

# Expression of Vascular Endothelial Growth Factor and Its Receptor, KDR, Correlates with Vascularity, Metastasis, and Proliferation of Human Colon Cancer<sup>1</sup>

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## Abstract

We studied the correlation between expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and their receptors with vascularity, metastasis, and proliferative index of human colon cancers. Immunohistochemical analyses using antibodies against VEGF, bFGF, their receptors (KDR, *flt-1*, *bek*, and *flg*), factor VIII, and proliferating cell nuclear antigen were carried out on archival specimens of 52 human colon carcinomas and 10 adenomas. Vessels were quantitated by light microscopy ( $\times 200$ ), and the intensity of staining for VEGF and bFGF was assessed on a scale of 0-3+. The presence or absence of immunostaining for KDR, *flt-1*, *bek*, and *flg* was evaluated in endothelial cells, and proliferation was determined by counting the number of proliferating cell nuclear antigen-positive cells per 500 tumor cells. Expression of VEGF and KDR was higher in metastatic than in nonmetastatic neoplasms and directly correlated with the extent of neovascularization and the degree of proliferation, whereas expression of bFGF, *flt-1*, *bek*, and *flg* did not differ among tumor types. Vessel counts were greater in metastatic tumors than in nonmetastatic tumors. These findings support the hypothesis that VEGF is an important angiogenic factor in primary and metastatic human colon cancer. VEGF expression and vessel counts may aid in predicting patients at risk for metastasis from colon cancer.

## Introduction

*In vivo* experimental studies (1, 2) have demonstrated that tumor growth and metastasis are dependent upon angiogenesis. Increased vascularity may allow for not only an increase in tumor growth but also a greater chance of hematogenous tumor embolization. Thus, it is hypothesized that inhibiting tumor angiogenesis will halt tumor growth and decrease metastatic potential. Identification of specific angiogenic factors may provide a target for antineoplastic therapy.

Two well-characterized peptides, VEGF<sup>3</sup> (3, 4) and bFGF (5, 6), are known to induce angiogenesis in rodent tumor models. Our laboratory has demonstrated that VEGF mRNA and protein are expressed in both primary and metastatic human colon cancer cell lines (7). When primary cell lines and their metastatic counterparts were studied, VEGF expression was higher in the metastatic cell line. Kitadai *et al.* (8) have demonstrated "hot spots" for bFGF in metastatic human colon cancer by *in situ* hybridization.

Weidner *et al.* (9) demonstrated a statistically significant correlation between the incidence of metastases and microvessel counts in invasive breast carcinomas. This finding has been confirmed in

numerous studies of breast cancer (10, 11) as well as melanoma (12), cervical cancer (13), prostate cancer (14), and other tumors. To date, however, no studies have examined the relationship between microvessel density and metastasis in colon cancer. The purpose of this study was to determine whether expression of the angiogenic factors VEGF and bFGF and their receptors correlate with vascularization, metastasis, and proliferation in human colon cancer.

## Materials and Methods

**Patients.** Paraffin-embedded tumor specimens from 52 randomly chosen patients with colon cancers and 10 with adenomas who had undergone surgery at The University of Texas M. D. Anderson Cancer Center were studied. The pathology reports and clinical histories at the time of surgery were reviewed to determine disease stage. The specimens studied were staged as follows: 10 patients, villous adenomas; 28 patients, nonmetastatic tumors [4 patients, Dukes, class A (Astler-Coller modification), T<sub>1</sub>N<sub>0</sub>M<sub>0</sub>; 24 patients, Dukes, B, T<sub>2-3</sub>N<sub>0</sub>M<sub>0</sub>]; and 24 patients, metastatic tumors (12 patients, Dukes, C, T<sub>x</sub>N<sub>1-2</sub>M<sub>0</sub>; 12 patients, Dukes, D, T<sub>x</sub>N<sub>x</sub>M<sub>1</sub>).

After an initial review of all available hematoxylin and eosin-stained slides of the surgical specimens, we selected one representative paraffin block from each case for further study. Blocks selected were those in which mucosa, invasive edge, and viable tumor were present. Consecutive 4- $\mu$ m sections were recut from each block. One section from each case was stained with hematoxylin and eosin, and additional sections were immunostained for VEGF, bFGF, factor VIII, and PCNA. Remaining sections from 55 cases were stained with antibodies against KDR, *flt-1*, *bek*, and *flg*.

