

Local increase of antiprotease and neutrophil elastase- α_1 -proteinase inhibitor complexes in lung cancer

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ABSTRACT: Tumour progression is dependent on many factors including antiproteases and proteases released by tumour cells or host cells infiltrating the tumour. In the present study, we evaluated the antiprotease content, namely α_2 -macroglobulin (A_2M) and α_1 -proteinase inhibitor (A_1PI) and neutrophil (PMN) elastase complexed with A_1PI , in limited and extended lung cancer patients compared to a nonsmoker and smoker control population. Data showed that A_2M and A_1PI were increased in the involved lung from limited cancer when compared to normals. In extended lung cancer, A_2M content was also increased in the uninvolved side. The concentration of PMN elastase- A_1PI complex was increased on both sides in lung cancer patients ($10.2 \text{ ng}\cdot\text{ml}^{-1}$ in the uninvolved side, $8.2 \text{ ng}\cdot\text{ml}^{-1}$ in the involved side) when compared to nonsmokers ($1.9 \text{ ng}\cdot\text{ml}^{-1}$, $p<0.001$) and smokers ($3.8 \text{ ng}\cdot\text{ml}^{-1}$, $p<0.005$). This increase was not solely due to the smoking habit. We conclude that antiproteases and PMN elastase complexed with antiprotease are increased in lung cancer area. This increase could result in extracellular changes in lung cancer.

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The nature of pulmonary defence against tumours is largely unknown. As in other organs, tumour progression is in part dependent on the presence of growth factors released by tumour cells or by host cells such as lymphocytes, granulocytes, mast cells and macrophages infiltrating the tumour [1]. Some of these factors are proteases known to modify the extracellular matrix [2, 3]. The matrix then becomes suitable for cellular proliferation, differentiation or migration. These proteases also act as intercellular messengers by carrying a mitogenic signal [2, 3]. Thus, the release of cathepsin-B activity has been positively correlated with malignancy and tumour metastases [4, 5].

The role of α_1 -protease inhibitor (A_1PI) and of α_2 -macroglobulin (A_2M), the two major antiproteases, is poorly understood in cancer disease. A_1PI accounts for over 90% of the serum trypsin inhibitory capacity [6] and is mostly produced by the liver. However, alveolar macrophages can also secrete A_1PI [7]. A_2M has the widest spectrum of protease inhibiting activity [8] but its role *in vivo* remains obscure. Synthesis and secretion of A_2M has been demonstrated in culture supernatants from alveolar macrophages [9] and from certain kinds of tumour cells [10-12].

In addition to its protease binding property, human A_2M has been shown to combine *in vitro* with a number of biologically active molecules different from proteases. In this respect, A_2M is considered as a major serum cytotoxic factor for tumour cells [13] and as an immunoregulator agent [14, 15]. In this way, A_2M could modulate

cell functions through the different molecules it carries and delivers to target cells.

Bronchoalveolar lavage (BAL) allows the recovery of alveolar macrophages and lymphocytes and their biochemical products surrounding the tumour process *in vivo* [16]. Macrophages [17-20] and lymphocytes [21-23] are considered as the main effector cells in tumour resistance although conflicting results are reported. The aim of this study was to evaluate the protease inhibitor content, namely A_1PI and A_2M and the neutrophil (PMN) elastase complexed with A_1PI in the surroundings of the tumour in comparison with the contralateral uninvolved side.

We observed a local increase of the two antiproteases in the lung segment involved by the tumour. PMN elastase- A_1PI complexes were found to be increased in the lung on the involved as well as the uninvolved side.

Materials and methods

Study population

Nineteen patients with cancer involving the lung were included in this study. The diagnosis of bronchogenic carcinoma or lymphangitic carcinomatosis suspected at the time of bronchoscopy was confirmed on the basis of histopathological study of tissue obtained either at bronchoscopy or by transparietal needle lung biopsy. Eleven

men and eight women were included in this study; eleven were smokers with a mean smoking habit evaluated at fifty pack yrs, eight were nonsmokers. The average age was 62 yrs ranging from 54–74 yrs. The Karnofsky scale mean was 95 and the mean weight loss over the last six months was 3%.

