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# Expression of insulin-like growth factors and their binding proteins by bronchoalveolar cells from children with and without interstitial lung disease

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*Expression of insulin-like growth factors and their binding proteins by bronchoalveolar cells from children with and without interstitial lung disease. K. Chadelat, M. Boule, S. Corroyer, B. Fauroux, B. Delaisi, G. Tournier, A. Clement. ©ERS Journals Ltd 1998.*

**ABSTRACT: The involvement of the insulin-like growth factor (IGF) system in lung growth and repair following injury is sustained by a number of studies. Based on this knowledge, the aim of the present work was to document the expression of the IGFs and their binding proteins by alveolar cells obtained by bronchoalveolar lavage (BAL).**

**Two groups were investigated: a control group of five children and a group of 11 children referred to the department for exploration of interstitial lung disease (ILD). Components of the IGF system studied included IGF-I, IGF-II and IGF-binding proteins (IGFBP). Expression of these factors was analysed at the level of messenger ribonucleic acid (mRNA) (by semi-quantitative reverse transcription polymerase chain reaction techniques), and of protein for the IGFBPs. In addition, expression of two major cytokines associated with the inflammatory process, tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), was also documented.**

**In children without parenchymal disease, the growth factor expressed was IGF-I, in association with the presence of mRNA for IGFBP-2 in all cases. In children with ILD, expression of IGF-I was observed in nine patients and of IGF-II in three patients, and the presence of IGFBP-2 was found in all extracts analysed (mRNA and proteins). Evaluation of IGFBP-2 expression indicated an increase in the group of children with ILD. Interestingly, a significant association was observed between the increase in IGFBP-2 expression and TGF-β expression.**

**The present data emphasize the presence on insulin-like growth factor-binding protein-2 in the BAL of all patients, and suggest that this protein may be an important factor of the injury/repair processes during the progression of alveolar inflammation.**

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Insulin-like growth factors (IGF) are polypeptides that are synthesized in many foetal and adult tissues. The family consists of two peptides, IGF-I and IGF-II, both of which share considerable structural homology both with each other and with insulin [1, 2]. IGFs have a broad range of biological activities, including stimulation of mitogenesis, as well as expression of differentiated functions. They act by binding to the type 1 IGF receptor and possibly the type 2 IGF/mannose 6-phosphate receptor, using autocrine, paracrine or endocrine mechanisms [3, 4]. The IGF system also includes IGF-binding proteins (IGFBP). At present, six IGFBP (termed IGFBP-1 to IGFBP-6) have been well characterized. They appear to be essential in the co-ordination and regulation of the biological activities of the IGF [5, 6]. At the tissue level, they provide a means of cell type-specific localization, they prolong the half-life of the IGFs and regulate their metabolic clearance. They are also essential factors for the control of the biological actions of the IGFs by directly modulating the interactions of the IGFs with their receptors. In addition, it was recently proposed that the IGFBPs may have direct effects on cellular functions such as proliferation by binding to specific cell surface receptors. Although the IGFBPs share relatively high amino acid sequence similarity, each has distinct structural and biochemical properties. In addition, each IGFBP appears to be subjected to differential tissue-specific expression, developmental and hormonal regulation.

The involvement of the IGF system in lung growth and development is sustained by several studies that have documented the expression of both IGF-I and IGF-II, as well as their receptors in the developing lung [7–12]. Furthermore, evidence was provided that the expression and distribution of these factors displayed developmental changes. The importance of the IGF system during development is illustrated by recent studies of type 1 IGF receptor null mutant mice, which showed that a lack of type 1 IGF receptors resulted in death at birth from respiratory failure [13]. In addition to its involvement in lung growth, recent reports suggest a role of the IGFs in the processes of repair following lung injury [14–17]. Indeed, increased IGF

molecules were documented in idiopathic pulmonary fibrosis in adult patients. Studies of the localization of IGF-I and the type 1 IGF receptor showed distinct patterns depending on the stage of the disease. At an early stage, these molecules were found to be expressed in various cell types including alveolar macrophages, type 2 epithelial cells, fibroblasts and endothelial cells. In lung tissue at a late stage of the disease, IGF-I was found to be expressed only in alveolar macrophages.

Alveolar cells are involved in a number of biological activities. Their numerous functions include keeping the surface of the alveoli sterile and providing defence against any invasive agents [18, 19] and, therefore, they are essential components for the maintenance of alveolar structure integrity in the mature lung. In addition, their presence within the alveolus strongly suggests a role in the process of lung development and maturation. From an extensive survey of the literature it is now well established that a large part of their action is mediated by the production of a number of polypeptide growth factors. The aim of the present study was to document the expression of the IGFs and their binding proteins by alveolar cells obtained from children without pulmonary diseases and to compare the results with the profile of expression observed in patients with interstitial lung diseases (ILD).

