Expression of the VEGF and angiopoietin genes in endometrial atypical hyperplasia and endometrial cancer

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Angiogenesis is critical for the growth and metastasis of endometrial cancer and is therefore an important therapeutic target. Vascular endothelial growth factor-A (VEGF-A) is a key molecule in angiogenesis, but the identification of related molecules and the angiopoietins suggests a more complex picture. We investigated the presence of transcripts for VEGF-A, VEGF-B, VEGF-C, VEGF-D, Angiopoietin-1 and Angiopoietin-2 in benign endometrium, atypical complex hyperplasia (ACH) and endometrioid endometrial carcinoma using *in situ* hybridisation. We confirmed the presence of VEGF-A mRNA in the epithelial cells of cancers examined (13 out of 13), but not in benign endometrium or ACH. We also demonstrate, using quantitative polymerase chain reaction, that levels of VEGF-B mRNA are significantly lower in endometrial cancer than benign endometrium. We conclude that loss of VEGF-B may contribute to the development of endometrial carcinoma by modulating availability of receptors for VEGF-A.
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Angiogenesis is a critical event in the growth and spread of tumours (Folkman, 1990). The vascular endothelial growth factor family (VEGFs) and the angiopoietins are key factors in this process (Fong *et al*, 1995; Shalaby *et al*, 1995; Ferrara *et al*, 1996; Maisonpierre *et al*, 1997; Thurston *et al*, 1999). Vascular endothelial growth factor-A is a dimeric glycoprotein existing as four main isoforms by alternative splicing from a single gene. The coding regions of the VEGF gene comprise eight exons. The first four are conserved but alternative splicing of the last four gives rise to mRNAs of 121, 145, 165, 189 and 206 amino acids, respectively ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) (Houck *et al*, 1991; Tischer *et al*, 1991; Charnock-Jones *et al*, 1993). The VEGF-A isoforms bind the tyrosine kinase receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) to promote endothelial cell proliferation, migration and increased vascular permeability. Elevated levels of VEGF-A are found in many tumours including cancers of the endometrium (Doldi *et al*, 1996; Fujimoto *et al*, 1998) and ovarian epithelium (Olson *et al*, 1994; Boocock *et al*, 1995; Abu-Jawdeh *et al*, 1996). When VEGF-A signalling is blocked with VEGF-neutralising antibodies (Olson *et al*, 1996) or dominant-negative receptors (Millauer *et al*, 1994), tumour angiogenesis and growth are impaired.

However, the family of VEGF genes has expanded with the discovery of other VEGFs, for example, VEGF-B (Olofsson *et al*, 1996a), VEGF-C (Joukov *et al*, 1996) and VEGF-D (Achen *et al*, 1998), which share significant sequence homology with VEGF-A. VEGF-B exists as two splice variants, a heparin binding isoform of 167 amino acids ($VEGF-B_{167}$) and a secreted isoform of 186 amino acids ($VEGF-B_{186}$) (Olofsson *et al*, 1996b). Whereas VEGF-A binds to both the VEGFR-1 and VEGFR-2 receptors, VEGF-B binds only to the VEGFR-1 receptor (flt-1) (Olofsson *et al*, 1998). Vascular endothelial growth factor-B forms homodimers and can hetero-dimerise with VEGF-A (Olofsson *et al*, 1996a) to promote endothelial cell proliferation *in vitro* (Olofsson *et al*, 1996a).

Both VEGF-C and VEGF-D are ligands for VEGFR-2 (KDR) and the third VEGF receptor VEGFR-3 (flt-4) (Joukov *et al*, 1996; Achen *et al*, 1998). VEGFR-3 is expressed by adult lymphatic endothelial cells but not by adult vascular endothelium. There is a strong association between lymphovascular density and VEGF-C expression in human malignant mesothelioma, suggesting this pathway as an important therapeutic target (Ohta *et al*, 1999).

A second family of growth factors, the angiopoietins, are ligands for the tyrosine kinase receptor Tie-2 (Davis *et al*, 1996; Maisonpierre *et al*, 1997). The two ligands share 60% sequence homology and bind with equal affinity to Tie-2. Angiopoietin-1 (Ang-1) maintains pericyte/endothelial cell interactions (Hanahan, 1997; Thurston *et al*, 1999) while Angiopoietin-2 (Ang-2) is a functional antagonist of Ang-1 and leads to marked vessel regression in tumours in the absence of VEGF-A (Holash *et al*, 1999). Compared to Ang-1, which is widely expressed in adult tissues, Ang-2 is selectively expressed at sites of active angiogenesis, for example, the corpus luteum of the ovary (Goede *et al*, 1998) and endometrium (Li *et al*, 2001).

