Transforming growth factor-β_1 in sarcoidosis

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Transforming growth factor-β_1 in sarcoidosis. F. Salez, P. Gosset, M.C. Copin, C. Lamblin Degros, A.B. Tonnel, B. Wallaert. ©ERS Journals Ltd 1998.
ABSTRACT: Transforming growth factor-β (TGF-β) is a cytokine that promotes extracellular matrix accumulation and inhibits matrix degradation. Although the natural course of sarcoidosis is usually favourable, granuloma healing in the lung may result in pulmonary fibrosis and respiratory impairment in some patients.

In this study TGF-β_1 was evaluated in bronchoalveolar lavage (BAL) fluid and culture supernatants of alveolar macrophages (AM) from 73 patients with biopsy-proven sarcoidosis. Disease activity was defined when patients recently developed or increased symptoms (cough, dyspnoea, systemic symptoms) and/or demonstrated increasing opacities on chest radiography. Pulmonary function tests were performed in all patients including forced expiratory volume in one second (FEV1), forced vital capacity (FVC), total lung capacity (TLC) and the diffusing capacity of the lung for carbon monoxide (DL,CO). Fourteen patients with idiopathic pulmonary fibrosis (IPF) and 14 healthy subjects were investigated as a control group. Immunohistochemistry was used to evaluate the cell distribution of TGF-β_1 on lung specimens.

TGF-β_1 levels in BAL and in AM supernatants were not different between sarcoidosis and healthy subjects, whereas they were markedly increased in IPF. However, the TGF-β_1 level was significantly increased in BAL fluid but not in AM supernatants from sarcoidosis with altered lung function, compared with patients with normal lung function. The TGF-β_1 level in BAL was increased in active sarcoidosis but this increased level was mainly related to the higher level observed in patients with altered lung function. TGF-β_1 levels in BAL correlated significantly with the lymphocyte percentage. TGF-β_1 staining assessed by immunohistochemistry was intense in epithelioid histiocytes comprising non-necrotizing granuloma and in bronchiolar epithelial cells, in hyperplastic type II pneumocytes and occasionally in AM.

This study supports the hypothesis that overproduction of transforming growth factor-β_1 is associated with functional impairment in patients with pulmonary sarcoidosis.
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Sarcoidosis is a chronic inflammatory disorder of unknown aetiology characterized by the formation of nonnecrotizing granulomas in affected tissues, particularly the lung. Although the natural course of the disease is usually favourable, granuloma healing in the lung may result in pulmonary fibrosis and respiratory impairment in some patients. The mechanisms by which granuloma healing results in lung fibrosis have not been fully established but may involve the release of cytokines and growth factors by inflammatory cells present within or around the granulomas [1–5].

Transforming growth factor-β (TGF-β) is an important profibrotic growth factor implicated in pulmonary fibrosis [6–9]. It is a 25 kDa disulphide-linked homodimer or heterodimer protein with a broad range of biological functions. In mammals, three isoforms exist: TGF-β_1, TGF-β_2 and TGF-β_3, with nearly identical biological properties, and the biological functions reported for TGF-β are generally those of TGF-β_1 [6]. Extensive *in vitro* data have demonstrated the essential role of TGF-β in fibrosis: it induces the synthesis of many extracellular matrix molecules including fibronectin, type I collagen and tenascin and

also decreases matrix degradation *via* differential effects on the expression of proteases and their inhibitors. TGF-β has immunoregulatory activities and is thought to act as a mediator regulating chemotaxis and proliferation of fibroblasts [6–9].

With this in mind, this prospective study was initiated to evaluate TGF-β_1 levels in bronchoalveolar lavage (BAL) fluid and alveolar macrophage (AM) supernatants from 73 patients with pulmonary sarcoidosis. In addition, the tissue distribution of TGF-β_1 in lung specimens was analysed by immunohistochemistry.

Methods

Patients

Seventy-three consecutive nonsmoking patients with pulmonary sarcoidosis were included in the study. The diagnosis of sarcoidosis was established on compatible clinical

and radiographic findings and histological evidence of noncaseating granulomas. Special stains and cultures for fungi and mycobacteria were negative in BAL fluid. According to clinical and radiological data, the disease was classified as active or inactive. Criteria of disease activity were: recently developed or increasing cough or dyspnoea and/or systemic symptoms such as cutaneous lesions, weakness, fever, arthralgia and/or increasing opacities on chest radiography [4, 10]. Patients with active disease also exhibited an increased T-lymphocytosis in BAL (table 1). Five patients with "active" sarcoidosis and five patients with "inactive" sarcoidosis received corticosteroid treatment at the time of BAL.

