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ORIGINAL ARTICLE

Expression of vascular endothelial growth factor D is associated with hypoxia inducible factor (HIF-1α) and the HIF-1α target gene DEC1, but not lymph node metastasis in primary human breast carcinomas

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Background: Vascular endothelial growth factor D (VEGF-D) induces angiogenesis and lymphangiogenesis. Nodal metastasis is recognised as a powerful prognostic marker in breast carcinoma, but the molecular mechanisms underlying this process are unknown. Although it has been suggested that VEGF-D may regulate nodal metastasis, this is based largely on animal models, its role in human disease being unclear.

Aims: To measure the pattern and degree of VEGF-D protein expression in normal and neoplastic human breast tissues.

Methods: The pattern and degree of VEGF-D expression was measured in normal tissue and invasive carcinomas, and expression was correlated with clinicopathological parameters, hypoxia markers, and survival. Because other VEGF family members are affected by oestrogen, whether VEGF-D is regulated by oestrogen in breast cancer cell lines was also assessed.

Results: VEGF-D was significantly positively associated with hypoxia inducible factor (HIF-1α) ($p = 0.03$) and the HIF-1α regulated gene DEC1 ($p = 0.001$), but not lymph node status, the number of involved lymph nodes, patient age, tumour size, tumour grade, lymphovascular invasion, oestrogen receptor, progesterone receptor, c-erb-B2, or tumour histology (all $p > 0.05$). There was no significant relation between tumour VEGF-D expression and relapse free ($p = 0.78$) or overall ($p = 0.94$) survival. VEGF-D expression was enhanced by oestrogen in MCF-7 and T47D breast cancer cells, and was blocked by hydroxytamoxifen.

Conclusion: These findings support a role for hypoxia and oestrogen induced VEGF-D in human breast cancer and also suggest that tamoxifen and related oestrogen antagonists may exert some of their antitumour effects through the abrogation of VEGF-D induced function.

The metastatic spread of tumour cells from the primary tumour is a characteristic of malignancy.[1] Recently, a new member of the vascular endothelial growth factor (VEGF) family has been implicated in metastatic spread via both the blood and lymphatic systems.[2–4] VEGF-D (the murine homologue is designated the c-fos induced growth factor) is most closely related to VEGF-C by sequence homology. The N-terminal and C-terminal extensions of VEGF-C and VEGF-D are proteolytically cleaved[5 6] after secretion to form mature ligands. These bind with increased affinity to VEGF receptor 2 (KDR) and VEGF receptor 3 (flt4) and induce phosphorylation.[2 7] VEGF-D is expressed in a wide range of normal tissues, being most abundant in heart, lung, and skeletal muscle.[2 4] Functionally, VEGF-D stimulates endothelial cell migration and proliferation in vitro,[2 8] and in animal tumour models VEGF-D induces both tumour angiogenesis and lymphangiogenesis, promoting lymphatic spread.[9]

> "Recently, a new member of the vascular endothelial growth factor (VEGF-D) family has been implicated in metastatic spread via both the blood and lymphatic systems"

In patients with breast cancer, lymph node metastasis is recognised as the most important prognostic indicator,[10] yet the role of VEGF-D in normal and neoplastic human breast has been investigated in only one study of 105 breast cancers.[11] Therefore, we examined the pattern and degree of VEGF-D protein expression in a large series of invasive breast cancers with longterm follow up. Our aims were to determine whether VEGF-D protein has a role in either normal and/or neoplastic breast and to correlate expression with standard clinicopathological characteristics and survival. Because hypoxia has recently been shown to upregulate VEGF-D activity in smooth muscle cells,[12] and given the oestrogen responsiveness of other VEGF family members,[13] we also measured hypoxia markers in breast cancers and assessed the effect of oestrogens on VEGF-D expression in breast cancer cell lines.

