Expression of Plasminogen Activator Inhibitors 1 and 2 in Lung Cancer and Their Role in Tumor Progression[1]

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ABSTRACT

The plasminogen activator cascade initiated by urokinase type plasminogen activator (u-PA) is involved in extracellular matrix degradation during the tumor invasion process. The plasminogen activator inhibitors 1 (PAI-1) and 2 (PAI-2) are two specific inhibitors of u-PA. We hypothesized that the balance between u-PA and its two inhibitors could be disrupted to favor plasminogen activation during lung cancer progression. Using immunohistochemistry, we analyzed the pattern of expression of u-PA, PAI-1, and PAI-2 in non-small cell lung carcinomas (NSCLC) and neuroendocrine (NE) lung tumors. u-PA and PAI-1 were both detected in stromal fibroblasts and in tumor cells. In 84 NSCLCs, their epithelial expression was strongly correlated and linked to the presence of node metastasis ($P = 0.008$), whereas their coexpression in fibroblasts was associated with larger tumor size ($P = 0,04$) and advanced stages ($P = 0.009$). In 72 NE tumors, u-PA and PAI-1 were more frequently expressed in fibroblasts in high-grade NE tumors (SCLC and large cell NE tumors) than in low- and intermediate-grade tumors (typical and atypical carcinoids). Comparison of *in situ* hybridization and immunohistochemistry in 14 cases showed that PAI-1 was consistently expressed by stromal fibroblasts, although the protein was also localized in tumor cells. In contrast, the expression of PAI-2 was restricted to fibroblasts and correlated with the absence of nodal involvement ($P = 0.005$). Considering NE tumors, the frequency of PAI-2 expression decreased along the NE spectrum from typical carcinoids to SCLCs. These data suggest that PAI-1acts in synergy with u-PA to favor tumor invasion process and connotes aggressivity, in contrast with PAI-2, which may block u-PA-mediated proteolysis and is inversely correlated with tumor progression.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in Europe and the United States. Unfortunately, therapeutic efforts have failed to produce a substantial improvement in outcome. On the basis of histological classification, ~75% of lung cancer are NSCLCs[3] pertaining to various forms of epithelial proliferations (squamous cell carcinomas, adenocarcinomas, and large cell carcinomas) without NE differentiation, and 25% are NE carcinomas displaying NE differentiation. NSCLCs have a rather unpredictable prognosis, although basaloid carcinomas, a variant of large cell carcinoma, bear a poor prognosis (1). NE carcinomas encompass a spectrum of four clinicopathological entities including typical carcinoid, a low-grade tumor, atypical carcinoid of intermediate grade, and large cell NE carcinomas and SCLCs, both high-grade tumors. Large cell NE carcinoma has been described recently as a variant of large cell carcinoma with NE differentiation (2). Because this tumor spectrum is expected to be sustained by genetic abnormalities of gradually increasing severity, it thus provides a model for the evaluation of aggressivity markers. TNM is thus far proposed as the best predictive factor of prognosis in NSCLC (3). Still a better understanding of the key factors of tumor progression may be useful to clinical management in providing progression markers in patients at a given stage of the disease and to design new cellular targets for future therapies. Useful indicators of lung cancer progression belong to five main categories of genetic deregulation reflecting oncogene activation, tumor suppressor gene invalidation, escape from apoptosis, cell-to-cell and cell-to-extracellular matrix adhesion disruption, and ECM degradation.

Indeed, ECM degradation, in which the plasminogen system is involved, is one of the major keys in the tumor invasion process. The plasminogen activation is a proteolytic cascade that leads to effective degradation of ECM components and is initiated by the generation of active u-PA. The pro-uPA linked to its receptor (u-PAR) at the cell membrane (4) is activated in u-PA (5). The active u-PA converts the plasminogen to plasmin, which plays both a direct role on the ECM degradation and an indirect role by activating other matrix metalloproteinases (6). The proteolytic activity of u-PA is negatively regulated by PAI-1 and PAI-2, which belong to the serine protease inhibitor (serpin) superfamily. PAI-1 secreted in the vicinity of cells is

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; NE, neuroendocrine; TNM, Tumor-Node-Metastasis; ECM, extracellular matrix; u-PA, urokinase type plasminogen; u-PAR, U-PA receptor; PAI, plasminogen activator inhibitor; LCNEC, large cell NE carcinoma.

found in high concentration in blood, where it plays a role in fibrinolysis (7). PAI-1 inhibits the u-PA-bound u-PAR and activates internalization of the complex u-PAR/u-PA/PAI-1. PAI-2 was initially found in placental tissue and is localized in cell cytoplasm. As shown for PAI-1, exogenous PAI-2 can react and inhibit proteolysis-dependent u-PA linked to its receptor.

