Direct measurement of IGF-I and IGFBP-3 in bronchoalveolar lavage fluid from idiopathic pulmonary fibrosis

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ABSTRACT. Idiopathic pulmonary fibrosis (IPF) is characterized by the rearrangement of extracellular matrix and progressive increase in the amount of fibrotic tissue in the lung. IGF-I is a potent profibrogenic molecule and its bioavailability is dependent on at least 6 binding proteins called IGFBPs. Among these, IGFBP-3 is the most represented in serum and in different connective tissues. The purpose of this study was to identify and characterize IGFBP-3 in bronchoalveolar lavage (BAL) fluids. We studied 11 patients with IPF and 6 normal subjects by performing baseline pulmonary function test and BAL. IGF-I and IGFBP-3 were measured by RIA in BAL and serum. No significant differences were observed between serum IGF-I and IGFBP-3 from IPF patients and normal subjects. Instead, the direct measurement in BAL revealed a significant increase of IGF-I and IGFBP-3 in IPF patients compared to normal subjects. BAL IGF-I and IGFBP-3 concentrations were significantly related to inspiratory vital capacity (IVC) and carbon dioxide partial pressure ($PaCO_2$): the higher the value of IVC and the lower the value of $PaCO_2$, the higher the level of IGF-I and IGFBP-3. In conclusion, IGFBP3 and IGF-I could be important local mediators of IPF. Their direct measurement in BAL in IPF patients could be used as a clinical marker of the disease, since high levels of IGFBP-3 and IGF-I in BAL are associated to the initial phase of the disease.

(J. Endocrinol. Invest. *24:* 856-864, 2001)

INTRODUCTION

The IGF-I and IGF-II peptides are heavily involved in cellular differentiation, mitogenesis, and metabolic control both in different experimental models (1). One of the particular characteristics of IGFs is the fact that they exist in the circulation, in the conditioned medium from different cell culture types and in many biological fluids, associated with specific IGFBPs (2). So far, six IGFBPs (IGFBP-1 to IGFBP-6) have been cloned (3). Recently, new IGFBP-related proteins with low affinity for IGFs and low molecular weight (around 30 kDa) have been reported (4). Although all the IGFBPs are represented in human serum, the predominant form is a 42-39 kDa doublet, characterized as IGFBP-3, that binds both IGF (7.5 kDa) and an acid-labile subunit of 85 kDa, determining a final ternary complex of 150 kDa (5). On the contrary, in the cellular culture medium and in the biological fluid, all the different types of IGFBPs, with a different degree of representation, are found. IGFBPs may have both a stimulatory and an inhibitory effect on autocrine, paracrine or endocrine IGF action depending on the experimental condition, tissues and cellular types (6-9), and from the control of IGF receptors binding (10). These IGFBPs can also be affected by specific IGFBP proteases and by different degrees of phosphorylation and glycosylation, modifing in this way the affinity for IGFs (2). In particular for IGFBP-3, which is mostly regulated by IGF-I, a partial proteolysis has been described in pregnancy plasma (11) and subsequently in plasma from post-surgical patients (12). Several recent

Key-words: Idiopathic pulmonary fibrosis, BAL, IGFBPs.

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Accepted April 3, 2001.

studies suggested that IGFBP-3 could have an IGF-independent effect (13).

Together with its physiological actions, the IGF system has been reported to also play an important role in several pathological status such as chronic interstitial fibrotic diseases (14-17) and systemic sclerosis, where an increased levels of IGF-I has been reported in bronchoalveolar lavage (BAL) fluids (18). In particular, idiopathic pulmonary fibrosis (IPF) represents a chronic inflammatory interstitial disease, leading to low oxygen tension in the blood, in which increased production of growth factors, activated alveolar macrophages, proliferation of epithelial cells and altered production of extracellular matrix components by connective cells play a relevant pathophysiological role (16). Recently, it has been reported by using *in situ* hybridization studies that IGF-I is largely expressed in IPF lung biopsies (19). Moreover, an enhanced expression of IGFBP-rP2 (20) and a significant increase of spontaneous release of IGFBP-3 (19) in cultured cells harvested from BAL fluids of patients affected by IPF, were observed.

