Platelet-Derived Growth Factor-AA Is an Essential and Autocrine Regulator of Vascular Endothelial Growth Factor Expression in Non–Small Cell Lung Carcinomas

Yasunori Shikada,1,2 Yoshikazu Yonemitsu,1 Takaomi Koga,1 Mitsuhiro Onimaru,1 Toshiaki Nakano,1 Shinji Okano,1 Shihoko Sata,1 Kazunori Nakagawa,1 Ichiro Yoshino,1 Yoshihiko Maehara,2 and Katsuo Sueishi1

1Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
2Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract
It is widely accepted that angiogenesis is required for tumor progression. Vascular endothelial growth factor (VEGF) is a key molecule for tumor angiogenesis; however, its expression regulation is not well understood during all stages of tumorigenesis. Using cell lines and surgical specimens of human non–small cell lung cancers (NSCLCs), we here show that platelet-derived growth factor-AA (PDGF-AA) is an essential autocrine regulator for VEGF expression. To directly assess the expression of PDGF-AA–dependent VEGF and its roles in tumorigenesis, we stably transfected established cell lines with their antisense genes. In addition, the levels of PDGF-AA and VEGF expression in surgical sections were measured and compared with clinicopathologic findings such as tumor size and patient prognosis. PDGF-AA tightly regulated VEGF expression and had a greater effect on tumor size and patient prognosis than did VEGF in both cell lines and surgical sections. PDGF-AA expression was not seen in the atypical adenomatous hyperplasia at all, whereas VEGF was occasionally seen. Furthermore, the frequency of VEGF expression was higher in advanced NSCLCs than in precancerous lesions, which was tightly correspondent to the results for PDGF-AA. These results indicate that PDGF-AA is an important regulator of the frequency and level of VEGF expression during the transition from a precancerous lesion to advanced cancer. The PDGF-AA/VEGF axis, therefore, may be a ubiquitous autocrine system for enhancing angiogenic signals, and PDGF-AA, and its related pathways could be a more efficient target of antiangiogenic therapy for cancers than VEGF and its pathways. (Cancer Res 2005; 65(16): 7241-8)

Introduction
Angiogenesis is required for tumor progression, as supported by a number of studies showing a reduction in tumor growth by antiangiogenic agents (1–3). Folkman et al. proposed the concept of an "angiogenic switch" that is required for the progression to advanced cancer from an occult tumor (4). It is now widely accepted that the angiogenic switch is "off" when the effect of proangiogenic molecules is silent and "on" when the net balance is tipped in favor of angiogenesis (5, 6). Relatively less attention, however, has been paid to defining which molecule is the angiogenic switch or how it acts.

According to the original proposal (1), a molecule must have the following characteristics before it can be considered as a possible angiogenic switch: (a) it must be expressed specifically in advanced cancer but not in precancerous or early cancerous lesions of <2 mm3; and (b) it must determine the size, and probably the malignant potentials, of cancers by controlling the expression of angiogenic growth factors. In the last decade, various signals that trigger tumor angiogenesis, including angiogenic factors, have been identified (7), and among these, vascular endothelial growth factor-A (VEGF-A, commonly called VEGF) has been recognized as one of the most potent mediators of tumor angiogenesis (8). A logical question followed: is VEGF an angiogenic switch for tumors? Unfortunately, this is not likely, because VEGF is abundantly expressed in cancers, precancerous lesions, and their originating tissue, although VEGF is essential for tumor angiogenesis and its frequency of expression is higher in advanced malignancies than in noncancerous tissue (9, 10). These theoretical considerations suggest the existence of upstream regulator(s) that mediate the expression of VEGF in tumors as molecular candidates for the angiogenic switch; however, the regulatory mechanism of the expression of VEGF in each tumor has not been fully elucidated.

