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TGF-β type II receptor in pulmonary arteries of patients with very severe COPD

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Running Head: TGF-β and very severe COPD

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Abstract

A mild-moderate increase of pulmonary artery pressure is often associated with severe chronic obstructive pulmonary disease (COPD). Transforming growth factor-β is a cytokine involved in the maintenance of integrity of vasculature. To investigate whether the TGF-β pathway may be involved in the development of pulmonary hypertension associated with COPD, we examined surgical specimens from 14 patients undergoing lung transplantation for very severe COPD (FEV_1 17±2% predicted) and from 7 donors. With immunohistochemistry, we quantified the expression of TGF-β1 and TGF-β type II receptor (TGF-βRII), the cell proliferation index and structural changes in pulmonary arteries. In severe COPD patients, we observed an increased expression of TGF-βRII ($p<0.05$) both in tunica media and intima, which was associated with a normal proliferation index in both layers. Conversely, we observed a significant thickening in tunica intima, that was not present in tunica media, suggesting that mechanisms other than cell proliferation may be involved in intimal thickening. In conclusion, in pulmonary arteries of patients with severe COPD, there is an upregulation of TGF-βRII associated to a normal proliferation index. These findings suggest the activation of an antiproliferative pathway, which might explain the relative low degree of pulmonary hypertension observed in these subjects.

ABSTRACT WORDS COUNT: 197

Key Words: Lung transplantation, emphysema, pulmonary hypertension, vascular remodelling, cytokines

Introduction

Chronic obstructive pulmonary disease (COPD) is a disorder characterized by airflow limitation that is slowly progressive and not fully reversible [1]. Severe and very severe COPD are usually associated with an increased pressure of pulmonary artery [2]. This pulmonary hypertension secondary to COPD, albeit usually much milder than primary hypertension, is nonetheless an important risk factor for hospitalization and shorter life expectancy [3]. Pulmonary hypertension secondary to COPD may be due to hypoxic pulmonary vasoconstriction, emphysematous destruction of the capillary bed, excessive shear stress, inflammation and remodelling of pulmonary arteries acting on a background of genetic susceptibility [4, 5].

The most common morphological change in pulmonary arteries of patients with COPD is the thickening of intimal layer. By contrast, at variance with primary hypertension, the medial layer of the arteries is normal or only mildly thickened [6]. These structural changes are often associated with a functional impairment of the endothelium [7] and with an inflammatory process in adventitial layer [8,9]. The mechanisms underlying the development of these structural abnormalities in pulmonary arteries are still unknown.

The role of the transforming growth factor-β (TGF-β) pathway has been investigated in COPD. An increased expression of TGF-β1 in the airways of smokers with COPD as compared to smokers without COPD has been reported [10-12], suggesting a role for TGF-β1 in the development of COPD. By contrast, recent studies on genetics of COPD suggest a protective rather than a predisposing role of TGF-β1 for COPD [13-15]. The TGF-β pathway has been poorly investigated in pulmonary hypertension secondary to COPD, even if a down regulation of TGF-βRII has been reported both in primary and secondary pulmonary hypertension [16,17].

TGF-β is a pleiotropic cytokine with a wide variety of effects on cell proliferation and differentiation and on inflammation. In humans, the TGF-β family includes 3 isoforms (TGF-β1, TGF-β2, TGF-β3) with great structural and functional similarities [18]. TGF-β regulates cellular processes by binding to 3 receptors known as types I, II and III. In particular, TGF-β binds either to type III receptor, which then presents it to type II receptor (TGF-βRII), or directly to TGF-βRII. Once activated, TGF-βRII recruits and activates the type I receptor (RI). The activated RI phosphorylates Smad, 2 or 3, which then binds to Smad4. The resulting Smad complex is able to translocate into the nucleus where, interacting with various transcription factors, regulates the transcription of many genes. The final effect depends on the specific ligand, heteromeric receptor type, downstream signals and transcriptional activation [19].

