Relationship Between Vessel Density and Expression of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in Small Cell Lung Cancer in Vivo and in Vitro

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ABSTRACT
In 21 human small cell lung cancer (SCLC) cell lines, we determined the expression of mRNA and secreted protein levels of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The VEGF expression was highly variable between cell lines, with a >100-fold variation, under identical in vitro conditions. The bFGF expression in cell lines was generally very low. Nine of the cell lines were further analyzed during growth as solid tumor xenografts in nude mice (in vivo). A more uniform VEGF protein expression was present in vivo. Compared with the variable in vitro expression, VEGF was relatively up-regulated in the tumor lines CPH 54A and CPH 54B and down-regulated in GLC 3. One line, DMS 79, had a high VEGF expression in vivo as well as in vitro. The vessel density was determined by Chalkley point counting on CD31 immunostained cryosections of tumors of each of the nine SCLC lines. We found a strong positive correlation between vessel density and tissue VEGF protein expression ($r_s = 0.75$; $P = 0.02$) and a comparatively strong negative correlation ($r_s = -0.80$; $P = 0.01$) between vessel density and tissue bFGF expression. No significant correlation was present between vessel density and in vitro VEGF expression. We conclude that VEGF and bFGF expression is dependent on environmental conditions, as well as cell line-specific factors, and that a strong positive correlation exists between in vivo VEGF expression and vessel density, whereas high tissue levels of bFGF are not correlated with higher vessel densities in SCLC xenografts.

INTRODUCTION
High vessel density in tumor tissue is associated with poor prognosis in many solid tumors, including non-SCLC (1) and breast cancer, where the vessel density estimated by Chalkley counts is an independent prognostic marker (2). VEGF is an endothelial cell-specific mitogen, with a pivotal role in angiogenesis. High pretreatment serum levels of VEGF have been shown to be associated with poor outcome in SCLC (3), as well as non-SCLC (4). bFGF is also angiogenic, but less specific, and it may act synergistically with VEGF in vivo (5).

In the present study, we investigated whether levels of VEGF and bFGF in vitro in a panel of SCLC lines reflected the levels in the corresponding solid tumor xenografts upon inoculation in nude mice. We then examined the relationship between the actual tissue levels of angiogenic factors and the vessel density in the solid tumors. This information is important for the understanding of the effect of endothelial growth factors (angiogenic factors) and their inhibitors in SCLC.

MATERIALS AND METHODS
Cell Lines.

Twenty-one SCLC cell lines were included. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in 175 cm² (650 ml) CELLSTAR culture flasks. The medium contained 10% FCS and no antibiotics, and cells were tested free of Mycoplasma. CPH 54A and CPH 54B, originally established in our laboratory as subclones from the same patient tumor (6), were grown in Eagle’s MEM. DMS tumors, established at Dartmouth Medical School (Hanover, NH; Ref. 7) were grown in Waymouth’s medium, except DMS 79, which were grown in RPMI 1640. The following cell lines were all grown in RPMI 1640: NCI-H69 and NCI-N417 from the National Cancer Institute and Naval Hospital (Bethesda, MD; Ref. 8); 24 H and 86 M1 from the Philipps University (Marburg, Germany; Ref. 9); GLC-2 and GLC-3 (10); GLC-14, GLC-16, and GLC-19 established from the same patient during longitudinal follow-up (11); and GLC-26 and GLC-28, established from one patient. All GLC tumors were established at the University Hospital of Groningen, Groningen, the Netherlands.

Hypoxic conditions were established in anaerobic culture jars using hydrogen- and carbon dioxide-generating envelopes with a palladium catalyst according to the manufacturer’s instructions (BBL GasPak Anaerobic System; Becton Dickinson).

Tumor Xenografts.

Thirty male athymic nude mice (NMRI-nu/nu), 8 weeks of age, obtained from M&B (Ry, Denmark) were used. The mice were kept in laminar air-flow conditions. 

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3 The abbreviations used are: SCLC, small cell lung cancer; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.
Vessel Density and VEGF Expression in SCLC

**Fig. 1** VEGF protein in culture medium from 21 SCLC measured by ELISA. Mean values derived from three independent experiments for each cell line. The VEGF content was measured in doublets for each experiment. Bars, SD. For 54A and 54B, no VEGF could be detected in medium from six independent experiments, including three with cells grown under hypoxic conditions for 24 h.

benches. They received sterile food pellets and water ad libitum. Institutional guidelines for animal welfare and experimental conduct were followed.

