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Expression of vascular endothelial growth factor (VEGF) and association with microvessel density in small-cell and non-small-cell lung carcinomas

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Summary. Recent studies have demonstrated that tumor angiogenesis is a prognostic factor for various malignant neoplasms. Specifically, in non-small-cell lung carcinomas (NSCLCs) most reports show an association between neovascularization and vascular endothelial growth factor (VEGF) expression as well as the presence of metastases and survival, although a few reports do not agree with these findings. Angiogenesis is not clearly characterized in small-cell lung carcinomas (SCLCs), since they are rarely treated by surgery, and thus the available tissue for biological characterization is sparse. The aim of the present study was to investigate angiogenesis and the expression of VEGF in lung tumors. We examined 88 non-small-cell and 39 small-cell lung carcinomas. Angiogenesis was estimated by determining microvessel counts, with the use of anti-CD31 and anti-factor VIII antibodies and expression of VEGF was also evaluated immunohistochemically. Our data showed that in NSCLCs angiogenesis was more prominent in poorly-differentiated neoplasms and correlated with VEGF expression, therefore it is at least in part mediated by the latter. Interestingly, in SCLCs a higher vascularization was noted. However, there was no strong association with VEGF expression. Thus, small-cell lung carcinoma may represent a suitable neoplasm for testing antiangiogenic drugs in combination with chemotherapy. Nevertheless, antiangiogenic therapy should not be targeted specifically to the VEGF pathway, since in SCLCs other mediators of angiogenesis may be important as well.

Key words: VEG, Angiogenesis, Lung, Carcinoma

Introduction

Studies on tumor growth have shown that it is absolutely dependent on vascularization. Seminal work performed by Folkman three decades ago revealed that tumors beyond the size of 2 mm require new vessel formation for subsequent growth and development (Folkman, 1990). Angiogenesis, which is the generation of blood vessels from preexisting vasculature, provides nutrients to growing tumor cells, and it is also critical for the distant spread of neoplastic cells. The number/density of microvessels (MVC, microvessel count/MVD, microvessel density), as a measure of tumor angiogenesis, represents the neovascularization ability of the tumor, and is an important prognostic factor for increased metastatic potential and worse prognosis in human carcinomas (Weidner et al., 1991; Tomisaki et al., 1996; Sanz-Ortega et al., 2000; Papamichael, 2001). Regarding non-small-cell lung carcinoma, the vast majority of reported studies showed that high angiogenesis influences the prognosis (Giatromanolaki, 2001). However, other studies in the literature reported an association between high MVD and better prognosis (Chandrashud et al., 1997; Duarte et al., 1998).

In order to achieve a functional vasculature, tumors cells produce (or induce the production from other cells) a large number of angiogenic factors (Diaz-Flores et al., 1994). Several growth factors and cytokines have been identified, some stimulating and some inhibiting the angiogenic process. VEGF is a secretory glycoprotein that acts as a specific endothelial mitogen and can induce vascularization around actively growing tumor cells (Ferrara et al., 1992; Ferrara and Davies-Smyth, 1998). Recent studies have demonstrated the presence of increased VEGF mRNA and protein and correlated it with worse prognosis in several carcinomas, such as gastric, colorectal, bladder and lung (Tanigawa et al., 1996; Takanashi et al., 1997; Streeter and Crew, 2001). Regarding lung neoplasms such as squamous cell carcinoma, non-small-cell carcinoma, and stage I adenocarcinoma, the prognostic value of this expression is controversial (Takanami et al., 1997; Volm et al., 1997; Giatromanolaki et al., 1988; Shibusha et al., 1998; Yano et al., 2000; Ballie et al., 2001; Fontanini et al., 2002; Tsoli et al., 2002).

The non-small-cell lung carcinoma is a common

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neoplasm, with an incidence of approximately 120 new cases/100,000 each year. In initial stages the therapeutic approach is mostly surgical. However, in most cases the primary neoplasm has already spread at the time of diagnosis, and radiotherapy or chemotherapy improve survival only slightly.

The small-cell lung carcinoma represents a specific entity, that has worse prognosis, despite the initial response to chemotherapy. A detailed biological characterization is not feasible, since such neoplasms are rarely treated by surgery, and thus only a few surgical specimens are available (Lucchi et al., 2002).

The aim of the present study was to determine the levels of VEGF expression in small-cell and non-small-cell lung carcinomas and to associate them with vascularity.