To investigate whether the TGF-β pathway is involved in pulmonary hypertension in COPD patients, in this study we examined the expression of TGF-β1 and TGF-β type II receptor in pulmonary arteries of patients undergoing lung transplantation for very severe COPD. Given the complexity of the TGF-β pathway we selected to examine the expression of TGF-β1 and TGF-βRII as they have been already implicated in the pathogenesis of COPD or of pulmonary hypertension, respectively [10-17].

Methods

Subject characteristics

We examined lung specimens obtained from a group of patients undergoing lung transplantation because of very severe COPD [20]. A $FEV_1 < 25\%$ predicted without reversibility and/or a $PaCO_2 \geqslant 55$ mmHg (and/or pulmonary hypertension) with progressive deterioration were the main criteria for transplantation. Fourteen COPD patients (11 males and 3 females) ranging in age from 45 to 64 years (mean 55 years) were included.

The study conformed to the Declaration of Helsinki, and informed written consent was obtained from each subject undergoing transplantation. Each patient underwent preoperative interview, pulmonary function tests, chest radiography, ECG, echocardiography, right heart catheterization and routine blood tests. As control group, we analyzed specimens obtained from the unused lung of 7 donors whose other lung was used for transplant. Four were males and 3 females, with age ranging from 15 to 60 yrs (mean 34 years), all died of suicide or subarachnoid hemorrhages . No clinical data were available for the control group.

Lung tissue preparation

Lung tissue used for the present study included material from 14 patients undergoing lung transplantation for very severe COPD. Cold ischemia preservation was 60 minutes and 120 minutes, respectively, for single and double lung transplantation. Small-sized pieces from all lobes were cut and immediately fixed in Karnovky's solution for electron microscopy. The lungs were gently fixed in 10% phosphate-buffered formalin by airway perfusion and processed for sectioning (3 μm).

Samples were selected from specimens that showed features of excellent tissue preservation and adequate lung inflation. In particular, large thin blocks approximately 30 x 25 mm were cut from the subpleural areas of the apical anterior and lingular segments of the upper lobes, as well as the apical and basal segments of the lower lobes. A more centrally placed block was taken to sample the segmented airways and blood vessels. The right lung was sampled in the same way with the middle lobe being treated in the same way as the lingula [21]. Adult control lungs obtained from unused donor lung transplantation were not treated with prostacyclin before preparation.

The Local Research Ethics Committee approved the study.

Immunohistochemistry and morphometric analysis

Randomly selected tissue blocks were taken from the subpleural parenchyma, fixed in 4% formaldehyde, embedded in paraffin wax and processed for immunohistochemical analysis of TGF-β1 and TGF-βRII. Briefly, sections underwent antigen retrieval by heating in a microwave oven on high power for 8 minutes in 0.01 mol/L citrate buffer (pH 6.0) and then incubated with a mouse monoclonal antibody anti–TGF-β1 (dilution 1:20; Genzyme Diagnostics, Cambridge, MA), with a polyclonal antibody anti–TGF-βRII (dilution 1:200; Biotechnology Inc. Santa Cruz, CA) and the monoclonal MIB-1 antibody (1:50 Dako, Santa Barbara, CA, U.S.A.) which recognizes the Ki-67 antigen, a marker of proliferation. The TGF-β1 antibody has high affinity for human TGFβ-1, however it may cross-react with other members of the TGF beta family. The antibody used was raised against synthetic peptides corresponding to segments of the aminoterminal ends of the respective, biologically active TGF-β. Before incubation with primary antibody, the sections were treated with a biotin blocking kit (Vector Laboratories, Peterborough, UK) to inhibit endogenous biotin. The detection system was performed using the Vectastain ABC kit (Vector Laboratories) with 3-amino-9-ethylcarbazole as the chromogenic substrate. Sections were counterstained with Mayer's hematoxylin.