METHODS

Patients and tumours

Between 1990 and 1993, normal tissues derived from breast reductions (n = 15) and tissues from 207 consecutive patients with invasive breast carcinoma undergoing surgery at the John Radcliffe Hospital (Oxford, UK) were collected. Patients with cancer who had distant metastases were excluded from the analysis; table 1 details the clinical data

Abbreviations: CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; DC, dextran charcoal stripped; ER, oestrogen receptor; ERE, oestrogen receptor response element; FCS, fetal calf serum; HIF, hypoxia inducible factor; RT-PCR, reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor

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regarding tumour size, grade, and oestrogen receptor status of these patients. Tumours were treated by simple mastectomy (n = 38) or wide local excision (n = 169). All patients with tumours had axillary node status confirmed histologically. The grading of ductal carcinomas was performed by specialist breast pathologists trained at a single institution (John Radcliffe Hospital) according to the Bloom and Richardson method. Repeat follow up was performed every three months for the first 18 months and every 18 months thereafter; clinical parameters, relapse free survival, and overall survival were recorded from the date of surgery. In patients < 50 years old, adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) was administered if tumours were node positive, or oestrogen receptor (ER) negative, and/or ⩾ 3 cm. Patient who were ⩾ 50 years who had ER negative, node positive tumours also received CMF. Radiotherapy was given according to accepted practice at the time. The median follow up was 7.5 years (range, 0.6–11.2), during which time there were 66 relapses (local or distant) and 44 deaths from breast cancer.

VEGF-D immunohistochemistry

The pattern of VEGF-D expression was assessed in whole tissue sections taken from 15 normal breast tissues and 15 invasive carcinomas. The degree of VEGF-D expression was measured semiquantitively in 207 invasive breast carcinomas using tissue microarrays. The tissue microarrays were constructed by first assessing haematoxylin and eosin stained whole tissue sections of each tumour to select the representative areas of the tumour from which the core biopsies were taken. Cores (1 mm) were then removed from the designated donor block using a precision instrument (Beecher Instruments, Silver Spring, Maryland, USA) and were placed into the recipient paraffin wax block. Sections (5 µm thick) were then cut, placed on polylysine coated slides, and stained with a goat polyclonal antibody (SC-7602; Santa Cruz Biotechnology, Santa Cruz, California, USA),[14] a rabbit antigoat Ig (Dako, Ely, Cambridgeshire, UK) bridge, and the EnVision (Dako) detection system. Antigen retrieval was performed in 0.1M EDTA with 0.1% Tween (pH 6.0) in an 800 W microwave for 20 minutes. Omission of the primary antibody was used as a negative control. Scoring of invasive carcinomas was performed blinded and was graded according to the intensity and extent of epithelial staining, as reported previously, namely[14]: negative, 0; weak focal staining, 1; strong focal/widespread moderate staining, 2; or strong widespread staining, 3. Grade 2 and 3 tumours were considered positive. This was performed by two observers over a conference microscope.

Table 1 Comparison of the expression of angiogenic ligand VEGF-D protein and the clinicopathological variables

	VEGF-D negative	VEGF-D positive	p Value
Total no of patients	75	132	
Age			
<50	19	41	0.38
⩾50	56	91	
Nodal status			
Negative	44	84	0.48
Positive	31	48	
Tumour size			
⩽2 cm	47	87	0.64
>2 cm	28	45	
Grade			
I	15	32	0.12
II	22	32	
III	10	33	
ER			
Negative	19	46	0.16
Positive	56	86	
EGFR			
Negative	31	50	0.64
Positive	42	78	
HIF-1α			
Negative	61	93	0.03*
Positive	6	25	
DEC1			
Negative	33	26	0.0001*
Positive	26	93	

*Significant.
EGFR, epidermal growth factor receptor; ER, oestrogen receptor; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor.

