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VEGF-A, VEGF-C, and VEGF-D in Colorectal Cancer Progression

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Abstract

We aimed to assess the relationship of the angiogenic cytokines VEGF-A, VEGF-C, and VEGF-D and their receptors VEGFR-2 and VEGFR-3 in the adenoma–carcinoma sequence and in metastatic spread of colorectal cancer (CRC). mRNA expression levels were measured using semi-quantitative reverse transcription polymerase chain reaction in 70 CRC (35 with paired mucosae) and 20 adenomatous polyps. Immuohistochemistry and ELISA assessed protein expression. VEGF-D mRNA expression was significantly lower in both polyps and CRCs compared with normal mucosa (*P*=.0002 and .002, respectively), whereas VEGF-A and VEGF-C were significantly raised in CRCs (*P*=.006 and .004, respectively), but not polyps (*P*=.22 and *P*=.5, respectively). Receptor expression was similar in tumor tissue and normal mucosae. Tumors with lymph node metastases had significantly higher levels of VEGF-A compared with non-metastatic tumors (*P*=.043). There was no association between VEGF-C or VEGF-D and lymphatic spread. The decrease in VEGF-D occurring in polyps and carcinomas may allow the higher levels of VEGF-A and VEGF-C to bind more readily to the VEGF receptors, and produce the angiogenic switch required for tumor growth. Increased expression of VEGF-A within CRCs was associated with lymphatic metastases, and therefore, this member of the VEGF family may be the most important in determining metastatic spread. *Neoplasia* (2001) **3,** 420–427.

Keywords: angiogenesis, immunohistochemistry, staging, lymphatic, adenoma–carcinoma.

Introduction

Neoangiogenesis, the formation of new capillaries from preexisting blood vessels, is essential for tumor development beyond a diameter of 2 to 3 mm^3 [1]. This process is mediated by angiogenic cytokines and provides tumors not only with nutrients for growth, but also increases the opportunity for tumor cells to enter the circulation and metastasize [2]. The most potent of these cytokines is vascular endothelial growth factor (VEGF-A), a heparin-binding glycoprotein with potent angiogenic, mitogenic, and vascular permeability–enhancing activities specific for endothelial cells.

The gene for human VEGF-A is organised into eight exons and located on chromosome 6 [3]. As a result of alternative splicing, at least four transcripts encoding mature monomeric VEGF containing 121, 165, 189, and 206 amino acid residues ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) have been detected. The secretion pattern of the four isoforms differs markedly. $VEGF_{121}$ is a weakly acidic polypeptide that does not bind to heparin, and is freely soluble. $VEGF_{165}$, the predominant form secreted by a variety of normal and transformed cells [4], is a basic heparin-binding glycoprotein. Although secreted, a significant portion remains bound to the cell surface or extracellular matrix. The $VEGF_{189}$ isoform includes 24 additional amino acids and is not freely secreted, but instead remains predominantly bound to the cell surface and/or extracellular matrix [5]. $VEGF_{206}$ is a rare isoform, so far identified only in a human fetal liver cDNA library.

VEGF-C and VEGF-D, two more recently discovered cytokines, have not only angiogenic properties but also a lymphangiogenic action. VEGF-C is 48% identical to VEGF-D with long NH_2- and C-terminal extensions, which set VEGF-C and VEGF-D apart as a subfamily of VEGF-related proteins.

VEGF-A acts through the tyrosine kinase receptors Flt-1 (fms-like tyrosine kinase)/VEGFR-1 and KDR (kinase insert domain-containing receptor)/VEGFR-2, which are expressed on vascular endothelium. VEGF-C and VEGF-D act through VEGFR-2 and Flt-4/VEGFR-3 [6,7], a receptor that in adult normal tissues is restricted to the lymphatic endothelium and high venular endothelium of lymph nodes. The pattern of expression of VEGF-C in relation to VEGFR-3 during the sprouting of the embryonic lymphatic endothelium confirms its importance in developing lymphatics [8,9], with overexpression of VEGF-C resulting in lymphatic endothelial proliferation and selective hyperplasia of the lymphatic vasculature [10]. Thus, the association of VEGFR-3 and its two ligands with lymphangiogenesis has provided a

Abbreviations: CRC, colorectal cancer; RT-PCR, reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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possible mechanism for primary tumors to metastasize *via* newly formed lymphatics and has led to studies investigating VEGF-C and lymphatic spread [11–13].

