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Increased Plasma Levels of Serine Proteinase Inhibitors in Lung Cancer Patients

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Abstract. *Background: Tumor growth and invasiveness occur through infiltration of tumor cells into the host cells and by angiogenesis, which is modulated by proteinases and antiproteinases released from tumor cells that carry out tissue remodelling. A number of studies have revealed variations in the plasma levels of serine proteases and their inhibitors among tumor types. Patients and Methods: By immunological methods we analysed the levels of serine protease inhibitors AAT, ACT and SLPI in newly diagnosed lung cancer patients (n=14) compared to non-smoker and smoker, age- and gender-matched control groups (n=16), and also in an expanded group of lung cancer patients with local tumors (n=14) and with metastasis (n=18). Results: Our data show that plasma levels of AAT, ACT and SLPI were elevated in lung cancer patients by 1.43-fold, p<0.01, 2.57-fold, p<0.01 and 1.6-fold, p<0.001, respectively when compared to controls. In addition, we found that levels of AAT and ACT were higher by 1.47-fold, p<0.001 and 2.27-fold, p<0.001, respectively in lung cancer cases with metastasis compared to localized tumor. Conclusion: These inhibitor levels may provide measures of cancer progression in individual patients and possibly offer useful information for an understanding of the mechanisms of metastasis.*

Under physiological conditions, a balance exists between proteolytic activity and the regulated inhibition of proteolysis. In pathological conditions like tumorogenesis, the balance between proteolysis and its inhibition is disrupted (1, 2). Tumor progression is generally associated with extensive tissue remodelling to provide a suitable environment for tumor growth, invasion and metastasis, and it is known that proteinases expressed by cancer cells and/or host cells play a key role in this process (3, 4). At least four classes of proteinases are frequently involved in tumor growth and invasiveness: MMPs, serine, aspartic and cysteine proteinases (5-9). Of these, MMPs and serine proteinases have been most closely linked to tumor progression (10, 11).

Among serine proteinases, leukocyte elastase, plasmin, thrombin, trypsin and cathepsin G are suggested to be particularly important in lung tumor progression (12-18). For example, cathepsin G not only actively degrades the extracellular matrix, but also can control cell growth and activate pro-MMPs, which are typically released in a latent form (6). Neutrophil elastase is the only proteinase that is able to degrade insoluble elastin and it can also hydrolyse other proteins, including collagens, fibronectins and proteoglycans (19-21). It was reported that elastase is produced and secreted by various types of tumor cells, including non-small lung cancer cells, and that the local production of elastase is associated with poor disease prognosis (13, 21, 22). The regulation of proteolytic enzyme activity in the respiratory airways by endogenous inhibitors is a prerequisite for the maintenance of tissue integrity and for the repair of tissue damage. Proteinase inhibitors that provide protection against the extracellular activity of serine proteinases include AAT, ACT, SLPI and SKALP. Whereas AAT and ACT are produced mainly by the liver and reach the tissue *via* passive diffusion (23), SLPI and SKALP are produced locally (24, 25). Because of their ability to inhibit proteinases, proteinase inhibitors have generally been considered to counteract tumor progression and metastasis (26). However, it has also become increasingly apparent that inhibitors of proteinases may express other than inhibitory functions and therefore may have multiple effects on tumor growth. Indeed, it has been

Abbreviations: AAT, α1-antitrypsin; ACT, α1-antichymotrypsin; SLPI, secretory leukoprotease inhibitor; MMPs-matrix metalloproteinases; proMMP-2, progelatinase A; SKALP, elafin/skin–derived antileukoproteinase; NSCLC, non-small cell lung carcinoma; SCLS, small cell lung carcinoma; ELISA, quantitative sandwich enzyme immunoassay.

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demonstrated that over-expression of a number of serine proteinase inhibitors from the serpin and kunitz families results in enhancement of cancer cell malignancy (27-29). Several studies have reported that increased expression of SLPI, a member of the kazal-type serine proteinase inhibitory family, promotes the tumorogenic and metastatic potential of cancer cells (30). Moreover, serum SLPI levels in patients with primary lung carcinoma were found to correlate with tumor stage and response to therapy (31). Similarly, a direct relationship was shown between lung cancer progression and the levels of the serpins, AAT and ACT, which are the principal inhibitors of neutrophil elastase and cathepsin G, respectively. In addition, it has been found that high AAT and ACT expression levels in lung adenocarcinoma are associated with enhanced tumor progression (32, 33).