## Materials and methods

### *Study population*

*Control group.* Five children were included in this group. Bronchoalveolar lavage (BAL) was performed in three cases during a screening endoscopy for the suspected inhalation of a foreign body. In the other two children, it was part of the pulmonary investigations undertaken because of uveitis and therefore suspicion of sarcoidosis [20]. All patients were retrospectively considered free of lung disease. This was established based on the results of complete pulmonary explorations. None had a history of acute lung disease during the 4 weeks preceding the BAL. None received therapy either at the time of investigation or within the previous month. All patients had a normal physical examination and chest radiograph [21]. Functional tests were within the range of predicted values for height-matched children. Microbiological (bacteriological and viral) analysis of the BAL fluid showed sterile results.

*Group of children with interstitial lung diseases.* This group included 11 children (6–17 yrs old; four females and seven males) who were referred to the pulmonary paediatric department for the exploration of ILD which was suspected on clinical symptoms including cough and/or dyspnoea, and the presence of a reticular or reticulonodular pattern of lung opacities on the chest radiograph. In addition to a review of the patient's history and a physical and radiological evaluation, the initial pulmonary investigation included bronchoscopy with BAL and pulmonary function tests. The results of dynamic lung compliance ($CL_{dyn}$) and lung transfer factor for CO ($T_{L,CO}$), expressed as the percentage of predicted values for height-matched children, were considered as significantly decreased if the values were <75% of predicted. Other investigations orientated by patient presentation included blood cell counts and routine biochemical tests, complete immunological studies, sweat chloride concentrations, microbiological studies, cardiac evaluation, barium swallow and histological analysis of biopsy specimens [20].

In four patients, the diagnosis of sarcoidosis was established on the basis of compatible clinical and radiographic findings, histological evidence of noncaseating epithelioid cell granulomas in liver biopsies, and exclusion of other granulomatous lung disorders. In four patients, the diagnosis of idiopathic lung fibrosis was based on histological examination of lung biopsies. In the other three patients, known causes of ILD could be eliminated, but the diagnosis remained uncertain as lung biopsy could not be performed.

Patient characteristics with the results of BAL cell counts and pulmonary function tests are listed in tables 1 and 2. Informed parental consent was obtained in each case before the procedures, which were approved by the Human Experimentation Committee of Cochin University (Paris, France).

### *Bronchoalveolar lavage and BAL cell studies*

BAL was performed during bronchoscopy under local anaesthesia, as described previously [22]. In brief, patients were premedicated with midazolam (0.3 mg·kg$^{-1}$ body weight)

Table 1. – Results of bronchoalveolar lavage cell counts from children in the control group

| Patient No. | Age yrs | Total cells·mL$^{-1}$×10$^3$ | AM % | L % | N % |
|---|---|---|---|---|---|
| 1 | 4 | 460 | 81 | 17 | 2 |
| 2 | 4 | 160 | 74 | 14 | 2 |
| 3 | 3 | 150 | 79 | 18 | 3 |
| 4 | 11 | 200 | 85 | 13 | 2 |
| 5 | 7 | 80 | 90 | 10 | 0 |

AM: alveolar macrophages; L: lymphocytes; N: neutrophils.

Table 2. – Characteristics of patients with interstitial lung disease

| Patient No. | Age yrs | Total cells·mL$^{-1}$ ×10$^3$ | AM % | L % | N % | E % | CLdyn % | $T_{L,CO}$ % |
|---|---|---|---|---|---|---|---|---|
| 1 | 17 | 230 | 70 | 29 | 1 | 0 | 40 | 57 |
| 2 | 13 | 220 | 57 | 37 | 6 | 0 | 39 | 121 |
| 3 | 15 | 210 | 81 | 16 | 3 | 0 | 68 | 98 |
| 4 | 13 | 240 | 55 | 42 | 2 | 1 | 28 | 95 |
| 5 | 7 | 160 | 88 | 9 | 0 | 3 | 67 | 63 |
| 6 | 9 | 260 | 91 | 6 | 3 | 0 | 43 | 46 |
| 7 | 13 | 310 | 69 | 29 | 2 | 0 | 59 | 90 |
| 8 | 9 | 450 | 27 | 67 | 2 | 4 | 24 | 42 |
| 9 | 6 | 800 | 56 | 16 | 24 | 4 | 63 | 65 |
| 10 | 11 | 160 | 56 | 43 | 1 | 0 | 53 | 86 |
| 11 | 15 | 180 | 76 | 22 | 2 | 0 | 75 | 61 |

Bronchoalveolar lavage (BAL) studies: total cell count per millilitre; AM, L, N and E indicate the relative proportion of alveolar macrophages, lymphocytes, neutrophils and eosinophils in BAL. Lung function tests: dynamic lung compliance (CLdyn) and the lung transfer factor for CO ($T_{L,CO}$) were expressed as a percentage of predicted values for height-matched children.

and local anaesthesia was achieved with topical lidocaine. The bronchoscope was introduced into a lower right lobe segment. The volume of sterile normal saline instilled was equivalent to 10% of the functional residual capacity. The sterile solution at 37°C was injected in six aliquots, and only the last five aliquots of aspirated fluid were collected and pooled. The first portion of the BAL was not examined.