The expression of VEGF-D in tumors has not been extensively studied, but in adenocarcinoma of the lung, VEGF-D levels were found to be significantly lower than in normal tissue [14], whereas others have been unable to detect VEGF-D expression in either tumor biopsies or the colorectal cell line SW 480 [15].

This study shows the changes in expression of VEGF-A, VEGF-C, and VEGF-D and their receptors VEGFR-2 and VEGFR-3 through the adenoma–carcinoma sequence, and their association with metastatic spread in colorectal carcinoma.

Materials and Methods

Tissue Samples

Twenty adenomatous polyps, removed either surgically or at colonoscopy, and 70 sporadic colorectal cancers (CRCs), of which 35 had a paired normal mucosa sample taken 10 cm from the primary tumor, were studied. There were 7 with Dukes A, 22 with Dukes B, 27 with Dukes C, and 14 with Dukes D carcinoma. Seven of the Dukes C/D tumors had a positive lymph node taken from the mesentery for comparison with the primary tumor. Colonic mucosa from five benign resections was also taken. All samples were snap-frozen and stored in liquid nitrogen until RNA extraction.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the tumor samples using SV Total RNA Isolation System (Promega, Madison, WI, USA). Two micrograms of total RNA was diluted in 16 μl of double distilled water (DDW) and denatured at 95°C for 5 minutes; oligo (dT) primer was added (5 μl of 5× RT buffer and 2 μg of oligo (dT)) and incubated for 15 minutes at 4°C. The primed RNA was reverse-transcribed and incubated for 60 minutes at 42°C [5 μl of M-MLV RT 5× buffer (Sigma, St. Louis, MO, USA), 5 μl of 1 mg/ml bovine serum albumin (Sigma), 1 μl of 5 U/μl human placental ribonuclease inhibitor (Promega rRNasin), 2 μl of 350 mM β-mercaptoethanol, 2 μl of 25 mM dNTP (Sigma), 1 μl of 200 mM sodium pyrophosphate, 8 μl of DDW, and 1 μl of M-MLV reverse transcriptase (Sigma)]. The cDNA was denatured for 5 minutes at 95°C and stored at −20°C until PCR.

PCR conditions were optimized such that the number of cycles lays within the linear range of amplification. Five microliters of cDNA was used for PCR reactions in a total volume of 50 μl of PCR mixture [1 μl of 0.2 μM sense and antisense primer; 5.0 μl of 10× PCR buffer (Sigma REDTaq PCR reaction buffer 10×), 5.0 μl of 2.5 mM dNTP (Sigma), 1.5 μl of DNA polymerase (Sigma REDTaq DNA polymerase)]. Primers used are shown in Table 1. PCR conditions were as follows: 28, 30, 32, 34, or 36 cycles of 1 minute at 94°C, 2 minutes at 50°C, and 1.0 minute at 72°C for Beta-actin, VEGF-A, VEGFR-2, VEGF-C, and VEGF-D, respectively. VEGFR-3 required a hot start at 96°C for 8 minutes, 56°C for 3 minutes with addition of 1.5 μl of DNA polymerase, then 31 cycles of 1 minute at 94°C, 2 minutes at 56°C, and 1.0 minute at 72°C. Each PCR finished with an extension step of 10 minutes at 72°C.

PCR was performed on 70 CRCs (35 with paired normal mucosae), 20 adenomatous polyps, and 5 colonic mucosae from benign resections in the case of VEGF-A, VEGF-C, VEGF-D, and VEGFR-3. Due to insufficient cDNA, PCR for VEGFR-2 was performed on 40 CRCs (14 with paired normal mucosae), 13 adenomatous polyps, and 4 further benign colonic mucosal samples.

PCR products were run on a 2% agarose gel with 0.1 μg/ml ethidium bromide. The gel was exposed to ultraviolet light and the image captured using a Polaroid MP-4 Land camera. Band intensity was analyzed by image analysis (GelPro; Media Cybernetics, Silver Spring, MD, USA). The intensity of the PCR products was semi-quantitated with the intensity of their respective beta-actin band intensity, and is expressed as arbitrary units.

Immunohistochemistry

VEGF-C and VEGF-D Four normal colonic resections, 11 adenomatous polyps, and 59 CRCs were examined. Paraffin sections (5 μm) were deparaffinized and placed in solution of absolute methanol and 3% hydrogen peroxide for 30 minutes. After washing in distilled water and rinsing in phosphate-buffered saline, slides were blocked with 1:10 diluted rabbit serum for 20 minutes. Slides were incubated overnight at 4°C in a humidified chamber with anti–VEGF-C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti–

Table 1. Sequences of Primers Used for RT-PCR with Basepair Size.

