Vascular Endothelial Growth Factor/KDR Activated Microvessel Density versus CD31 Standard Microvessel Density in Non-Small Cell Lung Cancer

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INTRODUCTION

Vascular endothelial growth factor (VEGF) is an important angiogenic factor, linked to poor outcome in human malignancies including non-small cell lung carcinoma (NSCLC). We used the 11B5 monoclonal antibody recognizing the VEGF/KDR complex (R. A. Brekken et al., Cancer Res., 58: 1952–1959, 1998) to assess the VEGF expression in cancer cells and the VEGF/KDR activated microvessel density (aMVD) in early operable NSCLC. The JC70 anti-CD31 monoclonal antibody was used to assess the standard MVD (sMVD). The aMVD was significantly higher in the invading front of the tumors and in the normal lung adjacent to the tumors as compared with normal lung distant to the tumor or to inner tumor areas (P < 0.0002). The sMVD was higher in the normal lung and decreased from the invading front to inner tumor areas (P < 0.0001). However, the vascular activation (aMVD:sMVD) was 4–6 times higher in the tumor areas as compared with lung from normal individuals (36–58% versus 9%; P < 0.0001). 11B5 fibroblast reactivity, noted in 25% of cases, correlated with high aMVD and sMVD in the inner tumor areas. Multivariate analysis showed that aMVD was the most potent and independent prognostic factor (P = 0.001; t-ratio, 3.28). It is concluded that intense VEGF/KDR angiogenic pathway activation is a tumor-specific feature in more than 50% of NSCLC cases and is associated with poor postoperative outcome. Clinical trials involving targeting of the VEGF/KDR-positive vasculature with specific antibodies, such as 11B5, are, therefore, encouraged.

ABSTRACT

Vascular endothelial growth factor (VEGF) is an important angiogenic factor, linked to poor outcome in human malignancies including non-small cell lung carcinoma (NSCLC). We used the 11B5 monoclonal antibody recognizing the VEGF/KDR complex (R. A. Brekken et al., Cancer Res., 58: 1952–1959, 1998) to assess the VEGF expression in cancer cells and the VEGF/KDR activated microvessel density (aMVD) in early operable NSCLC. The JC70 anti-CD31 monoclonal antibody was used to assess the standard MVD (sMVD). The aMVD was significantly higher in the invading front of the tumors and in the normal lung adjacent to the tumors as compared with normal lung distant to the tumor or to inner tumor areas (P < 0.0002). The sMVD was higher in the normal lung and decreased from the invading front to inner tumor areas (P < 0.0001). However, the vascular activation (aMVD:sMVD) was 4–6 times higher in the tumor areas as compared with lung from normal individuals (36–58% versus 9%; P < 0.0001). Fibroblast 11B5 reactivity, noted in 25% of cases, correlated with high aMVD and sMVD in the inner tumor areas. Multivariate analysis showed that aMVD was the most potent and independent prognostic factor (P = 0.001; t-ratio, 3.28). It is concluded that intense VEGF/KDR angiogenic pathway activation is a tumor-specific feature in more than 50% of NSCLC cases and is associated with poor postoperative outcome. Clinical trials involving targeting of the VEGF/KDR-positive vasculature with specific antibodies, such as 11B5, are, therefore, encouraged.

Several factors, such as hypoxia and cytokines that induce VEGF expression, also induce the expression of the VEGF receptors on endothelial cells (10, 11). Moreover, VEGF per se has been shown to up-regulate its receptors (12, 13). After VEGF/KDR binding, conformational changes seem to induce an epitope on the NH2 terminus that is not present on free VEGF or KDR molecules. Ke-Lin et al. (14) showed that rabbit polyclonal antibodies that are directed against the NH2 terminus of VEGF selectively stain tumor vessels. Brekken et al. (15) recently developed Mabs directed against peptides that correspond to the NH2-terminal 26 amino acids of human VEGF. Some of these Mabs were shown to react preferentially with the VEGF/KDR complex. Injection of such antibodies into nu/nu mice bearing NCL-H358 human NSCLC resulted in preferential localization to tumoral vascular endothelium. The 11B5 Mab was shown to have a very high preference for the VEGF/KDR complex.