Several important studies have indicated that some oncogenes determining the malignant potentials of cancer, including Ras (11) and HER-2/neu (12), up-regulate VEGF expression, suggesting that the "angiogenic switch" might contain multiple molecules and signal transduction pathways. Furthermore, knowledge of important related mechanisms, such as that of the extracellular regulatory system for enhancing the expression of VEGF, is also limited.

Recently, we showed that the platelet-derived growth factor-AA (PDGF-AA)/PDGFα-receptor (PDGFRα)/p70S6K pathway in mesenchymal cells (fibroblasts and vascular smooth muscle cells) was essential for therapeutic and tumor angiogenesis in vivo (13). We also showed that PDGF-AA, which does not target the vascular endothelial cells, dominantly regulates the expression of VEGF, essentially contributing to the angiogenic process in vivo (13).

A previous study revealed that the expression of PDGF-AA is strictly limited in mesenchymal cells but not in cells of epithelial lineage, because of the methylation of GC-rich sequences of the PDGF-A promoter in epithelial cells (14). Demethylation can result in dysregulated expression of multiple genes and is now
suggested to be an essential step for carcinogenesis (15, 16). These facts may support the hypothesis that a gain-of-function in cancers causes the dysregulated expression of PDGF-AA, which in turn causes the enhanced expression of VEGF by an autocrine mechanism, a system would be similar to that observed in mesenchymal cells. Using established cell lines and surgical specimens of non–small cell lung cancers (NSCLCs), which are a major cause of death in Western countries and Japan, we here show that PDGF-AA is a critical autocrine regulator for VEGF, which may be involved in the “angiogenic switch”–related pathways in malignancies.

Materials and Methods

Cells. The cancer cell lines, QG56 (human lung squamous cell carcinoma), A549 (human lung adenocarcinoma), and SAS and TF (human oral squamous cell carcinoma), were maintained in continuous culture in RPMI 1640 supplemented with 100 units/mL penicillin/streptomycin and 10% FCS. These cell lines were washed, and cell pellets were collected and then frozen in liquid nitrogen.

Reverse transcriptional-PCR. The gene expression of full-length PDGF-A was determined from MRC5 cells (a human fibroblast cell line). Total RNA of cultured cells growing exponentially was extracted using ISOGEN (Nippon Gene Inc., Toyama, Japan) according to the manufacturer's protocol. Total RNA of cultured tissue was isolated using the same method. Synthesis of cDNA was done with 5.0 μg total RNA using a first-strand cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA).

Construction of AS-PDGF-A/AS-VEGF plasmid. Primers incorporating the EcoRI site for PCR of PDGF-A were as follows: forward 5'-aGATCCACGAGGTCGATCTGGTATTTTACTTATTACTTTTTTTTTTTTACACATC-3' and reverse 5'-GAGGATCCTGTCATGATCTGGTTATTTTACTTATTACTTTTTTTTTTTTACACATC-3'. PCR amplicons were directly inserted into a plasmid vector (PCR II) by the TA cloning method according to the manufacturer's protocol (Invitrogen). The whole sequence was determined to be completely matched to a reported sequence (Genbank accession no. X03795). The amplicons were abstracted using an EcoRI restriction enzyme and then inserted into the vector for mammalian cell expression, pcDNA3.1(+)-I (Invitrogen).

The human VEGF165 cDNA expression plasmid vector was previously obtained (17). The AS-VEGFgene expression plasmid based on pcDNA3.1(+)-I was constructed as described above.

Establishment of stable transfomant. Stable transfections were done using LipofectAMINE 2000 reagent (LF2000; Invitrogen) according to the manufacturer's instructions. Briefly, logarithmically growing cells were transfected with plasmid-LF2000 complex at a 1:2 weight ratio. Forty-eight hours later, the selective medium containing 0.5 mg/mL geneticin antibiotics (G418; Promega Corp., Madison, WI) was replaced, and the cell clones were selected and identified. Next, the transfected cells were spread onto 96-multwell plates and cultured to confluence, and the cell clones that reduced PDGF-AA or VEGF expression were selected using the ELISA kit included in the Human PDGF-AAImmunooassay System and the ELISA kit included in the Human VEGFIImmunooassay System, which detect VEGF121 and VEGF165 (both from R&D Systems, Inc., Minneapolis, MN).