Gene (bp)	Sense Primer	Antisense Primer	Size
β-Actin	5′-TCGACAACGGCTCCGGCA-3′	5-AAGGTGTGGTGCCAGATT-3′	239
VEGF-A*	5′-CTCACCAAGGCCAGCACATAGG-3′	5′-ATCTGGTTCCGAAAACCCTGAG-3′	159, 291, 363, 414
VEGF-C	5′-GTCTGTGTCCAGTGTAGATG-3′	5′-AGGTAGCTCGTGCTGGTGTT-3′	360
VEGF-D	5′-CAGTGAAGCGATCATCTCAGTC-3′	5′-TACGAGGTGCTGGTGTTCATAC-3′	397
KDR/VEGFR-2	5′-GGAAATCATTATTCTAGTAGGCAC-3′	5′-CCTGTGGATACACTTTCGCGATG-3′	793
Flt-4/VEGFR-3	5′-AGCCATTCATCAACAAGCCT-3′	5′-GGCAACAGCTGGATGTCATA-3′	298

*Four isoforms of VEGF-A 121, 165, 189, and 206, respectively.

VEGF-D (Santa Cruz Biotechnology) antibody diluted 1:100 with phosphate-buffered saline.

Slides were then incubated with biotinylated rabbit anti-goat IgG for 20 minutes (Vectastatin ABC kits; Vector Laboratories, Burlingame, CA, USA) and then with pre-mixed ABC (Vector Laboratories) reagent for 20 minutes. Immunostaining was performed by incubating the slides in diaminobenzidine (Vector Laboratories). After chromogen development, the slides were washed, counterstained with hematoxylin, dehydrated with alcohol and xylene, and mounted in routine fashion.

Negative controls were performed in all cases by omitting the primary antibody. Cases with at least 20% immunoreactive tumor cells were considered positive, as described previously [13].

VEGF-A Paraffin sections were deparaffinized and heated in a microwave oven for 10 minutes in 10 mM sodium citrate buffer, pH 6. Four normal colonic resections, 11 adenomatous polyps, and 59 CRCs were examined. The slides were then treated with a commercially available immunostaining kit (ImmunoCruz; Santa Cruz Biotechnology) using a mouse monoclonal antibody [VEGF (C-1)]. After chromogen development with diaminobenzidine, slides were counterstained with Gill's hematoxylin. Negative controls were performed in all cases omitting the primary antibody. Cases with at least 20% immunoreactive tumor cells were considered positive.

VEGFR-3 Fourteen randomly selected paraffin sections were deparaffinized and heated in a microwave oven for 15 minutes in 10 mM sodium citrate buffer, pH 6. Endogenous peroxidase was blocked using absolute methanol and 3% hydrogen peroxide for 30 minutes. Slides were incubated with 10% rabbit blocking serum, after which a mouse monoclonal antibody 9D9 (a kind gift from Professor Kari Alitalo, Helsinki, Finland) was added at a concentration of 1.1 μl/ml for 2 hours at room temperature. Slides were then incubated with biotinylated secondary antibody for 45 minutes (R&D Systems, Abingdon, UK), and then for 30 minutes with HSS-HRP (R&D Systems). Immunostaining was performed by incubating the slides for 20 minutes with AEC (3-amino-9-ethylcarbazole). After chromogen development, the slides were washed, counterstained with hematoxylin, and mounted in aqueous medium. Negative controls were performed in all cases omitting the primary antibody.

Quantitative Measurement of VEGF-A and VEGF-D Protein Concentration

The total protein concentration of 25 tumor samples (16 with paired normal mucosae) was measured using GeneQuant (Pharmacia, Cambridge, UK). These samples were then assayed for VEGF-D protein (Figure 1) by ELISA (R&D Systems), with 14 paired samples assayed for VEGF-A (R&D Systems). The ELISA concentration (pg/ml) obtained was divided by its total protein concentration (μg/ml) to correct for variation between samples, producing a normalised ratio for each sample. No ELISA for VEGF-C is commercially available at present.

