

Expression of insulin-like growth factor-I (IGF-I) in alveolar macrophages and lymphocytes obtained by bronchoalveolar lavage (BAL) in interstitial lung diseases (ILD). Assessment of IGF-I as a potential local mitogen and antiapoptotic cytokine

Piotr Kopiński¹, Krzysztof Śladek², Jerzy Szczeklik³, Jerzy Soja², Artur Szlubowski², Barbara Balicka-Ślusarczyk², Bożena Lackowska⁴, Marta Plato¹ and Adam Szpechciński¹

¹Chair of Gene Therapy, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz

²Department of Occupational Medicine and Environmental Diseases, Jagiellonian University Medical College, Kraków

³2nd Chair of Internal Medicine, Jagiellonian University Medical College, Kraków

⁴Department of Immunology, Center of Oncology, Kraków, Poland

Abstract: Little is known about IGF-I expression in the alveolar lymphocytes (AL), and about local role of IGF-I in physiological conditions and in interstitial lung diseases. Bronchoalveolar lavage was carried out in patients with silicosis, asbestosis, idiopathic pulmonary fibrosis (IPF) and sarcoidosis, as well as in control subjects (n=13, 9, 12, 56, 15, resp). Alveolar macrophages (AM) and lymphocytes (AL) were studied for (1) IGF-I, BCL-2, Fas and Fas Ligand expression and (2) cell cycle (incl. sub-G₁ peak of late apoptosis) with propidium iodide (PI). Flow cytometry (FC) and immunocytochemistry were used. AL early apoptosis was detected by Annexin V FITC/PI staining. IGF-I was present in AL of all tested groups. The number of IGF-I positive AL was significantly higher in IPF (52 ± 6.7%) and in later (II and III) stages of sarcoidosis (39 ± 7.8 vs 16 ± 4.0% in controls, p<0.05). Increased BCL-2 expression in AL was detected in IPF and sarcoidosis. In all tested groups, AL were almost exclusively Fas+ T cells. Generally, a low number of AL entered apoptosis; no significant differences were found between patient groups, except decreased apoptosis rate in sarcoidosis (0.60 ± 0.17 vs 1.15 ± 0.33% in controls, p<0.05). Proportion of AL positive for IGF-I was significantly correlated with parameters reflecting AL and AM cell proliferation and BCL-2 expression (e.g. AL IGF-I+ vs AM in S phase of cell cycle: r_s = +0.50, p=0.001), but not with apoptosis. The results show that human alveolar lymphocytes express IGF-I in normal conditions, as well as in ILD. The proportion of IGF-I+ lymphocytes was significantly increased in IPF and at later stages of sarcoidosis. In our material there was no evidence for profibrogenic or antiapoptotic activity of IGF-I. We suggest that IGF-I originating from AL may be locally active as a mitogen for alveolar macrophages and lymphocytes in ILD.

Key words: Alveolar macrophages - Apoptosis - Asbestosis - Cell cycle - Insulin-like growth factor-I - Idiopathic pulmonary fibrosis - Lymphocytes - Sarcoidosis - Silicosis

Introduction

Insulin-like growth factor-I is encoded by IGF-I gene, composed of 6 exons. Its biological functions

are mediated by the activation of a specific receptor (IGF-IR). IGF-I exerts a series of significant effects, including remarkable proliferation and differentiation of target cells [27]. It also participates in the control of apoptosis, protecting both normal and tumor cells from this process. Its promoting role has been demonstrated in a number of human malignancies [25]. Increased serum IGF-I levels have been shown to be carcinogenesis predictor in some human

Correspondence: Piotr Kopiński, Chair of Gene Therapy, Collegium Medicum, Nicolaus Copernicus University, M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, Poland; e-mail: mpkopins@consoft.com.pl

organs, such as brain, breast, ovary, prostate, colon and lung [30].

In the lower airways, IGF-I is permanently present. Its concentration in homogenized lung tissue of healthy subjects is about 234 mU/g [5]. Biological role of IGF-I in lungs includes tissue developmental growth, repair processes and compensatory growth of healthy lung, which appears after contralateral pneumonectomy [8, 19]. Alveolar macrophages are usually considered to be the main local source of IGF-I: the commonly used former name of insulin-like growth factor-I was alveolar macrophage derived growth factor, AMDGF [18].

IGF-I is regarded to participate in the pathogenesis of many interstitial lung diseases (ILD), including sarcoidosis, idiopathic pulmonary fibrosis (IPF) and pneumoconioses (silicosis and asbestosis). It potentially acts as a mitogen for lung fibroblasts and, subsequently, as an activator of pulmonary fibrosis [10, 23].

In sarcoidosis, increased IGF-I level is considered to stimulate collagen synthesis by pulmonary fibroblasts [2]. In IPF, the enhanced local IGF-I expression seems to affect fibroblast growth and local collagen synthesis [31]. Increased IGF-I secretion was also observed in alveolar macrophages originating from silicosis and asbestosis patients [9, 21]. High surface expression of IGF-I receptor on AM could explain intensive macrophage proliferation observed in this subgroup of ILD [26]. It should be emphasized that all the mentioned disorders might be complicated by disseminated pulmonary fibrosis, resulting in severe respiratory failure and fatal outcome [24]. However, both in ILD patients and in normal conditions, little is known about IGF-I expression in cells other than alveolar macrophages.

Apoptosis seems to be a powerful mechanism participating in the pathology of lower airways in ILD [1]. The imbalance of pro-survival and pro-apoptotic signaling pathways in such cells as alveolar macrophages (AM) and lymphocytes (AL) may play an important role in the onset, progress and remission of ILD [22]. For example, the reduced apoptosis of alveolar lymphocytes was demonstrated in inflammatory diseases, characterized by high BAL lymphocytosis, such as extrinsic alveolitis and sarcoidosis [17, 28]. Recently, we reported similar findings in lung pneumoconioses [29].

In order to explain the dysregulation of programmed cell death observed in ILD, many investigators focused their attention on different components of anti-apoptotic pathways, *e.g.* on interleukine-2 (AL in sarcoidosis), transforming growth factor- β (immune cells in pulmonary fibrosis), intracellular BCL-2 or BCL-X_L expression (AL in sarcoidosis and extrinsic alveolitis), transcription factor NF- κ B (AM in pneumoconioses) or surfactant protein D (experiments on murine AM) [4, 6, 17]. It should be emphasized, however, that the mechanisms responsible for normal immune cell apoptosis (the event that seems to be not

Table 1. Anti-human monoclonal antibodies used for immunocytochemical staining (ICS) and flow cytometry (FC)

Monoclonal Ab – dilution	Manufacturer	Cat. No	Application in the study
Anti-BCL-2, mouse, 1:50	DAKO Cytomation	M 088701	ICS, FC
Anti-Fas (CD95), mouse, 1:200	DAKO Cytomation	M 3554	ICS
Anti-IGF-I, mouse, 1:50	Cymbus Biotech	CBL67	ICS, FC
Anti-CD45 FITC, mouse	BD Pharmingen	345808	FC
Anti-CD3 FITC/CD16+56 PE, mouse	BD Pharmingen	342403	FC
Anti-CD4 FITC, mouse	BD Pharmingen	345768	FC
Anti-CD8 FITC, mouse	BD Pharmingen	345772	FC
Anti-CD5 FITC/CD19 PE, mouse	BD Pharmingen	340396	FC
Anti-CD95 PE (Fas), mouse	BD Pharmingen	335037	FC
Anti-CD178 (FasL) PE, mouse	R & D	FAB1261P	FC

very common in physiological conditions), as well as for its extremely reduced rate in some lower airway disorders, are poorly understood. The role of IGF-I, which is probably active locally as a mitogen, profibrotic cytokine and anti-apoptotic agent together, needs also more accurate explanation.

