Published in : Cytokine (2017), vol. 99.
Status : Postprint (Author's version)

Increased production of TGF-β1 from sputum cells of COPD: Relationship with airway obstruction

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a chronic airway disease characterized by a profound airway remodelling that leads to airway obstruction. A role for transforming growth factor-β1 (TGF-β1) has been proposed in airway remodelling of COPD. Regarding the TGF-β1 production at local level, the results seemed to be controversial. In this study, an original model of sputum cell culture thought to maintain important cells interactions, was used.

We investigated the production of TGF-β1 from sputum cell culture in 33 COPD encompassing the whole severity spectrum and compared the results with those found in 39 healthy controls. Sputum was induced by inhalation of saline, the cellular fraction cultured for 24 h and the spontaneous production of total TGF-β1 was assessed by ELISA. Using, a TGF-β1 reporter cell assay, we also compared the levels of active and total TGF-β1 in the sputum cell culture supernatants of COPD and controls. Moreover, as a combination of tumor necrosis factor-α (TNF-α) and TGF-β1 have been shown to have a cumulative impact on the severity of airflow limitation in COPD, the TNF-α release was also measured in a representative subgroup of patients.

Our results indicated that the use of sputum cell culture was a reliable and reproducible method to assess TGF-β1 production at airway level. Sputum cells from COPD produced greater amount of total TGF-β1 than those of healthy controls ($p < 0.001$). This result was confirmed using the cell reporter assay which also showed a higher level of active TGF-β1 in the COPD group compared to controls. In addition, total TGF-β1 production was increased according to GOLD stage and was inversely related to FEV1/FVC ratio ($p < 0.05$). By contrast, the production of this growth factor was not correlated with the functional markers of emphysema nor with demographic characteristics such as age, BMI or smoking status. Interestingly, the production of total TGF-β1 was inversely related to that of TNF-α ($r = -0.53$, $p < 0.05$) which was decreased in COPD. In summary, COPD patients displayed a raised production of total and active TGF-β1 from their airway cells. Total TGF-β1 correlates with the severity of airway obstruction without evidence of a link with emphysema.

Keywords : COPD ; Sputum ; TGF-β1 ; TNF-α ; Airway remodelling

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a pulmonary obstructive disease where airflow limitation is not fully reversible. Functional definition is based on spirometry and a post-bronchodilator FEV1/FVC ratio less than 70% is the usual cut-off. Origin of airflow obstruction is thought to be related to a persistent bronchial inflammation due to noxious particles and gas. The most often incriminated etiologic factor is tobacco smoking [1].

However, beside inflammation, functional alterations partly relate to airway remodelling the histological substrate of which is a subepithelial fibrosis [2-4].

Transforming Growth Factor-βl (TGF-β1), a dimeric polypeptide of 25 kDa, is one of the main factor implicated in airway remodelling [5]. TGF-β1 is virtually produced by all the human cells and its production can be induced by reactive oxygen intermediates and some proteases such as neutrophil elastase [6]. It is secreted as an inactive form by linking with latency associated peptide (LAP) and latent TGF-β binding protein (LTBP). TGF-β1 associated with its "carrier proteins" needs to be activated to binds its receptor. Several activation factors have been defined including proteases, pH, reactive oxygen species and integrins [7]. There are three different

receptors for TGF-β1: TGFβ-RI, RII, RIII, and in the same way as their ligand, they are potentially expressed by all cells in human.

TGF-β1 is known to play a central role in the pathophysiology of COPD [8,9]. However, few studies have looked at the TGF-β1 across the whole severity spectrum of COPD and have looked at the cellular production of this growth factor *"ex vivo"*. To this end, we enrolled 33 COPD including stages 2, 3 and 4 and compared them to 39 healthy subjects. We assessed the TGF-β1 production by using the sputum cell culture model, a non-invasive and validated human model [10-12] that preserves some cell interactions that may occur *in vivo*.

In addition, Tumor Necrosis Factor-α (TNF-α), a proinflammatory cytokine, was also shown to be involved in the local and systemic manifestations of COPD [13]. A recent study indicated that TNF-α may have cumulative effect on COPD severity when combined with TGF-β1 [14]. Indeed, lower FEV1 values were observed in the COPD groups of patients with low TGF-β1/high TNF-α and high TGF-β1/low TNF-α compared to the group with low TGF-β1/low TNF-α. But the lowest FEV1 values were found in the group of COPD patients with the highest serum values of TGF-β1 and TNF-α.

Consequently, we also measured the TNF-α release by sputum cells using the sputum cell culture model.