**Immunohistochemical Staining.** Immunohistochemical staining was performed by the immunoperoxidase technique following predigestion and trypsinization. Antibodies used were a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution for VEGF, a rabbit polyclonal antibody (Sigma Chemical Co., St. Louis, MO) at a 1:30 dilution for bFGF, a rabbit polyclonal antibody (Dako Co., Carpinteria, CA) at a 1:250 dilution for factor VIII, a mouse polyclonal antibody (Dako Co.) at a 1:50 dilution for PCNA, a rabbit polyclonal antibody (Santa Cruz Biotechnology) at a 1:100 dilution for KDR, a rabbit polyclonal antibody (Santa Cruz Biotechnology) at a 1:200 dilution for *flt-1*, a rabbit polyclonal antibody (Santa Cruz Biotechnology) at a 1:200 dilution for *bek*, and a rabbit polyclonal antibody (Santa Cruz Biotechnology) at a 1:200 dilution for *flg*. For positive controls, normal mucosa was stained for VEGF, bladder tumor for bFGF, umbilical vein for KDR and *flt-1*, and normal liver tissue for *bek* and *flg*. Negative controls used all reagents except the primary antibody.

**Evaluation of Immunostaining and Vessel Counting.** Intensity of staining for VEGF and bFGF was graded on a scale of 0-3+, with 0 representing no detectable stain and 3+ representing the strongest stain. The presence or absence of KDR, *flt-1*, *bek*, and *flg* was evaluated on tumor endothelial cells.

Vessel counts were assessed both in the tumor itself and at the invasive edge by light microscopy after staining for factor VIII. Areas containing the highest numbers of capillaries and small venules were identified by scanning tumor sections at low power ( $\times 40$  and  $\times 100$ ). After the area of highest neovascularization was identified, individual vessel counts were performed at  $\times 200$  magnification ( $\times 20$  objective and  $\times 10$  ocular, 0.739 mm<sup>2</sup> per field). Based on the criteria Weidner *et al.* (9), vessel lumens were not necessary for a structure to be defined as a vessel, and RBC were not used to define a vessel lumen.

Received 6/5/95; accepted 6/5/95.

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<sup>1</sup> This work was supported in part by American Cancer Society Cancer Development Award 94-21 (L. M. E.).

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<sup>3</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PCNA, proliferating cell nuclear antigen.

Vessel counts were assessed without knowledge of patient outcome and were performed by two investigators (Y. T., Y. K.) simultaneously using a double-headed light microscope. Only one tumor was excluded from this study because of poor staining. Proliferation (PCNA index) was evaluated by counting the number of PCNA-positive-staining cells out of 500 tumor cells at the invasive edge.

**Statistical Analyses.** Differences in mean vessel counts, mean staining, intensity of VEGF, and bFGF among tumor stages were analyzed by Student's *t* test. Correlations between vessel counts and intensity of VEGF and bFGF staining were examined by the Spearman rank correlation coefficient. Differences in KDR positivity among stages were analyzed by  $\chi^2$  analysis. For all statistical analyses, the level of significance was set at 0.05. Statworks statistical software (Cricket Software, Inc., Philadelphia, PA) was used for all analyses.

## Results

**Correlation between VEGF, bFGF, and Stage of Disease.** The intensity of VEGF and bFGF staining was homogeneous within tumors, with no detectable "hot spots." The intensity of VEGF staining was significantly higher in the tumors of patients with metastases than in nonmetastatic tumors and adenomas ( $P < 0.001$  and  $P < 0.001$ , respectively). However, there was no difference in the intensity of VEGF staining between nonmetastatic tumors and adenomas. bFGF staining intensity did not correlate with stage of disease, vessel count, or VEGF staining intensity (Fig. 1; Table 1).

Fig. 2 shows the percentage of patients with metastatic disease plotted against the intensity of VEGF staining. The prevalence of metastatic disease increased as VEGF expression increased.

**Correlation between Expression of KDR and Stages of Disease.** Ten % (1 of 10) of adenomas, 28.6% (6 of 21) of nonmetastatic tumors, and 62.5% (15 of 24) of metastatic tumors were positive for KDR. The presence of KDR-positive-staining endothelial cells was more frequent in metastatic tumors than in nonmetastatic tumors ( $P = 0.02$ ), while no differences were observed between adenomas and nonmetastatic tumors (Fig. 1). There were no differences in positivity of the other receptors (*flt-1*, *bek*, and *flg*) on tumor endothelium studied between differentiated tumors and undifferentiated tumors.