Angiogenesis is an important feature of endometrial cancer as microvessel density is an independent prognostic indicator of both progression and overall survival (Kaku *et al*, 1997). Furthermore, there is a progressive increase in microvessel density from benign

endometrium through the cancer precursor, atypical complex hyperplasia (ACH) to invasive disease (Abulafia *et al*, 1995).

While VEGF-A mRNA and protein are present in endometrial cancer (Doldi *et al*, 1996; Guidi *et al*, 1996; Fujimoto *et al*, 1998), its expression in ACH is unknown. Vascular endothelial growth factor-C (Hirai *et al*, 2001) and VEGF-D (Yokoyama *et al*, 2003) have both been correlated with a poor prognosis in endometrial cancer, but the expression of VEGF-B has not been described. In order to further investigate a possible role for other angiogenic factors in endometrial cancer, we investigated the presence of transcripts for VEGF-A, VEGF-B, VEGF-C, VEGF-D, Ang-1 and Ang-2 in benign postmenopausal endometrium, ACH and endometrioid endometrial carcinoma by *in situ* hybridisation and used quantitative polymerase chain reaction (PCR) to determine the levels of VEGF-B and Ang-2 mRNA in benign and malignant endometrium.

MATERIALS AND METHODS

Patients

A total of 13 carcinoma specimens were examined using *in situ* hybridisation. All were endometrioid-type carcinomas. Five benign postmenopausal endometrial specimens and five complex atypical hyperplasias were also examined for comparison. The age range of the patients was 51–87 years (median 57 years). Histopathological findings and clinical data are shown in Table 1. Presence or absence of lymphovascular space involvement is indicated, where reported, in the table. The survival status for each patient was noted at the time of preparation of this manuscript. Ethical committee approval was obtained from the committee of Addenbrookes Hospital NHS Trust.

Tissue samples

In situ hybridisation and immunohistochemistry were performed on formalin-fixed, paraffin-embedded endometrial tissue. Tissue used for reverse transcription–PCR (RT–PCR) and quantitative PCR was snap-frozen from endometrium removed during endometrial curettage or total hysterectomy, after examination by a histopathologist.

Production of radiolabelled riboprobes for *in situ* hybridisation

Antisense and sense (control) RNA probes were generated against VEGF-A, VEGF-B, VEGF-C, VEGF-D, angiopoietin-1 and angiopoietin-2 (Sowter *et al*, 1997). For VEGF-A, probes were designed to span the first four exons thus detecting the four commonly occurring isoforms. The sequences were amplified in pCRscript SK (Stratagene Ltd., Cambridge, UK) and pBluescript II KS vectors (Stratagene Ltd., Cambridge, UK). Radioactive, single-stranded probes incorporating ^{33}P-UTP (Amersham International plc, Little Chalfont, Buckinghamshire, UK) were carried out with 1 μg plasmid template using the Ambion MAXIscript transcription kit (Ambion Inc., Austin, TX, USA) before phenol extraction and ethanol precipitation.

Identification of mRNA encoding VEGF-A, VEGF-B, VEGF-C, VEGF-D, angiopoietin-1 and angiopoietin-2 by *in situ* hybridisation

Tissue sections (6 μm) were cut onto slides coated with 3-aminopropyltriethoxy-silane (Sigma Chemical, Poole, Dorset, UK). The hybridisation procedure was performed as described by Clark *et al* (1996). The riboprobe was incubated with the tissue sections for 18 h at 55°C. The sections were washed and treated with RNAse A (Sigma Chemicals, St Louis, USA), before being coated with autoradiographic emulsion (Amersham Pharmacia Biotech UK Ltd, Aylesbury, UK) and exposed for 21 days at 4°C. Sections were developed with photographic developer (Kodak D19) and counterstained with Mayer's haemalum (BDH Chemicals, Lutterworth, UK) before viewing.

Immunohistochemistry for cytokeratin and CD-68

Immunohistochemistry was performed on serial sections to identify the cell types expressing the transcripts. Mouse antibodies to human cytokeratin (clone MNF116; Dako, Ltd., High Wycombe,

Table I Clinical and histopathological details for patient samples used in the study