Morphometric measurements of pulmonary arteries were performed using a computerized image analyzer (Casti Imaging SC processing, Venice Italy) as previously reported [22]. At least ten muscular pulmonary arteries with a perimeter of less than 1.5 mm (corresponding to a diameter of about 0.5 mm) and a double elastic lamina visible for at least half the circumference were selected for each patient. To avoid measurements in tangentially cut vessels, muscular pulmonary arteries with a short/long diameter ratio less than one-third were excluded from the analysis. The areas occupied by the tunica intima and media were measured on sections stained with anti smooth muscle actin (dilution 1:50, M0851 Dako Ltd, High Wycombe, UK), revealed with horseradish peroxidase and diaminobenzidine. External and internal elastic laminas were outlined and the areas occupied by the tunica intima and media were computed and were expressed as the percentage of the area encompassed by the external elastic lamina as previously described [22].

The positive cells for TGF-β1 and TGF-βRII were counted in the media of pulmonary arteries. The results were expressed as number of positive cells per square millimeter of tissue examined. The final result (per patient) is the average of the number of TGF-β1 and TGF-βRII positive cells present in each artery of that patient. Moreover, the results were expressed as percentage of positive arteries defined as arteries with at least one TGF-β1 and TGF-βRII positive cell in the media over total number of arteries examined. The proliferation index was calculated as percentage of positive arteries, defined as arteries with at least one Ki67 positive cell in the tunica media and intima over total number of arteries examined.

To evaluate the intensity of staining for expression of TGF-βRII in tunica intima and media a semiquantitative score was applied as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining.

The cases were coded and the measurements were made without knowledge of clinical data.

Statistical analysis

Group data were expressed as means ± standard error (SEM), or as medians and range when appropriate. Differences between groups were analyzed using the non-parametric Mann-Whitney U-test for morphological data, and the unpaired Student's t-test for clinical data. Correlation coefficients were calculated using Spearman's rank method. Probability values of $p \leq 0.05$ were accepted as significant.

Results

Clinical findings

Clinical characteristics, lung function and haemodynamic parameters of patients with very severe COPD are shown in table 1. Five out of 14 patients with severe COPD had α1-antitrypsin deficiency emphysema. No significant differences in lung function and haemodynamics were observed between patients with or without α1-antitrypsin deficiency. All patients with very severe COPD were ex-smokers with a mean packs-years of 43±13. No data are available about the presence of chronic bronchitis symptoms. The mean value of FEV_1 was 17±2 % predicted. The average DLco was 26±6 %. The mean pulmonary artery pressure (PAP) was 26±3 mmHg as measured by right heart catheterization. The left ventricular ejection fraction values, obtained on heart catheterization were 66±5%, while right ventricular ejection fraction values obtained on echocardiogram were 50±3.

Before lung transplantation, all COPD patients were treated with inhaled anticholinergic and/or β_2-agonists and seven of them with oral steroids (methylprednisolone, 4 to 8 mg/d). All patients were clinically stable without evidence of lung infection at the time of surgery.

Immunohistochemical and morphometric findings

We examined on average 13±2 pulmonary muscular arteries per subject. The arterial perimeter was similar in patients with very severe COPD and donors (647±34 *vs* 701±55μ), indicating that arteries of similar size were compared.

TGF-β1 and TGF-βRII were expressed both in the tunica intima and in tunica media, with a very intense staining in the intimal layer which precluded a precise quantification of number of positive cells in this layer, where we were able to perform only the semiquantitative score for staining intensity.

Both the number of TGF-βRII+ve cells and the % of TGF-βRII+ve arteries were increased in tunica media of patients with severe COPD as compared to donors (median, range 158, 61-324 *vs*. 56, 7-216 cells/mm^2; p =0.05 and 100, 62-100% *vs* 83, 13-100% of positive arteries; p=0.03)(Figures 1 a-b and 2). Furthermore, there was a marked difference in median values of TGF-β1+ve cells between severe COPD and donors, but this difference did not reach statistical significance (median, range: 17.2, 0.6-395 *vs* 5, 0-87 cell/mm^2 ; p= ns). Finally, the percentage of TGF-β1+ve arteries was similar in severe COPD and donors (median, range:19, 7-100 *vs* 18, 0-58% of positive arteries; p= ns).