In the present study, we identified and characterized the IGFBP-3 in concentrated BAL pathological sample by using Western ligand- and immunoblotting analysis. In order to better represent the *in vivo* biological condition of patients affected by IPF, we directly measured IGFBP-3 and IGF-I levels into the concentrated BAL samples. The data obtained by the analysis of BAL samples were then compared with those investigated in serum of the same normal as well as pathological subjects. The main purpose of this study was to find a possible correlation between bioumoral data of IGF-I and IGFBP-3 directly measured in BAL fluids and the degree of IPF by clinical data.

METHODS

Patients

A group of 11 patients with diagnosis of IPF (9 males and 2 females) were studied. Mean age was 61.9±3.3 yr. The diagnosis was obtained from clinical history, chest radiograms (computed tomography scan in 9 patients), which demonstrated diffuse reticolonodular opacities, and from histologic evaluation. Nine patients were treated with prednisone. Six normal no-smoker volunteers (5 males, 1 female) (Table 1), mean age 59±1.4 yr, had normal physical examinations, chest radiograms and pulmonary function tests. IPF was characterized by the increased percentage of eosinophils in BAL compared to controls (Table 2). Informed consent was given by each subject and the study was approved by the Local Ethics Committee.

Lung function

Baseline pulmonary function testing was performed by measuring static (inspiratory vital capacity: IVC, and total lung capacity: TLC) and dynamic pulmonary lung volumes with a water-sealed spirometer (Pulmo-

Table 1 - *Clinical characteristics of the subjects.*

Patients	Sex	Age (yr)	Smoke	Therapy	PaO_2	$PaCO_2$	pH
a	M	73	S	P	57	38	7.45
b	M	59	S	N	128	41	7.39
c	M	75	Ns	P	59	37	7.44
d	M	57	S	N	81	53	7.39
e	M	47	Ns	P	44	46	7.41
f	F	57	Ns	N	38	55	7.45
g	M	65	S	N	54	51	7.38
h	M	61	S	P	97	29	7.41
i	M	59	S	P	85	40	7.42
l	M	46	S	N	55	45	7.37
m	F	82	Ns	P	115	50	7.38
Mean±SE		61.9±3.3			73.8±8.9	43±2.3	7.41±0.1
Controls (no.=6) Mean±SE	5 M/1 F	59±1.4	Ns	-	92±5.6	41.5±0.7	7.42±0.1
p		NS			0.01	NS	NS

F: female; M: male; N: none; Ns: non-smoker; P: prednisone; PaO_2: oxygen partial pressure; $PaCO_2$: carbon dioxide partial pressure; S: smoker.

Table 2 - *Results of bronchoalveolar lavage.*

Patients	M (%)	L (%)	N (%)	E (%)
a	56	19	7	8
b	63	20	6	9
c	49	34	8	9
d	58	24	7	11
e	48	1	6	35
f	60	31	5	4
g	26	65	6	3
h	23	43	4	30
i	32	34	6	28
l	15	44	7	32
m	37	58	3	2
Mean±SE	42.4±5	33.9±5.5	5.9±0.4	15.5±3.8
Controls (no.=6) Mean±SE	84.5±13	12.6±10	1.4±1	0
p	0.02	NS	NS	0.001

E: eosinophils; L: lymphocites; M: macrophages; N: neutrophils.

net Godart), as previously reported (21). The normal values for lung volumes are those proposed by the European Community for Coal and Steel (22). Blood gas analysis, including oxygen partial pressure (PaO_2) and dioxyde partial pressure ($PaCO_2$), and pH were measured in the arterial blood at rest.

Bronchoalveolar lavage

BAL was performed with a flexible fiberoptic bronchoscope by instillation of saline (three 50 ml aliquots) into the bronchoalveolar tree. The fluid was recovered by gentle suctioning, filtered through sterile gauze and then centrifuged at 1200 rpm for 15 min at 4 C. The supernatant was aspirated and ten 3-ml aliquots were lyophilized for later detection of IGF-I and IGFBPs. The cell pellet was resuspended in saline to obtain 300 cells/ml and cell differential count was performed on cytocentrifuged slides stained with May-Grumwald Giemsa. Four hundred cells were counted.
Protein concentration was estimated in the concentrated BAL supernatant using the Pierce protein assay reagent (Pierce Chemical Co., Rockford, IL, USA) based on the method of Bradford (23), with BSA as standard. Results of BAL in the populations investigated are reported in Table 2.