Assessment of HIF-1α and the hypoxic target gene, DEC1

The hypoxia inducible factor (HIF-1α) protein was detected using the monoclonal antibodies ESEE 122 (IgG1; dilution 1/40)[15] and the HIF target gene DEC1,[16] with CW27 rabbit polyclonal antibody and the Envision horseradish peroxidase kit (Dako, Glostrup, Denmark), and assessed in tissue microarrays. Two observers assessed the localisation and degree of cellular staining using a conference microscope. The intensity of nuclear staining was compared with that seen in parallel stained control sections. The evaluation of HIF-1α was based on the intensity and extent of nuclear and cytoplasmic reactivity, whereas DEC1 was based on the proportion of nuclei staining.[17] Omission of the primary antibody was used as a negative control and the cell line HeLa as a positive control.

The effect of oestrogen on VEGF-D gene expression in breast cancer cell lines

Because oestrogen responsiveness is an important prognostic indicator in breast cancer and other members of the VEGF family are regulated by oestrogen,[13] we screened a series of ER positive (MCF-7 and T47D) and negative (MDA-MD-231, MDA-MD-435, MDA-MD-453, MDA-MD-468, BT20, and SKBR3) breast cancer cell lines (all from ATCC, Manassas, Virginia, USA) for VEGF-D gene expression using reverse transcription polymerase chain reaction (RT-PCR) and the VEGF-D primers (see below).[18] We then went on to investigate the potential regulation of VEGF-D expression in the ER positive breast cancer cell lines MCF-7 and T47D.

MCF-7 or T47D cells (1×10^6) were grown to 30% confluence in 75 cm^2 culture flasks in 15 ml RPMI 1640 (Gibco BRL, Gaithersburg, Maryland, USA), supplemented with 10% heat inactivated fetal calf serum (FCS), 2mM L-glutamine, 100 µg/ml streptomycin (Gibco BRL), and 60 µg/ml benzylpenicillin (CSL Ltd, Victoria, Australia) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After 24 hours the cells were washed with phosphate buffered saline, and the medium replaced with phenol red free RPMI 1640 (Gibco BRL) supplemented with 10% dextran charcoal stripped FCS (DC-FCS),[19] 2mM L-glutamine, 100 µg/ml streptomycin (Gibco BRL), and 60 µg/ml benzylpenicillin. The cells were then cultured in DC-FCS hormone free medium for two to three days until approximately 80% confluent, then fresh DC-FCS medium containing 10^{-9}M 17β oestradiol (Sigma, Poole, Dorset, UK),[20] 10^{-6}M 4-hydroxytamoxifen (Sigma),[21] or a combination of 10^{-9}M 17β oestradiol and 10^{-6}M 4-hydroxytamoxifen was added to the cells. Cells were pretreated with the antioestrogen compound 4-hydroxytamoxifen for two hours before the addition of 17β oestradiol. Cells were harvested two or 18 hours after 17β oestradiol treatment, together with control cells grown in parallel in DC-FCS medium without hormone treatment. Cells were harvested into TRIzol™ (Gibco BRL; Life Technologies Inc, Gaithersburg, Maryland, USA) and total RNA was extracted according to the manufacturer's

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instructions. Cell culture experiments were repeated in triplicate.

Relative RT-PCR was used to measure the changes in VEGF-D gene expression in MCF-7 and T47D cells. VEGF-D primers used in cell culture experiments were as follows: forward, GTATGGACTCTCGCTCAGCAT; reverse, AGGCTCTCT TCATTGCAACAG.[22] Pilot experiments determined that an 18S primer to 18S Competimer™ (Ambion, Austin, Texas, USA) primer ratio of 1 : 9 was required to coamplify VEGF-D and 18S. Thirty PCR cycles were required to maintain the PCR reactions in the midlinear range (data not shown).

As a positive control, expression of the oestrogen regulated gene pS2 was determined.[23 24] Pilot experiments determined that an 18S primer to 18S Competimer™ primer ratio of 3 : 7 was required to coamplify pS2 and 18S. PCR conditions were as described above, with an annealing temperature of 55°C and 30 PCR cycles.

