Cancer growth and spread are saltatory and phase-locked to the reproductive cycle through mediators of angiogenesis

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Abstract

The frequency of breast cancer metastatic spread is affected by the menstrual cycle phase of its resection. Breast cancer growth, post-resection spread, and cure frequency are each modulated by the estrous cycle in C3HeB/FeJ mice. Tumor metastases are 2- to 3-fold more frequent when the resection is done during diestrus as compared with estrus. Tumor angiogenesis is essential for both cancer growth and lethal metastatic cancer spread. The balance between vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) modulates new blood vessel formation and blood vessel permeability. Sex hormones modulate the expression of these key angiogenesis regulators in the endometrium and uterus. We, therefore, asked whether the estrous cycle modulates the density of CD31-positive vessels within the tumor, the permeability of tumor blood vessels, levels of VEGF and bFGF immunoreactive protein in normal breast and breast cancer, and whether expression of these genes are modulated by the estrous cycle stage in C3HeB/FeJ mice. We find that tumor blood vessel density and blood volume do not vary throughout the cycle; however, tumor capillary permeability is regulated by the estrous cycle stage being highest in diestrus, the cycle stage associated with the highest cancer growth rate and the highest frequency of post-resection cancer metastasis. VEGF protein levels in breast cancer are >100-fold higher than in normal breast. VEGF protein in this mammary tumor varies with the estrus cycle with highest levels in proestrus. In a non-breast tumor, methylcholantrenene A sarcoma, from CD2F1 mice, tumor VEGF protein also varies with the estrus cycle with highest levels in proestrus and diestrus. VEGF gene expression in the mammary tumor does not change significantly across the cycle, but is modulated by the cycle in normal breast tissue. bFGF protein concentration is 6-fold higher in normal breast than in breast cancer. bFGF protein pattern in both tumor and breast are similar, opposite to VEGF, and affected by oophorectomy. bFGF message is modulated by the cycle in both breast cancer and normal breast. The changes in breast cancer capillary permeability, VEGF, and bFGF that occur during each fertility cycle, in breast tissue and breast cancer, putatively in response to cyclical changes in sex hormones, might contribute, at least in part, to both the modulation of cancer growth and post-resection breast cancer spread by the fertility cycle. These fertility cycle-induced effects on tumor biology also seem to extend to non-breast cancer biology. [Mol Cancer Ther 2005;4(7):1065–75]

Introduction

The mammalian fertility cycle affects breast cancer growth and spread (1–3). In a transplantable mouse breast cancer model, tumor growth is consistently slower during estrus than during diestrus (1). In this model, the timing of resection of equal sized breast cancers, within the estrous cycle, determines the frequency with which the cancer metastasizes following resection. Two to three times as many mice are cured by primary tumor resection done at or near estrus, as compared with when cancers are resected at diestrus (3, 4). Clinical data indicate that the timing of breast cancer surgery during the menstrual cycle meaningfully affects breast cancer control (5–11). In aggregate, the most high-quality retrospective clinical studies, two metaanalyses and the single prospective study done to date, show an average absolute 25% 10-year disease-free survival advantage for premenopausal women whose breast cancers are resected during early luteal phase of their menstrual cycle, as compared with the follicular phase (12–14).

We do not know how the estrous and menstrual cycles modulate cancer growth and post-resection metastatic potential. We do know, however, that tumor blood vessel permeability and angiogenesis are each essential for cancer growth and spread (15). We also know that progesterone and estrogen modulate new blood
vessel formation and capillary permeability in the uterus and ovary (16). These sex hormones might, therefore, regulate the growth and post-resection spread of breast cancer cells, at least in part, by stimulating the production of angiogenesis modulating molecules such as vascular endothelial growth factor (VEGF) and/or basic fibroblast growth factor (bFGF) within breast cancers. There is reason to believe that the balance or the ratio between these two molecules may be largely responsible for microvasculature changes essential for both tumor growth and metastasis following primary cancer resection (16).

VEGF is a mitogen specific for vascular endothelial cells, and a known enhancer of vascular permeability (17). VEGF mRNA and protein levels are regulated by estrogen and progesterone, in rat uterus (18, 19). VEGF is produced by human and rodent breast cancer cells (20). Increased tumor cell VEGF expression and increased microvessel density in primary breast cancer are each associated with decreased patient survival (20, 21). In mice, shutting down VEGF effects, either with antisense VEGF oligonucleotides or monoclonal antibody to VEGF, decreases tumor blood vessel density and tumor growth rate and diminishes the frequency of metastasis (22, 23). Conversely, overexpression of VEGF enriches tumor vasculature, growth, increases tumor vessel permeability, and enhances metastatic cancer spread (24). Tumor vascular permeability is reduced within hours by VEGF antibody, strongly suggesting that maintenance of tumor vessel integrity requires the presence of VEGF within the tumor microenvironment (25). Successfully metastatic tumor cells must traverse these vessels at least twice during metastatic transposition. Therefore, the VEGF modulation of vascular integrity may be necessary for cancer spread.

bFGF is both a mitogen for a variety of cell types, including the endothelial cell, and, in many circumstances, a negative modulator of angiogenesis (26). bFGF mRNA and protein levels are regulated by estrogen and progesterone, in rat uterus (18). bFGF is present in human mammary tumor cytosol (27). High tumor bFGF levels are associated with high breast cancer estrogen receptor concentrations, low grade (good prognosis) histopathology, and small primary tumors (27). Patients whose tumors contain high concentrations of bFGF protein show better breast cancer survival. This is also true when patients’ tumors show high bFGF mRNA levels (28, 29). A low bFGF level in breast carcinoma is an independent indicator of poor prognosis, adumbrating early disease recurrence and death.

