In NSCLC, VEGF-A Response to Hypoxia May Differ between Squamous Cell and Adenocarcinoma Histology

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Abstract. *Aim: To investigate if hypoxia induces vascular endothelial growth factor (VEGF)-A and VEGF-C secretion in non-small cell lung cancer (NSCLC) cells and if the secretion is cell type-dependent. Materials and Methods: Adenocarcinoma (AC) (H522, PAC) and squamous cell carcinoma (SCC) (H520) cell lines were exposed to hypoxia and normoxia. Supernatants were analysed with enzyme-linked immunosorbent assay (ELISA). Tissue microarrays, from 304 patients diagnosed with stage I-IIIA NSCLC, were immunohistochemically-stained and scored for VEGF-A and VEGF-C. Results: In vitro, VEGF-A expression in hypoxic AC cells was significantly higher than that in normoxic cells (H522: p=0.004, PAC; p=0.007). In contrast, hypoxia led to significantly reduced VEGF-A production in the SCC cell line compared to normoxic cells (p=0.005). Conclusion: In vitro, AC and SCC exhibit different VEGF-A responses to hypoxia. Hypoxia mediates a pro-angiogenic response in AC, but apparently not in SCC.*

Lung cancer is the leading cause of cancer death in the Western world (1). The 5-year survival rate for all stages combined is only 11-15%. As a consequence, new therapies are highly warranted.

In lung cancer, as in many other types of solid tumours, hypoxia evolves when malignant cells outgrow their original blood supply (2). To survive in the hostile hypoxic environment, the tumour cells must be able to induce angiogenesis to supply the growing tumour with both oxygen and nutrients (3). In addition, tumour cell survival depends on the removal of CO_2 and other metabolic waste products. To address these metabolic needs tumour cells induce angiogenesis, mainly through secretion of vascular endothelial growth factor-A (VEGF-A). VEGF-A binds to VEGF receptor (VEGFR)-1 and VEGFR-2, on endothelial cells in the surrounding environment, which in turn induces vessel sprouting (3).

Lymphangiogenesis may also give rise to potential routes for the spread of lung cancer cells (4). Tumours are able to induce lymphangiogenesis (5), mainly through VEGF-C and VEGF-D secretion, which in turn activate VEGFR-3, expressed in lymphatic endothelium (6). In addition, VEGF-C can induce angiogenesis after proteolytic processing (7, 8).

Lung cancer is divided into two histopathological subgroups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is further divided into three main subgroups based on histology: adenocarcinoma (AC), squamous cell carcinoma (SCC) and large-cell carcinoma (LCC). Previously, NSCLC was therapeutically regarded as one disease (9). However, after clinical trials with novel chemotherapeutic and molecular-targeted agents there is now a rationale for a histology-based treatment approach (9, 10).

As lung tumours are not composed only of malignant transformed cells, cells of the tumour environment may be equally important as targets for future cancer therapies (11). Fibroblasts, among other tumour stromal cells, have been shown to support angiogenesis and tumour growth, and facilitate metastatic dissemination (12). In addition, fibroblasts are reported to be the main source of VEGF-A secretion in many tumour types (13).

The monoclonal antibody bevacizumab, targeting VEGF-A, has shown dissimilar clinical responses in AC *versus* SCC (9). Moreover, SCC is also known to be more hypoxic and necrotic than AC (14). These observations may imply that AC and SCC have different angiogenic profiles.

In vitro comparison of the angiogenic potential between these two major NSCLC cell types, AC and SCC, has not been previously performed to our knowledge. The aim of this study was thus to investigate if hypoxia-induced VEGF-A and VEGF-C secretion by NSCLC cells is dependent upon histological subtype.

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Table I. *Cell cultures used in the in vitro experiments.*

Cell line	Histology	Origin	Producer
H520	Lung squamous cell carcinoma	Human	ATCC, Manassas, VA, USA
H522	Lung adenocarcinoma	Human	
PAC	Lung adenocarcinoma	Human	In-house
CAF	Lung cancer-associated fibroblasts	Human	

PAC, Primary adenocarcinoma; CAF, cancer-associated fibroblasts.