Patient	Histology	Age	Status	FIGO stage	Grade	LVS involved	Tamoxifen use
1	Benign	51	Alive	—	—	—	N
2	Benign	57	Alive	—	—	—	N
3	Benign	80	Alive	—	—	—	N
4	Benign	63	Alive	—	—	—	N
5	Benign	68	Alive	—	—	—	N
6	ACH	56	Alive	—	—	—	N
7	ACH	58	Alive	—	—	—	N
8	ACH	52	Alive	—	—	—	N
9	ACH	69	Alive	—	—	—	N
10	ACH	52	Alive	—	—	—	N
11	Endometrioid cancer	41	Alive	1C	1	N	N
12	Endometrioid cancer	56	Alive	2B	2	N/R	N
13	Endometrioid cancer	48	Alive	2B	2	Y	N
14	Endometrioid cancer	46	Alive	1B	2	N	N
15	Endometrioid cancer	71	Alive	3A	3	N/R	N
16	Endometrioid cancer	87	Alive	1B	3	N	N
17	Endometrioid cancer	54	Alive	1B	3	N	Y
18	Endometrioid cancer	78	Deceased	1C	3	N	N
19	Endometrioid cancer	84	Deceased	4A	3	Y	N
20	Endometrioid cancer	56	Alive	3B	3	N/R	N
21	Endometrioid cancer	52	Alive	1B	3	N/R	Y
22	Endometrioid cancer	68	Deceased	4A	3	Y	Y
23	Endometrioid cancer	81	Alive	1B	3	Y	N

ACH = atypical complex hyperplasia; LVS = lymphovascular space involvement N/R = not reported N = never, Y = yes.

Buckinghamshire, UK) and human CD68, a macrophage marker, (clone PG-1; Dako, Ltd.) were used as described previously (Clark *et al*, 1996). Mouse IgG (Dako, Ltd.) incubated with serial sections provided negative controls.

Reverse transcription – PCR

RNA from benign postmenopausal endometrium, ACH and endometrioid endometrial carcinoma was analysed by RT – PCR to determine whether mRNA for VEGF-C was present (Figure 1). Total RNA was extracted from snap-frozen tissue using acid phenol purification (Chomczynski and Sacchi, 1987). Samples for PCR were unrelated to those used for *in situ* hybridisation. RNA was annealed with oligo-dT primers (Pharmacia Biotech LTD., St Albans, UK) and cDNA was synthesised using reverse transcriptase (HT Biotechnology LTD., Cambridge, UK). Polymerase chain reaction for VEGF-C was performed using the upstream primer 5′-TGCCGATGCATGTCTAAACT-3′, and the downstream reverse complement primer 5'-TGAACAGGTCTCTTCATCCAGC-3′ for 40 cycles (15 s at 95°C and 60 s at 60°C).

Quantitative PCR

RNA was extracted and purified and cDNA was prepared as above. Quantitative, real-time PCR was performed in triplicate using the ABI PRISM 7700 Sequence Detector (Applied Biosystems, War-rington, UK) according to the manufacturer's instructions and the resultant data were averaged. Non-template controls were included in each experiment. Specific oligonucleotide primers and probes were designed for VEGF-B using Primer Express® 1.5 software (Applied Biosystems, Warrington, UK). Specific primers and probe for Ang-2 were a gift from Mrs K Day of The Reproductive Molecular Research Group at the University of Cambridge Department of Obstetrics and Gynaecology. Details for VEGF-B primers and probe are shown below.

Forward primer, 5′ – AGCACCAAGTCCGGATG – 3′
Reverse primer, 5′ – GTCTGGCTTCACAGCACTG – 3′
Probe, 5′-6FAM – AGATCCTCATGATCCGGTACCCGT – 3′

A 1 μl volume of each cDNA was amplified using PCR Master Mix (Applied Biosystems, Warrington, UK) under the following PCR conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. β-Actin and 18 s RNA were used as endogenous control sequences for VEGF-B and Angiopoietin-2, respectively. Preliminary experiments confirmed that the endogenous control sequences were amplified at the same rate as the target sequences (data not shown). The threshold cycle (C_T) was determined for each sample. C_T value is the cycle number at which the level of reporter fluorescence rises above a preset threshold level. Normalisation was achieved by subtracting the mean C_T value for each endogenous control triplicate from the mean C_T value for each target gene triplicate. Target gene abundance was calculated for each tissue type.

Figure 1 Reverse transcription PCR analysis of VEGF-C in samples of benign endometrium (A−G), ACH (H−J) and endometrioid endometrial carcinoma. Well differentiated carcinoma (K−M), moderately differentiated carcinoma (N−S) and poorly differentiated carcinoma (T−W). Lane r represents an RNA control in which the RT step was omitted and lane c represents a negative control in which the cDNA was replaced by water. The size of the transcripts (shown on the right of the figure) was estimated by co-electrophoresis of a 1 kb ladder (lane I).

