Angiogenic Growth Factors in Preinvasive Breast Disease

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ABSTRACT

Recently, we showed that preinvasive breast pathologies, such as usual hyperplasia, atypical hyperplasia, and carcinoma in situ, have an increased vascularity when compared with normal breast tissue (S. C. Heffelfinger et al., Clinical Cancer Res., 2: 1873–1878, 1996). To understand the mechanism of this increased vascularity, we examined by immunohistochemistry each of these pathological lesions for the expression of angiogenic growth factors. These studies showed that normal breast tissue contains numerous angiogenic agents, particularly vascular endothelial cell growth factor and basic fibroblast growth factor. At the transition from normal epithelium to proliferative breast disease, insulin-like growth factor (IGF) II expression was increased, primarily in the stroma and infiltrating leukocytes. However, among proliferative tissues, IGF I decreased with increasing vascularity. Finally, both epithelial vascular endothelial growth factor and epithelial and leukocytic platelet-derived endothelial cell growth factor increased at the transition to carcinoma in situ, whereas stromal and leukocytic basic fibroblast growth factor were elevated only in invasive carcinoma. Therefore, during histological progression there is also a complex progression of angiogenic growth factors. For CIS, two forms of vascularity are found: stromal microvascular density (MVD), and vascularity associated with the epithelial basement membrane (vascular score). There was 35% discordance between these two measurement systems. Among carcinoma in situ cases, decreases in stromal IGF II were associated with increasing vascular scores but not MVD, and increases in platelet-derived endothelial cell growth factor were associated with increasing MVD but not the vascular score. The presence of discordance and differential association with specific angiogenic agents suggests that these two forms of vascularity may be differentially regulated.

INTRODUCTION

The growth of invasive carcinoma beyond 1–2 mm cubic requires the induction of a new blood supply (1). However, even before a tumor reaches the invasive state, neovascularization of the tissue may occur. We and others have shown that during breast tumorigenesis, tissue vascularity increases very early in the process of transformation, potentially before histopathological changes have occurred and certainly by the stage of usual hyperplasia (2–6). In our study of tissue vascularity in archival tissues, we also found that histologically normal epithelium was more vascular in women who had or would soon have a diagnosis of invasive breast cancer than in women who did not have invasive disease (2). Others have recently confirmed these findings in vivo (7). Furthermore, the vascularity of UH, AH, and various grades of CIS increased with disease progression and correlated with those histological features that predict relative risk of subsequently developing invasive carcinoma (2).

Tumor vascularity is thought to be regulated by the opposing forces of angiogenic stimulators and inhibitors (8). These, in turn, are regulated, at least in part, by genetic changes resulting in tumor progression and altered local environmental conditions, such as hypoxia (9, 10). Which of the many known angiogenic regulators identified in invasive tumors may operate at the preinvasive stage of disease is completely unknown, and to date, there has not been any systematic examination of angiogenic factors in preinvasive breast pathologies. To better understand how the increase in tissue vascularity with breast tumorigenesis may be regulated, we examined archival tissues of preinvasive breast pathologies for proteins that are known to stimulate angiogenesis. Just as tissue microvasculature is of prognostic significance in invasive disease (11) the expression of angiogenic factors, such as VEGF (12, 13), bFGF (14), and PD-ECGF (15), have been associated with a worse prognosis in patients with invasive breast disease. These studies suggest that the regulation of an angiogenic phenotype may vary considerably among breast cancers. Therefore, in our examination of preinvasive breast disease, we chose to examine a broad spectrum of angiogenic growth factors. Included in this study were VEGF, bFGF, IGFs I and II (16, 17), PDGF-B (18), and PD-ECGF, which is also known as thymidine phosphorylase (19). Immunohistochemical examination of these proteins indicate that angiogenic factors are present in abundant amounts in normal breast tissue, primarily in the epithelium. With disease progression, the presence of several growth factors increases in the epithelium, as well as in other cell types. Therefore, several angiogenic growth factors derived from multiple cell types may

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3 The abbreviations used are: UH, usual hyperplasia; AH, hyperplasia with atypia; CIS, carcinoma in situ; DCIS, ductal CIS; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; MVD, mean vascular density; NL, normal tissue.
be important in regulating the graded increase in tissue vascularity seen in breast tumor formation.