Therefore, high VEGF tumor levels and low bFGF levels independently predict poor breast cancer outcomes. The relationship between the concentrations of these two potentially sex hormone–regulated angiogenesis modulators with, in some circumstances, opposite action may, help to explain the fertility cycle stage dependence of breast cancer growth and post-resection spread. Therefore, we asked whether VEGF and/or bFGF concentrations and levels of gene expression in mammary tumors and normal mammary tissue are modulated rhythmically by the mouse fertility cycle in ways that might help explain the dependence of post-resection breast cancer spread upon the murine estrous and, by analogy, the human menstrual cycle.

Materials and Methods

C\textsubscript{3}H Mammary Tumor Model

Sexually mature, female C\textsubscript{3}HHeB/FeJ mice (The Jackson Laboratory, Bar Harbor, ME), 10 to 14 weeks of age, were housed four per cage alongside singly housed male mice, to enhance estrous cycling as in our previous studies (3, 4). All procedures were done in the same quadrant (14 hours after lights on) of the circadian cycle (time of day) because of the known variation of the immune response, surgical response, and tumor behavior with circadian time (30). All animals were kept on lighting schedules with 12 hours light alternating with 12 hours of dark with food and water freely available. In a subgroup of mice (see Table 1, row 1), bilateral oophorectomy was done (n = 10) at 10 weeks of age. Confirmation of oophorectomy was accomplished through serial vaginal cytology, described below. The primary mammary tumor (B. Fisher, University of Pittsburgh, Pittsburgh, PA) originated spontaneously in a female C\textsubscript{3}H mouse and has subsequently been passed in vivo in C\textsubscript{3}HeB/FeJ female mice (31). Tumors were harvested under sterile conditions and tumor cell suspensions made by gentle grinding of minced tumor pieces over a stainless steel mesh into Medium 199 (Life Technologies, Gaithersburg, MD). Tumor cells were inoculated s.c. at 2 \times 10^7 viable cells in the right hind flank. Tumor sites were palpated and subsequently measured twice daily at 12-hour intervals (length, width, and height) by the same individual, using calipers, and estrous cycle stage was concurrently determined by vaginal smear. Average tumor growth rates were then computed for each estrus cycle stage. Tumors were excised from animals in one of four estrous stages (n = 12/stage) and ovariectomized animals (n = 15/stage), at an average size of 1,300 mm\textsuperscript{3}. Serum was recovered and stored at −80°C. Lower mammary gland from the opposite side of s.c. tumor was isolated from C\textsubscript{3}H tumor–bearing animals. Histologic examination confirmed the tissue as normal mammary gland devoid of any tumor. All resections were done without any knowledge of the estrous cycle phase at the time of that resection (blinded).