Xenografts were established by s.c. injection of cells and maintained by serial transplantation. Prior to transplantation, the mice were anesthetized by a s.c. injection of ketamine (10 mg/kg) and xylazine (1 mg/kg) in 0.9% NaCl solution. Through a 1-cm incision in the dorsal skin, 1-mm³ tumor blocks were s.c. implanted into both flanks. For the experiments described here, passage number seven and higher were used.

**Northern Blotting.** Triplicate cultures of each cell line were harvested in exponential growth phase. Cell lines growing attached to the culture flasks were harvested with a cell scraper (GLC 2, DMS 53, DMS 92, DMS 114, DMS 273, DMS 406, DMS 456, 54A, and 54B). Harvested cells were washed in DEB buffer (150 mM NaCl, 10 mM Na₂-EDTA, and DMS 406, DMS 456, 54A, and 54B). Harvested cells were washed in DEB buffer (150 mM NaCl, 10 mM Na₂-EDTA, and 10 mM Tris-HCl), spun down, frozen in liquid nitrogen, and stored at −80°C. Tumor tissue blocks of ~0.25 g were frozen in liquid nitrogen. Polyadenylated RNA was extracted and transferred to nylon membranes as described previously (12). For the hypoxia study, we used total RNA, isolated from cells by TRIzol Reagent (Life Technologies). Membranes were prehybridized for 3 h in 5% dextran sulfate, 50% formamide, 1% SDS, 1 mM NaCl, and 100 μg/ml salmon sperm DNA at 42°C and hybridized with 32P-labeled cDNA probes overnight at 42°C. The mRNA expression was visualized on a PhosphorImager (STORM 840; Molecular Dynamics Inc.). Glyceraldehyde-3-phosphate dehydrogenase expression was used as loading control in mRNA membranes. The VEGF expression in the hypoxia study was corrected for the 28S expression and quantified by the software program Image Quant version 5.0 (Molecular Dynamics, Inc.). We used cDNA probes encoding VEGF, bFGF, 28S, and glyceraldehyde-3-phosphate dehydrogenase (Clontech).

**ELISA.** Cell culture medium was changed to 20 ml of fresh medium 48 h before harvesting. All media from cell lines were harvested when the cells were in an exponential growth phase. The cell number was counted at the time of harvesting. The medium was briefly spun, a proteinase inhibitor mixture (Complete; Boehringer Mannheim) was added, and medium was frozen in liquid nitrogen.

**Immunohistochemistry.** Tissue slices from tumors of 10 mm in diameter were frozen in cooled isopentane. Frozen sections were fixed in acetone. CD31 immunostaining was performed on sections from four tumors in each group. Sections were washed in PBS and TBS and incubated with 10% rabbit serum for 30 min. They were then incubated with a mixture of two monoclonal rat antimonoclonal CD31 antibodies at a dilution of 20 μg/ml overnight at 4°C. The antibodies used were clone 390 (Serotec, Ltd.) and MEC 13.3 (PharMingen). Rat IgG2a (Serotec, Ltd.) was used as a negative control. Sections were incubated with biotin-conjugated rabbit antirat immunoglobulin (DAKO), at a dilution of 1:600 (2.3 μg/ml) for 30 min, washed, and incubated with alkaline phosphatase-conjugated Streptavidin (DAKO) at a dilution of 1:200 (1.5 μg/ml) for 30 min. As substrate for the alkaline phosphatase reaction, we used freshly prepared Fast Red Substrate System (DAKO), followed by a 10-min wash in tap water. After this procedure, sections were counterstained with hematoxylin and mounted with aqueous mounting media.

**Vessel Density.** Vessel density was recorded as the number of point counts of CD31-positive vessels/field at ×200, viewed through an ocular Chalkley Point Array (Graticules Limited, Tonbridge, United Kingdom). Ten fields/section, randomly selected from nonnecrotic areas of tumors were examined with a Leica DMRB microscope. Vessel density counts were recorded independently by two observers in sections from four tumors of each cell line.