BAL fluid was centrifuged at 300×g for 10 min at 4°C and the cell pellet was resuspended in phosphate-buffered saline solution (PBS). An aliquot was used for cytological studies, which included total cell counts determined using a haemocytometer and differential cell counts using a Wright-Giemsa-stained cytocentrifuge preparation. Another aliquot was used for microbiological studies.

### *Cytokine expression studies*

*RNA extraction of BAL cells and reverse transcription (RT).* BAL fluid was centrifuged at 300×g for 10 min at 4°C and the cell pellet was resuspended in 10 mL of PBS. An aliquot was used for the cell studies noted above and the remaining fluid was again centrifuged at 300×g for 10 min. Ribonucleic acid (RNA) was extracted as described previously using the guanidium isothiocyanate method after the addition of 10 μg of transfer RNA (tRNA) (10 $mg \cdot mL^{-1}$; Gibco BRL, Grand Island, NY, USA) [23]. RNA was resuspended in a volume of $dH_2O$ adjusted to the cell number (8 μL for $1 \times 10^6$ cells) [24, 25].

RNA was reverse transcribed into complementary deoxyriboncleic acid (cDNA) after its denaturation at 65°C for 10 min. The RT was performed at 37°C for 60 min. The 20 μL reaction included 8 μL of the denatured RNA, 1 μL ribonuclease (RNase) inhibitor (40 $U \cdot mL^{-1}$; Boehringer Mannheim Biochemicals, Germany), 1 μL 200 mM dithiothreitol (DTT), 1 μL oligo-deoxythymidine (dT) primer (0.5 $\mu g \cdot \mu L^{-1}$; Boehringer Mannheim), 4 μL RT buffer (5 × RT buffer; Gibco BRL), 2 μL of 5 mM deoxyri- bonucleoside triphospahte (dNTP) mix (Boehringer Mannheim), 1 μL of Moloney Murine Leukaemia Virus reverse transcriptase (MMLV-RT, 20 $U \cdot \mu L^{-1}$; Gibco BRL) and 2 μL $dH_2O$. The reaction was stopped by incubation at 65°C for 5 min. The cDNA was then stored at -20°C [20].

*Amplification of cDNAs by polymerase chain reaction (PCR) and analysis.* The following cDNAs were amplified using 2 μL of cDNA preparation for each cDNA: cDNAs to β-actin (actin), tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), IGF-I, IGF-II, IGFBP-2 and IGFBP-3. Amplification was performed by preparing a cDNA-containing reaction mix for each patient as described previously [20]. The nucleotide sequences for oligonucleotide 5' and 3' primers used in the PCR were as follows. For TNF-α: 5'-GCA CTG AAA GCA TGA TCC GG-3', 5'-AGA GAG GAG GTT GAC CTT GG-3'; TGF-β: 5'-GCC CTG GAC ACC AAC TAT TGC-3', 5'-GCT GCA CTT GCA GGA GCG CAC-3'; IGF-I: 5'-GAT GCA CAC CAT GTC CTC CT-3', 5'-CAA TCT CCC TCC TCT GCT CT-3'; IGF-II: 5'-ATG GGA ATC CCA ATG GGG AAG-3', 5'-GAA GAA CTT GCC CAC GGG GTA-3'; IGFBP-2: 5'-ATG AAG GAG CTG GCC GTG TT-3', 5'-AAG AGA TGA CAC TCG GGG TC-3'; IGFBP-3: 5'-GCT AAA GAC AGC CAG CGC TA-3', 5'-ATC CAC ACA CCA GCA GAA GC-3'; and actin: 5'-CAT GCC ATC CTG CGT CTG GA-3', 5'-CCA CAT CTG CTG GAA GGT GG-3' [16, 20, 26–29]. Amplification was carried out in a PCR thermocycler standard (Perkin-Elmer, Cetus, Foster City, CA, USA). The PCR conditions were: denaturation at 92°C for 1 min, reannealing at 55°C for 1 min, and primer extension at 72°C for 2 min, for 40 cycles (TNF-α, TGF-β, IGF-I, IGF-II, IGFBP-2 and IGFBP-3) and 25 cycles (actin). These cycle numbers were established from preliminary experiments so that amplifications remained within the exponential phase [16, 20]. A 30 μL aliquot of the PCR mixture was analysed on a 1.5% agarose gel and visualized by ethidium bromide staining. Comparison with the molecular weight (*M*r) confirmed the predicted sizes for amplified products, which were 510 base pairs (bp) for TNF-α, 336 bp for TGF-β, 501 bp for IGF-I, 341 bp for IGF-II, 374 bp for IGFBP-2, 218 bp for IGFBP-3 and 546 bp for actin. To confirm the specificity of the amplification products, the DNA was then transferred on to a nylon membrane and hybridization with a nested oligonucleotide probe was performed as described previously [20]. The results were analysed semiquantitatively [16]. The amplified signals were scanned by laser densitometry to estimate band intensity. All TNF-α, TGF-β, IGF-I, IGF-II, IGFBP-2 and IGFBP-3 values were indexed to actin and expressed as arbitrary units [16]. A representative gel of amplification of the cDNAs derived from BAL cells from a patient with ILD (patient 10) is shown in figure 1.