Materials and Methods

Cell culture (Table I). Cell lines: Human NSCLC cell lines were cultivated in Nunc EasyFlasks™ Nunclon™ Δ (Nalge Nunc International, Rochester, NY, USA) in RPMI-1640 medium supplemented with 10% heat-deactivated fetal bovine serum (FBS), 4 mM L-glutamine and antibiotics. Prior to the experiments, the cultures were kept in humidified incubators at 37˚C with 5% CO_2.

Commercial cell lines: Human NSCLC cell lines H522 (cat.no. NCI-H522 AC) and H520 (cat.no. NCI-H520, SCC) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured as described above. Cell authentication tests and certificates were provided by the manufacturer.

In-house primary cell lines: Human carcinoma-associated fibroblasts (CAFs) and primary adenocarcinoma (PAC) cells were harvested from freshly-resected NSCLC tumour tissues. All patients participated after informed consent, and the study was approved by the Regional Ethical Committee. Tumour biopsies were collected and cut into 1-1.5-mm^3 pieces. Enzymatic digestion was carried out for 1.5 h in 10 ml DMEM/Ham's F-12, containing 0.8 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO, USA). Digested tissue was spun-down, and re-suspended in fresh growth medium (DMEM/Ham's F-12), supplemented with 10% FBS. Pure fibroblast cultures were obtained by selective cell detachment with enzyme-free cell dissociation solution (Millipore, Billerica, MA, USA), and by further cell propagation in the presence of 10% FBS. After serial elimination of fibroblasts, remaining adherent tumour epithelial cell colonies were grown in a specially tailored serum-free medium that favors the growth of epithelial cells. Tumour epithelial cell cultures that tolerated subculturing were established as continuous cell lines. The resulting CAF and tumour cell cultures (PACs) were characterized for purity and cell identity, by flow cytometry, using Fluorescein isothiocyanat (FITC)-conjugated anti-human α-smooth muscle actin (α-SMA) (Abcam, Cambridge, UK), and anti-human pan cytokeratins (Sigma-Aldrich, St. Louis, MO, USA).

Experimental conditions. Subconfluent cells were trypsinized, counted and plated in growth medium in 12-well plates (BD Falcon, Bedford, MA, USA) at the following densities 300,000 cells/well for H520; 200,000 cells/well for H522; 70,000 cells/well for CAF; and 90,000 cells/well for PAC. Three parallels for each cell line were seeded into three separate series (n=9). Prior to each experiment, the cells were seeded onto six different plates, three plates for normoxic (6, 12, 24 h) and three plates for hypoxic conditions (6, 12, 24 h). All plates were initially maintained under normoxic conditions (5% CO_2 and 21% O_2) for 48 h to allow for cell adherence and equilibrium. After 48 h, fresh starvation medium was added (0.5% FBS) and the cells were then exposed to either hypoxia (2.5% O_2) or normoxia (21% O_2). Hypoxia was induced by infusing nitrogen gas in the incubator (Hera Cell150; Thermo Scientific, Waltham, MA, USA). At the end of each incubation period the supernatant was harvested, centrifuged, transferred to new eppendorf tubes and stored at –80˚C prior to analysis.

Table II. *Comparison of VEGF-A and VEGF-C secretion after exposure to normoxia and hypoxia: adenocarcinoma (AC) (H522) versus squamous cell carcinoma (SCC) (H520) and AC (H522) versus primary AC (PAC).*

	Mean pg/ml		*p*-Value	Mean pg/ml		*p*-Value
	H520	H522		H522	PAC	
VEGF-A						
Normoxia						
6 h	208	56	0.0003	56	75	0.15
12 h	297	94	<0.0001	94	104	0.60
24 h	484	148	<0.0001	148	178	0.19
Hypoxia						
6 h	211	84	<0.0001	84	100	0.41
12 h	261	165	0.02	165	138	0.38
24 h	298	283	0.75	283	256	0.51
VEGF-C						
Normoxia						
6 h				285	630	<0.0001
12 h				499	820	0.001
24 h				797	1554	<0.0001
Hypoxia						
6 h				307	569	<0.0001
12 h				564	770	0.12
24 h				645	1022	0.003

ELISA. Cell supernatants were analysed for VEGF-A and VEGF-C concentrations using ELISA (Quantikine, R&D Systems, Abingdon, UK), following the manufacturer's instructions. Assay sensitivity (MDD) was 5,0 pg/ml for VEGF-A and 13,3 pg/ml for VEGF-C. The ELISA measurements were normalised to total protein concentrations using the DC Protein Assay (Bio Rad, Hercules, CA, USA), following the manufacturer's recommendations.

