Angiostatin Expression in Non-Small Cell Lung Cancer

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ABSTRACT
Angiostatin, a potent inhibitor of angiogenesis, tumor growth, and metastasis, was examined in a panel of human lung cancer cell lines with Western blot analysis and in 143 primary non-small cell lung carcinomas with immunohistochemistry. Thirty-four of 143 cases (24%) stained positively. Patients with angiostatin-positive tumors survived longer (146 weeks) than patients with angiostatin-negative tumors (77 weeks; log-rank test: \( P = 0.07 \); rank-sum test: \( P = 0.02 \)). To determine whether combining stimulating and inhibiting factors might improve the prognostic capability, both angiostatin and vascular endothelial growth factor (VEGF) were analyzed together with respect to patient survival. The median survival time of patients with angiostatin-positive/VEGF-negative carcinomas was 184 weeks, whereas the median survival time of patients with angiostatin-negative/VEGF-positive tumors was only 52 weeks. The angiostatin-positive tumors exhibited an increased incidence of apoptosis and a reduced capability to be transplanted into nude mice, but these differences did not reach or were only of borderline statistical significance.

INTRODUCTION
Angiogenesis, the development and formation of new blood vessels, is important in a variety of processes such as growth and differentiation, wound healing, and the formation of neoplasms (1, 2). An avascular tumor grows to the size of 2–3 mm and only when it becomes vascularized does the tumor mass rapidly expand (3, 4). Angiogenesis is a highly regulated process that is the result of an increased production of stimulating factors and a concomitant decrease in the inhibitors of angiogenesis (5). VEGF2 and fibroblast growth factor are the angiogenic factors that are most commonly expressed in tumors (6, 7). Additionally, tumors may activate angiogenic inhibitors such as angiostatin and endostatin.

O’Reilly et al. (8) reported that angiostatin, produced by a primary Lewis lung carcinoma, suppressed the growth of lung metastases. Lannutti et al. (9) administered angiostatin to mice with hemangiendothelioma and found that angiostatin significantly reduced tumor volume and increased survival time when compared with the untreated controls. O’Reilly et al. (10) inhibited the growth of three human and three murine primary carcinomas in mice by systemically administering human angiostatin. They found that the apoptotic index was significantly increased in those tumors treated with angiostatin.

In the present study, we investigated the expression of angiostatin in lung cancer cell lines and the biopsies of lung cancer patients. We compared the expression of angiostatin in NSCLCs with the respective patient’s actual clinical outcome and examined whether the combined determination of VEGF and angiostatin expression in tumor biopsies can yield improved prognostic information for lung cancer patients.

Furthermore, we evaluated the effect of angiostatin expression on the transplantability into nude mice and also analyzed the histological sections of the primary human tumors for apoptosis.

MATERIALS AND METHODS
Cell Lines. The following lung cancer cell lines were used: H69, H128, H146, H209, RPMI-280, LXF-297, A-549, and LUTC-ML54. The cell suspensions were plated at moderate density and allowed to attain a confluence of 30–40%. The cultures were subcultured weekly by Accutase (PAA Laboratories, Linz, Austria). All cancer cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Paisley, Scotland), which contained 10% FCS, penicillin/streptomycin (100 units/ml), and amphotericin (100 units/ml).

Patients and Tumors. One hundred forty-three consecutive patients with previously untreated NSCLCs were admitted into this study. All patients (129 men and 14 women) underwent surgery in the Chest Hospital Heidelberg-Rohrbach. The morphological classification of the carcinomas was conducted according to the WHO specifications. Of the carcinomas, 81 were squamous carcinomas, 38 were adenocarcinomas, and 24 were large cell carcinomas. All patients were staged at the time of their surgery (lobectomy, \( n = 95 \); pneumonectomy, \( n = 28 \); partial resection, \( n = 20 \)) according to the guidelines of the American Joint Committee on Cancer. Forty-five patients had stage I or stage II tumors, and 98 patients had stage IIIA tumors. Ninety-eight patients were treated only by surgical procedures. 13 patients were additionally treated with cytotoxic drugs, and 31 patients were treated with radiation (one case could not be defined precisely). The different treatment procedures had no significant effects on the survival time of the patients in our study. The average age of the patients was 58 years. Follow-up data were obtained from hospital charts and by corresponding with the referring physicians. The survival times were determined from the day of surgery. Only patients who were alive \( 4 \) weeks after surgery (\( n = 134 \)) were included in the survival analysis.
Immunohistochemistry. The previously described biotin-streptavidin method was used to detect the proteins (11, 12). Briefly, formalin-fixed and paraffin-embedded tissues were deparaffinized and pretreated with proteinase K (Boehringer, Mannheim, Germany). After incubation with hydrogen peroxide and protein blocking solution, the primary antibodies were applied for 16 h at 4°C. After incubation with secondary antibodies, the streptavidin biotinylated peroxidase complex Strept AB Complex/HRP (Dako, Denmark) was added, and the peroxidase activity visualized with 3-amino-9-ethylcarbazole. Counterstaining was performed with hematoxylin. Negative and positive controls were conducted. As a further control for specificity, the antibodies were incubated with blocking peptides (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Without having any prior knowledge of each patient’s clinical data, three observers independently evaluated the results from the immunohistochemical staining.