**Statistics.** For evaluation of the relationship between expression of angiogenic factors and vessel density counts, we used the Spearman Rank Correlation Coefficient, rs, which does

**Fig. 2** VEGF mRNA expression in 54A and 54B cell cultures grown under hypoxic conditions for 12, 18, and 24 h, compared with normoxic controls. A 25-fold increase in VEGF mRNA expression in 54 A and a 10-fold increase in 54 B was found.
RESULTS

VEGF and bFGF Expression in Cell Lines in Culture.
The VEGF mRNA expression and the secreted amount of VEGF protein in the growth medium from the 21 SCLC lines (Fig. 1) were highly variable. The values for secreted protein varied from no expression in 54A and 54B to 183 pg/ml/10⁶ cells in DMS 79. The level of bFGF was very low in all cell lines in culture, both at the mRNA level and as secreted protein (results not shown). The mRNA expression agreed with the level of secreted protein in most cell lines. DMS 79, which had the highest level of secreted protein, also had a higher VEGF mRNA expression than all other cell lines. In cell culture of 54A and 54B, we found no VEGF protein in the culture medium, although a weak VEGF mRNA band was found on Northern blots. To test whether the VEGF protein production could be induced, we let the two cell lines grow under hypoxic conditions, because it is well established that hypoxia induces increased VEGF production (13, 14). We found an increased production of VEGF mRNA in both lines (Fig. 2) but still no VEGF protein in the culture medium, although a weak VEGF mRNA band was found on Northern blots. To test whether the VEGF protein production could be induced, we let the two cell lines grow under hypoxic conditions, because it is well established that hypoxia induces increased VEGF production (13, 14). We found an increased production of VEGF mRNA in both lines (Fig. 2) but still no VEGF protein in the culture medium, although a weak VEGF mRNA band was found on Northern blots.

VEGF and bFGF Expression in Xenografts.
On the basis of the VEGF findings in vitro, nine cell lines were selected for further analysis during growth as solid tumor xenografts in nude mice. We selected lines with a broad range of VEGF expression in vitro (Table 1). The level of VEGF protein in tumors was more uniform in xenografts than in cell culture with a 10-fold variation between cell lines with high VEGF protein (DMS 114) and low VEGF protein (GLC 3). Compared with the in vitro expression, VEGF was relatively up-regulated in CPH 54A and CPH 54B and down-regulated in GLC 3. One line, DMS 79, had a high VEGF expression in vivo as well as in vitro.

In contrast to the very low bFGF level in all of the SCLC lines, there was a considerable amount of bFGF in the xenograft tumors. An especially high level of bFGF protein was found in 54B, compared with all other lines (Table 1).

Vessel Density. The vessel density, estimated by Chalkley point counting, varied considerably between the different SCLC lines, whereas different tumors of the same lineage had a very similar vessel density (Table 1, Fig. 3).

Correlation between Vessel Density and Expression of Angiogenic Factors. We found a strong correlation between vessel density and VEGF protein level in xenografts with correlation coefficient $r = 0.75, P = 0.02$ (Fig. 4). No significant correlation was found between vessel density in the solid tumors and VEGF protein in the corresponding culture medium or

Table 1  Expression of angiogenic factors and vessel density in nine SCLC lines

<table>
<thead>
<tr>
<th>SCLC line</th>
<th>VEGF protein cell culture pg/ml/10⁶ cells</th>
<th>bFGF protein cell culture pg/ml/10⁶ cells</th>
<th>VEGF protein tumors pg/250 μg protein</th>
<th>bFGF protein tumors pg/250 μg protein</th>
<th>Vessel density counts/field</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPH 54A</td>
<td>0</td>
<td>0.7 (0.1–1.2)</td>
<td>71 (14–99)</td>
<td>860 (730–1537)</td>
<td>2.5 (2.1–2.8)</td>
</tr>
<tr>
<td>CPH 54B</td>
<td>0</td>
<td>0.1 (0.0–0.1)</td>
<td>59 (21–135)</td>
<td>7454 (5836–8500)</td>
<td>2.3 (2.2–2.5)</td>
</tr>
<tr>
<td>GLC 14</td>
<td>1.8 (1.6–2.1)</td>
<td>0.8 (0.3–0.6)</td>
<td>69 (40–86)</td>
<td>535 (379–688)</td>
<td>4.2 (4.0–4.2)</td>
</tr>
<tr>
<td>GLC 19</td>
<td>3.4 (3.1–3.5)</td>
<td>0.2 (0.1–0.2)</td>
<td>106 (66–143)</td>
<td>325 (198–379)</td>
<td>3.9 (3.6–4.2)</td>
</tr>
<tr>
<td>DMS 153</td>
<td>7.2 (6.4–8.7)</td>
<td>&lt;0.1 (0.0–0.1)</td>
<td>86 (18–245)</td>
<td>279 (87–421)</td>
<td>3.2 (3.0–3.4)</td>
</tr>
<tr>
<td>DMS 456</td>
<td>10.9 (9.0–13.2)</td>
<td>0.2 (0.2–0.2)</td>
<td>73 (19–148)</td>
<td>530 (308–976)</td>
<td>2.8 (2.8–2.9)</td>
</tr>
<tr>
<td>DMS 114</td>
<td>21.8 (19.2–23.4)</td>
<td>&lt;0.1 (0.0–0.1)</td>
<td>272 (230–314)</td>
<td>423 (407–440)</td>
<td>4.6 (4.3–5.0)</td>
</tr>
<tr>
<td>GLC 3</td>
<td>37.7 (37.7–37.8)</td>
<td>0.9 (1.0–0.7)</td>
<td>25 (5–44)</td>
<td>603 (495–702)</td>
<td>2.5 (2.0–2.8)</td>
</tr>
<tr>
<td>DMS 79</td>
<td>183.0 (101–273)</td>
<td>0.1 (0.1–0.1)</td>
<td>198 (58–268)</td>
<td>183 (134–150)</td>
<td>5.4 (5.1–5.9)</td>
</tr>
</tbody>
</table>