Fourteen patients with idiopathic pulmonary fibrosis (IPF) were also studied as a control group. The diagnosis of IPF was based on accepted criteria [11], which included evidence of diffuse parenchymal infiltrates (peripheral and reticular nodular with a lower lobe predominance) on chest radiography and restrictive lung function with an open lung biopsy demonstrating varying degrees of interstitial fibrosis and intra-alveolar inflammatory cells. Two patients were treated with steroids and one patient with steroids and cyclophosphamide at the time of the study.

A control group of 14 nonsmoking and healthy subjects was also studied. None of the control subjects was atopic or had taken corticosteroids in the year preceding the study.

Oral informed consent was obtained for BAL from all the patients and control subjects. The study protocol was approved by the hospital's ethics committee.

Bronchoalveolar lavage

BAL was performed by instillation of 200 mL sterile saline into the bronchoalveolar tree with fibreoptic bronchoscopy under local anaesthesia. The retrieved fluid was centrifuged and the cells were counted. The viability, as assessed by cellular exclusion of trypan blue, always exceeded 90%. The analysis of BAL cells, in per cent is summarized in table 1.

Cell isolation and alveolar macrophage culture

The lavage fluid was filtered through sterile surgical gauze and centrifuged at 400×g for 10 min at 4°C. After three washings, the pellet was resuspended at a cell concentration of $1.5 \times 10^6 \cdot mL^{-1}$ in RPMI 1640 containing 5% heat-inactivated foetal calf serum (FCS) and 2 mM L-glutamine (Gibco, Courbevoie, France). Cells were allowed to adhere to plastic Petri dishes (2 mL in 35 mm diameter well) for 2 h at 37°C. The nonadherent cells were removed by three washes with RPMI. Adherent cells contained >95% AM and <1% lymphocytes. The viability of the AM was evaluated by a trypan blue exclusion test and was never <90%. Lavage fluid and AM supernatants (10 mL) collected after 3 h incubation were stored at -20°C for further assays.

Cytokine assays

TGF-β_1 assays only detect the biologically active form of TGF-β_1. However, TGF-β_1 is usually present in an inactive latent complex form. To measure the amount of natural active TGF-β_1, the samples were assessed without acidification. To assay for total TGF-β_1 including the active and latent forms, all samples were treated by acidification. TGF-β_1 levels were evaluated by an enzyme-linked immunosorbent assay (ELISA) system (Promega, Madison, WI, USA) using an antibody sandwich format as reported previously [12]. The protocol was performed according to the manufacturer's instructions. The sensitivity was 16 pg·mL^{-1} of TGF-β_1.

Table 1. – Characteristics of the study populations

	Inactive sarcoidosis (n=28)	Active sarcoidosis (n=45)	IPF (n=14)	Controls (n=14)
Age yrs	38±2	41±2	57±3	45±2
Sex ratio M/F	18/10	28/17	4/10	7/7
Smokers	0	0	4	0
Chest radiographic stages†				
Stage 0	3	4	-	-
Stage I	5	13	-	-
Stage II	13	23	-	-
Stage III	7	5	-	-
FEV1 % pred	96±3.1	96.1±2.7	78±4*	-
FVC % pred	100.7±2.5	102.3±2.9	85±5*	-
TLC % pred	97.9±2.5	97.4±2.5	73±4.2*	-
$D_{L,CO}$ % pred	99.3±3.4	99.6±3.3	55±5.7*	-
Total BAL cell count $10^3 \cdot mL^{-1}$	212±28	249±27	577±148*	389±101
Alveolar macrophages %	84.6±1.3	52.1±1.9+	75±2.9*	86±5.7
Lymphocytes %	13.1±1.0	45.6±1.9+	14±2.6	5.1±13
Neutrophils %	1.1±0.5	1.4±0.2	7.6±0.8*	13±0.3
Eosinophils %	0.3±0.1	0.5±0.1	2.4±1.0*	0.4±0.2

Results are expressed as mean±SEM. †: stage 0: normal radiograph; stage I: bilateral lymphadenopathy; stage II: bilateral lymphadenopathy with parenchymal infiltration; stage III: solely parenchymal infiltration. IPF: idiopathic pulmonary fibrosis; M: male; F: female; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; TLC: total lung capacity; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide; BAL: bronchoalveolar lavage. +: p<0.0001, significantly different from inactive sarcoidosis, IPF and controls; *: p<0.05, significantly different from the other groups.