Although some *in vitro* experiments (reviewed in Ref. 8) showed that PAI-1 inhibited invasion and metastasis, coexpression of u-PA, u-PAR, and PAI-1 correlated with optimal invasiveness of cultured lung cells through Matrigel (9). *In vitro* results were less variable for PAI-2, which was found consistently to inhibit cell invasion (10, 11). In human malignancies *in vivo*, levels of u-PA, PAI-1, and PAI-2 were significantly higher than in the corresponding normal tissues and have been found to be related to patient prognosis (12, 13). Overexpression of u-PA, PAI-1, and PAI-2 have been reported in tissue extracts of lung cancer (14–18), but results on the clinical impact of these components remain controversial. Thus, high levels of u-PA mRNA studied using Northern analysis were associated with metastasis (14), whereas protein content by ELISA analysis did not show significant relationship with patient outcome (15, 16, 18). Patients with adenocarcinomas containing high levels of PAI-1 protein (15) or with squamous and large cell carcinomas containing both u-PAR and PAI-1 protein at high levels had a shorter survival than others, whereas no clinical correlation was found considering u-PAR and PAI-1 in extracts of NSCLC as well as of SCLC (18). In NSCLC, a low content of PAI-2 protein in tissue extracts was correlated with tumor dissemination (17). However, because of the variable proportion of stromal and epithelial cells in tumor tissue, tissue extracts could not provide information on the level of the proportion of the protein produced by each cellular compartment and on the relative contribution of these cell secretions to tumor progression. Moreover, some of the PAI-1 in tumor extracts may originate from blood platelets, which are rich in PAI-1. It was thus of interest to analyze and compare the exact localization of u-PA and its inhibitors in different histological classes of lung carcinomas. Using *in situ* hybridization and immunohistochemistry, we have previously reported a predominant stromal expression of u-PA in a large series of lung tumors (19, 20). The stromal mRNA expression of u-PA was correlated with tumor size and lymph node metastasis, and its epithelial expression was correlated with tumor size (20). The localization of PAI-1 and PAI-2 proteins has been investigated in only one previous series of NSCLC (17), where they were localized in cancer cells.

Using immunohistochemistry, we describe in the present study the combined patterns of u-PA, PAI-1, and PAI-2 expressions in a series of lung carcinomas and correlate their expression with clinicopathological parameters and patient outcome.

MATERIALS AND METHODS

Tissue Samples. The present study includes tumor tissues from 156 patients, obtained at the primary site at lung resection in 130 patients and from lymph node metastasic sites in 20 SCLCs and 6 non-SCLCs. According to the TNM classification, 65 patients were at stage I, 13 patients at stage II, 56 patients at stage III, and 22 patients at stage IV. Samples were for one part fixed in formalin for histological observation and for another part directly frozen in isopentan cooled liquid nitrogen and stored at −80°C. The tumors consisted of 84 NSCLC non-NE carcinomas and 72 NE carcinomas, according to the new lung carcinoma classification (21). NSCLCs included 39 squamous carcinomas, 35 adenocarcinomas, 10 large cell carcinomas, comprising 7 basaloid carcinomas according to Brambilla *et al.* (1) and 3 usual large cell carcinomas. NE carcinomas consisted of 14 typical carcinoids, 7 atypical carcinoids according to Arrigoni *et al.* (22) and Travis *et al.* (23), 24 LCNECs, high-grade NE lung tumors described by Travis *et al.* (2, 23), and 27 SCLCs. Five normal lung parenchyma and three samples of pulmonary fibrosis were also examined as non-tumor tissue controls.

Immunohistochemistry. Immunohistochemical analysis and *in situ* hybridization were performed on adjacent frozen sections to compare mRNA and protein localization. The mouse monoclonal antibody directed against human M_r 54,000 u-PA was purchased from Biodesign International (Kennebunk, Me) and does not cross-react with tissue type plasminogen activator. The mouse monoclonal antibodies directed against human PAI-1 and PAI-2 were purchased from American Diagnostica, Inc. (Greenwich, CT) and do not cross-react. Because of limited availability of a specific and reactive anti-PAI-2 antibody, 87 of the series of 156 cases could be immunostained with PAI-2. The indirect immunoperoxidase technique was performed on frozen sections fixed in acetone at −20°C for 10 min for the anti-PAI-1 and in paraformaldehyde 4% in PBS (pH 7.4) for 10 min for the anti-PAI-2, based on comparison of both procedures of fixation for immunodetection of these proteins. Non-specific protein binding was blocked using 2% newborn donkey serum in PBS with 1% BSA for 30 min. The primary antibodies anti-PAI-1 and anti-PAI-2 diluted in PBS-BSA at 5 μg/ml were applied and incubated at 4°C overnight. Slides were then exposed for 60 min to the secondary biotinylated donkey anti-mouse immunoglobulin at 1:500 (Jackson Immunoresearch Laboratories, West Grove, PA), followed by the amplification system streptavidin-biotin-peroxidase complex (Dakopatts, Glostrup, Denmark). PBS (pH 8.6) was used to rinse sections between steps. The slides were then treated with the diaminobenzidine and hydrogen peroxide and counterstained with hematoxylin. The primary antibodies were replaced by non-immune mouse immunoglobulin for negative control.

Scores were calculated using percentage of labeled cells and intensity of staining. Final score was obtained by multiplying percentage (1–100%) by intensity (1–3). The scores of immunostaining were assigned by three investigators independently (C. R., E. B., and I. B.), and a consensus was obtained.

Differences among the ratios were evaluated using the Fischer exact test. $P < 0.05$ was considered significant.

***In Situ* Hybridization.** *In situ* hybridization was carried out on cryostat sections of frozen samples. Seven-μm-thick sections were transferrred to aminopropyltriethoxy-silane-coated slides (Aldrich Chemical Co., Milwaukee, WI). Cryostat sections were immediately transferred to dry ice for 30 min, then fixed in formaldehyde (Formal-fixx; Shandon Lipshaw, Inc., Pittsburgh, PA) for 15 min at 4°C, and washed twice in PBS.

