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Vascular Endothelial Growth Factor-D Expression Is an Independent Prognostic Marker for Survival in Colorectal Carcinoma[1]

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ABSTRACT

The angiogenic factor vascular endothelial growth factor-D (VEGF-D) is a ligand for VEGF receptor-3 (VEGFR-3/Flt-4) and receptor-2 (VEGFR-2/KDR) and is implicated in the development of lymphatic vessels and promotion of lymphatic metastases. We assessed the expression of VEGF-D and VEGFR-3 in relation to microvessel density (MVD) in colorectal carcinomas (CRC), adenomas, and adjacent normal tissue by immunohistochemistry on consecutive archival sections. VEGF-D was detected in malignant and benign epithelium and in some smooth muscle of the colorectum. High-grade VEGF-D expression was observed frequently (74%) in CRC compared with adenomas (0%) and adjacent normal mucosa (22%). High-grade VEGF-D expression was not correlated with MVD, Dukes' stage (A to C), or tumor differentiation, but was associated with lymphatic involvement and patient survival. By multivariate analysis, VEGF-D expression was found to be an independent prognostic factor for both disease-free and overall survival. VEGFR-3 expression was detected in a subset of vessels, typically thin-walled and devoid of RBCs, in 89% of CRC cases examined. VEGFR-3-positive vessel densities increased progressively from normal mucosa to adenomas and carcinomas and were correlated with MVD, but not with Dukes' stage (A to C), tumor differentiation, or VEGF-D expression. VEGFR-3 expression was spatially associated with macrophage-rich inflammatory infiltrates, which were significantly more frequent among VEGFR-3-positive cases. We conclude that VEGF-D expression, but not that of its receptor VEGFR-3, is an independent prognostic indicator in CRC. VEGF-D expression may be associated with disease outcome through the promotion of lymphatic involvement/metastases.

INTRODUCTION

The VEGF[6] family of angiogenic growth factors includes VEGF-A, -B, -C, -D, and -E as well as placenta growth factor (1). Differences in the regulation and tissue distribution of these family members suggest that they have distinct roles both during development and in the adult vasculature. Relatively little is known about the physiological role of VEGF-D in the adult; however, it is a potent angiogenic factor *in vivo* and stimulates endothelial cell proliferation and migration *in vitro* (2, 3). In normal tissues, VEGF-D transcripts have been detected in lung, heart, skeletal muscle, skin, adrenal glands, and the gastrointestinal tract (4–6), whereas VEGF-D protein itself is frequently sequestered in dispersed neuroendocrine cells within those tissues (6). The gene that encodes VEGF-D is under the control of the *c-fos* proto-oncogene product, which is known to be essential for malignant progression (7). Interestingly, VEGF-D has been reported to be up-regulated in malignant melanoma as well as in melanoma cell lines (8) and in inflammatory breast carcinoma (9), although VEGF-D transcripts are down-regulated in adenocarcinoma of the lung (10). Recent evidence indicates that VEGF-D stimulates both angiogenesis and lymphangiogenesis in experimental tumors and, furthermore, that VEGF-D expression is required for the growth and establishment of lymphatic vessels within tumors (5). On the basis of these data, it has therefore been suggested that VEGF-D expression promotes metastatic spread of tumors via the lymphatic route (5).

In the human, VEGF-D activates VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) receptor tyrosine kinases (2, 11), both of which are essential for vascular development (1, 12). VEGFR-2, the main receptor through which VEGF-A signals in endothelial cells, is up-regulated in tumor angiogenesis (1). Although VEGFR-3 expression becomes restricted mainly to lymphatic and fenestrated endothelium in the adult (1, 6, 12), it may also play a critical role in the maintenance of the integrity of the endothelial lining during tumor angiogenesis (13). VEGFR-3 expression is up-regulated in breast cancer and a number of other tumor types (14–16).

Angiogenesis, as indicated by increased MVD and changes in expression of VEGF and its cognate receptors, has been shown in some studies to have prognostic significance in CRC (17–21), whereas lymphatic invasion and lymph node involvement are also recognized as important prognostic factors (22). VEGF-D has been functionally implicated in both angiogenesis and lymphatic development; however its exact biological role remains poorly understood. We have examined the expression of VEGF-D and VEGFR-3 in relation to MVD in archival samples of normal adjacent bowel mucosa, premalignant lesions, and CRC by immunohistochemistry to determine the distribution of this novel member of the VEGF family, its receptor VEGFR-3, and their relationship to known prognostic markers for CRC.