Western ligand blot

The procedure for IGFBP identification was carried out essentially according to the method of Hosselopp *et al.* (24). Briefly, 120 µl of 25-folds concentrated BAL, adjusted for protein concentration along with pre-stained mol wt marker proteins (Bio-Rad, Richmond, CA, USA), were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel; 18 x 12 cm) under non-reducing conditions. The electrophoresed proteins were blotted onto a nitro-cellulose membrane at 0.08 Amps for 16 h using a transblotting cell apparatus (Bio-Rad). The nitrocellulose membrane was pre-washed with buffers containing detergents and then incubated for 2 h at 25 C with $1x10^6$ cpm [^{125}I]IGF-II prepared as previously described (25). After the membrane was washed and dried, radioactive bands were visualized by autoradiography.

Immunoblotting

Concentrated BAL fluid was electrophoresed and blotted onto a nitro-cellulose membrane, as described for Western ligand blotting. Polyclonal antibodies against human IGFBP-1 to IGFBP-5 was purchased from UBI (Lake Placid, NY), and goat antirabbit immunoglobulin G conjugated with alkaline phosphatase was obtained from TAGO Immunological Co. (Burlingame, CA, USA). The nitro-cellulose membrane was incubated at 4 C with an appropriate dilution of the antibody in phosphate buffer solution containing 3% non-fat dry milk. The nitro-cellulose membrane was rinsed three times with PBS containing 3% non-fat dry milk and incubated at 25 C for 3 h with antirabbit immunoglobulin G-alkaline phosphatase conjugate at a final dilution of 1:20,000 in PBS containing 3% non-fat dry milk. The membrane was washed once with 0.1 mol/l Tris-HCl buffer, pH 9.5, containing 0.1 ml/l NaCl and 5 mmol/$MgCl_2$, and then incubated with bromochloroindole phosphate-nitro blue tetrazolium substrate solution, as previously described (26). The reaction was stopped with PBS containing 20 mmol/l EDTA.

IGFBP-3 and IGF-I measurements in concentrated BAL and serum

BAL IGFBP-3: The levels were determined by RIA using recombinant human IGFBP-3 (rhIGFBP-3) as standards and radioactive tracer and anti-human IGFBP-3 as polyclonal antiserum (27). The detectable range of this RIA was 5-160 ng/ml. The antiserum used in this study did not show significant cross-reactivity with other IGFBPs at concentrations up to 500 ng/ml. Inter- and intrassay variation of this assay was less than 10%.
Serum IGFBP-3: It was measured by RIA using a commercial kit (Mediagnostic, Tübingen, Germany) following the technical procedure as reported in Baxter *et al.* (28).

BAL and serum IGF-I: IGF-I concentrations in biological fluids were measured by RIA (29) using a commercial kit (Nichols Institute, San Juan Capistrano, CA, USA) after extraction of samples by ethanol 87.5%-2NHCl (12.5%) neutralized with 0.855M Tris-base. Only for BAL IGF-I (30) detection the protocol of the Company was slightly modified by using a 2-5-fold dilution of samples instead of a 15-fold dilution as recommended for the quantification of serum IGF-I.

Statistical analysis

All the correlations were evaluated using Spearman correlation coefficient (rho). Differences between groups were evaluated by using the Mann-Whitney test and differences in gender between groups were examined by using χ^2 test. $P<0.05$ was considered statistically significant.

RESULTS

IPF patients had pulmonary volumes (IVC and TLC) significantly reduced compared to normal subjects, and analysis of arterial blood gases revealed a significant difference in terms of PaO_2 between IPF patients and normal subjects. The cytological analysis of BAL harvested from patients (Table 2) showed a significant increase of eosinophils, compared to the normal individuals. This cellular profile confirmed the diagnosis of idiopathic interstitial lung disease.