Sections were incubated in 0.1 mol/l glycine in PBS for 3 min, treated with 0,3% Triton X-100 in PBS, and rinsed in PBS. Samples were treated with 0.2 mg/ml proteinase K (Boehringer

Table 1 u-PA and PAI-1 immunostaining in lung carcinomas

Histology	No. of cases	u-PA			PAI-1			
		Positives	F[a]	C	Positives	F	C	ECM
Non-NE carcinomas	84	73 (87%)	72 (86%)	30 (36%)	62 (73%)	37 (44%)	43 (51%)	17 (20%)
Squamous carcinomas	39	33 (85%)	32 (82%)	14 (36%)	30 (76%)	18 (46%)	23 (59%)	13 (33%)
Adenocarcinomas	35	30 (86%)	30 (86%)	11 (31%)	24 (69%)	13 (37%)	115 (43%)	2 (6%)
Basaloid carcinomas	7	7 (100%)	7 (100%)	3 (57%)	6 (86%)	5 (71%)	4 (57%)	2 (29%)
Large cell carcinomas	3	3 (100%)	3 (100%)	1 (33%)	2 (67%)	1 (33%)	1 (33%)	0 (0%)
NE carcinomas	72	51 (71%)	51 (71%)	5 (7%)	50 (69%)	35 (49%)	9 (13%)	21 (29%)
Typical carcinoids	14	5 (36%)	5 (36%)	0 (0%)	5 (36%)	2 (14%)	3 (21%)	0 (0%)
Atypical carcinoids	7	5 (71%)	5 (71%)	1 (14%)	3 (21%)	1 (14%)	2 (29%)	0 (0%)
LCNEC	24	20 (83%)	20 (83%)	1 (4%)	18 (75%)	16 (67%)	1 (4%)	12 (50%)
SCLC	27	21 (78%)	21 (78%)	3 (11%)	24 (89%)	16 (59%)	3 (11%)	9 (33%)
Total	156	124 (79%)	123 (79%)	35 (22%)	112 (72%)	72 (46%)	52 (33%)	38 (24%)

[a] F, fibroblasts; C, cancer cells; ECM, deposit in ECM.

Mannheim, Mannheim, Germany) in 0.1 mol/l Tris, 0.05 mol/l EDTA (pH 8.0) at 37°C for 20 min. They were fixed in formaldehyde at 4°C for 10 min, rinsed in PBS, and acetylated (0.25% acetic anhydride in 0.1 mol/L triethanolamine, pH 8.0) for 10 min. The sections were rinsed twice in 2× SSC (1× SSC contains 150 mmol/l NaCl, 15 mmol/l sodium citrate, pH 7.0) and then dehydrated in graded alcohols. Digoxigenin-labeled PAI-1 RNA probe was kindly provided by B. Sordat (Lausane, Switzerland), and the labeling protocol was done as described previously (24). PAI-1 RNA probes were applied (2 ng in 30 μl) in a solution of 2× SSC, 50% desionided formamide, 5% dextran sulfate, 1 mg/ml yeast tRNA (Boehringer Mannheim), 10 mmol/l DTT, and 1 mg/ml BSA (Sigma). Sections were covered by siliconized, autoclaved coverslips and hybridized at 52°C overnight in a chamber humidified with 80 ml of a mixture (formamide 50%, 2× SSC). After hybridization, slides were subsequently washed in 2× SSC, 50% formamide at 52°C for 10 and 30 min, respectively. Unhybridized transcripts were digested with 20 mg/ml of RNase A in 0.4 mol/l NaCl, 10 mmol/L Tris-HCl (pH 7.5), and 50 mmol/l EDTA at 37°C for 30 min. This was followed by washing in 2× SSC, 50% formamide at 52°C for 10 min. Sections were then washed in Tris-buffered saline [TBS; 0.1 mol/l Tris (pH 7.5), 150 mmol/L NaCl]. Slides were preincubated in TBS containing 2% sheep normal serum and 0.3% Triton X-100 for 30 min and incubated with 1:360 polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (750 units/ml; Boehringer Mannheim) in preincubation buffer for 4 h. Slides were washed with TBS (twice for 10 min each) and with 100 mmol/l NaCL, 100 mmol/l Tris (pH 9.5), and 5 mmol/l $MgCl_2$ for 10 min. Development of alkaline phosphatase was done using nitroblue tetrazolium (Fluka)/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) as substrate. Sections were counterstained with hematoxylin and fixed with an aqueous mounting solution (Glycergel; Dako, Glostrup, Denmark)

Sense control probes on serial sections gave no signal. In addition, H&E staining carried out on adjacent sections contributed to the precise identification of the different cell populations expressing a positive hybridization signal. The scores of *in situ* hybridization were evaluated with the same method as immunohistochemistry.

RESULTS

u-PA Pattern of Expression. In the 156 cases analyzed (Table 1), the u-PA protein was detected in 124 (79%) lung carcinomas. A score of 20 was required for positive staining, the range of scores recorded was 20 to 270. The u-PA protein was detected in fibroblasts in 79% of the cases and in cancer cells in 22%, but fibroblastic expression predominated in all histological types. The fibroblasts were stained more intensively and frequently at the periphery of tumor lobules than in their center. The frequency of u-PA epithelial expression did not vary significantly between the NSCLC histological subtypes. In NE tumors, the fibroblastic expression was significantly more frequent in high-grade tumors, SCLCs and LCNECs, than in low- and intermediate-grade tumors, typical and atypical carcinoids (41 of 51 *versus* 10 of 21; $P = 0.01$).

Epithelial expression of *u-PA* gene was significantly more frequent in NSCLCs than in NE carcinomas (30 of 84 *versus* 5 of 72; $P = 0.0001$). It was not observed in the low-grade NE tumors (typical carcinoids) and was detected in only 1 of 7 atypical carcinoids and in 4 of 51 high-grade NE tumors.

u-PA protein was studied previously in 89 of 156 cases in parallel with mRNA expression on serial sections (20). Eighty-five of 89 cases (95%) were positive for both protein and mRNA expression. A superimposition of mRNA and protein occurred in 44 cases (49%). In the other cases, mRNA expression in stromal and/or cancer cells was associated with a u-PA protein expressed in another compartment than that expressing mRNA. In 11 cases, u-PA mRNAs were localized in both tumor and fibroblastic cells, whereas the protein was exclusively detected in fibroblasts. In some cases, u-PA mRNA was expressed either in cancer cells (six cases) or in stromal cells (seven cases), whereas the corresponding protein was localized simultaneously in both cancer and stromal cells. In nine cases, the u-PA mRNA was detected in cancer cells, and the protein was localized in stromal cells. In eight cases, u-PA mRNA was not detectable, but protein was immunostained in stromal (often perivascular) cells.