MATERIALS AND METHODS

Study Population. The case reports of 84 patients diagnosed with CRC at Nottingham City Hospital between 1991 and 1992 were reviewed. After exclusion of patients with Dukes' stage D disease ($n = 6$) and perioperative ($n = 3$) and unrelated deaths ($n = 6$), 69 patients were available for full survival analysis over a median follow-up time of 7.5 years. Of the 69 tumors evaluated, 32% were rectal in origin. As was the practice in the United Kingdom at the time, these patients had not received any preoperative chemotherapy or radiotherapy. Mean age at diagnosis was 65 years (SD = 10.9 years) with approximately equal sex distribution: 26% were Dukes' stage A, 42% were stage B, and 32% were stage C. Vascular invasion was identified in 10% of cases, and 81% of carcinomas were moderately differentiated, 12% were poorly differentiated, and 7% were well differentiated. During a median follow-up period of 297 weeks, there were 46 deaths (54.8%) and 33 recur-

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[6] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; MVD, microvessel density; PBS/A, Dulbecco's calcium- and magnesium-free PBS; vWF, von Willebrand's factor; CRC, colorectal carcinoma.

rences (38.1%). The median overall survival of 6.9 years was significantly associated with Dukes' stages A to C. For Dukes' stage C, median overall survival was 180 weeks ($P < 0.0001$), but for stage A and B, it was not reached at 406 and 430 weeks, respectively. Nine patients had multiple recurrences, and the most common secondary sites were local (21.4%), liver (10.7%), lymph nodes (3.6%), lung (2.4%), and ovary (1.2%). H&E-stained sections of all of the archival specimens were reassessed by a pathologist (T. M.) to confirm stage, presence of viable tumor, and invasive edge. Twenty-three sections of adjacent normal mucosa and 20 adenomas were also examined.

Immunohistochemistry. Formalin-fixed, paraffin-embedded serial sections (4 μm) mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma Chemical Co., Poole, United Kingdom) were deparaffinized in Histolene (Cellpath, Herts, United Kingdom) and 1:1 Histolene/ethanol, and rehydrated. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6) in an 800 W microwave for 15 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 100% methanol for 30 min. Sections were washed in Dulbecco's calcium- and magnesium-free PBS (PBS/A), and blocked with either 1.5% normal rabbit serum or 10% horse serum and 0.3% BSA in PBS/A for 20 min (see Table 1). Primary antibodies were diluted as indicated in Table 1 in 0.3% BSA in PBS/A and incubated on the sections overnight at 4°C. After three washes in PBS/A (5 min), 1:200 dilutions of secondary antirabbit or antimouse biotinylated antibodies (Vector Laboratories Inc, Burlingame, CA) were added to the sections for 30 min. Antibody binding was visualized using the Avidin Biotin Complex (ABC) Elite Kit and 3,3-diaminobenzidine according to the manufacturer's instructions (Vector Laboratories). Sections were counterstained with Gill's hematoxylin and, after dehydration through graded ethanol solutions, mounted in DPX (BDH Lab Supplies, Poole, United Kingdom). For general negative controls, the primary antibody was replaced with PBS/A. In addition, sections stained with primary antibody incubated overnight at 4°C in the presence of 5-fold excess (by weight) of blocking peptide (sc7602; Santa Cruz Biotechnology) were used as negative controls for VEGF-D staining. Sections of normal heart were stained as positive controls for VEGFR-3 expression.

Microscopic Assessment of VEGF-D and VEGF-R3 Expression, MVD, and Inflammatory Infiltrates. All sections were scored blind by two independent observers (J. W. and P. W. H.), and in cases of scoring disagreement a third independent assessment was performed (J. C. M.). VEGF-D staining was graded according to the intensity and extent of staining of the epithelium as follows: negative = 0, weak/very limited moderate staining = 1, moderate widespread/strong localized staining = 2, or strong widespread = 3. VEGFR-3$^+$ vessels were assessed under $\times$100 magnification in a field area of 2.0 mm^2 in five areas with the highest VEGFR-3$^+$ vessel densities. For each section, (*a*) intratumor density of VEGFR-3$^+$ vessels within a "hotspot," (*b*) proximity of VEGFR-3$^+$ vessels to malignant epithelium, and (*c*) the presence and proximity of inflammatory infiltrates to VEGFR-3$^+$ vessels were examined. MVD assessment was determined on CD31- and vWF-stained sections under $\times$200 magnification in a grid area of 0.16 mm^2, using the criteria of Weidner (23). Five areas (three for adjacent normal bowel) of high vascular density (hotspots) were selected and counted on each section, and the MVD was determined. Further evaluation of vascularity was made using the Chalkley counting method (24) in the same five selected hotspots.

Statistical Analysis. All statistical calculations were performed using SPSS version 8.0 (SPSS Inc., Chicago, IL). Survival was measured from the date of surgery to the time of event (recurrence or death) or to the last census prior to closure of the study (May 5, 1999) and calculated by the Kaplan-Meier method. Deaths attributable to causes other than CRC were excluded from survival and prognostic analysis. For this analysis, VEGF-D antibody-stained sections were grouped as low (0 and 1) or high grade (2 and 3). The log-rank test was used to compare survival based on VEGF-D grade, VEGFR-3$^+$ vessel densities, and MVD. Differences in VEGF-D expression between groups were estimated using the χ^2 test for categorical data and independent-paired Student's *t* test for continuous variables. VEGFR-3$^+$ vessel densities or MVD between categorical variables were compared by ANOVA. Comparisons of MVD and VEGFR-3$^+$ vessel densities and their relationship to tumor size were made using the Pearson correlation coefficient. Differences between cases based on VEGFR-3$^+$ vessel densities or MVD above or below the median value were estimated using the χ^2 test for categorical data and the independent paired Student's *t* test for continuous variables. Cox regression analysis was used to estimate the prognostic influence of various factors.

