Breast cancer is the leading female cancer in the U.K. and U.S., and indeed in most Western countries. Although local spread and recurrence of the disease can be devastating, metastasis to distant organs is the leading cause of breast cancer–related death (1, 2). The regional lymph node basin is the first station in the metastatic spread from which further hematogenous dissemination to distant organs occurs (3). Both tumor growth and systemic dissemination are highly dependent on angiogenesis (4). For example, it has been established that a solid tumor would not grow >3 mm³ without an adequate new blood supply (5). Normal breast tissue microvascularity is typically maintained in a quiescent state by the balance between proangiogenic and antiangiogenic factors (6). Tumors require neovascularization for sustained growth and metastasis (4). For example, it has been established that a solid tumor would not grow >3 mm³ without an adequate new blood supply (5). Normal breast tissue microvascularity is typically maintained in a quiescent state by the balance between proangiogenic and antiangiogenic factors (6). Tumors require neovascularization for sustained growth and metastasis (4).

The aim of this study was to correlate the expression of pigment epithelium–derived factor (PEDF), a potent endogenous antiangiogenic molecule, with severity and prognosis in breast cancer.

Purpose: The purpose of this study was to correlate the expression of pigment epithelium–derived factor (PEDF), a potent endogenous antiangiogenic molecule, with severity and prognosis in breast cancer.

Experimental Design: To investigate the gene expression profile of PEDF in human breast cancer, we examined human breast cancer tissue (n = 119), background breast tissue (n = 33), and a range of cell lines for mRNA and protein levels of PEDF by using reverse transcription PCR, real-time quantitative PCR, immunohistochemistry, and ELISA.

Results: By using reverse transcription PCR, real-time quantitative PCR, immunohistochemistry, and ELISA, PEDF expression was found to be dramatically decreased in breast cancer. An overall outlook for the patients inversely correlated with PEDF mRNA levels. Exogenous PEDF inhibits endothelial tube formation induced by breast cancer cell–conditioned medium, in vitro.

Conclusion: These observations collectively support the hypothesis that a lack of PEDF expression is a potent factor for the enhancement of tumor growth and angiogenesis in breast cancer.
HT-115), human pancreatic cancer cells (MIA-PACA-2), human bladder cancer cells (EJ-138 and T-24), human melanoma cells (G-361), human lung carcinoma cells (PLC-PRF-5), human fibroblast cells (MRC-5), human epithelial cell line (ECV-304), and a human endothelial cell line (HHEC) were from the European Collection for Animal Cell Culture (Porton Down, Salisbury, United Kingdom). Human breast cancer cells were cultured with DMEM supplemented with 10% FCS.

Bovine microvascular endothelial cells were isolated and cultured as described previously (26). Briefly, freshly isolated bovine retinas in ice-cold Eagle’s minimal essential medium (MEM) with HEPES were homogenized by a Teflon glass homogenizer. After trapping on an 83 μm nylon mesh, the microvessels were transferred into 2 × MEM containing enzyme cocktail (500 μg/mL collagenase, 200 μg/mL Pronase, and Dnase) at 37°C for 20 minutes. The resultant vessel fragments were trapped on a 53 μm mesh, washed with cold MEM and centrifuged at 225 × g for 10 minutes. The cell pellets were resuspended in microvascular endothelial cell basal medium with growth supplement at 37°C, 5% CO2 for 3 days. Cells were used between passages 1 and 3.

Human retinal pigment epithelial (RPE) cells were isolated from three donor eyes as described previously (27). Briefly, after the lens and vitreous were removed, the residual eye cup was rinsed with PBS (M缓冲) and treated with 0.25% trypsin-EDTA. Detached cells were suspended in DMEM supplemented with 10% FCS for 3 days. Cells were used between passages 1 and 3.

**Human breast specimens.** Breast tissues (33 background normal breast tissue and 119 breast cancer tissue) were snap-frozen in liquid nitrogen immediately after mastectomies. All protocols were reviewed and approved by the ethical committee of the University Hospital of Wales. After obtaining written informed consent, cancer specimens and background normal breast tissues removed from the same patients were verified by the pathologists and the background samples were confirmed to be free from tumor deposits. Table 1 shows the patient clinical data.