Table 1 *Demographic and functional characteristics.*

	Healthy subjects (n = 39)	COPD patients (n = 33)
Sex (M/F)	21/18	24/9
Age (years)	39 ± 14	64 ± 9**
Smoking behaviour (NS/ExS/S)	25/8/6	0/21/12
Pack-years	4 ± 9	48 ± 29**
BMI (kg/m^2)	23.9 ± 3.5	25.0 ± 5.0
FEV1 (ml)	4104 ± 923	1260 ± 448**
FEV1 (% pred)	108 ± 22	45 ± 15**
FVC (ml)	4877 ± 1167	2258 ± 847
FVC (% pred)	111 ± 13	74 ± 17**
FEV1/FVC	85 ± 6	50 ± 12**
RV (% pred)	N.D.	187 ± 51
TLC (% pred)	N.D.	116 ± 18
RV/TLC (%)	N.D.	56 ± 10
DLCO (% pred)	N.D.	42 ± 17
KCO (% pred)	N.D.	57 ± 24
ICS	0 (0%)	22 (66%)
LABA	0 (0%)	23 (70%)
LAMA	0 (0%)	13 (40%)

Results are expressed as mean ± SD.
** $p < 0.01$ vs healthy subjects. BMI: body mass index; FEV1: forced expiratory volume in 1s; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; DLCO: diffusing capacity of the lung for carbon monoxide; KCO: gas transfer coefficient; ICS: inhaled corticosteroids; LABA: long acting beta 2 agonist, LAMA: long acting muscarinic antagonist.

2. Methods

2.1. Subjects

Thirty-three COPD patients were enrolled from our outpatient clinic and revalidation centre. Their characteristics are summarized in Table 1. Diagnostic of COPD was made according to GOLD criteria and the patients were divided in three groups according to GOLD classification of severity [15]. All patients had a Pa02 ≥ 60 mmHg and none was supplied by oxygenotherapy. All were recruited during stable state of the disease according to Anthonisen's criteria (no increase in dyspnoea, sputum production nor changes in sputum colour).

Thirty-nine healthy subjects were recruited from hospital staff member and local advertisement. None of them had an history of cardio-pulmonary diseases. All underwent a spirometry and a metacholine challenge to exclude undiagnosed latent asthma. Subjects were only selected if their provocative concentration of methacholine

inducing a 20% decrease in FEV_1 was > 16 mg/ml.

All subjects gave a written informed consent and our study was approved by the local ethic committee.

2.2. Lung function tests

Each subject underwent a lung function and Diffusion for carbon monoxide (DLCO) assessment as previously described [16] and according to ATS/ERS standard criteria [17-19].

2.3. Sputum induction and processing

The sputum was induced and processed as previously described [20,21]. Cell viability was determined by trypan blue exclusion and cytospins stained with May-Grünwald-Giemsa were used to assess the differential cell count by counting 500 non-squamous cells under a light microscope.

2.4. Sputum cell culture

Cell suspension containing 100,000 non-squamous cells was placed in 96 microwells plates containing 250 µl RPMI supplemented with antibiotics and cultured for 24 h at 37 °C and 5% CO_2. The supernatant from cell culture was collected and stored at -80 °C until immunoassay.

2.5. Immunoassay

Total TGF-β1 levels were measured by ELISA (R&D Systems, Abingdon, United Kingdom). The sensitivity of our assay was 4 pg/ml. In a representative subgroup of healthy controls (n = 15) and COPD patients (n = 28), TNF-α levels were measured by ELISA (Biosource, Fleurus, Belgium) with a sensitivity of 10 pg/ml.

2.6. TGF-β1 luciferase assay

In order to investigate the functionality of the TGF-β1 in the sputum cell culture model, we used a cell reporter assay. A sensitive and specific system, based on Transformed mink lung cells (TMLC, gift of Daniel Rifkin, New York University medical center, NY) stably transfected with plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene [22], was used to assess the bioactive TGF-β1 in the sputum cell culture supernatant of a new subgroup of patient (characteristics in Table 2). The TMLC were cultured in DMEM with 10% of FBS and were plated at the density of 15,000 cells/well in a 96-well plate. Both the active and total TGF-β1 present in the samples were evaluated. To activate the latent TGF-β1, the supernatants were incubated with 1 N H Cl for 10 min and neutralized by 1.2 N NaOH/0.5 M HEPES. The supernatants were then added (final dilution 3X) and incubated 16-20 h. Recombinant human TGF-β1 (Thermo Fisher Scientific, Aalst, Belgium) was used as positive control at the dose of 25 ng/ml. Each condition was done with and without anti-hTGF-β antibody (InvivoGen, Toulouse, France) at the dose of 4 µg/ml. Each sample was done in triplicate and the results were expressed as relative light units.

2.7. Statistical analysis

The data are expressed as mean ± SD or as median (range) when relevant. We performed "t" test or Mann-Whitney test according to the distribution of the data. Likewise, correlations were tested with Spearman's rank correlation analysis or Pearson's rank correlation analysis. Statistical analyses were performed by using Prism (Graphpad software, San Diego, USA). A p value < 0.05 was considered as statistically significant.