Statistical analyses

The presence or absence of specific *in situ* hybridisation for each transcript was compared across the different histological cate-gories using the two-tailed Fisher's exact probability test. This was computed using the *R* statistical computing programme (www.r-project.org). Statistical significance was accepted as $P < 0.05$. For results of quantitative PCR, normalised log-transformed transcript levels were compared between groups using the unpaired Student's *t*-test (two-tailed).

RESULTS

Expression of members of the VEGF family and angiopoietins by *in situ* hybridisation

The presence of VEGF-A was detected in all cases of endometrioid endometrial cancer (Table 2 and Figure 2) and was increased near areas of necrosis. Hybridisation for VEGF-A was not seen in cases of ACH or in sections of benign postmenopausal endometrium.

In contrast, hybridisation of the VEGF-B riboprobe was seen in all the five cases of ACH (Table 2 and Figure 2). There was continued expression for VEGF-B in low-grade tumours that diminished as the grade of the tumour increased. Vascular endothelial growth factor-B mRNA was also present in epithelial and stromal compartments (Figure 2), although, unlike VEGF-A, there was no apparent increase in hybridisation near areas of necrosis.

Messenger RNA encoding VEGF-C was not detected in any of the tissues examined using *in situ* hybridisation (Table 2 and Figure 1). A specimen of human lymphoma was used as a positive control and demonstrated specific hybridisation (Figure 2). However, PCR amplification of VEGF-C cDNA was seen in all the samples of endometrial cancer (13 out of 13) and ACH (three out of three) examined (Figure 1), in addition to six out of 7 benign postmenopausal endometria. These findings confirm the presence of VEGF-C in benign, premalignant and malignant endometrium (Hirai *et al*, 2001), although at levels beneath the detection threshold for *in situ* hybridisation.

Messenger RNA encoding VEGF-D was present in three of the five ACH specimens and in six of the 13 carcinomas (one moderately differentiated tumour and five poorly differentiated tumours). The distribution of silver grains did not correspond with epithelial tumour cells but did co-localise with tumour-associated macrophages (CD-68 positive cells) in serial sections (Figure 3).

Hybridisation for Ang-1 mRNA was seen in only one benign and one ACH specimen. Similarly, mRNA encoding Ang-2 was seen in no benign tissues and in only one of the five hyperplastic samples. In contrast, while hybridisation was absent in well/moderately differentiated cancers, hybridisation for Ang-2 mRNA was clearly demonstrated in four out of nine of the poorly differentiated tumours examined. Hybridisation was most marked in macro-phage-rich regions of these tumours and was seen to correspond with tumour-associated macrophages (CD-68) upon immunostain-ing of serial sections (Figure 3). In all of the experiments described, there was no specific hybridisation when the sense probes were used on adjacent sections.

Transcript abundance of VEGF-B and Ang-2 in benign endometrium and endometrial carcinoma assessed by quantitative PCR