Confirmation of a selective recognition of human tumoral vasculature by anti-VEGF/KDR antibodies is of importance because it demonstrates that tumor-specific antiangiogenic therapy would be feasible. Moreover, the patterns of expression assessed by such Mabs may prove of prognostic importance. VEGF expression does not always relate to increased tumor neo-angiogenesis (16). VEGF/KDR-combined assessment seems advantageous because it allows the assessment of VEGF expression in cancer cells together with the assessment of VEGF angiogenic activity (VEGF bound to endothelial cells). In the present study we evaluated the patterns of expression of the 11B5 in a series of NSCLCs. We also investigated the use of the 11B5 Mab as a prognostic tool.

MATERIALS AND METHODS

We examined 102 tumor samples from patients with early operable (T1,2 N0,1–M0 staged) non-small cell lung cancer (67 squamous and 35 adenocarcinomas). Histological diagnosis, grading, and N stage was done on H&E-stained sections. Eighty-two patients were male, and 20 were female, their ages ranging from 31 to 74 years (median, 61 years). Survival data were available in 93 of 102 patients. Patients dying within 60 days after surgery were excluded, so as to avoid bias from perioperative death. The follow-up of surviving patients at the time of analysis was 3–7 years (median, 56 months).

Immunohistochemistry for VEGF and VEGF/KDR Complex. The VEGF/KDR complex was assessed with the 11B5 Mab, an IgMx isotype produced using the VEGF Hu NH2 terminus as an immunogen (15). The VEGF expression was also assessed with the VG1 Mab (IgG isotype) recognizing the 121, 165, 189, and 206 amino acids, which are all encoded by a gene located on chromosome 6p21.3 (1). Two tyrosine kinase receptors for VEGF have been identified: the Flk-1 or VEGF receptor 1 and the KDR or VEGF receptor 2, which shares an 85% sequence identity with the murine flk-1 (6–8). Both of the receptors are expressed almost exclusively on endothelial cells, although monocyes and smooth muscle cells have been also reported to bear the receptors (9).

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3 The abbreviations used are: VEGF, vascular endothelial cell growth factor; Mab, monoclonal antibody; Flt-1, fms-like tyrosine kinase receptor; Flk-1, fetal liver kinase 1; TBS, Tris-buffered saline; APAAP, alkaline phosphatase/anti-alkaline phosphatase (procedure); MVD, microvessel density; sMVD, standard MVD; SECD, single-endothelial-cell density; aMVD, activated MVD; NSCLC, non-small cell lung carcinoma.

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Immunohistochemistry with a Panendothelial Marker. The JC70 Mab (DAKO) recognizing the CD31 panendothelial antigen (platelet/endothelial cell-adhesion molecule; Ref. 17) was used for microvesSEL and single endothelial cell staining on 5-μm paraffin-embedded sections. We used the APAAP procedure as described previously (18). Sections were dewaxed, rehydrated, and predigested with protease type XXIV for 20 min at 37°C. JC70 (1:50) was applied at room temperature for 30 min and washed in TBS. Rabbit antimouse antibody 1:50 (v/v) was applied for 30 min, followed by the application of mouse APAAP complex 1:1 (v/v) for 30 min. After washing in TBS, the last two steps were repeated for 10 min each. The color was developed by 20-min incubation with New Fuchsin solution.

Assessment of Cancer Cell VEGF Expression. The expression of the two Mabs in normal and neoplastic epithelium was recorded. The percentage of VEGF positive cancer cells (0–100%) was assessed by two independent observers (A. G., E. S.) at ×200. The tumoral invading front and inner tumor areas were separately examined and scored.

Assessment of Stromal VEGF Expression. The expression of VEGF in stromal fibroblasts was assessed at low (×100) and high (×200) power. Strong fibroblast reactivity in >50% of the examined fields was required to group cases as bearing high VEGF fibroblast reactivity. The tumoral invading front and inner tumor areas were separately examined and scored.

Assessment of the aMVD and the sMVD. The 11B5-positive microvesSels correspond to tumor vasculature found in an activated VEGF/KDR state at the time of surgery. The 11B5-positive MVD is, therefore, the aMVD as opposed to the JC70 (CD31)-positive MVD that corresponds to the entire tissue vascular network, activated or not. The CD31-positive MVD, being a measure of the entire vascular structure in a tissue, will, therefore, be briefly called sMVD.

