Angiogenic Profile of Breast Carcinoma Determines Leukocyte Infiltration

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ABSTRACT

To study the relationship between the angiogenic profile and leukocyte infiltration of tumors, single cell suspensions of archival frozen medullary and ductal breast cancer tissues were analyzed by flow cytometry. The amount of leukocytes and endothelial cells was measured, as well as the expression of intercellular adhesion molecule-1 (ICAM-1) on the endothelial cell fraction. A significantly higher number (3.2-fold) of infiltrating leukocytes was observed in medullary carcinoma. The composition of this infiltrate was similar to that seen in ductal carcinomas. The more intense infiltrate was explained by the ~3-fold enhanced endothelial ICAM-1 expression in medullary carcinoma. The angiogenic profile of all tumors was assessed by quantitative real-time reverse transcription-PCR analysis. Vascular endothelial growth factor (VEGF)-C and VEGF-D, but not VEGF-A, basic fibroblast growth factor, placental growth factor, and angiopoietins 1, 2, and 3 showed a relatively higher level of expression in ductal carcinoma than in medullary carcinoma. In vitro, both VEGF-C and VEGF-D were found to decrease endothelial ICAM-1 expression in the presence of basic fibroblast growth factor. These data suggest that in vivo angiogenic stimuli prevent the formation of an effective leukocyte infiltrate in tumors by suppressing endothelial ICAM-1 expression.

INTRODUCTION

An intense leukocyte infiltrate in tumors is thought to be a sign of an effective antitumor immune response and is consequently often related to an improved clinical outcome (1–3). We and others have previously demonstrated, both in vitro and in vivo, that leukocyte vessel wall interactions are regulated by tumor derived angiogenic factors (4–8). These factors downregulate the expression of endothelial adhesion molecules involved in leukocyte interactions, of which, intercellular adhesion molecule-1 (ICAM-1) is the most important one (9), ultimately leading to a suppressed infiltration and escape from immune surveillance. To study the relationship between angiogenesis, endothelial cell phenotype and leukocyte infiltration, we adapted the flow cytometric system which recently was described as a reliable and objective alternative for the assessment of microvessel density (10). This method was used to simultaneously determine the level and composition of leukocyte infiltrate in frozen breast cancer tissue, which allows retrospective analysis.

To investigate the leukocyte infiltrate in tumor tissue, we selected breast carcinoma. Among these tumors, a distinct type of disease has been identified, namely medullary breast carcinoma, which is defined, among a number of other characteristics, by an intense infiltration of leukocytes (11, 12). Interestingly, medullary breast carcinoma has a better clinical prognosis, which is thought to be related to this inflammatory infiltrate. We compared these rather rare tumors (5 to 8% of breast cancer) with the most common histologic diagnosis of breast cancer, which is not otherwise specified ductal carcinoma (60 to 70% of breast cancer; ref. 13).

We demonstrate here, with flow cytometry, that a relatively lower level of leukocyte infiltration in ductal type of breast carcinoma than in the medullary type can be explained by the relatively lower level of ICAM-1 expression on tumor vessels in the former case. This suppressed ICAM-1 expression can be explained by a higher expression of angiogenic factors by ductal breast carcinoma tumor cells. This report describes the correlation between angiogenic potential endothelial adhesion molecules and leukocyte infiltrate in human breast cancer.

MATERIALS AND METHODS

Patient Characteristics. Frozen tumor tissues of ductal (not otherwise specified, n = 14) and medullary breast tumors (n = 9) were obtained from the archive of the Department of Pathology (University Hospital Maastricht). Mean age, tumor size, and percentage of patients with positive lymph nodes were comparable in both groups (60 ± 14.55 versus 51 ± 7.97 years, 21.8 ± 5.31 versus 24.2 ± 13.62 mm in diameter, and 43 versus 31% for medullary versus ductal breast carcinoma, respectively).