Real-time, quantitative PCR was carried out using cDNA generated from benign postmenopausal endometria and endometrioid-type endometrial adenocarcinoma. The abundance of VEGF-B tran-script was greater in benign endometrium ($n = 7$) than in atypical hyperplasia ($n = 3$) ($P = 0.13$) and endometrial cancer ($n = 17$) ($P = 0.04$) (Figure 4A). Conversely, cDNA encoding Ang-2 was
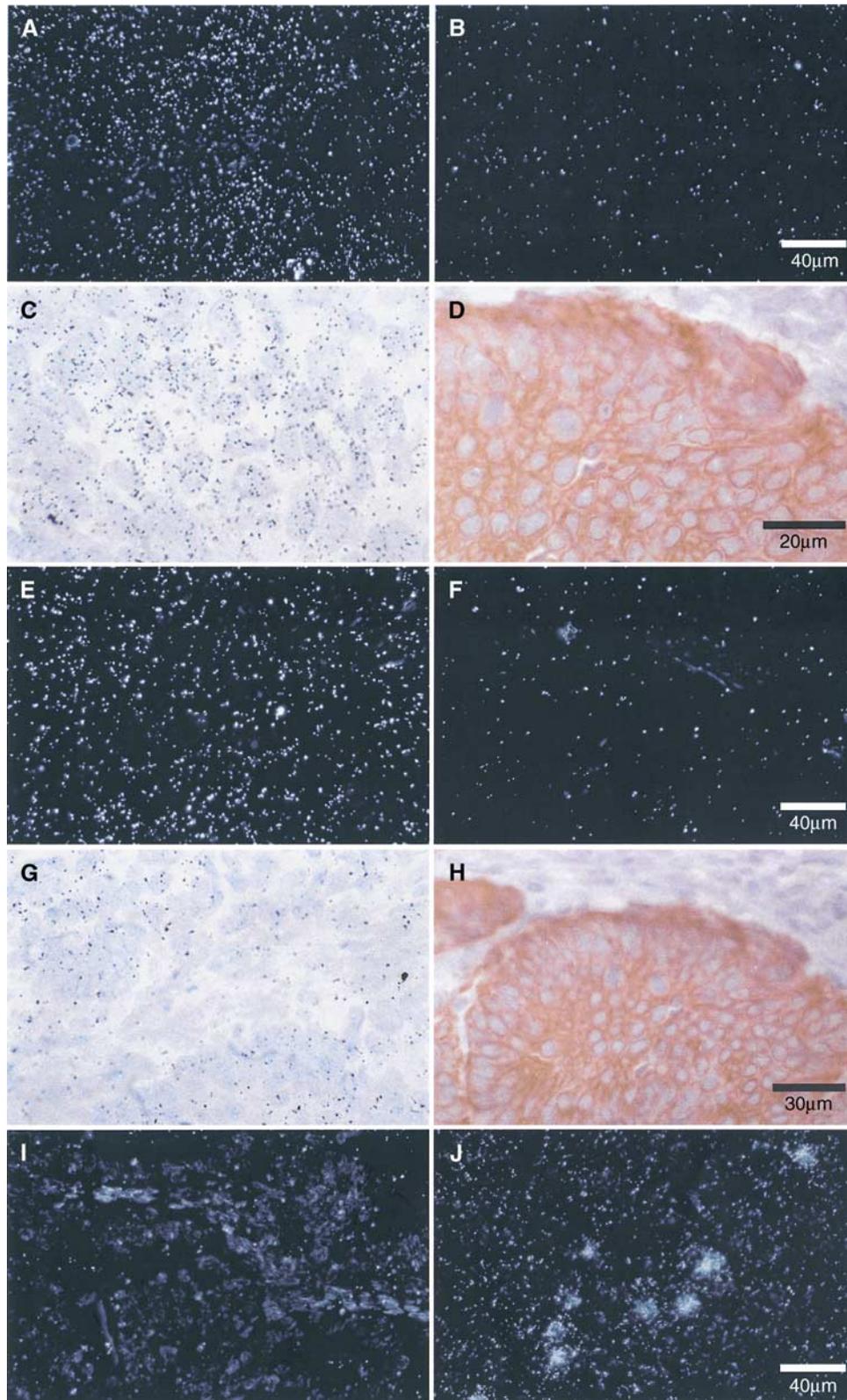
Figure 2 Expression of mRNA encoding VEGF-related factors in the epithelial cells of endometrioid endometrial carcinoma and ACH. *In situ* hybridisation with VEGF-A antisense probe in a moderately differentiated (FIGO grade 2) carcinoma shown under dark-field (**A**) and light-field (**C**) conditions shows hybridisation in epithelial carcinoma cells. There is no specific hybridisation when a sense probe (negative control) is used (**B**). The scale bar in (**B**) applies to (**A**), (**B**), (**E**), (**F**), (**I**) and (**J**). Immunostaining of a serial section with anti-human cytokeratin confirms the epithelial localisation of silver grains (**D**) (scale bar applies to ACH (**C**) and (**D**). *In situ* hybridisation with VEGF-B antisense probe in ACH shown under dark-field (**E**) and light-field (**G**) conditions shows diffuse hybridisation in epithelial and stromal cells. There is no specific hybridisation when a sense probe (negative control) is used (**F**). Immunostaining with anti-human cytokeratin (**H**) identifies the epithelial and stromal cells (**H**) (scale bar applies to (**G**) and (**H**). *In situ* hybridisation with VEGF-C antisense probe in a poorly differentiated (FIGO grade 3) carcinoma shown under dark-field conditions (**I**) shows no specific hybridisation when compared with a human B-cell lymphoma (**J**).

Table 2 Expression of VEGF-A, VEGF-B, VEGF-C, VEGF-D, Ang-1 and Ang-2 detected by *in situ* hybridisation in sections of endometrium