Statistical analysis

Tests of hypotheses on the location parameter (median) were performed using rank statistics (Mann-Whitney, Kruskal-Wallis, and adjusted Kruskal-Wallis for ordered groups). The χ^2 test was used to test for the independence of categorical variables, including categorised continuous variables. The log rank test was used to test for differences in survival. All statistics were performed using the Stata package release 7.0 (Stata Corporation, College Station, Texas, USA).

RESULTS

VEGF-D protein localisation and relations between VEGF-D protein expression, clinicopathological variables, and survival

Weak VEGF-D expression was seen in myoepithelial cells and in luminal ductal cells of glands and ducts in normal breast tissue derived from surgical reductions (fig 1). Most immunoreactivity was present in the luminal ductal cell layer, with more occasional staining in myoepithelial cells; interlobular and intralobular stromal and inflammatory cells also expressed VEGF-D (fig 1). Expression was enhanced in areas of cystic disease. Stronger expression of VEGF-D, which was predominantly in the malignant epithelium of breast cancers, was seen in both in situ and invasive cancers. In in situ cancers, there was variation in expression both within and between affected ducts, whereas in invasive disease, expression was generally homogeneous. Nevertheless, heterogeneity of expression was seen in different parts of some tumours, with peripheral accentuation. No enhancement of VEGF-D expression was seen in necrotic areas (fig 1), but consistent with recent findings, a strong granular pattern of VEGF-D staining was seen within the cytoplasm of malignant epithelium at the secretory pole of glands in well differentiated tumours[14] (fig 1). VEGF-D staining was also seen in non-neoplastic elements, including smooth muscle of arterial walls and endothelium, together with stromal cells and macrophages (fig 1). Expression of HIF-1α and DEC1 was both nuclear and cytoplasmic. In general, this was of equal intensity, although some cases demonstrated nuclear positivity alone.

A significant association was seen between VEGF-D and HIF-1α ($p = 0.03$) and the HIF regulated gene DEC1 ($p = 0.001$), but no significant associations were seen with lymph node status ($p = 0.48$), number of lymph nodes involved (categories: 0, 1–4, ⩾ 5 nodes; $p = 0.94$), patient age ($p = 0.38$), tumour grade ($p = 0.12$), tumour size ($p = 0.64$), ER ($p = 0.16$), or epidermal growth factor receptor ($p = 0.64$) (table 1). There was no significant difference in relapse free ($p = 0.78$) or overall survival ($p = 0.94$) when stratifying by VEGF-D expression.