Tissue microarray (TMA) analyses. Patients: Tissue from 304 patients diagnosed with stage I-IIIA NSCLC of SCC or AC histology were used in this study. Patients were diagnosed at the University Hospital of North Norway and Nordland Central Hospital

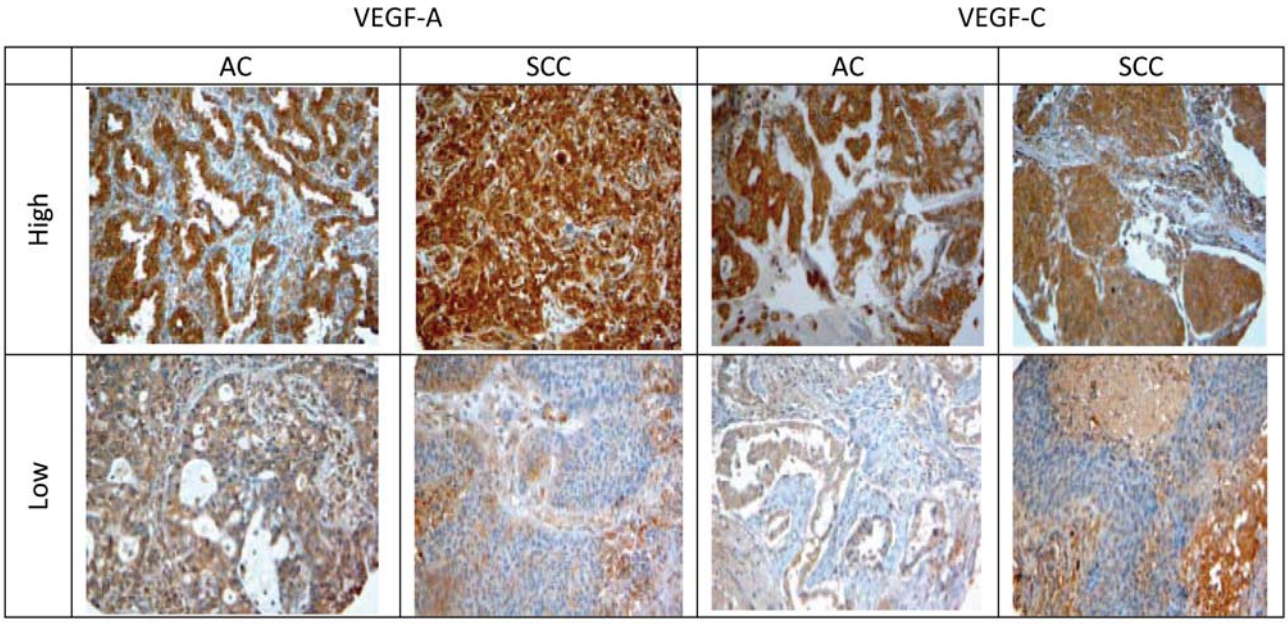

Figure 1. *Immunohistochemical analysis of vascular endothelial growth factor (VEGF)-A and VEGF-C in non-small cell lung cancer (NSCLC). Different cytoplasmic staining intensity scores for tumor cell expression are shown for adenocarcinoma (AC) and squamous cell carcinoma (SCC)(magnification ×200).*

from 1990 to 2004. Their primary tumour tissue samples were collected at surgery, and the complete demographic and clinicopathological data were retrospectively obtained. The tumours were staged according to the Union for International Cancer Control (UICC) TNM classification and histologically-classified according to WHO guidelines (15).

TMA construction: From each surgical specimen, duplicate 0.6-mm core biopsies from the most representative areas of tumour cells were obtained using a tissue arraying instrument (Beecher Instruments, Silver Springs, MD, USA). All collected cores were gathered in eight TMA blocks. The methodological details have been reported previously (16).

Immunohistochemistry. The applied antibodies were subjected to in-house validation by the manufacturer for immunohistochemical analysis on paraffin-embedded material. The antibodies used in this study were VEGF-A (1:10, rabbit polyclonal; RB-1678; Neomarkers, Fremont, CA, USA) and VEGF-C (1:25, rabbit polyclonal; 18-2255; Zymed Laboratories, SF, CA, USA). The detailed methodology has been previously reported (16).