**Correlation between KDR and VEGF.** The intensity of VEGF staining was significantly higher in KDR-positive tumor endothelia than in KDR-negative tumor endothelia ( $P = 0.005$ ; Table 2).

**Vessel Counts at the Invasive Edge and within the Tumor.** There was a direct correlation between vessel counts within the tumor and at the invasive edge ( $P < 0.001$ ). In nearly all patients, the vessel count at the invasive edge of the tumor was greater than the vessel count within the tumor. The median and mean vessel counts at the invasive edge were 34.0 and 35.4, respectively, which were nearly twice those within the tumor (median and mean, 18.0 and 19.6). The counts made by the two independent observers did not differ by more than 10% (Fig. 1).

**Vessel Count in Adenomas and Nonmetastatic and Metastatic Tumors.** The median and mean vessel counts for adenomas, nonmetastatic tumors at presentation, and metastatic tumors at presentation are shown in Table 1. The mean vessel count was not significantly greater in patients with nonmetastatic tumors than in those with adenomas. However, the mean vessel count was significantly greater, both in the tumor and at the invasive edge in patients with metastatic tumors than in patients with nonmetastatic tumors ( $P < 0.001$  and  $P < 0.001$ , respectively).

As shown in Fig. 2, the prevalence of metastatic disease increased as the vessel count within the tumor increased. A similar pattern was observed when vessels were quantitated at the invasive edge (data not shown).

**Correlation between VEGF, bFGF, and Vessel Count.** Vessel count was significantly correlated with VEGF expression both at the invasive edge and within the tumor ( $P < 0.001$  and  $P < 0.05$ , respectively). There was no correlation between bFGF staining and vessel count.

**Correlation between KDR and Vessel Count.** Tumor endothelia that were KDR positive were associated with higher vessel counts than were KDR-negative tumor endothelia. This finding held true both at the invasive edge and within the tumor ( $P = 0.001$  and  $P < 0.001$ ; Table 2). There were no differences in vessel counts, VEGF, or bFGF expression in tumors where endothelia stained positive for *flt-1*, *bek*, or *flg* as compared to negative-staining endothelia.

**Correlation between PCNA Index, Vessel Count, VEGF, and bFGF.** PCNA indices ranged from 35.6 to 90.8%, with a mean of 63.8%. PCNA indices in metastatic tumors were higher than in nonmetastatic tumors (Table 1;  $P < 0.001$ ). PCNA index was significantly correlated with vessel count, both at the invasive edge and within the tumor ( $P < 0.001$  and  $P < 0.001$ ). There were also significant correlations between the intensity of VEGF and PCNA index ( $P = 0.001$ ). bFGF staining and PCNA index were not correlated.

## Discussion

In the present study, we examined the expression of putative angiogenic factors that may be responsible for colon cancer angiogenesis. We found correlation between expression of the angiogenic factor VEGF and its receptor and colon cancer neovascularization, metastasis, and proliferation.

It is difficult to choose a histological site to study microvessel density in colon cancer, because colon cancer does not occur in a single layer of the bowel except in its earliest stages. Rather, the tumor grows from the mucosa inward, transgressing numerous layers of the bowel. Therefore, we chose to examine vessel counts both within the tumor and at the invasive edge. Consistently, we found that vessel counts were higher at the invasive edge of the tumor than within the tumor. Other studies (15) from our laboratory have demonstrated that satellite lesions at the invasive edge are hot spots for the expression of various metastasis-related genes. Similarly, we have found that the greatest angiogenic activity occurs at the invasive edge of the tumor, suggesting that the invasive edge of the tumor is the most active area in local invasion as well as metastasis.

Our study demonstrated that the vessel counts in patients with metastatic tumors at presentation were greater than those in patients with nonmetastatic tumors or adenomas. This was true for vessel counts both in the tumor and at the invasive edge. Since the majority of these tumors were procured within the past year, long-term follow-up data are not available. However, we plan to follow patients with Dukes class A, B, and C tumors to determine if higher vessel counts can predict which patients are likely to have recurrences.

We included patients with positive lymph nodes in our group of patients with metastasis-forming tumors. Although angiogenesis refers to an increase in blood vessel formation, others (10, 11) have found that angiogenesis also corresponds to increased lymph node metastases. Smith and Basu (16) demonstrated that neovascularization of the rabbit cornea following injection of India ink leads to the appearance of ink particles in ipsilateral lymph nodes. These findings indicate that lymphocapillary anastomoses are present and/or that angiogenesis correlates with the formation of new lymphatic vessels.