The cell cloning was done thrice, and the cell clone showing the lowest PDGF-AA or VEGF expression in each cell line was used for experiments.

Immunoprecipitation. For immunoprecipitation, cells were lysed with 500 μL of cell lysis buffer (Promega) containing a cocktail of protease inhibitors (1.5 mM/liter, oleptatin, 4 mM/liter, leupeptin, 0.01 mM/liter, aprotinin, and 50 mM/liter, phenylmethylsulfonyl fluoride), and the supernatant of the lysed cells was recovered for immunoprecipitation. The amount of protein was determined with a Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA), and aliquots of 500 μg of proteins were used for immunoprecipitation.

Non-specific proteins bound to magnetic beads were eliminated by exposure to protein-G magnetic beads (Pharmacia Biotech AB, Uppsala, Sweden). For immunoprecipitation, 3 μg of anti-human PDGFRA antibody (R&D Systems) was added to the tube, bead pellets were heat treated in 20 mL mercaptoethanol sample buffer, and the samples were loaded onto 10% SDS-polyacrylamide gel. After transfer to the nitrocellulose membrane, immunoprecipitated proteins were determined by immunoblotting with polyclonal anti-phosphorylated-PDGFRA antibody (p-PDGFRα; Tyr720, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Tissue samples. For immunohistochemistry, tissue samples were derived from 128 Japanese patients with NSCLC who underwent surgical resection at the Department of Surgery, Kyushu University Hospital (Fukuoka, Japan) between January 1990 and April 1995 for real-time quantitative PCR, samples were taken from 60 Japanese patients with NSCLC who underwent surgical resection in the same department between January 1996 and April 2000. No patient had received any antitumor treatment, including anticancer drugs or radiation therapy, before surgery.

Tumor and adjacent normal lung tissue samples were freshly obtained from resected lobes, immediately frozen in liquid nitrogen, and stored at –80 °C for real-time PCR and reverse transcription-PCR (RT-PCR), or embedded in paraffin for immunohistochemistry after formalin fixation. All patients gave their informed consent before the tissue sampling.

Real-time quantitative PCR. Gene expression was measured using the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Corp., Foster City, CA). The primer sequences were as follows: human PDGF-A, 636 bp, forward 5'-TCCACGCCACCTAAGCATT2-3'; reverse 5'-TCACTTGCTACCC- TGAAGAAT-3'; probe 5'-FAM-CTCGAGACGACGAGCTTACCATGATCT-3'; probe 5'-FAM-ACTGCCACATTGAGACCTGTT- TAMRA-3'.

The coefficient of correlation was r = 0.97, and the slope was constant in each experiment. As internal controls, the same samples were tested for 18s rRNA (Perkin-Elmer) in the same manner. Each sample was analyzed in duplicate.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections (diameter, 5 mm) were reacted overnight at 4 °C with goat anti-PDGF-A antibody (1:65 in PBS, R&D Systems) or mouse anti-VEGF antibody (1:200 in PBS, R&D Systems) as primary antibodies. Isotype-matched nonimmune antibodies were used for negative controls. The rinsed sections were subjected to peroxidase-labeled secondary antibody (1:250 in PBS) at room temperature for 30 minutes. PDGF-A or VEGF protein was visualized using diaminobenzidine, and nuclei were counterstained with hematoxylin. A positive reaction was defined as immunohistochemical positivity in cells that showed a stronger reaction than that seen in arterial vascular smooth muscle cells in the same specimen, and in at least 30% of tumor cells at a ×200 high-power field for five randomly selected areas. All sections were examined by two independent investigators (Y.S. and T.K.) who were blinded to the clinical data.

