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Increased TGFβ_1 plasma level in patients with lung cancer: potential mechanisms

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Abstract

Background Plasma transforming growth factor β_1 (TGFβ_1) levels are elevated in patients with lung cancer. As TGFβ_1 is mainly found in platelets and as nonmalignant pulmonary diseases (NMPD) are frequently associated with lung cancer, we investigated the potential contribution of platelet degranulation and/or of a concomitant NMPD to the increased plasma levels of TGFβ_1 reported in patients with lung cancer.

Materials and Methods Blood samples were collected in duplicate from 30 healthy subjects, 14 patients suffering from NMPD and 37 patients with lung cancer. The platelet count was determined and the samples were processed to obtain plasma. One sample was collected in EDTA (EDTA plasma) and the other in a mixture inhibiting platelet degranulation (PIM plasma). TGFβ_1 concentrations and β-thromboglobulin (βTG) levels, an index of platelet degranulation, were measured in both plasma samples.

Results TGFβ_1 and βTG plasma levels measured in PIM plasma were lower than those obtained in EDTA plasma. With respect to PIM plasma, both TGFβ_1 and βTG levels were higher in patients with lung cancer than those with NMPD and in healthy individuals. In patients with NMPD, only TGFβ_1 levels were increased as compared to healthy controls, βTG levels being similar.

Conclusion Methods for collecting and processing blood samples are critical in determining reliable circulating TGFβ_1 levels. Increased TGFβ_1 plasma levels observed in patients with lung cancer are related, at least partly, to concomitant NMPD and also to platelet degranulation as proved by increased βTG levels.

Keywords β-thromboglobulin, lung cancer, platelets, pulmonary disease, TGFβ_1.
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Introduction

In patients with lung cancer, blood levels of transforming growth factor beta 1 (TGFβ_1) are elevated. Therefore, TGFβ_1 has been suggested as a potential marker for monitoring lung tumour response to therapy [1,2]. The TGFβ superfamily includes several multifunctional regulatory polypeptides, which contribute to many physiological and pathological processes [3–9]. TGFβ is synthesized by several types of normal and malignant cells, including macrophages, lymphocytes, megakaryocytes, endothelial cells, chondrocytes as well as leukemia cells and cells from glioblastoma, lung and gastric carcinoma. At least five isotypes of TGFβ have been identified [8–10]. The isoforms TGFβ_1, TGFβ_2 and TGFβ_3 have been found in mammalian tissues, TGFβ_1 being the predominant isoform in human plasma [11]. In humans, TGFβ_1 is mainly found in the α-granules of platelets [4,12] and it can be released into the plasma in response to platelet activation [13]. Thus, platelet activation and degranulation should be avoided during blood sampling and processing in order to measure true circulating TGFβ_1 plasma levels. Because collection of plasma without causing platelet activation requires specific procedures, the possibility that previously reported levels of TGFβ_1 in plasma might

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derive in part from platelet degranulation due to inadequate blood drawing, preanalytical sample handling and/or processing can not be ruled out [11,13,14].

Elevated plasma $TGF\beta_1$ concentrations have been reported in various malignant and benign conditions, including fibrotic diseases such as hepatic venoocclusive disease or treatment associated pulmonary fibrosis [15–19]. Because lung cancer is frequently associated with nonmalignant pulmonary disease (NMPD), one may argue that elevated TGFβ levels in patients with lung carcinoma could also be related to concomitant NMPD. To date, the mechanisms responsible for the increased $TGF\beta_1$ levels reported in patients with lung cancer are unclear. Two mechanisms have been proposed previously, namely a direct production of $TGF\beta_1$ by the tumour itself or an interaction between tumour and normal pulmonary cells resulting in an increased production of $TGF\beta_1$ [3,17].

The present study investigated the potential contribution of platelet degranulation and/or of concomitant NMPD to increased $TGF\beta_1$ plasma levels observed in lung cancer patients.