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Preparation of Single Cell Suspensions and Fluorescence-activated Cell Sorting (FACS) Analysis. Single cell suspensions were made as described previously (10). In short, from each tumor, two 30-μm thick frozen tissue sections were fixed in 1 mL of 1% paraformaldehyde (Merck, Darmstadt, Germany) washed with PBS and centrifuged. Subsequently, the sections were digested by an incubation in collagenase and dispase (both 5 mg/mL; Life Technologies, Inc., Breda, the Netherlands), washed in PBS containing 0.5% BSA (Sigma, Zwijndrecht, the Netherlands), centrifuged, and additionally processed on ice. Cell pellets were incubated with appropriately diluted EN4 antihuman CD31 (Monosan, Sanbio, Uden, the Netherlands), HI30 antihuman CD45 (common leukocyte marker, PharMingen, San Diego, CA), T3-4B5 antihuman CD3 (T-cell marker, 0.225 mg/mL; DAKO), DJ130c antihuman CD68 (macrophage marker, Dako), MT310 antihuman CD4 (T-helper cell marker, 0.1 mg/mL; DAKO), HK3 antihuman CD8 (cytotoxic T-cell marker, 0.05 mg/mL; DAKO), or KP1 antihuman CD68 (macrophage marker, DAKO) antibodies. After the first incubation, the cells were washed and incubated with biotin-conjugated streptavidin (10 μg/mL; DAKO). Subsequently, the cells were washed and incubated with phycoerythrin-conjugated antimouse IgG (15 μg/mL; DAKO). Finally, the cells were washed and analyzed on a FACScalibur (Becton Dickinson, Mountain View, CA) with Cellquest software (Becton Dickinson). In some experiments propidium iodide (Calbiochem, San Diego, CA) staining was used to control for intact cells (10).

For double staining of the endothelial cell with CD31 and ICAM-1 antibodies, the CD31 staining was followed by incubation with FITC-conjugated 6.5B5 antihuman CD54 (ICAM-1, DAKO), or KP1 antihuman CD68 (macrophage marker, DAKO) antibodies. After the first incubation, the cells were washed and incubated with phycoerythrin-conjugated streptavidin (10 μg/mL; DAKO). Finally, the cells were washed and analyzed on a FACScalibur (Becton Dickinson, Mountain View, CA) with Cellquest software (Becton Dickinson). In some experiments propidium iodide (Calbiochem, San Diego, CA) staining was used to control for intact cells (10).

Immunohistochemistry. Serial cryosections (5 μm) were made adjacent to the sections used for flow cytometry and mounted onto organosilane-coated slides (Starfrost, Friedrichsdorf Germany), fixed in acetone (Merck) for 10 minutes at −20°C, and subsequently air-dried for 16 hours at room temperature. Endogenous peroxidase was blocked by immersing the slides in 3% H2O2 in methanol (or PBS in case of CD8 staining) for 30 minutes. Subsequently, the slides were washed in PBS, and nonspecific binding was blocked by an incubation with PBS containing 5% BSA for 30 minutes. Sections were washed again and incubated with the primary antibodies (see under FACS analysis) for 1 hour at room temperature. After washing the slides, biotin-conjugated horse antimouse IgG (1:200 dilution; Vector Laboratories, Burlingame, CA) was applied for 1 hour followed by washings and an incubation with freshly prepared avidin-biotin-peroxidase complex ( Vectastain, Brunschwig Chemie, Amsterdam, the Netherlands) for 30 minutes. Peroxidase activity was detected with diaminobenzidine (Sigma). Slides were counterstained with hematoxylin (Merck) and mounted in entellan (Merck). Microvessel density was assessed as previously described (14), and leukocyte infiltration was determined by two independent observers in four randomly selected high-power fields (×200). Results are expressed as number of vessels/mm² (CD31) or leukocytes/mm².