The mechanisms by which serine proteinase inhibitors promote tumor progression and whether these effects may counteract the inhibition of the degradation of extracellular matrix proteins during tumor invasion and metastasis remains to be investigated. It is likely that this may depend on several factors, including the proteinase activity and the profiles and/or molecular forms of the inhibitors released.

To our knowledge, the plasma levels of serine proteases such as AAT, ACT and SLPI have not been evaluated in the same experimental setting. Therefore, the objective of the current study was to measure plasma levels of AAT, ACT and SLPI in a group of newly diagnosed lung cancer patients (n=14) relative to age- and gender-matched healthy subjects (n=16). We then expanded the lung cancer patient group (n=32) and compared the levels of these inhibitors among cases without and with metastases.

Patients and Methods

Study population. A total of 32 newly diagnosed lung cancer patients and 16 healthy controls were included in this study. The study population was divided into two sets: the first was 14 lung cancer patients and 16 age- and gender-matched healthy controls, the second consisted of 32 newly diagnosed lung cancer patients with and without metastases. The first set included 14 selected lung cancer patients (among them 4 with metastases) with confirmed diagnosis at the Department of Respiratory Medicine, University Hospital Malmö, Sweden. NSCLC was diagnosed histologically in 11 cases and SCLC in 3 cases. Among these cases were 8 males and 6 females, mean age 62.0 (ranging from 56 to 67 years). The control group included 8 males and 8 females, mean age 55.5 (ranging from 43 to 64 years). There were 5 smokers among controls and 9 in the patient group. Smokers were defined as smoking at the time of diagnosis. Non-smokers we defined as never smoking or had quit smoking 1 year prior to lung cancer diagnosis.

The second study set included all 32 newly diagnosed lung cancer cases, which were subdivided into local tumor (n=14, 7 men and 7 women, mean age 65.8 (ranging from 56 to 78)) and metastatic tumor (n=18, 13 men and 5 women, mean age 70.3 (ranging from 49 to 83)). All cases with local tumors were NSCLC, while among the cases with metastatic tumor were 12 NSCLC, 5 SCLC and 1 mesothelioma. The cases with local tumor included 4 smokers and those with metastatic tumors 12 smokers.

All individuals gave a signed, informed consent to take part in this study, which has been approved by the research ethical committee of Lund University, Sweden.

Venous blood samples were taken from patients on initial diagnosis before treatment and in parallel from healthy volunteers. Plasma samples were obtained by centrifugation and were stored at -80˚C until assayed.

Assay for plasma SLPI. Plasma samples were analysed for SLPI by using a commercially available kit based on ELISA (BIOTRAK, Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. The intensity of color development is measured by using a microplate reader (Labsystems, USA). Duplicate readings for each standard and plasma sample were performed. The lower detection limit for SLPI is 25 pg/ml.

Assay for plasma AAT. Plasma samples were analysed for AAT based on an immunonephelometric method according to the manufacturer's instructions (Beckman image automatic machine). The nephelometric method measures the agglutination of particles by quantifying scattered light. The coefficient of variation (CV) for AAT is 7% at 1.5 g/l and 5% at 2.0 g/l.

Assay for plasma ACT. Individual plasma samples were analysed by rocket immunoelectrophoresis as described by Laurell (34) using monospecific antisera against ACT (DAKO; Denmark), 5.5 μg per square gel area. Plasma samples were diluted 40 times in sterile 0.9% NaCl. For the calibration curve, a standard plasma Seronorm™ (Sero AS, Norway) with known concentration of ACT was used. The immunoprecipitates were stained with Coomassie brilliant blue R-250.

Statistical analysis. All variables were log-transformed to achieve a normal distribution and all calculations were performed with normally distributed variables. The differences of the means in experimental results were analyzed for their statistical significance with the independent-two-samples (two-sided) *t*-test. Statistical Package for Social Sciences (SPSS for Windows, release 11) was used for the calculations.

Results and Discussion

In the present study we showed that plasma levels of three serine proteinase inhibitors, AAT, ACT and SLPI, are significantly higher in lung cancer patients compared to age- and gender-matched healthy controls (Table I). Moreover, in the extended lung cancer patient group (n=32), significantly higher levels of plasma AAT and ACT, but not SLPI, were found in cases with tumor metastasis (n=18) compared to cases with local tumor (n=14) (Table II). Our data are consistent with previous studies that demonstrated increased circulating levels of serine proteinase inhibitors in various types of tumors, including lung cancer (35, 36).