CD\textsubscript{2}F\textsubscript{1} Sarcoma Tumor Model

Female CD\textsubscript{2}F\textsubscript{1} mice 10 to 14 weeks old were purchased from Charles River (Portage, MI). The animals were housed four per cage and maintained in a 12-hour light, 12-hour dark cycle with food and water ad libitum. Ascitic tumor cell suspension of methylcholantrene A sarcoma was harvested from BALB/c female mouse, centrifuged and resuspended in DMEM and 5 \times 10\textsuperscript{5} cells were inoculated s.c. on the backs of mice during the activity cycle. Animals were sacrificed at mean tumor size of 990 mm\textsuperscript{3} at one of
<table>
<thead>
<tr>
<th></th>
<th>Proestrus</th>
<th>Estrus</th>
<th>Metestrus</th>
<th>Diestrus</th>
<th>Analysis results (across four estrous cycles)</th>
<th>Oophorectomized mice</th>
<th>t test (diestrus versus oophorectomy)</th>
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<tbody>
<tr>
<td><strong>Cancer biology</strong></td>
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<tr>
<td>Surgical cure (%)*</td>
<td>33 (n = 18)</td>
<td>96 (n = 26)</td>
<td>79 (n = 14)</td>
<td>44 (n = 10)</td>
<td>24.6† &lt;0.001</td>
<td>50 (n = 10)</td>
<td>nd</td>
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<tr>
<td>Cancer growth rate (mm³/d)^†</td>
<td>214.8 ± 30.1</td>
<td>308.4 ± 38.4</td>
<td>276.1 ± 33.5</td>
<td>636.2 ± 54.3</td>
<td>21.1 &lt;0.001</td>
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<td><strong>Hormones</strong></td>
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<td>Circulating estrogen (pg/mL)^x</td>
<td>10.3 ± 1.1</td>
<td>4.2 ± 1.0</td>
<td>3.1 ± 0.7</td>
<td>7.5 ± 1.3</td>
<td>nd</td>
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<tr>
<td>Circulating progesterone (ng/mL)^k</td>
<td>60</td>
<td>8</td>
<td>20</td>
<td>8</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td><strong>Growth factors</strong></td>
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<tr>
<td>VEGF Tumor protein (pg/mg)</td>
<td>24.7 ± 3.1</td>
<td>16.1 ± 1.2</td>
<td>14.9 ± 1.4</td>
<td>12.9 ± 1.8</td>
<td>6.5 0.001</td>
<td>12.6 ± 1.0</td>
<td>0.12</td>
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<tr>
<td>VEGF Tumor mRNA (PI units)^a</td>
<td>0.53 ± 0.13</td>
<td>0.70 ± 0.1</td>
<td>0.81 ± 0.06</td>
<td>0.75 ± 0.1</td>
<td>1.3 0.28</td>
<td>0.67 ± 0.09</td>
<td>0.56</td>
</tr>
<tr>
<td>VEGF Normal mammary protein (pg/mg)</td>
<td>0.03 ± 0.002</td>
<td>0.09 ± 0.03</td>
<td>0.05 ± 0.007</td>
<td>0.05 ± 0.008</td>
<td>2.4 0.08</td>
<td>0.03 ± 0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>VEGF Normal mammary mRNA (PI units)</td>
<td>0.35 ± 0.07</td>
<td>0.32 ± 0.03</td>
<td>0.17 ± 0.02</td>
<td>0.21 ± 0.04</td>
<td>3.3 0.03</td>
<td>0.25 ± 0.06</td>
<td>0.48</td>
</tr>
<tr>
<td>VEGF Serum protein (pg/mL)</td>
<td>1.58 ± 17.3</td>
<td>201 ± 51.1</td>
<td>134 ± 28.8</td>
<td>244 ± 74.6</td>
<td>0.98 0.42</td>
<td>98.9 ± 17.3</td>
<td>0.09</td>
</tr>
<tr>
<td>VEGF Methylcholantrenene A sarcoma VEGF Tumor protein absorbance**</td>
<td>0.054 ± 0.005</td>
<td>0.033 ± 0.006</td>
<td>0.043 ± 0.008</td>
<td>0.056 ± 0.005</td>
<td>3.42 0.024</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>bFGF Tumor protein (pg/mg)</td>
<td>0.29 ± 0.05</td>
<td>0.44 ± 0.06</td>
<td>0.52 ± 0.07</td>
<td>0.36 ± 0.09</td>
<td>1.2 0.34</td>
<td>0.24 ± 0.06</td>
<td>0.07</td>
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<tr>
<td>bFGF Tumor mRNA (PI units)</td>
<td>0.51 ± 0.11</td>
<td>0.33 ± 0.1</td>
<td>0.33 ± 0.05</td>
<td>0.2 ± 0.03</td>
<td>4.4 0.01</td>
<td>0.17 ± 0.02</td>
<td>0.46</td>
</tr>
<tr>
<td>bFGF Normal mammary protein (pg/mg)</td>
<td>1.9 ± 0.11</td>
<td>2.1 ± 0.42</td>
<td>3.3 ± 0.58</td>
<td>2.8 ± 0.32</td>
<td>2.6 0.06</td>
<td>1.7 ± 0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>bFGF Normal mammary mRNA (PI units)</td>
<td>0.19 ± 0.04</td>
<td>0.25 ± 0.03</td>
<td>0.14 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>4.1 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.67</td>
</tr>
<tr>
<td>Tumor CD31 blood vessel</td>
<td>8.2 ± 0.65</td>
<td>6.21 ± 0.75</td>
<td>7.51 ± 0.71</td>
<td>8.1 ± 0.38</td>
<td>1.85 0.15</td>
<td>7.3 ± 1.38</td>
<td>0.54</td>
</tr>
<tr>
<td>Tumor vascular volume††</td>
<td>0.043 ± 0.015</td>
<td>0.069 ± 0.013</td>
<td>0.049 ± 0.007</td>
<td>0.063 ± 0.009</td>
<td>1.1 0.35</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Tumor capillary permeability ††</td>
<td>0.11 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.16 + 0.01</td>
<td>3.9 0.01</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Abbreviation: nd, not done.

*Ref. 3.
†x² value.
|Ref. 1.
††Ref. 41.
‡‡Ref. 42.

VEGF methylcholantrenene A sarcoma (immunohistochemistry), density over a high power field (all other data in this table are from C3H tissue/tumor).

**PI units, relative phosphoimage units. Tissue-specific PCR samples are processed simultaneously for a specific gene so that comparisons could be made across estrous cycle and expressed relative to control gene for each sample.

††Milliliters of blood per gram of tissue. Extravascular plasma volume per gram of tissue per hour.

Extravascular plasma volume per gram of tissue per hour.
four estrous stages \((n = 10-20/\text{stage})\). Tumors were dissected away from skin and underlying muscle and fixed in 10% buffered formalin for 24 hours and embedded in paraffin blocks.

**Fertility Cycle Phase Determination**

Daily vaginal smears were done using sterile saline washings stained with Diff Quik (Baker, Newark, DE), and were read by one individual. Slides from each mouse were read in sequence to determine the orderly progression of cycling and to classify each of those smears as either proestrus (P), estrus (E), metestrus (M), or diestrus (D; refs. 3, 32). Estrous stage was determined daily, starting 4 days prior to tumor inoculation until sacrifice to confirm regular cycling in each mouse and to assign the most precise estrous stage determination at the time of sacrifice. In our previous studies with these tumor models, estrous cycling continues regularly with minimal perturbation until just prior to death when a slight prolongation of the cycle length is observed only in the last week of life (33).