The purpose of the present study was to determine the expression of IGF-I in the alveolar immune cells both lymphocytes and macrophages in ILD, such as sarcoidosis, idiopathic pulmonary fibrosis, asbestosis and silicosis. The results of IGF-I expression, calculated separately for AL and AM, were referred to the clinical data and to the parameters characterizing the apoptosis and proliferation of immune cell. Especially, in the material coming from relatively large group of patients, we tested the statistical correlations between the percentage of IGF-1-positive alveolar macrophages and lymphocytes and (1) the predicted value of vital capacity (VC), which decline in ILD serves as a clinical marker of lung fibrosis; (2) the percentage of apoptotic AM and AL (3) the rate of AM and AL proliferation (S and G₂M phases of the cells cycle). In this way we tried to assess the profibrotic, anti-apoptotic and mitogenic role of IGF-I in interstitial lung diseases.

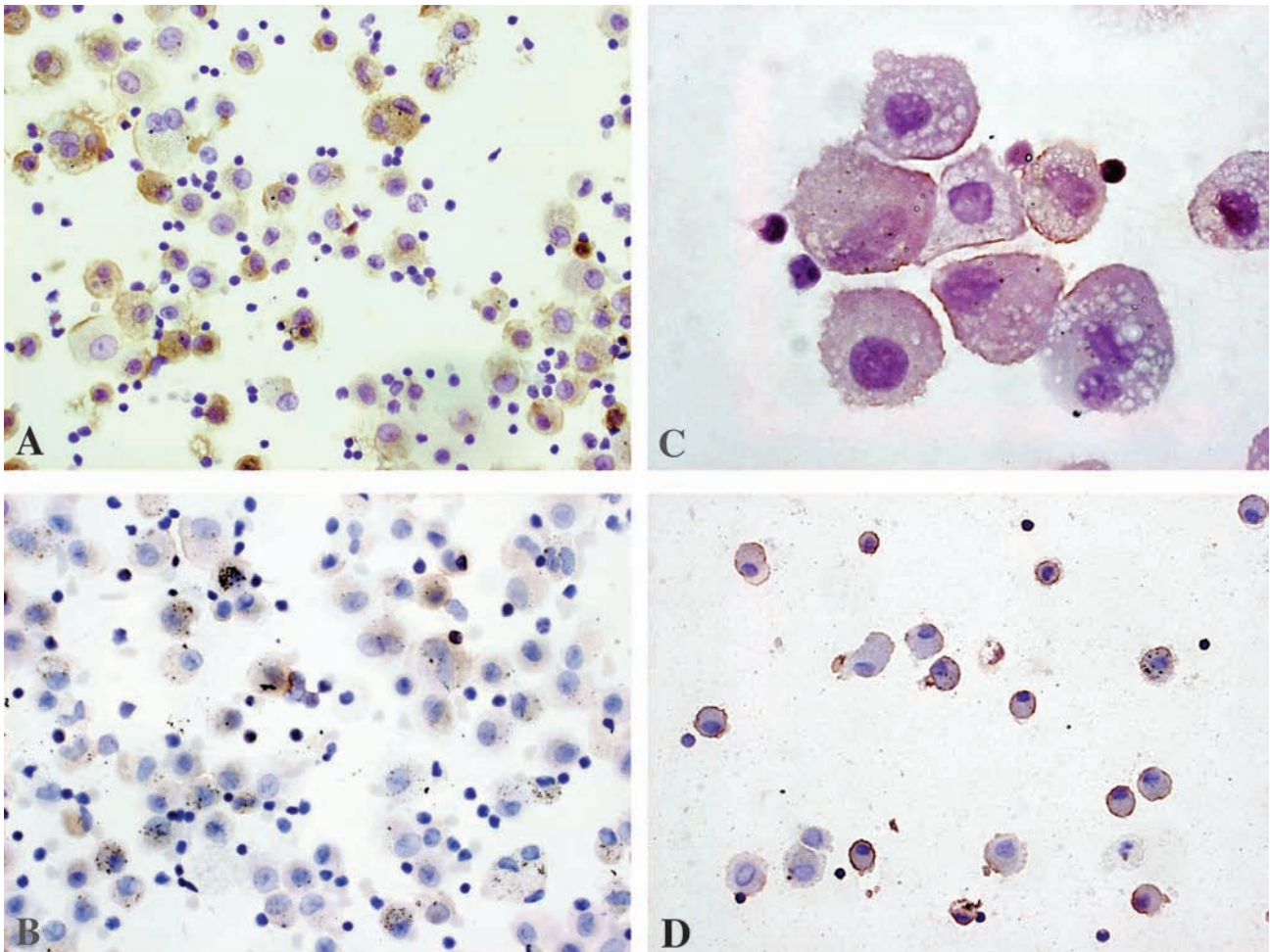


Fig. 1. Examples of BAL immunocytochemistry are presented in the micrographs. **A.** In contrast to alveolar macrophages, alveolar lymphocytes (AL) demonstrate moderate expression of IGF-I. Only some AL are intensely positive. Flow cytometry is more sensitive (see Fig. 2). Sarcoidosis, counterstained with Harris hematoxylin, $\times 200$. **B.** BAL cytospin preparation with relatively high percentage of positive lymphocytes. Asbestosis, counterstained with Harris hematoxylin, $\times 200$. **C.** One of four AL is positive. It should be emphasized that positive alveolar lymphocytes frequently adhere to macrophages. Some AM present both cytoplasmic and superficial IGF-I expression (they absorb cytokine from microenvironment by IGF receptors?). Control group, counterstained with Harris hematoxylin, $\times 600$. **D.** Common superficial expression of Fas (CD95) on BAL immune cells. Control group, counterstained with Harris hematoxylin, $\times 200$.

Materials and methods

Patients. The cytological material originating from bronchoalveolar lavage (BAL) was harvested from 90 patients with ILD, *i.e.* sarcoidosis (56 cases), asbestosis (9), silicosis (13) and idiopathic pulmonary fibrosis (12). A diagnosis of sarcoidosis was established in accordance with recently described criteria [12]. In all patients non-caseating granulomas were identified by transbronchial or endotransbronchial biopsy. None of patients included in the study had previously been treated with steroids. Patients were subdivided according to conventional chest X-ray staging.

A diagnosis of pneumoconiosis (asbestosis or silicosis) was established according to chest X-ray standards of International Labor Organisation in subjects with proven professional or environmental (as happened in some cases of asbestosis) exposition to the respective inorganic dust. In these patients the exposition was additionally determined by BAL cytology, including the presence of ferruginous bodies in asbestosis and silica dust light emission in polarized light in silicosis [15, 29].