Table I. *Levels of plasma serine protease inhibitors in 14 newly diagnosed lung cancer patients and 16 age- and gender-matched healthy controls.*

Measured parameters	Subject group				T-test
	Controls (n=16)		Patients (n=14)		
	Mean	95% CI[a)]	Mean	95% CI[a)]	*p*-value
AAT mg/ml	1.41	1.34-1.48	2.02	1.54-2.51	0.006
ACT mg/ml	0.42	0.36-0.47	1.08	0.62-1.53	0.003
SLPI pg/ml	34.38	29.17-39.58	55.3	44.36-66.14	0.001

a) CI = confidence interval for mean

Table II. *Serine protease inhibitors in plasma of 32 newly diagnosed lung cancer patients branched according to tumor type.*

Measured parameters	Tumor type				T-test
	Local (n=14)		Metastatic (n=18)		
	Mean	95% CI[a)]	Mean	95% CI[a)]	*p*-value
AAT mg/ml	1.68	1.50-1.87	2.48	2.05-2.90	0.001
ACT mg/ml	0.66	0.40-0.93	1.50	1.06-1.94	0.001
SLPI pg/ml	49.86	39.83-59.90	57.57	49.20-66.00	NS[b)]

a) CI = confidence interval for mean b) NS = not significant

Tumor cells themselves secrete serine proteinase inhibitors and it has been demonstrated that over-expression of these inhibitors favors tumor progression (37-39). Therefore, the observed elevated levels of serine proteinase inhibitors possibly represent a pool of exogenous (host cell) and endogenous (tumor cell) produced inhibitors that arise from the inflammation accompanying the cancer process.

It is well documented that tumor growth is often associated with an increased inflammatory response measured by increased secretion of growth factors and pro-inflammatory cytokines that are chemoattractants for macrophages and polymorphonuclear leukocytes/neutrophils (40). An inflammatory response is observed in lung cancer, both locally and systemically and inflammatory infiltrates are shown to correlate with greater tumor invasiveness (41). Proteinase inhibitors play an important role in modulation of the inflammatory response and are therefore involved in tumor growth and invasiveness (42, 43).

Elevated levels of AAT have been observed in patients with various tumor types, such as brain (44), gynecologic (45), colorectal (46), advanced gastric (47) and breast cancer (48). Recent data reported by Bata and co-workers show strongly elevated plasma AAT levels in patients with primary lung carcinoma compared to controls (49). However, contradictory data exist for the relationship between levels of AAT and cancer progression. Some investigators propose that AAT plays a protective role in tumorogenesis. For example, Finlay and co-workers showed that AAT might be directly involved in breast cancer progression by acting as a tumor suppressor (50). Serine proteinases, such as elastase, proteinase-3 and cathepsin G, released by neutrophils trigger a proteinase cascade that entails activation of proMMP-2, an enzyme involved in tumor invasion and angiogenesis. In an experimental model *in vitro*, AAT was found to block the activation of proMMP-2 and tumor cell invasion by inhibiting serine proteinase activity (6). However, earlier immunohistochemical studies revealed that patients with AAT-positive colon, gastric and lung adenocarcinomas had a worse prognosis than AAT-negative ones (51, 52, 27), suggesting that AAT may play other roles in carcinogenesis *in vivo*, in addition to its role as proteinase inhibitor.

AAT, a member of the serpin family of serine proteinase inhibitors, is the principal inhibitor of neutrophil elastase and proteinase-3 (53, 54). During the acute phase, AAT rises by 3- to 4-fold above normal (1.34 mg/ml), while local levels of AAT that are regulated by growth factors might be increased up to 11-fold (55). It has been reported that imbalance between AAT and neutrophil elastase may be a predisposing condition for lung cancer development (56). Patients who carry the deficiency allele of AAT are shown to have a significantly higher risk of developing squamous cell or bronchoalveolar carcinoma of the lungs (57). On the other hand, it was reported that strong expression of AAT in lung adenocarcinoma correlates with poor prognosis (33), although it was not shown whether the inhibitory activity of AAT was normal and whether elastase levels were high among this group of patients. Since uncontrolled activity of elastase raises the risk of developing lung cancer, it is plausible that the imbalance between AAT and elastase could be a predisposing condition for lung cancer development. On the other hand, AAT is produced by various tumor cells and is a good substrate for MMPs. Therefore, one can speculate that there might be a link between MMP activity, tumor cell propensity to produce and secrete AAT and tumor progression. Among MMPs, neutrophil collagenase, gelatinase-B, stromelysin-1 and -3 and matrylisin can effectively cleave AAT (58-62). The cleavages by these MMPs occur at peptide bonds within the AAT active site loop, resulting in the inactivation of AAT as a proteinase inhibitor and generation of cleaved forms of AAT. Recent studies provide good experimental evidence that the C-terminal fragment of AAT may enhance tumor growth and

invasiveness *in vitro* and *in vivo* (63). By using comparative proteome analysis to identify protein alterations in plasma of prostate, lung and breast cancer patients, investigators have found a significant elevation of AAT and its N-terminal fragment in patients with all analysed tumor types (64). These findings further indicate that different molecular forms of AAT in the circulation *in vivo* may express different biological activities and effects on cancer cells.