It is unlikely that any one factor is responsible for tumor angiogenesis. Recently, numerous peptide factors have been implicated in the process of tumor angiogenesis (5). The best characterized of

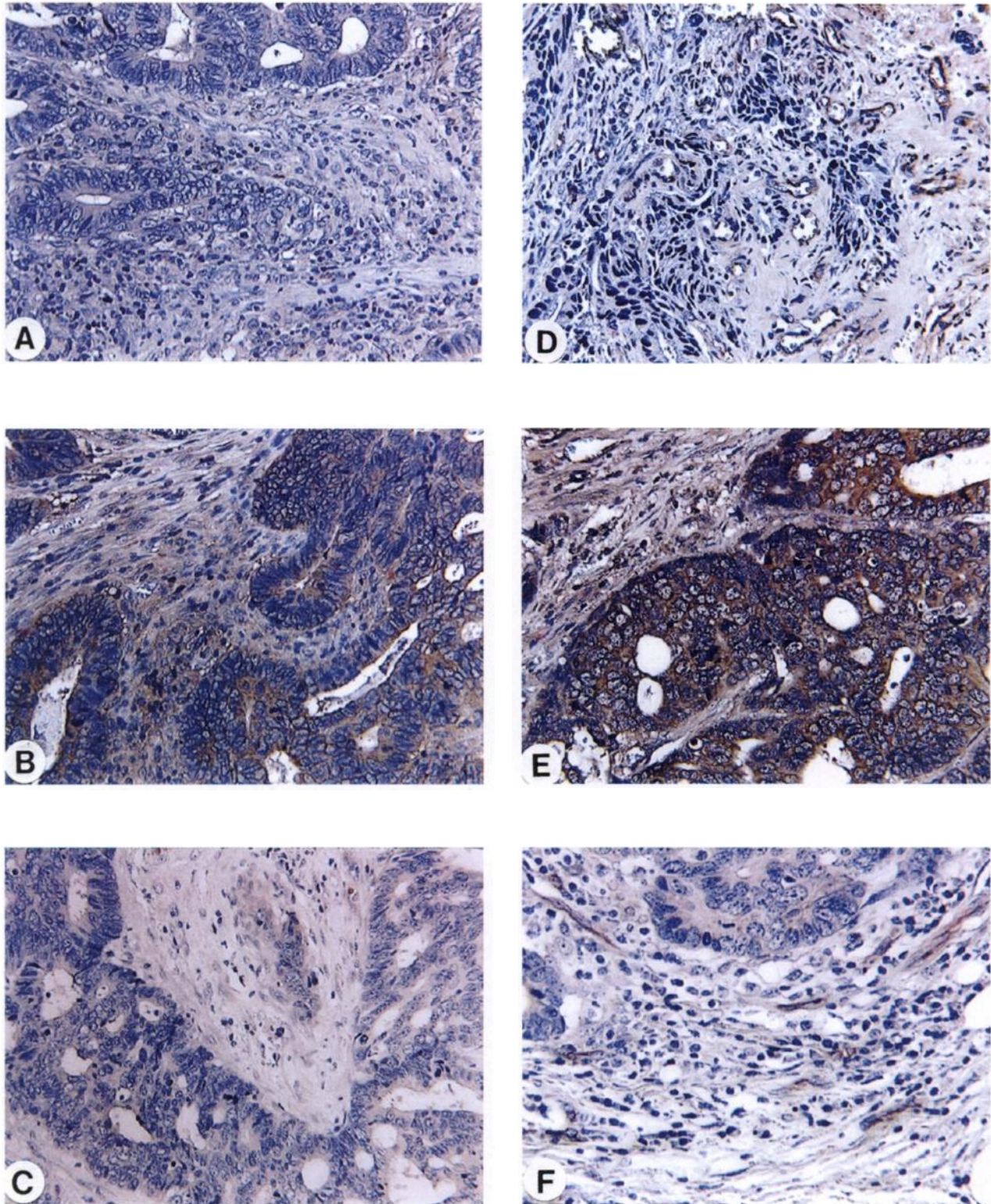


Fig. 1. Immunohistochemical staining of a nonmetastatic tumor (A, B, and C) and a metastatic tumor (D, E, and F) with antibodies to factor VIII (A and D), VEGF (B and E) and KDR (C and F).  $\times 200$ . Vessel counts in the metastatic carcinoma (D) were higher than those in the nonmetastatic carcinoma (A). The metastatic carcinoma (E) showed stronger positive staining for VEGF (3+) than did the nonmetastatic carcinoma (1+; B). The metastatic carcinoma (F) showed positive staining for KDR on endothelial cells, whereas there was no KDR expression in the nonmetastatic carcinoma (C).