RNA Isolation and cDNA Synthesis. Total RNA was isolated from 10 20-μm thick sections with the RNasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. High-quality total RNA could only be isolated from eight medullary and eight ductal tissue samples. Possible genomic DNA contamination was removed by on-column DNase treatment with the RNase-free DNase set (Qiagen). The purity and integrity of the RNA were checked by gel electrophoresis according to standard procedures. One μg of total RNA was incubated for 5 minutes at 70°C, and cDNA synthesis was performed for 1.5 hours at 42°C with 400 units of Moloney murine leukemia virus reverse transcriptase RNase H (Promega, Leiden, the Netherlands) in 20 μL of 1× first-strand buffer (Promega), and 1 mmol/L deoxynucleoside triphosphates in the presence of 10 units of RNase inhibitor rRNasin (Promega) and 0.5 μg of random primers (Promega). The reverse transcriptase activity was inactivated by incubation at 95°C for 5 minutes, and after addition of 1× 10 mmol/L Tris (pH 8)-1 mmol/L EDTA up to a final volume of 50 μL, the cDNAs were stored at −20°C.

Real-Time Quantitative Reverse Transcription-PCR (RT-PCR). The primers used for real-time quantitative RT-PCR (qRT-PCR) were targeted against β-actin, cyclophilin A, and the following angiogenesis factors (Table 1): vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, basic fibroblast growth factor (bFGF), angiopoietins 1, 2, and 4, and placental growth factor. Primers were human specific (checked previously described (14), and leukocyte infiltration was determined by two independent observers in four randomly selected high-power fields (×200). Results are expressed as number of vessels/mm² (CD31) or leukocytes/mm².

### Table 1

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>β-Actin</td>
<td>CATTCCAAATATGAGATGCATT</td>
<td>CCTGTTGGACTTGGGAGAG</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>CTCGAAATAAAGTTGACTTGGTT</td>
<td>CTTGGCATGGAGGGGACA</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>AAGGAGGAGGGGCAAGAACAT</td>
<td>CCAAGCCTGTCATTGGG</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>CCTGCCTACACACATTAACA</td>
<td>TGGGTTCAACAGCTGGCA</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>GCAGAGAGAAAATCACCATT</td>
<td>GGTTGCTGGATAGTTTGG</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>AGCTACCAACACAAACAGTT</td>
<td>CAAATGGTACAGGTTGGG</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>TGCCAGGTTGAAATAATTCAG</td>
<td>TTCCTTTTTAGAACAGTGGG</td>
</tr>
<tr>
<td>Angiopoietin-4</td>
<td>AACAGCGCGCTGCAAGGC</td>
<td>GCTTCGCTCTTTGTGCA</td>
</tr>
<tr>
<td>bFGF</td>
<td>CCGCCAGCGCGGGTACG</td>
<td>CACATTTGAGAACAGTGG</td>
</tr>
<tr>
<td>Placental growth factor</td>
<td>TGCAGCTCCTAAAGATCGT</td>
<td>GGAAACAGCATGCGGCA</td>
</tr>
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</table>
by the Basic Local Alignment Search Tool), and the specific melting point of the amplicons was analyzed with the ABI PRISM 7000 Dissociation curve software (Applied Biosystems, Foster City, CA). All primers were designed to meet several requirements concerning GC-content, annealing temperature, and amplicon length and synthesized by Sigma-Genosys (Cambridgeshire, United Kingdom). Real-time qRT-PCR was performed on an ABI PRISM 7000 Sequence Detection System apparatus (Applied Biosystems) with the SYBR Green PCR master mix (Applied Biosystems). The PCR reaction was performed in a 25-μL volume containing 1.5 μL of cDNA, 1× SYBR Green PCR master mix (Applied Biosystems), and 400 nmol/L of each primer. The PCR profile was as follows: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Data were analyzed with the Sequence Detection System software (Applied Biosystems). The parameter Ct (threshold cycle) was defined as the cycle number at which the fluorescent signal passed a fixed value (threshold) above baseline. The absolute mRNA copy numbers were calculated from standard curves generated with 10-fold dilution series of precisely quantified cloned template DNA. The copy number of each target gene was normalized to 1 million copies of the housekeeping gene β-actin.