Table 1 *Antibodies used for immunohistochemistry*

Antibody (supplier)	Antigen	Dilution
JC/70A. (Dako Ltd.)	CD31	1:1000
F8/86 (Dako Ltd.)	vWF	1:3000
NCL-CD3-PS1 (Novocastra Laboratories Ltd.)	CD3	1:100
KP1 (Dako Ltd.)	CD68	1:500
(25) Anti-VEGFR-3	VEGFR-3	1:2000
Anti-VEGF-D polyclonal (Santa Cruz Biotechnology, Inc.)	VEGF-D	1:2000

RESULTS

VEGF-D Expression in Normal Mucosa, Adenomas, and CRC. VEGF-D was detected mainly in the malignant epithelium of CRCs and less frequently in adenomas and adjacent normal tissues. Consistent with the recent findings of others (7), we also observed VEGF-D staining in some smooth muscle of the bowel and arterial walls and, occasionally, endothelium. The specificity of the VEGF-D staining was confirmed by preincubation of the anti-VEGF-D polyclonal antibodies with blocking peptide, which abolished both the epithelial and smooth muscle staining (data not shown). A strong granular pattern of VEGF-D staining was observed within the cytoplasm of malignant epithelium (Fig. 1*A*). Typical examples of the different intensities of VEGF-D staining observed in CRC are shown in Fig. 1, *A* and *B*. In comparison, VEGF-D staining of adenomas (not shown) and normal adjacent mucosa was generally weaker and less common (Fig. 1*C*). Normal heart, which is known to express VEGF-D, showed a mixture of granular and diffuse staining with this antibody (Fig. 1*D*).

VEGF-D staining was quantitatively assessed and grouped into high- or low-grade categories. There was significant agreement of VEGF-D grade between the two independent assessors (Kappa statistic = 0.758). A summary of the grading of VEGF-D expression in normal and neoplastic colorectal tissue is given in Table 2. VEGF-D expression was detected in all cases of CRC and in 50% of adenomas examined. Moreover, 74% of CRCs were assessed as high grade, whereas 100% of adenomas and 78% of normal mucosa had either no or low-grade VEGF-D staining. A significant difference in grade of VEGF-D expression was demonstrated between CRCs and adenomas and adjacent normal mucosa ($P < 0.001$).

Relationship of VEGF-D Expression with Survival and Prognosis in CRC. Patients were divided into two groups according to high- or low-grade VEGF-D expression, and the associated clinicopathological features of these groups were compared. There was no significant association between VEGF-D grade and MVD or VEGFR-3, nor was VEGF-D expression significantly associated with Dukes' stage (A to C) or tumor grade. High-grade VEGF-D expression was associated with tumor recurrence ($P = 0.003$), death ($P = 0.005$), and lymph node involvement at all stages of disease (21 of 23 patients with high-grade VEGF-D expression with lymph node involvement *versus* 30 of 46 with no nodal disease; $P = 0.02$).

Patients exhibiting high-grade VEGF-D expression had a significantly shorter overall survival compared with those with low-grade expression (median of 343 weeks *versus* median not reached at 430 weeks; $P = 0.0072$; Fig. 2*A*). The pattern of disease-free survival was similar, with a median survival of 309 weeks for patients with high-grade VEGF-D, whereas the median was not reached at 395 weeks in patients with low-grade expression ($P = 0.0041$; Fig. 2*B*). The significance of the association between shorter overall survival and high-grade VEGF-D expression was maintained when the analysis was restricted to Dukes' B patients (median overall survival not reached at 401 *versus* 430 weeks; $P = 0.0458$). By multivariate