these factors are bFGF (6, 7) and VEGF (3, 4). Our laboratory (8) has demonstrated the presence of bFGF in colon cancer specimens and cell lines with high metastatic potential. Therefore, we chose to investigate whether expression of bFGF correlates with vessel density and disease progression. Our laboratory (17) has also

demonstrated that VEGF is expressed in nearly all colonic epithelia, benign or malignant. As shown by Northern blot hybridization, VEGF mRNA is increased relative to that in the matching normal mucosa in a significant number of cases. Therefore, VEGF was also chosen for investigation as an angiogenic factor in colon

Table 1 VEGF and bFGF expression, vessel count, and proliferative index in human colon carcinomas and adenomas

Tumor type	Intensity of VEGF staining (mean ± SEM)	Intensity of bFGF staining (mean ± SEM)	Vessel count (mean ± SEM)		PCNA index (%) (mean ± SEM)
			Within tumor	At invasive edge	
Adenomas (n = 10)	1.7 ± 0.2	0.7 ± 0.2	10.0 ± 2.2	17.6 ± 3.5	53.7 ± 2.8
Nonmetastatic tumors (n = 28)	1.5 ± 0.1	1.1 ± 0.1	16.3 ± 2.0	29.2 ± 2.8	58.7 ± 1.5
Metastatic tumors (n = 23)	2.5 ± 0.1	1.3 ± 0.2	27.9 ± 2.3	48.7 ± 3.4	73.9 ± 2.1

cancer. Experimental evidence suggests that bFGF and VEGF are synergistic in inducing angiogenesis (18).

Our studies found no correlation between the expression of bFGF and vessel density. By *in situ* hybridization, our laboratory (15) has demonstrated hot spots for bFGF mRNA expression at the invasive edge of tumors. However, by Northern blot hybridization, very little bFGF mRNA is detected in human colon cancer specimens. Our immunohistochemical findings suggest that bFGF may be important only in angiogenesis at the invasive edge of the tumor, and our ability to detect this subtle finding may be limited by the sensitivity of immunohistochemical staining and/or Northern blot hybridization. bFGF has also been found to be a mitogen for other cell types besides endothelial cells, and its presence as detected by *in situ* hybridization does not necessarily implicate its role as an angiogenic factor. The fact that bFGF may not be an important angiogenic factor in colon cancer is supported by our findings that the most common receptors for bFGF were not frequently demonstrated on tumor endothelia (data not shown).

In contrast, a strong correlation was noted between VEGF expression and vessel counts. In addition, VEGF expression was found to be higher in patients with metastatic tumors than in those with nonmetastatic tumors. Moreover, VEGF expression correlated with PCNA index, which also correlated with vessel count.

Others have shown that VEGF is not a mitogen for tumor epithelium and that the major biological effect of VEGF is through its actions on endothelial cells. Our study supports this specificity of activity of VEGF since receptors for VEGF were not present on tumor epithelial cells. Therefore, any increase in proliferative index associated with VEGF is probably due to its angiogenic activity. To show that VEGF plays a role in the angiogenesis and progression of colon cancer, it must be demonstrated that receptors for this factor are present on tumor endothelium. Therefore, after we observed that VEGF expression correlated with vessel count and metastasis tumors, we stained the remaining slides for KDR. Tumor epithelium has an abundance of cytoplasm where VEGF is synthesized and stored. However, KDR, as a receptor, is limited to the cell membrane of the endothelium, and it is impossible for an observer to grade expression on a scale of 0–3+ as with other cytoplasmic and secreted proteins. Therefore, KDR expression was graded as either positive (present) or negative (absent). Staining of tumor endothelium was consistent and widespread when KDR was present, and no hot spots were detected. The correlation of KDR positivity with VEGF expression and vessel density adds credence to the hypothesis that VEGF is an important angiogenic factor in colon cancer. These data are consistent with the findings of Brown *et al.*, who demonstrated higher expression of mRNA (by *in situ* hybridization) for VEGF (vascular permeability factor) in malignant colonic epithelium as compared to benign colon epithelial tumors (19). In addition, this group demonstrated that the mRNA encoding both receptors for VEGF were present on adjacent tumor endothelial cells, but they were not expressed in endothelial cells away from the tumor.