When we evaluated the staining intensity for the expression of TGF-βRII, we found an increased intensity staining in patients with severe COPD compared to donors, both in tunica intima (2, 0-3 vs 1, 0-1; p=0.003) and in tunica media (1.05,0.5-3 vs 0.5, 0-1; p=0.001).

Morphometric analysis of pulmonary arteries in patients with severe COPD showed an increased thickness of the tunica intima (median, range 20.5,11-27 *vs* 11, 11-15 μ; p= 0.001), but not of the tunica media (median, range 42.5, 26-57 vs 48, 35-60 ; p= ns; Figure 3) as compared to donors. The proliferation index was similar in patients with severe COPD and donors both in tunica intima (median, range 0, 0-26 *vs* 0,0-0; p=ns) and media (median, range 40, 17-80 *vs* 30,0-60; p=ns).

No significant differences in morphometric measurements and in the expression of both TGF-β1 and TGF-βRII were observed between patients with or without α1-antitrypsin deficiency.

The mean intraobserver coefficients of variation for TGF-βRII positive cells were 0.12 , while for TGF-β1 positive cells were 0.08.

Correlations

A significant correlation was found between TGF-βRII expression in tunica media and the severity of airflow limitation ($p<0.05$; $r = 0.59$). No other correlations were found between TGF-βRII expression and any vascular remodelling parameter. Similarly, no correlations were found between vascular remodelling parameters and disease severity or smoking history.

Discussion

This study shows that in pulmonary arteries of patients with very severe COPD undergoing lung transplantation there is an increased expression of TGF-βRII both in tunica media and in tunica intima, which was associated to a normal proliferation index in both layers. Conversely, we observed a significant thickening only in tunica intima, that was not present in tunica media, suggesting that mechanisms other than cell proliferation may be involved in intimal thickening.

Since TGF-β1 has been implicated in the pathogenesis of COPD and TGF-βRII in that of pulmonary hypertension [10-17], the focus of our study was to examine the expression of these two molecules in pulmonary arteries of patients with very severe COPD.

Previous studies have shown an increased number of inflammatory cells [8, 9] in pulmonary arteries of patients with COPD. Inflammatory cells are a source of cytokines and growth factors that may contribute to the development of structural and functional abnormalities of the vessel wall [23]. Among different cellular pathways, transforming growth factor-beta 1 is a pleiotropic cytokine that, by binding to its receptors, might be involved in orchestrating both inflammatory and remodelling processes observed in pulmonary arteries of patients with COPD [24]. While TGF-β1 is often regarded as purely profibrotic factor [10], considerable evidence indicates that this molecule can exert diverse and potentially protective effects within vascular wall as it is able to inhibit the proliferation of vascular cells [25]. Recent studies have shown defects in growth suppressive genes

in plexiform lesions of patients with idiopathic pulmonary hypertension, including TGF-β type II receptor and the apoptosis-related genes, bax [16]. In 90% of plexiform lesions the TGF-βRII protein is not expressed, while only 8 % of arteries of secondary pulmonary hypertension did not express TGF-βRII receptor (in contrast to the abundant expression in the endothelial cells outside the lesions). Thus, it has been proposed that somatic mutations in growth regulatory genes allow clonal expansion of endothelial cells, that contribute to the formation of plexiform lesions and vascular obliteration. It's interesting to note that a decreased expression of type II receptors with the acquisition of a proliferative phenotype has been described in vascular cells within atheromas [26,27] and that the loss TGF-βRII has been associated with progression from adenoma to carcinoma [28] and with tumor progression toward metastasis in lung adenocarcinoma [29].Taken together, these studies assign the role of an important growth inhibitor to the TGF-β signaling system that, if impaired, leads to cells proliferation in cancer and vascular diseases [30]. Indeed, a loss of TGF-β signaling has recently been described in plexiform lesions of patients with idiopathic pulmonary hypertension [17]. The increased expression of the receptor associated with a normal thickness of tunica media and of cell proliferation observed in our study further suggests an antiproliferative profile of this cytokine, supporting its potential protective role in limiting the vessel wall disease observed in COPD. This might explain the mild pulmonary hypertension in the extremely severe COPD patients examined in our study. In fact, the average value of mean pulmonary artery pressure in our population is 26 mmHg, which is consistent with that observed by other authors in patients with COPD, in whom it rarely exceeds 35 mmHg [31].