3. Theory

Non-invasive techniques such as induced sputum have been used to assess the airway inflammation and remodelling in airway diseases like asthma and COPD. However, conflicting results exist regarding the airway TGF-beta release in COPD using the induced sputum supernatant method. This study is the first to assess the TGF-beta production by sputum cells in culture throughout the severity range of COPD. As this parameter has a potential use in the evaluation and management of COPD patients, the acquisition of a good investigation method for TGF-beta at local level is primordial and may be used to look at the whole TGF-beta pathway.

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Table 2 *Demographic and functional characteristics of the validation cohort used for the cell reporter assay.*

	Healthy subjects (n = 8)	COPD patients (n = 11)
Sex (M/F)	3/5	3/8
Age (years)	64 ± 4	68 ± 5
Smoking behaviour (NS/ExS/S)	3/4/1	0/5/6*
Pack-years	12 ± 7	43 ± 24*
BMI (kg/m^2)	26.7 ± 3.3	23.2 ± 3.8
FEV1 (ml)	2959 ± 771	1328 ± 529***
FEV1 (% pred)	109 ± 21	56 ± 19***
FVC (ml)	3841 ± 1032	2437 ± 876**
FVC (% pred)	116 ± 28	83 ± 21**
FEV1/FVC	77 ± 5	54 ± 11***
GOLD stage (I/II/III)		2/7/2

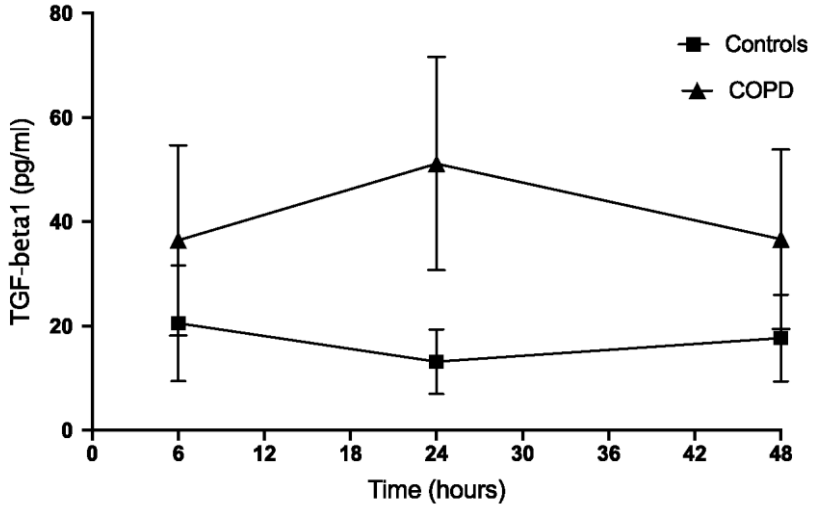
Results are expressed as mean ± SD.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.0001$ vs healthy subjects. BMI: body mass index; FEV1: forced expiratory volume in Is; FVC: forced vital capacity.

Table 3 *Sputum cell counts.*

	Healthy subjects (n = 39)	COPD patients (n = 33)
Sputum weight (g)	4.67 (0.69-10.10)	2.72 (0.62-6.8)**
Squamous cells (%)	9 (1-54)	8 (0-50)
Viability (%)	73 (12-91)	68 (30-97)
10^6cells/g	0.8 (0.3-10.8)	3.2 (0.2-29.8)**
Macrophages%	54 (11-88)	14 (1-45)
Macrophages 10^3/g	420 (79-2441)	400 (35-2744)
Neutrophils%	30 (1-78)	80 (7-99)**
Neutrophils 10^3/g	276 (6-7906)	2210 (143-28906)**
Eosinophils%	0.0 (0.0-1.6)	0.8 (0.0-60.7)*
Eosinophils 10^3/g	0 (0-10)	36 (0-1385)*
Epithelial cells%	4 (0-61)	2 (0-34)
Epithelial cells 10^3/g	50 (0-444)	56 (0-918)
Lymphocytes%	4 (0-8)	1 (0-14)
Lymphocytes 10^3/g	27 (0-444)	39 (0-312)

Results are expressed as median (range).
* $P < 0.05$.
** $P < 0.01$ vs healthy subjects.

Fig. 1. *Kinetic of TGF-β1 production from sputum cell culture from 10 subjects including 5 healthy subjects (full squares) and 5 COPD (full triangles). Results are expressed as mean (SEM).*



4. Results

4.1. Demographic and functional patient characteristics

The demographic and functional characteristics of the COPD and healthy subjects are summarized in Table 1. COPD patients were all current or ex-smoker but two subjects had a smoking history of less than 10 pack years. Overall there was no difference in BMI between the two groups. Two third of our COPD patients were receiving regular combination therapy and slightly less than one third was receiving regular tiotropium.