Scoring of immunohistochemistry. Two pathologists scored each core independently by light microscopy. The dominant staining intensity in tumour cells was 0=negative; 1=weak; 2=intermediate; and 3=strong (Figure 1). Only cytoplasmic staining was evaluated. In cases of disagreement, the slides were re-examined and consensus was reached by the observers. Interindividual variability was evaluated in a previous article (16).

Statistical methods. The statistical analyses of TMA data were carried out using the SPSS 16.0 package (SPSS, Chicago, IL, USA). ELISA data are expressed as the mean±s.e.m. The numerical outcome from both groups (normoxia and hypoxia group) was tested statistically with paired-sample *t*-tests. Significance was defined as $p<0.05$.

Results

Patterns of VEGF-A expression during normoxia versus hypoxia. Results from the different NSCLC cell lines under normoxic and hypoxic conditions are presented in Table II and Figure 2. For all cell types, there was a gradual accumulation of VEGF-A over time. The VEGF-A concentration after 24 h of hypoxia ranged between 256 pg/ml and 324 pg/ml for the different cell lines. When compared with normoxic conditions, a significant increase in VEGF-A secretion was observed after 12 and 24 h of hypoxic incubation for H522 cells (12 h, $p=0.04$; 24 h, $p=0.004$), PAC cells (12 h, $p=0.03$; 24 h, $p=0.007$) and CAFs (12 h, $p=0.0001$; 24 h, $p<0.0001$). In contrast, H520 cells had 38.4% lower VEGF-A secretion after 24 h of hypoxic incubation ($p=0.004$).

Measurements at 24 h revealed 1.9-, 1.4- and 2.8-fold increased VEGF-A expression in hypoxic vs. normoxic conditions for H522 cells, PACs and CAFs, respectively.

Comparison of VEGF-A expression between NSCLC cell lines under normoxic and hypoxic conditions (Table II). After exposure to normoxia, the VEGF-A secretion from H520 cells was significantly higher when compared to that of H522 cells (6 h; $p=0.0003$, 12 h; $p<0.0001$ and 24 h; $p<0.0001$). The VEGF-A secretion during hypoxia was significantly lower in