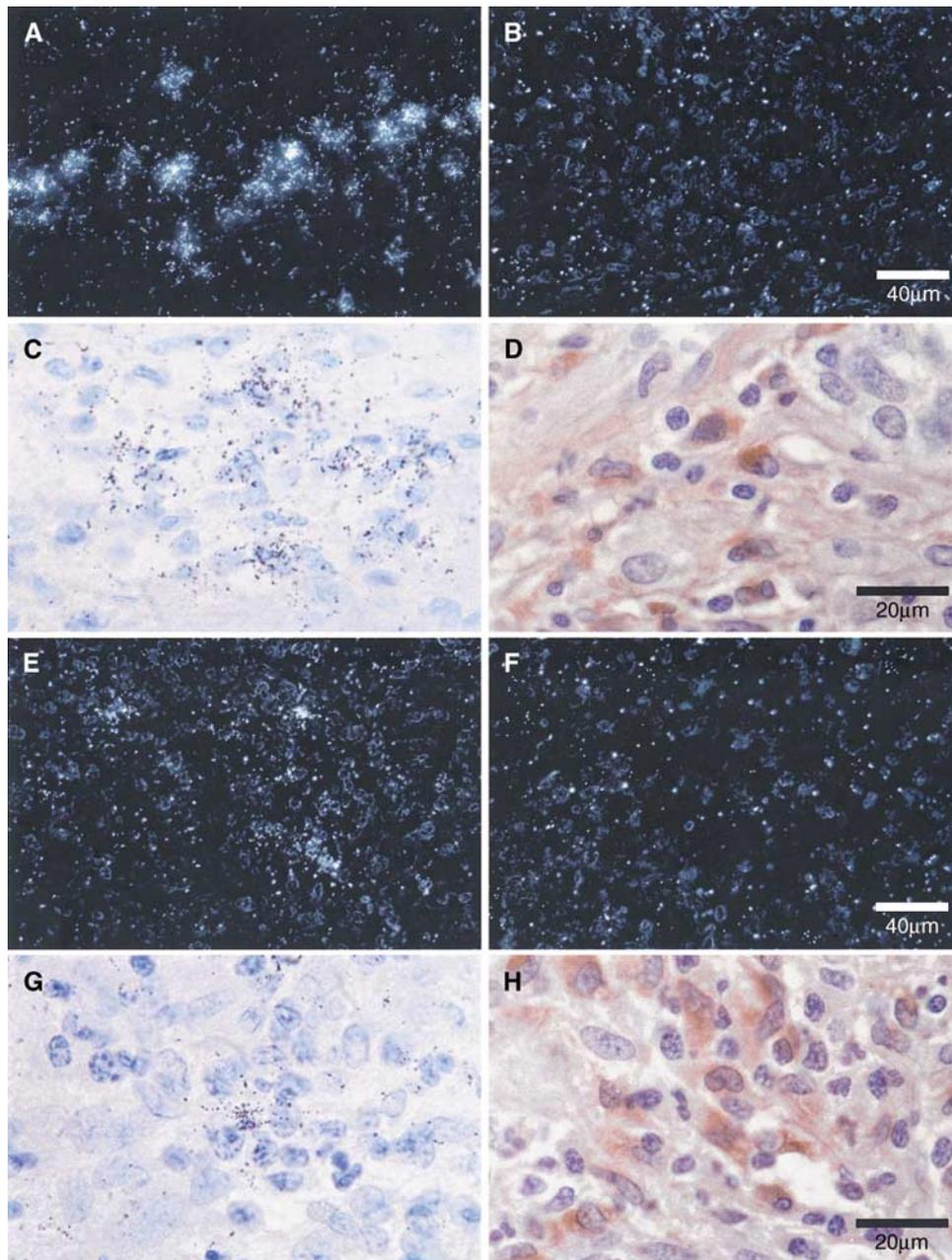| Endometrial histology	*n*	VEGF-A	VEGF-B	VEGF-C	VEGF-D	ANG-1	ANG-2
Benign	5	0/5	0/5	0/5	0/5	1/5	0/5
ACH	5	1/5	5/5*	0/5	3/5	1/5	1/5
FIGO G1/2 carcinoma	4	4/4*	3/4	0/4	1/4	0/4	0/4
FIGO G3 carcinoma	9	9/9*	4/9	0/9	5/9	0/9	4/9

*$P < 0.05$.

