Epithelial Expression of VEGF Receptors in Colorectal Carcinomas and their Relationship to Metastatic Status

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Abstract. *Background: Vascular endothelial growth factor (VEGF), originally identified as an endothelium-specific factor, can also bind to malignant cells, a mechanism by which a tumor could regulate its own progression. The biological effects of VEGF are mediated by three receptors (VEGFRs), VEGFR-1, VEGFR-2 and VEGFR-3. This study aimed at defining the expression of VEGFs in colorectal cancer (CRC) epithelia and their relationship to the metastatic status. Materials and Methods: Using immunohistochemistry, the levels of tumoral immunoreactivity for VEGFs in 105 non-metastatic, lymphogenously-metastatic and haematogenously-metastatic CRC specimens were assessed. Statistical analysis was performed using Fisher's exact probability test. Results: VEGFR-1 immunoreactivity was positive in only 50% of the cases. However lack of expression of VEGFR-1 was significantly associated with lymphogenous and haematogenous metastases. VEGFR-2 and VEGFR-3 were expressed in all investigated specimens to varying degrees. Low levels of VEGFR-2 were significantly associated with distant metastases. No significant changes were detected in VEGFR-3 expression. Conclusion: Epithelial expression of VEGFR-1 and VEGFR-2 appear to have a protective effect against tumor aggressiveness in CRC.*

Tumor metastasis is a critical step in determing the aggresiveness of cancer and is the principal cause of cancer death. A crucial event leading to metastasis is the endogenous production of growth factors which act on tumors through functional external receptors. The vascular endothelial growth factor (VEGF) family is particularly important because of its angiogenic (paracrine) and cell survival (autocrine) properties (1-3). Neoplastic cells secrete VEGFs by themselves to stimulate new vessel formation, thus providing oxygen and nutrients to the tumor but also allowing access to the circulation to facilitate metastasis. In addition, it appears that VEGF can also act as a survival factor for tumor cells to protect them from stress situations such as hypoxia, chemotherapy and radiotherapy (4).

The biological effects of VEGFs are mediated by three related receptor tyrosine kinases, VEGFR (VEGF receptor) -1, -2 and -3. The main tissue that expresses VEGFRs is the endothelium. Since tumor cells represent a major source of VEGFs, one emerging hypothesis is that these ligands may form an autocrine loop with VEGFRs expressed on tumor cells themselves, thereby enabling a tumor to regulate its own survival, growth and progression. Indeed, it has become increasingly apparent that VEGFRs can be expressed on cancer cells *in vitro*, such as human colorectal and small cell lung cancer cell lines (5, 6). Detection analyses of human neoplastic tissues including carcinomas of the female genital tract, pancreatic cancer and squamous cell carcinoma of head and neck provided evidence for expression of VEGFRs on malignant epithelia (7-10). However, the role of the tumor epithelial expression of VEGFRs is still unclear and the published data so far are controversial. Thus, the expression of VEGFRs in some studies was correlated with an increased risk for poor clinical outcome, whereas in other studies no direct contribution to tumor progression was found.

In the case of colorectal cancer, tumoral expression of VEGFRs has already been recognised (11-16). However, a clear association between their expression and the metastatic disease status has not been described so far. Our interest in this study was focused on the epithelial expression of VEGFR-1, VEGFR-2 and VEGFR-3 and their role concerning the metastatic behavior of colorectal cancer. In particular, our purpose was to ascertain whether the expession pattern of these receptors could determine the metastatic potential of colorectal carcinomas. For this reason,

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using immunohistochemistry we assessed the levels of their immunoreactivity in the malignant epithelia of 105 non-metastatic, lymphogenously-metastatic and haematogenously-metastatic colorectal carcinoma specimens.