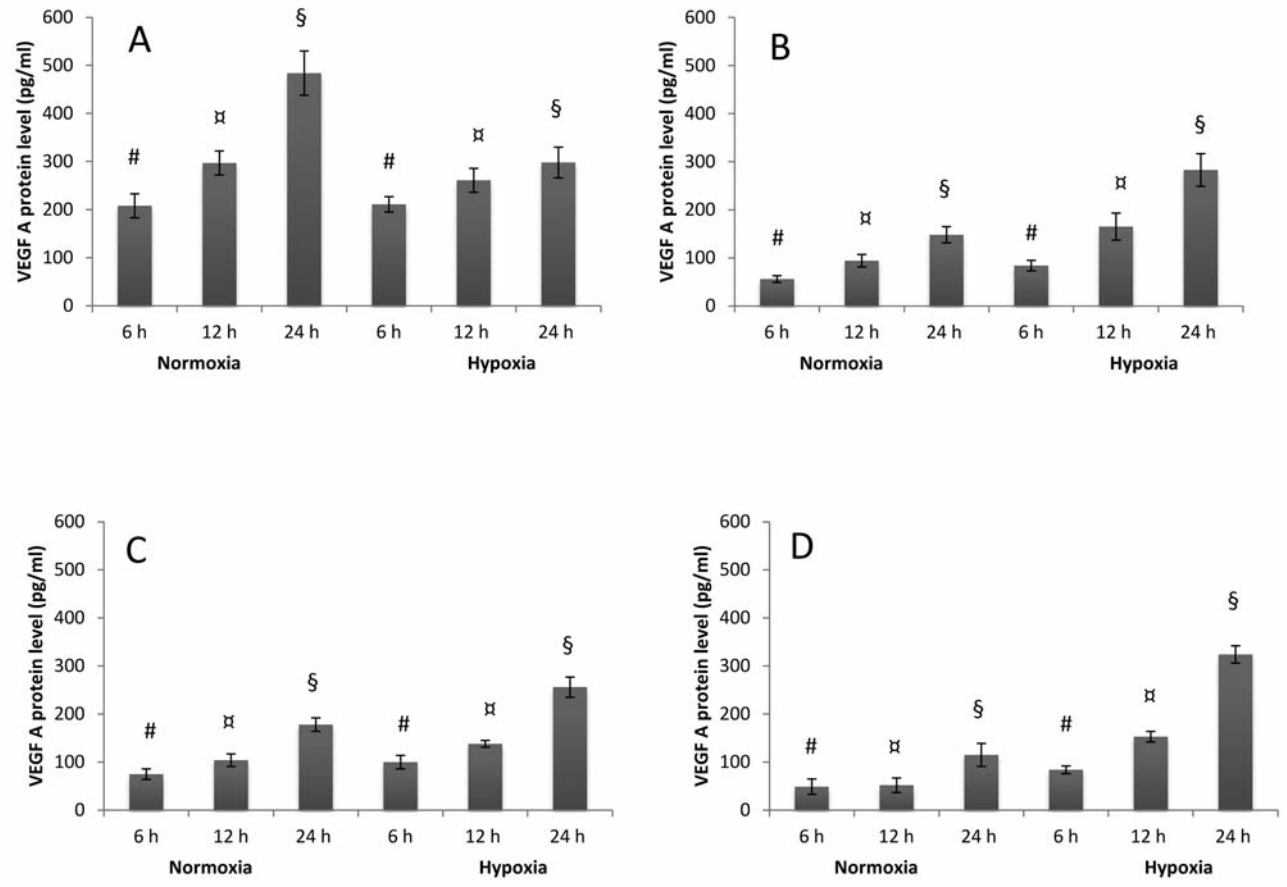

Figure 2. *Determination of VEGF-A concentration after exposure to normoxia and hypoxia after 6, 12 and 24 h. n=9. Concentrations are given as mean values ± s.e.m. A: H520; $^{\#}p=0.91$, $^{¤}p=0.32$, $^{§}p=0.005$ B:H522; $^{\#}p=0.052$, $^{¤}p=0.042$, $^{§}p=0.0041$ C: PAC; $^{\#}p=0.19$, $^{¤}p=0.03$, $^{§}p=0.007$ D:CAF; $^{\#}p=0.08$, $^{¤}$ $p=0.0001$, $^{§}p=0.000005$.*

H522 than H520 cells at 6 h ($p<0.0001$) and 12 h ($p=0.02$), but not at 24 h ($p=0.75$). There was no significant difference in response to normoxia and hypoxia between the in-house AC cell line (PAC) and the commercial AC cell line H522.

Patterns of VEGF-C expression during normoxia versus hypoxia. Results for the different NSCLC cell lines are presented in Figure 3. For H520 cells, VEGF-C secretion was not detectable at any scheduled sampling time. For H522 (AC) and PAC cells there was a gradual increase of VEGF-C secretion over time. CAFs exhibited a gradual accumulation of VEGF-C under normoxic condition, while under hypoxia there was a decrease in secretion from 6 to 12 h, followed by an increase from 12 to 24 h. Between 6 and 12 h of hypoxia there was no significant change in VEGF-C secretion in any of the examined cell lines. After 24 h of hypoxia, H522 cells and CAFs tended to have a lower secretion of VEGF-C compared to that under normoxia, while PAC cells had a significantly lower VEGF-C secretion ($p=0.0002$).

Comparison of the VEGF-C expression between NSCLC cell lines under normoxic and hypoxic conditions. Under normoxic condition there was a significantly higher VEGF-C production by the in-house PACs *versus* the commercial H522 cell line at all times (Table II). During hypoxia we observed the same pattern, except for no difference at 12 h. The total amount of VEGF-C produced by the in-house PACs was generally larger than that for H522 cells. The VEGF-C secretion was, in general, lower after exposure to hypoxia.

VEGF-A and VEGF-C expression in NSCLC tissues comparing AC and SCC histology. Results, including clinicopathological parameters, are presented in Table III. In SCC, 92% of the patients had a high or intermediate expression of VEGF-A, while 8% had low or no expression. In AC, 81% of the patients had a high or intermediate VEGF-A expression, while 19% had a low or no expression. The SCC tumours tended towards a higher VEGF-A expression when compared to AC tissues ($p=0.059$).