To further analyze the interaction between u-PA and its two inhibitors, 67 additional cases were analyzed using immunohistochemistry, knowing that it did not exactly reflect where

Table 2 Correlations of clinicopathological parameters with PAI-1 expression and high levels of u-PA expression in lung tumors

		Non-NE carcinomas						NE carcinomas		
		u-PA expression		PAI-1 expression				PAI-1 expression		
	No. of cases	F[a]	C	F + C	F	C	Nb	F + C	F	C
Tumor size (T of TNM)										
T_1–T_2	49	18 (37%)	16 (33%)	32 (65%)	18 (37%)	22 (45%)	44	21 (48%)	17 (39%)	5 (11%)
T_3–T_4	35	23 (68%)	14 (40%)	30 (88%)	19 (56%)	21 (62%)	27	20 (74%)	18 (67%)	3 (11%)
P		$P = 0.011$	NS	$P = 0.035$	NS	NS		$P = 0.053$	$P = 0.04$	NS
Node involvement										
N−	46	18 (40%)	12 (26%)	26 (58%)	16 (33%)	15 (33%)	28	12 (43%)	9 (32%)	3 (11%)
N+	38	23 (61%)	18 (47%)	36 (95%)	21 (55%)	28 (74%)	43	29 (67%)	26 (60%)	5 (12%)
P		NS	NS	$P = 0.0003$	NS	$P = 0.0006$		NS	$P = 0.04$	NS
Tumor stage										
I	34	14 (41%)	11 (32%)	20 (54%)	10 (27%)	13 (35%)	28	12 (43%)	9 (32%)	3 (11%)
II–III	41	24 (59%)	15 (37%)	37 (90%)	24 (59%)	25 (61%)	28	18 (64%)	16 (57%)	3 (11%)
P		NS	NS	$P = 0.0008$	$P = 0.01$	$P = 0.04$		NS	NS	NS

[a] F, fibroblasts; C, cancer cells; NS, not significant, mean of scores for u-PA fibroblastic expression, $n = 69$, for u-PA epithelial expression and PAI-1 expressions. A score >10 was considered positive.

the protein was synthesized, but where it was eventually functional.

Correlation of u-PA Expression with Clinicopathological Parameters. The correlations of u-PA protein expression with the clinicopathological variables are summarized in Table 2. The mean value of expression in fibroblasts ($n = 69$) was used as the cutoff value to divide the patients into groups with low and high scores of expression. In NSCLCs, high levels of fibroblastic u-PA expression were more frequent in T_3-T_4 tumors than in T_1-T_2 tumors ($P = 0.011$), whereas no correlation was found between cancer cell u-PA expression and any clinicopathological parameter. In NE carcinomas, there was no correlation between u-PA expression and pathological parameters.

PAI-1 Pattern of Expression. In normal and fibrotic lung, PAI-1 was detected in type II pneumonocytes and also in macrophages. Results are shown in Table 1. PAI-1 staining was heterogeneous in tissue sections, and scores were evaluated, considering a mean value for the overall tissue section. A score of 20 was required for positive staining, and the scores ranged from 20 to 240. PAI-1 protein was detected in 112 of 156 (72%) lung carcinomas. PAI-1-stained cells were fibroblasts in 72 of 156 cases (46%), epithelial cells in 52 cases (33%), and occasionally endothelial cells and macrophages. In 38 of 156 cases (24%), the ECM also presented a specific staining with the anti-PAI-1 antibody as compared with the negative control. The ECM PAI-1 staining was strongly correlated with the presence of fibroblastic PAI-1 expression ($P < 0.0001$). Epithelial and fibroblastic expressions of PAI-1 were higher at the tumor-stroma interface, where the staining was intense at the periphery of tumor lobules or in the adjacent fibroblasts. The PAI-1 staining was stronger at the invasive front of the tumor as compared with the centrally located tumor lobules. The epithelial localization of the PAI-1 protein was significantly more frequent in NSCLCs than in NE carcinomas ($P < 0.0001$), whereas no difference was noted between histological subclasses. Among NE carcinomas, fibroblastic expression of PAI-1 predominated in SCLCs and LCNECs (32 of 51 cases), whereas only 3 of 21 carcinoids (1 atypical and 2 typical) showed PAI-1 expression in fibroblasts ($P = 0.0001$). Deposits of PAI-1 in the ECM of NE tumors were only detected in high-grade SCLCs and LCNECs ($P = 0.001$). Among NSCLCs, ECM deposits were significantly more frequent in squamous and basaloid carcinomas than in other tumor types ($P = 0.005$).

In situ hybridization performed in 14 cases detected PAI-1 mRNA in fibroblasts in all cases and also in cancer cells in 3 cases. The *in situ* hybridization staining in fibroblasts was superimposed with immunostaining in 5 of 10 cases (Fig. 1). In one of those, mRNA was present in fibroblasts, and the protein was also deposited in the ECM. In the three cases where messengers were localized in fibroblasts and cancers cells, the protein was detected in one case in fibroblasts and deposited in the basement membrane and in two cases in cancer cells. In one case, transcripts were detected in fibroblasts and the protein in cancer cells. In the five remaining cases, the transcripts were localized in fibroblasts, and the protein was localized in both tumor cells and fibroblasts. The immunostaining of cancer cells was reinforced at the cell membrane.