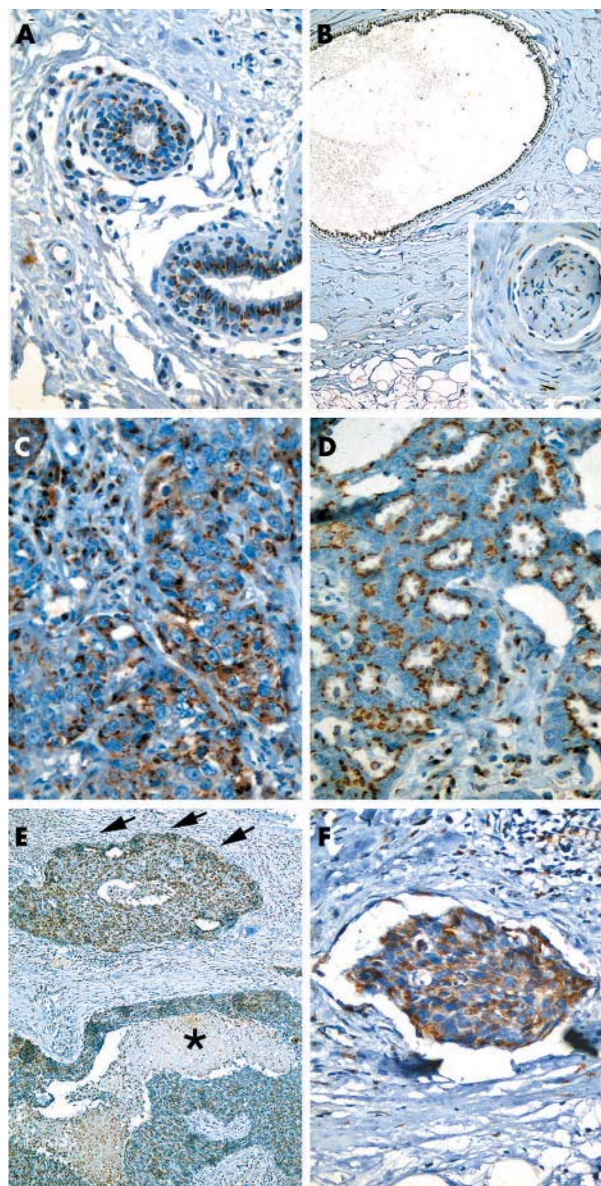

Figure 1 (A) Vascular endothelial growth factor D (VEGF-D) staining in luminal ductal cells with occasional staining in myoepithelial cells of normal breast. (B) Expression in areas of cystic disease, with enhanced VEGF-D expression in duct epithelial cells (inset: nerve and stromal cells that are also immunopositive). (C) Heterogeneous staining of VEGF-D in malignant epithelium in a poorly differentiated tumour and (D) a well differentiated tumour with granular staining at the apical pole of glands. (E) Enhanced VEGF-D immunostaining at the periphery of a tumour (arrows) without upregulation in an adjacent area of necrosis (asterisk). (F) Strong staining for VEGF-D in a lymphatic tumour embolus.

The effect of 17β estradiol on VEGF-D gene expression in oestrogen responsive breast cancer cell lines

VEGF-D was strongly expressed in both ER positive breast carcinoma cell lines (MCF-7 and T47D; fig 2), and was also expressed in five of the six ER negative breast carcinoma cell lines tested. VEGF-D gene expression was strong in MDA-MB-435, MDA-MB-468, and SKBR3, weak in MDA-MB-453, very weak in BT20, and undetectable by RT-PCR in MDA-MB-231 cells (fig 2).

The effect of 17β estradiol on VEGF-D expression was investigated in MCF-7 and T47D breast cancer cell lines. VEGF-D mRNA was upregulated in MCF-7 and T47D cells (fig 3) incubated for two and 18 hours in medium containing

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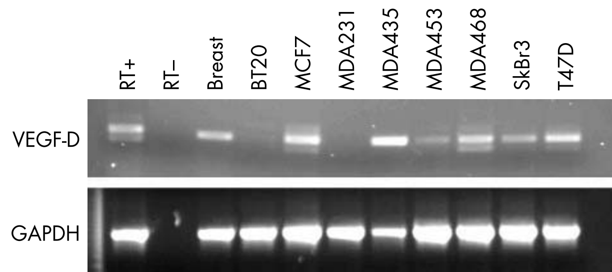

Figure 2 Representative reverse transcriptase (RT) polymerase chain reaction showing ethidium bromide stained vascular endothelial growth factor D (VEGF-D) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression in a panel of oestrogen receptor positive (MCF-7 and T47D) and negative (BT20, MDA-MD-231, MDA-MD-435, MDA-MD-453, MDA-MD-468, and SKBR3) breast carcinoma cell lines with human umbilical vein endothelial cell RT+ and RT− controls (2 μg total RNA used for each sample).

10^{-9}M 17β estradiol. To assess whether this effect was ER regulated, the experiment was repeated using T47D cells and the partial ER agonist 4-hydroxytamoxifen. At 18 hours, VEGF-D mRNA gene expression and the oestrogen responsive positive control gene pS2 were suppressed in response to 4-hydroxytamoxifen treatment (fig 4).