The same method of microvesSEL counting was used for angiogenesis assessment for both 11B5 and JC70 Mabs. Sections were scanned at low power (×40 and ×100). All of the areas of tumor adjacent to normal lung were identified. MicrovesSEL counting followed in four consecutive ×200 fields starting from the tumor tissue adjacent to the normal lung (t1 field; tumor-invading front), and moving twice the optical field toward the normal tissue (n1, adjacent to the tumor normal lung; n2, distant to the tumor normal lung). Fig. 1 shows schematically the followed procedure for the MVD assessment. The tumor peripheral layer (t1) cannot be clearly separated from the adjacent normal lung layer (n1) because tumor islets invade, and are frequently found in, the n1 area. We found that this area (t1/n1), which is covered by two ×200 optical fields (4-mm course), constitutes the “real invading front” of the tumor. The n2 area rarely contains cancer islets, but its angiogenic activity may be affected substantially by the invading front. To avoid biases, the CD31- and VEGF-positive MVD was also assessed in 10 lung samples (bronchial and alveolar) from normal individuals. The MVD was also assessed in all of the ×200 optical fields in inner tumor areas (t2 areas). The final MVD in n2, n1, t1, and t2 areas was the mean score obtained from three fields of the highest individual score. Vessels with a clearly defined lumen or well-defined linear vessel shape but not single endothelial cells were taken into account for MVD. The SECD was separately assessed in the same areas.

Assessment of Necrosis. The percentage of optical fields (×200) with necrosis was recorded by two observers (A. G., E. S.) separately. Necrotic areas in more than 50% of the examined fields (mean value of the score given by the observers) was scored as extensive; necrotic areas in less than 50% of the examined fields was scored as limited.

Statistical Analysis. Statistical analysis and graphic presentation were performed using the GraphPad Prism 2.01 package (GraphPad, San Diego CA). Fisher’s exact test of the unpaired two-tailed t test was used for testing relationships between categorical variables as appropriate. Linear regression analysis was used to assess the correlation between continuous variables. Survival curves were plotted using the method of Kaplan-Meier, and the log-rank test was used to determine statistical differences between life tables. A Cox proportional hazard model was used to assess the effects of patient and tumor variables on overall survival. P < 0.05 was considered significant.

RESULTS

Normal Lung Immunostaining. Alveolar epithelium was always negative, whereas bronchiolar and differentiated columnar cells showed persistently positive cytoplasmic reactivity for both VG1 and 11B5 Mabs. Alveolar macrophages were positive. The normal fibroblasts were not stained with the VG1 or 11B5 Mab. Normal endothelium was not stained with the VG1 Mab. Staining of the vasculature in the normal lung adjacent to the tumor was frequently observed with the 11B5 staining. However, not all of the vessels (morphologically identified) stained positively. In normal lung samples, there were stained vessels with the 11B5 occasionally but not with the VG1 Mab. On the contrary, there was a dense vascularization identified with the anti-CD31 staining. Fig. 2, a and b, show normal lung tissue stained with the JC70 and the 11B5 Mabs, respectively.

Cancer Immunostaining. The pattern of cancer cell staining with the 11B5 was granular cytoplasmic (Fig. 2, c and d), similar to the VG1. Immunoreactivity was heterogeneous with no differences between the central and marginal tumor areas. The intensity of tumor staining, when present, was equal or stronger than normal epithelial reactivity. Both squamous cell carcinomas and adenocarcinomas showed a varying degree of VEGF expression. Using the VG1 and the 11B5 Mabs, we found that the mean percentage of cells stained was 54.9 ± 32% (median, 70%; range, 0–100%) and 56.2 ± 32% (median, 60%; range, 0–100%) respectively. Linear regression analysis showed a significant correlation between the two Mabs (P = 0.0001; r = 0.67).

Tumor stromal fibroblasts and macrophages were only occasionally positive with the VG1 Mab. On the contrary, using the 11B5 Mabs, we found that 28 (27%) of 102 cases showed intense fibroblast cytoplasmic reactivity throughout the tumor (Fig. 2d) and 9 of 102 showed focal fibroblast expression.