### Table 1. Breast cancer patient clinical data details

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>Cell line</th>
<th>Sample no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue sample</td>
<td>Background</td>
<td>33</td>
</tr>
<tr>
<td>NPI</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Tumor node metastasis</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Survival status</td>
<td>Good prognosis</td>
<td>89</td>
</tr>
<tr>
<td>Histologic subtypes</td>
<td>Ductal</td>
<td>93</td>
</tr>
<tr>
<td>ER status</td>
<td>Negative</td>
<td>71</td>
</tr>
</tbody>
</table>

**Isolation of total cellular RNA.** Total cellular RNA was isolated from the breast samples and human cell lines using total RNA isolation reagent (Abgene UK, Epsom, Surrey, United Kingdom) as previously described (28). Spectrophotometric measurement was done to determine the concentration of RNA.

**RT-PCR analysis.** cDNA was used to produce cDNA using a reverse transcription kit (Sigma, Poole, Dorset, United Kingdom). PCR was done using a 2400 thermocycler (Perkin-Elmer, Norwalk CT) with conditions set to 40 cycles at 94°C, 60 seconds at 55°C, and 60 seconds at 72°C. The quality of DNA was verified by 0.59 kb β-actin PCR products using primers (5′-ATGATATCG-GCGCCGCTCCTC-3′; reverse 5′-CGCTGGTACGATCCTICA-3′). PEDF forward and reverse primers were 5′-GGTGCTACTCCTCTGCATT-3′ and 5′-ACTGACCTACCCGTTACAAAGAAAGATCCCTCCCTC-3′. PCR products were separated by 2% agarose gel and visualized under UV light following ethidium bromide staining.

**Real-time quantitative PCR analysis.** The mRNA levels of PEDF in the breast specimens were quantified (shown as copies/g). PEDF expression decreases in breast cancer. The results for the test molecules were normalized against the levels of β-actin. The level of the PEDF transcript from a given sample was determined by the software from an internal standard, a method previously described (29–31).

**Immunohistochemical staining of breast specimens for PEDF and microvascular density.** Tissue section acquisition and immunohistochemistry were undertaken as previously described (28). In brief, frozen sections of breast specimens at a thickness of 6 μm were fixed in 4% formaldehyde for 15 minutes, dehydrated in graded ethanol, and embedded in wax. Sections were cut at a thickness of 5 μm and placed on charged slides.

**Conditioned media preparation.** Near-confluent cells (MDA-MB-231 and MCF-7) were cultured with DMEM for 48 hours. The medium was removed and subjected to centrifugation at 1,000 × g for 5 minutes to remove debris and the supernatant was collected as the conditioned medium.

**ELISA of PEDF.** Concentrations of PEDF in conditioned medium derived from human breast cancer cells and human RPE cells were determined using a commercial PEDF ELISA kit (Chemicon Europe, Ltd., Hampshire, United Kingdom), according to the manufacturer’s instructions. Briefly, the culture medium was changed to serum-free DMEM. After 24 hours, the conditioned medium was treated with 8 mol/L urea on ice for 1 hour. The urea-treated samples were diluted 1:100 in assay diluent, added to the antibody-coated wells immediately, and incubated at 37°C for 1 hour. After four washes, 100 μL of the diluted biotinylated mouse anti-human PEDF monoclonal antibody was added to each well and incubated in the wells at 37°C for 1 hour. Then, 100 μL of diluted streptavidin-peroxidase conjugate was added.
and incubated at 37°C for 1 hour. After the addition of 3,3′,5,5′-tetramethylbenzidine for 5 to 10 minutes, 100 µL of stop solution was added, and the absorbance was measured immediately at 450 nm in a microplate reader. For standardization, the PEDF concentration was normalized to the protein concentration in the samples.