**MATERIALS AND METHODS**

**Tissue Characteristics.** Paraffin-embedded archival tissues from 89 patients were retrieved from the pathology files at the University of Cincinnati based on a search for specimens that contained either epithelial hyperplasia, atypical hyperplasia, or carcinoma *in situ*. Specimens included mastectomies, excisional biopsies, and reduction mammoplasties. Two observers (S. C. H. and R. Y.) independently confirmed each diagnosis based on the consensus criteria (20). Among the identified cases, 96% contained normal epithelium; 53%, UH; 12%, AH; 62%, CIS; and 29%, invasive carcinoma. Patients were seen between 1980 and 1995. The vascular scores for each tissue and patient demographics of the population have been reported previously (2).

**Immunohistochemistry.** All immunohistochemistry was performed on 4-μm, paraffin-embedded sections using the Ventana ES immunostaining system. After deparaffinization in xylene and any required pretreatments, slides were placed in the instrument that adds the primary antibody, the biotinylated antimouse or anti-rabbit secondary antibody, and avidin-conjugated peroxidase or alkaline phosphatase as dictated by a bar code. Primary antibodies were incubated for 32 min at 37°C. The instrument performed all washes. Primary antibodies were for VEGF (polyclonal A20, 1:400; Santa Cruz Biotechnology, Santa Cruz, CA), bFGF (polyclonal Ab-2, 1:50; Oncogene Research Products, Cambridge, MA), PDGF-B (N-30 anti-peptide polyclonal, 1:125; Santa Cruz Biotechnology), PD-ECGF (AB-1, PGF44, 1:200; Lab Vision Corp., Fremont, CA), IGF I and IGF II (polyclonal, 1:400; R and D Antibody, Berkeley, CA), and von Willebrand factor (polyclonal, 1:10,000; Dako, Carpinteria, CA). All tissues were trypsinized (Ventana Instruments, Tuscon, AZ) prior to addition of the primary antibody, except for those to be stained with anti-PD-ECGF. For this antibody, antigen retrieval using 0.01M citrate buffer (pH 8.0) was performed by two readers independently while performed by two readers independently while sitting at a multiheaded microscope) was 86% for all antibodies, ranging from negative (0) to extremely intense (4). Staining reactivity was tested for each as follows. Reactivity for VEGF and IGF I and IGF II were compared on a subset of 10–20 cases to slides stained using commercial reagents from sources other than those used in the primary study. For VEGF, the additional antibodies tested were a polyclonal antibody from Biogenex (San Ramon, CA; 1:20) and a monoclonal antibody from Lab Vision (Fremont, CA; clone JH12, 1:10). Additional reagents for IGF were polyclonals from Intergen (Purchase, NY; IGF I at 1:60 and IGF II at 1:40). Because the PDGF-B antibody was antipeptide, we tested for antigen specificity by peptide inhibition of antibody reactivity *in situ*. Briefly, 5–50-fold by weight purified peptide (N-30; Santa Cruz Biotechnology) was preincubated for 2 h at room temperature with the diluted antibody prior to immunohistochemistry. Finally, antibodies to bFGF and PD-ECGF were tested by Western blot against cell extracts known to be positive for these antigens by immunohistochemistry of the cell blocks (Cytoblocks; Shandon, Pittsburgh, PA). SK-Hep1 (a gift from Gretchen Darlington; Baylor College of Medicine, Houston, TX; Ref. 21) and A431 cells (American Type Culture Collection, Rockville, MD) were grown as recommended and scraped directly into SDS-PAGE buffer (22). Aliquots were boiled, and the proteins were separated on a 4–20% acrylamide gradient gel under reducing conditions, blotted onto nitrocellulose, and tested for immunoreactivity to either bFGF or PD-ECGF using 5 μg/ml of the primary antibody. Specificity of reactivity was determined by either molecular weight analysis or comigration with purified recombinant growth factor (bFGF; Becton Dickinson, Franklin Lakes, NJ).