Serum areas were constantly stained for VG1 and 11B5. Tumor infiltrating lymphocytes and macrophages were frequently but not always stained. The extend of necrosis was not associated with the cancer cell 11B5 staining nor with the fibroblast reactivity (data not shown).

Tumor Endothelium Immunostaining. VG1-positive tumor vesSels were occasionally observed, but MVD assessment was not performed because of the very low number of stained vessels. On the contrary, 11B5-positive vessels were observed mainly in the invading front but also in inner and normal lung areas adjacent to the tumor. MVD assessment was feasible with the 11B5 Mabs. Fig. 2b shows intense staining of the microvesSELs in the tumoral stroma. Of interest, staining of 11B5-positive vessels concerned not only the endothelial

Fig. 1. Schematic representation of the method used for microvesSEL assessment. Optical fields ×200 were used to assess the MVD in the normal lung distant (n2 area) and adjacent (n2 area) to the tumor-invading front, in the tumor-invading front (t1 area), and in inner tumor areas (n2 areas). The n1 area contained sparse foci of cancer tissue within normal lung tissue so that the real invading front was the n2n1 area.
cells but also the myoepithelial component when present. The lumen of the vessels was also stained positively in tumor and adjacent normal lung, probably showing a high VEGF plasma concentration in, and proximal to, the tumoral vessels.

Single endothelial cells, persistently identified (and counted) in CD31 staining, were not stained with the 11B5 Mab, showing that this Mab recognizes sprouting vessels and not migrating endothelial cells.

**MVD Assessment.** Microvessel counting of VEGF/KDR-positive vessels was performed in four different areas (t1, t2, n1, and n2 areas as described in “Materials and Methods”) and in tissue lung samples from normal individuals. Fig. 3a shows the 11B5-positive (VEGF/KDR aMVD) obtained in these areas. The tumor-invading front (t1) and the normal lung adjacent to the invading front (n1) had a very similar aMVD (mean aMVD, 15.4 ± 9 versus 15.06 ± 9; P = 0.79).

Both of these areas had a significantly higher mean aMVD as compared with the aMVD of inner tumor areas (t2; 4 ± 3), of normal lung distant to the tumor (n2; 10 ± 5), and of lung from normal individuals (6 ± 3; P < 0.0001, P < 0.0002, and P < 0.002, respectively). This shows an important up-regulation of the VEGF/KDR angiogenic pathway in the tumor-invading front and the adjacent normal lung (t1 + n1 areas).

Microvessel counting was also performed in the same areas in anti-CD31 stained samples. Fig. 3b shows the CD31-positive sMVD in these areas. The sMVD in the lung from normal individuals, in the normal lung area adjacent (n1), and in the normal lung area distant (n2) to the tumor-invading front had a similar sMVD (66 ± 6 versus 57 ± 14 versus 59 ± 16). On the contrary, this range of sMVD was significantly higher as compared with the sMVD in t1 (30 ± 21; 30 ± 12 versus 30 ± 21; P < 0.002).
P < 0.0001) and in t2 (13 ± 9; P < 0.0001) areas. The t1 area had a significantly higher sMVD as compared with the t2 area (P < 0.0001). These results show that the standard vasculature of normal alveolar lung is significantly more dense than that of the tumoral (either peripheral or inner) one.

SECD that was assessed with the anti-CD31 staining was high in the tumor-invading front and adjacent normal lung but was sharply decreased in inner tumor areas (18.2 ± 4 in n1/t1 area versus 5 ± 2 in t2 area; P < 0.0001). No single-endothelial cells stained positive for 11B5.

The VEGF/KDR Activation Ratio. We assessed the VEGF/KDR activation ratio (Ract), aMVD:sMVD, in all of the measured tissue areas. Fig. 3c shows the Ract in these areas. Both the adjacent normal lung (n1) and the inner tumor areas (t2) had a significantly higher Ract (0.28 ± 0.2 and 0.36 ± 0.3, respectively) as compared with n2 area (0.20 ± 0.1; P < 0.0009) and lung from normal individual (0.09 ± 0.02; P < 0.0001). The invading front (t1) area had the highest Ract (0.58 ± 0.3), which was significantly different from all of the others (P < 0.0001). The Ract in t2 areas was significantly higher (0.36 ± 0.3) as compared with normal tissue (P < 0.0001). This shows that, although the sMVD is two to four times higher in the normal lung, the VEGF/KDR aMVD is up to six times higher in the tumor.

