activities, consistent with MMP-9 and MMP-2, were significantly increased in involution MG matrix in comparison with nulliparous matrix. This observation demonstrates that MMP activity isolates with the ECM, and is in agreement with a large body of data demonstrating elevated stromal levels of MMPs in mammary tissue after weaning (Ref. 5 and the references therein). In vivo, elevated...
stromal MMP-9 and MMP-2 activity is highly correlated with increased metastatic potential in most epithelial cancers, including breast cancer (3, 4, 10). MMP-9 and MMP-2 are also present in the EHS tumor matrix used in these studies (13), further suggesting a causal role between elevated MMP activity and the increased tumor cell motility observed.

Increased fragmentation of FN was detected in mammary stroma isolated from involuting MGs in comparison with nulliparous MG matrix. Experiments aimed at investigating the potential role of FN fragments in modulating cell behavior identified significantly increased C-100 cell migration and invasion on FN120 as compared with intact FN. These observations are in agreement with reports by others demonstrating enhanced cancer cell motility and invasion on fragmented ECM glycoproteins in comparison with intact ECM glycoproteins, including FN (14) and laminin (15). In addition, cell invasion on FN fragments was found to be inhibited to a greater degree by MMP inhibitors than invasion on gelatin, suggesting that cell invasion on FN fragments may be more MMP-dependent than cell invasion on gelatin. Of potential relevance is the observation that FN120 fragments, but not intact FN, can induce MMP-9 activity in mammary epithelial cells in vitro (9). In vivo, induction of MMP activity by FN fragments may induce a feedback loop during MG involution, contributing to further matrix degradation, which results in an environment permissive for tumor cell dissemination. However, the magnitude of the enhancement of in vitro motility and invasion on FN fragments was less than that observed on invasion MG matrix. In addition, significant fragmentation of FN was not detected in EHS tumor matrix (data not shown), which also supports motility and invasion. These observations suggest that whereas FN fragments may contribute to the enhanced C-100 cell motility observed on invasion MG matrix, other ECM components are likely to be involved as well.

The in vitro work reported here raises the question of whether stroma may become permissive for tumor cell dissemination during MG involution in women. Based on our results, we hypothesize that a subset of women with preexisting malignant breast lesions may be at increased risk for tumor progression during postlactational regression of the MG. Our hypothesis that mammary involution might promote tumor progression appears to be in conflict with the demonstrable protective effect of pregnancy. Clearly, early first-term and multiple full-term pregnancies are associated with a lifetime reduction in risk for breast cancer in women, establishing that, overall, pregnancy is protective for breast cancer (16). However, of potential relevance to our hypothesis are the observations made as the “cross-over” and “dual” effects of pregnancy (see Refs. 16 and 17, for review). In younger women, breast cancer incidence for parous women is actually greater than that for nulliparous women for 20–30 years after the time of the first birth (16, 17). Starting at around age 50 years, a “cross-over” occurs, in which parous women experience a reduction in risk for breast cancer in comparison with aged-matched nulliparous women (16, 17). In addition, first full-term pregnancy in older women has been shown to be associated with a permanent increase in breast cancer risk (16). Because the protective effect of pregnancy is not constant but rather depends on the age of first pregnancy, the effect of pregnancy on breast cancer risk is considered “dual” (16, 17). Whereas it is widely recognized that pregnancy may promote breast cancer because of high levels of ovarian hormones, which stimulate tumor cell proliferation (18), we suggest that, in addition, postpregnancy-associated changes in mammary stroma may contribute to breast cancer promotion. Specifically, we propose that the period of active tissue remodeling that occurs during postlactational involution may be permissive for tumor cell dissemination. Our rationale is based on the observations that tissue remodeling provides a break in the natural stromal barriers that suppress tumor cell motility and invasion. In addition, mammary tissue remodeling may release and/or induce ECM components that can promote tumor cell progression, such as MMPs, transforming growth factor β, hepatocyte growth factor/scatter factor, and FN fragments (Ref. 5 and the references therein and Ref. 19).

In conclusion, we have demonstrated that ECM components of rat MG stroma, which can modulate in vitro measurements of the metastatic phenotype, are altered by reproductive state. The data support a large body of research demonstrating that the “unit of function” of the normal and malignant MG includes both the epithelial cell and its surrounding stromal component (20). Consequently, it becomes increasingly important to investigate the apparent complex relationships between reproductive status, MG stroma, and modulation of mammary tumor cell dormancy and dissemination.

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References

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