For evaluation of angiogenesis, the dehydrated slides were treated with 40 mg/mL of proteinase K (DAKO, Carpinteria, CA) for 10 minutes at room temperature. After washing in PBS, they were treated with 3% H2O2 and then 5% nonfat dry milk, and incubated with von Willebrand factor (vWF, 1:800; DAKO) overnight at 4 °C. The following procedures and visualization were done using the same methods described above. The microvessels in the tumor and adjacent mesenchyma, which was marginated at 2-mm distant from the tumor periphery, were labeled with vWF under light microscopy.

Animals. Male BALB/c nu/nu mice (5 weeks old) were from Kyudo Co., Ltd. (Tosu, Saga, Japan). All animal experiments were done under approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animal, Recombinant DNA, and Infectious Pathogen Experiments at Kyushu University and according to the law (no. 105) and notification (no. 6) of the Japanese Government.

Tumor implantation model. With mice under sufficient anesthesia by an i.p. injection of sodium pentobarbital, 1 × 106 tumor cells were injected s.c. into the abdominal region. Measurement of the tumor was done thrice per week. Tumor volumes were estimated by the formula V = π/6 × a2 × b, where a was the short and b was the long axis (11).
Statistical analysis. Results are presented as the mean ± SE. Differences between groups were determined by one-way ANOVA followed by an unpaired Student’s t test with Bonferroni correction for multiple comparisons. The correlation between PDGF-A and VEGF was analyzed using Spearman’s correlation analysis and χ²-statistic analysis. Survival curves were plotted using the Kaplan-Meier’s analysis, and the log-rank test was used to determine the statistical differences between life curves. The relationship between the expression of PDGF-AA and clinicopathologic factors was examined using the Mann-Whitney U test and Fisher’s exact test, and a positive ratio with respect to tumor size was examined using the Cochran-Armitage linear trend test. The relationship between the microvessel count was also examined using the Mann-Whitney U test. *P < 0.05 was considered significant.

Results

Platelet-derived Growth Factor-AA Is an Autocrine Regulator of Vascular Endothelial Growth Factor Expression in Human Non–Small Cell Lung Cancers

Established cell lines. To obtain direct evidence that PDGF-AA is an autocrine regulator for VEGF in human NSCLCs, we first established independent cell lines [i.e., QG56 (human lung squamous cell carcinoma) and A549 (human lung adenocarcinoma)] which were stably transfected with plasmid pcDNA3.1 (+) expressing full-length antisense human PDGF-A cDNA (AS-PDGFA). Control lines transfected with an empty vector or full-length...
antisense human VEGF165 cDNA (AS-VEGF) were simultaneously established. In addition, two other cell lines, SAS and TF (human oral squamous cell carcinoma lines), were also included to confirm the findings. These cells were maintained in G418, and single-cell cloning was done thrice using 96-well plates. The clone that showed the lowest secretion of the target protein in each cell line was used for the following experiments.

A preliminary experiment showed high expressions of PDGF-AA and VEGF in all cell lines subjected to the ELISA (data not shown). Using murine fibroblasts (NIH3T3), we next examined whether AS-PDGFA might be cross-reactive for human VEGF expression, because the nucleotide sequence of PDGF-A showed ~30% homology to that of VEGF. AS-PDGFA transfection did not affect the protein secretion level from cotransfected sense human VEGF165 expression vector [pcDNA3.1(+)/hVEGF165] in the culture medium of NIH3T3 (Fig. 1A), indicating that AS-PDGFA did not seriously affect the expression of human VEGF.

All cell lines expressed the known receptor of PDGF-AA, PDGFRα, which was detected by RT-PCR (data not shown). This was also confirmed by immunoprecipitation to PDGFRα, and as expected, the phosphorylation of PDGFRα seemed lower in cells that were stably transfected with AS-PDGFA than in those transfected with an empty vector in repeated experiments; this finding was later confirmed by densitometry (Fig. 1B).