Of 102 analyzed cases, 55 (53%) and 36 (35%) had more than 50 and 90%, respectively, of vessels stained with 11B5 in the invading front. In inner areas, 37 (36%) cases had more than 50% of the vessels stained with 11B5.

VEGF Cancer Cell Expression and MVD. Linear regression analysis showed that 11B5 cancer cell reactivity was not correlated with the sMVD in the invading front (t1 area; P = 0.15; r = 0.14), but there was an association with the sMVD in inner tumor areas (t2 area; P = 0.04; r = 0.20). However, a strong correlation of 11B5 cancer cell expression with aMVD was observed in the invading front and adjacent normal tissue (t1 area: P < 0.0001, r = 0.58; n1 area: P < 0.0001, r = 0.48). A significant, although less strong, correlation was also observed in the n2 (P = 0.04; r = 0.26) and t2 (P = 0.02; r = 0.22) areas. This shows that VEGF expression in cancer cells is directly related to VEGF/KDR angiogenic pathway up-regulation in the invading front and adjacent normal lung, but up-regulation of angiogenesis in the invading front may also occur through VEGF-independent pathways. Nevertheless, inner vascularization (both

Fig. 3. The aMVD (a), the sMVD (b), and the Ract (c) obtained in lung tissue from normal individuals (A), in the normal lung distant (n2 area; B) and adjacent (n2 area; C) to the tumor-invading front, in the tumor-invading front (t1 area; D), and in inner tumor areas (n2 areas; E).
standard and activated) is associated with the VEGF cancer cell expression.

**Stromal Fibroblast 11B5 Staining and MVD.** The 11B5- and CD31-positive MVD in t1 and t2 areas was studied according to the 11B5 immunoreactivity of cancer stroma fibroblasts. Fig. 2e shows strong fibroblast staining with the 11B5 Mab. Positive fibroblast reactivity was associated with high aMVD in both t1 and t2 areas ([19.9 ± 10 versus 13.6 ± 8 (P = 0.002) and 10.6 ± 7 versus 2.2 ± 1 (P < 0.0001), respectively]. On the contrary, fibroblast reactivity was related to high sMVD in t2 areas (17 ± 12 versus 12 ± 7; P = 0.01) but not in t1 area (34 ± 7 versus 30.9 ± 19; P = 0.44). In 9 of 102 cases with low 11B5 cancer cell and stroma reactivity, there was a focal expression in 20–30% of optical fields examined. This focal expression concerned both cancer cell and fibroblasts. Again, the aMVD in these fields was higher as compared with other tissue areas (mean aMVD, 10.6 ± 6 versus 2.5 ± 2; P < 0.0001).

No association of fibroblast staining with necrosis was noted.

**Grouping According to the MVD and R\_act.** The median value was used as a cutoff point to distinguish two groups of low versus high aMVD (median, 13 for the t1 area and 3 for the t2 area). The median R\_act (median, 58% for the t1 area and 36% for the t2 area) was also used to define cases with low or high VEGF/KDR pathway activation. The 33rd and 66th percentile of CD31 MVD was used to define low, medium, and high CD31-positive sMVD (20 and 30, respectively, for the t1 area and 7 and 14, respectively, for the t2 area). This splitting of sMVD in thirds was based on previous studies of ours showing that medium and low MVD have similar prognosis and association with pathological variables (18).

**Histological Correlation of aMVD.** High aMVD in the invading front (t1 area) was associated with node involvement (21 of 50 versus 12 of 52; P = 0.05). No other association of 11B5 expression in cancer cells or of aMVD in the t1 or t2 area with clinicopathological variables (histology, T stage, N stage, histology grade) was noted (data not shown).