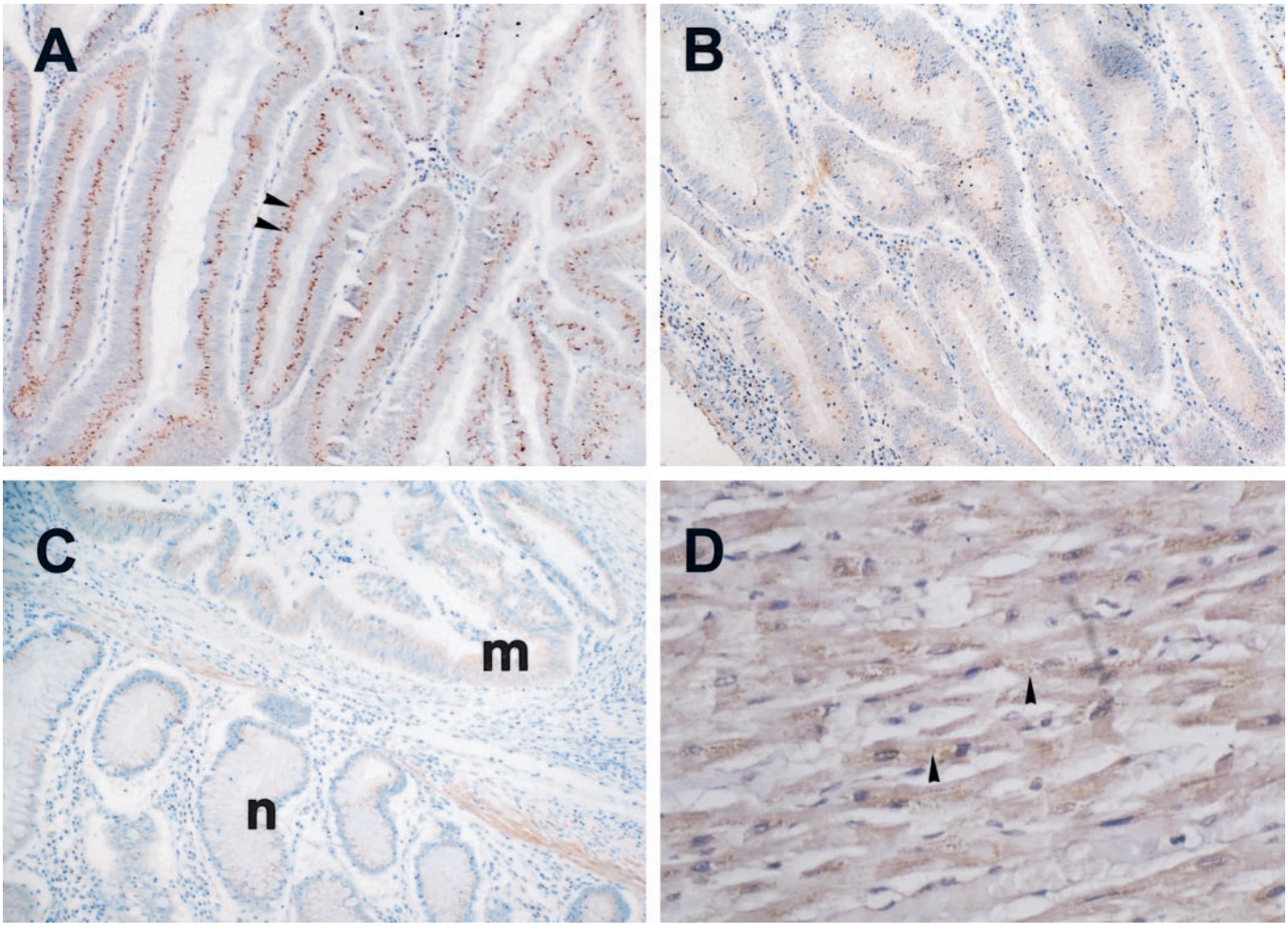

Fig. 1. Immunohistochemical staining for VEGF-D in normal and malignant colorectal epithelium. *A*, typical high-grade VEGF-D expression in a Dukes' B tumor. *B*, low-grade expression in a Dukes' B tumor. *C*, area of transition between normal mucosa (*n*) and malignant epithelium (*m*; magnification, ×200). *D*, section of normal heart as positive control for VEGF-D staining (magnification, ×400). Note the characteristic granular staining pattern in all tissues (*arrowheads*).

analysis, grade of VEGF-D expression was found to be an independent prognostic indicator of both disease-free survival ($P = 0.026$; hazard ratio, 4.133; 95% confidence interval, 1.181–14.460) and cause-specific overall survival ($P = 0.037$; hazard ratio, 3.811; 95% confidence interval, 1.087–13.362) in CRC. Dukes' stage (A to C) and vascular invasion were also identified as independent prognostic factors in this study.

VEGFR-3 Expression in Normal Bowel, Premalignant Lesions, and CRC. VEGFR-3 was detected in a subset of vessels, which were typically thin-walled and devoid of RBCs; occasionally leukocytes were also stained (Fig. 3*A*). Some vessels that stained for VEGFR-3 were also strongly positive for CD31 in consecutive sections (Fig. 3, *A* and *B*). In CRC, VEGFR-3 was restricted to the endothelium of vessels predominantly at the tumor periphery. Occasional VEGFR-3 staining of the myenteric plexus and platelets within blood vessels was also observed. In a few cases, malignant cells were visible within the lumen of VEGFR-3^+ vessels, suggesting lymphatic invasion (Fig. 3*C*).

Table 2 *VEGF-D expression in CRC, adenomas, and normal mucosa*

	VEGF-D expression, *n* (%)			
	Absent	Low	High	Total
CRC	0 (0)	22 (26)	62 (74)	84 (100)
Adenoma	10 (50)	10 (50)	0 (0)	20 (100)
Normal	5 (22)	13 (56)	5 (22)	23 (100)

VEGFR-3^+ vessel density was determined blind by two independent observers, and there was significant correlation ($P = 0.001$) between the individual VEGFR-3^+ vessel density assessments. Although the difference between the groups was not statistically significant, the mean VEGFR-3^+ vessel densities were 1.7/mm^2 (±2.0) for normal bowel samples and 2.0/mm^2 (±1.3) for adenomas (Fig. 4*A*). VEGFR-3 expression was detected in 89.3% of CRCs, with a mean VEGFR-3^+ vessel density of 3.9/mm^2 (±2.7; median, 3.5/mm^2). A significant difference in the mean VEGFR-3^+ vessel density was demonstrated between CRCs and either adenomas or normal bowel ($P < 0.001$; Fig. 4*A*). Within the group of malignant lesions, there was no significant difference in the mean VEGFR-3^+ vessel density as a function of Dukes' stages ($P = 0.960$), tumor differentiation ($P = 0.210$), or tumor size ($P = 0.823$). There was a weak association ($P = 0.027$) between VEGFR-3^+ vessel density and MVD (Fig. 4).