Fifteen persons diagnosed for ILD, in which complete clinical investigation (incl. lung function tests, chest X-rays, DLCO and

arterial blood gas analyses) finally excluded any lung pathology, served as the control group. They did not present any signs of infection or chronic lung disease and they were not treated with corticosteroids, immunosuppressive agents or any other drugs known as a potential ILD cause.

All involved subjects were nonsmokers. In this way we omitted the data interpretation problems concerned with distinct cytoimmunological pattern observed in the group of smokers [11].

Bronchoalveolar lavage (BAL). BAL was carried out according to criteria of European Respiratory Society [13]. In brief, the patients were premedicated with midazolam 2.5-5.0 mg *i.v.*, followed by local anesthesia of upper airways with 2% lidocaine solution. The Olympus Bf 20 bronchofiberscope was inserted into the middle lobe or into the left lung lingula, alternatively. Lavage with 200 ml of 0.9% NaCl sterile solution (37°C) was carried out by sequential instillation of four 50 ml aliquots of saline. The BAL fluid fractions were retrieved carefully by gentle suction, then pooled and filtered. The fluid recovery was calculated as the percentage of the instilled volume. The material was immediately sent to the laboratory.

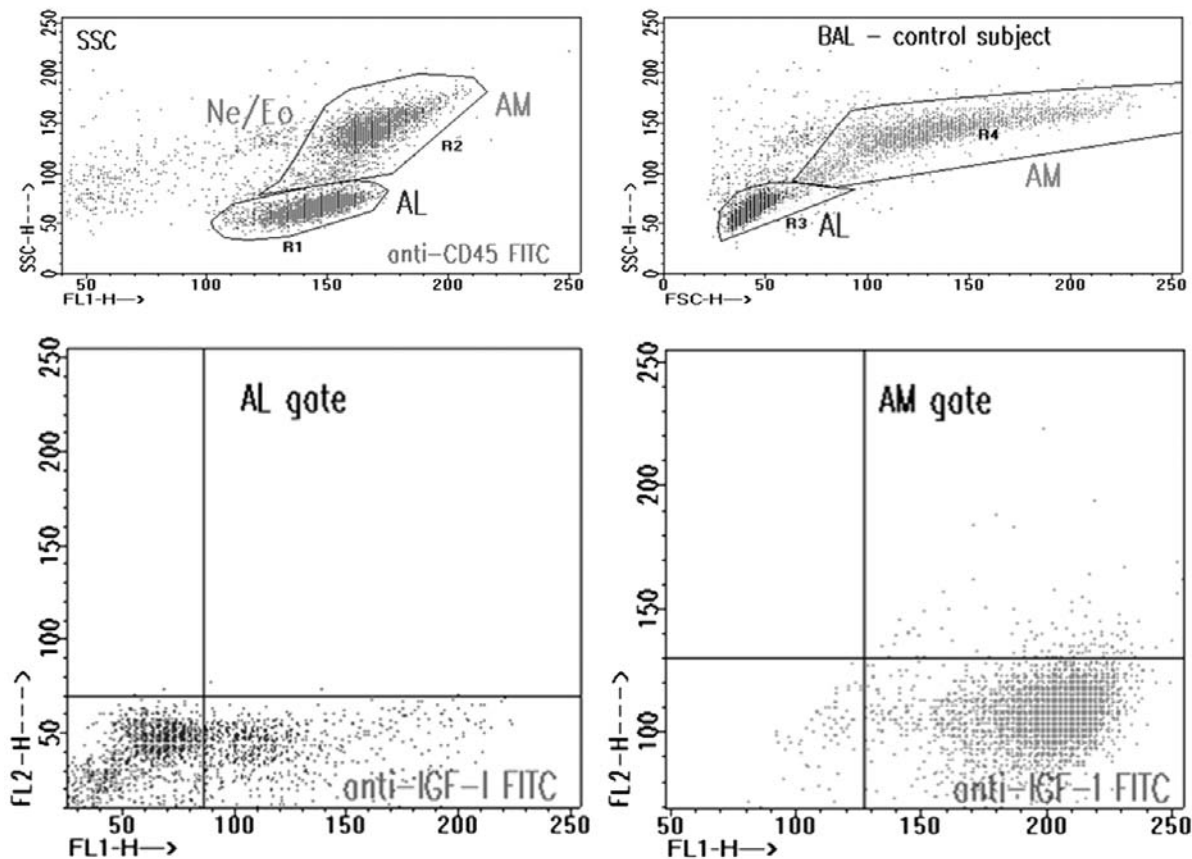


Fig. 2. FC analysis of IGF-I expression in AL and AM. Alveolar lymphocyte and macrophage gates are defined in flow cytometry due to cell CD45 expression and side scatter, SSC (upper left panel, gate R1 and R2 respect.) and then redefined, *i.e.* back-gated, acc. to FSC/SSC parameters (upper right panel, gate R3 and R4 respect.). Sample of AL (R3) gate analysis for IGF-I expression in left lower panel; parallel analysis of AM (R4) gate in right lower panel. About 30% of AL and > 95% of AM are positive. Markers set according to the negative isotype control. SSC presented in logarithmic scale in order to visualize dot plot of macrophages.

BAL routine cytology. The total cell count and cell viability (trypan blue exclusion test) were calculated, as described before. BAL cytospin smears were stained simultaneously with hematoxylin-eosin (HE) and May-Grünwald-Giemsa (MGG). In each sample the differential count of BAL reactive cells was calculated as the mean result of both methods (at least 500 cells were counted) [14].

BAL immunocytochemistry. The immunocytochemical procedure was performed on samples obtained by BAL material cyto-centrifugation (100-300 μ l of native BAL fluid, Shandon, Cytospin 3, 1000 rpm, 5 min followed by fixation in 70% ethanol, 10 min, stored at -80°C). The avidin-biotin-peroxidase method was used (LSAB 2 System, DAKO Cytomation, cat. no K0675). The immunostaining procedure was carried out using mouse anti-human monoclonal antibodies (MoAbs) directed against CD95, IGF-I and BCL-2 (details listed in Table 1), diluted in Antibody Diluent (Dako Cytomation, no S2022). As negative controls, we replaced primary antibodies with nonspecific mouse IgG1.

After rehydration, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in PBS (5 min). Cytospin smears were then washed in TBS, blocked with 3% bovine serum albumin (BSA, POCh, 30 min, room temperature) to inhibit nonspecific immunoreactivity and incubated overnight at 4°C with the monoclonal antibodies (data in Table 1). Alternatively, the wet chamber incubation with diluted MoAbs at room temperature, 1 h, was carried out. Consecutive incubations were con-

ducted with biotinylated anti-mouse secondary antibody (50 μ l) and horseradish peroxidase solution linked with streptavidin (Streptavidin-HRP, 50 μ l, LSAB 2 Systems, DAKO Cytomation, no K0675). Both incubations were carried out at room temperature for 15 min, and slides were rinsed $3\times$ in TBS before each incubation. Diaminobenzidine (DAB) was used as chromogen. The slides were rinsed with TBS buffer, pH 7.6 (Trismabase SIGMA, cat. no K5458) in NaCl solution, counterstained with Harris hematoxylin (2-5 min), rinsed in water, dehydrated with use of graded alcohols (50% 70% 90% and 99%, 5 min each), bathed in fresh xylene and covered with glycerol jelly. The cells were evaluated in light microscope. In each slide we counted at least 250 cells (500 on the average), calculating separately the percentage of positive macrophages and positive lymphocytes [14]. Intracellular expression of IGF-I and surface staining for Fas (CD95) are presented in Figure 1.