Correlation of PAI-1 Protein Expression with Clinicopathological Parameters. The correlation of PAI-1 immunostaining with clinicopathological variables is shown in Table 2. In NSCLCs, expression of PAI-1 in epithelial cells and/or fibroblasts was more frequent in T_3-T_4 tumors than in T_1-T_2 tumors (T of TNM; $P = 0.035$), in node positive tumors (N+ = N_1 to N_3) than in node-negative tumors (N− = N_0; $P = 0.0003$), and in stages II-III than in stage I ($P = 0.0008$). Considering epithelial *versus* fibroblastic localization of PAI-1 staining, the epithelial expression of PAI-1 was significantly more frequent in node-positive than in node-negative tumors ($P = 0.0006$) and in stages II-III than in stage I ($P = 0.04$). The fibroblastic expression was correlated with extended stages ($P = 0.01$). In NE carcinomas, only the fibroblastic expression was correlated with tumor size ($P = 0.04$) and with the presence of node metastasis ($P = 0.04$).

Relations between u-PA and PAI-1 Expressions. Considering all histological tumor types, epithelial expressions of u-PA and PAI-1 were directly correlated ($P = 0.0001$), whereas fibroblast expressions were not. The fibroblastic coexpression of

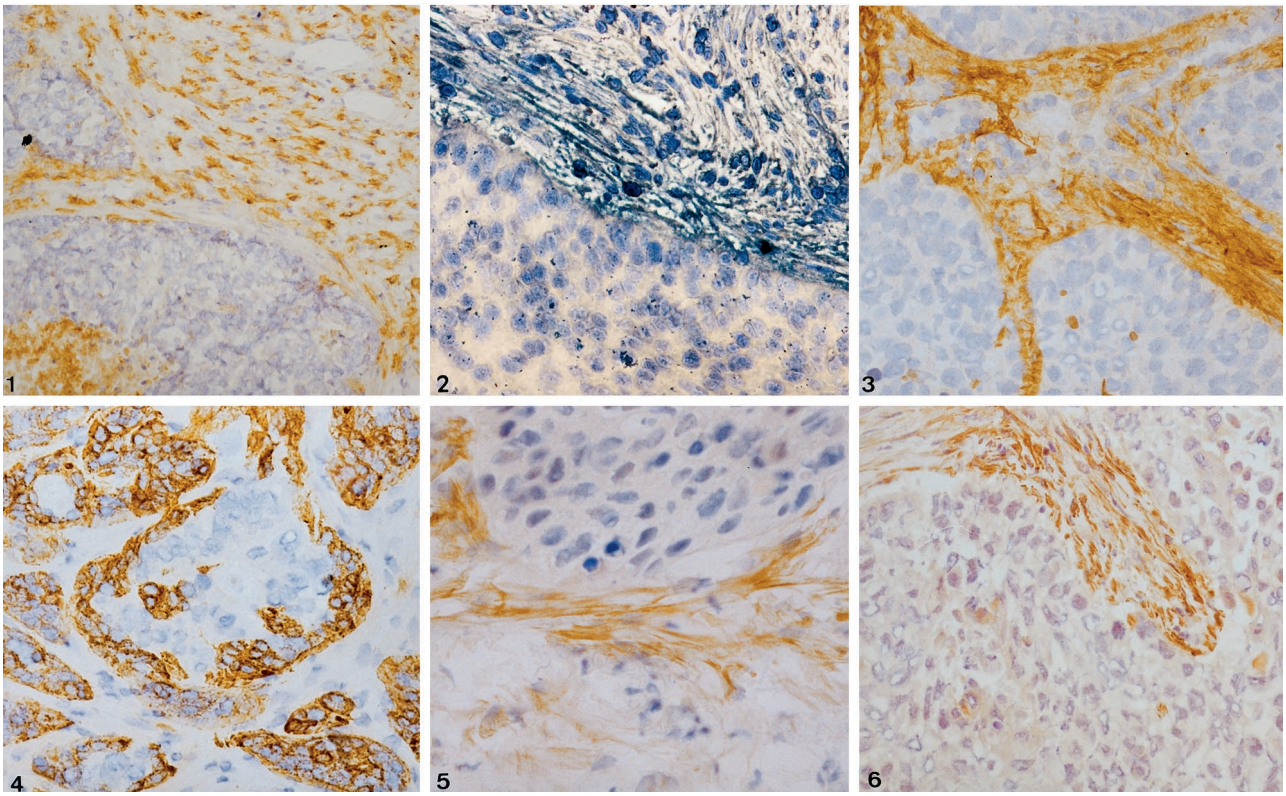

Fig. 1 *Panel 1*, immunostaining of u-PA in stromal fibroblasts of a basaloid carcinoma (×10). *Panel 2*, localization of PAI-1 mRNA, by *in situ* hybridization, in stromal fibroblasts of a squamous carcinoma (×10). *Panel 3*, immunostaining of PAI-1 in stromal fibroblasts and deposited in ECM in squamous carcinoma (×20). *Panel 4*, immunostaining of PAI-1 in cancer cells at the periphery of the lobule; the staining is reinforced at the cell membrane in adenocarcinoma (×20). *Panels 5* and *6*, immunostaining of PAI-2 in squamous carcinoma in perilobular fibroblasts (*Panel 5*; ×20) and in thin stromal trabeculae penetrating the lobules (*Panel 6*; ×20).