Relationship of VEGFR-3 Expression with Survival and Prognosis in CRC. Those patients with the highest VEGFR-3^+ vessel densities demonstrated a median overall survival of 329 weeks, compared with 308 weeks for those with lower VEGFR-3^+ vessels densities (Fig. 5*A*). This difference was not statistically significant. Similarly, in terms of disease-free survival, the median was >424 weeks for patients with high VEGFR-3^+ vessel densities compared

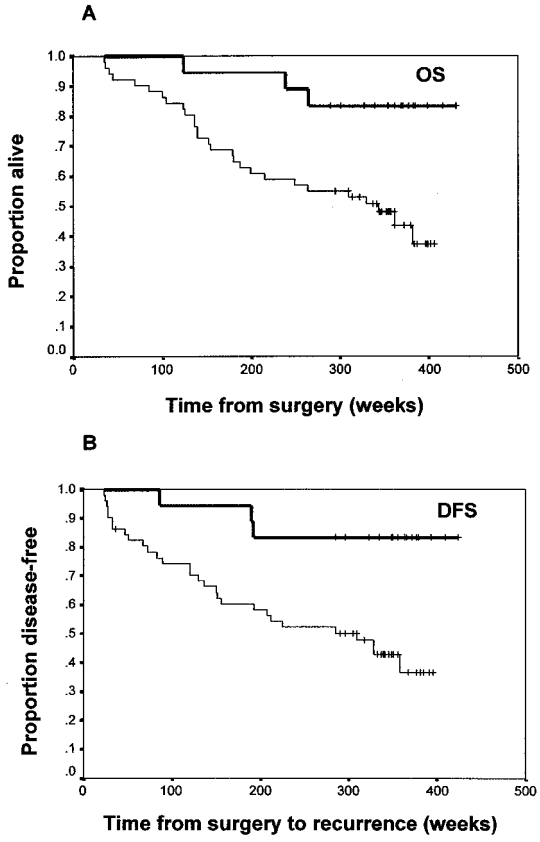

Fig. 2. Impact of VEGF-D expression on patient survival. Kaplan-Meier analysis indicating cause-specific overall survival (*OS*; *panel A*) and disease-free survival (*DFS*; *panel B*) of patients after surgery for CRC, according to grade of VEGF-D expression: *thin line*, high-grade; *thick line*, low-grade; +, censored observations.

with 358 weeks for patients with lower values; again, this difference did not achieve statistical significance. There was no increase in overall or disease-free survival demonstrated for individual Dukes' stages (A to C) on the basis of VEGFR-3^+ vessel densities.

Association of VEGFR-3 Expression with Inflammatory Infiltrates. Inflammatory infiltrates were seen in 88.3% of sections positive for VEGFR-3, but were not observed in VEGFR-3-negative sections. This association of VEGFR-3 with inflammatory cells was highly significant ($P < 0.001$). Furthermore, when the samples were subdivided by the median VEGFR-3^+ vessel density, inflammatory infiltrates were found to be more frequently associated with high VEGFR-3-expressing tumors ($P = 0.02$). Morphologically, these infiltrates consisted of a mixed leukocyte population surrounding VEGFR-3^+ vessels with a mean distance of 82.4 μm ($\pm$13.6 μm) from VEGFR-3^+ vessels. This contrasts with a mean distance of 205.3 μm ($\pm$203.4 μm) for adenocarcinoma cells from VEGFR-3^+ vessels. The nature of the inflammatory infiltrates was examined further by immunohistochemical staining for pan-T-cell (CD3) and macrophage (CD68) markers in 23 CRC specimens (data not shown). In 78.3% of cases examined, $CD68^+$ cells comprised >50% of the total infiltrate, indicating an excess of macrophages. We saw no evidence of a spatial association between expression of VEGFR-3 and that of its ligand, VEGF-D.

A significant difference in overall survival was demonstrated between those patients with or without inflammatory infiltrates. Median overall survival for patients with inflammatory infiltrates was 361 weeks, compared with 126 weeks for those without ($P = 0.007$; Fig. 5*B*).

MVD in Normal Bowel, Premalignant Lesions, and CRC. Most CRC were highly vascular, with hotspots occurring frequently within the mucosal folds of the malignant epithelium (Fig. 3*B*). In addition to endothelium, some inflammatory cells also stained for CD31 but were readily distinguishable on morphological grounds. MVD was determined blind by two independent observers on CD31-stained sections from five selected vascular hotspots under $\times$200 magnification in a graticule area of 0.16 mm^2. Chalkley counts (24) were also examined as an alternative measure of vascularity and were found to correlate well ($P < 0.001$) with MVD determined in the same vascular hotspots. MVD was also assessed on consecutive sections stained for vWF with good correlation ($P = 0.024$) to MVD determined using CD31 as a vascular marker.

A summary of MVD data for normal bowel, premalignant lesions, and CRCs is shown in Fig. 4*B*. There was no significant difference in MVD between normal mucosa (127.2 $\pm$ 53.7/mm^2) and premalignant lesions (158.2 $\pm$ 44.9/mm^2). However, there were highly significant differences between the mean MVD for CRC (237.4 $\pm$ 86.4/mm^2) and normal mucosa ($P < 0.005$) or premalignant lesions ($P < 0.0001$).