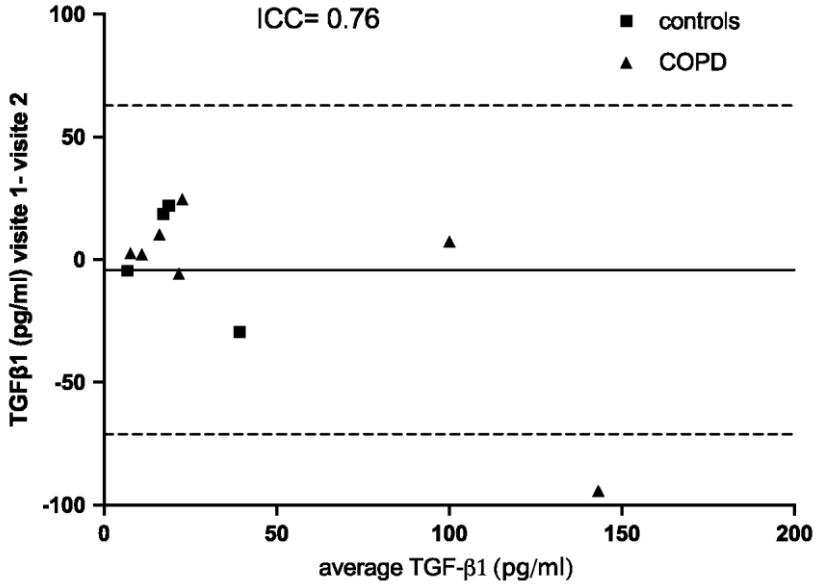
4.2. Sputum cell counts

Results are given in Table 3. COPD patients exhibited greater sputum total cell counts ($p < 0.01$) and a greater percentage and absolute value of neutrophils ($p < 0.01$) than healthy subjects. Consequently, the percentage of macrophages was lower in COPD ($p < 0.01$) but the absolute value was not different. There was also a slight increase in both the percentage and the absolute value of eosinophils ($p < 0.05$) in COPD whereas there was no difference in lymphocyte and epithelial cells counts.

4.3. Spontaneous total TGF-β1 production by sputum cells: kinetic and reproducibility

We first evaluated the kinetic of TGF-β1 production by assaying the growth factor level at 6 h, 24 h and 48 h. We didn't find any significance between the different time points even if there was a trend for a slight decrease at 48 h in the COPD group ($p = 0.06$). Likewise, no differences were seen between healthy controls and COPD patients. However, even if only 5 patients by group had results at each time point, we observed a trend of higher TGF-β1 release at 24 h in the COPD cohort ($p = 0.09$) (Fig. 1). We therefore chose the time point of 24 h for further experiments. Reproducibility of the 24 h production was also assessed between visits planned at 4 days interval in both COPD and healthy subjects. As shown in Fig. 2, the reproducibility was satisfactory with an intraclass coefficient of correlation (ICC) of 0.76.

Fig. 2. *Reproducibility of TGF-β1 production from sputum cell culture in 4 healthy subjects (full squares) and 7 COPD (full triangles). ICC: Intraclass coefficient of correlation. Continuous line represents the mean difference between visitl and visit 2 and dashed lines represent the interval of ± 2 SD.*



4.4. Spontaneous total TGF-β1 production by sputum cells: COPD versus healthy subjects

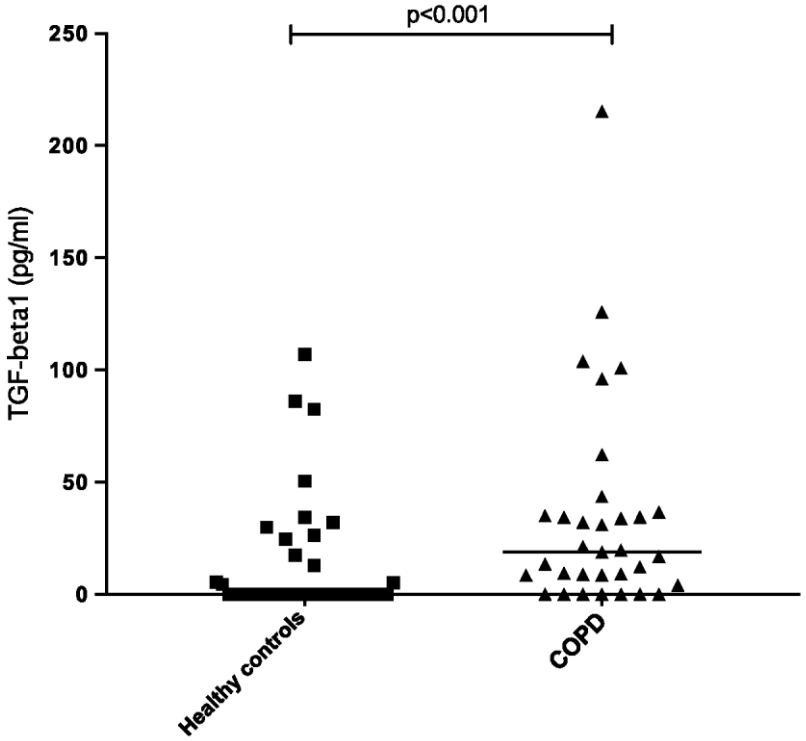
COPD patients had a marked increase in TGF-β1 production as compared to healthy subjects (19 pg/ml (0-215) and 0 (0-107) respectively, $p < 0.001$, Fig. 3). Within the COPD group, we found a relationship between the