Table 3 Correlations of clinicopathological parameters with PAI-1 and u-PA coexpression in non-NE carcinomas

	No. of cases	u-PA and PAI-1		
		C + F[a]	F	C
Tumor size (T of TNM)				
T_1–T_2	49	30 (61%)	15 (31%)	12 (25%)
T_3–T_4	35	30 (88%)	19 (56%)	16 (47%)
P		$P = 0.014$	$P = 0.04$	$P = 0.06$
Lymph node involvement				
N−	46	25 (54%)	14 (30%)	9 (20%)
N+	38	35 (92%)	20 (53%)	19 (50%)
P		$P = 0.0005$	$P = 0.08$	$P = 0.008$
Tumor stages				
I	34	19 (56%)	9 (26%)	8 (18%)
II–III	41	36 (88%)	23 (56%)	17 (41%)
P		$P = 0.001$	$P = 0.009$	NS

[a] F, fibroblasts; C, cancer cells; NS, not significant.

u-PA and PAI-1 were more frequent in NSCLC than in NE tumors (61 of 84 *versus* 34 of 72; $P = 0.002$). Considering NSCLCs, epithelial expressions of u-PA and PAI-1 were still correlated ($P = 0.009$; Table 3); the coexpression of u-PA and PAI-1, whichever compartment was considered, was significantly correlated with tumor size ($P = 0.014$), node involvement ($P = 0.0005$), and extended stages ($P = 0.001$). Their coexpression in fibroblasts correlated with tumor size ($P = 0.04$) and extended stages ($P = 0.009$), and their epithelial coexpression was correlated with the presence of node metastasis ($P = 0{,}008$) but not significantly with tumor size ($P = 0.06$). In NE tumors, the fibroblastic expressions of u-PA and PAI-1 were significantly associated ($P = 0.015$; Table 4), and their coexpression in fibroblasts was more frequently observed in high-grade than in low- and intermediate-grade tumors (28 of 51 *versus* 2 of 21; $P = 0.005$). However, no correlation could be established between PAI-1 and u-PA coexpression and classical clinicopathological parameters (size, node involvement, and stage) in NE tumors.

PAI-2 Pattern of Expression and Correlation with Clinicopathological Parameters. In normal and fibrotic lung, PAI-2 was localized in perivascular myofibroblasts of large vessels (up to the diameter of 500 μm). Eighty-seven of 156 lung carcinomas were immunostained for PAI-2. PAI-2 protein was detected exclusively in stromal cells (fibroblasts and endothelial cells), and scores ranged from 20 to 300 (Table 5). PAI-2 staining was heterogeneous and was stronger and more frequent at the periphery of tumor lobules. As observed for PAI-1, the signal was stronger at the invasive front of the tumor. PAI-2 protein was detected in fibroblasts (scores ≥20) in 58 of 87 lung carcinomas (67%) more frequently in NSCLC than in NE tu-

Table 4 Correlation between u-PA and PAI-1 expression[a]

	Non-NE tumors				NE tumors			
	Fibroblasts		Cancer cells		Fibroblasts		Cancer cells	
	u-PA−	u-PA+	u-PA−	u-PA+	u-PA−	u-PA+	u-PA−	u-PA+
PAI-1−	3	44	27	14	16	21	56	7
PAI-1+	2	34	15	28	5	30	9	0
	NS		$P = 0.009$		$P = 0.015$		NS	

[a] Correlation between u-PA and PAI-1 when they are expressed both in fibroblasts or both in cancer cells.

Table 5 PAI-2 expression and u-PA and PAI-2 coexpression according to histological classes

Histology	No. of cases	PAI-2	PAI-2 fibroblasts	
		F[a]	u-PA (F)	u-PA (C)
Non-NE carcinomas	64	47 (73%)	46 (72%)	22 (34%)
Squamous carcinomas	29	22 (78%)	21 (72%)	10 (45%)
Adenocarcinomas	25	15 (60%)	15 (60%)	8 (36%)
Basaloid carcinomas	7	7 (100%)	7 (100%)	3 (57%)
Large cell carcinomas	3	3 (100%)	3 (100%)	1 (33%)
NE carcinomas	23	11 (48%)	9 (39%)	1 (4%)
TC & AC	8	7 (88%)	5 (63%)	0 (0%)
LCNEC	6	1 (17%)	1 (17%)	1 (17%)
SCLC	9	3 (33%)	3 (33%)	0 (0%)
Total	87	58 (67%)	55 (63%)	23 (26%)

[a] F, fibroblasts; C, cancer cells; TC, typical carcinoid; AC, atypical carcinoid.

mors ($P = 0.018$). The fibroblastic expression of PAI-2 was correlated with the absence of node involvement, considering NSCLC and NE tumors together ($P = 0.005$). This correlation was maintained, considering only NSCLCs ($P = 0.02$; Table 6).

Among NE carcinomas, the frequency of PAI-2-positive cases was significantly higher in low-grade (carcinoids) than in high-grade NE tumors (LCNECs and SCLCs; $P = 0.004$).

Relations of u-PA and PAI-2 Expressions. PAI-2 was expressed exclusively in u-PA-positive cases with three exceptions (two carcinoids and one squamous lung carcinoma). No other correlation between u-PA and PAI-2 expressions was observed, considering either fibroblastic or epithelial expression of u-PA. However, the fibroblastic coexpression of u-PA and PAI-2 was more frequent in tumors without node metastasis ($P = 0.045$). In the same way, the association of u-PA epithelial expression with the fibroblastic expression of PAI-2 was more frequent in node-negative than in node-positive tumors, but this only tended toward significance ($P = 0.08$; Table 6).