BAL immune cell phenotyping. BAL material was centrifuged ($300\times g$, 10 min) and a cell pellet was resuspended in PBS to $2-10\times 10^6$ cells per ml. The samples containing 50 μ l of cell suspension were incubated with mixture of saturating amounts of MoAbs for 30 min in the dark. Double- or three-colour typing was performed, according to the percentage of AL (more or less than 5% of BAL reactive cells, respectively). Mouse anti-human MoAbs directed against superficial CD3, CD 4, CD8, CD16+56, CD19, CD45, CD95 and CD178 antigens, fluorochrome-conjugated (FITC or

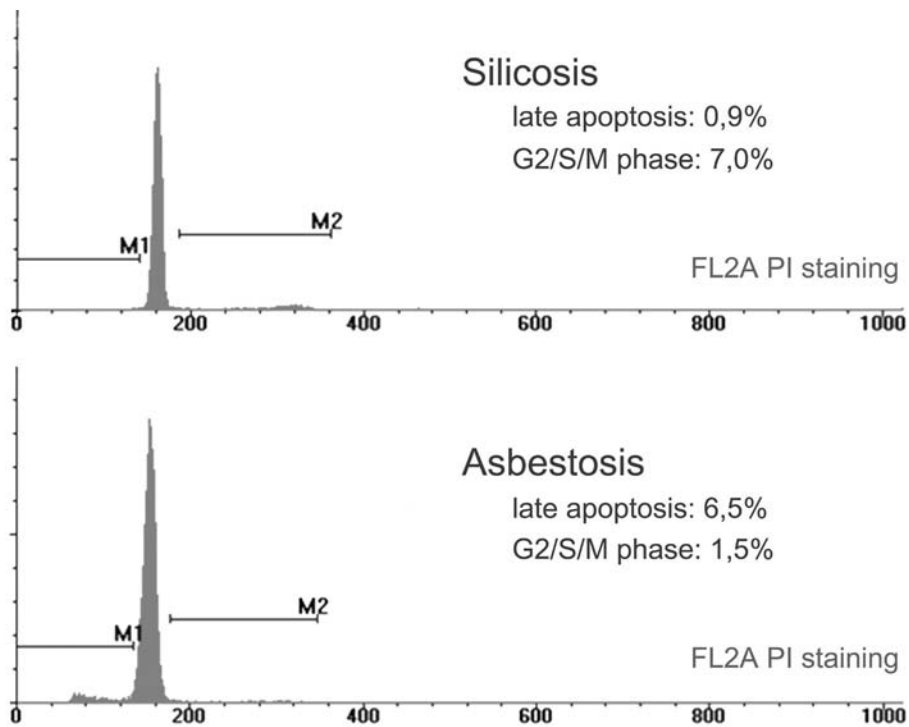


Fig. 3. Late apoptosis and cell cycle analysis of AL gate - samples of silicosis (the upper panel) and asbestosis (the lower panel). Generally, AL late apoptosis (marker 1, M1) in the majority of examined ILD patients is uncommon as determined with the use of PI; the relatively frequent AL apoptotic rate (>6% in presented asbestosis sample) occurs rarely. Another characteristic feature of BAL material is a relatively low percentage of proliferating AL (G₂/S/M phase, marker 2, M2).

PE) were used in the study (Becton Dickinson Immunocytometry Systems, BDIS, Mountain View, CA, USA; R & D Systems, data in Table 1). Negative isotype control was used in sample series of every patient. After incubation, cells were washed in PBS (300× g, 10 min) and resuspended in 300 µl of PBS containing 1% formaldehyde [3, 14].

Flow cytometry was also applied to test BAL cell IGF-1 and BCL-2 expression. The samples containing 50 µl of BAL cell suspension were preincubated with 0.3% saponin (200 µl, 10 min), washed in 0.1% saponin (1 ml, 300× g, 5 min) and incubated with saturating amounts of the respective MoAb (in 100 µl of 0.3% saponin, 30 min in the dark). Cells were washed and incubated with the secondary antibody (rabbit anti-mouse FITC conjugated serum, DAKO Cytomation, cat. no F031302) for 10 min in the dark. The same secondary antibody was used as negative control (primary MoAb was omitted). The cells were washed twice with PBS and resuspended in 300 µl of PBS with 2% formaldehyde for immediate flow cytometric analyses.

Early apoptosis detection with Annexin V FITC/PI staining.

Detection of early apoptosis in BAL lymphocytes was performed according to the instruction of manufacturer (APOTEST™-FITC, DAKO Cytomation, cat. no K2350). In brief, fresh BAL cells (10⁵-10⁶ cells/ml) were washed, resuspended in 96 µl of ice-cold diluted binding buffer and incubated with 1 µl Annexin V-FITC and 2.5 µl propidium iodide for 10 min in the dark. The cell sample was diluted with 250 µl of binding buffer and then measured immediately by flow cytometry. Negative control was carried out for each sample (incubation with binding buffer alone). For positive control, the cells were incubated on ice with 3% formaldehyde in binding buffer for 30 min [29].

Late apoptosis detection with cell permeabilization and PI staining.

BAL cells (1-2 × 10⁶) were washed in PBS, resuspended in 0.03% Nonidet solution with 0.005% propidium iodide (PI), centrifuged (400 × g, 5 min) and incubated with 250 µl of the Nonidet/PI solution (room temperature, 15 min in the dark). The next incubation was carried out for 15 min with 250 µl of RNase

solution (88 Kunitz U per 100 ml PBS, room temperature, in the dark). The cells were finally suspended in 1 ml volume (adjusted with PBS) and acquired in FACSCalibur flow cytometer (BDIS) within 24 h from staining [29].

Flow cytometry. All BAL materials included in the study fulfilled the precise criteria of cytometric analyses, which were commented on elsewhere [14]. FC data were acquired within 24 h after staining, using CellFit software and FACSCalibur cytometer (BDIS). An argon ion laser excitation 488 nm was used. The emitted light was detected by logarithmic amplification through barrier filters specific for the emission range of the different fluorophores: 530/22 nm for FITC (fluorescence channel FL1), 585/42 nm (FL2) for PE and >650 nm (FL3) for PE Cy5. In each sample 8000-12000 events (cells) were acquired.

Gates for AL and AM were determined due to the cell granularity (side scatter, SSC) and intensity of staining with CD45 FITC ("back gating"). The sample was presented in Figure 2. The results of lymphocyte typing and lymphocyte early apoptosis detection were yielded by quadrant analyses of FL1 vs FL2 channel dot plot and were presented as the percentage of gated lymphocytes [29].

For detection of late apoptosis, cells were acquired with the use of pulse processor. Cell cycle was assessed simultaneously. The conjunction of two gates was applied: forward scatter (characterizing cell size) vs side scatter (FSC vs SSC), as well as pulse width vs pulse area in a fluorescence-2 standard channel (FL-2W vs FL-2A). Data on 15 000-20 000 BAL cells were collected and analyzed with BDIS ModFit software. The sample was presented in Figure 3. Two distinct FSC/SSC gates were applied for separate AL and AM apoptosis/cell cycle FC analyses.