TGF-β1 production and the severity of the disease as reflected by the GOLD stages (Fig. 4). TGF-β1 levels were 0 pg/ml (0-107), 12 pg/ ml (0-126), 13 pg/ml (0-96) and 66 pg/ml (0-215) in healthy subjects and GOLD II, III, IV respectively. Furthermore, there was a negative correlation between TGF-β1 production and the ratio FEV1/FVC ($r = -0.37$, $p < 0.05$, Fig. 5). By contrast, the levels of TGF-β1 were not significantly related to the diffusion lung capacity ($r = -0.15$, p: NS).

A subgroup of patients (n = 9) with functional pattern of emphysema (TLC > 110% and KCO < 50%) was identified among all the COPD. No difference in TGF-β1 production was found between emphysematous ((10 pg/ml (0-215)) and the other COPD patients ((31 pg/ml (0-125)) (Fig. 6).

Likewise, TGF-β1 production failed to relate to any sputum cell type nor to the viability of the cell suspension.

Fig. 3. *Total TGF-β1 production from sputum cell culture at 24 h in healthy subjects (full squares) and COPD (full triangles).*



4.5. Spontaneous total TGF-β1 production: influence of age, smoking and BMI and treatment

Within each group, there was no correlation between TGF-β1 production and demographic characteristics of the subjects such as age, BMI and pack-years. Smoking behaviour did not influence TGF-β1 levels in COPD patients (current smokers: 10 pg/ml (0-125) vs ex-smokers: 24 pg/ml (0-215), p: NS) nor in healthy subjects. When we matched our patients according to age and tobacco habits (healthy controls: mean ± SD age: 55 ± 6 and pack-years: 21 ± 10, n = 6; COPD: mean ± SD age: 55 ± 7 and pack-years: 31 ± 10, n = 6), we still observed a significant difference between controls and COPD for the total TGF-β1 released ((median (range): 23 pg/ml (0-126) versus 0 pg/ ml (0-17) respectively, $p < 0.05$).

There was no significant difference between COPD patients treated by inhaled corticosteroids and those who were free of this treatment (18 pg/ml vs 23 pg/ml, p: NS).

4.6. Relationship between total TGF-β1 and TNF-a production

We also measured levels of TNF-α in representative subgroups of COPD patients (n = 28) and healthy subjects (n = 15). We found a marked decreased production of this cytokine in COPD compared to healthy subjects (55 pg/ml vs 1234 pg/ml; $p < 0.01$) and a negative correlation between TGF-β1 and TNF-α productions in COPD

patients (r = -0.53, p < 0.05) (data not shown).

4.7. Assessment of the TGF-β1 functionality

Using TMLC reporter cells, the functionality of the TGF-β1 released by the sputum cells in culture was assessed in a new cohort of patients matched for age and confirmed our results (Fig. 7A). Indeed, COPD patients produced more bioactive TGF-β1 before and after activation by acid. When patients were matched for age and tobacco habits (Fig. 7B), five patients were selected in each group (only ex and current smokers, pack-year: 12 ± 7 vs 22 ± 9 in controls and COPD respectively), the results remained significant.

We were not able to assess the culture supernatants in this bioassay according to disease severity due to the low number of patients with GOLD I and III.

Fig. 4. *Relationship between total TGF-β1 production and COPD severity as assessed by GOLD stages. GOLD stage II: n = 12, GOLD stage III: n = 14, GOLD stage IV: n = 7. Results are expressed as median (range).*