Cell Culture. Human umbilical vein-derived endothelial cells (HUVECs) were harvested from normal human umbilical cords by perfusion with 0.125% trypsin/EDTA. HUVECs were routinely cultured in RPMI 1640 (Life Technologies, Inc., Burlington, MA), supplemented with 20% heat-inactivated human pooled serum (University Hospital Maastricht), 2 mmol/L L-glutamine (Life Technologies, Inc.), 50 ng/mL streptomycin, and 50 units/mL penicillin (ICN Biomedicals BV, Zoetermeer, the Netherlands), and seeded into gelatin-coated (MERCK Eurolab BV, Amsterdam, the Netherlands) tissue culture flasks at 37°C and 5% CO₂. Confluent HUVEC cultures were passed 1:3. For the experiments, HUVECs in passage 2 or 3 were plated onto fibronectin-coated tissue culture plates (Costar Corp., Cambridge, MA). For analysis of the regulation of ICAM-1 expression by VEGF, HUVECs were cultured in fibronectin-coated 24-well tissue culture plates at a concentration of 20,000 cells per well for 3 days. Cells were stimulated with 10 ng/mL bFGF and/or VEGF-A (Peprotech London, United Kingdom), VEGF-C (a kind gift from Dr. Kari Alitalo, Helsinki, Finland), and/or VEGF-D (R&D Systems, Plymouth, MN) in various concentrations. After 3 days, the stimulated wells reached a confluence of 80 to 90%. HUVECs were trypsinized and harvested, washed in cold PBS, and fixed with 1% paraformaldehyde (Merck) during 30′ and stained for FACS analysis with mouse antihuman ICAM antibody (MEM111, Monosan, Uden, the Netherlands).

Statistical Analysis. Correlations between FACS analysis and immunohistochemistry, CD31, ICAM-1, and leukocyte infiltrate were tested with the Spearman correlation test. The Mann-Whitney U test was used to determine the difference between medullary and not otherwise specified breast carcinomas. The Student’s t test was used to determine the difference between the in vitro results. P values < 0.05 were considered statistically significant and all calculations were performed with SPSS.

RESULTS

Flow Cytometric Assessment of Leukocyte Infiltrate in Tumors. A recently described method for flow cytometric detection of endothelial cells in single cell suspensions made of frozen tumor tissues (10) was adapted for quantification and compositional characterization of tumor leukocyte infiltrate. Single color flow cytometry on CD45 (a pan-leukocyte marker) stained single cell suspensions of archival frozen breast cancer tissues easily quantified the total numbers of infiltrating leukocytes. A large variation in the amount of infiltrating WBCs in these breast carcinoma specimens was found, ranging from 12 to 91% of cells. Flow cytometric results were validated and confirmed by simultaneous quantification of infiltrate by conventional immunohistochemical analysis in adjacent 5-μm sections of the same tissues. A high correlation between detection of CD45 expressing cells by FACS analysis and immunohistochemistry was found (correlation coefficient r = 0.875, P < 0.0001; Fig. 1A). Similar results were found for quantification of T lymphocytes after staining with anti-CD3 antibody (correlation coefficient r = 0.906, P < 0.0001; Fig. 1B).

Enhanced Leukocyte Infiltrate and Vascularization in Medullary Breast Carcinoma. A characteristic of medullary carcinoma is the intense infiltration with small lymphocytes and...
plasma cells as compared with ductal carcinoma. Using the above described method, this characteristic of medullary carcinoma was readily detected by flow cytometry. A 3.2-fold higher level of infiltration was found in medullary carcinoma (Fig. 2A). This enhanced infiltration was also evident in the T lymphocyte subset as detected by CD3 expression (Fig. 2B). Immunohistochemical analysis showed similar results: 808 CD45+ cells/mm² in ductal carcinoma versus 1436 CD45+ cells/mm² in medullary carcinoma (P < 0.004); 533 CD3+ cells/mm² in ductal breast carcinoma versus 889 CD3+ cells/mm² in medullary breast cancer (P < 0.003). The higher number of T lymphocytes in medullary carcinoma was evident for both helper (CD4+) and cytotoxic (CD8+) T-cell subsets (Table 2). A significantly enhanced number of monocytes/macrophages (CD68+) was also observed to be present in medullary carcinoma as compared with ductal carcinoma. Although a statistically significant difference was not observed for the CD16 expressing leukocyte subset, a clear trend in the same direction was also noted for these cells (Table 2).