Materials and methods

Our material consisted of 88 non-small-cell carcinomas (48 squamous cell carcinomas, 30 adenocarcinomas, 10 large-cell carcinomas) and 39 small-cell lung carcinomas, that were examined in our Laboratory from 1994-2002. The median age of the patients was 53 years for non-small-cell carcinomas (range 45-61) and 58 years for small-cell carcinomas (range 51-65). The surgical specimens consisted of pneumonectomies, lobectomies and segmental resections. A representative formalin-fixed paraffin-embedded block of tumor tissue was selected from each case on the basis of containing viable tumor and surrounding non-neoplastic lung parenchyma.

Immunohistochemistry

We used the EnVision System and the monoclonal antibodies VEGF (Neomarkers), CD31 (DAKO) and Factor VIII (DAKO). Briefly, 5 μm-thick, histological sections were dewaxed in xylene, rehydrated through graded alcohols, immersed in 10 mM Tris and 0.5 M EDTA, pH 9.0, and microwaved twice for 5 minutes each. Subsequently, the sections were incubated with 0.3% H_2O_2 for 30 minutes to block endogenous peroxidase activity. The sections were then incubated overnight at 4 °C with the primary antibodies (dilutions: VEGF, 1:50; CD31, 1:50; and Factor VIII, 1:50). Non-specific binding was blocked by incubating the sections for 30 min with Blocking Solution (DAKO). Detection was carried out using the EnVision System kit (DAKO) with diaminobenzidine as chromogen. Counterstaining was performed with hematoxylin Harris.

Microvessel detection and counting

Vascularity was measured by the average number of CD31- and Factor VIII-positive vessels, as described previously (Lucchi et al., 2002). Briefly, for each slide, the three most intense regions of neovascularization were identified at low power (x10 objective lens and x10 ocular lens). In each of these selected areas a x200 field was counted (x20 objective lens, x10 ocular lens, 0.72 mm^2 per field), and the average count of the three fields was recorded (MVC: microvessel count). Although in most of the counted vessels a lumen was identified, this was not necessary. Vessels within muscular walls or lumens larger than approximately eight red blood cells were excluded from the count. Vessels had to be separated clearly from each other to be counted. No counts were performed in areas of necrosis or inflammation. In all cases MVC was determined independently by two pathologists. The median value of MVC with the use of the CD31 antibody was 62 microvessels (range: 28-250 microvessels). This count was used as the cut-off point to distinguish low from high MVC. The median value of MVC with the use of the factor VIII antibody was significantly lower (MVC=45, range: 18-159 microvessels).

VEGF expression

The percentage of tumor cells that exhibited a positive cytoplasmic immunoreactivity to VEGF was determined by counting at least 1000 neoplastic cells in each case. The median value (25%) was used as the cut-off point to distinguish low (0-25% positive cells) from high VEGF-(>25% positive cells) expressing tumors.

Statistical analysis

For the statistical analysis, VEGF expression and MVC were considered dichotomous variables, using the cut-off values described above. VEGF and MVC were compared in the NSCLC group with respect to histology and differentiation with the chi-squared test. In the SCLC group, VEGF expression and MVC were compared with Fisher's exact test. Analyses were performed in SPSS 10.0. P-values were two-taled.