**Tubule-forming assay.** Near-confluent bovine retinal microvascular endothelial cells (BRMECs) were pretreated with MDA-MB-231–conditioned medium and/or 25 ng/mL of PEDF for 48 hours. Cells were detached and plated sparsely (2.5 × 10^4/well) on 24-well plates coated with 12.5% (v/v) Matrigel (Collaborative Research, Bedford, MA) and left overnight. The medium was then aspirated and 250 µL/well of 12.5% Matrigel was overlaid on the cells for 2 hours to allow the polymerization of Matrigel, followed by the addition of 500 µL/well of basal medium MCDB131 (InVitrogen, Ltd., Paisley, United Kingdom) with 10% FCS for 24 hours. The following day, the culture plates were observed under a phase contrast microscope and photographed at random in five different fields (×10). The tubule length (mm/mm²) and number of capillary connections per microscope field were quantified.

To assay the effect of the conditioned medium from RPE cells on MDA-MB-231’s conditioned medium–induced tubule formation, near-confluent BRMECs were pretreated with the conditioned medium from MDA-MB-231 for 24 hours, followed by treatment of RPE conditioned medium–induced tubule formation with the conditioned medium and/or 25 ng/mL of PEDF for 48 hours. Cells were detached and plated sparsely (2.5 × 10^4/well) on 24-well plates coated with 12.5% (v/v) Matrigel, followed by the addition of 500 µL/well of basal medium MCDB131 (InVitrogen, Ltd., Paisley, United Kingdom) with 10% FCS for 24 hours. The following day, the culture plates were observed under a phase contrast microscope and photographed at random in five different fields (×10). The tubule length (mm/mm²) and number of capillary connections per microscope field were quantified.

**Results**

**Screening of human cell lines for PEDF.** A variety of 24 human normal and cancer cell lines were examined for PEDF expression using RT-PCR. PEDF was expressed in the majority of the human cancer lines examined. However, only five of the nine breast cancer lines examined revealed any detectable level of PEDF transcripts (Fig. 1A; Table 2). These breast cancer cell lines displayed weak to moderate levels of PEDF. Also, PEDF was found to be strongly expressed in the colorectal (no. 13, Fig. 1A) and human fibroblasts (nos. 15 and 16), whereas the colorectal (no. 14), lung (no. 20), and melanoma (no. 21) cell lines exhibited weak levels of PEDF. The pancreatic (no. 17), liver (no. 22), and epithelial cell lines (no. 23) expressed a moderate level of PEDF.

**Expression of PEDF in paired breast specimens.** We also did conventional RT-PCR on a panel of paired breast tissues (N, normal; T, tumor). Figure 1B showed that both normal breast tissue and breast cancer tissue expressed PEDF mRNA. Overall, PEDF mRNA was expressed to a higher level in the normal breast tissues when compared with breast cancer tissues (Fig. 1B).
Level of secreted PEDF in human breast cancer cell lines. The PEDF levels in the conditioned medium derived from human breast cancer cell lines (MDA-MB-231, MCF7, and ZR-75-1) were measured by ELISA with the conditioned medium from human RPE cells as a positive control. In Fig. 1C, ELISA revealed that PEDF proteins were found in all three conditioned media from human breast cancer cells with significantly lower levels than in the conditioned medium from human RPE cells, consistent with the results of RT-PCR, showing that the decreased PEDF mRNA results in decreased protein expression.

Immunohistochemical staining of human breast specimens. Anti-PEDF antibodies were used for immunohistochemical staining of frozen tissue sections. We showed that the PEDF protein levels were dramatically reduced in the breast tumor specimens compared with the normal breast tissue. In normal breast tissue, mammary epithelial cells displayed an intense and widespread staining. Stromal cells in normal tissues were largely negative. In contrast, cancer cells in the breast cancer tissues had virtually lost the staining for PEDF (Fig. 2, right). These results, together with our RT-PCR and quantitative-PCR data, show that breast cancer tissue has significantly reduced PEDF at both the protein and mRNA level. We were unable to show a significant correlation between the levels of PEDF transcript and microvascular density in tumor tissues (correlation coefficient, r = −0.120; P > 0.05).