Sixteen had primary lung cancer: 5 squamous cell carcinoma, (3 at stage 1 of the TNM classification, 2 at stage 3), 10 adenocarcinoma (3 at stage 1, 7 at stage 3) and 1 extended small cell carcinoma. Three had secondary lung cancer: 2 lymphangitic carcinomatosis (1 from gastric adenocarcinoma and 1 from endometrial cancer), 1 had a unique metastasis from breast cancer. All stage 1 patients were considered as limited cancer (n=6), all stage 3 patients, the small cell carcinoma patient and the secondary lung cancer patients were considered as extended cancer (n=13).

Control population

The control population included 14 nonsmoking normal subjects, 9 men and 5 women, mean age 30 yrs (range 22–46 yrs) and 10 smoking normal subjects, 7 men and 3 women, mean age 34.5 yrs (range 25–52 yrs) and a mean smoking habit evaluated at 16.3 pack yrs.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was carried out in all patients according to the following procedure. The upper respiratory tract was anaesthetized with 4% xylocaine, and a fiberoptic bronchoscope was passed through the mouth. Careful examination of the bronchial tree was performed and patients with macroscopic hyperaemia in the cancer area were excluded to avoid blood contamination and no endoscopic lesion was observed. The bronchoscope was securely wedged in a subsegmental bronchus of the right middle lobe for control subjects or in the lobe involved in the cancer process for cancer patients (n=19, involved side). In 12 patients (6 with stage 1 and 6 with stage 3 primary lung cancer) we performed a BAL on the contralateral uninvolved side with the same procedure. A total of 250 ml sterile 0.9% NaCl at room temperature was infused in five 50 ml aliquots and gently aspirated after each infusion. The first 50 ml lavage was discarded to decrease contamination by bronchial secretions and the next four aliquots were pooled and tested for the presence of blood by the Benzidin test. Several patients with blood positive BAL samples were excluded from the present study. The lavage fluid was filtered through a single layer of gauze to remove gross mucus, then centrifuged to separate the cells from the BAL fluid.

Sensitivity of the Benzidin test

The sensitivity of the Benzidin test was assessed by serial dilution of heparinized venous blood up to 1/1000 to reach a similar range of albumin concentration to that observed in BAL. Diluted blood samples (1/1000) were

still Benzidin positive suggesting that major blood contamination of BAL fluid could be detected by this method.

Cell count

A total cell count and a differential count were performed respectively, on a Coulter cell counter and on cytocentrifuged preparations stained by May-Grünwald-Giemsa stain. The results are expressed as total number of cells, macrophages and lymphocytes $\times 10^4 \cdot \text{ml}^{-1}$ of BAL recovered. Red blood cells (RBC) were counted on a Coulter cell counter in some BAL samples (normals, n=5; cancer, n=10). The lavage supernatant was stored at -20°C for protein analysis.

Protein assays

Serum levels of albumin, immunoglobulin G and M (IgG, IgM), A₁PI and A₂M were determined by immunonephelometry [24]. The immunoradiometric assay (IRMA) was used for the measurement of all proteins in BAL. This assay, previously described in detail [24], provided a sensitivity in the nanogram per millilitre range and does not require a concentration of the BAL fluid. BAL samples were diluted in 20% horse and goat serum in phosphate buffered saline, pH 7.4. Results of BAL are expressed as previously in coefficient of excretion relative to albumin (RCE) to correct for both serum concentration of the different proteins and variable dilution of BAL [24].

$$\text{RCE} = \frac{\text{BAL (protein)/BAL (albumin)}}{\text{Serum (protein)/Serum (albumin)}}$$

BAL protein data are also presented as their ratio to BAL albumin concentration.