MVD was not associated with Dukes' stage ($P = 0.088$), tumor differentiation ($P = 0.212$), or tumor size ($r = 0.057$; $P = 0.611$). Patients with a MVD greater than median (218.1/mm^2) had a significantly longer overall survival (median, 381 *versus* 187 weeks; $P = 0.030$; Fig. 5*C*). A difference in disease-free survival was also seen, although this did not achieve statistical significance (median, 424 *versus* 225 weeks; $P = 0.054$).

DISCUSSION

Increased MVD is a negative prognostic indicator in several types of cancer (25). However, this relationship is less clear in CRC, where high MVD has been reported to be associated with poor prognosis (17), to have little or no impact on survival (18), or to predict longer survival (19). In our study, high MVD appeared to favor longer overall survival. Given the importance of lymphatic dissemination in the progression of CRC, we therefore decided to examine the expression of the angiogenic growth factor VEGF-D, which has been shown to promote the development of lymphatic vessels in tumor models (5), to determine whether it might be associated with survival. High-grade VEGF-D expression was associated with lymph node involvement and was significantly more frequent among patients who subsequently had disease recurrence. Moreover, and most importantly, VEGF-D expression was of independent prognostic significance for both disease-free and overall survival. We did not find a significant correlation between VEGF-D expression and either MVD or vascular invasion. Taking these data together, we suggest that VEGF-D does not act as an angiogenic factor in the context of CRC, but may play a role in the development and/or function of lymphatic vessels thereby promoting lymphatic metastases.

In the same population of patients, we examined the expression of VEGFR-3, one of two endothelium-associated tyrosine kinase receptors that bind VEGF-D (1, 2). Through the spectrum of disease, VEGFR-3^+ vessel densities showed a similar pattern to MVD measurements determined using CD31 as an endothelial cell marker. There was a significant increase in CRC compared with normal bowel and adenomas, but no further increase with tumor stage. Indeed, MVD appeared to decrease slightly with increasing Dukes' stage. De Waal *et al.* (26) have reported that lymphatic counts correlate well with MVD in normal skin and early (horizontal) melanomas until the late (vertical) growth phase, which is normally accompanied by an exponential increase in blood vessel number. In contrast, a recent study of breast cancer found no correlation between MVD and VEGFR-3^+ vessel densities (27).

The specificity of the anti-VEGFR-3 monoclonal antibody used has been extensively characterized and found to be restricted to lymphatic