Next to the infiltrate, the amount of endothelial cells was also quantified by flow cytometry according to the earlier described method (10). Interestingly, a significantly higher number of endothelial cells were observed in ductal breast carcinoma (P < 0.01, Fig. 2C). This result was confirmed by the classical assessment of microvessel density by CD31 staining and counting of blood vessels in adjacent cryosections (medullary 95 vessels/mm² versus ductal 58 vessels/mm², P < 0.02). Also for the detection of endothelial cells, a significant correlation between flow cytometric and histochemical techniques was observed (r = 0.658, P < 0.0001), as was described before in other tissues (10).

A low but significant correlation (r = 0.451, P < 0.05) was observed for the amount of CD31+ cells (endothelial cells) and CD45+ cells (leukocytes).

**Endothelial ICAM-1 Expression Is Lower in Ductal Breast Carcinoma Than in the Medullary Type.** ICAM-1 is generally accepted to be one of the most important adhesion molecules for infiltration of leukocytes. To investigate the relationship between leukocyte infiltration and endothelial ICAM-1 expression, the above described method was applied for double staining with CD31 (phycoerythrin) and ICAM-1 (FITC) antibodies. This approach showed that there was significantly more ICAM-1 present on endothelial cells obtained from medullary breast carcinoma as compared with endothelial cells from ductal breast carcinoma (P < 0.01; Fig. 3). The ICAM-1 expression on endothelial cells in medullary and ductal breast carcinoma correlated with the amount of leukocyte infiltrate as detected by CD45 (r = 0.715, P < 0.001), as well as by all used leukocyte subset antibodies.

**Ductal Breast Carcinoma Is More Angiogenic Than Medullary Carcinoma.** We have demonstrated before that angiogenic factors down-regulate endothelial adhesion molecules such as ICAM-1 (5), leukocyte vessel wall interactions

### Table 2: Leukocyte infiltration in medullary and ductal breast carcinoma determined by immunohistochemistry

<table>
<thead>
<tr>
<th>Leukocyte Subset</th>
<th>No. of cells in medullary carcinoma</th>
<th>No. of cells in ductal carcinoma</th>
<th>Significance (P) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45 (all leukocytes)</td>
<td>1437 ± 584†</td>
<td>809 ± 341</td>
<td>0.004</td>
</tr>
<tr>
<td>CD3 (T lymphocytes)</td>
<td>888 ± 416</td>
<td>534 ± 419</td>
<td>0.030</td>
</tr>
<tr>
<td>CD4 (helper T lymphocytes)</td>
<td>624 ± 387</td>
<td>373 ± 418</td>
<td>0.048</td>
</tr>
<tr>
<td>CD8 (cytotoxic T lymphocytes)</td>
<td>548 ± 284</td>
<td>323 ± 214</td>
<td>0.036</td>
</tr>
<tr>
<td>CD16 (granulocytes/natural killer cells)</td>
<td>793 ± 321</td>
<td>533 ± 161</td>
<td>0.063</td>
</tr>
<tr>
<td>CD68 (monocytes/macrophages)</td>
<td>490 ± 124</td>
<td>343 ± 146</td>
<td>0.018</td>
</tr>
</tbody>
</table>