**Vascular Score.** Vessels were defined by immunohistochemistry for von Willebrand factor. Vascularity was examined by two methods. In all tissues, the vascular score was determined for each histological type of epithelium, as described previously (2). Briefly, this is a quartile score representing the proportion of basement membrane for each alveolar or ductal unit that is surrounded by vessels: 1, less than 1/3; 2, 1/3–2/3; 3, greater than 2/3 of the circumference being surrounded by vessels; and 4, complete encircling. Within a tissue, a mean vascular score is calculated from the ductal or alveolar units within each histological category. In addition to the vascular score, we determined a subjective MVD on a 0–4 scale based on observation of von Willebrand factor-stained vessels in the adjacent stroma, as defined by others (23).

**Statistics.** Mean immunoreactive scores among groups were compared by ANOVA on ranks and Tukey test (SigmaStat; SPSS Science, Chicago, IL). Data for the percentage of positive cases were compared by Z test (SigmaStat, SPSS Science).

**RESULTS**

**Specificity of Immunoreagents and Concordance in Interpretation.** Each primary antibody was tested for specific immunoreactivity by either comparing the staining pattern with commercial antibodies of the same specificity in a subset of cases (VEGF, IGF I, and IGF II), inhibiting immunoreactivity with excess peptide immunogen (PDGF-B), or Western blot analysis of cell extracts (bFGF and PD-ECGF). Fig. 1 shows the results of these studies, which support the antigen specificity of these commercial reagents.

Fig. 2 shows an example of immunostaining intensity values ranging from negative (0) to extremely intense (4). Staining concordance was determined in 10% of cases for each antibody. Interobserver variability in determining stain intensity of the same cells (*i.e.*, performed by two readers independently while sitting at a multiheaded microscope) was 86% for all antibodies, varying from 78–96%. The highest variability was during the discrimination of intensities as either 3 or 4 and therefore was
the poorest for bFGF. On the other hand, weak staining was rarely disputed, showing the best concordance for PDGF and IGF I. In no case did either intraobserver or interobserver values differ in intensity by more than 1. Intraobserver variability was determined by an individual rereading cases at an interval of 9–12 months; therefore, these data test not only consistency in determining intensity scale but also selecting the most intensely stained cell(s) within each histological tissue type for each antibody tested. Mean concordance within one intensity value for all antibodies was 84%.

**Epithelial VEGF Increases with Progression.** Table 1 shows the mean relative intensity and percentage of cases positive for VEGF immunoreactivity at all stages of progression in all cases tested. Table 1 also shows the mean immunoreactivity for VEGF, excluding negative cases. The relative intensity of VEGF immunoreactivity in the epithelium increased with progression to invasion, being statistically greater in CIS and invasion than in normal epithelium. VEGF staining of invasive epithelium was also statistically greater than that of UH. VEGF staining was present to some extent in nearly all types of epithelium, indicating that the increase in mean relative intensity is attributable to a true increase in immunoreactivity per cell type with progression and not simply due to an increase in the number of cases showing immunoreactivity. Stromal mean staining intensity for VEGF was considerably lower than in the epithelium, and although there was a trend for stromal staining to increase with progression, this was not statistically significant. This lower mean staining intensity was due both to less staining of individual cases in the stroma relative to the epithelium and fewer positive cases. There was no change in vascular or leukocyte VEGF immunoreactivity with progression, with relative intensity means of 1.61 and 1.08, respectively; staining was fairly ubiquitous in the endothelium, whereas it was identified in leukocytes in only a one-third to one-half of cases.