PMN elastase- α_1 -proteinase inhibitor complex levels in BAL

The PMN elastase-A₁PI complex concentration was determined on the concentrated fluids obtained with dialysis bags under vacuum with a cut-off of 12,000 Da molecular weight using the enzyme-linked immunosorbent assay (ELISA) described in detail by NEUMANN *et al.* [25] and purchased from Merck (D-6100 Darmstadt). Briefly, the samples were added to microtitration plates coated with sheep anti-PMN elastase IgG. This antibody does not cross-react with cathepsin-G and other neutrophil proteinases. After incubation and washing, the solid phase-bound elastase-A₁PI complexes were further incubated with alkaline phosphatase-labelled rabbit anti-A₁PI IgG. After further washings, p-nitrophenylphosphate was added to measure the amount of solid phase-bound elastase-A₁PI complexes. This assay was calibrated using a standard solution of known elastase-A₁PI concentration. The BAL volumes observed before and after the concentration process were recorded and their ratio was multiplied by the elastase-A₁PI complex value in concentrated

BAL to express the data per millilitre of unconcentrated BAL. PMN elastase-A₁PI complexes were not measured in the serum.

Statistical methods

Data were tested for normality and are expressed as mean±SD. Paired t-tests were used to compare data from BAL of the uninvolved and the involved side in lung cancer patients. For other comparisons, unpaired t-tests were used; p values of less than 0.05 were considered as significant.

Results

Volume and cellular population recovery

BAL volumes, total cell counts and total number of macrophages and lymphocytes are given in table 1. As expected, the total number of macrophages is increased in the normal smokers (p<0.01) compared to the nonsmokers. In limited lung cancer, the same increase (p<0.01) is observed irrespective of the side involved compared to the normal nonsmokers. This increase may also be related to the smoking habit. In extended lung cancer,

Table 1. – Volumes and cellular components of BAL

	Normal nonsmokers (n=14)	Normal smokers (n=10)	Limited cancer (n=6)		Extended cancer (n=13)	
			uninvolved	involved side	uninvolved	involved side
Volume recovered ml	161±19 (130–190)	129±27 (67–120)	92±25 (50–130)	105±42 (30–150)	104±39 (45–160)	94±44 (40–172)
% BAL recovered	81±10 (72–95)	67±9 (58–81)	43±12 (33–50)	55±21 (20±75)	57±18 (30–80)	51±18 (26–55)
Total cell count ×10 ⁴ ·ml ⁻¹	165±44 (113–252)	435±304* (158–1026)	453±365* (192–1098)	437±184* (173–710)	473±360* (140–1160)	240±160* (80–600)
Macrophage count ×10 ⁴ ·ml ⁻¹	146±38 (68–238)	424±302* (155–1015)	400±328* (170–977)	370±163* (152–617)	418±363* (120–1130)	205±128 (65–420)
Lymphocyte count ×10 ⁴ ·ml ⁻¹	15±17 (1.6–57)	6.3±6.03 (0.5–23)	52±35* (17–110)	55±46* (72–140)	18.6±8.2 (13–35)	8±6 (2–60)
PMN count ×10 ⁴ ·ml ⁻¹	0.5±1.6 (0–3.5)	2.9±3.2* (2–11)	4.0±7.6 (0–19.5)	6.7±10.5* (0–27)	3.6±8.0 (0–20)	10.6±21 (0–75)

Results expressed as mean±SD, range in brackets; BAL: bronchoalveolar lavage; PMN: polymorphonuclear neutrophils; *: p<0.05 when compared to normal nonsmokers.

Table 2. – Protein component in BAL and in serum

		Normal nonsmokers	Normal smokers	Limited cancer		Extended cancer	
				uninvolved	involved side	uninvolved	involved side
BAL	Alb μg·ml ⁻¹	17.7±12.3 (9–55)	17.5±7.5 (9.8–29.4)	30.4±18.4 (16–67)	75.9±72.3 (25–210)	36.1±15.2 (19–60)	170.4±233.6 (20–820)
	IgM (RCE)	0.08±0.05 (0.01–0.2)	0.03±0.01* (0.01–0.06)	0.02±0.02* (0.01–0.07)	0.1±0.06† (0.03–0.13)	0.13±0.07† (0.01–0.23)	0.27±0.28* (0.05–1.08)
	IgG (RCE)	0.71±0.3 (0.13–1.4)	0.94±0.38 (0.52–1.23)	0.83±0.45 (0.11–1.7)	1.1±0.38 (0.35–1.5)	1.17±0.3 (0.3–1.9)	1.02±0.6 (0.2–3.2)
Serum	IgM mg·ml ⁻¹	0.62±0.4 (0.03–1.2)	1.73±1.5* (0.38–6)	2.1±1.7** (0.85–5.2)		1.6±1.17** (0.7–2.7)	
	A ₁ PI	2.4±0.54 (1.8–4.1)	1.7±1* (0.8–4.4)	2.3±1.1 (0.7–3.8)		2.65±1.0 (0.52–3.8)	
	A ₂ M	1.66±0.7 (0.97–3.5)	1.66±0.4 (1–2.3)	1.6±0.76 (0.57–2.8)		1.72±0.63 (0.6–2.3)	