We believe that the strength of this study is two-fold. First, the fact that numerous parameters associated with angiogenesis is associated with metastasis suggests that the association of these factors with metastasis is not by chance alone. Second, the use of immunohistochemistry on archival paraffin-embedded tissues to assess vascularity and related factors can be readily performed in any clinical pathology laboratory. If these data can be reproduced in other laboratories, then it is possible that these parameters of angiogenesis may be used in a clinical setting to help predict those patients at risk for metastasis who may benefit from adjuvant therapy.

Several issues still need to be addressed in regard to the biology of this ligand-receptor interaction. Does VEGF confer a growth advantage to endothelial cells that express KDR, or does VEGF up-regulate its own receptor? We are currently carrying out *in vitro* experiments to answer this question. Although most tumors with high levels of VEGF were associated with KDR positivity in their endothelial cells,

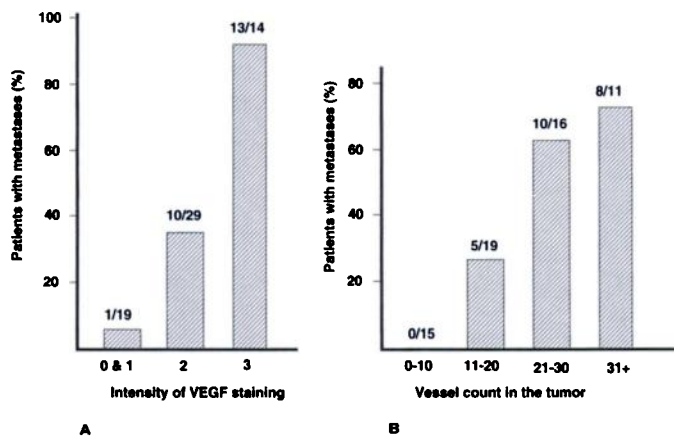


Fig. 2. Relationship between VEGF expression and metastasis (A) and vessel count and metastasis (B). The prevalence of metastatic disease increases as the intensity of VEGF expression or vessel count increases.

Table 2 Correlation between expression of flk-1 and expression of VEGF, vessel count, and proliferative index in human colon carcinomas

KDR expression	Intensity of VEGF staining (mean ± SEM)	Vessel count (mean ± SEM)		PCNA index % (mean ± SEM)
		Within tumor	At invasive edge	
Positive (n = 22)	2.3 ± 0.2	26.6 ± 1.8	47.2 ± 2.5	68.4 ± 2.5
Negative (n = 33)	1.7 ± 0.1	17.7 ± 2.0	29.4 ± 2.6	59.9 ± 2.1

there were some tumors high in VEGF that were negative for KDR and *vice versa*. These observations may be due to the limitations of immunohistochemistry. In addition, there may be redundancy in the process of angiogenesis similar to that in other biological processes. If VEGF expression is low or KDR is not expressed, tumors may still grow, with growth being dependent on other angiogenic factors and receptors. Indeed, it is unlikely that VEGF is the only angiogenic factor in colon cancer. It is possible that if expression of VEGF or KDR is inhibited, then other angiogenic factors and receptors may be up-regulated to compensate for the decrease in VEGF activation. Although *flt-1* is also known to be a receptor for VEGF, recent experiments have demonstrated that *flt-1* is not associated with endothelial cell mitogenicity, chemotaxis, or actin reorganization (20).

Our study has demonstrated a relationship between vessel density and tumor metastasis in colon cancer. In addition, VEGF expression has been associated with advanced disease as well as vessel density. The findings from this study could have numerous implications. It may someday be possible to predict which patients with intermediate-stage colon cancer (Dukes B and possibly Dukes C) are at risk for metastasis formation. We are in the process of quantitating vessels in patients with Dukes B carcinoma who have been followed for at least 5 years to determine if vessel density and VEGF expression can predict which patients will have a recurrence. If vessel density and VEGF expression prove to be reliable prognostic factors, patients at high risk for developing metastatic disease can be selected for adjuvant therapy. In addition, the identification of factors that correlate with angiogenesis in colon cancer may provide a basis for experiments targeting these angiogenic factors to inhibit vascularization of tumors. If these factors are indeed responsible for colon cancer angiogenesis, then therapeutic strategies to inhibit their activity using either specific antibodies or antisense RNA may allow clinicians to treat not only the malignant cells within a tumor but also the vascular supply of the tumor.

#### Acknowledgments

We thank Ricky Sanchez and Velma Harris for technical assistance.

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