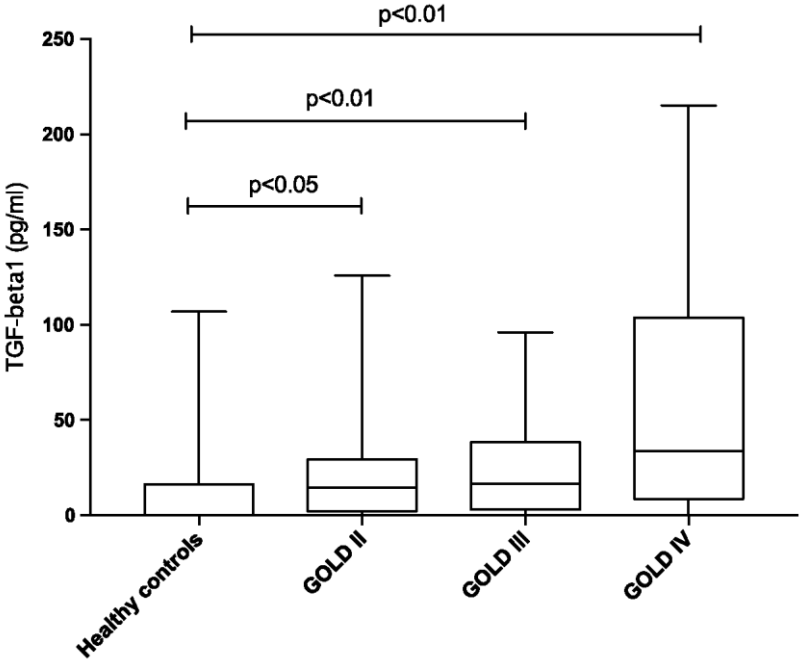


Fig. 5. *Correlation between TGF-β1 production and airway obstruction in COPD reflected by the ratio FEV_1=/FVC.*

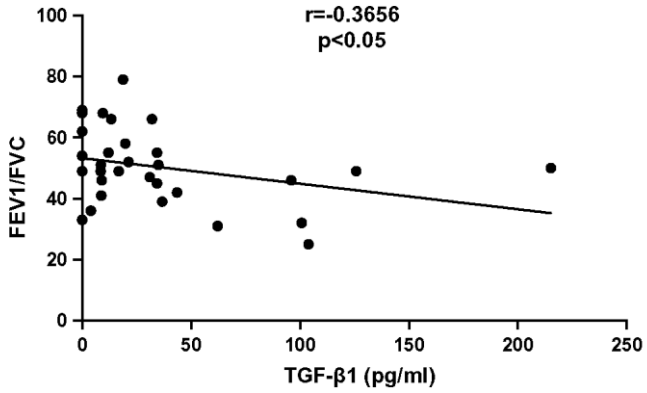
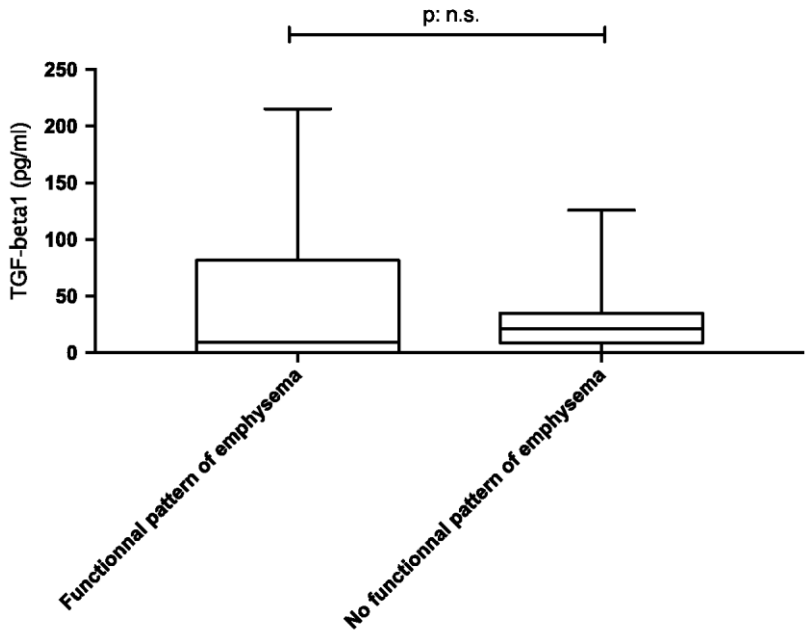


Fig. *6. Production of TGF-β1 in COPD patients with or without functional pattern of emphysema. Results are expressed as median (range).*