Materials and Methods

Tissue samples. The colorectal tissue samples used in this study came from 105 patients undergoing elective surgery for colorectal cancer at the University of Mainz during the years 1995-1999. The investigation of these tissues was in accordance with the rules of the responsible state ethical committee of Mainz University. The morphological classification of the carcinomas was conducted according to WHO specifications (17). All tumors were staged following the guidelines of the TNM Classification of Malignant Tumors (17). With respect to the T status, all tumors investigated were T3 and moderately differentiated (G2), and were separated into three groups according to metastatic status. The first group comprised 42 cases without tumor metastasis to regional lymph nodes or distant organs (N0/M0). Among the remaining 63 metastasizing cases, 30 were characterized by lymphogenous (N+) and 33 by haematogenous metastases (M+). For all samples investigated, follow-up data were obtained from hospital charts and by corresponding with the physicians in charge during a period of 5 years after surgery.

Antibodies. Primary antibodies: rabbit polyclonal flt-1/VEGFR-1 (Acris; Hiddenhausen, Germany), mouse monoclonal flk-1/VEGFR-2 (A3; Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and rabbit polyclonal flt-4/VEGFR-3 (C-20; Santa Cruz Biotechnology, Inc.). Secondary antibodies: horse anti-mouse biotinylated IgG (Vector Laboratories, Inc., Burlingame, CA, USA), rabbit anti-goat biotinylated IgG (Vector Laboratories, Inc.).

Immunohistochemstry. All immunohistochemical reactions were conducted using formalin-fixed and paraffin-embedded samples. After deparaffination, the samples were treated in a microwave oven in EDTA buffer for 15 minutes. Incubation with the primary antibodies to the three VEGFRs, and the secondary antibodies, horse anti-mouse biotinylated IgG (for VEGFR-2) and rabbit anti-goat biotinylated IgG (for VEGFR-1 and VEGFR-2) were carried out in accordance with standard protocols using the Vectastain Elite reagent (Vector Laboratories, Inc.). Anti-VEGFR-1, anti-VEGFR-2 and anti-VEGFR-3 were used at a dilution of 1:100, 1:100 and 1:200, respectively. All secondary antibodies employed in this study were used at a dilution of 1:200. Sections were counterstained with Mayer's hematoxylin. To prove the specificity of the immunoreactions, a sample from every colorectal carcinoma sample (n=105) was stained solely with the secondary antibody omitting the primary antibody as negative control. Immunostaining reactions of each sample were evaluated by three authors independently (N.S., C.J., M.T.) without knowledge of the clinical data. The intensity of tissue staining was scored on a semi-quantitative scale from 0 to 2 (0: no staining, 1: weak staining, 2: strong staining). VEGFR-1 staining was either negative or positive, whereby the intensity of the positive staining did not differ and was always strong. VEGFR-2 and VEGFR-3 expression was positve in all investigated cases and the intensity of immunorection was classified as "low" for weak staining and "high" for strong staining. In most cases the staining was homogeneous. In those cases where heterogeneous staining was observed within the same sample, that level of staining which was visible in more than 50% of the cells was chosen for the classification into a defined group.

Statistics. The evaluation of data concerning association of immunostaining reaction with tumor stage was assessed using χ^2 (Fisher's exact test). $P<0.05$ was considered to be significant in all statistical analyses.

Results

To elucidate the relevance of VEGFRs for colorectal cancer metastasis, immunohistochemistry was used to determine their expression in 42 non-metastatic (N0/M0) and 30 lymphogenously-metastatic, as well as 33 haematogenously-metastatic, colorectal carcinomas. With respect to the T status, all tumors investigated were defined as T3 (subserosa infiltration) and moderately differentiated (G2). This selection was performed with regard to a potential relationship between the expression level of VEGFRs and the metastatic disease status. For all non-metastatic cases investigated, a follow-up for at least five years after surgery was carried out.

The topological staining distribution of the three VEGFRs was homogenous. No differences of staining intensity between the superficial tumor fraction and the invasive tumor edge were observed. VEGFR staining showed a predominantly membranous and in some cases also a cytoplasmatic distribution in the tumors. A nuclear expression was not found. Because of this staining pattern, the expression levels of the corresponding non-neoplastic mucosa were not determined. In our experience, the mucus content of the colonic goblet cells leads to a non-specific immunoreactivity.