Materials and methods

Population

After approval of the study protocol by the hospital ethics committee, 30 blood donors and 51 patients gave their informed consent and were included in the study. The 30 healthy blood donors, used as controls, filled in a medical questionnaire and underwent a physical examination to exclude any disease. The age, gender and smoking history of each individual were recorded. Among the 51 patients, 37 had a diagnosis of primary lung cancer (LC group) while 14 had pulmonary non malignant pulmonary disease (NMPD group). NMPD patients were carefully examined to rule out any malignant lesion and/or thrombotic process. Among those, seven patients had chronic obstructive disease (COPD) according to the American Thoracic Society criteria [20], five others had moderate fibrotic pattern with no sign of evolutive disease and two were heavy smokers with a mixed obstructive/fibrotic pattern. In the LC group, there were 23 squamous-cell carcinoma, eight adenocarcinoma, four anaplastic carcinoma and two unclassified tumours. After assessment of the extent of the neoplasm according to the TNM classification [21], five LC patients were assigned to stage II, eight to stage III_A, 13 to stage III_B and 10 to stage IV. In one case, the clinical stage could not be determined because the patient died before the extent of the disease was assessed. All tumours were active at the time of blood sampling. Blood was collected before any treatment in 25 patients and after chemotherapy but before radiotherapy in 12 patients.

Pulmonary function tests

In the 51 patients, measurements of lung volumes and ventilatory mechanics were performed using a pneumotachograph and a body plethysmograph. Pulmonary diffusing capacity was also assessed using the single breath carbon monoxide test ($DL_{CO}SB$). Vital capacity (VC), forced expiratory volume in 1 s (FEV_1), FEV_1/VC ratio and the carbon monoxide diffusing capacity per unit of alveolar volume (T_kCO) were reported as percent of the predicted value for normal subjects with identical characteristics.

Blood sampling and processing

Blood samples were taken using a rigorous technique to avoid *ex vivo* platelet degranulation. To minimize blood stasis and turbulences, tourniquets, vacutainers and syringes were not used. A 21G needle connected to silicone tubing (butterfly needle) was inserted in an antecubital vein. Blood was allowed to flow freely by gravity through the needle and the silicone tubing into the collection tubes. The first 3 mL of blood were discarded to avoid contamination of the samples by tissue thromboplastin. Subsequent 3 mL of blood were collected into two prechilled tubes, one containing EDTA and the other a mixture that aimed at preventing platelet degranulation. This platelet degranulation inhibiting mixture (PIM) consisted of ACD (formule A) anticoagulant (0·5 mL) containing potassium EDTA (1 mM), adenosine (1 mM) and prostaglandin E_1 (1 μM). The tubes were gently mixed while sampling the blood. Each tube was immediately placed in a slurry of ice and water. At least 15 min after sampling and within 1 h, blood was centrifuged at 4000 *g* for 30 min. The middle third of the plasma supernatant was collected and aliquots were stored at –70 °C until assay [22]. All assays were performed within 3 months.

$TGF\beta_1$ assay

After activation of latent $TGF\beta_1$ by acidification, $TGF\beta_1$ measurements were performed by ELISA using the Quantikine kit (R & D Systems, R & D Systems Europe Ltd, Abingolon, UK) which specifically detects $TGF\beta_1$, but not the other isoforms. $TGF\beta_1$ levels were expressed in ng mL^{-1}.

Platelet marker assay

Plasma levels of β-thromboglobulin (βTG, ng mL^{-1}), used to evaluate the extent of platelet degranulation, were measured by ELISA using the commercially available kit Asserachrom βTG (Diagnostica Stago, Franconville, France).

Statistical analysis

Results are expressed as mean ± SD or as proportions. For some variables, a log transform was used to normalize the distribution. Mean values were compared by one-way analysis of variance followed by multiple testing, whereas paired data were compared by Student's *t*-test. Proportions were compared using the Chi-square test for contingency tables.