5. Discussion

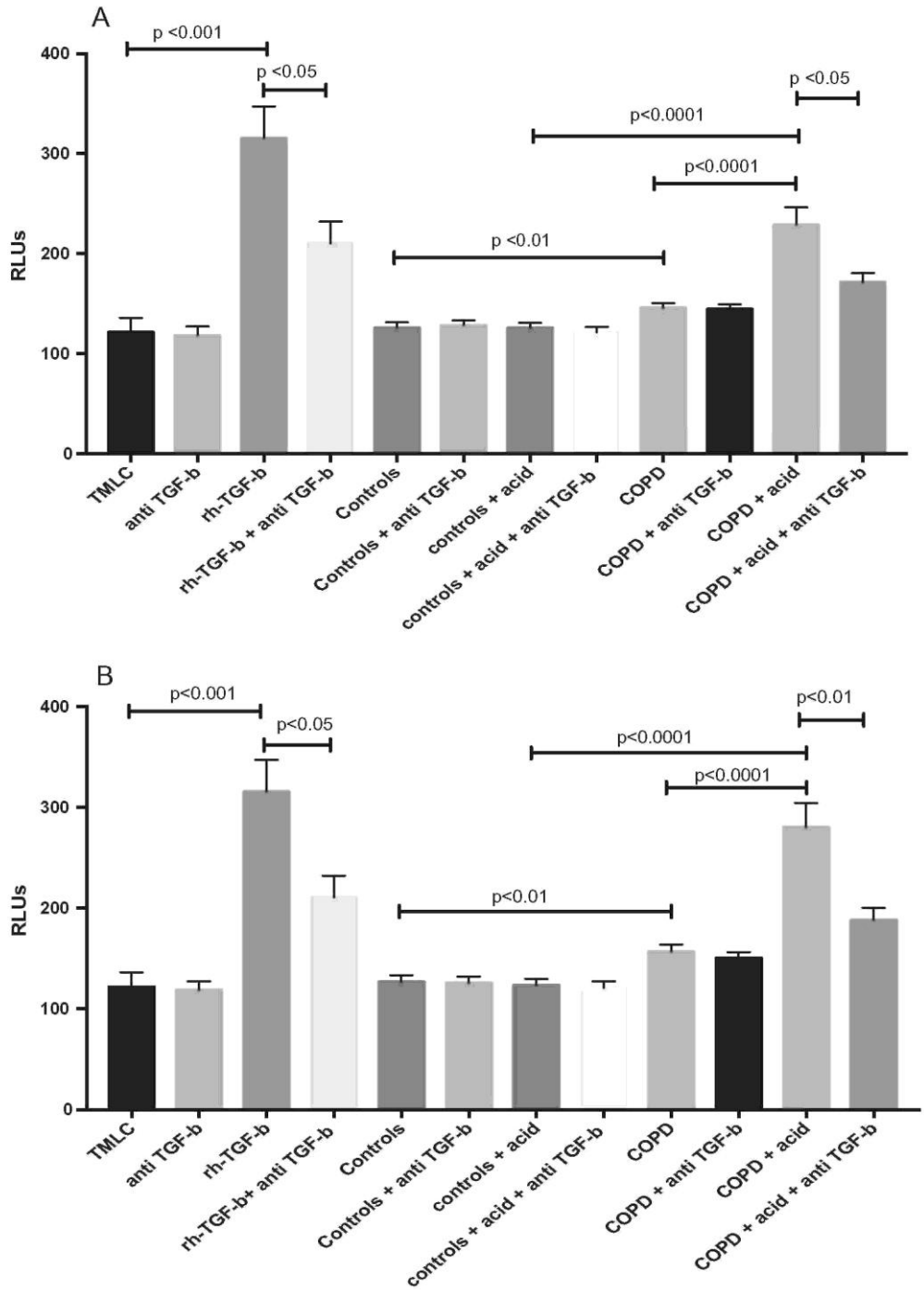
TGF-β1 is a growth factor known to be involved in airway remodelling in COPD. Our study displayed that airway cells from COPD patients produced greater amount of total TGF-β1 as compared to healthy subjects and that the production was increased with the disease severity according to GOLD stage. The levels of active and total TGF-β1 appeared also higher in the COPD group compared to controls in a sensitive and specific bioassay system.

The role of TGF-β1 in COPD has been described by several studies [23-25]. However, only few studies looked at the release of TGF-β1 from airway cells. An increased release of TGF-β1 from alveolar macrophages isolated from bronchoalveolar lavage fluid of COPD patients compared to healthy subjects has been showed [26] but this observation was controversial in another study [27]. As TGF-β1 is known to be released by several cell types including macrophages, epithelial cells, fibroblasts and eosinophils, we believe that it is pertinent to assess the overall production of this growth factor by a mix of cells present in the airways as found in the induced sputum model. However, sputum supernatant measurements appeared to be inconsistent. Some authors found a higher concentration of TGF-β1 in the induced sputum of patients with COPD than in controls [28,29], a result not observed in other studies [30,31]. These conflicting results attest of the variability of the cellular TGF-β1 sources in the sputum supernatant model. Therefore, we choose here an original model that focused on the airway cell ability to spontaneously produce TGF-β1 when cultured *"ex vivo"*. This model was found to be reliable as it yielded a satisfactory reproducibility when the total TGF-β1 production was assessed a few days apart.

The simultaneous assessment of active and total TGF-β1 by the bioassay showed that both fractions had higher levels in the sputum cell culture supernatant of patients with COPD versus controls. To our knowledge, this method of TGF-β1 measurement has never been used in a sputum cell culture model. It allowed us to detect low level of mature and soluble TGF-β1, indeed, it has been shown to be more sensitive than ELISA in more complex biological samples [32].