VEGFR-1 immunoreactivity was positive only in 50% of the cases (Figure 1 A, B). The positive endothelial VEGFR-1 expression served as an internal positive control for the negative tumoral staining. Absence of expression of VEGFR-1 was significantly associated with lymphogenous ($p=0.02$) and haematogenous metastasis ($p=0.005$) (Table I). VEGFR-2 and VEGFR-3 were expressed in all investigated specimens to varying degrees. The tumors were classified with respect to the staining intensity into two groups with low and high expression levels respectively (Figure 1 C-F). Low levels of VEGFR-2 expression were significantly associated with the cases of distant metastasis ($p=0.02$) (Table II). There were no identified significant differences in the expression intensity of VEGFR-2 between non-metastatic tumors and carcinomas with lymph node metastasis (Table III). A uniform distribution of VEGFR-3 immunostaining in the non-metastatic and metastatic tumors was observed.

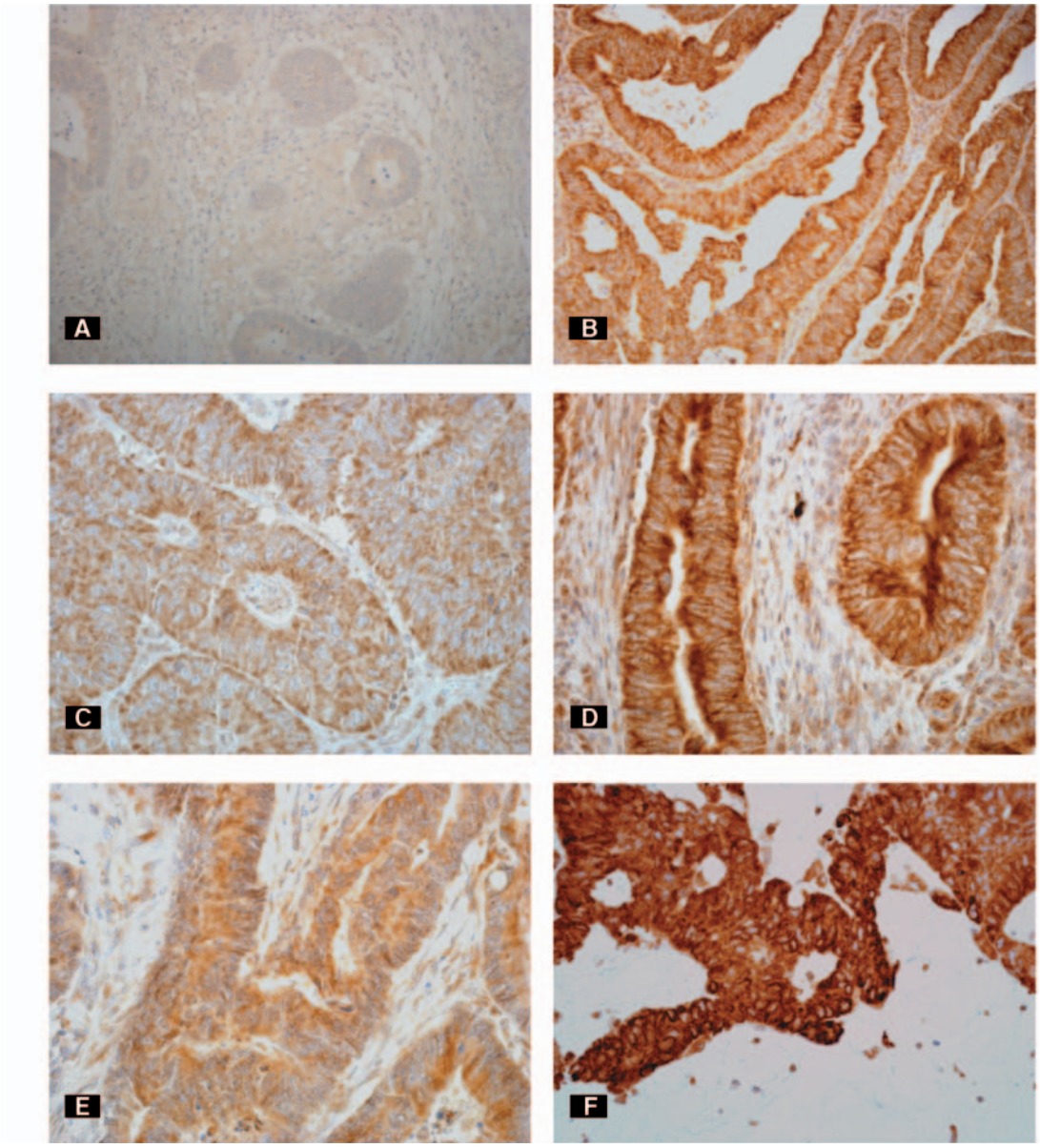

Figure 1. *Expression of VEGFRs in colorectal carcinomas. A, B: Expression of VEGFR-1, negative (A) and positive (B) staining. C, D: Expression of VEGFR-2, low (C) and high (D) staining intensity. E, F: Expression of VEGFR-3, low (E) and high (F) staining intensity. Magnification: x400.*

Discussion

Given the prevalence of colorectal cancer and the high morbitity and mortality associated with lymphogenous and haematogenous metastasis, an understanding of the underlying mechanisms is essential for the development of effective treatments for these conditions. In this context, VEGF and its receptors are becoming of increasing interest as major regulators of colon cancer metastases. *In vitro* and *in vivo* studies have suggested a possible autocrine effect for VEGF and VEGFRs by demonstrating the presence of these receptors in tumor colonic cells (5, 11-16). In the present study, we also found a positive tumoral expression in all investigated colorectal carcinoma specimens with respect to VEGFR-2 and VEGFR-3 and in half of the cases with respect to VEGFR-1. These observations imply the existence of a potential, functional VEGF/VEGFR loop that controls tumor cell behavior directly by a non-endothelial-related pathway. Concerning the topological staining distribution of the three VEGFRs, a homogenous

Table I. *Expression and statistical significance of VEGFR-1 in non-metastatic (N0/M0), lymphogenously-metastatic (N+) and haematogenously-metastatic (M+) colorectal carcinomas. Considering the expression levels, the cases examined were separated into two groups, characterized by a negative or positive expression of VEGFR-1 (cf. Figure 1A and 1B) (n=105 cases).*