At variance with TGF-βRII receptor, the expression of TGF-β1 was not significantly different between the two groups of subjects, even if the median value of TGF-β1 was higher in severe COPD patients, showing a parallel trend of the expression of the ligand and of its type II receptor. This observation, together with the finding of a normal thickness of tunica media, further supports the antiproliferative role of TGF-β pathway in COPD. This lack of medial thickening in pulmonary arteries of patients with severe COPD is not completely unexpected, as a similar finding

has been reported by Santos and coworkers in patients undergoing LVRS for severe emphysema [6].

In contrast with the medial layer, we found an increased thickness of the tunica intima in patients with severe COPD as compared to donors. However, when we analysed the cell proliferation in tunica intima, we found that it was similar in the two groups of subjects. Based on these findings, we could hypothesise that different mechanisms, besides cell proliferation, may be involved in intimal thickening, such as extracellular matrix deposition and smooth muscle cell migration. In particular, a latent form of TGF-β, LTB1 (latent transforming growth factor β binding protein 1), could play a role in intimal remodelling favouring smooth cells migration as recently described [32,33]. Furthermore, cytokines other than TGF-β might be involved for the development of intimal remodelling.

Since TGF-β pathway seems to be involved not only in the development of pulmonary hypertension, but also of COPD itself, it is intriguing to note that genetic studies pointed toward a protective role of TGF-β1 in preventing the development of the disease. Indeed, it has recently been described that high producer genotype for TGF-β1 may protect against the development of COPD [14,15], even if the mechanisms through which this molecule acts remain to elucidate.

In our study the structural changes as well as the expression of TGF-β1 and TGF-βRII in pulmonary arteries were similar in patients with and without α1-antitrypsin deficiency, indicating that the extent of remodelling of pulmonary arteries is similar in the two types of emphysema. However, it is also possible that the sample size was too small (particularly in the group of patients with α1-antitrypsin deficiency) to detect a significant difference. Furthermore, it should be highlighted that all patients with α1-antitrypsin deficiency were heavy smokers and it is possible that smoking itself had influenced the results.

Although we are well aware of the poor clinical characterization of the donors in our study, we can be confident that these subjects were free from any kind of major lung diseases, as the lungs were carefully checked by the pathologist for transplant. Indeed, lung specimens from donors gives

the unique opportunity to analyze a large amount of lung tissue in relatively healthy individuals, nonetheless there are still potential biases. In particular, since smoking history is not an exclusion criteria for selection of lung donors [34], it is possible that some of our donors were smokers. Moreover, some of them would have been ventilated before lung transplantation. Because it is known that smoking and ventilation may induce structural changes in pulmonary arteries, this may have influenced the results [9,35-36].

In conclusion, in pulmonary arteries of patients with very severe COPD, there is an upregulation of TGF-β family signaling which is associated with a normal thickness of tunica media and a normal cell proliferation. This may suggest an antiproliferative, and thus protective, role of this cytokine in the development of pulmonary hypertension observed in end-stage COPD. Further studies are required to explore the complexity of the TGF-β pathway and its involvement in this heterogeneous disease.