### *Protein studies*

*Protein electrophoresis and immunoblotting.* Cellular proteins were analysed as described previously [30]. The cells were washed with cold PBS and scraped in 2 × Laemmli buffer, the volume of buffer used being adjusted to the cell number. In some conditions proteins were also extracted from the cell-free supernatant after trichloroacetic acid precipitation and solubilization in 2 × Laemmli buffer [30]. The proteins were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (11% acrylamide). Western blots were prepared by transferring the proteins on to 0.45 μm nitrocellulose membranes, (BioRad, Richmond, CA, USA) for 1 h at 130 V.

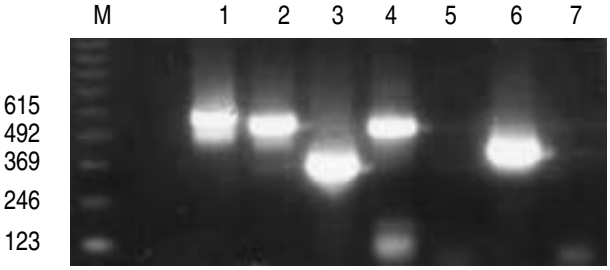


Fig. 1. – Messenger ribonucleic acid (mRNA) expression of components of the insulin-like growth factor (IGF) system and cytokines by bronchoalveolar lavage (BAL) cells. Reverse transcription-polymerase chain reaction was performed on RNA extracted from BAL cells from patient 10. Amplification products obtained with the specific primers for β-actin (lane 1), tumour necrosis factor-α (lane 2), transforming growth factor-β (lane 3), IGF-I (lane 4), IGF-II (lane 5), IGF-binding protein (IGFBP)-2 (lane 6) and IGFBP-3 (lane 7) are shown. M: size markers (base-pair ladder).

Immunoblotting was performed by first saturating the nitrocellulose sheet for 4 h at room temperature in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.2% Tween (TBS-T) and 10% powdered milk. This was followed by incubation with diluted antiserum in 5% milk-Tris-buffered saline (TBS) for 20 h at 4°C. The rabbit anti-bovine IGFBP-2 and IGFBP-3 antisera were used at a 1:2,000 dilution (UBI, Lake Placid, NY, USA). The membranes were then washed three times in TBS-T buffer and incubated for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated goat antirabbit immunoglobulin G (IgG) (Amersham, Bucks, UK), diluted 1:3,000 in milk-TBS. The membranes were then washed three times in TBS-T, after which they were incubated for 2 min at room temperature in chemiluminescence reaction detection reagents (ECL Western blotting, Amersham). The membranes were then exposed to autoradiography film (Hyperfilm-ECL; Amersham).

*Ligand blotting.* Proteins were resuspended in 2 × Laemmli buffer adjusted to the cell number. After boiling, the samples were analysed on SDS-PAGE (11% polyacrylamide) under nonreducing conditions. The proteins were then electrotransferred on to a nitrocellulose filter and the membranes were washed for 45 min in TBS containing 1% Tween. The blots were incubated in TBS containing 1% bovine serum albumin and $^{125}$I-labelled IGF-I and IGF-II (200,000 counts per minute (cpm) each for 48 h at 4°C). After washing, the binding proteins were visualized by autoradiography. Relative molecular weights (*M*r) were estimated by running a prestained *M*r standard [30].

### *Statistical analysis*

Results are presented as individual data, median and mean±SEM. A Mann-Whitney U-test was used to assess BAL parameter differences between the control group and the group of children with ILD. Correlations were evaluated using Spearman's test. A p-value $<0.05$ was considered statistically significant.