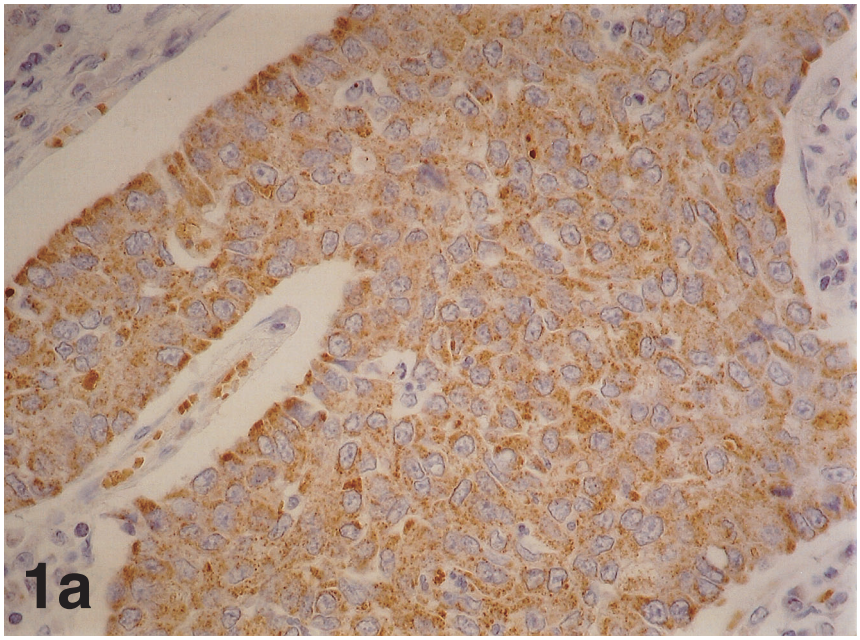
Results

VEGF expression in non-small-cell carcinomas

High expression of VEGF was detected in 68/88 (77.27%) of non-small-cell carcinomas (Fig. 1a). Histologically different subtypes and different grades expressed various intensities in VEGF staining. Specifically, 36 from the 48 squamous cell carcinomas (75%), 22 from the 30 adenocarcinomas (73.3%), and all large-cell carcinomas (100%) showed high expression of VEGF (Table 1). Cytoplasmic immunostaining was detected in all cases. Besides tumor cells, in many cases tumor stromal cells and vascular endothelium stained as well, although the staining intensity was very weak. There was no significant correlation between VEGF expression and age, sex, tumor subtype or vessel invasion. Increased expression of VEGF was significantly associated with low differentiation ($p<0.001$). The better differentiated carcinomas showed

Table 1. Microvessel counts and VEGF expression in NSCLC.

HISTOLOGY	n	MVC	HIGH MVC	p	HIGH VEGF	p
Subtype						
SCCs	48	28-190	36	0.188	36	0.188
ADCs	30	35-200	22	exact 0.199	22	exact 0.199
LCCs	10	70-203	10		10	
Differentiation						
Well	20	28-170	8	<0.001	8	<0.001
Moderate	28	45-195	22	exact <0.001	22	exact<0.001
Poor	40	70-233	38		38	



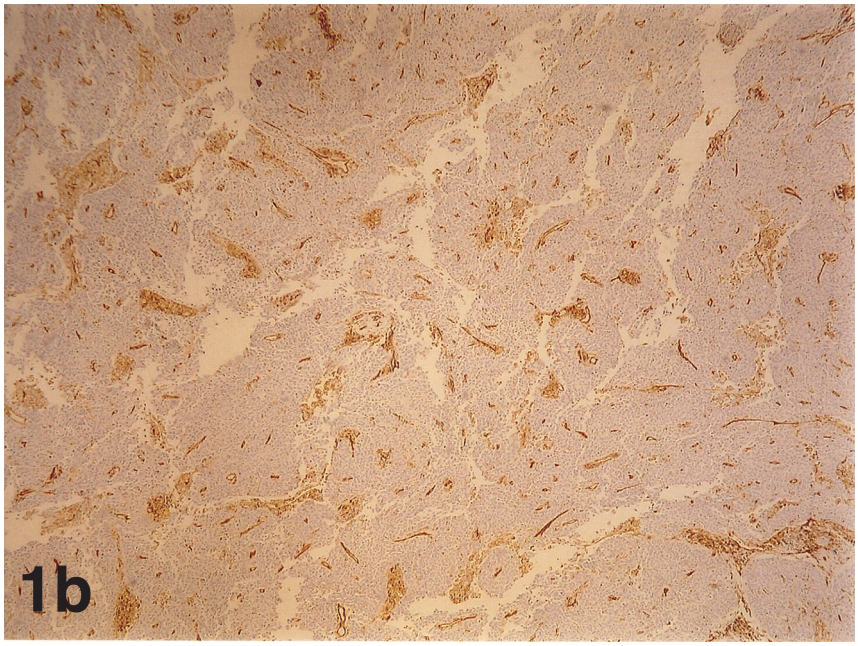
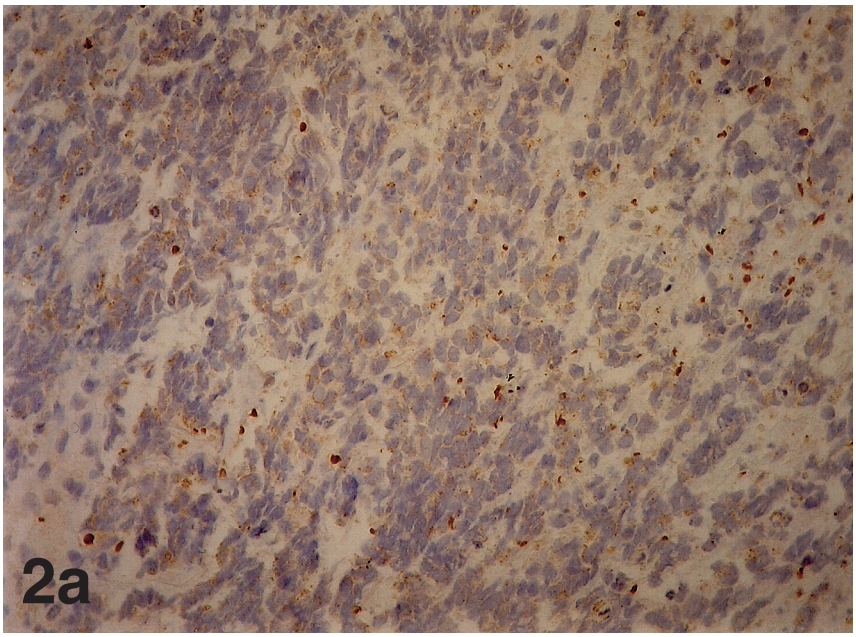