Statistical analysis. All BAL cytology and phenotype results were presented as medians ± SEM (due to nonparametric distribution of values) [20]. The Mann-Whitney U-test was used to compare the data obtained in analyzed groups (ILD patients vs controls). The Spearman's rank correlation coefficient r_s was applied to test the correlation between two random variables. P-values < 0.05 were considered statistically significant.

Table 2. Patients' data and BAL cytoimmunology in interstitial lung diseases (ILD)

Group		Controls	Silicosis	Asbestosis	IPF	Sarcoidosis
N		15	13	9	12	56
Sex	MF	6/9	11/2	7/2	6/6	24/32
Age #	Years	45 ± 10.9 (23-68)	44 ± 11.3 (25-68)	49 ± 8.3 (39-71)	53 ± 11.1 (35-75)	39 ± 11.6 (25-75)
BAL fluid recovery	%	50 ± 4.2 (33-65)	51 ± 4.2 (30-69)	44 ± 8.1 (36-73)	48 ± 4.2 (41-74)	51 ± 1.5 (30-80)
VC #	% pred. value	109 ± 7.0 (82-114)	80 ± 8.4 (39-111)	74 ± 8.6 (46-112)*	77 ± 14.5 (64-93)*	96 ± 4.5 (65-122)
FEV1/VC #	%	87 ± 8.3 (72-98)	70 ± 4.3 (65-93)*	86 ± 5.8 (72-99)	89 ± 7.9 (75-94)	74 ± 2.9 (65-92)
BAL total cell no	× 10 ³	125 ± 33 (50-290)	370 ± 26 (101-703)*	425 ± 63 (121-890)*	505 ± 91 (103-1150)*	425 ± 49 (75-1220)**
Macrophages	%	84.5 ± 2.7 (76-93)	91 ± 4.2 (65-95)*	82 ± 3.5 (70-89)	52 ± 6.7 (7-80)*	55 ± 2.1 (23-88)*
Lymphocytes	%	13.3 ± 2.0 (7-23)	6 ± 3.8 (1-35)*	11 ± 3.1 (9-25)	38 ± 7.4 (7-66)*	43 ± 2.1 (11-77)**
Neutrophils	%	0.3 ± 0.6 (0-3)	1.5 ± 0.5 (0.9-4.1)	1.8 ± 1.3 (0-13)*	9 ± 4.1 (0-36)**	0.6 ± 0.2 (0-6)
Eosinophils	%	0 ± 0.1 (0-0.2)	0 ± 0.2 (0-0.2)	0	1.9 ± 6.4 (0-40)*	0.5 ± 0.2 (0-5)*
T cells CD3+	%	89 ± 1.8 (78-96)	91 ± 1.1 (85-95)	92 ± 1.6 (85-95)	89 ± 1.6 (80-94)	94 ± 0.6 (82-99)
Th/Tc CD4/CD8		2.2 ± 0.4 (0.8-5.1)	0.8 ± 0.1 (0.2-1.7)*	3.1 ± 1.7 (0.6-10.9)	0.7 ± 0.3 (0.2-2.8)*	7.9 ± 0.9 (1.6-32.3)**

M - male, F - female; # Data presented as mean ± SD (range); other results presented as median ± SEM (range);

*P < 0.05 as compared to controls; **P < 0.01 as compared to controls

Results

The basic information about groups included in the study, as well as their BAL cytological and immunological data, are presented in Table 2. Increase in BAL total cell number was found in all tested groups, as compared with controls. Silicosis was characterized by higher percentage of alveolar macrophages and a relative decline in BAL lymphocytes. In asbestosis, increased percentage of AM and neutrophils together with high CD4/CD8 ratio was found. IPF was characterized by increase in proportion of BAL lymphocytes, neutrophils and eosinophils; CD4/CD8 ratio was low. BAL distinct lymphocytosis together with mild eosinophilia and typical high CD4/CD8 ratio was characteristic for sarcoidosis patients.

AL population, in contrast to the respective peripheral blood results, was dominated by T cells with only few NK and B cells, regardless of the tested group (data not shown). Additionally, in all groups, up to 100% of AM, Th (CD4) and Tc (CD8) cells expressed CD95 marker, suggesting high AM and AL suscepti-

bility to proapoptotic stimuli. The results of Fas Ligand (CD178) expression on AL are presented in Figure 4. The percentage of CD178-positive AL was increased in asbestosis, and in IPF (for Tc cells only); it was decreased in total sarcoidosis, as compared with controls. However, we did not observe any association between Fas Ligand expression on AL and the number of apoptotic BAL immune cells.

Almost all AM, according to immunocytochemical staining, were positive for IGF-I: from 88 ± 4.9 (77-100)% in IPF to 93 ± 5.5 (44-100)% in sarcoidosis and 95 ± 3.8 (75-100)% in silicosis. The respective values for asbestosis and controls were 91 ± 3.7 (81-99)% and 94 ± 1.3 (91-97)%. There were no significant differences in IGF-I and BCL-2 expression in AM between the tested groups.

Results of intracellular expression of IGF-I and BCL-2 in alveolar lymphocytes (data from flow cytometry) were presented in Figure 5. Sarcoidosis was subdivided according to the disease stages.

A noticeable number of alveolar lymphocytes expressed IGF-I. The percentage of IGF-I-positive

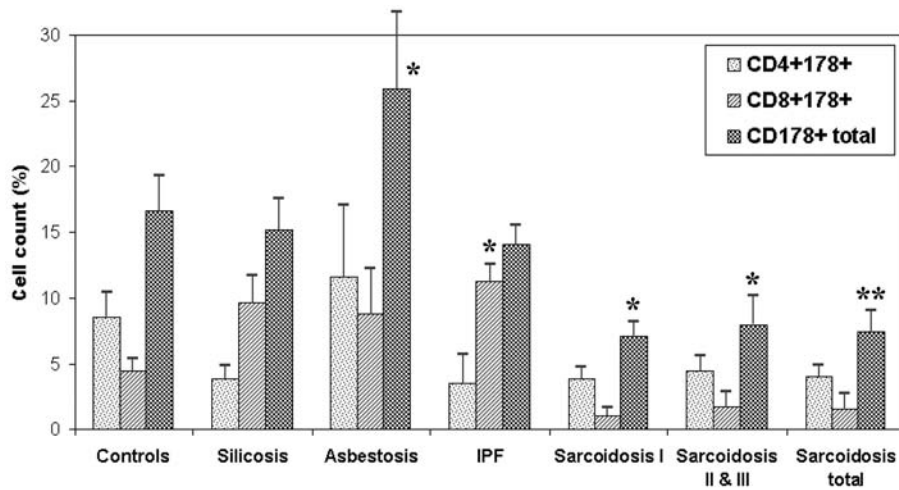


Fig. 4. Expression of Fas Ligand (CD178) - alveolar lymphocytes in ILD. Flow cytometry. Data presented as medians \pm SEM of AL positive for CD178; * $p < 0.05$; ** $p < 0.01$ as compared to controls.