## Results

### *Analysis of IGFBP synthesized by BAL cells*

The first step was to determine the profile of IGFBP synthesized by freshly isolated BAL cells. For this purpose cellular extracts were prepared from three patients (patients 4, 6 and 7) and analysed by ligand blotting using labelled IGF-I and IGF-II. The results are shown in figure 2. In all three patients, a 34,000 kDa band could be observed. In addition to this band, in one patient, three species of IGFBP were found with estimated *M*r, of 39,000–42,000, 30,000 and 24,000 kDa. To identify these IGFBP species further, immunoblot experiments were performed. The 34,000 kDa band found in the extracts of all patients was identified as IGFBP-2 (fig. 3a). In the extracts from patient 4 containing several IGFBP species, the 39,000–43,000 kDa band appeared to correspond to IGFBP-3 (data not shown). From data reported in other cell systems, the 30,000 kDa band may correspond to IGFBP-1 and the 24,000 kDa band to IGFBP-4 [5]. However, this remains to be determined in BAL cells. In patients 4 and 6, sufficient amounts of protein could be extracted from the BAL supernatant and analysed by immunoblotting using anti-IGFBP-2 antiserum. The results confirmed the accumulation of IGFBP-2 in the BAL fluid of these patients (fig. 3b).

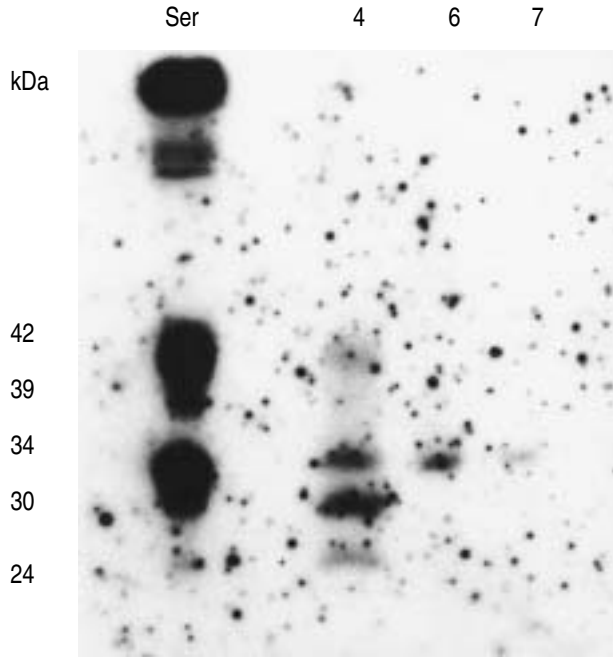


Fig. 2. – Ligand blot analysis of insulin-like growth factor-binding proteins (IGFBP) synthesized by bronchoalveolar lavage (BAL) cells. BA: proteins from three patients (patients 4, 6 and 7) were analysed on sodium dodecyl sulphate-polycrylamide gel electrophoresis under non-reducing conditions. After transfer to nitrocellulose, blots were incubated with $^{125}$I-labelled IGF-I and IGF-II, and the IGFBP were detected by autoradiography. For comparison, analysis of IGFBP was also performed in serum (Ser). Molecular weights (kDa) are indicated.

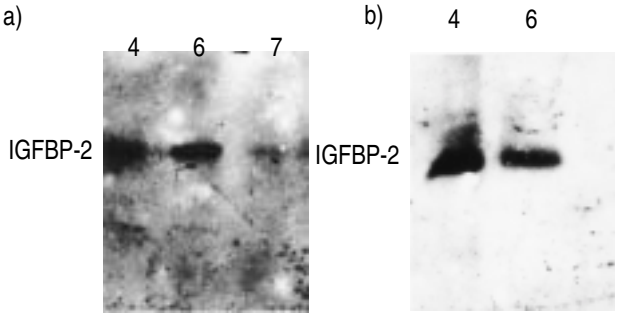


Fig. 3. – Immunoblot analysis of insulin-like growth factor-binding protein (IGFBP)-2. The proteins were prepared from: a) bronchoalveolar lavage (BAL) cells obtained from three patients (patients 4, 6 and 7), and b) BAL supernatants from two patients (patients 4 and 6). They were separated by sodium dodecyl sulphate-polycrylamide gel electrophoresis (11% acrylamide), transferred to nitrocellulose and probed with an antibody specific to bovine IGFBP-2.

Based on several considerations, including: 1) the present results of ligand blotting and immunoblotting experiments; 2) the documented involvement of IGFBP-2 and IGFBP-3 in the control of epithelial and mesenchymal cell proliferation [5, 30, 31]; and 3) the limited amounts of BAL material available in children, the study of IGFBPs was then focused on the expression of IGFBP-2 and IGFBP-3 by BAL cells.