Western Ligand and Immunoblot analysis of IGFBP in concentrated BAL fluid

Concentrated BAL fluids obtained from these patients and from normal subjects were equally loaded on 12% SDS gel acrylamide after protein determination in order to apply the same amount of protein (100 µg/ml) per well during the electrophoresis migration. The autoradiogram reported in Figure 1 shows that all the samples from patients with IPF (*a* to *m*) present one intact band of protein migration of about 43-49 kDa. The various intensity of the radioactive dots obtained during the Ligand blot analysis among the samples may represent the different concentrations of this IGFBP band in the IPF population studied. In the same gel were not observed other identified bands between 24 and 49 kDa (the molecular weight range where all the IGFBPs from IGFBP-1 to IGFBP-6 have been identified). Moreover, IGFBP proteolitic bands lower than 24 kDa were not observed on the gel increasing the time of exposure of the film to 2 wk. On the contrary (Fig. 2), the electrophorethic analysis of BAL fluid specimens harvested from 6 normal subjects (*n* to *s*) did not show any detectable signal neither at 49 kDa size nor at any other IGFBP band levels even at very long exposure of the autoradiogram (2 wk). In order to exclude the presence of any IGFBP proteolitic fragments in samples from controls, Western immunoblot analysis was performed by using polyclonal antibodies against IGFBP-1 to IGFBP-5 (since antibody for IGFBP-6 is not commercially available for immunoblot purposes). Although we used low dilution of IGFBP antisera (1:200) we did not observed any positive immunobands (data not shown). Instead, higher dilution of IGFBP-3 antibody (1:400) was capable to characterize the 49 kDa IGFBP band observed in IPF patients as IGFBP-3, which was

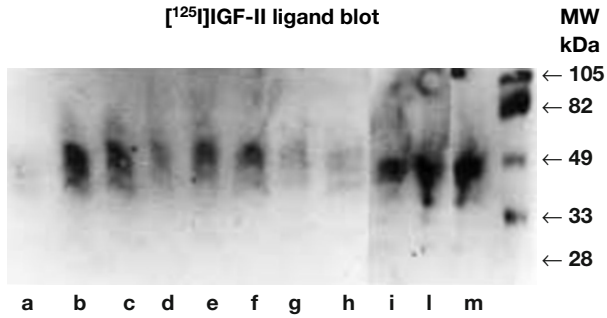

Fig. 1 - *[125I]IGF-II Western ligand blot of concentrated bronchoalveolar lavage fluids obtained from patients affected by idiopathic pulmonary fibrosis identified with the letters from* a *to* m *in the bottom of the Figure (see the Method section). On the right the protein molecular weights (MW) expressed in kilodalton (kDa) are reported. The autoradiogram is the result of three days exposure of the gel. A representative gel is shown.*

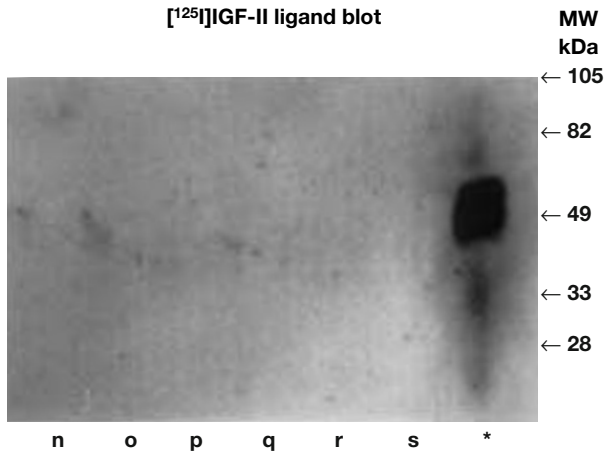

Fig. 2 - *[125I]IGF-II Western ligand blot of IGFBPs of concentrated bronchoalveolar lavage fluids obtained from normal subjects (Table 1). A positive control (*) was represented by patient* m. *On the right of the Figure the protein molecular weights expressed in kDa are reported. The autoradiogram is obtained after two weeks of exposure of the gel.*

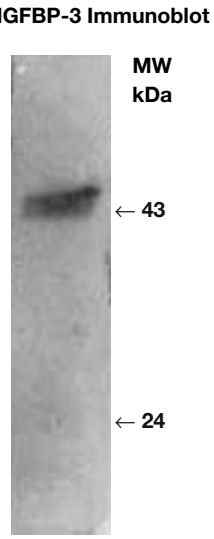

Fig. 3 - *Immunoblot analysis of concentrated bronchoalveolar lavage fluid from a significant patient affected by idiopathic pulmonary fibrosis (patient* m*) obtained using a IGFBP-3 polyclonal human antiserum. The antiserum was diluted at 1:400. The 43 kDa band was identified as IGFBP-3.*

represented by its classical glycosylated doublet shape. A representative BAL of patient m has been used during the immunoblot study reported in Figure 3, to confirm the data obtained by the Ligand blot studies and the highly sensitive RIA. The other IGFBP-antibodies used did not show positive signals (data not reported).

