Vascularity of Proliferative Breast Disease and Carcinoma in Situ Correlates with Histological Features

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ABSTRACT

The level of vascularity within an invasive breast carcinoma is a predictor of metastatic potential and survival. However, little is known about the vascular potential and prognostic value of angiogenesis in preinvasive breast pathology. Women with proliferative breast disease or carcinoma in situ are at increased risk of developing invasive breast cancer. This relative risk increases in correlation with defined histopathological features. We asked whether these early proliferative lesions and carcinoma in situ were capable of inducing a vascular supply. Vascularity in preinvasive archival paraffin-embedded breast tissue from 90 patients was quantified by immunohistochemical identification of vessels using anti-von Willebrand factor. Vascular scores were analyzed with respect to histopathological diagnosis, age at diagnosis, and presence of coincident invasive disease. These data indicate that: (a) the vascularity of histopathologically normal epithelium is greater in breasts containing invasive disease than in breasts lacking invasive disease; (b) simple proliferative breast disease induces a vascular supply greater than that of normal breast epithelium; and (c) vascularity increases in proportion to epithelial lesion progression and relative risk of invasion. These studies indicate that the vascularity of preinvasive breast pathology may be a clinically useful predictor of invasive breast cancer.

INTRODUCTION

Breast tumorigenesis can be characterized by a succession of histologically defined “precursor states” in which there is epithelial growth (1). These include epithelial hyperplasias (PBD) and CIS. Women diagnosed with these forms of pathology are at increased risk of having invasive disease, especially if there is a family history of breast cancer (2-5).

All tumors require an adequate blood supply to grow beyond a size of 2–3 mm (6), and quantification of tumor-induced vascularity is of prognostic value. This was first demonstrated for breast cancer by Weidner et al. (7), who showed that the number of vessels in areas of intense vascularity correlates with metastatic potential and patient survival. These observations have now been confirmed by others (8-15). Since the ability of tumor cells to induce a vascular supply occurs early in the process of cell transformation (16), it seemed reasonable that preinvasive breast lesions may also induce a vascular supply. Indeed, in 1975, in vivo assays by Gimbrone et al. (17) showed that murine preinvasive breast tissues contain the ability to induce angiogenesis in rabbit irises. Since that time, others have documented that the vascular supply in preinvasive breast disease is greater than in normal breast (18-20). Furthermore, one study indicated that vascular density in the tissue of fibrocystic change was a predictor of progression to invasive disease (20).

The studies reported here examine the vascularity of preinvasive breast disease to test the hypothesis that vascularity increases with progression toward invasive disease.

Quantification of vascularity in invasive tumors is not a determination of “new vessels” but a measure of local vessel density (21). In tumors, the local vessel density is focally greater than in normal tissue, and therefore by implication, new vessels must have been produced or migrated into the tumor by tissue remodeling (22). A similar approach has been taken by several groups to quantify vessels in preinvasive breast disease (19-20, 23, 24). We chose a different approach for our studies for two reasons: (a) the distribution of vessels in breast stroma is very heterogeneous and difficult to relate to individual ducts or lobules by position, especially in breasts with marked epithelial proliferation; and (b) ducts and lobules in close proximity can display more than one pathological diagnosis. Since capillaries rarely touch the basement membrane of normal breast epithelium, we used this feature to identify and quantify “new” vessels by restricting our assessment to vessels that touched the basement membrane of epithelium defined as either normal, proliferative, or in situ carcinoma. Here we report that compared with normal breast tissue, vascularity is increased in preinvasive breast pathology, that the degree of vascularity correlates with histological features that are predictive of invasion, and that histologically normal breast tissue from cancerous breasts is more vascular than similar tissue in normal breasts.

MATERIALS AND METHODS

Immunohistochemistry. Specimens were routinely processed by the surgical pathology service of each hospital submitting tissues. Tissue processing included formalin fixation and paraffin embedding. Four-μm sections from each specimen were deparaffinized with xylenes and hydrated through graded alcohol. Vessels were stained using the Ventana automated immunohistochemistry stainer 320ES. Briefly, sections were pretreated with trypsin and then incubated with polyclonal anti-von Willebrand factor 1:3000 (Dako Corp, Carpenteria, CA) at 37°C for 30 min. Next, the slides were incubated with a bioti-
Table 1. Criteria for angiogenic grade

Vascularity of ductal or alveolar epithelium was determined on slides stained by immunohistochemistry for von Willebrand factor. The proportion of basement membrane touched by vessels for each epithelial unit was scored according to this formula. Up to 18 ducts or cross-sections of lobular alveolae were examined per slide per diagnostic category and averaged. Therefore, each slide will have a mean vascular score for each diagnostic category identified.