*Expression of IGF and IGFBP by BAL cells from control subjects*

To determine whether IGF-I, IGF-II, IGFBP-2 and IGFBP-3 were expressed in BAL cells obtained from children considered to be free of lung parenchymal disease, semiquantitative RT-PCR experiments were performed after the extraction of RNA. The presence of transcripts for IGF-I and IGFBP-2 was documented in alveolar cells from these patients (table 3). The levels of expression of IGF-I and IGFBP-2 determined by densitometric analysis indicated a mean value of 31.8 arbitrary units (±5.9) with a median of 27 for IGF-I, and a mean value of 83.8 arbitrary units (±8.7) with a median of 74 for IGFBP-2. By contrast, no expression of either IGF-II or IGFBP-3 was observed.

*Expression of IGF and IGFBP by BAL cells from children with ILD*

Results of the expression of IGF-I, IGF-II, IGFBP-2 and IGFBP-3 in BAL cells obtained from children with inflammatory alveolar processes are indicated in table 4. In this group, transcripts for IGF-I were detected in nine patients. For this growth factor the mean value was 29.1 arbitrary units (±11.9) with a median of 5. IGFBP-2 messenger (m)RNA was documented in all cell preparations with a mean value of 235.5 arbitrary units (±55.8) and a median of 125. Comparison with the values observed in the control group indicated significantly higher expression of IGFBP-2 in the group of children with ILD ($p<0.0038$). Transcripts for IGF-II were detected in three patients, albeit at a low level. IGFBP-3 expression was only found in one patient.

Table 3. – Reverse transcription-polymerase chain reaction (RT-PCR) data from children in the control group

| Patient No. | TNF-α au | TNF-β au | IGF-I au | IGF-II au | IGFBP-2 au | IGFBP-3 au |
|---|---|---|---|---|---|---|
| 1 | 0 | 63 | 21 | 0 | 105 | 0 |
| 2 | 0 | 32 | 27 | 0 | 105 | 0 |
| 3 | 0 | 38 | 45 | 0 | 74 | 0 |
| 4 | 0 | 34 | 47 | 0 | 66 | 0 |
| 5 | 0 | 40 | 19 | 0 | 69 | 0 |

The expression of tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, insulin-like growth factor (IGF)-I, IGF-II, IGF-binding protein (IGFBP)-2 and IGFBP-3 was evaluated using RT-PCR analysis. All values were indexed to actin and expressed as arbitrary units (au).

Table 4. – Reverse transcription-polymerase chain reaction (RT-PCR) data from patients with interstitial lung disease

| Patient No. | TNF-α au | TNF-β au | IGF-I au | IGF-II au | IGFBP-2 au | IGFBP-3 au |
|---|---|---|---|---|---|---|
| 1 | 132 | 121 | 5 | 0 | 113 | 0 |
| 2 | 110 | 228 | 60 | 0 | 175 | 0 |
| 3 | 109 | 208 | 3 | 0 | 152 | 0 |
| 4 | 11 | 398 | 0 | 0 | 469 | 58 |
| 5 | 169 | 256 | 0 | 0 | 239 | 0 |
| 6 | 772 | 1056 | 6 | 1 | 657 | 0 |
| 7 | 104 | 126 | 47 | 1 | 113 | 0 |
| 8 | 93 | 133 | 1 | 0 | 79 | 0 |
| 9 | 215 | 334 | 4 | 1 | 361 | 0 |
| 10 | 117 | 148 | 91 | 0 | 121 | 0 |
| 11 | 112 | 110 | 103 | 0 | 111 | 0 |

The expression of tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, insulin-like growth factor (IGF)-I, IGF-II, IGF-binding protein (IGFBP)-2 and IGFBP-3 was evaluated using RT-PCR analysis. All values were indexed to actin and expressed as arbitrary units (au).

*Expression of the IGF system and lung development*

From the current knowledge of the various stages of postnatal lung development, the hypothesis of changes in the levels of expression of the components of the IGF system could be suggested during lung growth.The data collected in the present study indicated that the levels of expression of either IGF-I or IGFBP-2 were relatively homogeneous in the control group (table 3). The size of this group could not allow any conclusions to be drawn. However, knowing the ethical problems of collecting BAL samples from children without pulmonary diseases, the observation that expression of IGF-I and IGFBP-2 appeared to be relatively similar in the five children aged 3–11 yrs old is of interest.