Fig. 1. a. High expression of VEGF in squamous cell carcinoma. x 400. **b.** Immunohistochemical detection of CD31 in squamous cell carcinoma. x 100

a predominantly low expression of VEGF.

Microvessel counts in non-small cell carcinomas

By staining with two different antibodies, we noted differences in microvessel staining. It became apparent that CD31 was superior to Factor VIII in microvessel detection, since the latter revealed only 71% of the microvessels highlighted by CD31. Therefore, for further analysis, counts obtained with CD31 staining were utilized. No differences in CD31 immunoreactivity were noted between the central and marginal tumor areas. Among the 88 non-small-cell carcinomas, 68 (77.27%) exhibited increased microvessel count (MVC>62) (Fig. 1b). Specifically, 75% of the squamous cell carcinomas (36/48), 73.3% of the adenocarcinomas (22/30), and all large-cell carcinomas showed a high microvessel count (Table 1). There was no significant association between microvessel count and age, sex, tumor subtype or vessel invasion. Increased MVC was significantly associated with low differentiation ($p<0.001$). The better differentiated carcinomas showed



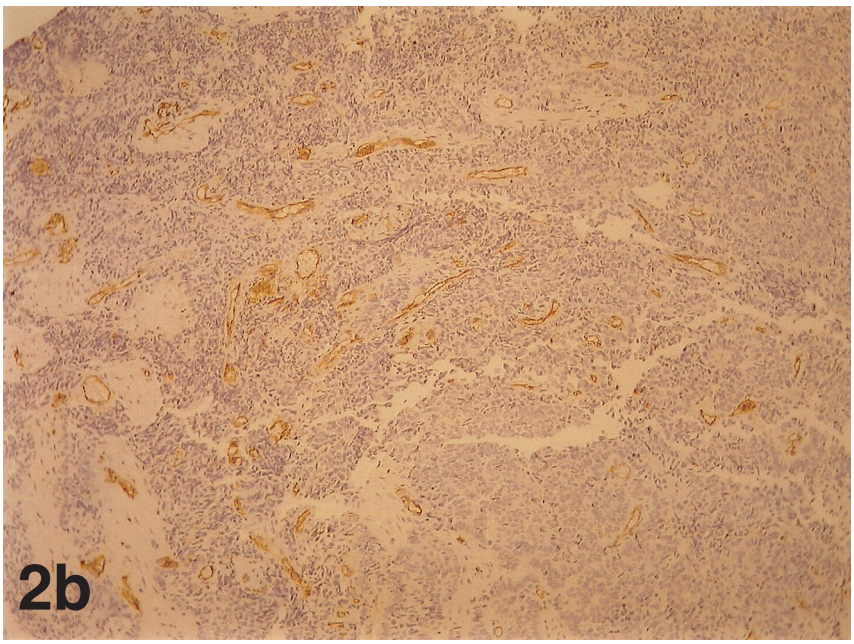


Fig. 2.a. Low expression of VEGF in SCLC. x 400. **b.** Immunohistochemical detection of CD31 in SCLC. x 100

predominantly low MVC.

Assessment of the relationship between VEGF expression and angiogenesis revealed a statistically significant relationship between VEGF staining and MVC ($p<0.001$).