DISCUSSION

Immunohistochemistry was used in the present study to analyze the specific localizations of u-PA and its two specific inhibitors (PAI-1 and PAI-2) in a large panel of lung carcinomas including NE and non-NE tumors. u-PA, PAI-1, and PAI-2 were found to be overexpressed in lung cancer as compared with normal or inflammatory lung in agreement with previous results in tissue extracts (15–18). Although we localized u-PA and PAI-1 proteins in both cancer cells and stromal fibroblasts, we detected PAI-2 exclusively in fibroblasts. This is in contrast with two other reports, where a tumor cell exclusive expression of u-PA and PAI-2 was described (17, 25), using immunohistochemistry on fixed paraffin sections. However, using *in situ* hybridization, we have demonstrated previously the existence of u-PA transcripts in both epithelial and stromal compartments (20). Such discrepancies could be explained by the difference of material used for these studies (paraffin section *versus* frozen sections). Interestingly, PAI-1 and PAI-2 were described in both tumor cells and fibroblasts in breast cancer using immunohistochemistry on frozen sections (8, 26–28), suggesting that differences in sources of PAI-1 and PAI-2 might exist between different tumor types and that methodological differences might affect the recognition of PAI-2 in fibroblasts.

In our previous study on NSCLCs (20) using *in situ* hybridization, we reported that high levels of u-PA mRNA in fibroblasts were correlated with tumor size ($P = 0.01$), presence of node metastasis ($P = 0.001$), and extended stage III ($P = 0.04$), and that high levels of u-PA mRNA in tumor cells were more frequent in T_3-T_4 tumor than in T_1-T_2 tumor ($P = 0.04$). However, in the present study, u-PA protein expression assessed by immunohistochemistry showed a correlation between high levels of u-PA protein in fibroblasts and tumor size ($P = 0.011$). Actually, we had observed previously (20) that the protein expression was superimposed with that of u-PA mRNA in only half of the tumors. Moreover, the range of score values was smaller for protein expression than for mRNA expression. Thus, a possible explanation for these discrepancies could reside in the high turnover of the protein as compared with relative stability of mRNA expression and/or in the lack of sensitivity of u-PA antibodies in detecting low protein levels that might otherwise be biologically active.

This is the first report using immunohistochemistry to compare expression of u-PA with its two specific inhibitors, PAI-1 and PAI-2. Interestingly, the patterns of expression of PAI-1 and PAI-2 were different not only in their compartment of expression but also divergent in their relation with u-PA expression. Whereas PAI-1 expression was strongly and directly correlated with u-PA expression in cancer cells of NSCLC and with u-PA in fibroblasts of NE tumors, PAI-2 was not correlated with the u-PA tumor cell expression. However, only three cases presented PAI-2 expression in fibroblasts in absence of u-PA expression, indicating that PAI-2 was elicited in tumors in response to u-PA. In a previous study, we have reported that u-PA expression was more frequent in NSCLCs than in NE cancer cells (20), and Papot *et al.* (18) have reported higher level of plasminogen system components (u-PA and PAI-1) in NSCLCs than in SCLCs, using an ELISA technique. The results

Table 6 Correlations of clinicopathological parameters with PAI-2 expression and u-PA and PAI-2 coexpression

	NE and non-NE tumors		Non-NE tumors		NE tumors		Non-NE and NE tumors PAI-2 fibroblasts			
	No. of cases	PAI-2 F[a]	No. of cases	PAI-2 F	No. of cases	PAI-2 F	No. of cases	u-PA F	No. of cases	u-PA C
Tumor size (T of TNM)										
T_1–T_2	56	40 (71%)	39	31 (80%)	17	9 (53%)	56	37 (66%)	23	15 (65%)
T_3–T_4	31	18 (58%)	25	16 (64%)	6	2 (33%)	31	18 (58%)	17	8 (47%)
P		NS		NS		NS		NS		NS
Node involvement										
N−	47	38 (81%)	36	31 (86%)	11	7 (64%)	47	35 (74%)	17	13 (76%)
N+	40	20 (50%)	28	16 (57%)	12	4 (33%)	40	20 (50%)	23	10 (44%)
P		$P = 0.005$		$P = 0.02$		NS		$P = 0.045$		$P = 0.08$
Tumor stage										
I	43	34 (79%)	32	27 (84%)	11	7 (64%)	43	31 (72%)	16	12 (75%)
II–III	32	20 (63%)	27	17 (63%)	5	3 (60%)	32	20 (63%)	19	10 (53%)
P		NS		NS		NS		NS		NS

[a] F, fibroblasts; C, cancer cells; NS, not significant.

of our *in situ* analysis allow interpretation of theses differences in indicating that stromal fibroblasts are the main source of u-PA and PAI-1. Indeed, the low cell fraction represented by fibroblasts in SCLC helps to explain the results of ELISA analysis. Considering only the epithelial expression of u-PA, our results give credit to the conclusions drawn by Papot *et al.* (18), that epithelial cells with NE differentiation are less prone to express urokinase and its inhibitor PAI-1 than cells without NE differentiation. Nevertheless, the epithelial synthesis of plasminogen components is a minor source of proteases in invasive cancers.

Considering all tumor types, PAI-1 expression was correlated directly with tumor size, presence of node metastasis, and tumor stages. Although PAI-1 is considered as an u-PA inhibitor and should counteract u-PA activities and its effects on tumor progression, PAI-1 was described as a poor prognosis factor in several malignancies (8, 29, 30). In the present study, we have shown that in NSCLCs, the expression of PAI-1 was associated with the presence of node metastasis. PAI-1 also seems to play an independent role on tumor growth and cell migration as deduced from its correlation with tumor size and presence of node metastasis in NE tumors. Consistent with our results, Pedersen *et al.* (15) have shown that the high levels of PAI-1 was an independent factor of poor prognosis in lung adenocarcinomas, using an ELISA analysis of tissue extracts. Moreover, we showed that u-PA and PAI-1 expressions were strongly correlated in tumor cells, and their coexpression was associated with the presence of node metastasis. In NE tumors, we found that PAI-1 and u-PA fibroblastic expressions were frequently coexpressed in high-grade tumors, giving support to the concept of a synergistic effect of both proteins in favoring tumor progression. Furthermore, in ovarian cancer, PAI-1 alone was not an independent prognostic marker, whereas the combination of both u-PA and PAI-1 was, using multivariate analysis (31). Together, these results reinforce the hypothesis of a facilitating role of PAI-1 in cell migration in synergy with u-PA. In this context, PAI-1 might play a role in cell migration independent of its inhibitory effect on u-PA proteolysis.