IGF-I and IGFBP-3 measurement in serum and BAL fluid

The RIA values of the concentration of IGF-I (A) and IGFBP-3 (B) in serum obtained from IPF and normal subjects are reported in Figure 4A and B, respectively. As clearly shown, no significant difference was observed between serum IGF-I from IPF patients (mean±SE 99±39 ng/ml) and normal subjects (mean±SE 68±66 ng/ml). The same result (not statistically significant) was observed when the IGFBP-3 serum levels in IPF patients (mean±SE 1.8±0.5 µg/ml) were compared to the normal individuals (mean±SE 1.15±0.1 µg/ml).

On the contrary, the direct measurement of IGF-I and IGFBP-3 in BAL fluids revealed a significant difference between the two groups studied. In fact, in IPF patients IGF-I (A) (mean±SE 20±9 ng/ml) and IGFBP-3 (B) (mean±SE 14.8±0.4 ng/ml) were significantly higher (p=0.05 and p=0.03, respectively), compared to normal subjects (IGF-I mean±SE 4.95±2.5 ng/ml and IGFBP-3 mean±SE 2±0.5 ng/ml). In Figure 5 single values and means of IVC and $PaCO_2$ are reported.

When we plotted in IPF patients, the values of BAL IGF-I (Fig. 6) and IGFBP-3 (Fig. 7) concentration *vs* IVC (percent predicted value) and $PaCO_2$, a significant correlation was found: the higher the value of IVC and the lower the level of $PaCO_2$, corresponding to the higher the level of IGF-I and IGFBP-3 (p=0.02 to 0.05; rho=0.55 to -0.68).

DISCUSSION

IPF is a chronic inflammatory lung disease characterized by an intralveolar fibrosis mediated by the migration of interstitial cells and their differentiation in myofibroblasts and smooth muscle cells (31), a rearrangement of extracellular matrix component, and a progressive increase in the amount of fibrotic tissue leading to a general hypoxia (32). Although several growth factors have been demonstrated to be responsible for fibrosis and modified cellularity in IPF (16), the ethiopathological mechanisms of this disease still remain unknown. Several reports addressed attention to the IGF system as a relevant driver in different chronic interstitial fibrotic diseases including

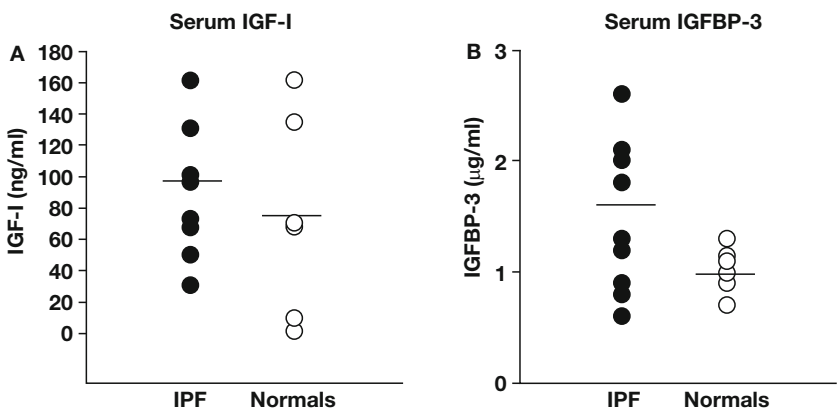

Fig. 4 - *Measurement of serum IGF-I (A) and IGFBP-3 (B) in patients with idiopathic pulmonary fibrosis (IPF) and in normal subjects. Bars represent mean.*

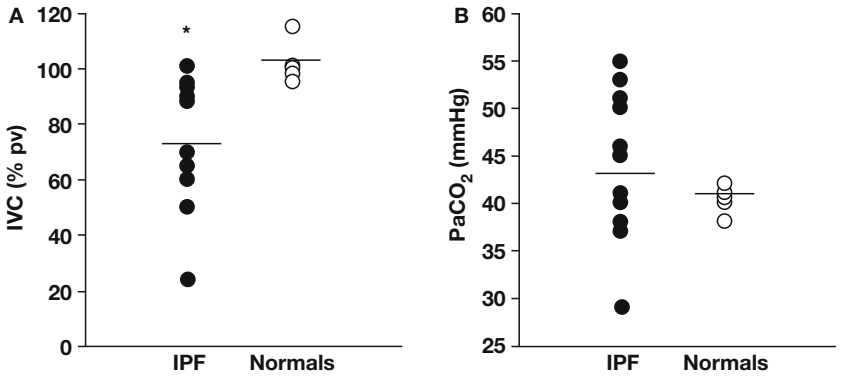

Fig. 5 - *Measurement of inspiratory vital capacity (IVC) (A) and $PaCO_2$ (B) levels in patients with idiopathic pulmonary fibrosis (IPF) and in normal subjects. Bars represent mean. *p=0.001. PV: predicted value.*