**Reverse Transcription-PCR**

Tissues were rapidly collected, homogenized and total RNA recovered (Trizol, Life Technologies). First-strand cDNA was generated from 1.0 \(\mu\)g of total RNA using SuperScript II reverse transcriptase (Life Technologies). Quantitative PCR was done using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer, Norwalk, CT) with \(^{32}\)P-labeled dCTP. Oligonucleotide primed primers for mouse VEGF, bFGF, ribosomal protein S16, and histone H1 were purchased from Life Technologies. PCR samples were fractionated by electrophoresis on an 8% PAGE and quantitated by phosphorimage analysis (STORM 860, Molecular Dynamics, Sunnyvale, CA). The linear range of amplification was determined for each tissue and each primer pair. Results are expressed as the ratio of the gene of interest to control gene for each sample (ribosomal S16 for tumor samples, and histone H1 for normal mammary tissue samples as levels of S16 varied significantly across the fertility cycle in normal mammary tissue but not in tumor samples).

**VEGF/bFGF Immunoassay**

Concentrations of VEGF in mouse serum and tissue homogenates were quantified using a “QuantikineM” mouse VEGF immunoassay (R&D Systems, Inc., Minneapolis, MN). Concentrations of mouse bFGF in tissue homogenates were quantified using a Quantikine human bFGF immunoassay (R&D Systems). Tissues were homogenized on ice in buffer containing 50 mmol/L Tris-HCl, 0.5% NP40, 1 mmol/L DTT, 100 mmol/L NaF, 0.1 mmol/L Na3VO4, and protease inhibitor cocktail (Mini-protean, Boehringer Mannheim (Indianapolis, IN)). The 12,000 \(\times\) g supernatant was collected and assayed.

**Tumor Tissue Array**

A trained clinical and experimental pathologist examined H&E sections from each tumor and marked the most viable areas of the tumor tissue. These areas were aligned with the tumor specimen within each tissue block for tissue array core sampling. A tissue array instrument (Beecher Instruments, Inc., Sun Prairie, WI) was used to sample and transfer the paraffin-fixed tissue cores into predrilled holes on a recipient paraffin block. For each tumor block, a tissue core was taken, labeled by position, and arrayed side by side in the recipient block. Multiple 5-\(\mu\)m sections were cut from the array block and mounted on the positively charged glass slides (SurgiPath, Richmond, IL) for histopathologic and immunohistochemical examination.

**VEGF Protein Immunohistochemistry**

The histopathologically selected tissue array sections, after deparaffinization and hydration, were digested using pepsin (4 mg/mL in 0.01 N HCl solution) for 40 minutes and washed in PBS twice (pH 7.2) for 5 minutes. Endogenous peroxidase activity was blocked by 3% \(\text{H}_2\text{O}_2\) in PBS for 15 minutes. Slides were incubated in normal goat serum for 1 hour at room temperature. The primary antibody (anti-VEGF mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA), was applied to sections at 1:400 dilution and incubated overnight at 4°C. The secondary biotinylated rabbit anti-mouse antibody and ExtrAvidin-peroxidase (B-6398 and E-8386, Sigma, St. Louis, MO) were applied for 45 and 30 minutes at room temperature, respectively. Between incubations, slides were washed thrice (5 min/each) in PBS. The color was developed by AEC substrate (AEC-101 kit, Sigma). The sections were finally counterstained with 1% methyl green solution (Sigma). The same tissue array slides were stained without primary antibody as negative controls.

**CD31 Immunohistochemistry**

CD31 is expressed in new blood vessels. Tumors were collected, fixed in 10% buffered formalin for <24 hours and embedded in paraffin for immunohistochemical analysis (CD31 staining) as previously described (21, 34, 35). Blood vessels staining positively for CD31 were then morphometrically enumerated.

**Quantitation of Immunostain**

The immunostained tumor tissue array sections were viewed under the Axioskop microscope. A digital image was taken from each of tumor tissue core using AxiosVision (Carl Zeiss, Germany) and analyzed using SigmaScan Pro4 (SPSS, Inc., Chicago, IL). The target objectives in this image were defined and selected by a preset intensity. The average intensity of the objectives \((\text{Obj}_{\text{intensity}})\) was measured. Images were also taken from the coordinated negative control stain sections to estimate background intensity \((\text{B}_{\text{intensity}})\). The final formula for calculating specific VEGF immunostain intensity was (36–38):

\[
\text{Obj}_{\text{intensity}} = (\log 255 - \log \text{Obj}_{\text{intensity}}) - (\log \text{B}_{\text{intensity}})
\]

**Tumor Blood Volume and Capillary Permeability Determination**

\(^{59}\)Fe-labeling of homologous RBC was accomplished by i.p. injection of 0.5 \(\mu\)Ci \(^{59}\)Fe-chloride (New England Nuclear, NEN, Newton, MA) into non–tumor-bearing C3HeB/FeJ blood donor mice followed 48 hours later by exsanguination under methoxyflurane anesthesia and euthanasia. The blood was washed twice with PBS and
of 125I-labeled bovine albumin (1-4 μCi, NEN) and given by tail vein injection to tumor-bearing mice 1 hour before euthanasia. Following exsanguination, the radioactivity of each isotope per volume of central blood and per gram wet weight of tissue was determined in the tumor using a gamma counter. Blood content and capillary leak was determined by dilution, as previously described (39, 40).