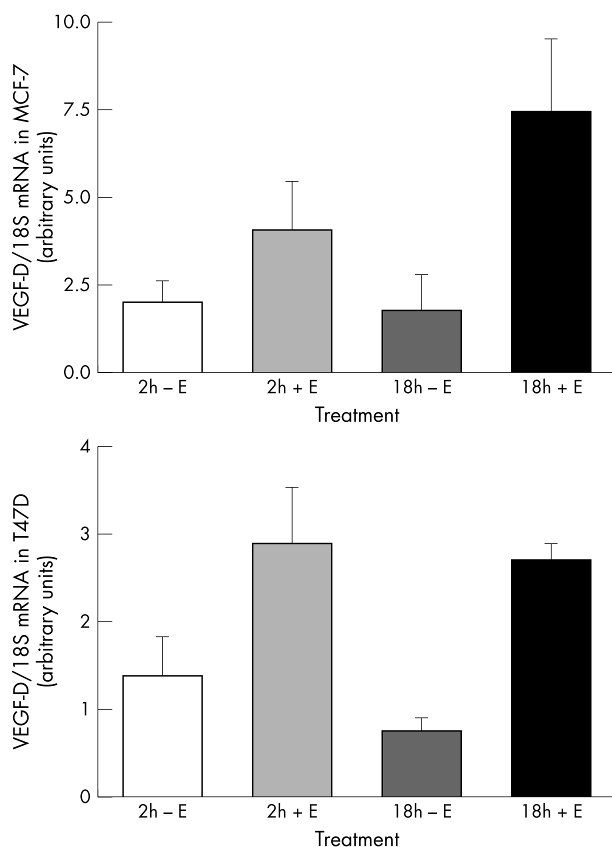

Figure 3 Vascular endothelial growth factor D (VEGF-D) mRNA expression in MCF-7 and T47D breast carcinoma cell lines incubated in hormone free RPMI 1640 medium or hormone free RPMI 1640 medium supplemented with 17β estradiol (E; 10^{-9}M) for two and 18 hours. Gene expression was measured by relative reverse transcriptase polymerase chain reaction, standardised to 18S, and expressed as mean (SEM).

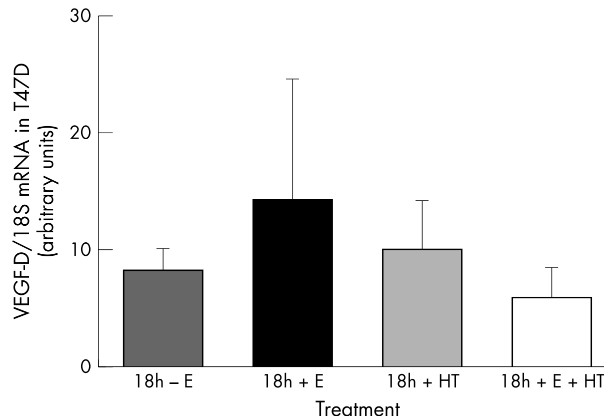

Figure 4 Vascular endothelial growth factor D (VEGF-D) in T47D breast carcinoma cell lines incubated in hormone free RPMI 1640 medium or hormone free RPMI 1640 medium supplemented with 17β estradiol (E; 10^{-9}M) and/or 4-hydroxytamoxifen (HT; 10^{-6}M) for 18 hours. Gene expression was measured by relative reverse transcriptase polymerase chain reaction, standardised to 18S, and expressed as mean (SEM).

DISCUSSION

We have investigated the pattern and degree of VEGF-D protein expression in a series of normal and malignant breast tissues. We found that VEGF-D is present in normal and neoplastic breast tissue, suggesting a role for VEGF-D in both physiological and pathological situations. In physiological situations, this cytokine may have a role in the vascular and lymphatic remodelling associated with changes that occur during the menstrual cycle, and in pathological situations it may be involved in changes that occur during tumour progression.