ACT is another serine proteinase inhibitor that specifically inactivates serine proteases, such as neutrophil cathepsin G, mast cell chymase and pancreatic chymotrypsin (65). In addition, the major fraction of human prostate-derived proteases belonging to the kallikrein family of enzymes, which are used clinically to monitor patients with prostate cancer, are found in complex with ACT (66, 67). It was also reported that ACT, which is known to bind DNA *in vitro*, inhibits DNA synthesis in permeabilized human carcinoma cells, suggesting that it may inhibit DNA polymerase and/or DNA primase activity (68). ACT expression has been shown in various tumor types, but its biological and clinical significance in tumor tissues are obscure. A significant increase in plasma ACT was previously found in gastric and in breast cancer patients (69, 35) Higashiyama and collaborators have shown that ACT is synthesised by lung adenocarcinomas and that its expression in surgically resected lung adenocarcinomas is closely associated with T factor, tumor size and mitotic grade. These findings led to the suggestion that ACT expression might be involved in tumor progression and especially tumor growth (32). We also found that plasma levels of ACT are significantly elevated in lung cancer patients relative to controls, and also among lung cancer patients with metastasis compared to local tumor.

It is important to point out that ACT, like AAT, under certain circumstances can interact with other molecules such as DNA, non-target proteases and peptides, which will result in loss of inhibitory activity of ACT as well as the genesis of new molecular forms of ACT. Therefore, the role of ACT in tumor growth and progression might depend not only on its levels, but also on its molecular form. These findings suggest a role for ACT in tumorogenesis, but more detailed investigations are required to establish precisely what this role is.

SLPI is found at significant levels in nasal, bronchial and cervical mucous and in saliva (70, 71). There is increasing evidence that SLPI has numerous functions that are not related to its protease-inhibitory activity. SLPI is a non-glycosylated, hydrophobic, cationic 12 kDa protein, consisting of two homologous cysteine-rich domains of 53 and 54 amino acids (72) The carboxyl-terminal domain of SLPI manifests inhibitory activities against chymotrypsin, trypsin, granulocyte and pancreatic elastase, cathepsin G and mast cell chymase (73-77), while anti-inflammatory, anti-bacterial and anti-fungal activities are associated with the amino-terminal domain (78, 79). Historically, SLPI was first purified from secretions of patients with chronic, obstructive pulmonary disease and cystic fibrosis (73) and it was suggested that SLPI, being a major anti-elastase inhibitor of the bronchi, is an important molecule for protecting the respiratory epithelium (71, 80). In contrast to AAT, SLPI blocks elastin-bound elastase in the alveolar walls, which might also protect against the development of lung emphysema (81).

Several studies have reported a direct correlation between SLPI expression levels and tumor progression. It has been shown that patients with non-small cell lung carcinoma have higher serum SLPI levels than healthy individuals, and these levels were found to correlate with tumor stage and response to treatment (31). Moreover, several *in vitro* studies demonstrated that the malignant characteristics of various cancer cells are directly associated with elevated levels of SLPI expression (82, 83). In addition, it was suggested that the pro-malignant activity of SLPI is related to its capacity to inhibit proteinase, but not to its pro-proliferative properties (84). In the present study, we also show that SLPI levels are significantly higher in lung cancer patients compared to controls, but we found no difference in these levels among patients with local and metastatic lung tumors.

In conclusion, a number of studies have reported that serum levels of specific serine proteinase inhibitors are elevated in patients with several types of cancer, including breast, gastric, colorectal and lung cancers (35, 85, 44). In this report, we showed that plasma levels of the serine proteinase inhibitors AAT, ACT and SLPI are increased in lung cancer cases relative to controls, and that AAT and ACT levels are significantly higher in metastatic lung cancer cases compared to cases with localized tumor. Increased levels of antiproteinases may arise from the inflammatory reactions accompanying the cancer process. Further studies are needed to establish the precise role of these inhibitors in lung and other cancers and to reveal the mechanisms that may regulate levels of these inhibitors among tumor types.

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