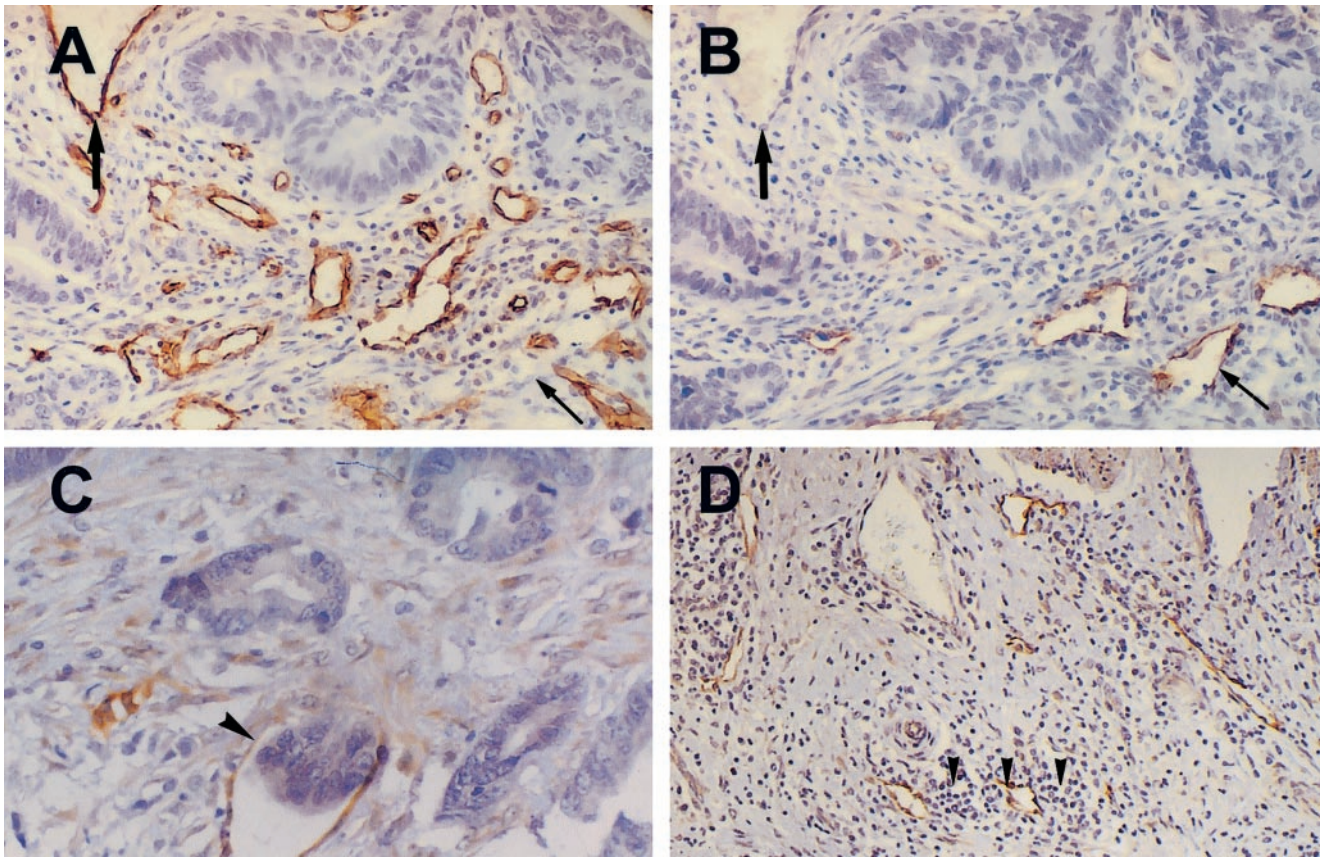

Fig. 3. Immunohistochemical staining of consecutive sections of CRC (Dukes' stage C) for CD31 (*A*) and VEGFR-3 (*B*). A blood vessel containing erythrocytes and positive for CD31 but negative for VEGFR-3 is indicated by a *thick arrow*, whereas a vascular channel positive for VEGFR-3 but negative for CD31 is indicated by a *thin arrow* (magnification, ×200). *C*, section of CRC (Dukes' stage C) showing a VEGFR-3$^+$ vessel containing malignant epithelial cells within the lumen (*arrowhead*; magnification, ×200). *D*, typical section of CRC (Dukes' stage C) showing VEGFR-3$^+$ vessels surrounded by inflammatory cells (*arrows*; magnification, ×200).

endothelium, fenestrated endothelium, and some angiogenic blood vessels (6, 14, 15, 28, 29). We detected VEGFR-3 expression on endothelium of both normal and abnormal colorectum. Consistent with lymphatic origin, these vessels were typically thin-walled and devoid of RBCs. In both normal and tumor tissue, only a small proportion of vessels expressed VEGFR-3. A similar pattern of vessel staining in the colon has recently been reported in a study using antibodies against LYVE-1, a CD44 homologue expressed on lymphatic endothelial cells (30). A proportion of VEGFR-3$^+$ vessels also strongly expressed the endothelial marker CD31, supporting the contention that VEGFR-3 may be expressed on a subset of angiogenic vascular endothelial cells. In a recent study of breast carcinoma, VEGFR-3 was detected in proliferating vascular endothelium as well as in lymphatic vessels (14). Furthermore, although not associated with tumor stage, VEGFR-3 expression was closely associated with the presence of inflammatory infiltrates in CRC. These findings raise important issues, as yet unresolved, concerning the relationship between the proliferation of vascular and lymphatic endothelium.

In a recent study of malignant mesothelioma, expression of VEGFR-3 (and VEGF-C) was correlated with lymphatic density, although this correlation did not have prognostic significance (31). Similarly, in gastric carcinoma, VEGFR-3 expression was shown to be spatially associated with VEGF-C, but not related to other clinical parameters (32). However, in head and neck cancer, high VEGFR-3 expression is a predictor for the presence of lymphatic metastases (33). We could not demonstrate any difference in survival based on

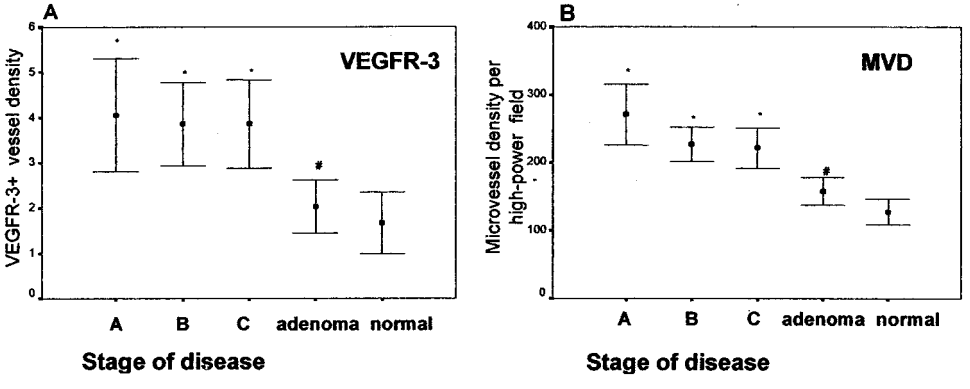

Fig. 4. Mean VEGFR-3$^+$ vessel densities (*A*) and MVD (*B*) grouped according to pathological group in CRC, adenoma, and adjacent normal mucosa (*normal*). Mean VEGFR-3$^+$ vessel densities for all Dukes' stages A to C were significantly different from that of adjacent normal mucosa (*, $P < 0.001$) and from that of adenomas (#, $P < 0.01$). MVD for all Dukes' stages A to C was significantly different from that of adjacent normal mucosa (*, $P < 0.001$) and that of adenomas (#, $P < 0.005$). All values represent means ± SE (*bars*).