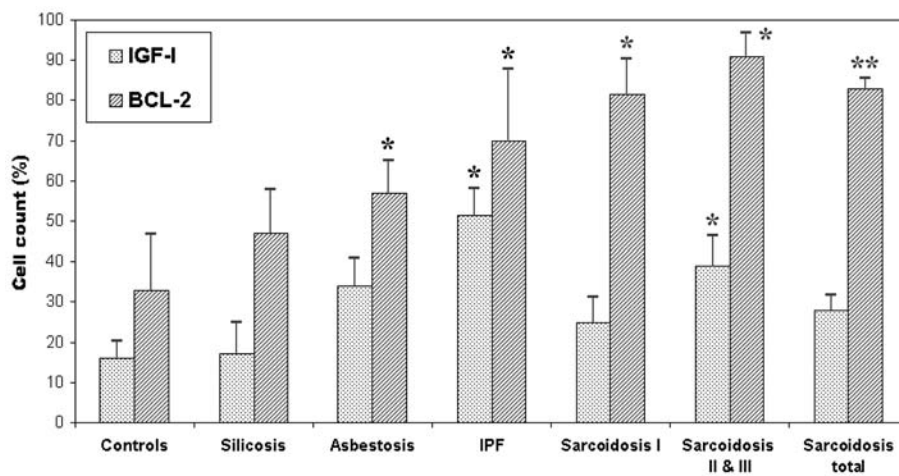


Fig. 5. Expression of IGF-I and BCL-2 - alveolar lymphocytes in ILD. Flow cytometry. Data presented as medians \pm SEM of AL positive for BCL-2; * $p < 0.05$ as compared to controls; ** $p < 0.01$ as compared to controls.

lymphocytes was significantly higher in IPF (52 ± 6.7 , range: 5-67%) and in later radiological sarcoidosis stages (39 ± 7.8 , range: 6-88%) than in controls (16 ± 4.0 , range: 6-18%, $p < 0.05$). Changes observed for stage I of sarcoidosis (25 ± 6.7 , range: 2-65%), silicosis (17 ± 7.8 , range 5-38%) and asbestosis (34 ± 7.2 , range 3-50%) were statistically insignificant. IGF-I expression was found in both major T cell subsets (Th and Tc, data not shown).

Significantly increased percentage of AL expressing BCL-2 was detected in IPF and sarcoidosis.

The results of cell cycle and apoptosis examination are summarized in Table 3. Decreased apoptosis rate - both early and late one - was characteristic for AL in sarcoidosis, regardless of the stage of the disease ($p < 0.05$). IPF was characterized by significantly higher rate of proliferating AL (cells of $G_2/S/M$ phase).

The percentage of IGF-I-positive alveolar lymphocytes was strongly and positively correlated with parameters reflecting proliferation of both AL and AM (Fig. 6). However, the percentage of alveolar

macrophages expressing IGF-I was correlated only with the respective data concerning AM alone and it was not related to any parameters of alveolar lymphocyte proliferation. Surprisingly, despite the positive correlation between the percentage of IGF-I-positive AL and the proportion of BAL cells carrying BCL-2, no relation between immune cell IGF-I expression and any parameters characterizing their apoptosis was found.

The percentage of AL and AM expressing IGF-I was not correlated with any values of lung function tests, including VC and FEV_1/VC .

Discussion

It has been known for many years that almost all AM secrete IGF-I as so called alveolar macrophage-derived growth factor, AMDGF. In the present study we have proven that the expression of IGF-I is also present in alveolar lymphocytes. In opposite to alveolar macrophages, which are almost all IGF-I+ in every

Table 3. Cell cycle and apoptosis in interstitial lung diseases (ILD)

Group		Controls	Silicosis	Asbestosis	IPF	Sarcoidosis
G ₀ /G ₁	%	97.0 ± 0.3 (89.7-99.1)	96.9 ± 1.1 (84.7-98.9)	97.8 ± 1.0 (96.3-98.8)	96.0 ± 1.3 (89.4-98.3)	96.0 ± 0.6 (85.0-99.7)
S	%	0.75 ± 0.78 (0.2-7.3)	0.8 ± 0.5 (0.4-6.3)	1.2 ± 0.2 (0.6-1.4)	2.2 ± 1.3 (0.6-4.9)	0.7 ± 0.4 (0.1-8.6)
G ₂ /M	%	0.7 ± 0.3 (0-1.7)	0.5 ± 0.4 (0-3.2)	0.6 ± 0.4 (0.2-1.0)	0.9 ± 0.2 (0.3-1.7)	1.1 ± 0.4 (0-10.6)
S/G ₂ /M	%	1.25 ± 2.9 (0.2-8.5)	1.9 ± 0.8 (0.8-6.7)	1.8 ± 0.5 (0.8-2.5)	3.3 ± 0.7 (1.5-5.2)*	2.8 ± 0.6 (0.1-16)
Late apoptosis sub-G ₁ peak	%	1.15 ± 0.33 (0.1-2.4)	1.99 ± 1.10 (0.3-9.2)	0.70 ± 0.33 (0-1.5)	1.05 ± 0.60 (0-5.4)	0.60 ± 0.17 (0-5.8)*
AM late apoptosis sub-G ₁ peak	%	1.21 ± 1.0 (0.1-6.8)	0.55 ± 0.3 (0.2-6.3)	0.80 ± 0.4 (0.2-1.6)	0.74 ± 0.3 (0-2.7)	0.81 ± 0.2 (0-4.6)
AMS phase	%	1.55 ± 0.3 (0.6-7.4)	3.2 ± 1.1 (1.0-19)	2.45 ± 0.4 (0.6-17)	3.7 ± 0.6 (0.1-8.5)	2.1 ± 1.1 (0.3-21.4)

All data concern alveolar lymphocytes, except the last two rows concerning alveolar macrophages (AM). Results are presented as median ± SEM (range), *P < 0.05 as compared to controls

tested individual, our results obtained in alveolar lymphocytes were different in some groups of patients, *i.e.* percentage of IGF-I-positive AL was significantly higher in IPF and in the later stages of sarcoidosis as compared to the control group.

It should be established what is an actual importance of IGF-I, including AL-derived IGF-I in the lower airways.

First of all, it has been considered for many years that IGF-I is active as a local fibroblast stimulator and profibrotic factor [8]. The secretion of IGF-I as well as expression of IGF-receptor were increased in BAL macrophages of patients with pneumoconioses and in AM of experimental animals exposed to silica [9, 26]. However, it should be emphasized that in large group of patients analyzed in the current study we did not observe any negative correlation between IGF-I expression and predicted value of VC. These results were in general consistent with some recent studies. In one of them, Krein *et al.* [16] suggested that IGF-I in lower airways, contrary to TGF-β, protects epithelial cell layer from injury and apoptosis. They questioned the link between IGF-I and interstitial fibrosis in ILD. The profibrotic role of IGF-I has not been confirmed in two other studies by Mustaers *et al.* [21] and Vanhee *et al.* [32], who examined statistical correlations in BAL fluid of asbestosis and coal workers' pneumoconiosis patients. Furthermore, in BAL of IPF patients analyzed by Pala *et al.* [23], IGF-I level was positively correlated with the results of lung function tests. We conclude that IGF-I does not stimulate lung fibrosis in clinical conditions, despite findings from experimental studies.