Furthermore, all cell clones stably transfected with AS-PDGFA showed significantly reduced PDGF-AA secretion, as well as reduced VEGF. On the other hand, transfection of AS-VEGF contributed only to the reduction of VEGF and not to the expression of PDGF-AA (Fig. 1C), indicating that PDGF-AA is an autocrine regulator for the expression of VEGF in these cell lines.

Surgical specimens. For further evidence regarding the role of PDGF-AA in VEGF expression, we did real-time quantitative RT-PCR to test the correlation of mRNA levels between PDGF-A chain and VEGF in surgically resected fresh NSCLC samples from 60 patients (adenocarcinoma, n = 32; squamous cell carcinoma, n = 20; others, n = 8). The expressions of PDGF-A mRNA and VEGF mRNA in the tumor were tightly correlated (Spearman's correlation test, \( r = 0.838, P < 0.001 \)), and there was a similar finding in adjacent normal lung tissue (Spearman's correlation test, \( r = 0.705, P < 0.001 \); Fig. 2A), suggesting that similar to our previous findings in noncancerous mesenchymal cells, the expression of PDGF-A was correlated to that of VEGF in NSCLCs.

Further retrospective analysis by immunohistochemistry using 128 formalin-fixed tissue sections (adenocarcinoma, n = 68; squamous cell carcinoma, n = 55; others, n = 5) also supported these findings; the immunohistochemically positive reaction of PDGF-AA was tightly correlated with that of VEGF (Fig. 2B and C; \( \chi^2 \)-statistic analysis, \( \chi = 0.325; P = 0.0002 \)).
Significance of Platelet-Derived Growth Factor-AA and Vascular Endothelial Growth Factor Expression in Human Non–Small Cell Lung Carcinomas

Established cell lines. We next returned to animal studies to examine the tumorigenesis in established cell lines. As a first step, we examined the proliferative activities of each cell transfected with an empty plasmid, antisense VEGF (AS-VEGF), or antisense PDGF-A (AS-PDGFA) were spread on dish plate, and the number of tumor cells were counted at days 3, 5, and 7. No significant difference on the tumor proliferation was found in all groups. Right, in vivo. In turn, these tumor cells were s.c. injected into the abdominal wall, and the tumor growth was measured at each time point. Points, mean; bars, ± SE. Differences between the groups were compared using one-way ANOVA followed by an unpaired Student’s t test. *, P < 0.01.

Reduction of VEGF expression resulted in mildly to moderately disturbed tumorigenesis in \( \text{nu/nu} \) mice in all four cell lines tested, as expected. On the other hand, stable transfection of AS-PDGFA caused considerably and significantly disturbed tumor growth in three of four cell lines, compared with those with an empty vector or AS-VEGF (Fig. 3, in vivo experiment). These results indicate that tumor growth is more dependent on the expression of PDGF-AA than that of VEGF.

We next assessed the tumor-induced angiogenesis in vivo, visualized by labeling with vWF, using tumor sections obtained at 28 days after tumor implantation (Fig. 4A, arrows, and B). None of the sections from QG56 tumor transfected with AS-PDGFA showed any histopathologic evidence of solid tumor; thus, these sections were excluded from the evaluation. In all tumor types, the number of intratumor microvessels was significantly decreased in the AS-VEGF group and even more significantly decreased in the AS-PDGFA group (Fig. 4 B, intratumor). Similar findings were also observed for angiogenesis at the tumor periphery of SAS and TF tumors, but the effect was rather mild in A549 tumors (Fig. 4B, peripheral), probably reflecting the milder antitumor effect of antisense gene expression seen in Fig. 3.