**bFGF Increases in Nonepithelial Cell Types in Invasive Disease.** Table 2 shows the mean relative intensity of immunoreactivity for bFGF and the percentage of positive cases for each histological cell type during progression. Epithelial immu-
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...significantly at the stage of CIS. The percentage of positive cases showing bFGF immunoreactivity in normal tissue was 2.20 ± 0.67, and this increased to 2.97 ± 0.68 in invasive carcinoma. Although there was some variation in the percentage of positive cases within each tissue type, this increase was due almost entirely to the increase in percentage of positive cases. IGF II, on the other hand, was much more prevalent in the epithelium. IGF II also showed slight increases with progression, but in this case, the increase was attributable to the large influx of inflammatory cells in invasive disease and not a change in relative staining intensity of individual cells at different stages of progression.

PDGF-BExpressionIsConstantDuringProgression. PDGF-B immunoreactivity was found primarily in the epithelium with low level staining in the endothelium and stroma and essentially no staining of leukocytes. Table 3 shows the mean relative level of immunoreactivity in the epithelium, endothelium, and stroma for all cases. Although there appears to be an increase in endothelial staining in invasive disease, it is not statistically different from the other stages of progression and is due entirely to the increase in the percentage of positive cases.

Epithelial and LeukocyticPD-ECGF Increases Significantly at the Stage of CIS. PD-ECGF staining was identified essentially only in the epithelium and in leukocytes. Mean levels of immunoreactivity for all cases were higher in CIS and invasive disease in both of these cell types relative to normal tissue or UH, as shown in Table 4. The increase with progression in epithelial staining is accounted for entirely by the increase in the percentage of positive cases with progression (Table 4). However, the increase in leukocyte mean reactivity is due to both an increase in staining intensity per cell and the influx of leukocytes with progression (Table 4).

IGF II Increases Early in Progression, Whereas IGF IRemainsConstant. Table 5 shows mean IGF I and IGF II immunoreactivity, percentage of positive cases, and mean immunoreactivity, excluding negative cases. Epithelial IGF I immunoreactivity was found in roughly one-half of the cases, with a slight increase in mean staining intensity with progression. This increase was entirely due to an increase in the percentage of positive cases. IGF II, on the other hand, was much more prevalent in the epithelium. IGF II also showed slight increases with progression, but in this case, the increase was attributable to more intense staining within individual cases with progression. Endothelial immunoreactivity for IGF I was rare; IGF II, however, was identified on the endothelium in between a one-third and one-half of cases, independent of histology (Table 5). Among those cases positive for endothelial IGF II staining, the relative intensity varied from 1.7 to 2.0. Stromal staining for both IGF I and IGF II was found in one-third to one-half of cases, independent of histology. Similar to the epithelium, IGF
specimens with the greatest vascularity (vascular score of mal IGF I immunoreactivity was decreased in those proliferative specimens. Surprisingly, both epithelial and stroma, whereas with IGF I, the stromal staining was fairly stable in these same tissues. Therefore, we compared IGF II also increased in stain intensity during progression in the stroma, whereas with IGF I, the stromal staining was fairly constant. Finally, leukocyte staining was identified in a similar number of cases for both IGF I and IGF II. However, stain intensity was constant for IGF I and increased for IGF II.

Growth Factor Expression, Vascularity, and the Presence of Invasion. Because we had shown previously that normal breast tissue from women with invasive disease was more vascular than tissue from women without invasive disease (2), we compared the presence of each angiogenic factor with immunoreactivity in normal epithelium and stroma between these two populations. No correlation between invasive disease and the presence of individual angiogenic factors in normal epithelium or stroma was identified. Similarly, there was no correlation for any of the growth factors between epithelial or stromal staining and the level of vascularity in normal tissue.

As noted above, only IGF II showed significant changes early in progression, with significant increases being found in stromal and leukocyte staining. IGF I staining remained essentially stable in these same tissues. Therefore, we compared IGF I and IGF II staining with the degree of vascularity among all proliferative specimens. Surprisingly, both epithelial and stromal IGF I immunoreactivity was decreased in those proliferative specimens with the greatest vascularity (vascular score of >1 versus <1, with the range of vascular scores being 0–1.67). As shown in Fig. 3a, this was significant (P = 0.021) in the epithelium. IGF II, on the other hand, showed slight to no decrease with increased vascularity (Fig. 3b). None of the other angiogenic factors showed any change with vascularity among the proliferative lesions.