VEGF expression and microvessel scores in small-cell carcinomas

Among the 39 small cell carcinomas only 12 (30.76%) exhibited high expression of VEGF (Fig. 2a). In contrast, most small-cell carcinomas had a high MVC (range 45-250, mean 78) (Fig.2b), the highest being in neoplasms with increased VEGF expression. Assessment of the relationship between VEGF expression and angiogenesis revealed no statistically significant relationship between VEGF staining and MVC ($p=0.333$).

Discussion

Compelling evidence suggests that tumor angiogenesis plays a pivotal role in tumor growth, maintenance and metastatic potential. Tumor microvessels differ from those of non-neoplastic tissues. They are more fragile and irregular, with increased permeability and higher proliferation rate than that of normal endothelial cells. In current studies angiogenesis is preferentially assessed by immunodetection of the endothelial marker CD31, which recognizes the PECAM-1 endothelial membrane antigen. Immunostaining for CD31 is more specific for capillary detection than Factor VIII, which does not stain all capillary endothelia in tumor tissues (Giatromanolaki et al., 1997) and from CD34, which displays lymphatic immunostaining as well (Tsoli et al., 2002).

Much research has focused on the key molecules that regulate the new vessel formation. One of the most important angiogenetic molecules is VEGF, also known as VPF (vascular permeability factor), a potent cytokine that acts as an endothelial-cell specific mitogen and survival factor (Ferrara and Davis-Smyth, 1997). There are four isoforms of VEGF: $VEGF^{121}$, $VEGF^{165}$, $VEGF^{189}$ and VEG^{F206}, of which $VEGF^{165}$ is the most abundant in human tissues (Yano et al., 2002). In the present study we used an antibody that recognizes all isoforms. Several studies have shown that VEGF is closely correlated with the process of neovascularization and prognosis in many solid tumors (Fontanini et al., 1997a,b; Decaussin et al., 1999).

In our study, detectable VEGF staining was observed in all cases. In non-small-cell carcinomas it appeared to be a correlation with an increase in the expression of VEGF and microvessel density. Higher expression of all parameters was noted in poorly-differentiated carcinomas. Our data indicated that the angiogenetic phenomenon may have an important role in the clinical behavior of these neoplasms, and this finding is in agreement with most reported studies (Takanami et al., 1997; Volm et al., 1997; Giatromanolaki et al., 1988; Shibusha et al., 1998; Yano et al., 2000; Ballie et al., 2001; Fontanini et al., 2002; Tsoli et al., 2002).

A positive correlation was noted between tumor angiogenesis (estimated by accessing tumor vasculature, by CD31 detection) and VEGF expression. This is in accordance with previous studies, where tumor vasculature was accessed by CD31, CD34 or Factor VIII immunodetection, and reflects the impact of VEGF on the angiogenetic process (Fontanini et al., 1997; Giatromanolaki, 2001; Tsoli et al., 2002).

Angiogenesis seems to play a role in the neoplastic process of small-cell lung carcinomas, as well. Regarding microvessel count, we observed a higher mean value in SCLCs than in NSCLCs. Thus, it appears that SCLC differs from NSCLC both from the pathological and the angiogenetic points of view. Moreover in this subtype of lung tumors VEGF seems to play a less important role in neovascularization. Specifically, in a high percentage of small-cell lung carcinomas a high microvessel count was correlated with low VEGF expression. This observation suggests that additional factors also participate in the angiogenetic process. Along these lines, several studies have shown that angiogenesis is a complex process that besides VEGF involves several factors such as: nitric oxide synthase (NOS-2), cyclooxygenase-2 (COX-2) (Marrogi et al., 2000), hypoxia-inducible factors H1F1a and H1F2a (Giatromanolaki et al., 2001), fibroblast growth factor (FGF) and thrombospondin (Sheibani and Frazier, 1999) etc. Studies on the role of bFGF, as an angiogenic and prognostic factor in non-small-cell lung carcinomas, have provided conflicting results (Volm et al., 1997; Gouddo et al., 1999). This complexity of angiogenesis may explain the modest results observed in angiogenesis therapy, which targets a single protein, such as the use of VEGF-VEGF receptor antagonists (Shepherd, 2001; Herbst et al., 2002).

In conclusion, our results suggest that VEGF is an important angiogenic inducer in non-small-cell lung carcinomas. They further indicate the necessity for further analysis of the complex phenomenon of vascularization in small-cell lung carcinomas, in order to determine whether it may represent a plausible target for testing new antiangiogenic drugs (Kerbel and Folkman, 2002), other than those specifically directed against VEGF function, in association with chemotherapy.

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