Quantification of PEDF expression in human breast tissues. We quantified the PEDF transcript expression in the breast specimens (tumor, n = 119; background, n = 33) using real-time quantitative PCR (all values are displayed as mean transcript copies). We show that the PEDF expression value in tumors was 0.273 ± 0.103, compared with 0.83 ± 0.358 in the normal tissue. Therefore, our results reveal that the PEDF expression was significantly decreased in the breast cancer tissue (0.0171) compared with that in the normal background breast tissues (Fig. 3A).

PEDF expression in relation to prognosis. We examined the PEDF levels in patients with different prognosis, by using the Nottingham prognostic index (NPI) as an indicator. The NPI-1 group (n = 67) represent patients with a good prognosis and an NPI < 3.4, the NPI-2 group (n = 37) contained patients with a moderate prognosis and an NPI = 3.4 to 5.4, whereas the NPI-3 patients (n = 15) had a poor prognosis with a NPI > 5.4. The patients assigned to the NPI-1 group had a PEDF transcript value of 1.228 ± 0.372, compared with the NPI-2 group with 0.498 ± 0.164, and NPI-3 with 0.203 ± 0.152. A Kruskal-Wallis test showed that there was a significant association between decreased levels of PEDF transcript and high NPI status (P = 0.013). An increased degree of lymph node involvement was associated with reduced levels of PEDF. Overall patients with node involvement had reduced levels of PEDF compared to those without any degree of node involvement (Fig. 3B). Furthermore, this correlation between PEDF and prognosis reached statistical significance in these instances (P = 0.024; Fig. 3D).

PEDF and breast tumor grade. Mean PEDF values in the moderate grade 2 (0.778 ± 0.295, n = 41) and poorly differentiated grade 3 (0.507 ± 0.258, n = 23) tumors seemed to be reduced compared with well-differentiated grade 1 tumors (0.998 ± 0.256, n = 55); however, these values did not reach statistical significance (grade 1 versus grade 2, P = 0.49; grade 1 versus grade 3, P = 0.27; Fig. 3C).

PEDF expression and survival status. We assessed the survival status of patients with breast cancer in association with PEDF levels, with an average of a 6-year follow-up period. Patients were divided into two groups, the patients who remained disease-free were assigned to the good prognosis group (n = 89), whereas the patients who had recurrence, metastasis to a distant site, or had died as a result of breast cancer, were allocated to the poor prognosis group (n = 30). The quantity of PEDF from each tumor specimen was assessed and we revealed that the patients with a poor prognosis had dramatically reduced levels of PEDF (P = 0.024). Our results show that the good prognosis group had high levels of PEDF (1.033 ± 0.275) compared with the statistically low levels observed in the poor prognosis group (0.331 ± 0.136; Fig. 3D).

Tumor node metastasis classification of patients. PEDF expression inversely correlated with patient outlook through tumor node metastasis (TNM) grouping (TNM-1, n = 68; TNM-2, n = 39; TNM-3, n = 8; TNM-4, n = 4). Levels of PEDF tended to be reduced in tumors of patients who had an overall poor outlook (TNM-3, 0.621 ± 0.392; TNM-4, 0.389 ± 0.223), compared to those patients with a relatively good prognosis (TNM-1, 1.78 ± 1.7; TNM-2, 1.323 ± 0.7). However, these differences did not reach statistical significance (TNM-1 versus TNM-2, P = 0.63; TNM-1 versus TNM-3, P = 0.62; TNM-1 versus TNM-4, P = 0.59; Fig. 3E).

PEDF treatment decreased the vascular endothelial cell tubule-forming activity of conditioned medium from the breast cancer cell line MDA-MB-231. To assess whether exogenous
PEDF treatment could suppress human breast tumor–induced angiogenesis, we treated microvascular endothelial cells with the conditioned medium from MDA-MB-231 or 25 ng/mL of PEDF for 48 hours followed by the tubule formation assay. The conditioned medium significantly increased both tubule length and capillary connections in the endothelial cell tubule formation assay. Although PEDF alone did not produce a statistically significant difference in endothelial cell tubule formation, PEDF significantly inhibited MDA-MB-231’s conditioned medium–induced endothelial cell tubule formation (Fig. 4).