Because most of the changes in angiogenic factor expression were found among the carcinomas in situ, we compared these data with the degree of vascularity. Vascularity was assessed as we have reported previously (2), basically restricting the quantification to those vessels that actually touch the epithelial basement membrane. In addition, we assessed the microvascular density in the adjacent connective tissue, as has been reported by others (23). The first we refer to as the “vascular score,” and the second, as MVD. Thirty-five % of cases showed discordance between these methods, however, as shown in Fig. 4, both mechanisms of assessment showed statistically significant differences between non-comedo ductal CIS and comedo carcinoma. Epithelial VEGF, bFGF, PD-ECGF, and IGF II immunoreactivity were compared against vascular score and MVD among all CIS. No differences were detected using vascular score (Fig. 5a). However, PD-ECGF was significantly higher in those cases of DCIS with the highest MVD (Fig. 5b). Although comedo DCIS is more vascular than non-comedo DCIS, the difference in PD-ECGF among the more vascular tumors was

<table>
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<tr>
<th>Table 3</th>
<th>PDGF-B immunoreactivity</th>
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<tr>
<td>Mean PDGF-B immunoreactivity in all cases by histological type</td>
<td>% positive cases that are PDGF-B immunoreactive</td>
</tr>
<tr>
<td>Epi</td>
<td>Endo</td>
</tr>
<tr>
<td>NL</td>
<td>1.05 ± 0.86</td>
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<tr>
<td>UH</td>
<td>1.37 ± 0.97</td>
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<tr>
<td>AH</td>
<td>1.58 ± 1.16</td>
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<tr>
<td>CIS</td>
<td>1.24 ± 1.09</td>
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<tr>
<td>Inv</td>
<td>1.07 ± 1.09</td>
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<tr>
<th>Table 4</th>
<th>PD-ECGF immunoreactivity</th>
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<tr>
<td>Mean PD-ECGF immunoreactivity in all cases by histological type</td>
<td>% positive cases that are PD-ECGF immunoreactive</td>
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<tr>
<td>Mean PD-ECGF immunoreactivity among positive cases</td>
<td></td>
</tr>
<tr>
<td>Epi</td>
<td>WBC</td>
</tr>
<tr>
<td>NL</td>
<td>0.78 ± 1.02</td>
</tr>
<tr>
<td>UH</td>
<td>1.25 ± 0.96</td>
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<tr>
<td>AH</td>
<td>1.17 ± 1.17</td>
</tr>
<tr>
<td>CIS</td>
<td>1.82 ± 1.22&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inv</td>
<td>1.48 ± 1.22&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup>Mean relative staining intensity ± SD.
<sup>b</sup>Percentage of positive cases showing PDGF-B immunoreactivity in each tissue type.
<sup>c</sup>Statistically greater than values for normal tissue; P < 0.001.
<sup>d</sup>Statistically greater than values for normal tissue and UH; P < 0.001.
<sup>e</sup>P < 0.001, relative to normal tissue.
<sup>f</sup>P = 0.043, relative to normal tissue.
<sup>g</sup>P = 0.034, relative to normal tissue.
<sup>h</sup>P = 0.008, relative to UH, and P < 0.001, relative to normal tissue.
<sup>i</sup>P = 0.007, relative to UH, and P < 0.001, relative to normal tissue.
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DISCUSSION

Increases in tissue vascularity are attributable to numerous processes including tissue remodeling, angiogenesis, and vasculogenesis (24). The contribution of each of these processes and their regulation in invasive cancer are still poorly understood. Even less well-understood are the mechanisms of increased tissue vascularity in preinvasive tumor formation.