**Tumor Growth Rate throughout the Estrus Cycle**

We borrowed data on tumor size measurements and fertility cycle stage of C3HeB/FeJ female mice in our previous study (1). In that study, MTP breast tumor cells were inoculated s.c. into the right flank and three-dimensional tumor size (TS = length × width × height) was measured by calipers from the time of tumor appearance until sacrifice. Measurements were made once daily at 14 hours after lights on. Vaginal smears were obtained from mice at the time of tumor inoculation and at each tumor measurement. Different estrous cycle stages, successive cycle numbers (cycle number 1, 2, 3, etc.) and estrous stages within each of these cycles (P1, E1, M1, D1; P2, E2, M2, D2; etc.) were assigned starting from the time of tumor inoculation until the last measurement. Daily tumor growth rate is then obtained as the increment in tumor size from the preceding tumor size measurement. Average growth rate is then obtained as the increment in tumor size tumor inoculation until the last measurement.

**Sex Hormone Concentrations during the Estrous Cycle**

These values were obtained from two reports (41, 42). Frequent serum measurements of estrogen and progesterone were made in each of these papers by sacrificing groups of mice at frequent intervals (up to every 20 minutes). For our purposes, the values during each vaginal smear identifiable cycle phase were averaged and/or extrapolated. These averages appear in Table 1 and Fig. 1 presented as the percentage of overall estrous cycle mean value.

**Statistical/Parametric Analyses**

Numerical values were contrasted across the four estrous cycle phases using one way ANOVA with a commercially available statistical program (SuperANOVA). When two groups were compared with one another (i.e., diestrous stage obtained tissues versus tissues from ovariectomized animals), Student’s two-tailed t test was done (SuperANOVA). A P ≤ 0.05 is considered statistically significant. Patterns of hormones and growth factors are double-plotted along the estrous cycle. Double plotting of rhythmic patterns is a standard chronobiological technique that allows visualization of recurring patterns. In order to visually examine rhythmic covariations of tumor growth, hormone, and growth factor values were correspondingly standardized and plotted simultaneously. Standardized rates were obtained as (x – μ) / SD, where x is the estrous stage mean, μ the overall mean across all estrous stages.

**Results**

**Angiogenic Growth Factor Expression**

**VEGF Serum Levels in Tumor-Bearing C3H Mice**

Because a prior study in humans reported plasma VEGF protein levels to vary throughout the menstrual cycle in normal women (43), we wondered if serum VEGF levels would be affected by the estrous cycle. Serum VEGF protein levels did not vary significantly in C3H mammary tumor bearing mice with fertility cycle stage or oophorectomy state, as determined by immunoassay (P = 0.42, Table 1). We could not obtain unclotted blood from these mice for practical reasons, and thereby have no data on plasma concentrations of this molecule. The amount of serum VEGF produced by blood clotting–associated platelet activation and aggregation is substantial, and could thereby mask estrus cycle differences.

**VEGF Expression in Tumors (C3H Mammary Tumor and Methylcholanthrene A Sarcoma)**

**VEGF Protein Levels.** Overall, mammary tumor VEGF protein levels were >100-fold higher than normal mammary tissue levels in C3H mice (12.9-24.7 versus 0.03-0.09 pg/mg). VEGF protein levels in mammary tumor samples from proestrus mice (estrogen- and progesterone-rich) were nearly 2-fold higher (24.7 ± 3.1) compared with tumors obtained from the estrogen-poorer stages of metestrus (14.9 ± 1.4) and diestrus (12.9 ± 1.8, F = 6.5, P = 0.001; Table 1; Fig. 2). Oophorectomy did not further diminish tumor VEGF levels compared with tumors obtained during the lowest estrogen and progesterone
VEGF mRNA Levels. In the normal mammary tissue, VEGF mRNA levels varied significantly across the fertility cycle ($F = 3.3, P = 0.03$). Two-fold higher message levels were found in mammary tissue samples obtained from mice in proestrus ($0.35 \pm 0.07$) versus metestrus ($0.17 \pm 0.02$, Table 1). VEGF message and protein each peaked in normal mammary gland at the same estrous cycle stage, proestrus, when both progesterone and estrogen levels are each high. VEGF mRNA levels in normal mammary tissue from oophorectomized animals were, however, not significantly different from breast tissue levels obtained from low estrogen state diestrus animals ($P = 0.48$; Table 1).

bFGF Protein Levels. Immunoassay detection of bFGF protein in mammary tumors revealed that bFGF levels did not vary significantly across the estrous cycle ($F = 1.2, P = 0.34$; Table 1; Fig. 2). bFGF protein levels in tumors from oophorectomized animals are lower than in each cycle stage, but not statistically different from tumors obtained during diestrus ($P = 0.07$, Table 1).

bFGF mRNA Levels. bFGF mRNA levels are higher in mammary tumors from mice during the estrogen- and progesterone-rich proestrus stage compared with tumors from estrus, metestrus, or diestrus ($F = 4.4, P = 0.01$). Oophorectomy did not further diminish tumor bFGF levels compared with tumors from low-estrogen state diestrus animals ($P = 0.46$, Table 1).

bFGF Protein Levels. bFGF protein levels were higher, although not significantly at 0.05 error level, in normal mammary tissue obtained during the metestrus stage (the estrogen withdrawal portion of the cycle) compared with samples obtained at the other stages ($F = 2.6, P = 0.06$; Table 1). However, bFGF protein levels in normal breast tissue from oophorectomized and diestrus animals were significantly different ($P = 0.01$, Table 1).

bFGF mRNA Levels. Normal mammary tissue bFGF mRNA levels varied across the fertility cycle, with significantly higher levels in the mammary tissue obtained during estrus, the cycle stage associated with the most frequent cure and lowest tumor growth rate, compared with those obtained at other estrous cycle stages ($F = 4.1, P = 0.01$, Table 1). bFGF mRNA levels in normal mammary tissue from oophorectomized animals were not significantly different from tissues obtained during diestrus ($P = 0.67$, Table 1).