Surgical specimens. Using 128 tissue sections immunohistochemically labeled for PDGF-AA (93 positive cases and 35 negative cases), we investigated the clinicopathologic role of PDGF-AA expression in NSCLCs. Among the clinicopathologic variables, only the tumor diameter was significantly larger in PDGF-AA-positive cases than in PDGF-AA-negative cases (Supplementary Table S1). Furthermore, as tumor size increased, the PDGF-AA-positive ratio was raised significantly (Cochran-Armitage test for linear trend, \( P < 0.0001 \)), as was the VEGF-positive ratio (Cochran-Armitage test for linear trend, \( P < 0.0001 \)).
Table 1). Interestingly, all tissue sections of noninvasive adenocarcinoma, bronchioalveolar carcinoma (n = 7; all cases were included in 20 ≤ φ < 30), and high-grade atypical adenomatous hyperplasia (AAH, n = 13), which are now considered to be precancerous lesions (18, 19), were negative for PDGF-AA. The seven cases of bronchioalveolar carcinoma included three VEGF-positive cases (42.9%). These results thus indicate that the expression of PDGF-AA may be essentially related to the size and progression of tumors.

Finally, a comparison of the 5-year survival rates of patients with NSCLCs who were VEGF positive (81 cases) or negative (47 cases) did not show a significant difference between the two groups (Fig. 5A; 38.6%, confidence interval [CI] = 15.6 versus 43.6%, CI = 11.6, respectively), whereas that of patients who were PDGF-A positive was significantly lower than that of those with a negative reaction (P < 0.05; Fig. 5B; 36.1%, CI = 17.8 versus 56.5%, CI = 11.0, respectively).

**Discussion**

The key observations obtained in the present study were as follows: (a) similar to our earlier observations in noncancerous mesenchymal cells, PDGF-AA was found to be an autocrine regulator for VEGF in NSCLCs, indicating that the PDGF-AA/VEGF axis may be a ubiquitous autocrine system for enhancing angiogenic signals; (b) the expression level of PDGF-AA was more critical for experimental tumor growth than that of VEGF in vivo, and the expression of PDGF-AA was rarely seen in precancerous or early cancer lesions of surgical sections; and (c) PDGF-AA expression was a prognostic indicator for individuals with NSCLCs. These results strongly suggest that PDGF-AA and its related pathways may be a more efficient target of antiangiogenic therapy for cancers than VEGF and its related pathways.

Recent studies have identified various signals related to tumor angiogenesis, including metabolic and/or mechanical stress, immune/inflammatory response, and factors that genetically
activate oncogenes and control the production of angiogenic regulators such as VEGF, angiopoietins, fibroblast growth factors, hepatocyte growth factor, etc. (7). There is no longer doubt that these signals contribute to tumor progression; however, relatively less attention has been paid to the critical question of which molecules and signal transduction pathways are critically involved in the angiogenic switch and how they function.

VEGF is an essential mediator for tumor angiogenesis, and that conclusion has been supported by the findings of a number of experimental and clinical studies, including promising early results of a humanized anti-VEGF antibody, Avastin (20–22). VEGF, however, is not likely to meet the definition of an angiogenic switch, and this assertion was supported by the present finding that AAH occasionally expressed VEGF (Table 1). Therefore, we should be able to identify an upstream regulator that controls the expression of VEGF in tumors. In the current study, we identified PDGF-AA as a possible candidate for one of the molecules involved in the angiogenic switch that meets the definition noted above, and the relationship between the role of PDGF-AA and tumorigenesis has been summarized in Supplementary Fig. S1A.