PEDF is a functional angiogenic inhibitor in normal human epithelial cells. To evaluate the function of PEDF in medium conditioned by normal epithelial cells on tumor-induced angiogenesis, we used the vascular endothelial cell tubule formation assay. The addition of anti-PEDF antibody to the conditioned medium from MDA-MB-231 did not affect its proangiogenic inductive activity (Fig. 4C and D). As expected, the conditioned medium from human RPE cells inhibited MDA-MB-231’s conditioned medium–induced tubule formation. However, the addition of neutralizing anti-PEDF antibody to the conditioned medium from human RPE cells eliminated the inhibitory effect of the RPE cell–conditioned medium (Fig. 4C and D).

Discussion

Angiogenesis is essential for the growth of solid tumors and tumor metastasis. It is now widely accepted that the
angiogenic cascade is initiated when the net balance between the effects of proangiogenic and antiangiogenic molecules is tipped in favor of angiogenesis. Endogenous angiogenic inhibitors are believed to be essential for maintaining the homeostasis of angiogenesis in the breast tissue (2). A potent natural inhibitor of angiogenesis is PEDF, a 50 kDa secreted glycoprotein expressed ubiquitously in many tissues (16, 34, 35). Although we were unable to detect PEDF in stromal cells in normal breast tissues by immunostaining, surprisingly, we observed very high mRNA levels of PEDF in two normal human fibroblast cell lines. In the current study, we do not have enough data to rule out the possibility that the expression level of PEDF in the stroma could be tissue-dependent. However, a recent study showed increased PEDF expression in early-passage fibroblasts in G0 phase, whereas the loss of PEDF expression was found in their senescent counterparts (36). We speculate that in the normal human fibroblast culture, the PEDF might be involved in the contact inhibition of growth of normal fibroblasts, and its expression may decline along with an increase in the number of passages of the culture.

PEDF is a potent inhibitor of the proangiogenic factor VEGF, at least in part, via the regulated intramembrane proteolysis of VEGFR-1 (19). In addition, PEDF also functions to promote neuronal cell survival and acts as a neurotrophic factor for retinoblastoma cells (34). Recently, Bard et al. (25) reported the presence of PEDF in effusions from patients with breast cancer, implicating a role for PEDF in breast tumor pathology.

Breast cancer is typically characterized by rapid growth and a pronounced propensity for invasion and metastasis. This study is the first to quantify PEDF expression in human breast tissue samples from patients with breast cancer and to correlate PEDF levels with prognosis, tumor grade, metastasis, and survival because any additional prognostic indicator to improve therapeutic intervention would be extremely useful.

We found that PEDF mRNA was absent or at very low levels in the breast cancer tissues compared with the normal

---

**Fig. 3.** Quantitative PCR analysis of PEDF expression in human breast cancer tissues. A, PEDF expression significantly decreased in human breast cancer tissues compared with normal background breast tissue (P = 0.0171). B, overall patients with node involvement had reduced levels of PEDF compared with those without any degree of node involvement with a significant association between decreased levels of PEDF transcript and high NPI status (P = 0.013). C, levels of PEDF seemed to correlate with differentiation with the observation that the lowest levels of PEDF were found in the poorly differentiated grade 3 tumors, although these values did not reach statistical significance. D, patients without recurrence were allocated to the good prognosis group, whereas those with recurrence, metastasis, or having died as a result of breast cancer were allocated to the poor prognosis group. The patients in the poor prognosis group had significantly decreased levels of PEDF (P = 0.024). E, a trend toward reduced PEDF levels was observed in tumors of patients who had an overall poor outlook (TNM-3 and TNM-4), compared to those patients with a relative good prognosis (TNM-1 and TNM-2).
breast tissue from patients with breast cancer. Via immunohistochemical staining, we confirmed that PEDF was dramatically decreased at the protein level in breast cancer tissue compared with the higher levels observed within normal breast tissue. Furthermore, in support of our ex vivo data, the results from screening a panel of 24 human cell lines showed that lower PEDF mRNA and protein levels were expressed in a large variety of human cancer cell lines, including breast, colorectal, bladder, and prostate cancer cell lines, compared with normal human cell lines. These show a strong correlation between decreased PEDF expression and progression of breast cancer.