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Table 1. Subject characteristics

	Severe COPD	Donors
Subjects M:F	11:3	4:3
Age, years	55 ± 2 §	34 ± 8
FEV_1 % predicted	17 ± 2	-
FEV_1 /VC %	37 ± 4	-
PaO_2, mmHg	78 ± 6*	-
$PaCO_2$,mmHg	49 ± 2*	-
pH	7.4 ±0.1	-
DL_{CO} % predicted	26 ± 6	-
K_{CO} % predicted	30 ±7	-
PAP mean, mmHg	26 ± 3	-
LVEF cat %	66 ± 5	-
RVEF echo %	50 ± 3	-

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; FEV_1 = forced expiratory volume 1 s; VC= vital capacity; DL_{CO} = carbon monoxide diffusing capacity; K_{CO}= transfer coefficient of carbon monoxide; PAP = pulmonary artery pressure; RVEF = right ventricular ejection fraction; LVEF = left ventricular ejection fraction; cat = right heart catheterization; echo= echocardiography

Values are expressed as mean ± SEM.

*Blood gas values measured with oxygen supplementation.

DL_{CO}, LVEF and RVEF measurements were available in 11 out of 14 patients.

§ : significantly different from donors (p<0.01)

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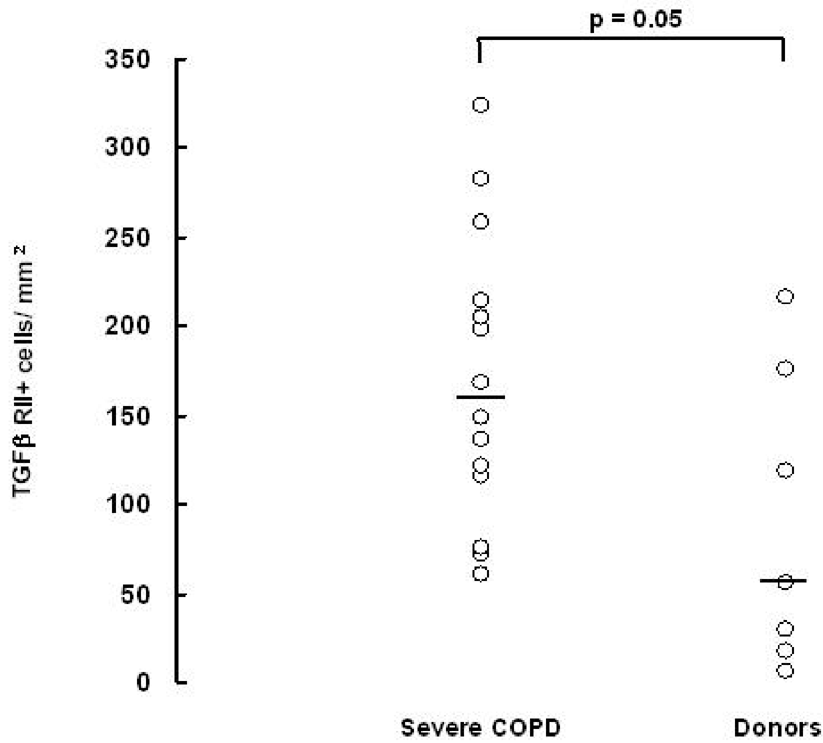
Fig.1. a) Individual counts of TGF-βRII$^+$ cells in the tunica media of pulmonary arteries in patients with severe COPD and donors. The results are expressed as the number of cells per square millimeter of tissue examined; b) 3 Individual counts of TGF-βRII$^+$ positive arteries in patients with severe COPD and donors. The results are expressed as percentage of positive arteries, defined as

arteries with at least 1 positive cell in tunica media, over the total number of arteries examined. Horizontal bars represent median values.