Secondly, since IGF-I protects many cell types from apoptosis [27], we have tried to relate the results

of IGF-I expression in BAL immune cells to data characterizing apoptosis of AL and AM. High proportion (up to 100%) of both T lymphocytes and alveolar macrophages expressed superficial Fas molecules. Additionally, many of these cells co-expressed Fas ligand. Alveolar cells are susceptible then to apoptosis induced by membrane stimuli. Surprisingly, we found a relatively low apoptotic rate in both AM and AL, especially in BAL lymphocytes originating from sarcoidosis patients. In general, our results obtained in patients with sarcoidosis were consistent with the hypothesis on apoptosis resistance of alveolar lymphocytes in this disorder [28]. However, account should be taken of the relatively low AL apoptosis rate obtained in the current study in other interstitial lung diseases, as IPF and asbestosis.

To our surprise, in our material there was no correlation between IGF-I expression in immune cells and the rate of AL and AM apoptosis. The only finding suggesting the influence of IGF-I on regulation of apoptosis in the lower airways was a significant positive correlation between the percentage of alveolar lymphocytes carrying IGF-I and expression of BCL-2 in both AL and AM. Finally, we have not proven the antiapoptotic activity of IGF-I in ILD immune cells. This lack of relation could be explained by the complex character of local interactions between prosurvival and proapoptotic factors [4, 6]. The mechanisms of this interaction have not been yet fully understood. For example, decreased BCL-2 expression can occur together with very low AL apoptotic rate in some lung disorders [17].

Thirdly, we expected that IGF-I expression in lower airways might affect cell proliferation in lower airways of ILD patients. Actually, the percentage of AL carry-

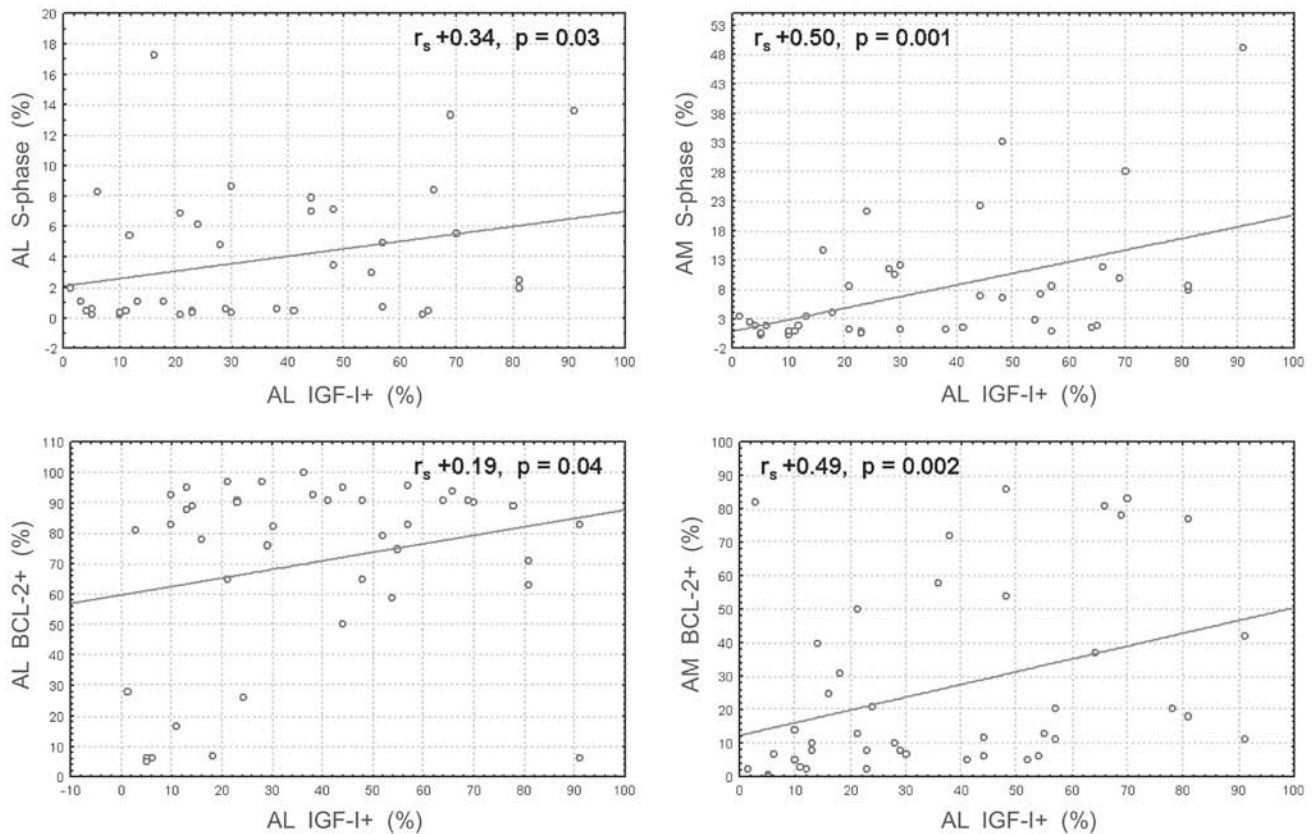


Fig. 6. Correlations between percentage of IGF-I positive alveolar lymphocytes and (1) proportion of AL and AM in S phase of the cell cycle (upper panels) and (2) BCL-2 expression in AL and AM (lower panels).

ing IGF-I was positively correlated with proliferation rate of BAL immune cells. This relation was statistically significant for both S and G2M phase of cell cycle in AL as well as AM. However, it should be emphasized that the immune cell proliferation rate observed in lung alveoli was in general low, if we take into consideration the active, extensive inflammation commonly present in sarcoidosis and other ILD. This infrequent proliferation may be explained by local activity of surfactant proteins and other potent mitosis inhibitors that appear in alveoli [7]. IGF-I seems to be unable to overcome them.

The cytological and immunological results obtained in the present study are in line with data reported by other authors and with our previous findings, described elsewhere [14, 15, 29].

Summing up, human lymphocytes in the lower airways express IGF-I both in normal conditions and in ILD. The proportion of IGF-I+ lymphocytes is significantly increased in IPF and in later (II and III) stages of sarcoidosis. The local role of IGF-I in normal conditions, as well as in ILD, needs further investigation. IGF-I seems to be a potent mitogen of alveolar lymphocytes and macrophages, but in our material no evidence for its antiapoptotic or profibrotic function in ILD has been found.

Acknowledgements: The work presented in the paper was performed in Krakow, Bydgoszcz and Bialystok, Poland. The study was supported by the grant of State Committee for Scientific Research no. 3P05D 15322 and by NATO grant no. LST.CLG 980617.