The association between variables was evaluated using the classical correlation coefficient. Results were considered to be significant at the 5% critical level ($P < 0{\cdot}05$). All calculations were carried out using the SAS statistical software (version 6·12 for Windows) (SAS Institute, Cary, NC, USA).

Results

The characteristics of the study groups are described in Table 1. Control subjects were significantly younger than patients. Lung cancer patients were also significantly older than those with nonmalignant pulmonary disease. The three groups were similar with respect to gender. Although the proportion of current smokers was similar in the three groups, all NMPD and LC patients had a smoking history, whereas only 12 (40%) of healthy subjects had such a record. The pulmonary function tests did not differ between the NMPD and LC groups. Patients from both groups had mild restrictive impairment, moderate obstructive disease and moderately reduced TkCO. Platelet count was significantly higher in the LC group than in the NMPD and control groups.

TGFβ$_1$ and βTG levels measured in EDTA plasma were significantly higher than those observed in PIM plasma regardless of the study group (Table 2). In EDTA plasma, TGFβ$_1$ and βTG were similar in the LC and NMPD groups but higher than in the control group. A statistically significant correlation was found between TGFβ$_1$ and βTG in the control group ($r = 0{\cdot}57$, $P < 0{\cdot}001$), NMPD group ($r = 0{\cdot}71$, $P < 0{\cdot}01$) and LC group ($r = 0{\cdot}64$, $P < 0{\cdot}0001$). By contrast, in PIM plasma, TGFβ$_1$ and βTG levels in the LC group were significantly higher than those in the control and NMPD groups (Table 2). Moreover, in the latter group, TGFβ$_1$ concentrations were also higher than in controls, whereas βTG levels were similar. A significant correlation was found between TGFβ$_1$ and βTG levels in the control group ($r = 0{\cdot}52$, $P < 0{\cdot}005$) and LC group ($r = 0{\cdot}34$, $P < 0{\cdot}05$) but not in the NMPD group ($r = 0{\cdot}09$, $P = 0{\cdot}76$).

In both plasma, TGFβ$_1$ and βTG were unrelated to platelet count, despite the fact that platelets were significantly higher in LC patients. Moreover, no significant correlation was found between TGFβ$_1$ nor βTG plasma levels and age. TGFβ$_1$ and βTG plasma levels were similar in smokers and nonsmokers.

In EDTA as well as in PIM plasma, levels of TGFβ$_1$ and of βTG were comparable across the various cancer cell types (Table 3). Likewise, no difference in TGFβ$_1$ and βTG plasma levels were observed between the different clinical stages of lung cancer (Table 4).

Discussion

Two salient findings emerged from our investigation on the mechanisms potentially responsible for the elevated TGFβ$_1$ plasma levels reported in patients with lung cancer. Firstly,

Table 1 Characteristics (mean ± SD) of the study groups

Variable	Control (n = 30)	Non Malignant Pulmonary Disease (n = 14)	Lung Cancer (n = 37)
Age (years)	44 ± 9	60 ± 11*	68 ± 9†
Sex ratio (M/F)	21/9	11/3	33/4
Smokers (%)	33	57	46
VC (%)		79 ± 19	74 ± 19
FEV$_1$ (%)		65 ± 29	61 ± 20
FEV$_1$/VC (%)		63 ± 21	71 ± 18
TkCO (%)		59 ± 24	69 ± 30
Platelet count ($\times 10^3$ mL^{-1})	256 ± 50	248 ± 115	341 ± 124†

Results of pulmonary tests are expressed as percentages of predicted values.
*$P < 0{\cdot}05$ (vs. control); †$P < 0{\cdot}05$ (vs. NMPD and control).