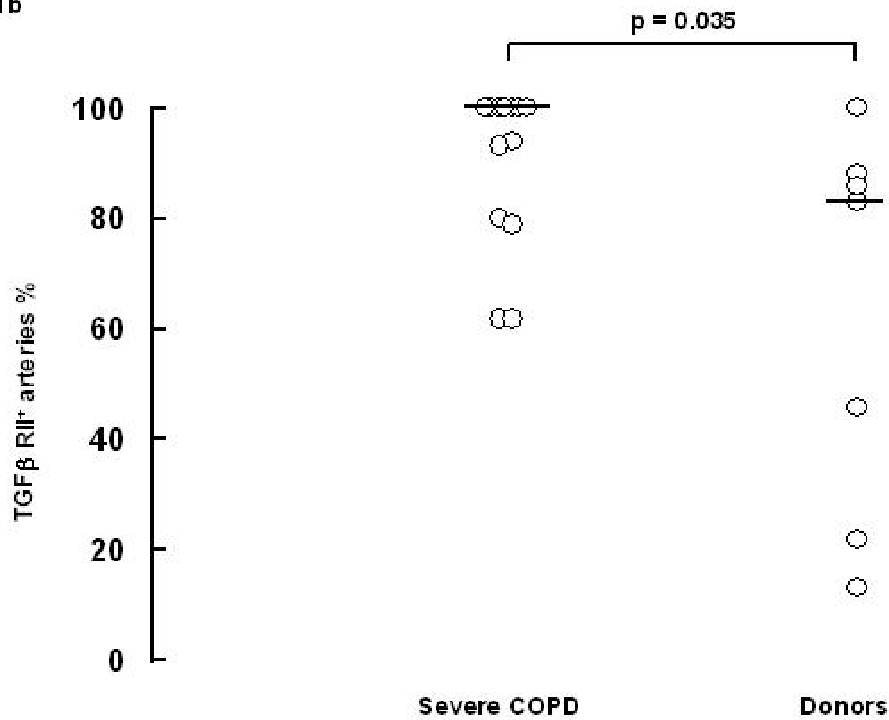


Fig.2. Microphotograph showing an overview of TGF-βRII expression in the tunica media of a pulmonary artery of a patient with severe COPD (panel A) and of a donor (panel B). Original magnification 200X. Panel A, upper right corner: detail showing a TGF-βRII positive cell stained in brown (arrow). Original magnification 630X

Fig. 2

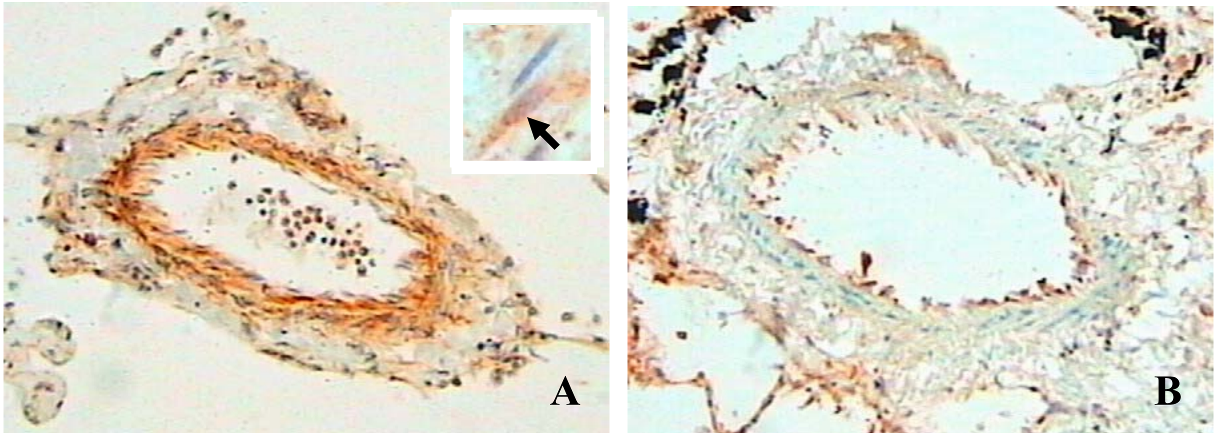


Fig.3. Measurements of tunica intima and media thickness in pulmonary arteries of patients with severe COPD and donors. Results are expressed as percentage of the area encompassed by the external elastic lamina. Horizontal bars represent median values.

Fig. 3