VEGF, bFGF protein, and RNA Relationships between Tissues Across the Estrous Cycle

VEGF is a secreted protein; therefore, in tumor-bearing mice, VEGF concentrations are five times higher in serum than in breast cancer cells, and they are several hundred times higher in cancer cells than in normal mouse breast cells. The concentration of this molecule is modulated by the estrous cycle most prominently in cancer, where it peaks in proestrus. It, however, decreases precipitously to much lower values in estrus, when surgical curability is surest. Unlike in breast cancer, VEGF peaks in estrus in normal breast cells. bFGF concentrations in normal breast are six...
times higher than in breast cancer. The concentration of this protein is most prominently modulated by the estrous cycle in normal breast in which it peaks during metestrus. The VEGF and bFGF RNA both cycle in normal breast tissue. The bFGF RNA in breast cancer cells cycles prominently, whereas the VEGF RNA expression changes less robustly in these cancer cells throughout the estrous cycle.

**Physiologic Endpoints**

**Mammary Tumor Vessel Density, Blood Content, and Capillary Permeability**

To determine if the variations observed in tumor VEGF protein levels over the fertility cycle were associated with differences in tumor vascularity, we examined mammary tumor CD31-positive blood vessel density, blood volume, and capillary permeability in tumors obtained across the estrous cycle. There were no significant differences in the density of tumor blood vessels across the cycle (Table 1). The pattern of mammary tumor blood volume was higher in estrus (0.069 ± 0.013 mL blood/g tissue), lowest in proestrus (0.043 ± 0.015 mL blood/g tissue) but these differences did not, however, reach statistical significance (P = 0.35, Table 1). Mammary tumor capillary permeability, however, did vary with the cycle and was nearly 50% higher in diestrus, the cycle phase associated with the highest frequency of post-resection metastasis (F = 3.9, P = 0.01; Table 1; Fig. 4).

**Circulating Sex Hormones**

Estrogen and progesterone, expressed as percentage of the mean, are double-plotted along with surgical cure rates (frequency) at each estrous cycle phase in Fig. 1. Estrogen and progesterone concentrations changes occurring throughout the mouse estrous cycle have been well documented. We did not measure these hormones in our mice, however, we have retrieved published values (41, 42). The mean values and SEs for estrogen are (in pg/mL): proestrus, 10.3 ± 1.1; estrus, 4.2 ± 1.0; metestrus, 3.1 ± 0.7; and diestrus, 7.5 ± 1.3 (41). Progesterone levels at onset of activity are extrapolated from data published by Michael (42), proestrus, 60; estrus, 8; metestrus, 20; and diestrus, 8 (in ng/mL). Both estrogen and progesterone are highest during proestrus and decrease rapidly to low levels between proestrus and estrous. They remain at lower levels during metestrus and estrogen increases, whereas progesterone continues to decrease in diestrus (Fig. 1).

**Tumor Growth Rate**

Growth rates were calculated from serially assessed mammary tumor sizes of C3H mice as a function of biological time (estrous cycle stage and cycle number) of measurement from our previous study (1). Standardized tumor growth rates vary significantly with fertility cycle phase (Table 1; Fig. 4). This cyclic effect is consistent across three separate studies of the C3H mammary tumor. Average tumor growth rates across many estrous cycles are significantly different (P < 0.001) and are 2- to 3-fold higher in diestrus (636.2 ± 54 mm³/d), as compared with other cycle stages (proestrus, 214.8 ± 30; estrus, 308.4 ± 38; metestrus, 276.1 ± 33 mm³/d; Table 1). Estrous stage tumor growth across all cycles showed that tumor growth rate is highest in diestrus, and that cycle phase was associated with low surgical curability (Fig. 4). A similar dependence of tumor size/growth rate on fertility cycle is seen in the methyl cholantrene A sarcoma (1), which also shows fertility cycle dependence of tumor VEGF protein.

**Estrous Cycle Pattern of Post-Resection Breast Cancer Spread**

Two large, independent studies to determine the optimal time of breast cancer resection within the fertility cycle have recently been published (3). In these studies, 33% of mice (6 of 18) resected during proestrus remained free of metastases and were apparently cured, 96% of mice (25 of 26) resected during estrus were apparently cured, 79% of mice (11 of 14) resected in metestrus remained metastasis-free, and 44% of those (11 of 25) resected in diestrus were apparently cured (Table 1; Fig. 1). These
surgical cure proportions are significantly different across the four estrous stages ($\chi^2 = 24.6, P < 0.001$). Oophorectomy also impacted cure with a cure frequency of 50% (5 of 10; $\chi^2 = 24.9, P < 0.001$; ref. 3).