<table>
<thead>
<tr>
<th>Circumference surrounded by vessel</th>
<th>Angiogenic grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>&lt;1/3</td>
<td>1</td>
</tr>
<tr>
<td>≥1/3 → &lt;2/3</td>
<td>2</td>
</tr>
<tr>
<td>≥2/3 → &lt;3/3</td>
<td>3</td>
</tr>
<tr>
<td>Complete encircling</td>
<td>4</td>
</tr>
</tbody>
</table>

Evaluation of Vascularity. Slides were examined independently by R. Y. and S. C. H. Histopathological diagnoses of preinvasive disease were ascertainment for each duct or lobule by the consensus criteria (25). Categories of epithelium included the following: normal, proliferative (including florid ductal hyperplasia and lobular hyperplasia), atypical hyperplasia (ductal and lobular), and carcinoma in situ (ductal and lobular; Ref. 26). Simple hyperplasia (less than a four-cell thickness per individual ductal unit) and all forms of adenosis were excluded from examination. DCIS was subcategorized as either micropapillary, cribriform, solid, or comedo, as per consensus criteria in Ref. 27, and by two recently suggested criteria for the classification of DCIS (28, 29). The classification system by Silverstein et al. (28) is dependent upon nuclear grade and the presence of necrosis. The classification system by Holland et al. (29) depends upon epithelial polarity and nuclear size and cytological features. Only the positively stained vessels in direct contact with either ductal or lobular basement membrane were considered. Each duct or cross-section of a lobular unit was graded as shown in Table 1. Up to 18 ducts or cross-sections of lobular alveolae within a single diagnostic category on each slide were given a vascular score, and the mean was determined for the final vascular score. Therefore, a unique vascular score was determined for each diagnostic category found on a slide. The level of vascularity around ducts or lobules containing epithelial proliferation or in situ carcinoma was scored independently by two pathologists. Normal epithelium was scored by S. C. H. or R. Y. When present, invasive breast cancer was graded according to the criteria of Bloom and Richardson (30).

Clinical Characteristics. Specimens from 90 patients were examined: 34% premenopausal (ages 32–50) and 65% postmenopausal (ages 51–81). Follow-up data were available for a mean of 4.7 years after diagnosis (range, 1–16 years). Cases were accessioned from the surgical pathology case files at the University of Cincinnati Hospital and from surrounding regional hospitals. Specimens included mastectomies, excisional biopsies, and reduction mammoplasties. Fig. 1 illustrates the relationship of PBD, CIS, and invasive disease among these cases. As shown in this Venn diagram, 58 (64%) patients had at least one form of PBD, with or without atypia, 61 (68%) individuals had some form of CIS, and 56 (62%) patients had invasive carcinoma. All but one case of invasion was coincident with the preinvasive pathology.

Statistics. All statistics were performed using SigmaStat (Jandel Scientific, San Rafael, CA). Among histological subtypes, means for each patient were compared by ANOVA on ranks and multiple comparison testing by Dunn’s method. Variation between pathologists and comparison of multiple slides from one specimen were tested by Wilcoxin sign ranks (31).

RESULTS

Interobserver differences were evaluated for each diagnostic category on 136 slides. Ninety-one% showed no significant differences between the two pathologists (P > 0.05). The discrepant comparisons were equally distributed between proliferative and in situ lesions. No significant differences were found between different slides from the same patient.