*Expression of the IGF system and inflammatory alveolar processes*

To evaluate the inflammatory processes present in the alveolar compartment of children with ILD, the expression was documented of two major cytokines associated with the inflammatory response: TNF-α, which is known to be induced very early during inflammation, and TGF-β, which is more involved in the repair mechanisms. Expression of these molecules was assessed in each patient. As indicated in tables 3 and 4, TNF-α was not detected in the control group and various levels of expression were found in the group of patients with ILD, with a mean value of 176.7 arbitrary units (±61.5) and a median of 112. For TGF-β, transcripts were documented in all patients. Differences in the TGF-β values were observed, with expression

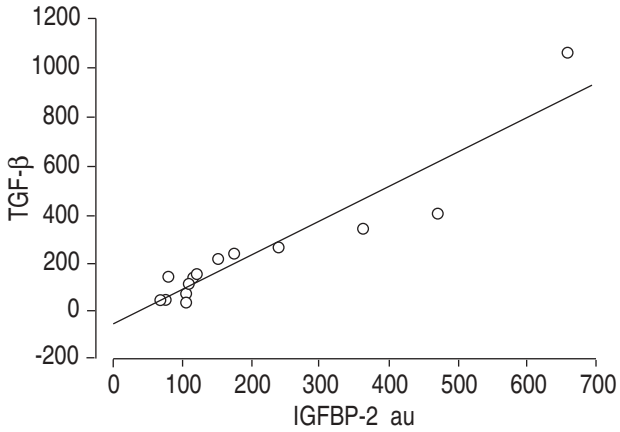


Fig. 4. – Relationship between insulin-like growth factor-binding protein (IGFBP)-2 and transforming growth factor (TGF)-β expression by bronchoalveolar lavage cells. The correlation coefficient (r)=0.921 and probability value (p) <0.001. au: arbitrary units.

being significantly lower in children free of parenchymal disease, *i.e.* a mean value of 41.4 arbitrary units (±5.5) with a median of 38 in the control group, and a mean value of 283.5 arbitrary units (±82.4) with a median of 208 in the group with ILD.

To determine whether a relationship between the expression of IGF-I or IGFBP-2 and the expression of TNF-α or TGF-β could be documented, statistical analyses were performed. A significant association between the increases in IGFBP-2 and TGF-β values was observed ($r=0.921$, $p<0.001$; fig. 4). By contrast, no correlation could be documented between changes in the levels of IGF-I and changes in the levels of TNF-α and TGF-β, or of IGFBP-2 and TNF-α.

## Discussion

The results presented in this report provide information on the expression of IGF and their binding proteins by alveolar cells in paediatric populations. In children without parenchymal disease, the growth factor expressed was IGF-I, and its expression was associated with the presence of mRNA for IGFBP-2. In pathological situations with interstitial lung processes, in addition to transcripts for these factors, the presence of IGF-II could be documented in some patients. Furthermore, the levels of expression of IGFBP-2 were found to be increased in the group of children with ILD.

The protocol used in this study was chosen to provide information on the various components of the IGF system present in the alveolar space and the experiments were performed on freshly isolated BAL cells. The major interest of this protocol is that it eliminates phenotypic modifications of BAL cells that may be induced by cell manipulation and culture [32]. The data indicated that IGF-I was the main IGF expressed by BAL cells. These results are in agreement with several reports which have shown that IGF-II transcripts appeared to be more abundant in foetal tissues and IGF-I was predominantly expressed in postnatal tissues.

As indicated above, because of the limited number of cells available in some paediatric patients, a semiquantitative RT-PCR method was used. The major limitation of this technique is that it provides information on the IGF burden within the alveolar space of patients but it does not specify which cells express the IGF molecules. From other studies, it is likely that alveolar macrophages are the major source [19, 33]. This is sustained by several reports documenting the production of IGF-I by macrophages under various experimental conditions. Lymphocytes may also participate in IGF synthesis, but it is likely that their involvement in IGF production is dependent on their degree of activation [34]. The other cells that can be found in the alveolar space are the neutrophils. Recently, much attention has been focused on the capacity of these cells to produce a number of cytokines [35]. The molecules produced by neutrophils include TNF-α, interleukins (IL) and TGF-β. At present, no evidence of IGF production by neutrophils has been reported.

The data presented herein lead to a discussion on the potential role of IGF-I in the alveolar space of children. Although the experimental protocol used did not indicate whether the corresponding protein was produced and active, an analysis of IGF RT-PCR results suggested that the level of expression of IGF-I was relatively homogeneous in children without inflammatory alveolar processes. In these situations, IGF-I may be viewed as a molecule required for lung growth and homeostasis. This can be explained by the observations that a wide variety of cell types demonstrate a mitogenic response to IGF-I, and that IGF-I is involved in the differentiation processes of a number of cells. By contrast, in the group of patients with ILD differences in the levels of IGF-I expression were observed, which may correspond to distinct aspects of the repair processes. IGF-I has been shown to play a key role in normal wound healing induced by various agents [5]. Clearly, IGF-I has multiple points of action and several mechanisms maintain a high wound IGF-I level. It is now well established that many factors, including inflammatory cytokines, can modulate IGF-I expression. Lake *et al.* [36] documented the role of TNF-α in stimulating the synthesis of macrophage IGF-I. Other factors, including IL-1 and interferon-γ, have been reported to affect IGF-I production in different cell systems [13, 37].