* Statistical significance was determined with the Mann Whitney U test.
† Mean number of tumor infiltrating leukocytes/mm² (± SD).
(4), and leukocyte infiltration (15). To see whether there was also a difference in the angiogenic potential between ductal and medullary breast tumors, we investigated the expression profile of eight angiogenic factors by quantitative real-time RT-PCR. Two housekeeping genes were included in the analysis, and because there was a strong correlation between the expression levels of both housekeeping genes (correlation coefficient, \( r = 0.965, P < 0.001 \)), the expression level of each target gene was normalized only to \( \beta\)-actin expression. VEGF-A was found to be the predominant angiogenic factor in both tumor types, whereas bFGF was expressed at very low levels (Fig. 4A). There was no significant difference in expression of both factors between ductal and medullary tumors. Interestingly, the expression of VEGF-C and VEGF-D was found to be at least 200-fold higher in ductal carcinomas (Fig. 4B). Angiopoietins 1, 2, and 4 and placental growth factor were all expressed at relatively low levels, and although the only significant differences between the two breast cancer types were observed for VEGF-C and VEGF-D, the trend of enhanced expression in ductal breast cancer was noted for all angiogenic factors.

**Endothelial ICAM-1 Regulation by VEGF-A, VEGF-C, and VEGF-D.** Because VEGF-C and VEGF-D were overexpressed in ductal breast carcinoma, we tested whether these growth factors are involved in the regulation of ICAM-1 in blood vessels, as we have previously published for VEGF-A and bFGF (5). To that end, the effect of these angiogenic proteins on ICAM-1 was investigated with cultured endothelial cells. Unlike VEGF-A, VEGF-C and VEGF-D alone had no effect on ICAM-1 expression on these cells (Fig. 5). However, in combination with 10 ng/mL bFGF, a significant synergistic down-regulation of ICAM-1 expression was observed (Fig. 5A).

Earlier reports by us and others have demonstrated a bi-phasic response of ICAM-1 expression by bFGF, with an early up-regulation and a marked down-regulation after longer time points (16). Also a transient up-regulation by VEGF has been reported (8). Therefore, we performed a temporal study with both VEGF-C and VEGF-D in comparison with VEGF-A and bFGF. The bi-phasic response as observed for bFGF and VEGF-A was not present using VEGF-C and VEGF-D. At best, a transient up-regulation can be observed for VEGF-C (Fig. 5B). In combination with bFGF, VEGF-C and VEGF-D act similarly as VEGF-A (Fig. 5C).

**DISCUSSION**

A flow cytometric approach was used to reliably quantify and identify the composition of the leukocyte infiltrate in archival frozen breast tumor tissues. This retrospective study indicated that the relatively rare medullary carcinoma of the breast, which is widely known to be characterized by an intense infiltration, has on average a 3.2-fold increased leukocyte infiltration as compared with ductal (not otherwise specified) breast carcinoma. The enhanced infiltrate in medullary carcinoma is composed of similar leukocyte subsets as present in ductal breast carcinoma. The demonstration that endothelial expression of ICAM-1, which is the most important adhesion molecule for leukocyte extravasation [both necessary and sufficient (9)], is lower in ductal carcinoma suggests that the absence of adhesion molecules on the tumor vessels is the possible reason for the low
Immune Infiltrate/Angiogenesis in Breast Cancer

It is known that leukocyte infiltration can positively influence the prognosis of cancer (1, 14), and the present results may therefore serve as an explanation for the better prognosis of medullary over ductal carcinoma.

Although tumor-infiltrating leukocytes can contribute to the antitumor immune response, there are also reports describing a role of immune cells in promoting tumor growth by the production of angiogenic factors. The presence of tumor associated macrophages, known to produce angiogenic factors such as VEGF, was correlated to vascular grade and to clinical outcome in breast cancer (23–25). Similarly, in the present study, we observed a higher microvessel density and leukocyte (and macrophage) infiltration in medullary carcinoma as compared with ductal carcinoma. Nevertheless, medullary carcinoma has a better prognosis (13). This apparent discrepancy might also be explained by the comparison of two different tumor types in the present study.