We identified an increase in tissue vascularity in preinvasive breast pathologies that correlated with histological features of disease progression (2). In this study, we surveyed growth factors known to be angiogenic in vitro and in vivo to begin looking at mechanisms of vascular growth regulation during the process of tumorigenesis. Surprisingly, even normal breast tissue is awash in angiogenic proteins, independent of the presence of progressive disease. Therefore, the mere presence of angiogenic growth factors is insufficient to initiate an angiogenic phenotype. This phenomenon has also been seen by others and is thought to be attributable to the high level of angiogenic inhibitors in normal tissues (25). To date, we have not examined these tissues for angiogenic inhibitors. However, it is clear that during progression, the expression of numerous angiogenic agents increases, and these agents can be found in many cell types. Because we confined these studies to immunohistochemical stains, we cannot determine the cell of origin for each angiogenic factor; however, the presence of the protein in the tissue is clearly significant. Furthermore, because multiple histological stages are present on a single slide, we can directly compare changes in expression within individual cases and among different cell types.

Three of the growth factors examined (VEGF, bFGF, and PD-ECGF) showed significant increases at the transition from AH to CIS. Generally, these elevated levels were maintained in the invasive component of the tumor. VEGF showed a continuous slow increase with progression, which was only statistically significant at the stage of CIS. Clearly, VEGF expression is not suddenly turned on at this level of progression. Interestingly, VEGF in particular has been thought to mediate significant changes in angiogenesis with small changes in expression (26). The mean increase in expression in VEGF from normal tissue to invasion is on the order of 25% (assuming a linear

Table 5 IGF I and II immunoreactivity

<table>
<thead>
<tr>
<th></th>
<th>Epi</th>
<th>Str</th>
<th>WBC</th>
<th>Mean IGF I and II immunoreactivity in all cases by histological type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Epi</th>
<th>Str</th>
<th>WBC</th>
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<tbody>
<tr>
<td>NL</td>
<td>0.85 ± 0.87</td>
<td>0.46 ± 0.69</td>
<td>0.41 ± 0.87</td>
<td>1.89 ± 1.17</td>
<td>0.48 ± 0.67</td>
<td>0.59 ± 1.11</td>
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<tr>
<td>UH</td>
<td>1.13 ± 1.00</td>
<td>0.44 ± 0.65</td>
<td>0.26 ± 0.77</td>
<td>2.15 ± 1.22</td>
<td>0.58 ± 0.85</td>
<td>0.42 ± 0.96</td>
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<tr>
<td>AH</td>
<td>0.69 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23 ± 0.83</td>
<td>0.15 ± 0.55</td>
<td>1.85 ± 1.34</td>
<td>0.31 ± 0.85</td>
<td>0.15 ± 0.55</td>
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<tr>
<td>CIS</td>
<td>1.34 ± 1.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.55 ± 0.75</td>
<td>0.30 ± 0.80</td>
<td>2.42 ± 1.42</td>
<td>0.64 ± 0.91</td>
<td>0.62 ± 1.21</td>
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<tr>
<td>Inv</td>
<td>1.21 ± 1.29</td>
<td>0.68 ± 0.90</td>
<td>0.39 ± 1.03</td>
<td>2.61 ± 1.26</td>
<td>0.89 ± 1.07</td>
<td>0.50 ± 1.11</td>
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% positive cases that are IGF-I and II immunoreactive<sup>b</sup>