Mean values are shown. Numbers in parentheses, range. The range represents three cell culture experiments and four tumors of each cell line.
DISCUSSION

In some tumors, the VEGF protein level was relatively up-regulated in xenograft tumors compared with cell culture, whereas others were relatively down-regulated (Table 1). These relative differences in VEGF production between cell cultures and solid tumors of the same tumor line demonstrate the importance of the microenvironment for VEGF production.

CPH 54A and CPH 54B are subpopulations of the same patient tumor. They represent different metabolic phenotypes (15–17) in vivo and in vitro. When grown as solid tumors, significant differences in blood flow and dispersion kinetics are present (17–19). Both the metabolic and physiological differences between CPH 54A and CPH 54B were found previously to be mainly the result of intrinsic cellular differences (20). Correspondingly, the different radiosensitivities of 54A and 54B are present both in tumors and in cell cultures (21). How such phenotypic features are translated or maintained from the cellular to the organ level is not fully explained. In this regard, it is interesting that when it came to VEGF and bFGF in the present study, there was no such coupling between in vitro and in vivo phenotypes. It is well established that VEGF can be up-regulated by hypoxia, through the induction of the transcription factor hypoxia inducible factor-1 (22), as well as by various growth factors such as epidermal growth factor and transforming growth factor β (23). In some tumor lines, VEGF is constitutively up-regulated (14). In the tumor microenvironment, other cell types, such as stromal cells, inflammatory cells, and platelets, also contribute to the amount of VEGF present, both directly and indirectly, through the stimulatory effect of other growth factors on tumor cell VEGF production.

Despite a significantly increased RNA production during hypoxic stress, there was no detectable VEGF in CPH 54A and CPH 54B in cell culture. This seems to represent an interesting translational defect restricted to VEGF in 54A and 54B, because the two cell lines produce another hypoxia-dependent protein, glucose transporter-1, in response to hypoxia.4 From comparison of the fold increase in VEGF, it appears that hypoxia induces a larger increase in 54A than in 54B. This further adds to an increasing pool of evidence that these two subclones from the same individual genome represent different metabolic and physiological phenotypes, where 54B is predominantly glycolytic and thus less sensitive to changes in the microenvironmental oxygen supply (15–20). In neuroblastoma cell lines, an increase in both secreted VEGF protein and cell-associated VEGF protein has been found upon hypoxic stimulation (24).

Angiogenesis can be induced and modulated by a large number of growth factors, including VEGF, bFGF, angiopoietins, platelet-derived growth factor, and others (25). VEGF is a key factor for angiogenesis in development, and lack of one allele of the VEGF gene causes embryonal death (26). bFGF is not an endothelial cell-specific growth factor such as VEGF; it acts as a growth factor for many different cell types. bFGF levels do not seem to be important for the degree of tumor vascularization in our xenograft SCLC tumors, because there is a negative correlation between bFGF in tumors and the vessel density. The expression of bFGF in cell culture was very low in all SCLC lines, whereas considerable amounts were found in xenograft tumors. Whether this difference is attributable to up-regulation of bFGF production in tumor cells or attributable to bFGF production in stromal cells is not extractable from the current data. The two sister lines CPH 54A and CPH 54B had comparable VEGF levels and vessel density, but the bFGF level

in tumors was significantly different, with a 9-fold higher protein level in 54B than in 54A.

In conclusion, the cellular production in vitro of VEGF in 21 SCLC lines did not reflect the tissue content of VEGF in vivo, nor the vascular density, whereas the actual tissue content of VEGF is significantly and strongly correlated to the vessel density in human SCLC in nude mice.

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