We observed a higher angiogenic potential in the tumors with lower microvessel density. An explanation for this apparent contradiction can be found in the progression of tumor growth. In ductal breast carcinoma, angiogenesis cannot keep up with the aggressive tumor growth, which may result in a lower microvessel density, subsequent ischemia, and a continuously higher expression of angiogenic factors. If the latter is true, the assessment of microvessel density in tumors may just reveal the opposite of angiogenic potential. This may explain the difficulty of linking microvessel density to prognosis in many previous reports. In addition, this also places previous reports in the literature, describing an inverse relationship between microvessel density and prognosis, in a new perspective (26, 27).

Next to the differential expression of ICAM-1 on the endothelium in these tumors, we also found that there is a difference in angiogenic potential between ductal and medullary breast carcinoma cells. Both these VEGFs have been described to induce proliferative responses in endothelial cells either alone or in combination with other stimuli (21, 22). In our studies, both growth factors alone did not down-regulate ICAM-1 expression on HUVECs after longer time points. The transient up-regulation of ICAM-1, as published for bFGF and VEGF-A before, was not observed for VEGF-D, whereas a moderate trend in the same direction was noted for VEGF-C. Interestingly, in combination with bFGF and possibly also with other growth factors (not tested), VEGF-C and VEGF-D synergistically down-regulated ICAM-1 similarly as is seen for VEGF-A. Thus, the angiogenic stimulation of endothelium in ductal carcinoma suppresses the expression of adhesion molecules leading to the prevention of leukocyte infiltration. It is known that leukocyte infiltration can positively influence the prognosis of cancer (1, 14), and the present results may therefore serve as an explanation for the better prognosis of medullary over ductal carcinoma.

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remains to be investigated, whether angiogenic profiling may understand antitumor immune responses. It is suggested, but potential than by measuring microvessel density but also to of cancer, not only to have a better insight into the angiogenic factors was investigated. VEGF-A was found to be the predominant angiogenic factor, of which, mRNA expression was detected at high levels in both tumor types. All other angiogenic factors were expressed at relatively low levels. However, although all angiogenic factors showed a trend of overexpression in ductal breast carcinoma, we did find a marked overexpression of both VEGF-C and VEGF-D in ductal carcinomas. We suggest that the enhanced expression of angiogenic factors is responsible for the suppression of ICAM-1 in ductal carcinoma endothelial cells.

The angiogenic profile was determined on total RNA from a whole tissue section. The problem then arose as to whether the increased expression of these angiogenic factors could be explained by differences in the leukocyte infiltrate. A number of factors make this explanation unlikely: (a) although leukocytes can be producers of angiogenic factors, the medullary carcinomas were found to have lower expression of these factors. (b) Most factors were found to be equally expressed, indicating that not all factors follow this trend, and the differences found in VEGF-C and VEGF-D expression may be specific for these factors. (c) If the different amounts of leukocytes in the two tumor types is assumed to generate differences in the real-time qRT-PCR, then the factors of two to three difference in infiltration can never explain the 200-fold difference in cytokine expression. (d) For prognosis, it may be considered irrelevant where these cytokines come from as long as they contribute to a more aggressive growth characteristic of the tumor.

In line with our observations, Zhang et al. (1) recently demonstrated that the absence of intratumor T cells in ovarian carcinoma was associated with increased expression of VEGF and a significantly worse clinical outcome. We are aware of the fact that VEGF-C and VEGF-D are claimed to be mainly involved in lymphangiogenesis (28, 29), which might explain the difference in clinical outcome between medullary and ductal breast cancer. However, a recent study reported the absence of lymphangiogenesis and intratumoral lymph vessels in metastatic breast cancer (30). Together with the observation that VEGF-C and VEGF-D down-regulated endothelial ICAM-1 expression, we suggest that in ductal breast cancer, VEGF-C and VEGF-D are involved in the down-regulation of the tumor infiltrate.

In conclusion, we favor the view that the measurement of a large number of angiogenic factors, which we called angiogenic profiling, is a necessary tool in determining the prognosis of cancer, not only to have a better insight into the angiogenic potential than by measuring microvessel density but also to understand antitumor immune responses. It is suggested, but remains to be investigated, whether angiogenic profiling may help to predict the success of immunotherapy as an anticancer strategy.

REFERENCES
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