Table 2 TGFβ$_1$ and βTG levels (ng mL^{-1}, mean ± SD) in plasma from blood collected in EDTA and in platelet degranulation inhibiting mixture (PIM) for the three study groups

	TGFβ$_1$ (ng mL^{-1})		βTG (ng mL^{-1})	
Group	EDTA	PIM	EDTA	PIM
Control (n = 30)	2·4 ± 1·5	1·4 ± 0·5‡	103 ± 75	18·3 ± 14·7‡
NMPD (n = 14)	10·6 ± 5·2*	2·2 ± 0·5*‡	209 ± 92*	22·0 ± 8·2‡
Lung cancer (n = 37)	10·3 ± 5·5*	3·2 ± 1·0†‡	227 ± 74*	39·9 ± 22·5†‡

*$P < 0{\cdot}05$ (vs. control); †$P < 0{\cdot}05$ (vs. NMPD and control); ‡$P < 0{\cdot}05$ (vs. EDTA).

Table 3 TGFβ_1 and βTG levels (mean ± SD) in EDTA and PIM plasma of cancer patients according to cell type

	TGFβ_1 (ng mL^{-1})		βTG (ng mL^{-1})	
Cell type	EDTA	PIM	EDTA	PIM
Squamous cell carcinomas (*n* = 23)	11·2 ± 5·4	3·0 ± 0·9	232 ± 52·7	41·3 ± 23·8
Adenocarcinomas (*n* = 8)	8·1 ± 4·1	3·5 ± 0·8	197 ± 88·9	31·6 ± 9·5
Anaplastic carcinoma (*n* = 4)	11·2 ± 9·1	3·2 ± 0·6	269 ± 144	43·5 ± 35·6

Table 4 TGFβ_1 and βTG levels (mean ± SD) in EDTA and PIM plasma of cancer patients according to clinical stage

Clinical stage*	TGFβ_1 (ng mL^{-1})		βTG (ng mL^{-1})	
	EDTA	PIM	EDTA	PIM
II (*n* = 5)	9·0 ± 4·3	3·9 ± 0·6	219 ± 72·7	59·6 ± 41
IIIA (*n* = 8)	10·3 ± 6·7	2·8 ± 0·7	202 ± 81·2	28·3 ± 9·6
IIIB (*n* = 13)	11·5 ± 5·8	3·1 ± 1·0	270 ± 55·7	42·0 ± 20·2
IV (*n* = 10)	9·8 ± 5·3	3·4 ± 0·8	201 ± 77·2	37·1 ± 17·4

*According to the TNM classification of the UICC 1997 [21].

TGFβ_1 plasma levels critically depend on the technique used for blood sampling and processing. Secondly, patients with lung cancer or non malignant pulmonary disease had similarly elevated TGFβ_1 and βTG levels in EDTA plasma. Under carefully controlled sampling conditions (PIM plasma), both TGFβ_1 and βTG levels were increased in patients with lung carcinoma as compared to the two other groups, while NMPD patients had only elevated TGFβ_1 levels.

A large number of proteins are found in the α-granules of the platelets. They include platelet-derived growth factor (PDGF), platelet factor 4 (PF4), β-thromboglobulin (βTG) and transforming growth factor β_1 (TGFβ_1). All these substances are released from the α-granules into the plasma when platelets are activated by various stimuli. TGFβ is released by the platelets in an active form and quickly binds to a carrier protein identified as being α_2-macroglobulin. In our study, TGFβ_1 was released in an active form from its complex with α_2-macoglobulin by acid treatment before measurement [23]. Thus, high TGFβ_1 or βTG plasma levels may be related to their release into plasma during degranulation of activated platelets. To assess the extent of platelet degranulation, specific markers can be used. For that purpose and taking into account its stability in plasma (halflife > 90 min), βTG was selected [24].

In the present study, agents preventing platelet release reaction were used in PIM plasma to prevent an *ex vivo* increase of βTG in collected samples. They resulted in lower βTG plasma levels in PIM than in EDTA plasma. This procedure was also associated with lower TGFβ_1 plasma levels, strongly suggesting that *ex vivo* platelet degranulation significantly contributed to the high TGFβ_1 plasma levels observed in plasma from blood collected into EDTA. This is further supported by the significant relationships observed between βTG and TGFβ_1 levels. Our results are consistent with other reports suggesting that strict protocols have to be used for the measurement of TGFβ_1 in blood plasma samples [13,14]. Therefore, assessment of platelet degranulation might be useful in studies reporting TGFβ_1 levels.