Fig. 2 shows an example of normal, proliferative, and atypical proliferative epithelium, respectively. The proliferative epithelium partially fills the duct with swirls of cells that are irregular in size and shape (Fig. 2b). Fig. 2c shows proliferative epithelium with atypia in which some of the cells have an increased nuclear:cytoplasmic ratio with prominent nucleoli (arrow) and focal gland formation similar to cribriform DCIS (+). An example of in situ carcinoma that is highly vascular is illustrated in Fig. 3. Immunological staining of vessels (Fig. 3, brown) demonstrates the extent of vascularity along the basement membrane of a duct filled with in situ carcinoma. Because the sum of all the vessels around the duct would cover greater than two-thirds of the circumference, this duct would have a vascular score of “3” (see Table 1). In some cases, numerous vessels were prominent in the adjacent periductal connective tissue; however, these were not considered part of the vascular score. The vascular score of normal epithelium, PBD, and CIS is summarized in Table 2. This table indicates the number of cases in which each diagnostic category was identified, the mean vascular score for each diagnostic category, the S.E., and the range of vascular means found for each diagnostic category overall of the slides examined. These data show that PBD has an increased level of vascularity relative to normal epithelium.
Fig. 2 H&E-stained sections of: a, normal breast epithelium; b, PBD; and c, proliferative breast disease with atypia. Diagnostic criteria were as described in Ref. 27. Note that the epithelium in c has features of both simple proliferative epithelium and carcinoma in situ. In particular, there are foci of cells with cytological atypia including an increased nuclear:cytoplasmic ratio and prominent nucleoli (arrow) and lumen formation with cribriform-like palisading of the cells (*).

(0.836 versus 0.187), with \( P < 0.0001 \). Similarly, CIS is more vascular than PBD (1.525 versus 0.836), with a \( P < 0.0001 \). Analysis of vascularity among histological subtypes of PBD and CIS was difficult because of the small number of cases in some of the categories, particularly LCIS and micropapillary and comedo DCIS. However, the levels of vascularity differed sufficiently that even using a nonparametric ANOVA on ranks we found a statistically significant difference among some groups with a \( P < 0.0001 \) (Table 3). All forms of PBD and CIS differed from normal epithelium, confirming the previous pooled analyses. Among these subtypes, levels of vascularity fell into four groups. In order of increasing vascularity, these were: normal epithelium; all proliferative breast disease, LCIS, and micropapillary DCIS; cribriform and solid DCIS; and comedo DCIS. Detailed analysis indicated that further separation of groups would be statistically significant, but the power to discern these differences was insufficient in this preliminary study. In particular, there was a separation of PBD from a group containing atypical PBD/LCIS/micropapillary DCIS and a separation of cribriform from solid DCIS.

By this classification system (27), subtypes of DCIS were related to the Bloom and Richardson grade of invasive ductal cancer (30). Grades of invasion were associated with DCIS as follows: cribriform, 1.818 ± 0.603; solid, 2.286 ± 0.644; and comedo, 2.500 ± 0.577. These data show a trend for less vascular forms of DCIS to develop into low-grade invasive carcinoma, whereas the highly vascular comedo carcinoma tends to develop into high-grade carcinoma. More cases need to be analyzed to acquire enough power for statistical analysis.

Several new classification systems for DCIS have recently been published, and these have been shown to have predictive value for clinicopathological features and patient outcome. With the increasing use of molecular profiling, it is likely that these classification systems will be further refined to better reflect disease biology and provide more meaningful prognostic and therapeutic information.
Table 3  Data shown in Table 2 displayed according to diagnostic subtype

Diagnostic categories include normal epithelium; PBD; proliferation with atypia; microcystic, cribriform, solid, and comedo DCIS; and LCIS. Note the marked difference between vascularity in PBD without atypia and normal breast, indicating that vascularity is increased very early in the progression to invasive disease. In addition, note that comedo DCIS is markedly more vascular than any other form of CIS.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Mean Vascularity ± SD</th>
<th>SE</th>
<th>No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.187</td>
<td>0.0183</td>
<td>83</td>
</tr>
<tr>
<td>Proliferative</td>
<td>0.814*</td>
<td>0.0502</td>
<td>50</td>
</tr>
<tr>
<td>Atypia</td>
<td>0.889*</td>
<td>0.0940</td>
<td>20</td>
</tr>
<tr>
<td>Microcystic</td>
<td>0.962*</td>
<td>0.1455</td>
<td>9</td>
</tr>
<tr>
<td>Cribriform</td>
<td>1.418*</td>
<td>0.1670</td>
<td>15</td>
</tr>
<tr>
<td>Solid</td>
<td>1.616*</td>
<td>0.1345</td>
<td>28</td>
</tr>
<tr>
<td>Comedo</td>
<td>2.216*</td>
<td>0.2582</td>
<td>7</td>
</tr>
<tr>
<td>Lobular</td>
<td>1.341*</td>
<td>0.2014</td>
<td>4</td>
</tr>
</tbody>
</table>

* P < 0.0001 compared with normal.