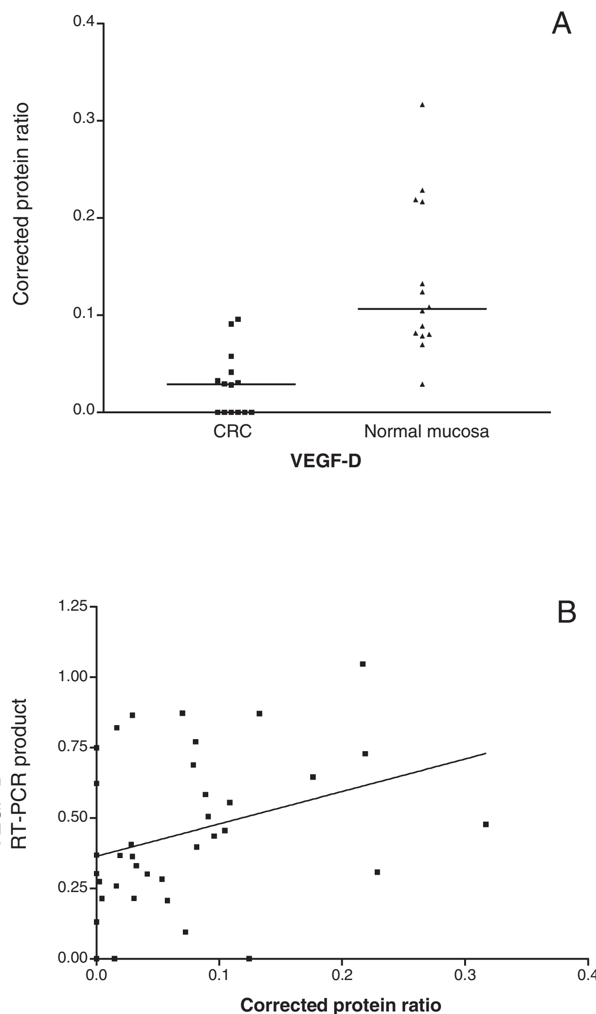

Figure 1. *(A) Comparison of corrected VEGF-D protein ratios in colorectal cancers and paired normal mucosa (horizontal bar represents median). (B) Correlation of corrected VEGF-D protein ratios with RT-PCR products (r=0.36, P=.03).*

Serum VEGF-D ELISA

Samples were obtained from 26 control patients who had undergone a normal colonoscopy, 11 adenomatous polyp patients, and 59 patients with CRC (7 Dukes A, 20 Dukes B, 18 Dukes C, and 14 Dukes D). These samples were assayed for VEGF-D by ELISA (R&D Systems).

Statistics

The data were non-normally distributed and therefore non-parametric tests were used. Comparison between different data sets used an unpaired *t*-test (Mann-Whitney) and comparison of paired data was calculated using Wilcoxon's rank test. The median value with interquartile range (IQR) is reported. Correlations (two-tailed) were calculated using the Spearman test. Statistical analysis was performed on Prism 3 (GraphPad Software, San Diego,

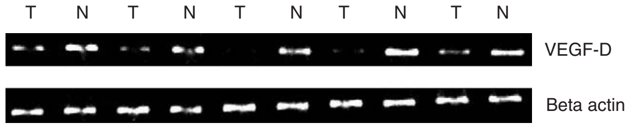

Figure 2. *VEGF-D RT-PCR products of five paired tumors (T) and paired normal mucosa (N).*

CA). A *P* value of <.05 was considered statistically significant.

Results

mRNA Expression of VEGF-A, VEGF-C, VEGF-D, KDR/VEGFR-2, and Flt-4/VEGFR-3

All CRCs, polyps, and normal mucosa expressed the VEGF-A 121 and 165 isoforms, with only one cancer also expressing the 189 isoform. A total of 67/70 (95.7%) CRCs, 18/20 (90%) polyps, and 38/40 (95%) normal mucosa expressed VEGF-C; whereas 64/70 (91.4%) CRCs, 15/19 (78.9%) polyps, and 38/40 (95%) normal mucosa expressed VEGF-D. A total of 29/40 (72.5%) CRCs, 10/13 (76.9%) polyps, and 10/17 (58.8%) normal mucosa expressed VEGFR-2; whereas 46/69 (66.6%) CRCs, 17/20 (85%) polyps, and 33/39 (84.6%) normal mucosa expressed VEGFR-3.