Because TGFβ_1 released during platelet activation contributes significantly more to levels of TGFβ_1 measured in EDTA plasma than in PIM plasma, subsequent discussion is exclusively based on measurements in plasma from blood collected in PIM. Under these conditions, TGFβ_1 levels were found to be significantly increased in patients with lung cancer and, to a lesser extent, in patients with NMPD. This is unlikely to be related to the age of these patients, our results indicating that age and TGFβ_1 plasma levels are not correlated. This lack of correlation has been previously reported [1].

Our results are in agreement with previous studies reporting that plasma TGFβ_1 levels are increased in many patients with lung cancer [1,2]. As suggested in previous reports, increased plasma levels of TGFβ_1 in patients with lung cancer may be related to direct production of TGFβ_1 by the tumour and more specifically by the tumour-associated stromal cells [3,17]. However, our results suggest the involvement of additional mechanisms to explain the increased TGFβ_1 plasma levels found in these patients.

A second possible explanation for increased TGFβ_1 in patients with lung cancer could be the presence of a concomitant pulmonary disease, as patients with NMPD also showed increased TGFβ_1 plasma levels. Similar results of pulmonary function tests in both groups of patients (NMPD and LC) and high levels of TGFβ_1 in patients with NMPD disease support this possibility. The similar βTG levels observed in controls and in NMPD patients suggest that platelet activation was not responsible for the increased TGFβ_1 level we found in the latter group. Increased plasma TGFβ_1 observed in patients with nonmalignant pulmonary disease suggest that TGFβ_1 might be involved in the process leading to nonmalignant lung disease. Also, they are consistent with studies showing that TGFβ_1 may have a role in several benign conditions, including fibrotic lung disease in humans and experimentally induced lung fibrosis in animals [25–32].

However, in our series, TGFβ_1 plasma levels were significantly higher in patients with lung carcinoma than in those with nonmalignant pulmonary disease. This suggests that at least one other mechanism is involved in the increased TGFβ_1 plasma levels observed in patients with lung cancer. A third additional cause may be an increased *in vivo* release of TGFβ_1 from platelet α-granules. This is suggested by βTG levels that were exclusively increased in patients with lung cancer. Increased platelet release of TGFβ_1 could result from the thrombocytosis we found in patients with lung cancer. Thrombocytosis is frequently observed in patients with lung cancer and has even been proposed as a prognostic factor in such patients [33–38]. The lack of correlation between TGFβ_1 plasma levels and platelet counts does not disclose this hypothesis. It is possible that the increased plasma TGFβ_1 in lung cancer patients results from a higher rate of degranulation of the platelets. This observation has been previously reported in patients suffering from lung cancer [39]. Mechanisms potentially involved include increased generation of thrombin, a strong activator of platelet degranulation, induced by procoagulant substances released from the tumour and the associated inflammatory cells [40–43]. Moreover, high platelet degranulation rate could be related to the production of young and more reactive platelets into the blood that occurs in patients with thrombocytosis [43].

Conclusion

Adequate blood sampling and processing conditions are critical to obtain reliable and valid measurements of TGFβ_1 plasma levels. *Ex vivo* platelet activation and contamination of plasma by TGFβ_1 released from platelet α-granules must be prevented and should be assessed in studies reporting TGFβ_1 blood levels as even the best methods of plasma collection may result in some platelet degranulation on occasion. TGFβ_1 might be involved in the process leading to nonmalignant lung disease as this category of patients was found to have elevated TGFβ_1. Increased plasma TGFβ_1 measured in patients with lung cancer may be related to concomitant nonmalignant pulmonary disease and to an increased release of TGFβ_1 from the platelet α-granules in addition to the previously suggested increased production of TGFβ_1 associated with the pulmonary tumour itself.

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