Results are expressed as mean±SD, range in brackets; BAL: bronchoalveolar lavage; Alb: albumin; IgG, IgM: immunoglobulin G and M, respectively; RCE: coefficient of excretion relative to albumin; A₁PI: α₁-protease inhibitor; A₂M: α₂-macroglobulin; *: p<0.05 when compared to normal nonsmokers; **: p<0.01 when compared to normal nonsmokers; †: p<0.05 when compared to normal smokers.

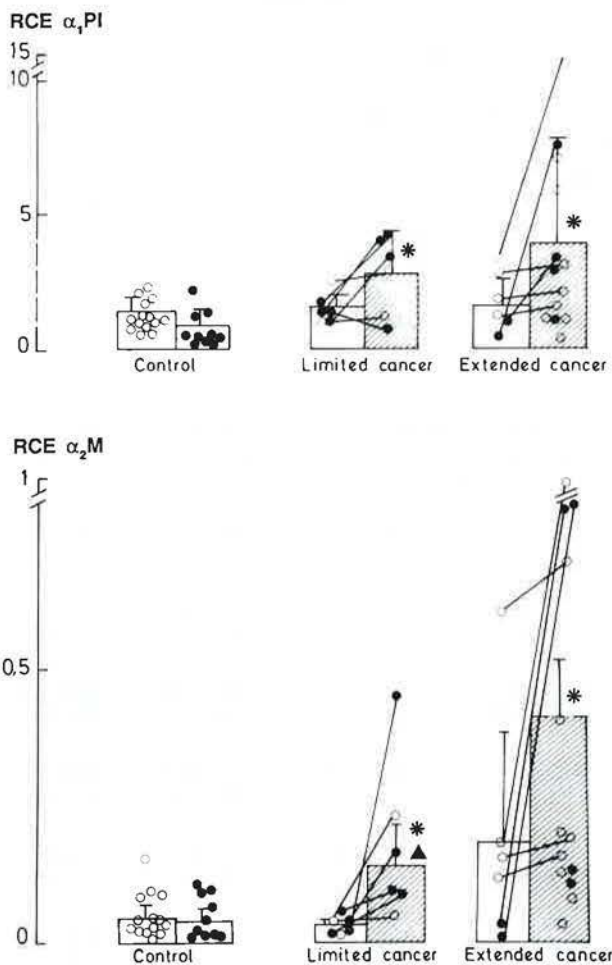


Fig. 1. - Coefficient of excretion relative to albumin (RCE) of α_2 -macroglobulin (A_2M) and α_1 -protease inhibitor (A_1PI) in normal population (control), in limited and extended cancer; comparison between uninvolved (open columns) and involved side (hatched columns); *: $p < 0.05$ when compared to the control group; #: $p < 0.05$ when compared to the control nonsmokers; \blacktriangle : $p < 0.05$ when compared to the uninvolved side, paired t-test. Columns represent means, vertical bars represent SD. Open circle: nonsmoker; closed circle: smoker.

only the uninvolved side shows an increase of macrophages. The total number of BAL lymphocytes tends to decrease in the smoker group compared to the nonsmokers. In contrast, the total number of BAL lymphocytes is increased in both involved and uninvolved sides from limited cancer patients ($p < 0.05$) without change in the T-helper and T-suppressor subpopulations compared to the normal group (data not shown). No change is observed in the blood from these patients in the different groups.

As expected, the total number of neutrophils in BAL is increased in the normal smokers ($p < 0.02$) compared to the nonsmokers. This increase of neutrophils is also observed in limited lung cancer and may be related to the smoking habit as well as to the age of the patients. The mean count of RBC was 40 per microlitre (ranges from 0-200) in lung cancer and 10 per microlitre (ranges

from 0-40) in normal subjects. High RBC counts were not associated with increased levels of albumin in BAL.