The Relationships of Sex Hormone Concentrations and Angiogenesis Modulators to Capillary Permeability, Tumor Growth, and Post-Resection Cancer Spread throughout the Estrus Cycle

The ratio of serum estrogen to progesterone, capillary permeability of tumor vasculature, the rate of tumor growth, and surgical curability at each of the four estrous cycle phases. These coordinate relationships across the estrous cycle indicate that they are each tightly controlled by that cycle and perhaps by one another. The possibility that the estrogen/progesterone ratio controls the bFGF/VEGF ratio, which, in turn, influences capillary permeability and cancer growth rate, is raised by these covariations. Stages: P, proestrus; E, estrus; M, metestrus; D, diestrus.

Discussion

We have previously shown that the fertility cycle alters mammary tumor growth and post-resection spread in both mice and human beings (1–3). Others have shown how hormone concentrations vary throughout the estrous cycle (41, 42). During each estrous cycle, the concentrations of estrogen and progesterone peak concurrently in proestrus in response to pulsatile hypothalamic secretion of follicle-stimulating hormone, luteinizing hormone, and other hormones. Estrogen and progesterone decrease rapidly during estrus, after follicular rupture, when the ova are available for fertilization. These highly compressed murine cycle phases are roughly comparable to the luteal phase of the menstrual cycle. If fertilization does not occur, a new crop of estrogen-secreting follicles are built and estrogen increases in the absence of progesterone during metestrus and decreases again in diestrus. The adjacent estrous cycle phases of highest cure frequency (estrus and metestrus) are preceded by rapid declines of both estrogen and progesterone. The cycle phases associated with most frequent postsurgical cancer spread are those phases preceded by and associated with the most rapid increase of each of these hormones.

We now show that those cycle phases associated with high and/or rapidly increasing VEGF and low and/or decreasing bFGF are those associated with the highest risk of post-resection breast cancer spread, leaky tumor capillaries, and fastest cancer growth rate (diestrus and proestrus). We further show that these fertility cycle effects on tumor VEGF protein are not limited to mammary tumors, because we find similar cycle-dependent differences in a sarcoma.

Human breast cancer VEGF and bFGF message and protein expression patterns are known independently to

Figure 4. Covariation of the serum estrogen/progesterone ratio, capillary permeability of tumor vasculature, the rate of tumor growth, and surgical curability at each of the four estrous cycle phases. These coordinate relationships across the estrous cycle indicate that they are each tightly controlled by that cycle and perhaps by one another. The possibility that the estrogen/progesterone ratio controls the bFGF/VEGF ratio, which, in turn, influences capillary permeability and cancer growth rate, is raised by these covariations. Stages: P, proestrus; E, estrus; M, metestrus; D, diestrus.

Figure 5. Relationship between the ratio of bFGF and VEGF protein levels within breast cancers and the likelihood of surgical cure of these tumors, as a function of when in the estrous cycle the tumors are resected. Those times of the cycle associated with the greatest bFGF/VEGF ratios are those same times associated with the highest rate of surgical cure/lowest metastatic potential: estrus (96% cure) and metestrus (79% cure). The estrous cycle stage associated with lowest probability of cure/highest metastatic potential, proestrus (33% cure), is associated with lower tumor bFGF/VEGF ratio. Stages: P, proestrus; E, estrus; M, metestrus; D, diestrus.
predict for metastatic cancer recurrence (15, 16, 22–24). Low levels of bFGF are associated with poor prognosis (15). High VEGF levels signal poor prognosis (22) and have been associated with high capillary permeability. In other mammary tumor studies, tumor VEGF protein levels have proven to be a more reliable predictor of tumor stage or aggressiveness than microvessel density or serum VEGF levels (25–27). We find that the highest VEGF levels occur during proestrus when cures are least frequent. VEGF levels are lower in the cycle stages associated with slower cancer growth and lower metastatic potential (estrus and metestrus). Buteau-Lozano et al. (44) have recently shown that VEGF can be transcriptionally regulated by estradiol and tamoxifen through an interplay of estrogen receptors-α and -β in transfected cancer cells. Manders et al. (45) have shown that high concentrations of VEGF predict breast cancer relapse and that progesterone receptor concentration is inversely correlated with both VEGF concentration and poor outcome. These data further support the connection among sex hormones, VEGF, and tumor outcome.

VEGF also influences other vascular events that are relevant to cancer metastasis. Recent work shows almost immediate nitric oxide–dependent effects of VEGF, mobilizing and remodeling preexisting host-derived latent vessels in growing tumors (46). VEGF also promotes adhesive interactions between the endothelium and tumor cells, white cells, and platelets. VEGF is likewise involved with the initiation of the deposition of a fibrin matrix necessary for promoting fibroblast and endothelial cell and successful tumor cell migration (22). In this capacity, VEGF could help establish the cellular foundation required for the boost of tumor growth we observe at each cyclical transition from estrus to metestrus (3), as well as the increased cancer spread following surgery in proestrus and diestrus.