IPF (14-16, 33-35). Rom *et al.* (36) demonstrated that alveolar macrophages from patients with IPF were able to release IGF-I. Successively, in lung tissue in IPF, Homma *et al.* (33) demonstrated, by using immunoistochemical analysis, the presence of IGF-I (and other growth factors) and its receptor in macrophages, alveolar type II cells, endothelial cells, vascular smooth-muscle cells and fibroblasts.

Also the IGF carrier proteins (IGFBPs), which have been demonstrated to modulate the IGFs effect in different experimental models belong to the IGF system. Among these, the IGFBP-3 is mostly represented in serum (37) and in different connective tissue cells such as fibroblasts (8) and macrophages (19) and in endothelial cells (38), all of them widely represented in lung tissue.

To our knowledge, although the IGFBPs have been associated to the progression and development of fibrogenesis in other interstitial lung disease (17), the only report concerning a pathophysiological role of IGFBP-3 in IPF was recently published by Aston *et al.* (19). In this paper the investigators showed the *in vitro* arise of IGFBP-3 in 24 h conditioned media obtained from BAL cells recovered by BAL from IPF patients. In the present study, we extended these previous observations by measuring IGF-I and IGFBP-3 level directly in BAL fluids. The potential advantage of this *in vivo* approach is to obtain more direct information about the lung biological milieu conditioning the development of IPF and, consecutively, the possibility to correlate clinical and functional status of the patients with biological parameters.

We observed a significant increase of IGF-I level in BAL fluid in IPF patients, as previously reported in bleomycin-induced mice (39), while IGF-I level measured in serum in IPF patients did not differ from normal subjects. However, in contrast to our data, a previous report (19) showed no difference between IPF and controls in terms of BAL IGF-I levels. Although our experimental model is not able to quantify the exact production of IGF-I among the different cells participating in the fibrotic process, this discrepancy could be explained: in fact, in that experimental model, IGF-I was measured in 24-h supernatants from cultured BAL macrophages, excluding other potential sources of IGF-I, as reported by Homma *et al.* (33) who found deposits of IGF-I peptide in macrophages, fibroblasts, alveolar Type II cells, vascular endothelial cells and vascular smooth muscle cells. Treatment with corticosteroid (40) and hypoxia (41) are *per se*

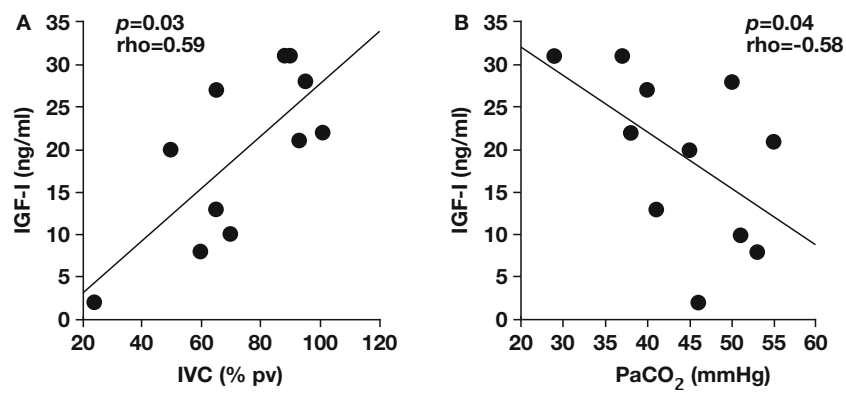

Fig. 6 - *A: relationship between IGF-I levels in concentrated bronchoalveolar lavage from patients and inspiratory vital capacity (IVC). B: relationshp between IGF-I levels in concentrated bronchoalveolar lavage from patients and carbon dioxyde partial pressure ($PaCO_2$).*