**Overall Survival Analysis.** Using the above cutoff points, we performed a univariate analysis of survival. Both the sMVD and the aMVD in the invading front (t1 area) were significant variables of poor prognosis (P = 0.009 and IP < 0.0001, respectively; Fig. 4, a and b. The R\_act in the t1 area was also related to poor prognosis (P = 0.05). A similar analysis using the assessment in the t2 area (inner area) did not show any significant prognostic results. N stage and T stage were also of prognostic importance (P = 0.0004 and 0.05, respectively).

Several multivariate prognostic models were evaluated (Table 1). Among the angiogenesis-related variables (sMVD, aMVD, and R\_act; model 1), only the aMVD had an independent prognostic significance (P = 0.005; t-ratio, 2.88). In a bivariate model including the R\_act and the sMVD (model 2), both variables were significant independent factors, showing that both the number and the activation status of vessels are important. Including T and N stages in two different models, the aMVD (and not the sMVD) remained a significant independent prognostic variable (models 3 and 4).

In a previous study, we showed that, although VEGF expression was associated with high angiogenesis and poor prognosis, about one-half of the cases expressing VEGF were poorly vascularized or had a good prognosis. We, therefore, examined the prognosis of patients stratifying for VEGF cancer cell expression and for aMVD (Fig. 5). Cases with high VEGF expression and intense VEGF/KDR angiogenic pathway up-regulation had a very poor prognosis as compared with patients with low VEGF/KDR angiogenesis, regardless of the VEGF expression status (P < 0.0002). Moreover, up-regulation of VEGF/KDR, even in the presence of low VEGF cancer cell expression, was associated with poor prognosis (P < 0.02). This shows VEGF/KDR angiogenic pathway activation, which does not occur in all VEGF-expressing tumors. In cases with low tumor VEGF expression, VEGF/KDR pathway activation is also associated with poor prognosis, which shows that VEGF/KDR is a much more potent prognostic factor as compared with the VEGF expression.

**DISCUSSION**

VEGF is involved in vascular permeability, endothelial cell migration, and proliferation as well as in vessel maturation (1–3). VEGF exerts its activity on endothelial cells by binding to two VEGF receptor tyrosine kinases, namely KDR (Flk-1) and Flt-1 (6–9). VEGF released by cancer cells up-regulates the expression of these receptors on endothelial cells (12, 13). However, several other factors such as hypoxia and some cytokines directly up-regulate the expression of both VEGF and its receptors (10, 11). Several pathology studies suggest that VEGF receptor mRNA is detected in stromal vessels adjacent to carcinoma and not in benign lesions or normal tissue (19–22). VEGF expression is, therefore, thought to induce a specific VEGF/VEGF-receptor angiogenic pathway that becomes up-regulated, as compared with steady-state activation levels, in malignancy.

In a recent study (16), we showed that VEGF expression was associated with increased angiogenesis in non-small cell lung cancer. However, about one-half of the cases expressing VEGF had a poor vascular density, which showed that VEGF-induced angiogenesis is dependent on other positive and/or negative regulators. This observation suggests that VEGF production by cancer cells does not always...
produce VEGF/VEGF-receptor angiogenic activity. Similarly, expression of VEGF receptors by the tumor endothelium does not necessarily mean a functional VEGF angiogenic pathway. Brekken et al. (15) recently reported the production of novel Mabs that recognize the VEGF bound to its receptor KDR. Conformational changes induce an epitope on the NH₂ terminus absent on free VEGF or KDR molecules but present on VEGF/KDR complex. Indeed, these Mabs seemed to selectively localize in tumoral vessels. Such Mabs may, therefore, be of importance for both diagnosis and therapy, because they would allow a direct assessment of the VEGF/KDR angiogenic pathway. If such a pathway is selectively activated in a tumor, this could become a target of a specific anti-VEGF/KDR therapy.