Comparison of CRC and Normal Mucosal Samples

VEGF-A 121 and 165 isoform expression was significantly raised in CRCs compared to paired normal mucosae [median 0.49 (interquartile range 0.29–0.66) and 0.30 (0.19–0.42) *vs* 0.34 (0.25–0.49) and 0.22 (0.19–0.32), *P*=.015 and *P*=.0045, respectively]. As a consequence, the total VEGF-A expression was significantly higher in CRCs compared with normal mucosae [median 0.79 (0.48–1.04) and 0.55 (0.44–0.83), *P*=.005, respectively], and was confirmed by ELISA measurement of normalised tumor protein (0.478 *vs* 0.237, *P*=.0017, respectively). There was a significant increase in VEGF-C expression in tumors

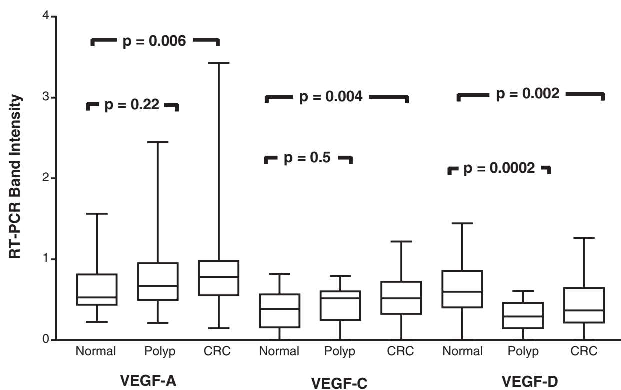

Figure 3. *mRNA expression of VEGF-A, VEGF-C, and VEGF-D through the adenoma–carcinoma sequence. Median values with interquartile range (box) and range (whiskers).*

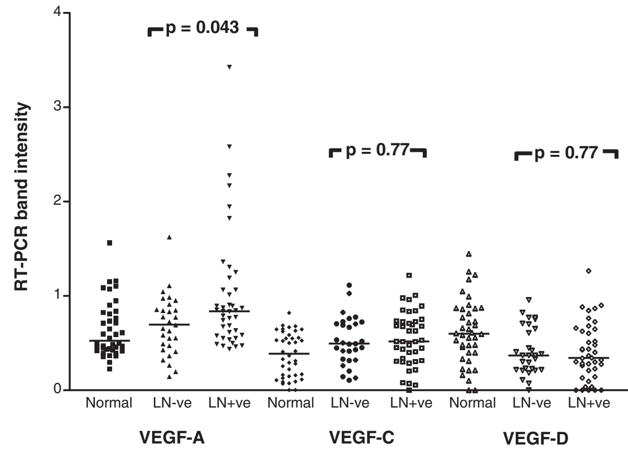

Figure 4. *Scatter plot of mRNA expression in normal mucosa, node-negative, and node-positive tumors (horizontal bar represents median).*

compared with paired normal mucosae [median 0.45 (interquartile range 0.32–0.68) and 0.37 (0.15–0.57), *P*=.04]. However, there was a significant decrease in VEGF-D expression in tumor tissue compared with paired normal mucosae [median 0.32 (0.22 0.44) and 0.60 (0.40–0.87), *P*=.0012].

The RT-PCR findings for VEGF-D were confirmed by ELISA, which correlated with the RT-PCR data (*r*=0.33, *P*=.035), and showed that the expression of VEGF-D protein in normal mucosa was significantly higher than the paired tumors (median 0.107 and 0.288, respectively, *P*=.0002) (Figures 1 and 2).

VEGFR-2 [median 0.19 (0–0.5) and 0.14 (0–0.32), *P*=.45] and VEGFR-3 [median 0.23 (0.16–0.41) and 0.19 (0.09–0.32), *P*=.09] expressions were similar in tumor tissue and normal mucosa.

VEGF-A, VEGF-C, VEGF-D, and the Adenoma–Carcinoma Sequence

There was an increase in total VEGF-A from normal mucosa to adenoma [median 0.53 (interquartile range 0.43–0.82) and 0.67 (0.49–0.96), respectively, *P*=.22] with a further increase in total VEGF-A in CRCs [0.78 (0.55–0.96), *P*=.006, compared to normal mucosa]. VEGF-C expression was higher in both polyps and CRCs with very similar levels of expression [0.52 (0.24–0.61) and 0.52 (0.32–0.73) respectively], but the differences were only statistically significant between normal mucosa and CRCs (*P*=.004). VEGF-D mRNA expression was signifi-

Table 2. VEGF-A, VEGF-C, and VEGF-D Immunohistochemistry.