Figure 3 Expression of mRNA encoding VEGF-D and Ang-2 in macrophages infiltrating a poorly differentiated (FIGO grade 3) endometrioid endometrial carcinoma. *In situ* hybridisation with VEGF-D antisense probe shown under dark-field (**A**) conditions and light-field conditions (**C**). *In situ* hybridisation with VEGF-D sense (control) probe shows no specific hybridisation (**B**) (scale bar applies to (**A**), (**B**), (**E**) and (**F**)). Immunostaining with anti-human CD68 in a serial section (**D**), localises the silver grains to tumour associated macrophages (TAMs) (scale bar applies to (**C**), (**D**), (**G**) and (**H**)). *In situ* hybridisation with Ang-2 antisense probe shown under dark-field (**E**) and light-field (**G**) conditions. *In situ* hybridisation with Ang-2 sense (control) probe (**F**) shows no specific hybridisation. Anti-human cytokeratin staining (**H**) localises the hybridisation to TAMs.
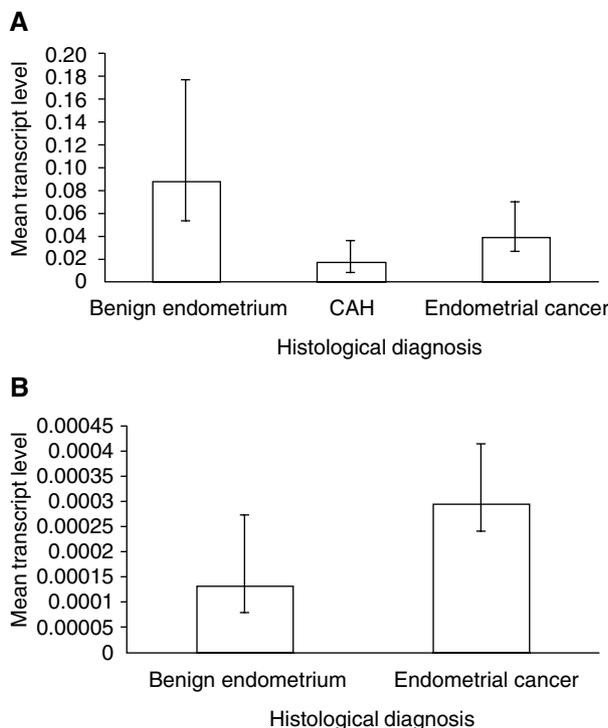
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A

B

Figure 4 Quantitative PCR for gene transcripts in benign and malignant endometrium. Levels of gene transcript are shown normalised to an endogenous control sequence. Transcript levels are expressed as arbitrary units and represent the mean for each histological group. Experiments for each sample were performed in triplicate. (**A**) The level of mRNA encoding VEGF-B is generally higher in benign postmenopausal endometrium than in CAH ($P = 0.13$) and endometrial cancer ($P = 0.04$). (**B**) mRNA encoding Ang-2 is generally present at higher levels in endometrial cancer than in benign postmenopausal endometrium.

more abundant in the cancer samples ($n = 17$) than in benign postmenopausal endometrium ($n = 4$) (Figure 4B), although this did not reach statistical significance ($P = 0.49$).

DISCUSSION

These findings confirm the expression of VEGF-A in endometrial cancer (Doldi et al, 1996; Guidi et al, 1996; Fujimoto et al, 1998; Fine et al, 2000), although in contrast to other published reports (Fujimoto et al, 1998; Fine et al, 2000), we did not demonstrate VEGF-A mRNA in benign specimens. This may be explained by the inclusion of both pre- and postmenopausal endometrium in other studies (Fujimoto et al, 1998). Messenger RNA for VEGF-A is abundant in premenopausal endometrium (Charnock-Jones et al, 1993). Our findings support those of Guidi et al (1996), who demonstrated little or no mRNA expression in benign atrophic endometrium. We demonstrated VEGF-A mRNA in only one out of 5 specimens of ACH compared with 13 out of 13 endometrioid cancers. These differences may be due to necrosis within the malignant tissue. Hypoxia, in necrotic areas of cancer, leads to the upregulation of transcription (Tischer et al, 1991) and increased stability of VEGF-A mRNA (Levy et al, 1996).

Messenger RNA and protein for the related growth factor VEGF-B have previously been identified in other tumours including ovarian carcinoma (Sowter et al, 1997). We demonstrate, using quantitative PCR, that mRNA encoding VEGF-B is more abundant in benign postmenopausal endometrium than in endometrial adenocarcinoma. The results also suggest that there may be a relative loss of VEGF-B mRNA in ACH although this was not

statistically significant and may reflect small sample numbers. We show, using in situ hybridisation, that where present, VEGF-B mRNA is expressed in both epithelium and stroma of ACH and some endometrial cancers. These findings contrast with the overexpression of VEGF-A seen in endometrial cancers (Doldi et al, 1996; Fujimoto et al, 1998). The distribution of VEGF-B in both epithelial and stromal compartments of atypical hyperplasia and some cancers is similar to that observed in ovarian cancer (Sowter et al, 1997), but in contrast to VEGF-A, hybridisation was not increased near areas of necrosis. Vascular endothelial growth factor-B is not upregulated by hypoxia (Silins et al, 1997) and this may explain the differences seen here.