Protein components

1) A_1PI , A_2M , IgM and IgG in limited and extended lung cancer. When proteins are measured in the serum (all except PMN elastase- A_1PI complexes), data are expressed as coefficient of excretion relative to albumin (RCE; table 2, fig. 1). Data are also expressed as an albumin relative concentration ratio (AR ng per ng BAL albumin $\times 10^{-3}$; table 3).

Comparisons between the normal and the cancer group demonstrated that the coefficient of excretion relative to albumin (RCE) was similar for IgG but not for A_1PI , A_2M and IgM. In lung cancer a significant increase of RCE for A_2M is observed on the involved side in both limited (mean 0.17, $p < 0.005$) and extended cancer (mean 0.36, $p < 0.005$) and even on the uninvolved side in extended cancer (mean 0.18, $p < 0.005$) compared to the normal group (mean 0.05) and without change in serum concentrations. For A_1PI RCE, no difference is observed in BAL between smokers and nonsmokers. In contrast, BAL A_1PI ratio to albumin (AR) and serum concentration are significantly decreased in the smoker group (mean A_1PI AR 16.9; mean A_1PI serum concentration 1.7 $ng \cdot ml^{-1}$) compared to the nonsmoker group (mean A_1PI AR 69.1, $p < 0.05$; mean A_1PI serum concentration 2.4 $mg \cdot ml^{-1}$, $p < 0.05$). In the lung cancer group, a significant increase of A_1PI RCE is observed on the involved side in both limited (mean 2.7, $p < 0.005$) and extended cancer (mean 3.8, $p < 0.02$) compared to the normal group without difference in A_1PI serum concentration. In contrast to A_2M , no increase is observed on the uninvolved side in extended cancer. In normals, smoking habit is associated with a decrease of IgM RCE (mean 0.03) and AR (mean 0.6) compared to the normal nonsmokers (mean RCE 0.084, $p < 0.01$; mean AR 1.3, $p < 0.05$). This contrasts with the increase of IgM serum concentration in smokers (mean 1.74 $mg \cdot ml^{-1}$) compared to the nonsmokers (mean 0.62 $mg \cdot ml^{-1}$, $p < 0.02$). In the lung cancer group, smoking habit may also explain the decrease of BAL IgM observed on the uninvolved side in limited cancer (mean RCE 0.02, $p < 0.05$) compared to the normal group (nonsmokers and smokers). On the involved side, there is an increase of RCE in both limited (mean 0.09) and extended cancer (mean 0.27, $p < 0.02$).

In contrast, in both the extended (mean 1.6, $p < 0.02$) and the limited (mean 2.1, $p < 0.01$) lung cancer patients, we observed an increase of IgM serum concentrations, as in smoking patients, compared to nonsmoking patients (mean 0.62, $p < 0.02$ and $p < 0.01$, respectively).

2) PMN elastase- A_1PI complexes in lung cancer. Data are expressed as absolute concentration ($ng \cdot ml^{-1}$, fig. 2) and albumin relative concentration (ng per ng BAL albumin $\times 10^{-3}$, table 3). Increased levels and ratio are observed in smokers (mean 3.8 $ng \cdot ml^{-1}$) compared to nonsmokers (mean 1.9 $ng \cdot ml^{-1}$, $p < 0.02$). Moreover, PMN elastase- A_1PI complex levels are increased much more in BAL from the lung cancer patients regardless of the

Table 3. — Albumin ratios (AR) of proteins in BAL

BAL protein/BAL Alb	Normal nonsmokers (n=14)	Normal smokers (n=10)	Lung cancer (n=19)	
			uninvolved	involved side
A ₁ PI ng·ngAlb × 10 ⁻³	69.1±34.8 (30–170)	16.9±13.4* (4–40)	125±66.7† (20–240)	328±692.8† (4–3200)
PMN Elastase-A ₁ PI	0.06±0.01 (0.04–0.09)	0.32±0.13* (0.2–0.6)	1.7±2.3* (0.2–6.2)	3.4±5.3* (0.4–14)
A ₂ M	1.6±1.3 (0.4–4.4)	2.6±4.5 (0.4–16)	3.7±5.3 (0.2–20)	20.9±43.4† (0.7–200)
IgG	192.6±88 (50–340)	225±108 (130–480)	251±153 (20–550)	450±761 (4–3600)
IgM	1.3±0.8 (0.1–2)	0.6±0.5* (0.2–1.6)	3.6±5.5 (0.1–20)	27.5±81.3 (1–370)

See legend to table 2. *: p<0.05 when compared to normal nonsmokers; †: p<0.05 when compared to normal smokers.