To detect angiostatin, a rabbit polyclonal antibody that was generated by immunizing rabbits with purified angiostatin protein (Ab-1; Oncogene Research Products, Cambridge, MA) was used (dilution, 1:150). Detection of VEGF was carried out using a rabbit polyclonal anti-VEGF antibody (Ab-2, dilution 1:10; Dianova, Hamburg, Germany). To detect caspase-3, we used the mouse monoclonal IgG1 antibody CPP32 p20 (E-8, dilution 1:8, dilution 1:500; Santa Cruz Biotechnology).

Three observers independently evaluated the results from the immunohistochemical staining without any prior knowledge of each patient’s clinical data. The few cases with discrepancies among the investigators were reevaluated and then classified according to the classification given most frequently by the observers. To evaluate the protein expression, the percentages of positive cells and the staining intensity were established. The tumors were classified into three groups: tumors without staining, tumors with weak staining, and tumors with moderate to strong staining. Tumors without staining or weak staining were classified as negative.

Western Blot Analysis. Protein was isolated with the TRI reagent (MRC, Cincinnati, OH). After electrophoresis on a 12% polyacrylamide gel in the presence of SDS and transfer to a polyvinylidene difluoride membrane (DuPont NEN, Boston, MA) by electroblotting, the transferred protein and molecular weight markers were detected with 0.3% Ponceau S. Blocking in 1% blocking solution (Western Blocking Reagent; Boehringer Mannheim, Mannheim, Germany) preceded the 1-h long incubation with the mouse antimannan antibody (clone C9–1; PharMingen, Becton Dickinson, Hamburg, Germany) and the rabbit polyclonal antibody (Ab-1; Oncogene Research Products) diluted in 0.5% blocking solution. Thereafter, peroxidase-conjugated streptavidin secondary antibodies Western-Blotting Kit (Boehringer Mannheim) were used to detect the proteins. All incubations were conducted at room temperature, and several washing steps followed each incubation. Signals were detected with chemiluminescence.

Assessment of Apoptosis. Apoptotic cell death was detected with a nonradioactive 3’ end DNA-labeling technique using the in situ cell death detection kit (Boehringer Mannheim). The procedure was described previously (13). Briefly, the paraffin-embedded specimens were dewaxed and then treated with proteinase K. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide, and the specimens were incubated with the labeling reaction mixture containing fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase. The peroxidase activity was made visible with 3-amino-9-ethylcarbazole. Negative controls were executed by omitting terminal deoxynucleotidyl transferase. DNase-treated specimens were used as positive controls. The apoptotic index was calculated as the ratio of apoptotic cells to the total number of tumor cells.

Xenotransplantation into Nude Mice. Of the 143 lung carcinomas, 106 tumors could be heterotransplanted into nude mice (BALB/c nu/nu, females, 6–10 weeks of age). Animals were maintained by conventional methods in Macrolon cages at 27°C and at 50% humidity. Autoclaved feed and acidified water were provided ad libitum. For transplantation, the tumor specimens were finely minced with scissors and suspended in tissue culture medium (TCM 199). Medium was added to reach a tissue:medium ratio of 1:3 by volume. Three hundred μl of each suspension (>10^7 cells/mouse) were injected s.c. into the flanks of three nude mice each with a 1.4-mm trocar needle. Tumor take was assumed when the presence of growing nodule(s) was noted within 3 months and the tumor histology was confirmed.