The function of VEGF-B is not clear. Vascular endothelial growth factor-1 binds VEGFR-1 (flt-1) but not VEGFR-2 (KDR) (Olofsson et al, 1998), both of which are present in peritumoral endothelial cells of endometrial cancers (Guidi et al, 1996). Mouse 'knockout' experiments suggest that the VEGFR-1 plays a role in endothelial differentiation and/or interactions between the endothelium and extracellular matrix (Fong et al, 1995), whereas signalling through VEGFR-2 leads to endothelial cell proliferation and migration (Waltenberger et al, 1994). Vascular endothelial growth factor-B is therefore unlikely to promote endothelial cell proliferation or migration directly. However, VEGF-B signalling via VEGFR-1 may participate in the organisation of endothelial–matrix interactions as VEGF-B increases the expression of both uPA (urokinase-type plasminogen activator) and PAI-1 (plasminogen activator inhibitor-1) in endothelial cell cultures (Olofsson et al, 1996a). These play a role in matrix degradation and its inhibition, respectively.

Vascular endothelial growth factor-B could contribute to the maintenance of host–tumour responses. Vascular endothelial growth factor-A, produced by tumour cells, inhibits the functional maturation of immune dendritic cells from haemopoietic progenitor cells (Gabrilovich et al, 1996). This effect is mediated by binding to VEGFR-1 (Oyama et al, 1998). Therefore, loss of VEGF-B may contribute to the escape of tumour cells from the host immune response by making more VEGFR-1 binding sites available for binding by VEGF-A. Vascular endothelial growth factor-B may thus act to suppress changes that favour the development of endometrial cancer.

We confirmed the presence of VEGF-C mRNA in endometrial carcinoma shown by Hirai et al (2001), but did not demonstrate this by in situ hybridisation. Intense hybridisation for VEGF-C mRNA is seen in endometrial natural killer cells (Li et al, 2001), hence the VEGF-C transcript may be present in non-tumour cells that were under-represented in our endometrial tissues.

Vascular endothelial growth factor-D binds to VEGFR-2 and VEGFR-3 (Achen et al, 1998), both of which are present in endometrial cancer (Guidi et al, 1996). In colon cancer, VEGFR-3 is associated with inflammatory infiltrates of TAMs (White et al, 2002) although VEGF-D has not been demonstrated in TAMs themselves. We have shown hybridisation for VEGF-D mRNA in ACH and both well differentiated and poorly differentiated tumours (Table 2), although not all cases in each group were positive. Recently, positive immunostaining for VEGF-D has been demonstrated in both epithelium and stroma of endometrial cancers with some cases of ACH also displaying immunopositivity (Yokoyama et al, 2003). In the latter study, 42 and 87% of stage I/II and stage III/IV tumours, respectively, showed significant immunostaining. These findings may reflect differences in the degree of inflammatory infiltration between individual tumours as well as increased expression of VEGF-D by the tumour cells. There is a tendency to higher TAM density in microvessel-rich tumours (Salvesen and Akslen, 1999), and our findings indicate that TAMs may assist in promoting angiogenesis in endometrioid endometrial carcinoma by the secretion of VEGF-D.

Angiopoietin-1 and Ang-2 are previously unreported in endometrial cancer. We demonstrated higher levels of Ang-2

mRNA in endometrial cancers than benign endometrium, although this did not reach statistical significance. The latter may reflect the small numbers of samples in the benign group. Nonetheless, the distribution of mRNA for Ang-2, assessed by *in situ* hybridisation, was similar to that of VEGF-D in TAMs (Figure 3). Both Ang-2 and VEGF-D were only seen in association with CD68-positive cells, although CD68-positive cells, without hybridisation for either of these factors were also present.

Angiopoietin-2 is an antagonist of Ang-1, acting through the Tie-2 receptor and disrupting pericyte-endothelial cell interactions. In the presence of VEGF-A, Ang-2 facilitates vessel sprouting while in its absence, regression of vessel sprouts occurs (Maisonpierre *et al*, 1997). However, in active tumour angiogenesis, a large proportion of the blood vessels lack pericytes and can be selectively ablated by withdrawal of VEGF-A alone (Benjamin *et al*, 1999). In our series, survival did not differ significantly where hybridisation for both VEGF-A and Ang-2 was seen and this may reflect a high proportion of immature tumour vessels lacking pericyte coverage. Angiopoietin-2 may also alter the response of destabilised endothelial cells to cytokines, thus modulating angiogenesis (Laurén *et al*, 1998).

We have shown that gene transcripts for the related growth factors, VEGF-A and VEGF-B, are differentially expressed in benign postmenopausal endometrium and endometrioid endometrial cancer. The reduced expression of VEGF-B in endometrial cancers compared with benign endometrium suggests that VEGF-B may play a role in maintaining normal cellular interactions in endometrium and that loss of expression could contribute to endometrial tumorigenesis.

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