Table 4  Vascularity versus the classification system for DCIS

Comparison of vascular score versus DCIS classified according to two recently proposed classification systems. The DCIS on each slide was classified according to the proposed criteria of Silverstein et al. (28), listed as either Class 1, 2, or 3, or by the criteria of Holland et al. (29), listed as either Class 1, 2, or 3. The mean vascularity ± SD was calculated for each diagnostic category. The mean vascularity showed no statistically significant association within either classification system.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Mean Vascularity ± SD</th>
<th>SE</th>
<th>No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1a</td>
<td>1.483 ± 0.723</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2a</td>
<td>1.165 ± 0.603</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3a</td>
<td>1.695 ± 0.748</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 1b</td>
<td>1.349 ± 0.578</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 2b</td>
<td>1.473 ± 0.711</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 3b</td>
<td>1.699 ± 0.818</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a According to the proposed criteria of Silverstein et al. (28).

b According to the criteria of Holland et al. (29).

Table 5  Mean vascularity versus menopausal status

Premenopausal women were defined as patients <50 years old at the time of diagnosis. Postmenopausal women were ≥50 years old. Thirty-one patients were premenopausal, and 59 were postmenopausal. Differences in the vascular score for each diagnostic category were not statistically different.

<table>
<thead>
<tr>
<th>Age</th>
<th>Normal</th>
<th>Proliferative</th>
<th>In situ</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤50 years old</td>
<td>0.202</td>
<td>0.842</td>
<td>1.445</td>
</tr>
<tr>
<td>&gt;50 years old</td>
<td>0.180</td>
<td>0.803</td>
<td>1.600</td>
</tr>
</tbody>
</table>

Table 6  Vascular score versus coincident invasion

The vascular score was calculated for each diagnostic category and segregated according to whether the patient had coincident invasive disease or had no evidence of invasion. The vascularity of normal epithelium was significantly more vascular when derived from a breast with invasive disease (P = 0.006). None of the other comparisons reached statistical significance. Numbers given are the mean vascular score ± SD.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Normal</th>
<th>Proliferative</th>
<th>In situ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No invasion</td>
<td>0.127 ± 0.134*</td>
<td>0.805 ± 0.301</td>
<td>1.366 ± 0.574</td>
</tr>
<tr>
<td>With invasion</td>
<td>0.221 ± 0.176*</td>
<td>0.830 ± 0.416</td>
<td>1.596 ± 0.756</td>
</tr>
</tbody>
</table>

* P = 0.006.

DISCUSSION

The angiogenic potential of preinvasive breast disease of murine origin was first shown over two decades ago (17–18). Since that time, several groups have quantified the vascularity of PBD or DCIS. Ottinetti and Sapino (19) examined vascular number and size within a 100-μm perimeter of normal epithelium, PBD, and DCIS. These studies showed an increased mean vascular size in PBD and DCIS relative to normal epithelium. Fregene et al. (23) examined vascular size in PBD and DCIS relative to normal epithelium but no increase in vessel number. Using the same approach, Guinebretiere et al. (20) examined the vascularity of fibrocystic disease and showed an increased relative risk for developing subsequent invasive breast cancer. Other methods were used by Fregene et al. (23) and Guidi et al. (24). Fregene et al. (23) quantified the number of vessels per ×400 field in the most intensely vascular regions near benign breast epithelium of both proliferative and nonproliferative categories. Although this is similar to the methods used in invasive cancers (reviewed in Ref. 33), when applied to small intraductal pathology, it is more difficult to assess how much stroma to include relative to the lesion size. These studies showed no difference in vascularity between proliferative and nonproliferative benign pathology but a significant increase in the vascularity of PBD in postmenopausal women versus premenopausal women (23). Finally, Guidi et al. (24) used both a 1–3+ estimate of vascularity and quantitative counts of microvessels within 500 μm of DCIS (24). These data showed that vascularity is greater in comedo than non-comedo DCIS, proportional to nuclear grade, and correlated with Her2/neu expression. All of these methodologies examined vessel number or size within a predetermined field size or within the connective tissue 100–500 μm from the