In the present study, we investigated the patterns of expression of one of these VEGF/KDR-recognizing Mabs, namely the 11B5, in non-small cell lung cancer. The 11B5-staining patterns of cancer cells were quite similar to the patterns obtained with a VEGF (VG1)-recognizing Mab (16). However, unlike VG1, the 11B5 Mab clearly recognizes the tumor vasculature as well as the stromal fibroblasts. Vessels in the invading front and in the normal lung adjacent to the tumor had an intense, although varying, reactivity. We, therefore, assessed the MVD separately in the invading front, in the inner tumor areas, and in the normal lung adjacent to the tumor as well as in lung areas away from the tumor or in lung samples from normal individuals. Anti-CD31 panendothelial staining was used to assess the sMVD, and 11B5-positive MVD would represent the VEGF/KDR aMVD. The sMVD in the normal lung and in the lung adjacent to the cancer was quite similar, whereas it rapidly decreased moving from the invading front to inner tumor areas. In contrast, the aMVD was high in the tumor-invading front and was rapidly extinguished in inner areas. This shows that endothelial cell migration does not contribute to the maintenance of the inner tumor vasculization. Although the sMVD in inner tumor areas was very low as compared with the invading front or to normal lung, the VEGF/KDR activation status was higher than that of the normal lung. These observations strongly support the theory that inner tumor vasculature is undergoing a continuous remodeling as a result of a balance between the active proliferation of the endothelial cells that compose the already formed vasculature and the activity of the apoptosis-related pathways. Normal tissues maintain pathways related to a better survival of their continuously remodeling vasculature, whereas such pathways seem to be less effective within the tumoral tissue.

Stromal fibroblasts in the tumor also showed an intense staining with the 11B5 Mab in about 25% of cases. Up-regulation of VEGF in human fibroblasts is well known to occur after exposure to hypoxia (24), to cytokines such as interleukin 1β (25), or even to hormonal manipulation (26). Although KDR is an endothelial cell-specific receptor, several reports suggest that KDR may also be found in cancer cells (27, 28). To our knowledge, there is no report showing a KDR up-regulation in fibroblasts in pathological conditions. It is, therefore, suggested that fibroblast staining is a result of VEGF overexpression, presumably a response to hypoxia or released cytokines by inflammatory cells. Fibroblast VEGF reactivity was strongly associated with both the sMVD and aMVD in inner tumor areas, which may indicate a role for fibroblasts in the intratumoral angiogenic process. Because VEGF inhibits apoptosis of endothelial cells through KDR activation (29), VEGF of fibroblast origin may influence the remodeling of inner vasculature in a positive direction.

Expression of VEGF has been correlated with increased vascularization and poor prognosis in a variety of human tumors. In a recent study (16), we showed that VEGF expression was associated with poor overall survival in non-small cell lung cancer, which is in accordance with three other published studies (30–32). The degree of sMVD, assessed with various Mabs, has also been shown to be a

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Fig. 5. Kaplan-Meier overall survival curves stratified for both cancer cell VEGF expression and for aMVD.
potent prognostic variable in operable non-small cell lung cancer (18, 33, 34). In the present study, the 11B5 Mab allowed the assessment of the VEGF/KDR-activated sMVD, the prognostic role of which was compared with the CD31-assessed sMVD and the VEGF production by cancer cells. In multivariate analysis, the aMVD was a much more potent independent prognostic variable as compared with the sMVD. The VEGF/KDR activation ratio of vessels in the invading front was also an independent variable as compared with the sMVD. This shows that the degree of vasculature activation is a prognostic variable quite different from MVD.

Survival analysis in the VEGF subgroups showed that activated VEGF/KDR angiogenic pathway, even in tumors with low VEGF expression, is strongly associated with poor prognosis. This once again stresses the importance of research toward assessing intrinsic inhibitory pathways of VEGF angiogenic activity. Platelet factor 4 has again stressed the importance of research toward assessing intrinsic inhibitory pathways of VEGF angiogenic activity. Platelet factor 4 has increased several-fold in both the invading front and the inner areas of tumors as compared with normal lung. Targeting VEGF/KDR-positive vasculature with specific antibodies such as 11B5 may result in a tumor-specific destruction of vasculature. Effective activation of VEGF/KDR angiogenesis is strongly related to poor prognosis and is independent of the sMVD as well as of the levels of VEGF expression by cancer cells. Because highly angiogenic tumors are unlikely to be eradicated with currently used radiotherapy and chemotherapy, the present study urges the evaluation of VEGF/KDR-specific antiangiogenic therapies.

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