Tumor status	VEGFR-1-negative	VEGFR-1-positive	*P*-value
N0/M0	13	29	
N+	18	12	0.02
M+	21	12	0.005

Table II. *Expression and statistical significance of VEGFR-2 in non-metastatic (N0/M0), lymphogenously-metastatic (N+) and haematogenously-metastatic (M+) colorectal carcinomas. Considering the expression levels, the cases examined were separated into two groups, characterized by a low or high expression of VEGFR-2 (cf. Figure 2A and 2B) (n=105 cases).*

Tumor status	VEGFR-2-high	VEGFR-2-low	*P*-value
N0/M0	17	25	
N+	11	19	NS
M+	23	10	0.02

NS, not significant.

pattern was seen, without differences in immunostaining intenstity between the superficial tumor fraction and the invasive tumor edge. These data suggest that VEGFR expression in the primary colorectal carcinomas is a consistent phenomenon within the tumour tissue independent of the infiltrative pattern at the site of deepest penetration. In contrast, Duff *et al.* found an increased tumoral VEGFR-2 expression throughout the tumour with a maximum at the invasive tumour edge (16).

The principal aim of this study was to determine if tumoral expression of VEGFRs is associated with the metastatic disease status in colorectal cancer. To our knowledge, studies involving a large number of cases have not yet been published on the topic of the relationship between expression of VEGFRs and the metastatic disease status in colorectal cancer. The results presented here clearly show that the absence of epithelial expression of VEGFR-1 was associated with lymphogenous and haematogenous metastatic disease status. In addition, low tumoral VEGFR-2 expression was associated with cases of distant metastases. We suggest that the tumoral expression of both receptors may have a protective effect and could act as negative regulators for processes facilitating metastasis. The ligand for VEGFR-1 and VEGFR-2 is VEGF, an angiogenic and pro-survival endothelial factor (1-3). We assume that VEGF could also act as an autocrine survival factor for colonic tumor cells themselves. Thus, if tumor cells do not express the receptors for this ligand, its pro-survival function is ineffective leading to cell death and tumor necrosis, which promote tumor progression (18). Indeed, adenocarcinomas of the gut are characterized by a typical morphological feature, namely abundant central necroses within the neoplastic glands.