Statistical Analysis. Patient survival time was determined from the date of surgery until the last follow-up visit or reported death and was evaluated by using life table analyses according to the method of Kaplan and Meier. Groups were compared by using the log-rank test and rank sum test. The correlations between clinical and molecular parameters were statistically evaluated by using Fisher’s exact test. This test was used as a statistical hypothesis test for the presence or absence
of a relationship between two factors. The results were regarded as statistically significant if \( P \leq 0.05 \).

**RESULTS**

**Expression of Angiostatin.** To confirm the specificity of the angiostatin antibodies used in this study and to test the expression of angiostatin in human lung cancer, two commercially available antibodies were tested by Western blotting in a panel of human lung cancer cell lines. Western blotting using the mouse monoclonal antibody clone C9-1 and the rabbit polyclonal antibody Ab-1 detected protein bands with an estimated molecular mass of 38 kDa in two of four NSCLC cell lines (LUTC-ML54 and A-549), whereas all four SCLC cell lines (H69, H128, H146, and H209) exhibited no reaction (Table 1). Fig. 1 presents the immunoblots of the two positive lung cancer cell lines and of three biopsies of human primary, NSCLCs. Using formalin-fixed, paraffin-embedded specimens, immunostaining was detected only with the antibody Ab-1 but not with the clone C9-1. Therefore, the antibody Ab-1 was used for the analysis of angiostatin expression in NSCLCs.

Expression of angiostatin was found in 34 of 143 NSCLCs (24%). As shown in Table 2, age, gender, histology, stage, lymph node involvement, tumor extent, and surgery procedures had no relationship to angiostatin expression.

**Combination of VEGF and Angiostatin.** To determine whether a combination of stimulating and inhibiting factors can provide improved prognostic information, we analyzed the expression of VEGF in these tumors. To this purpose, we evaluated all possible combinations of VEGF and angiostatin expression and correlated these data with patient survival. Fig. 3 and Table 3 show the survival curves and the median survival times according to the expression of angiostatin and VEGF. The median survival time of patients with angiostatin-negative/VEGF-positive tumors was only 52 weeks. Thus, the equilibrium between angiostatin and VEGF within lung cancer is important in controlling its overall growth. These factors may prove useful in assessing a patient’s prognosis.

**Fig. 2** Survival curves of patients (Kaplan-Meier estimates) with NSCLCs according to the expression of angiostatin (ANG; \( n = 134 \) patients). Log-rank test: \( P = 0.07; \) rank-sum test: \( P = 0.02 \).

**Fig. 3** Survival curves of patients with NSCLCs according to the expression of angiostatin and VEGF (\( n = 143 \) patients). ANG, angiostatin. Log-rank test: \( P = 0.07; \) rank-sum test: \( P = 0.019 \).
significant). \( \text{[62x238]} \)

**DISCUSSION**

Angiogenesis is a highly regulated process that is required for a number of physiological and pathophysiological processes. The progression of solid tumors is regulated by the equilibrium that exists between the stimulators and inhibitors of angiogenesis (5, 8). O’Reilly et al. (8) reported the identification and purification of a fragment of human plasminogen with endothelial inhibitory activity. They named this fragment angiostatin. Furthermore, they demonstrated that a primary mouse tumor can generate angiostatin, which almost completely suppresses the growth of primary tumor metastases (8, 14). It has also been shown that angiostatin inhibits primary tumor growth and induces a regression of primary murine tumors. Furthermore, a shift in the balance of tumor angiogenesis by the gene transfer of a cDNA coding for mouse angiostatin into murine T241 fibrosarcoma cells suppresses primary and metastatic tumor growth in vivo (4). The implantation of stable clones that express mouse angiostatin into mice inhibits primary tumor growth by an average of 77%. Apparently, tumor cells do not express angiostatin molecules per se (4) but rather produce proteases that subsequently cleave circulating plasminogen to generate angiostatin (15).

In the current study, we evaluated the expression of angiostatin in human lung cancer cell lines and biopsies of human NSCLCs. We determined that angiostatin is expressed in human lung carcinomas.