	VEGF-A	VEGF-C	VEGF-D
Normal mucosa (*n*=4)	0 (0%)	0 (0%)	0 (0%)
Polyps (*n*=11)	1 (9%)	0 (0%)	2 (18.2%)
Dukes A (*n*=11)	0 (0%)	1 (9.1%)	1 (9.1%)
Dukes B (*n*=18)	5 (27.7%)	12 (66.6%)	7 (38.8%)
Dukes C (*n*=22)	7 (31.8%)	8 (36.4%)	10 (45.5%)
Dukes D (*n*=8)	0 (0%)	1 (12.5%)	0 (0%)
Total number of cancers (*n*=59)	12 (20.3)	21 (35.6%)	18 (30.5%)

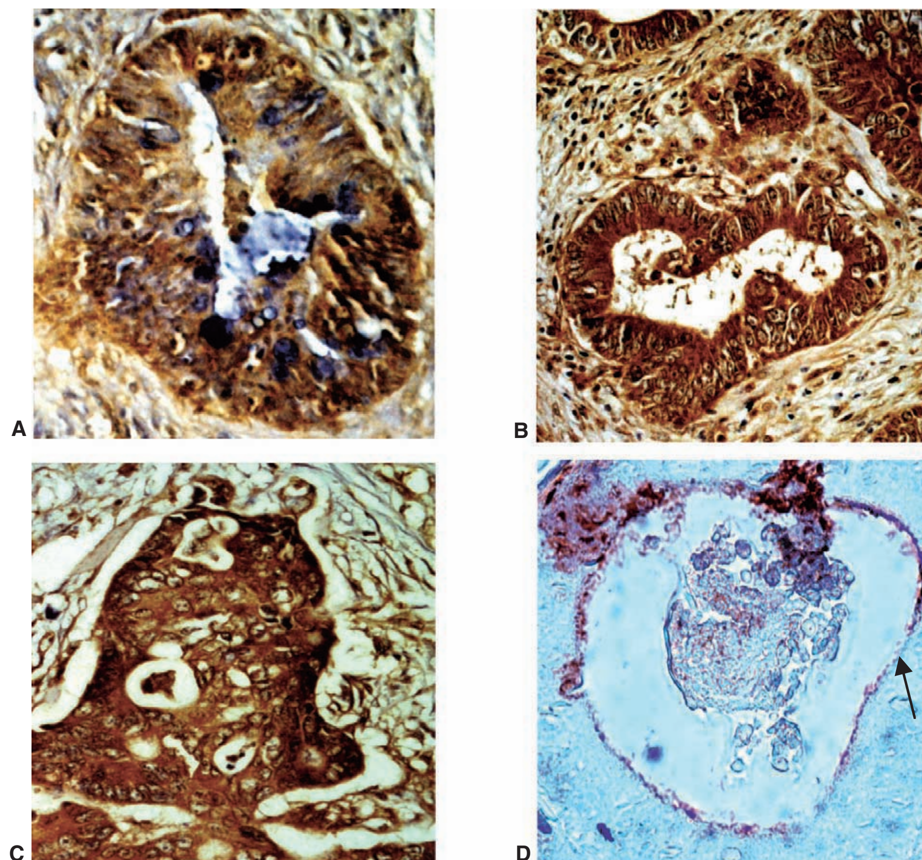

Figure 5. *(A–D) Immunostaining of tumor cytoplasm with VEGF-A (A), VEGF-C (B), VEGF-D (C), and tumour endothelial cells with VEGFR-3 (indicated by black arrow) (D).*

cantly lower in both polyps and CRCs compared to normal mucosa [0.29 (0.14–0.47), 0.37 (0.21–0.65), and 0.6 (0.4–0.86), respectively, *P*=.0002 and .002] (Figure 3).

There was no significant difference through the adenoma–carcinoma sequence in the expression of the two VEGF receptors.

VEGF-A, VEGF-C, VEGF-D, and Lymphatic Spread

The expression of VEGF-A and VEGF-C was significantly higher in both node-negative and node-positive tumors compared to normal mucosa. Comparison of the tumors with reference to nodal status shows that node-positive tumors had significantly higher VEGF-A mRNA levels compared with node negative tumors [0.84 (0.58–1.13) and 0.69 (0.45–0.89), respectively, *P*=.043]. However, there was no difference in VEGF-C expression between node-negative and node-positive tumors (0.49 and 0.52 respectively, *P*=.77) (Figure 4).