The inverse relationship between the FEV1/FVC ratio and TGF-β1 is in keeping with previous findings and lends support to a role for TGF-β1 in airway remodelling in COPD. By using immunochemistry and in situ hybridization, Vignola et al. found an overexpression of TGF-β in bronchial mucosal biopsies that was correlated to the thickness of the basal membrane [23]. De Boer and co-workers found similar results with a correlation between TGF-β1 expression and FEV1 alteration [25]. Finally, more recent studies found a stage-dependent association between the serum TGF-β 1 level and lung function in patients with COPD [14,33].

Fig. 7. *TGF-β1 luciferase assay. Comparison of controls (n = 8) and COPD patients (n = 11) matched for age (A) and for tobacco habits and age (n = 5 in each group) (B). Results are expressed in relative light units (RLUs) and showed as mean (SEM). Recombinant human TGF-β1 was used as positive control at the dose of 25 ng/ml and anti-hTGF-β antibody was used at the dose of 4 μg/ml.*



Of interest is the fact that TGF-β1 production did not relate to the diffusing capacity impairment as assessed by the DLCO and KCO. Furthermore, patients with increased lung volumes and severely altered coefficient transfer, deemed to have pronounced emphysema, did not produce greater amounts of TGF-β1. This suggests that TGF-β1 production from sputum cells may not be involved in the remodelling process affecting the alveolar vasculature that characterises emphysema. This finding is in accordance with a study that failed to highlight an association between the TGF-β1 serum concentrations with markers of emphysema as residual volume (RV) and DLCO [33].

Neither in COPD nor in healthy subjects did we find any relationship between TGF-β1 production and the age. This indicates that the production of TGF-β1 does not rise as a consequence of the aging of the tissue itself. Surprisingly, no association was found between active smoking and the current production of TGF-β1. This observation, which contrasts with Takizawa's finding [34], may be explained by the differences in the model used. While Takizawa assessed the production from pure epithelial cells, we measured the production from a cell mixture containing a minority of epithelial cells (< 5%) and other potential sources of TGF-β1 like macrophages. Our study shows that the release of TGF-β1 rather relates to the COPD itself than to the smoking behaviour. This is in keeping with the observation that airways inflammation [35] may persist after smoking cessation together with a progressive worsening of the disease.

As we used a mixed cell model we cannot anticipate which cell is responsible for the raised production found in COPD. The fact that we did not find any relationship between TGF-β1 production and a particular cell type would suggest that TGF-β1 production results from different cell sources. This would be in keeping with the recognised ability of several cell types to produce this growth factor [36] even if alveolar macrophages are thought to be a major source. It is noteworthy that the fraction of macrophages in sputum cell culture of COPD (14%) was twice to three times less than in healthy subjects (54%). Thus, it would suggest that macrophages of COPD display a markedly enhanced production of this growth factor as it was previously reported [26]. Another study showed that circulating monocytes isolated from COPD, the precursor of alveolar macrophages, produce more TGF-β1 than those of healthy subjects whereas this did not hold true for T-cells [37]. Whatever the cell source, it is important to consider the overall production of TGF-β1 from several airway cells as this may preserve some cell interactions that likely better reflect what happens in the airways *in vivo*.

An interesting finding of our study is the inverse relationship between TNF-α and TGF-β1. Our previous findings of a reduced TNF-α production in COPD observed using the same culture model are confirmed here and supports the concept of a negative feedback of TNF-α on TGF-β production [11,20]. There is also evidence that TGF-β may inhibit the activation of macrophages [38] and consequently, the release of TNF-α, and therefore alter the local defence against infectious agents that often colonize COPD airways [39]. Some previous publications showed higher serum and sputum levels of TNF-α in COPD patients but mainly in patients with weight loss [40], more severe disease [41] and in exacerbation [42], which is not the case in our COPD patients (normal BMI, majority of GOLD II and III and stable state).

One limitation of our work is the fact that the group of healthy and COPD patients were not well matched regarding the age and tobacco habits. However, when we selected patients according to these parameters, our result was still significant. Furthermore, when using the TGF-β1 reporter system, the patients were perfectly matched and our observations were confirmed.

6. Conclusions

We conclude that sputum cells from stable COPD release greater amounts of total TGF-β1 the levels of which are proportional to the severity of airway obstruction. This overproduction appears to be independent of the age, the current smoking status and the functional markers of emphysema. Together with the total TGF-β1, the production of active form of TGF-β1 from sputum cells is also increased in COPD compared to controls, attesting the involvement of this mediator in the COPD pathogenesis.

Conflict of interest

Authors have no conflicts to disclose.

Acknowledgments

The authors would like to thank Daniel Rifkin and Rifkin Lab from New York University for the TMLC reporter cells.

This work was supported by the IAP P7/30 from the Belgian Federal Government and FNRS (Fond National de la Recherche Scientifique).

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