In addition to pertinent clinical data, new risk factors at the molecular and cellular level are the subject of many ongoing studies. Predictive and prognostic factors can serve many purposes. They are used to understand the natural history of cancer, to identify homogeneous patient populations, to characterize subsets of patients with a potentially favorable or unfavorable outcome, to predict the success of therapy, or to generate follow-up strategies. Therefore, we assessed whether the expression of angiostatin could be used as a prognostic factor for patients with NSCLCs. We found that patients with angiostatin-positive lung cancer survived longer than patients with angiostatin-negative tumors. To determine whether a combination of stimulating and inhibiting factors may result in improved prognostic information, we examined all of the possible combinations of angiostatin and VEGF with regard to patient survival. Our results indicate that evaluating both factors together may very well be more important for the prognostic information than the isolated assessment of either. Therefore, our results demonstrate that the balance of angiogenesis-promoting and angiogenesis-inhibiting factors plays a crucial role in the control of tumor growth.

Tumors are complex cell populations in which cellular gain and loss occurs concurrently (16). Apart from proliferation, apoptosis or programmed cell death is one of the most important regulatory mechanisms of cellular homeostasis in organisms. The suppression of angiogenesis by angiostatin results in a characteristic pattern in which apoptosis and tumor cell proliferation create a dynamic equilibrium (5, 14). It has been suggested that angiogenic inhibitors control growth by indirectly increasing apoptosis in tumor cells (14). In our lung cancer study, we detected increased caspase-3 in angiostatin-positive lung carcinomas. The apoptotic indices were also higher in angiostatin-positive tumors, as compared with angiostatin-negative tumors, but these results were not statistically significant.

To study the effect of angiostatin expression on the transplantability into nude mice, we compared the angiostatin ex-

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**Table 3** Median survival times (MST) of patients with non-small cell lung carcinomas according to the expressions of angiostatin (ANG) and VEGF \( (n = 134 \text{ patients})^{a} \)

<table>
<thead>
<tr>
<th>Combinations</th>
<th>Patients/Deaths</th>
<th>MST (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG-positive/VEGF-negative</td>
<td>10/6</td>
<td>184</td>
</tr>
<tr>
<td>ANG-positive/VEGF-positive</td>
<td>21/14</td>
<td>144</td>
</tr>
<tr>
<td>ANG-negative/VEGF-negative</td>
<td>39/25</td>
<td>123</td>
</tr>
<tr>
<td>ANG-negative/VEGF-positive</td>
<td>64/50</td>
<td>52</td>
</tr>
</tbody>
</table>

\( ^{a} \text{Log-rank test: } P = 0.07; \text{rank-sum test: } P = 0.019. \)

**Table 4** Relationship between angiostatin expression and xenotransplantability in nude mice \( (n = 106)^{a} \)

<table>
<thead>
<tr>
<th>Angiostatin</th>
<th>Growth of tumors</th>
<th>Negative</th>
<th>Positive (%)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41</td>
<td>16 (28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>40</td>
<td>9 (18)</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>

\( ^{a} \text{Transplantation was not carried out in all cases because tumor material was not available.} \)

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**Table 5** Relationship between angiostatin expression and caspase-3 expression \( (n = 113)^{a} \)

<table>
<thead>
<tr>
<th>Angiostatin</th>
<th>Caspase-3</th>
<th>Negative</th>
<th>Positive</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>28</td>
<td>3 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>61</td>
<td>21 (38)</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

\( ^{a} \text{Caspase-3 was not determined in all cases because tumor material was not available.} \)
pression in the primary tumors with the take rate into nude mice. We found that the expression of angiostatin is associated with the growth of human lung cancer after heterotransplantation into nude mice. It was discovered that the take rate of human angiostatin-positive lung carcinomas was reduced compared with the take rate of angiostatin-negative carcinomas; however, this result does not reach statistical significance.

In conclusion, angiostatin is expressed in a subgroup of human NSCLCs. These tumors are characterized by an elevated incidence of apoptosis and a reduced transplantability into nude mice, and the presence of angiostatin in primary lung tumors is associated with longer patient survival.

The discovery of specific endothelial inhibitors such as angiostatin not only increases our understanding of the function of these molecules but also provides an important strategy for cancer treatment. Recent studies have demonstrated that application of angiostatin significantly suppresses the growth of a variety of mouse tumors (9, 10, 17). However, the dosages of angiostatin used in these animal studies is apparently too high for use in clinical trials (18). Additionally, long-term treatment with angiostatin is required to achieve an antitumor effect. Nevertheless, antiangiogenic substances are another promising tool in anticancer therapy.

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