A tumor implantation assay indicated that inhibition of tumor growth was more pronounced in AS-PDGFA than in AS-VEGF, suggesting that the expression of PDGF-AA may not only contribute to the regulation of VEGF but also exert other effects on tumor progression. With regard to tumor angiogenesis, we hypothesize that (a) in addition to VEGF, PDGF-AA may regulate other factors advantageous to tumor growth, including other angiogenic factors; and (b) in addition to the indirect angiogenic effect using VEGF, PDGF-AA itself may directly contribute to the angiogenic responses. At present, little is known regarding the former point, which we are currently investigating via microarray analysis. The latter point may be supported by our previous study indicating that PDGF-AA stimulates and maintains the local VEGF expression in mesenchymal cells (13). Such a paracrine mechanism of PDGF-AA for angiogenesis, which is supported by several studies (23–27), may reflect the difference of tumor growth between AS-PDGFA and AS-VEGF. From this point of view, PDGF-AA is likely to be an autocrine and paracrine angiogenic switch in solid tumors (Supplementary Fig. S1B). Regarding the nonangiogenic actions, the paracrine mode of PDGF-AA causes a desmoplastic reaction (28), which is an important feature of advanced NSCLCs (29), by activating mesenchymal myofibroblasts.

Might a PDGF-AA-related signal transduction pathway be a more effective molecular target for antitumor therapy than a pathway related to VEGF? Although it seems premature to draw such a conclusion, the current study suggests this possibility, and some recent studies may also support it. For example, in a previous study that measured the levels of various angiogenic factors, including VEGF, in the tumors of neuroblastoma patients, only the expression level of PDGF-AA was significantly correlated to patient survival, even when a high level of expression of various angiogenic factors was detected (30). On the other hand, an inhibitor of tyrosine kinases, including PDGFRs, is likely to be effective in patients with malignant tumors (31), suggesting that PDGF-AA and its related pathways may be important targets for tumor

### Table 1. Relationship between PDGF-AA/VEGF expression and tumor diameter of human NSCLCs

<table>
<thead>
<tr>
<th>Diameter</th>
<th>AAH (n = 13)</th>
<th>Tumor diameter (ϕ) of NSCLCs (n = 128)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AA-positive rate (%)</td>
<td>0 (0.0)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>VEGF-positive rate (%)</td>
<td>4 (30.7)</td>
<td>2 (40.0)</td>
</tr>
</tbody>
</table>

*Includes seven bronchioalveolar carcinomas.

\[ P < 0.0001, \text{Cochran-Armitage test for trend.} \]

\[ P < 0.001, \text{Cochran-Armitage test for trend.} \]

**Figure 5.** Comparison of 5-year survival of patients with NSCLCs. Kaplan-Meier curves indicating a 5-year survival of patients with VEGF-positive or -negative cases (A), PDGF-A-positive or PDGF-A-negative cases (B). The log-rank test was used to determine the statistical differences between life curves. A probability value of \( P < 0.05 \) was considered significant.
angiogenesis. Among these tyrosine kinases, it has been suggested in a recent experimental study that PDGF-AA and PDGF-Bβ might be one of the essential regulators for tumor angiogenesis (32); some thalidomide analogues markedly inhibited tumor growth and angiogenesis in vivo via a marked reduction of PDGF-AA without apparent changes of the expression levels of other angiogenesis-related factors. Together, these findings strongly suggest that the PDGF-AA/PDGF-Bβ signal transduction pathway warrants further study for the potential treatment of patients with intractable malignancies.

An important limitation of the present study was the lack of direct evidence of the demethylation status of the PDGF-A promoter in NSCLCs; because of this, the scheme shown in Supplementary Fig. S1A is still hypothetical, not definitive. For precise assessment regarding this issue, careful laser dissection of cancer cells should be done without contamination of mesenchymal cells, because the PDGF-A promoter of nontumoral mesenchymal cells should be demethylated. We have started this delicate assessment, and the data will be available in the near future.

In conclusion, we identified PDGF-AA as an autocrine, and probably a paracrine, regulator that made an essential contribution to the expression of VEGF in NSCLCs, affecting tumorigenesis revealed by experimental and clinicopathologic studies. Therefore, PDGF-AA and its related pathways, which determine the expression of VEGF in tumors, may have potential as molecular targets for an antitumor strategy for disrupting tumor angiogenesis.

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