thelium was greater when associated with invasive disease (0.221 versus 0.127; P = 0.006).
epithelial basement membrane. Because of the tremendous heterogeneity of vascular density in the connective tissue of benign breast epithelium, the influence of fibroplasia (24), and the inability to discriminate induced from preexistent vessels, we developed a method based on quantifying vessels in a location where vessels rarely exist, along the epithelial basement membrane.

As explained in Table 1, the vascularity scoring system is based upon the proportion of an individual duct or lobular cross-section that is surrounded by vessels. The 0–4+ grading system was readily reproduced by two pathologists and showed a clear distinction in vascularity among normal epithelium, PBD, and CIS. This is contrary to the results of Ottinetti and Sapino (19) and Fregene et al. (23), who showed no correlation between vascular number and histological features, but consistent with Guidi et al. (24), who demonstrated a difference in vascularity between comedo and non-comedo DCIS. Furthermore, our vascular scores correlated well with the previously defined relative risk of invasion for each histopathological subtype (4). Since two new classification systems for DCIS have been proposed recently, we evaluated vascularity for each proposed DCIS subtype (Table 4; Refs. 28 and 29). Vascularity was not statistically different among DCIS classes using either system. However, the system proposed by Holland et al. (29) showed a trend of increased vascularity with increasing grade of DCIS.

Previous studies on vascularity of DCIS described two patterns of vascularization: an increase in stromal vessels; and "cuffing" of the affected ducts (24). We also saw heterogeneity in stromal vascularity, which as described above, has been measured by others using multiple different methodologies. Guidi et al. (24) showed cuffing in 38% of DCIS (24), whereas Weidner et al. (7) reported cuffing in 23% of DCIS. Using the criteria of Guidi et al. (24), cuffing was found in 30% of DCIS in our study.

In 1982, using a rabbit iris model system, Jensen et al. (34) demonstrated that normal breast epithelium is more angiogenic when derived from a cancerous breast than a noncancerous breast. Our data on normal epithelium is consistent with this study (Table 6). However, this difference was not found with disease progression to PBD or CIS.

Fregene et al. (23) showed a statistically significant increase in vascularity in PBD of postmenopausal patients, whereas in invasive tumors, vascularity is significantly higher in premenopausal women (35). Our data showed no statistical difference in vascularity of normal, PBD, or CIS versus age (Table 5).

One study has shown that vascularity of fibrocystic disease is predictive of subsequent invasion (20). This study examined 48 women with fibrocystic disease, one-half of which developed invasive disease. Although our study demonstrates a correlation between vascularity and recognized histopathological features that are predictive of invasion, this study cannot test this hypothesis because most of the invasive disease in our patient population was concurrent with the preinvasive disease. Furthermore, the follow-up time interval for patients with benign diagnoses (2.14 years) was insufficient for such an analysis.

The relation of vascularity to noninvasive breast epithelium has two clinical implications: (a) if our data and the work of Guinebretiere et al. (20) are confirmed by others, vascularity could become a powerful tool to discriminate who is at high risk for invasive disease. Discrimination of vessels that are undergoing angiogenesis from nascent vessels will potentially make the characterization of vessels in preinvasive disease an even better marker for subsequent invasion. Factors such as endothelial cell tissue factor, specific integrins and other adhesion molecules, and transcription factors are all potential markers for the angiogenic phenotype (36–39); and (b) inhibition of vascularization of these premalignant lesions may inhibit progression to invasion. It is well understood that tumor growth is vessel dependent, and numerous antiangiogenic agents have been tested in vitro and in vivo (reviewed in Ref. 33). However, studies have yet to show that preinvasive progression to malignancy is vessel dependent. If proven, antiangiogenic agents may provide a new class of tumor-preventive therapy.

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Vascularity of proliferative breast disease and carcinoma in situ correlates with histological features.

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