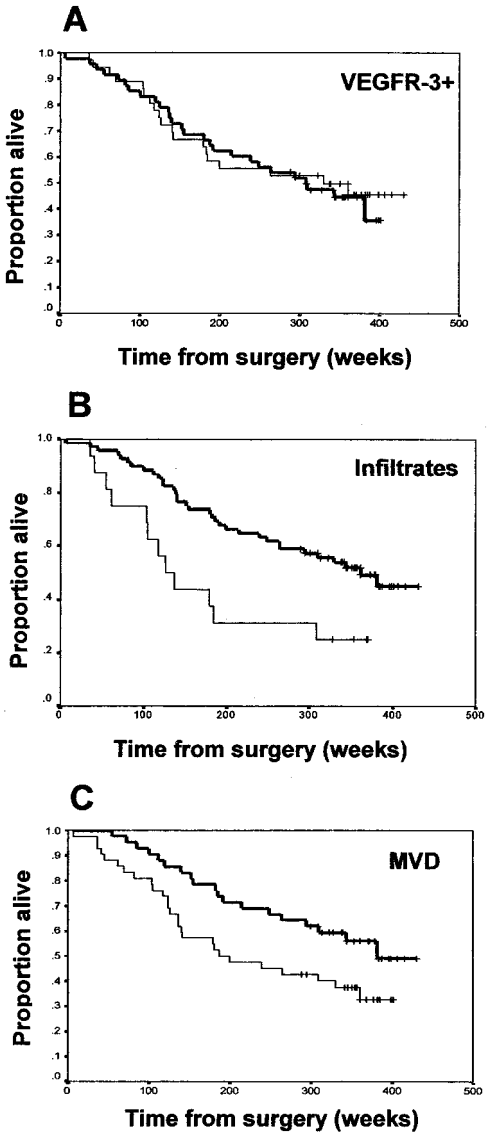

Fig. 5. Kaplan-Meier analysis of overall survival of patients after surgery for CRC according to median VEGFR-3$^+$ vessel densities (*A*) and the presence of inflammatory cell infiltrate (*B*). The presence of immune cell infiltrates is indicated by a *thick line*, and the absence by a *thin line*. *C*, median MVD. In *A* and *C* values less than median are indicated by a *thin line*, and values greater than median by a *thick line*; +, censored observations.

VEGFR-3$^+$ vessel density, which concurs with a recent study of VEGFR-3 expression in breast cancer using the same monoclonal antibody (27).

We also found a progressive increase in MVD from normal mucosa through adenomas to CRC, but no further increase with advancing Dukes' stage or tumor differentiation. In CRC, MVD has been associated with poorer prognosis in some studies (17, 20), and in another to be of no prognostic significance (34). In one study, however, high MVD was associated with longer survival (19), as we report here. Potential explanations for such differences include the grade of CRC examined, the endothelial cell marker used, and field area/magnification under which the vessels were counted. To confirm our findings we examined consecutive sections for CD31 and vWF expression and found a good correlation between MVD determined with these endothelial markers. MVD determined with anti-CD31 was consistently higher than when anti-vWF was used, probably reflecting the greater sensitivity of this marker for the detection of blood vessels (35).

This is the first report documenting a significant association between VEGFR-3$^+$ vessels and inflammatory cell infiltrates in tumors. The relationship between VEGFR-3 and inflammation has been noted previously in rodent model systems of wound healing (36). Moreover, expression of VEGFR-3 is transient in experimental wounds, but persists at low levels in human inflammatory skin ulcers (36). Interestingly, the density of VEGFR-3$^+$ vessels observed in our study is within a range similar to that seen in experimental wound models (36). Inflammatory infiltrates predict increased survival in rectal cancer patients, and lymphocytic infiltration around these tumors is associated with a stage-related improvement in prognosis (37). The pattern of inflammatory cell infiltrates we observed in CRC is similar to that seen in other studies of tumor angiogenesis (38). Associations between increased macrophage count and MVD have been noted in breast and endometrial cancer and lymphoma (39–41). Macrophages are an important source of angiogenic growth factors such as VEGF (42) and also produce inflammatory cytokines. These cytokines may regulate the expression of VEGF family members and their receptors, *e.g.*, interleukin-1β and tumor necrosis factor α, which up-regulate VEGF-A in CRC cell lines and VEGF-C in fibroblasts (43, 44). The same cytokines up-regulate expression of VEGFR-2, but not VEGFR-3, in endothelial cells (44). At this time there is no clear candidate molecule that might up-regulate the expression of VEGFR-3.

The mechanistic link between VEGF-D expression and disease outcome in CRC remains unclear: One interpretation of these data is that, in CRC, VEGF-D expression plays a greater role in lymphatic function and trafficking of leukocytes than in neovascularization *per se*. In agreement with our findings, enhanced VEGF-D expression was recently reported to be associated with lymph node involvement in inflammatory breast carcinomas (9). Moreover, VEGFR-3 has been shown to be expressed at major sites of hematopoiesis or blood cell trafficking in the sinusoidal endothelium of the liver, spleen, and bone marrow, which may be related to the regulation of hematopoietic cell translocation (6). Collectively these data suggest an important role for VEGF-D and its receptors at the interface of the immune system and the tumor.

We conclude that expression of VEGF-D, but not that of its receptor VEGFR-3, is associated with poor survival in CRC. Because expression of VEGF-D is not closely correlated with MVD, it appears that VEGF-D may not act primarily as an angiogenic factor in this setting, but rather is implicated in lymphatic development and function, facilitating lymphatic dissemination of disease.

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