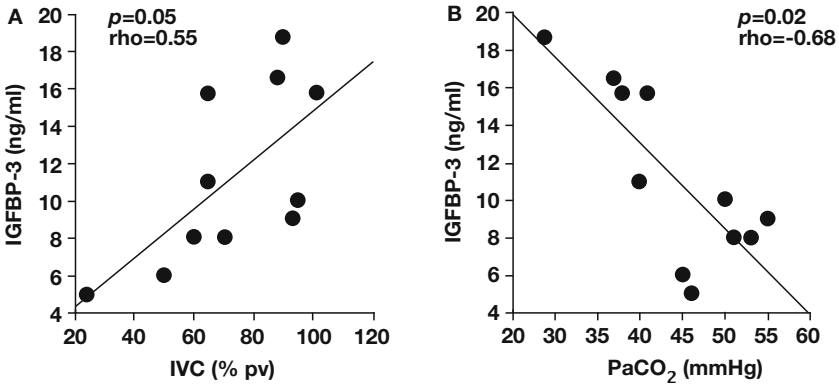

Fig. 7 - *A: Relationship between IGFBP-3 levels in concentrated bronchoalveolar lavage (BAL) from patients and inspiratory vital capacity (IVC). B: Relationship between IGFBP-3 levels in concentrated bronchoalveolar lavage (BAL) from patients and carbon dioxyde partial pressure ($PaCO_2$).*

factors able to activate the IGF-axis increasing the levels of IGF-I and IGFBP-3. However, although some of the patients studied undergone a chronic therapy with corticosteroid and all were affected by systemic hypoxia, the levels of serum IGF-I were not statistically significantly increased. Thus, as for IGF-I, IGFBP-3 measurements in plasma harvested from control and IPF patients did not show any difference, suggesting a local autocrine or paracrine regulation of the IGF system on the target cells of pulmonary fibrosis. According to this, we found a significant rise of IGFBP-3 in IPF BAL as previously reported in conditioned media from BAL macrophages of IPF patients (19). In normal subjects, instead, we did not observe any IGFBP band or their proteolitic fragments even at very long exposure of the gel (2 wk). In a recent paper (17), the variable presence of IGFBP fragments in normal subjects was reported. Even if the age of our normal population was clearly older, thus risking the hypothesis of a possible effect of age on IGFBPs production, this discrepancy could also be due to the different BAL processing method before loading the sample on electrophoresis. Thus, the absence of any IGFBP bands in our control subjects could depend on the generation of IGFBP proteolitic bands with low affinity for IGFs and then not detectable in IGF ligand blot analysis.

In BAL from sarcoidosis, together with IGFBP-2 and IGFBP-4, a proteolitic band of IGFBP-3 has been described (17), suggesting that the potential fibrogenic action of sarcoidotic BAL could be related to the reduced affinity of IGF-I for the IGFBP-3 fragments as previously reported (42, 43). The ligand blot and immunoblot analysis of BAL in our IPF showed a different profile of IGFBPs secretion. In fact, only an intact and glycosylated form of IGFBP-3 has been detected. Since the intact form of IGFBP-3 might positively or negatively modulate the bioavailability of IGF-I, it is possible to hypothesize a biological stimulatory effect on IGF-I action by the fact that the glycosylated form of IGFBP-3 has a reduced affinity for IGF-I (42) determining an increase of IGF-I bioavailable in free form.

The functional evolution of the fibrotic process in IPF is the reduction of pulmonary volumes like IVC (44). We observed, in our patients, a significant decrease of IVC compared to normal subjects with a parallel increase of $PaCO_2$ and found a significant correlation between BAL IGF-I and IGFBP-3 concentration on the one hand and IVC and $PaCO_2$ on the other. The regressions found, indicated that in patients with worse IVC and $PaCO_2$, BAL levels of IGF-I and IGFBP-3 were lower. The progressive reduction in cell number, with a consequent increase of extracellular matrix components is the marker of the evolution of the fibrotic process. We hypothesize that, in the initial phase of the IPF, the amount of IGF components produced by different cell lung populations (33) may be higher than in advanced lung fibrosis, where the cells/matrix ratio becomes progressively reduced.

In conclusion, we confirm that IGFBP-3 and IGF-I may play an important role in mediating fibrogenesis in idiopathic pulmonary fibrosis. Moreover, we propose that IGFBP-3 and IGF-I directly measured in BAL in IPF could be used as a marker to evaluate the clinical status of the disease, since the high levels of IGFBP-3 and IGF-I are associated to the initial phase of the disease. However, further studies in a larger population of IPF patients are needed to understand the exact mechanism by which IGF-I and its binding proteins are involved in the development of pulmonary fibrosis.

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