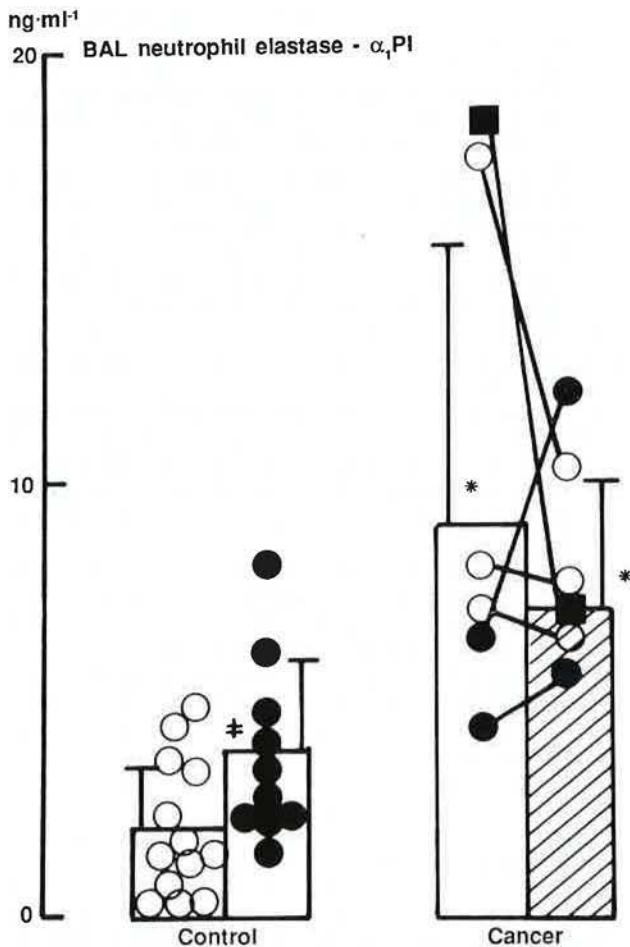


Fig. 2. — Bronchoalveolar lavage (BAL) neutrophil elastase α_1 -protease inhibitor (A₁PI) complex levels in the control nonsmokers (open circle) and smokers (closed circle) and in five limited lung cancer and one extended (■) lung cancer patients; comparison between uninvolved (open columns) and involved side (hatched columns); ‡: p<0.05 when compared to the nonsmoker control group; *: p<0.05 when compared to the control group.

side (uninvolved, mean 9.3 ng·ml⁻¹) compared to both the nonsmokers (p<0.001) and the smokers (p<0.005). This increase can be partly related to the smoking habit but is generally higher in lung cancer than in normal smokers and high levels are observed in nonsmoker lung cancer patients.

Discussion

In the present study we observed a local increase of antiproteases and PMN elastase-A₁PI complexes in BAL from patients with lung cancer. When the tumoural process is limited, as observed in stage 1 patients, the increase of both A₁PI and A₂M is restricted to the lung segment involved by the cancer and is not observed in the contralateral uninvolved side (fig. 1). This local increase is not associated with an increase in serum concentrations. In the extended lung cancer patients, as in stage 3 patients and in lymphangitis carcinomatosa, A₂M is increased on both the involved and uninvolved side, whereas A₁PI is only increased on the involved side. The increase of PMN elastase-A₁PI complexes observed in lung cancer is not related to the side of involvement and not solely related to the smoking habit since high levels are also observed in nonsmoker lung cancer patients. No correlation was observed with the PMN count in BAL. The highest level is observed in a case of unique metastasis from breast carcinoma and the lowest in a case of limited lung cancer.