> "Although enhanced vascular endothelial growth factor D expression was not seen adjacent to areas of necrosis, we did find a significant association with HIF-1α and the HIF target gene DEC1, consistent with non-necrotic viable areas of tumour also being hypoxic"

Previous studies using animal tumour models have shown that VEGF-D induces lymphangiogenesis and promotes tumour cell metastasis via the lymphatic system.[9] We detected VEGF-D protein expression in all histological types of breast cancer and not solely inflammatory breast cancers, as reported previously.[25] Furthermore, in contrast to the one published study examining VEGF-D in 105 breast cancers,[11] we were unable to demonstrate an association with lymph node metastasis. This discrepancy may result from the relative weakness of the reported association (44% of VEGF-D positive tumours were lymph node negative), together with the different antibodies and cutoff values used in each study; in addition, VEGF-D derived from stromal components may also contribute to lymph node spread.[26] Nevertheless, in view of the numerous pathways that are involved in tumour dissemination, it is unlikely that a single factor is responsible for the presence of lymph node metastasis.

Although enhanced VEGF-D expression was not seen adjacent to areas of necrosis, we did find a significant association with HIF-1α and the HIF target gene DEC1,[27–29] consistent with non-necrotic viable areas of tumour also being hypoxic. Furthermore, this suggests that tumour cells may have similar promoter elements to vascular smooth muscle cells, where hypoxic induction of VEGF-D has been reported.[12] Although the precise mechanism of this hypoxic

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Take home messages

- Vascular endothelial growth factor D (VEGF-D) was significantly positively associated with hypoxia inducible factor (HIF-1α) and the HIF-1α regulated gene DEC1 but there was no significant relation between tumour VEGF-D expression and relapse free or overall survival
- VEGF-D expression was enhanced by oestrogen in oestrogen receptor positive breast cancer cells and was blocked by hydroxytamoxifen
- These findings support a role for hypoxia and oestrogen induced VEGF-D in human breast cancer and also suggest that tamoxifen and related oestrogen antagonists may exert some of their antitumour effects through the abrogation of VEGF-D induced function

induction is yet to be defined, our findings suggest that it might partly be mediated through HIF-1α, and that hypoxia targeted treatments, such as blocking of HIF-1α, may be a mechanism to reduce VEGF-D.[30]

Nakamura *et al* reported the absence of an association between VEGF-D and ER and suggested that VEGF-D is unlikely to be regulated by oestrogen.[11] We also found no association between VEGF-D and ER. However, because other VEGF family members are oestrogen regulated,[20] and we have identified two potential oestrogen response elements (ERE) in the 5′ UTR (33–45 bp) and exon 5 (1173–1185 bp) of VEGF-D, which show 54% and 62% homology, respectively, with ERE,[31] we examined the effect of oestrogens on breast cancer cell lines. These investigations showed that VEGF-D expression was upregulated by 17β estradiol and that the active metabolite of tamoxifen, 4-hydroxytamoxifen, suppressed this induction. Tamoxifen is used in the treatment of metastatic breast cancer, where it leads to disease regression in approximately 30% of cancers.[32] Thus, 4-hydroxytamoxifen may have an additional anti-cancer role through suppression of the lymph(angiogenic) factor, VEGF-D. Nevertheless, further studies are required to unravel the molecular pathways underlying ER regulation of VEGF-D to determine whether this effect is mediated by ERα and/or ERβ (see Simak and Coombes for a review[32]).

Unlike the study of Nakamura *et al*,[11] in our larger series we did not find a patient survival disadvantage when stratifying by VEGF-D. This, together with the high expression of VEGF-D in some node negative tumours, may be the result of incomplete proteolytic processing of C-terminal and N-terminal domains.[5] The mature form of VEGF-D greatly enhances receptor binding, which immunohistochemistry would not specifically identify,[33] and further work examining these post-translational effects is needed.

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