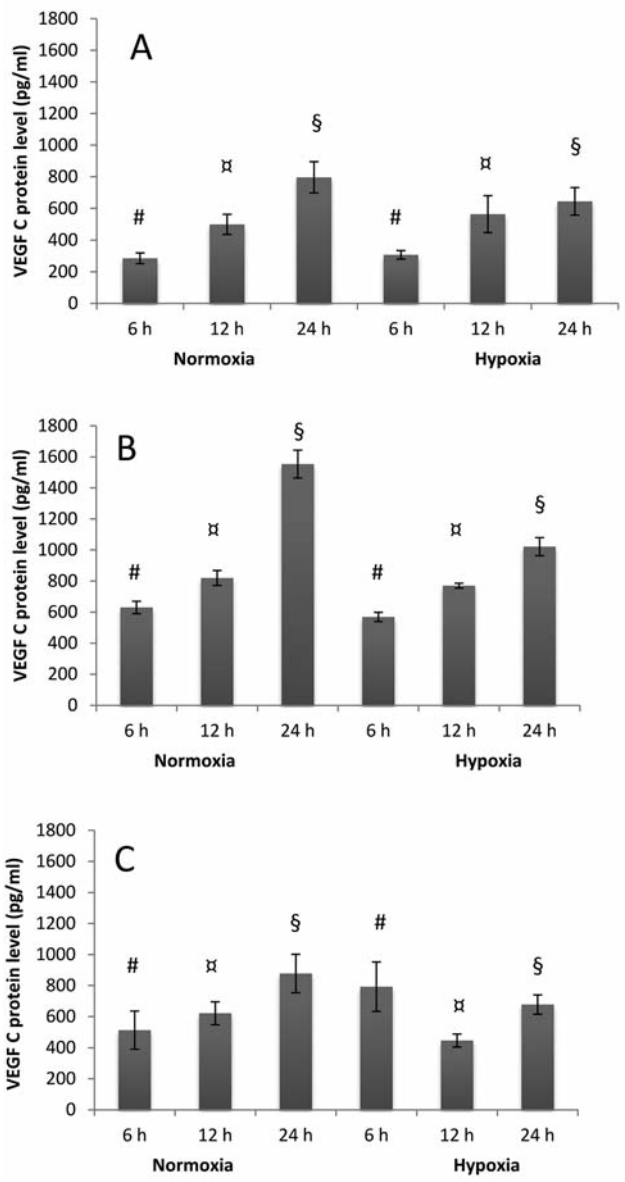

Figure 3. *Determination of VEGF-C concentration after exposure to normoxia and hypoxia after 6, 12 and 24 h. n=9, mean values ± s.e.m. H520; VEGF-C was not with in detectable levels in any of the culture times. A: H522; #p=0.62, ¤p=0.64, §p=0.27 B: PAC: #p=0.24, ¤p=0.35, §p=0.0002 C: CAF; #p=0.19, ¤p=0.06, §p=0.17.*

The VEGF-C expression was high or intermediate in 51% and low or absent in 49% in both SCC and AC (p=0.50).

Discussion

In vitro, it was observed that hypoxia, when compared to normoxia, led to a significantly higher VEGF-A secretion over time in the AC cell lines. In contrast, the SCC cell line secreted less VEGF-A under hypoxic than normoxic conditions. With respect to VEGF-C secretion, hypoxia led to lower secretion in one of the AC cell lines when compared to normoxia. The SCC cell line did not secrete VEGF-C at detectable levels.

A strength of the *in vitro* data is the large number of parallels (n=9). Moreover, our definition of hypoxia at 2.5% O_2 made our *in vitro* set-up similar to *in vivo* O_2 levels in solid tumours (2). Besides the commercial cell lines, we included one in-house AC cell line (PAC) and one in-house carcinoma-associated fibroblast cell line (CAF). A limitation of this study is that only one commercial SCC cell line (H520) was available at the time of experiments. On the other hand, the additional comparison of VEGF-A and VEGF-C expression levels between AC and SCC tumours from a large unselected NSCLC cohort adds strength to this study.

An increase in the VEGF-A production after exposure to hypoxia has previously been reported for different AC cell lines (17-19). To our knowledge, this is the first study of *in vitro* hypoxia-induced VEGF-A expression in SCC. Fukuyama *et al*. studied cytokine production by SCC and AC cell lines (20), but this was performed only under normoxic conditions. Our observation of lower VEGF-A secretion by hypoxic SCC cells was surprising. Is hypoxia not an inducer of VEGF-A secretion in SCC? Does hypoxia lead to a down-regulation of the angiogenic response in SCC? This finding may, in part, explain the observation that SCCs appear to be more hypoxic and necrotic than ACs (14).