VEGF-D expression was significantly reduced in both node-negative and node-positive tumors, but there was no significant difference between these categories.

A positive lymph node was harvested from seven patients and compared with their matched primary tumor. There was no significant difference between primary or lymphatic metastasis expression of VEGF-A mRNA [median 0.91 (0.71–1.51) and 0.83 (0.72–1.52), *P*=.81, respectively], VEGF-C mRNA [median 0.63 (0.46–0.78) and 0.57 (0.4–1.16), *P*=.94], or VEGF-D mRNA [median 0.44 (0.38–0.64) and 0.03 (0.0–0.47), *P*=.31].

Immunohistochemistry

VEGF-A, VEGF-C and VEGF-D were expressed in 20%, 35%, and 31% of tumors, respectively, with staining seen mainly in tumor cytoplasm (Table 2 and Figure 5). VEGFR-3 was localised to the endothelial lining of vessels around tumor cells. There was no correlation of positive immunostaining and lymphatic spread.

Serum VEGF-D

Serum levels of VEGF-D were similar in normal controls, polyp patients, and CRC patients [median 494 (303–744) pg/ml, 416 (351–938) pg/ml, and 463 (291–745) pg/ml, respectively]. There was no statistical difference between these groups.

Discussion

The role of the VEGF family in CRC has, to date, mainly concentrated on VEGF-A, but the newer members of the family of VEGF-C and VEGF-D may have important roles to play in both angiogenesis and lymphangiogenesis. VEGF-C and VEGF-D have been shown to have an angiogenic action

on endothelial cells both *in vitro* and *in vivo* [16,17], and are regulated by proinflammatory cytokines [18]. However, the evidence that these two ligands both bind to VEGFR-3, a tyrosine kinase receptor which is restricted to lymphatic endothelium in normal adult tissues, has suggested that VEGF-C and VEGF-D play an important role in lymphangiogenesis [6,7,19].

VEGF-C mRNA expression was first reported in a mixed population of tumors [20], and the functional association of VEGF-C and VEGF-D with lymphangiogenesis has provided a possible mechanism for primary tumors to metastasize *via* newly formed lymphatics. Subsequent clinical studies have reported a correlation between VEGF-C expression and lymphatic spread in prostate [12], gastric [13], thyroid [11,21], and neuroblastoma tumors [22].

In one study in CRC, the VEGF-C gene was found to be poorly expressed with only moderate overexpression in CRCs compared to control tissue [23]; however, numbers were small (n=12). In a larger series of patients, protein expression of VEGF-C (as measured by immunohistochemistry) correlated with lymph node spread [24].

The association of VEGF-C with lymphatic metastasis is not universally seen in all tumor types. Our study demonstrated no difference in VEGF-C mRNA expression relative to lymph node status, similar to earlier reports in adenocarcinoma of the lung [14], breast cancer [25], and malignant mesothelioma [26]. Interestingly, there was no significant difference between VEGF-C mRNA expression of the primary tumor and their paired positive lymph node metastasis; however, numbers are small and comparison with uninvolved lymph nodes is also required.

VEGF-C protein was expressed in 35% of CRCs as detected by immunostaining and was localised to the tumor cytoplasm. This is a lower frequency than reported in a previous study in which 55 of 99 CRCs stained positive for VEGF-C [24]. Unlike the previous study, we were unable to show a correlation between VEGF-C and lymphatic spread immunohistologically, confirming our mRNA expression results. The difference in VEGF-C expression between immunohistochemistry (35%) and RT-PCR (96%) is most likely due to differing sensitivity between the methods, rather than lack of translation of mRNA into protein.

VEGF-D mRNA is strongly expressed in normal tissue such as skeletal muscle, heart, lung and intestinal mucosa [7,15]. Early studies into VEGF-D expression in tumor cell lines and tumor biopsy samples found expression to be either weak or absent [15], and this has been confirmed in a large study of lung adenocarcinomas [14].

Our study is the first to report on VEGF-D expression in CRC. VEGF-D mRNA expression was significantly reduced in tumors compared with normal tissue and this was confirmed with quantitative protein measurements, similar to head and neck [27]. Immunohistochemistry was positive in 31% of tumors with staining localised to tumor cytoplasm, similar to VEGF-C patterns.

Serum VEGF-A levels have been shown to correlate with disease stage in CRC, with increasing levels being associated with more advanced disease [28]. Serum VEGF-D levels were not significantly different among control, polyp, or CRC patients, and there was no association between serum VEGF-D or VEGF-D mRNA expression and lymphatic spread. Analysis of serum VEGF-C levels will be interesting, but at present, there is no ELISA available.