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<thead>
<tr>
<th></th>
<th>Epi</th>
<th>Str</th>
<th>Endo</th>
<th>WBC</th>
<th>Mean IGF I and II immunoreactivity among positive cases&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Epi</th>
<th>Str</th>
<th>Endo</th>
<th>WBC</th>
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<tbody>
<tr>
<td>NL</td>
<td>59</td>
<td>36</td>
<td>8</td>
<td>20</td>
<td>1.44 ± 0.65</td>
<td>0.48 ± 0.67</td>
<td>0.59 ± 1.11</td>
<td>2.29 ± 0.86</td>
<td>0.58 ± 0.70</td>
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<tr>
<td>UH/AH</td>
<td>69</td>
<td>35</td>
<td>15</td>
<td>11</td>
<td>1.64 ± 0.78</td>
<td>0.58 ± 0.85</td>
<td>0.42 ± 0.96</td>
<td>2.51 ± 0.90</td>
<td>1.47 ± 0.70&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>CIS</td>
<td>72</td>
<td>42</td>
<td>0</td>
<td>25</td>
<td>1.29 ± 0.49</td>
<td>0.31 ± 0.85</td>
<td>0.15 ± 0.55</td>
<td>2.67 ± 0.50&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.00 ± 1.41</td>
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<tr>
<td>Inv</td>
<td>61</td>
<td>46</td>
<td>18</td>
<td>18</td>
<td>1.69 ± 0.98</td>
<td>0.64 ± 0.91</td>
<td>0.62 ± 1.21</td>
<td>3.03 ± 0.82&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.40 ± 0.87&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> Mean relative staining intensity ± SD.
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<sup>b</sup> Percentage of positive cases showing IGF I or II immunoreactivity in each tissue type.
<br>
<sup>c</sup> Mean relative staining intensity ± SD among cases that are positive for IGF I or IGF II.
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<sup>d</sup> Statistically greater than values for normal tissue, UH, and AH; P = 0.042.
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<sup>e</sup> Statistically greater than values for normal tissue and AH; P < 0.001.
<br>
<sup>f</sup> Statistically greater than values for normal tissue; P < 0.001.
scale). If true during breast tumorigenesis, then VEGF may play a significant role in regulating the marked up-regulation of vascularity seen at the stage of CIS. Numerous groups have examined VEGF expression in CIS and invasive breast tumors. For instance, Anan et al. (27) tested breast tumors and adjacent nontumorous tissues for VEGF expression by reverse transcription-PCR. They found VEGF message in only 17% of nontumorous tissues, whereas we found fairly ubiquitous protein expression. Brown et al. (28) also found high expression of VEGF message by in situ hybridization in comedo DCIS and invasive and metastatic cancers. Similarly, Toi et al. (29) showed that VEGF protein was predominately in the tumor cytoplasm. As in our study, they found low levels of expression in normal tissues. Guidi et al. (30) also found high VEGF expression in DCIS. Therefore, we and others agree that VEGF increases in the epithelium at the time of DCIS formation.

Unlike VEGF, which was primarily epithelial, bFGF and PD-ECGF increases were seen in nonepithelial components, particularly in tissues with either CIS or invasive disease. Even normal epithelium stained intensely for bFGF, but the changes during progression were in the stroma and leukocytes. This is consistent with the fact that bFGF is known to be sequestered in the stroma (32). Potentially, these growth factors are available for stimulation of endothelium upon the local release of proteolytic enzymes. Anan et al. (27) also measured bFGF in nontumorous breast tissue. They examined tissues by reverse transcription-PCR and found that bFGF message was present in the majority of both tumor and nontumor specimens, consistent with what we report here. Both Colomer et al. (33) and Gomm et al. (34) also showed the expression of bFGF in normal breast tissue. In addition, de Jong et al. (35) showed that in breast tumors, bFGF expression correlated with microvascular density. We examined bFGF expression in our cases of CIS and saw no correlation with MVD or vascular score.

PD-ECGF is thought to be involved in vascular remodeling during wound healing (36). In our study, the total load of PD-ECGF was so tightly related to the intensity of leukocyte infiltration that it seems clear that leukocytic induction of angiogenesis may, in a subset of cases, be similar in mechanism to inflammatory states. Moghaddam et al. (19) found that PD-ECGF levels were increased in breast tumors relative to normal breast tissue. Toi et al. (37) examined PD-ECGF immunostaining in invasive carcinomas and found that the presence of this growth factor correlated with microvascular density. Engels et al. (38) also examined PD-ECGF in breast pathologies, looking primarily at DCIS. They found no correlation with total microvascular density but did identify some correlation with the presence of a dense vascular rim around the DCIS. As in our studies, they saw only weak staining for normal breast epithelium. In our cases, PD-ECGF is positively correlated with MVD but not with “rimming,” as in the determination of vascular score. Although the criteria for rimming in their study differ from our criteria for vascular score, we attempted to more closely match criteria by correlating our most vascular DCIS
tissues with PD-ECGF immunoreactivity. We found no relationship. Why these two studies yield opposite results is not clear; further work is required to address these issues.