Different studies have reported an increase of proteolytic activity in cancer and have suggested a role of proteases in tumoural progression and metastasis [4, 5]. The local increase of PMN elastase-A₁PI complexes demonstrates indirectly the local increase of PMN elastase in the lung cancer area. However, the ELISA used in our study only detected the portion of elastase complexed to A₁PI and not the elastase bound to A₂M or the free elastase present in the lower respiratory tract.

A₁PI is a true antiprotease that can neutralize proteolytic activity such as neutrophil elastase [26] known to be able to destroy the elastin component of the extracellular matrix. The increase of PMN elastase-A₁PI complexes observed in our study confirms the antiproteolytic role of A₁PI. This effect can be prevented by the formation of a complex between PMN elastase and A₂M [27]. However, functional studies are required to determine whether the bronchoalveolar increase of A₁PI observed in lung cancer can efficiently inhibit the increased levels of elastase in the presence of increased levels of A₂M.

Contrary to JOCHUM *et al.* [28], we found different levels of PMN elastase-A₁PI complexes between the healthy nonsmokers and smokers. This discrepancy could be explained by an acute effect of smoking as previously reported by FERA *et al.* [29], since our healthy smokers continued to smoke right up to the time of the BAL procedure, whereas the patients of JOCHUM *et al.* stopped 24 h beforehand [28].

In our study, the local increase of A₂M seems to be closely related to the cancer process and to its spread supporting the possible competition between A₂M and A₁PI and subsequently the lesser inactivation of PMN elastase. However, although we corrected BAL values for A₂M serum concentration (when data were expressed in RCE), we cannot completely rule out that part of the A₂M rise in BAL is related to increased transudation of high molecular weight proteins in diseased patients. In this respect, a local increase of IgM is also observed in the lung involved by the cancer. Since these two proteins, A₂M and IgM, have a similar molecular weight, their local increase could be attributed only to a passive transudation. However, we did not find any correlation between the RCE of A₂M and IgM supporting the fact that their increase in BAL is not solely related to passive transudation but could be due either to a local secretory process or to an impairment of A₂M uptake [30].

Increased levels of antiproteases have previously been reported in BAL from interstitial lung disease patients [31] and A₂M appears to be a sensitive index of activity in lung inflammatory disease [32]. The increased levels of A₂M observed in lung cancer could be attributed to the inflammatory reaction accompanying the cancer process.

In conclusion, in the present study we observed a local enrichment of antiprotease and protease complexed to antiprotease in lung cancer. This increase could result in extracellular matrix changes in lung cancer.

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Augmentation locale des antiprotéases et des complexes élastase- α -antiprotéase dans le cancer du poumon. F.X.P. Marchandise, B. Mathieu, C. Francis, Y. Sibille.

RÉSUMÉ: La progression tumorale dépend de nombreux facteurs, parmi lesquels les antiprotéases et les protéases produites par les cellules tumorales ou les cellules hôtes infiltrant la tumeur. Nous avons évalué, dans la présente étude, le contenu en antiprotéases et en particulier en α -₂-macroglobuline (A_2M) et en α -₁-antiprotéase (A_1PI), ainsi que l'élastase des neutrophiles complexés avec l' α -₁-antiprotéase, chez des patients atteints de cancer du poumon limité ou étendu, par comparaison avec une population contrôlée de non fumeurs et de fumeurs. Nos observations montrent que, par comparaison avec les normaux, il y a une augmentation de A_2M et de A_1PI dans le poumon atteint en cas de cancer limité. Dans les cancers étendus, le contenu en A_2M est augmenté en outre du côté non atteint. La concentration du complexe PMN élastase- A_1PI est augmentée des deux côtés chez les patients atteints de cancer du poumon (10.2 ng·ml⁻¹ du côté sain, 8.2 ng·ml⁻¹ du côté atteint), par comparaison avec les non fumeurs (1.9, p<0.001) et avec les fumeurs (3.8, p<0.005). Cette augmentation n'est donc pas due exclusivement à l'habitude tabagique. Nous concluons que les antiprotéases ainsi que l'élastase des polynucléaires complexés avec l' A_1PI sont augmentées dans la zone du cancer pulmonaire. Cette augmentation pourrait entraîner des modifications extra-cellulaires dans le cancer du poumon. *Eur Respir J.*, 1989, 2, 623–629.