Elevated production of proangiogenic molecules, such as the VEGF family members, in breast cancer is considered to be one of the major signals triggering the “angiogenic switch” (37, 38). Endothelial cell tubule formation assay has been described as being the preferred variable for in vitro quantification of angiogenesis. We were able to show that conditioned medium derived from MDA-MB-231, a highly metastatic breast cancer cell line, induced a significant increase in in vitro angiogenesis and is likely to be attributed to increased VEGF levels in MDA-MB-231 (39). The addition of PEDF caused a substantial decrease in the breast cancer cell line conditioned medium–induced tubule formation after the addition of PEDF. PEDF is known to be a potent inhibitor of VEGF-induced angiogenesis (16) and we have previously shown that PEDF can block the angiogenic effect of VEGF via VEGFR-1 inhibition of VEGFR-2 signaling (19). The observation that conditioned medium from normal epithelial cells could inhibit tumor cell–induced tubule formation reinforces the idea that decreased PEDF levels may play a permissive role in breast tumor angiogenesis. Thus, the local tumor microenvironment and a shift in the proangiogenic and antiangiogenic balance is likely to influence the breast tumor growth pattern and the degree of tumor angiogenesis. A recent in vivo study (40) showed that in PEDF knockout mice, both prostate and pancreas developed hyperplasia with hyper-vascularization, indicating that angiogenesis has a critical role in maintaining tissue homeostasis. These data show that PEDF might (a) directly act on tumor cells via rendering a less aggressive subset of poorly differentiated human tumors or suppressing tumor growth and/or (b) inhibit tumor-induced angiogenesis leading to suppression of tumor growth. The inverse correlation between the levels of the PEDF transcript, and the degree of lymph node involvement, tumor differentiation, and patient outlook suggests that PEDF may play more than just an antiangiogenic role and may also contribute to cell survival and differentiation (34).

Despite the established antiangiogenic role for PEDF, the current study failed to show a significant correlation between

![Fig. 4. Endothelial cell in vitro tubule formation assay.](image-url)

A, B. Tubular length of endothelial cells

C. Photomicrographs of representative microscope fields showing endothelial tubule formation with the conditioned medium, in the presence or absence of neutralizing anti-PEDF (bar, 400 μm).

D. Morphometric quantitative analysis of tubule formation. Morphometric quantitative analysis of in vitro tubule formation of tubule length (mm/mm²) and capillary connections per microscope field were done using the NIH image analysis program (bars, SE).
the levels of PEDF transcript and microvessel density in breast cancer. It is therefore likely that PEDF may work in concert with other regulators of angiogenesis present in breast tumors, e.g., IL-8 (12), basic fibroblast growth factor (13), and thrombospondin (15). A simple correlation may therefore be unable to establish such a complex regulation.

In summary, our observations suggest that PEDF expression might be used as a prognostic marker for human breast tumor, indicating that the lack of PEDF expression is a potent factor for the enhancement of tumor growth and angiogenesis in breast cancer.

Acknowledgments

Breast Cancer Campaign, Cancer Research Wales, Wellcome Trust, United Kingdom, and the National Eye Research Centre, Bristol, United Kingdom.

References

4. Hansen S, Grabau D, Sorensen F, Bak M, Vach M, et al. Angiogenesis as a potential factor for human breast tumours, e.g., IL-8 (12), basic fibroblast growth factor (13), and thrombospondin (15). A simple correlation may therefore be unable to establish such a complex regulation.
10. Ferrara N, and the National Eye Research Centre, Bristol, United Kingdom. PEDF Expression Decreases in Breast Cancer

www.aacrjournals.org

Clin Cancer Res 2006;12(11) June 1, 2006

3517

Downloaded from clincancerres.aacrjournals.org on June 12, 2021. © 2006 American Association for Cancer Research.
Decreased Pigment Epithelium–Derived Factor Expression in Human Breast Cancer Progression


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/11/3510

Cited articles  This article cites 40 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/11/3510.full#ref-list-1

Citing articles  This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/12/11/3510.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/12/11/3510.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.