References

- [1] Agostini C, Perin A, Semenzato G (1998) Cell apoptosis and granulomatous lung diseases. *Curr Opin Pulm Med* 4: 261-266
- [2] Allen JT, Bloor CA, Knight RA, Spiteri MA (1998) Expression of insulin-like growth factor binding proteins in bronchoalveolar lavage fluid of patients with pulmonary sarcoidosis. *Am J Respir Cell Mol Biol* 19: 250-258
- [3] BAL Cooperative Steering Committee (1990) Lymphocyte surface antigen phenotypes in bronchoalveolar lavage fluid and peripheral blood. In: *Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis and selected comparison groups*. *Am Rev Respir Dis* 141: S169-S202
- [4] Bingisser R, Stey C, Wellev M, Groscurth P, Russi E, Frei K (1996) Apoptosis in human alveolar macrophages is induced by endotoxin and is modulated by cytokines. *Am J Respir Cell Mol Biol* 15: 64-70
- [5] Bloor CA, Allen JT, Knight RA (2001) Differential mRNA expression of insulin-like growth factor-I splice variants in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. *Am J Respir Crit Care Med* 164: 265-272
- [6] Borges VM, Lopes MF, Flaca H, Leite-Junior JH, Rocco PRM, Davidson WF, Linden R, Zin WA and DosReis GA

- (2002) Apoptosis underlies immunopathogenic mechanisms in acute silicosis. *Am J Respir Cell Mol Biol* 27: 78-84
- [7] Borron P, Veldhuizen RAW, Lewis JF, Possmayer F, Caveney A, Inchley K, McFadden RG, Fraher LJ (1996) Surfactant associated protein-A inhibits human lymphocyte proliferation and IL-2 production. *Am J Respir Cell Mol Biol* 15: 115-121
- [8] Cambrey AD, Kwon OJ, Gray AJ, Harrison NK, Yacoub M, Barnes PJ, Laurent GJ, Chung KF (1995) Insulin-like growth factor I is a major fibroblast mitogen produced by primary cultures of human airway epithelial cells. *Clin Sci* 89: 611-617
- [9] Chen F, Deng HY, Ding GF, Houg DW, Deng YL, Long ZZ (1994) Excessive production of insulin-like growth factor-I in silicotic rat alveolar macrophages. *APMIS* 102: 581-588
- [10] Fujimura N (2000): Pathology and pathophysiology of pneumoconiosis. *Curr Opin Pulm Med* 6: 140-144
- [11] Hoser G, Kawiak J, Domagała-Kulawik J, Kopinski P, Droszcz W (1999) Flow cytometric evaluation of lymphocyte subpopulations in BALF of healthy smokers and nonsmokers. *Folia Histochem Cytobiol* 37: 25-30
- [12] Hunninghake GW, Costabel U, Ando M (1999) ATS/ERS/WASOG statement of sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. *Sarcoidosis Vasc Diffuse Lung Dis* 16:149-173
- [13] Klech H, Pohl W (1989) Technical recommendations and guidelines for bronchoalveolar lavage (BAL). *Eur Resp J* 2: 561-585
- [14] Kopiński P (1997) Flow cytometry applied in cytological changes assessment in interstitial lung diseases (in Polish). *Folia Med Cracov* 38: 69-115
- [15] Kopiński P, Soja J, Czunko P, Jedynek U, Lackowska B, Szczeklik J, Śladek K, Chłap Z (2000) Cytoimmunological alterations in bronchoalveolar lavage (BAL) obtained in non-symptomatic individuals chronically exposed to silica dust (in Polish). *Pneumonol Alergol Pol* 68: 109-119
- [16] Krein P, Winston B (2002) Roles of insulin-like growth factor-I and transforming growth factor- β in fibrotic lung disease. *Chest* 122: 289-293
- [17] Laflamme C, Israël-Assayag E, Cormier Y (2003) Apoptosis of bronchoalveolar lavage lymphocytes in hypersensitivity pneumonitis. *Eur Respir J* 21: 225-231
- [18] Lohman-Matthes ML, Steinmüller C, Franke-Ullman G (1994) Pulmonary macrophages. *Eur Respir J* 7: 1678-1689
- [19] McAnulty RJ, Guerreiro D, Cambrey AD, Laurent GJ (1992) Growth factor activity in the lung during compensatory growth after pneumonectomy: evidence of a role for IGF-I. *Eur Respir J* 5: 739-747
- [20] Merchant RK, Schwartz DA, Helmers RA, Dayton ChS, Hunninghake GW (1992): Bronchoalveolar lavage cellularity. The distribution in normal volunteers. *Am Rev Respir Dis* 146: 448-453
- [21] Mustaers SE, Harrison NK, McAnulty RJ, Liao JY, Laurent GJ, Musk AW (1998) Fibroblast mitogens in bronchoalveolar lavage (BAL) fluid from asbestos-exposed subjects with and without clinical evidence of asbestosis: no evidence for the role of PDGF, TNF-alpha, IGF-1, or IL-1 beta. *J Pathol* 185: 199-203
- [22] Pabst R, Binns RM (1996) Lymphocytes migrate from the bronchoalveolar lavage space to regional bronchial lymph nodes. *Am J Respir Crit Care Med* 151: 495-499
- [23] Pala L, Giannini S, Rosi E, Cresci B, Scano G, Mohan S, Duranti R, Rotella CM (2001) Direct measurement of IGF-I and IGFBP-3 in bronchoalveolar lavage fluid from idiopathic pulmonary fibrosis. *J Endocrinol Invest* 24: 856-864
- [24] Pirożyński M, Pirożyńska E (1999) Bronchoalveolar lavage (in Polish). In: *Bronchofiberoskopia*, Pirożyński M [Ed], α -Medica Press, Bielsko-Biała, pp 118-127
- [25] Pollak M (2000) Insulin-like growth factor physiology and cancer risk. *Eur J Cancer* 36: 1224-1228
- [26] Rom WN, Paakko P (1991) Activated alveolar macrophages express the insulin-like growth factor-I receptor *Am J Respir Cell Mol Biol* 4: 432-439
- [27] Rubin R, Baserga R (1995) Insulin-like growth factor-I receptor. Its role in cell proliferation, apoptosis, and tumorigenicity. *Lab Invest* 73: 311-331
- [28] Stridh H, Planck A, Gigliotti D, Eklund A, Grunewald J (2002) Apoptosis resistant bronchoalveolar lavage (BAL) fluid lymphocytes in sarcoidosis. *Thorax* 57: 897-901
- [29] Szczeklik J, Trojan J, Kopiński P, Soja J, Szlubowski A, Dziejzina S, Iwaniec T, Woś M (2004) Apoptosis in bronchoalveolar lavage lymphocytes (L-BAL) in pneumoconiosis (in Polish). *Przegl Lek* 61: 235-240
- [30] Trojan LA, Kopinski P, Wei MX, Ly A, Glogowska A, Czarny J, Shevelev A, Przewlocki R, Henin D, Trojan J (2002): IGF-I: from diagnostic to triple-helix gene therapy of solid tumors. *Acta Biochim Pol* 49: 979-990
- [31] Uh ST, Inoue Y, King TE Jr, Chan ED, Newman LS, Riches DW (1998) Morphometric analysis of insulin-like growth factor-I localization in lung tissues of patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 158: 1626-1635
- [32] Vanhee D, Gosset P, Wallaert B, Voisin C, Tonnel AB (1994) Mechanisms of fibrosis in coal workers' pneumoconiosis. Increased production of platelet-derived growth factor, insulin-like growth factor type I, and transforming growth factor beta and relationship to disease severity. *Am J Respir Crit Care Med* 150: 1049-1055

Received: March 23, 2005

Accepted after revision: April 28, 2006