Next, we sought to study the VEGF-C secretion after exposure to hypoxia. There are three somewhat contradicting *in vitro* reports on this subject (17, 19, 22). In the most recent publication, Min *et al*. stated that hypoxia can induce VEGF-C production through CCAAT/enhancer binding protein (C/EBP)-δ and hypoxia-inducible factor 1α (22). Simiantonaki *et al*. showed that lung AC cells increase VEGF-C expression after exposure to hypoxia (19), while Enholm *et al*. stated that serum-alone, and not hypoxia, can induce VEGF-C expression (17). The study by Simiantonaki *et al*. had an experimental setup which did not differentiate between serum containing 10% FBS and starvation medium (0.5% FBS). Enholm and co-workers did argue that VEGF-C is regulated mainly by growth factors, which are components of added serum (17). The growth factors implicated in the stimulation of VEGF-C production are platelet-derived growth factor, epidermal growth factor, transforming growth factor-β and the tumour promoter phorbol myristate 12, 13-acetate (23). In hypoxic tumour areas, nutritional delivery may also be impaired because the tumour outgrows its blood supply and increased interstitial fluid pressure compresses existing vessels (24). Therefore, we believe that our experimental set-up, with starvation medium, better parallels the intra-tumoural conditions *in vivo*. Our findings that hypoxia does not increase the VEGF-

Table III. *Prognostic clinicopathological variables as predictors of disease-specific survival for the 113 patients with adenocarcinoma (AC)* and 191 patients with squamous cell carcinoma (SCC) (univariate analyses; log-rank test) and student t-test for equality of VEGF-A and VEGF-C means in AC and SCC‡.*

Characteristics	Patients N (%)		Median survival (months)		5-Year survival (%)		*p*-Value	
	SCC	AC	SCC	AC	SCC	AC	SCC	AC
Age							0.17	0.77
≤65 years	82 (43)	61 (54)	NR	51	62	40		
>65 years	109 (57)	52 (46)	NR	54	69	47		
Gender							0.19	0.21
Female	36 (19)	38 (34)	NR	60	77	53		
Male	155 (81)	75 (66)	NR	43	63	38		
Smoking status							0.75	0.76
Never	6 (3)	8 (7)	NR	18	60	38		
Previous	68 (36)	74 (66)	NR	54	64	40		
Present	117 (31)	31 (27)	NR	48	67	45		
WHO PS							0.51	**<0.001**
0	107 (56)	69 (61)	NR	NR	68	57		
1	74 (39)	39 (35)	127	40	66	22		
2	10 (5)	5 (4)	25	25	46	0		
Weight loss							0.89	**0.031**
<10%	170 (89)	106 (94)	NR	54	66	45		
>10%	21 (11)	7 (6)	NR	18	69	14		
Differentiation							**<0.001**	**0.002**
Poor	66 (35)	41 (36)	42	30	49	30		
Moderate	102 (53)	42 (37)	NR	52	78	36		
Well	23 (12)	30 (27)	NR	NR	62	71		
Surgical procedure							0.11	**<0.001**
Wedge	3 (2)	7 (6)	NR	NR	100	100		
Lobectomy	124 (65)	83 (74)	NR	54	70	45		
Pneumectomy	64 (34)	23 (20)	84	18	57	12		
pStage							**<0.001**	**0.001**
pI	79 (41)	62 (55)	NR	190	83	55		
pII	92 (48)	35 (31)	74	25	57	30		
pIIIA	20 (11)	16 (14)	13	24	33	21		
T-status							**0.001**	**0.023**
1	46 (24)	34 (30)	NR	190	88	61		
2	105 (55)	63 (57)	NR	47	65	38		
3	40 (21)	16 (14)	30	19	44	17		
N-status							**<0.001**	**<0.001**
0	130 (68)	79 (70)	NR	190	73	52		
1	51 (27)	22 (19)	71	21	55	17		
2	10 (5)	12 (11)	13	24	22	24		
Surgical margins							0.30	0.16
Free	171 (90)	106 (94)	NR	54	67	45		
Not free	20 (10)	7 (6)	63	23	53	20		
Vascular infiltration							0.11	**0.022**
No	160 (84)	100 (88)	NR	54	68	46		
Yes	31 (16)	13 (12)	71	22	53	14		
Adjuvant RT							0.11	**0.005**
Yes	36 (19)	96 (85)	127	57	56	48		
No	155 (81)	17 (15)	NR	21	68	17		
Marker expression								
VEGF-A								0.059‡
Negative	0 (0)	2 (2)	NP	18	NP	50		
Low	15 (8)	19 (17)	NR	NR	75	72		
Intermediate	91 (48)	47 (41)	71	54	75	49		
High	84 (44)	45 (40)	NR	44	56	34		
Mean expression	2.17	2.02						
Missing data	1	0						
VEGF-C								0.50‡
Negative	2 (1)	3 (3)	6	NR	50	67		
Low	91 (48)	52 (46)	NR	62	62	53		
Intermediate	77 (40)	39 (34)	NR	47	72	39		
High	21 (11)	19 (17)	70	54	64	37		
Mean expression	1.46	1.51						
Missing data	0	0						