Because CIS is nearly as genetically advanced a lesion as invasive carcinoma (39), it is not surprising that many of the angiogenic factors, known to be involved in regulating invasive angiogenesis, also are present in CIS. However, these factors appear to play a limited role in the earliest stages of tumorigenesis. Only IGF II showed a clear difference in staining pattern between histologically normal tissue and hyperplasia. Interestingly, the increases were not in the epithelium but were confined to the stroma and infiltrating leukocytes. Whether this indicates that local factors regulating angiogenic potential are more important than genetic changes in the epithelium at this stage of progression, we do not yet know. Because IGF II is produced by mesenchymal cells, these data may reflect a true increase in synthesis. On the other hand, the epithelial staining may be due to IGF binding to specific IGF binding proteins. These proteins are synthesized by breast epithelium in both estrogen-dependent and -independent pathways (40). Immunohistochemistry cannot discriminate among these possibilities but raises the question of IGF binding protein regulation of angiogenesis. Several groups have reported that by in situ hybridization, IGF I is an indicator of benign stroma, whereas the presence of IGF II is more commonly associated with the stroma of malignant tumors (41, 42). Similarly, Giani et al. (43) showed, by in situ hybridization and immunohistochemistry, that IGF II is primarily in tumorous stroma and only rarely in the epithelium. However, reports by others show both IGF I and IGF II in the epithelial and stromal compartments (44–48), consistent with the hypothesis that these growth factors may function via paracrine growth

Fig. 5 Mean epithelial growth factor (GF) expression (bars, SD) sorted by either vascular score (a) or MVD (b). All specimens are CIS. Individual growth factors are indicated in the legend. Note in b the increase in PD-ECGF in the CIS tissues with the highest MVD. However, there is no increase in PD-ECGF in cases with high vascular score, as shown in a.

Fig. 6 Mean stromal growth factor (GF) expression (bars, SD) sorted by either vascular score (a) or MVD (b). All specimens are CIS. Individual growth factors are indicated in the legend. Note in a the progressive decrease in IGF II with increasing vascular score. Similar changes are not associated with MVD (b).
regulation (49, 50). Our data indicate that IGF II increases early in progression. However, equally significant is the observation that total IGF I does not change with progression, but among proliferative lesions, IGF I decreases as vascularity increases. Together, these data suggest that the ratio of IGF I:IGF II may be important.

Our data regarding PDGF-B is consistent with other reports in that it is expressed primarily in the epithelium in both normal and malignant breast epithelium (51, 52). Coltrera et al. (51) reported that expression of PDGF-B correlated with cell proliferation. Our data indicate that in breast tumor progression, PDGF-B has no role in tissue vascularity.

As reported previously (2), vascular score increases with disease progression, indicating that vascularity immediately adjacent to the epithelial basement membrane occurs very early. At the level of CIS, increases in vascularity of the adjacent stroma are also clearly present in a subset cases. Although this aspect of vascularity correlates generally with vascular score, there are numerous cases of discordance, indicating that vessel growth in these two regions may be differentially (or temporally) regulated. For instance, our data show that PD-ECGF correlates with MVD but not vascular score. On the other hand, stromal IGF II inversely correlates with vascular score. Additional studies of growth factor expression in CIS are needed to resolve how these two aspects of vascularity are regulated.

In summary, just as the process of disease formation in breast tissue is complex, both morphologically and genetically, so the regulation of vascularity is multifactorial. Clearly, local conditions regulating stromal and immune reactions play some role. The genetic pathways of epithelial transformation must also be considered. Our data provide a groundwork on which to examine the interactions of these mechanisms with tissue vascularity. Knowledge of the regulatory processes that control vascularity in these tissues may provide targets for chemopreventive strategies in women with preinvasive breast pathologies.

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