VEGFR-3 mRNA expression was found in 74% of CRCs, which is higher than the 40% reported in thyroid adenocarcinomas [29] and 53% in gastric adenocarcinomas [13]. In our study, there was no difference in VEGFR-2 and VEGFR-3 mRNA expression among CRC tumors, polyps, or surrounding normal mucosa. Immunostaining localised VEGFR-3 to the endothelial lining of blood vessels surrounding the tumor, as reported in previous experimental [30] and clinical studies [31,32]. Recently, it has been shown that solid tumors are largely devoid of lymphatic capillaries, but are surrounded by functional lymphatics in the adjacent normal tissue and tumor periphery [33].

The expression of VEGF-A mRNA in normal colonic mucosa is variable, ranging from 12% [34] to 73–100% [35–37]. We detected the $VEGF_{121}$ and $VEGF_{165}$ isoforms in all normal mucosal samples, and showed that both the 121 and 165 isoforms were significantly raised in CRCs compared to normal mucosa. This was confirmed by measuring tissue protein levels quantitatively using ELISA. VEGF-C and VEGF-D mRNA were expressed in 96% and 90% of CRCs, respectively, and in 95% of normal mucosae. VEGF-C, similar to VEGF-A, was significantly higher in CRCs, but VEGF-D was significantly reduced in tumors compared to normal mucosa.

Significant upregulation of VEGF-A mRNA expression in adenomas compared to normal tissue, with a further increase during development of adenocarcinomas, has been reported previously [36], suggesting that angiogenesis is stimulated early in colorectal tumorigenesis but continues to increase during progression. Our results showed that both VEGF-A and VEGF-C are increased in adenomas, but the difference did not achieve statistical significance; however, there was a significant decrease in VEGF-D mRNA. The role of VEGF-D in tumors is largely unknown, but if VEGF-D acts as a competitive agonist with VEGF-A and VEGF-C, the fall in VEGF-D levels may allow VEGF-A and VEGF-C, which are more potent angiogenic cytokines, increased access to the two receptors. With VEGF-D remaining persistently low in carcinomas compared with adenomas, and with constant levels of the two receptors' expression, if VEGF-A and VEGF-C increase further within an adenoma, then the angiogenic switch may occur later. This change in the balance of different VEGF family members appears to be significant, and others have shown that the ratio of VEGF-D and VEGF-C is important for lymphatic invasion and metastasis, with low levels of VEGF-D and high levels of VEGF-C being associated with lymph node metastasis in lung cancer [14].

The evidence that VEGF-A increases occur late in the adenoma–carcinoma sequence is supported by immunohistochemistry studies [38]. In our study, only a single polyp stained for VEGF, whereas others have failed to demonstrate any positive staining [39,40]. Previous stud-

ies have used polyclonal VEGF-A antibodies for immunostaining and reported expression in 30% to 50% of colorectal tumors [41–43]. Ours is the first study to use a monoclonal VEGF-A antibody for immunohistochemistry, which demonstrated VEGF-A positivity in 20% of CRCs, and the increased specificity of the monoclonal antibody may explain the lower percentage of positive tumors compared to previous studies.

The aberrant isoform $VEGF_{189}$ has been correlated significantly with liver metastasis, vein involvement, and poor prognosis in CRC cancer [34], and it has been suggested therefore that the cell-associated isoform $VEGF_{189}$ is mainly associated with colon cancer progression. We detected the 189 isoform in only one tumor and we and others have shown that it is the increase in the $VEGF_{121}$ and $VEGF_{165}$ isoforms which are associated with cancer progression and lymphatic spread [35,37].

This study has shown that differences in the expression of the VEGF family are important in the adenoma–carcinoma sequence and subsequent cancer progression and metastasis. The decrease in VEGF-D occurs both in polyps and carcinomas and may allow the increased levels of VEGF-A and VEGF-C to bind more freely with the VEGFR-2 and VEGFR-3. These findings need to be explored in *in vitro* studies by varying the balance of these three angiogenic cytokines (recombinant human VEGF-C is not yet available), and measuring the subsequent angiogenic response. Increased expression of VEGF-A rather than VEGF-C within CRCs was associated with lymphatic metastases, and therefore, this member of the VEGF family may be the most important in determining metastatic spread.

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