*18 of these patients had bronchioalveolar carcinomas; ‡Student *t*-test for equality of means in different histologies. NP, No patients; NR, not reached; PS, performance status; RT, radiotherapy.

C secretion, and may even reduce it (H522 and CAF), is consistent with previously published data (17).

To further assess whether there is a difference in the VEGF-A and VEGF-C expression between AC and SCC, we used NSCLC tumour tissues (TMAs) and immunohistochemistry to evaluate the VEGF-A and VEGF-C expression. The expression of VEGF-A tended to be higher in SCC than in AC (p=0.059), as intermediate and high expression was seen in 92% and 81% of tumours, respectively. On the other hand, intermediate and high VEGF-C expression levels were similar in AC and SCC subgroups (p=0.50). The tendency for a higher expression of VEGF-A in SCC than in AC tumour tissues is not unanimously consistent with our *in vitro* results. The *in vitro* results indicate a trend towards a higher amount of VEGF-A in AC beyond 24 h of hypoxia when compared with SCC. On the other hand, Pajares *et al*., recently reported that the VEGF-A expression is higher in AC tissues than in SCC (25), which is consistent with our *in vitro* results.

Arinaga and colleagues reported a higher mean VEGF-C expression in AC than SCC tissues from lung, which is consistent with our *in vitro* observation (26). However, we did not observe a difference in VEGF-C expression between our AC and SCC tumour samples, consistent with two other studies (27,28). The explanation for the discrepancy between the *in vitro* and tissue data may be that factors, other than hypoxia induce VEGF-C expression in the more complex *in vivo* environment. Hence, the previously published data support our findings both *in vitro* and in tumour tissues.

Is there a difference in angiogenic potential between ACs and SCCs? VEGF-A is one of the main inducers of angiogenesis (3), and the mean vascular density (MVD) reflects *in situ* angiogenesis. Three former studies on this subject have all reported a higher MVD in ACs than SCCs (29-31). Consequently, it has been postulated that ACs have a higher angiogenic potential than SCCs. In contrast, Lee *et al*. observed that MVD was significantly higher in hypoxia-inducible factor 1α-positive cells of SCC rather than AC type (32).

The observed difference in the VEGF-A and VEGF-C response between AC and SCC *in vitro* is hypothesis-generating and points to a possible difference in the angiogenic response to hypoxia between these two histological subgroups. There is an apparent need for further studies to confirm these findings. If SCC cells, as indicated in our study, down-regulate their VEGF-A expression as a response to hypoxia, hypoxic markers rather than VEGF-A should possibly be targeted in these cells (33). In addition, the significant difference in the VEGF-C response between ACs and SCCs in our study may explain why serum VEGF-C is reported to be a better marker for lymphatic spread in AC (34).

Conclusion

Our *in vitro* results suggest a difference in the angiogenic response to hypoxia between the two major NSCLC histologies. AC, but not SCC, exhibited a VEGF-A response to hypoxia. The lack of VEGF-C production by SCC cells during both normoxia and hypoxia further highlights the diverging responses between the histological entities. The fact that SCC histology appears to be a subpopulation with an inferior response to bevacizumab, when compared to AC (35) may, at least in theory, be related to the difference in angiogenic potential between these two major NSCLC cell types. Further *in vitro* and *in vivo* studies comparing angiogenic indicators in histological subgroups of NSCLC are highly-warranted, as novel anti-angiogenic therapies are entering pre-clinical studies.

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