VEGF also induces a member of the inhibitor of apoptosis family, survivin, which preserves cellular integrity by stabilizing the microtubular network (19, 20). Stehbens *et al.* postulated that dynamic microtubules control the regional distribution of the adhesion molecule E-cadherin

Table III. *Expression and statistical significance of VEGFR-3 in non-metastatic (N0/M0), lymphogenously-metastatic (N+) and haematogenously-metastatic (M+) colorectal carcinomas. Considering the expression levels, the cases examined were separated into two groups, characterized by a low or high expression of VEGFR-3 (cf. Figure 3A and 3B) (n=105 cases).*

Tumor status	VEGFR-3-high	VEGFR-3-low	*P*-value
N0/M0	10	32	
N+	7	23	NS
M+	10	23	NS

NS, not significant.

at the cell surface, which is responsible for tissue integrity (21). It is also known that reduced tumor adhesiveness is necessary for tumor invasion and metastasis. In this context, we suggest that dysfunction of VEGF binding to its receptors could be an initial event associated with an increased migratory capacity of neoplastic cells on the basis of a disordered tumor tissue cohesion. Our results are in accordance with these of Hanrahan *et al.* who found a significant increase in VEGFR-1 mRNA in T3/T4 colorectal carcinomas compared with lymphogenously metastasizing tumours, together with a tendency towards a decrease in VEGFR-2 mRNA in nodal positive carcinomas (14). Chung *et al.* showed that low tumor VEGFR-1 expression was associated with worse survival and was correlated with advanced disease status in pancreatic carcinomas (9). VEGFR-2 expression was not clearly associated with outcome. In breast cancer, an association of low VEGFR-1 with worse prognosis was shown (22). In contrast, in esophageal squamous cell carcinomas VEGFR-1-positive cancers tended to be associated with poorer nodal status but VEGFR-2 expression did not correlate with clinicopathological factors or prognosis (23).

Taking together the controversial data published to date, tumor expression of both receptors seems to have differing effects, that is, both positive and negative, in tumor

progression behavior. These findings reflect the complexity of the pathophysiological events promoting the process of tumor metastasis. For example, although the crucial role of tumor necrosis in tumor progression is generally accepted, aberrant cell survival and resistance to apoptosis are hallmarks of tumor invasion and progression to metastatic disease (17, 24). In this context, Bates *et al.* demonstrated in a colon carcinoma spheroid model that tumor invasiveness was dependent on the autocrine VEGF/VEGFR-1 signaling-controlled survival of the migrating cells (25).

Epithelial expression of VEGFR-3 was observed in all investigated cases and had a uniform distribution in the non-metastatic and metastatic colorectal carcinomas. These results are in accordance with the conclusion of other research groups who found a positive tumoral expression of VEGFR-3 in most colorectal carcinomas but no correlation with lymph node metastasis (11-14). Onogawa *et al.* detected VEGFR-3 mRNA in all investigated colorectal carcinomas (15). In 16 of the 20 cases, VEGFR-3 mRNA expression was high in the tumors in comparison to the normal colorectal mucosa. In our study, the expression levels of the corresponding non-neoplastic mucosa were not determined; in our experience the mucus content of the colonic goblet cells leads to a non-specific immunoreactivity. In contrast to the findings in the case of colorectal cancer, in endometrial carcinomas high expression levels of VEGFR-3 in neoplastic cells were significantly related to myometrial invasion and lymph node metastasis (7). This discrepancy may be due to the different histogenetic origin of the tumors.

In conclusion, the present results demostrated that all three VEGFRs are expressed by colorectal carcinoma cells. A significant association was found between the absence of expression of VEGFR-1, as well as low levels of VEGFR-2, and tumor metastasis, suggesting that expression of these receptors on malignant epithelia has a protective effect against tumor aggressiveness in the case of colorectal cancer.

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