Although tumor VEGF protein levels are modulated by the fertility cycle, VEGF RNA levels do not change markedly across the estrous cycle, which suggests that these sex hormone-induced increases in VEGF protein level occur through translational or posttranslational means. Differences across the fertility cycle/oophorectomy state in mammary tumor bFGF mRNA levels were observed. Highest bFGF mRNA levels were detected in tumor samples obtained during the proestrus stage of the fertility cycle, suggesting an estrogen- and/or progesterone-induced increase. This is further supported by the depression of tumor bFGF protein associated with oophorectomy. These estrous cycle changes in bFGF protein are relatively greater in normal breast than in breast cancer cells. bFGF concentrations are also several fold higher in normal breast. High levels of bFGF in breast cancer confer good prognosis as contrasted with VEGF (29). The balance between VEGF and bFGF and their rates of change during the cycle might be more important than the absolute levels of bFGF or VEGF. The ratio of bFGF and VEGF during the cycle shows the best covariation with surgical curability. The ratio of the bFGF to VEGF is highest in tumors resected during estrus and metestrus, when metastatic potential is lowest. The ratio is lowest among tumors resected during proestrus and diestrus, when resected tumors are most likely to spread.

We found no difference in serum VEGF levels across the fertility cycle or with oophorectomy state in tumor-bearing mice. This is in contrast to a published report demonstrating alterations in VEGF plasma levels in premenopausal breast cancer patients across the menstrual cycle, with lower serum VEGF levels in the luteal phase showing an obverse covariation with progesterone concentration (20). This discrepancy could be explained by the differences between the dynamics of the menstrual and estrous cycles between these two mammalian species and the serum half-lives of these proteins. The menstrual cycle is six times longer and hormone profiles, although similar, are not identical to those characterizing the estrous cycle. It may also be explained by the fact that we studied serum, not plasma. The contribution of blood platelets to serum VEGF levels is also significant. This may be an important source of species difference because blood cannot be obtained easily from mice (but could easily be obtained in women) without profound platelet activation (47).

There are interesting differences between RNA and protein patterns of both VEGF and bFGF between tumor and normal mammary gland. Although both the tumor and mammary gland show relatively high VEGF protein levels at or around the time of ovulation, only the normal mammary tissue shows fertility cycle–induced increases in VEGF mRNA. Conversely, both the tumor and mammary tissue show high bFGF mRNA levels at or around the time of ovulation, whereas fertility cycle variations in bFGF protein were only found in normal breast. In our system, peak VEGF protein levels were 200-fold greater in the tumor (proestrus) than in the mammary tissue (estrus), demonstrating a potentially pivotal difference in the role of VEGF in physiologic versus pathologic VEGF mediated processes. It is interesting to note that even though gene expression was several hundred fold greater in tumor than normal breast, both were equally well regulated by the estrous cycle. Conversely, peak bFGF protein levels were some 6-fold greater in the mammary tissue (metestrus) than in the tumor (metestrus), suggesting an important role for this growth factor in normal mammary gland physiology. Differences also exist in the “kinetics” of the fertility cycle–induced changes. VEGF protein levels were greatest in the mammary tumor during proestrus, whereas increases in normal mammary tissue VEGF protein levels “shifted” one stage and peaked in estrus. An identical “shift” in stages was seen with bFGF mRNA levels. These peak shifts suggest tissue-specific hormonal control of the same genes in subtly different host tissues (benign and malignant breast cells).

Estrous and circadian cycles each affect the host-cancer balance and are physiologically linked. Circadian coordination of estrous cycle events have long been proven (e.g., timing of ovulation to the activity stage). Lesioning the
SCN (central circadian clock) in female mice, rats and hamsters, greatly disturbs the reproductive cycle (48). Circadian Clock gene mutant mice more recently have been shown to have very abnormal estrous cycles (49). Vessel remodeling is essential for cancer growth and we have shown that cancer growth rate is modulated substantially by both the circadian and estrous cycles. We have shown that post-resection metastatic potential is influenced by the estrous cycle (3). VEGF tumor levels have been shown to correlate with metastatic behavior in a wide range of murine and human cancers (20, 22). Here we show that the estrous cycle modulates pro- and antiangiogenic molecules (VEGF and bFGF) within cancer cells. The ratio of bFGF and VEGF covaries with post-resection freedom from metastasis and the VEGF/bFGF ratio accurately predicts a high rate of post-resection metastatic cancer cell spread. Recent observations tie the molecular circadian clock within tumor cells to angiogenesis-modulated tumor progression, via circadian clock-mediated changes in VEGF and methionine aminopeptidase tumor cell gene expression (50, 51). Because the circadian clock regulates the estrous cycle, it is probable that the molecules modulating angiogenesis on the circadian scale may also be relevant to the tumor biology we are describing on the estrous cycle scale.

Discontinuous, intermittent, in fact, saltatory, growth seems to us characteristic of how biological systems organize growth. Traverse through the cell cycle is a necessarily saltatory process at the microscopic level; whereas salutation and stasis characterize human growth at the organismal level (52). Our data show that relevant negative and positive molecular regulators of angiogenesis and tumor capillary permeability, cancer growth rate, and post-resection metastatic cancer spread are each modulated by the mammalian fertility cycle. These covariances do not prove causation. Conclusions about causation await specific blockade of estrogen, progesterone, VEGF, and/or bFGF. If that specific blockade eliminates or multiplies the fertility cycle dependence of cancer growth and spread, and at the same time diminishes or increases average cancer growth rate and enhances or diminishes the post-resection breast cancer cure frequency, causation will be likely and might lead to the testing of periresection antiangiogenic therapies to improve breast cancer cure frequency. The presence of fertility cycling of VEGF in a sarcoma raises the possibility that the biology of other cancers, and the host-cancer balance, may also be affected by the female reproductive cycle.

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References

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