The present study also provides new information on the expression of IGFBP by BAL cells, which has been examined by very few reports in the literature. Focusing on IGFBP-3, Aston *et al.* [16] measured the accumulation of this binding protein in the supernatant of BAL cells cultured for 24 h in serum-free medium. They reported an increase in the amounts of IGFBP-3 produced by cells from patients with ILD compared to those from control subjects. However, they did not provide information on the expression of IGFBP-3 by freshly isolated cells. In the present study, using ligand blotting experiments, the profile of IGFBPs synthesized by alveolar cells was characterized, and the results indicated that IGFBP-2 seemed to be the IGFBP predominantly synthesized by alveolar cells. Only in one patient could other proteins be observed on the pattern of ligand blotting.

The present findings focus the discussion on IGFBP-2. The role of this protein in controlling IGF action is incompletely defined [5]. Purified human IGFBP-2 has been reported to inhibit the ability of IGF-II to stimulate DNA synthesis in lung carcinoma cell lines. In fibroblasts IGFBP-2 also appeared to be a potent inhibitor of DNA synthesis and to reduce both IGF-I and IGF-II-stimulated mitogenesis. However, in other studies IGFBP-2 has been shown to enhance the effects of IGF-I. In addition to acting as a modulator of IGF actions, recent reports strongly suggest that IGFBP can have direct cellular effects that are not dependent on the IGFs [5]. The presence of the arginine-glycine-aspartic acid (RGD) integrin recognition sequence in IGFBP-2 has led to the suggestion that this protein may bind to cell surfaces *via* one or more integrin receptors, leading to the transmission of intracellular signals. This observation shares similarities with the finding that IGFBP-1 also contains the RGD sequence and that the interaction of IGFBP-1 with the α5β1-integrin receptor stimulated cell migration independently of IGF-I and IGF-II [31]. Taken together, these data strongly suggest that IGFBP may play an important role in the control of various cellular functions through IGF-dependent and IGF-independent mechanisms.

The cellular structure directly in contact with the cells present in the alveolar space is the alveolar epithelium [38]. This epithelium is composed of two cell types, type

1 and type 2. Type 1 cells are terminally differentiated cells and are unable to proliferate. By contrast, type 2 cells retain their ability to divide and can undergo transition into type 1 cells [39, 40]. Therefore, type 2 cells are considered as the stem cells of the alveolar epithelium, and their proliferation is crucial not only during lung development but also during lung repair after injury. Any delay in the re-epithelialization process may alter repair by allowing fibroblast replication and constitution of fibrotic changes. Focusing on the mechanisms that control type 2 cell proliferation, the involvement of the IGF system in this process has been documented. Using various models of modulation of type 2 cell proliferation, common mechanisms in the process of growth arrest have been reported, including a dramatic induction of IGFBP-2 [30, 41, 42]. These findings share similarities with recent reports indicating that in other cell types, such as fibroblasts, inhibition of cell proliferation was associated with an accumulation of IGFBP-3 [6, 43]. Moreover, study of the mechanisms involved in the stimulation of IGFBP-2 and IGFBP-3 during the process of growth arrest indicated that TGF-β may play a dominant role. Indeed, treatment of type 2 alveolar epithelial cells with TGF-β resulted in a dramatic induction of IGFBP-2 and an inhibition of proliferation [41]. Oh *et al.* [44] reported that IGFBP-3 was a major anti- proliferative factor and a key element in TGF-β-induced growth inhibition in human breast cancer cells. In addition, it is likely that stimulation of IGFBP expression represents one of the earliest effects of TGF-β and therefore may be necessary for TGF-β-induced inhibition of cell growth. This is suggested by the results of experiments using either anti-IGFBP-2 antibody or IGFBP-3 antisense oligonucleotide [5].

In the present study, an increased expression of IGFBP-2 was observed in the BAL cells of patients with ILD. Moreover, a correlation was documented between the expression of TGF-β and the expression of IGFBP-2. Taken together with the other reports in the literature, the present data lead to the suggestion that IGFBP-2 may be an important factor in the injury and repair processes during the progression of alveolar inflammation. IGFBP-2 may participate in the transient growth arrest of alveolar epithelial cells, thus allowing the repair of cellular components. However, at high levels of production and by delaying the process of re-epithelialization, IGFBP-2 may alter the normal repair programme of the alveolar structure and may contribute to the development of fibrotic changes.

To conclude, characterization of the components of the insulin-like growth factor system expressed by bronchoalveolar lavage cells suggests the involvement of insulin-like growth factor-binding protein-2 in the inflammatory processes associated with interstitial lung diseases. The relation of this protein to transforming growth factor-β and its potential role in the development of fibrosis have been discussed. Clearly, further studies need to focus on insulin-like growth factor-binding protein-2 in order to document its major cellular sources and to make progress in the understanding of the mechanisms regulating both its production and its mode of action.

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