Different hypotheses have been proposed to elucidate the mechanism involving the plasminogen system components. PAI-1 was shown to bind specifically to the somatomedin domain of vitronectin and to compete with and inhibit u-PA receptor-mediated cell attachment to vitronectin (32), thus favoring cell detachment independently of u-PA. *In vivo* excess of PAI-1 may thus facilitate metastasis by preventing cell adhesion or promoting cell detachment, especially at the trailing edge of cancer cells. Conversely, PAI-1 combined with u-PA may play a direct role in dynamic cell migration in the matrix. In this respect, Stefansson *et al.* (33) showed *in vitro* that formation of a complex between PAI-1 and u-PA on u-PA receptor resulted in loss of PAI-1 affinity for vitronectin, thus exposing RGD cell-attachment sites to specific integrins necessary for cell migration on the matrix. Accordingly, Planus *et al.* (34) showed that anchorage of myogenic cells to the ECM via immobilized PAI-1 and further cell mobilization required the presence of both u-PA, uPAR, and integrins and should occur at the leading cell edge. Transmembrane integrins connect the complex PAI-1, u-PA, and uPAR to the cytoskeleton and creates a traction force necessary for cell motility (8). Thus, migrating tumor cells may be alternatively attached to and tracted on the ECM components at one edge and simultaneously detached from it at the opposite edge. Although apparently producing contradictory effects alternatively, such as cell attachment or detachment, PAI-1 might allow cell migration on ECM, and an optimum concentration of PAI-1 is required to equilibrate u-PA-mediated ECM degradation and cellular adhesion. Compelling evidence has been provided that coexpression of u-PA, PAI-1, and uPAR is necessary for optimal invasiveness of cultured lung cancer cells (9). Most interestingly, using an *in vivo* assay of tumor progression on PAI-$1^{-/-}$ knock-out mice, Bajou *et al.* (35) could demonstrate that PAI-1 produced by host fibroblasts was a limiting and crucial factor for cell migration and metastasis.

We have also demonstrated that u-PA and PAI-1 fibroblastic expressions were both associated with tumor size. The ECM is considered as a reservoir of growth factors (36, 37), which are released by matrix protease-mediated ECM degradation. Growth factor activation could in turn be driven by the same matrix proteases. Indeed, the activation of plasminogen-induced

proteolysis is involved in the release and activation of numerous growth factors as well as transforming growth factor β involved in invasion and angiogenesis (36). Then, transforming growth factor β could stimulate u-PA and PAI-1 synthesis by both fibroblasts and epithelial cells (38).

We reported here that the presence of PAI-2 indicates a good prognosis because its expression was inversely correlated with lymph node metastasis in all tumors ($P = 0.005$) or in NSCLC ($P = 0.02$). Accordingly and although less frequent in NE tumors than in NSCLC, PAI-2 was more frequently detected in low-grade tumors (carcinoids) than in high-grade NE tumors (SCLCs and LCNECs; $P = 0.004$). Our results are in agreement with the demonstration that a low content of PAI-2 was associated with tumor dissemination (17), and that negative expression of PAI-2 antigen was significantly correlated with lymph node metastasis in lung cancer (25). Results on the prognostic impact of PAI-2 in other human malignancies are more controversial. Although increased levels of PAI-2 were related to good prognosis in breast and endometrium cancer (30), it was correlated with aggressive disease in colorectal cancer (39). Nevertheless, our results suggest that PAI-2 may inhibit uPA-mediated cancer cell migration. This hypothesis is sustained by the correlation found between the coexpression of u-PA and PAI-2 with the absence of node metastasis. PAI-2 was shown to inhibit the activity of receptor bound-uPA (40–42). Owing to its persistence at the cell surface environment, PAI-2, which is not interanalyzed (42), may play a major role in the inhibition of u-PA proteolytic activity. At least *in vitro*, PAI-2 was able to inhibit ECM degradation initiated by uPAR-bound u-PA (43). *In vivo*, PAI-2 was reported to suppress pulmonary metastases of a rat mammary carcinoma (11), and transfection of a human melanoma cell line with PAI-2 cDNA correlated with a reduced rate of lung metastasis from tumors established from these cells in *scid/scid* mice (10). There are also suggestions for an alternate biological function of PAI-2, because PAI-2 has antiapoptotic properties through inhibition of a proteinase involved in tumor necrosis factor-α-induced macrophage apoptosis (44). In this regard, the localization of PAI-2 in stromal cells of lung cancer is striking. One can speculate that PAI-2 could protect stromal cells endowed with antigen presentation properties from apoptosis in a tumor necrosis factor-α-rich microenvironment, thus favoring the immune response against cancer cells. However, further investigations are required to understand how PAI-2 can inhibit cancer cell invasion in addition to inhibiting matrix degradation.

In summary, we have shown that PAI-1 and u-PA are strongly correlated and linked with tumor progression parameters, suggesting a synergistic effect on tumor cell migration. Despite an inhibitory role of PAI-1 on u-PA-mediated ECM degradation, an optimal concentration of both u-PA and PAI-1 may modulate the level of ECM proteolysis in allowing cell migration. Alternatively, our findings on the second inhibitor, PAI-2 showed a correlation with favorable prognostic parameters, suggesting that PAI-2 could play a direct role in u-PA inhibition. However, the mechanisms of PAI-2 action need further investigation. In addition to providing markers of tumor progression, plasminogen system components might be optimally counteracted or substituted therapeutically to circumvent tumor migration and progression.

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