Lung function

The pulmonary function tests (PFT) consisted of standard spirometry using a Medical Graphics 1070 system (St Paul, MN, USA) and measurement of lung volumes *via* body plethysmography using a Medical Graphics 1085 system. A single-breath diffusing capacity of the lung for carbon monoxide ($D_{L,CO}$) was performed using the Medical Graphics 1070 system. The measurements of the lung function were performed using standard protocols. Results were expressed as a percentage of predicted values [13]. For the purpose of this study, the patients were divided into two groups: group 1 (n=48) exhibited normal pulmonary function (forced vital capacity (FVC): 106±2.1% pred, forced expiratory volume in one second (FEV1) 101.7±1.9%, total lung capacity (TLC) 100.9±1.9% and $D_{L,CO}$ 106±2.4%) and group 2 (n=25) exhibited altered pulmonary function corresponding to a decrease of >20% of the expected value of one of the four lung function parameters: FVC, FEV1, TLC or $D_{L,CO}$ (FEV1 80±3.8% pred, FVC 89±3.7%, TLC 87.5±3.6% and $D_{L,CO}$ 83.1±4.4%); all mean values were significantly different from those in group 1.

Immunohistochemistry on lung tissue

The antibody used for immunohistochemical staining was a commercially available mouse monoclonal antibody directed against TGF-β₁ (epitope corresponding to amino acids 328–353 mapping at the C-terminus of the precursor form of human TGF-β₁; Santa-Cruz Biotechnology, CA, USA). The immunohistochemical procedure has been described previously [14]. All sections were treated with 0.3% hydrogen peroxide in methanol for 30 min. The sections were digested with 1 mg·mL⁻¹ of hyaluronidase in 0.1 M sodium acetate in 0.15 M NaCl (pH 5.5) for 30 min at 37°C in a humid staining box and rinsed with Tris-buffered saline. Nonspecific protein staining was blocked by 1.5% horse serum and 48.5% human antibody for 30 min. The sections were incubated with 150 mL of 20 mg·mL⁻¹ of antibody for 20 min at 37°C. The sections were rinsed with Tris-buffered saline and incubated with biotinylated horse antimouse immunoglobin (Vector Laboratories, Burlingame, CA, USA) for 1 h, washed extensively, incubated with streptavidin–biotin–peroxidase complex for 1 h and developed according to the manufacturer's directions. For control experiments, mouse anti-TGF-β₁ was replaced by nonimmune mouse immunoglobulin G (IgG); the same technique was also used with omission of the primary antibody.

Lung biopsy specimens were obtained by thoracotomy performed for the diagnosis of diffuse lung disease in two untreated patients with sarcoidosis. Lung tissue fragments from controls were obtained by lobectomy performed for lung cancer and positive control lung tissue fragments were obtained from lung biopsies performed in two untreated patients with IPF as described previously [12]. Careful examination showed the absence of staining in nonepithelial structures on the slide (vessels, muscle, pleura), strengthening the specificity of the staining.

Statistical analysis

Results of TGF-β₁ are expressed as median and interquartile range (IQR). Multiple comparisons of mean data among the groups of patients were performed by analysis of variance (ANOVA) followed by Scheffé's test. Comparisons were also performed using the Mann-Whitney U-test. The correlation between different parameters was analysed using Spearman's rank correlation coefficient. Multiple regression analysis was performed to assess the contribution of activity, lung function and treatment to TGF-β₁ levels. A p-value <0.05 was considered significant.

Results

TGF-β₁ in sarcoidosis, idiopathic pulmonary fibrosis and healthy controls

Total and active TGF-β₁ levels in both BAL fluid and AM supernatants were not significantly different between sarcoidosis and healthy controls (p=0.5, table 2). In contrast, the total TGF-β₁ but not the active TGF-β₁ level in both BAL fluid and AM supernatants was significantly increased in IPF compared with controls and sarcoidosis (p=0.03). No

Table 2. – Transforming growth factor (TGF)-β₁ in bronchoalveolar lavage (BAL) fluid and alveolar macrophages (AM) supernatants from patients with sarcoidosis, patients with idiopathic pulmonary fibrosis (IPF) and healthy controls

	n	BAL fluid		AM supernatant	
		Active TGF-β₁	Total TGF-β₁	Active TGF-β₁	Total TGF-β₁
Controls	14	60 (42)	96 (91.5)	158 (40)	228 (68)
Sarcoidosis	73	132 (78)	199 (273)	96 (53)	372 (141)
According to disease activity					
Active sarcoidosis	45	183 (257)*	273 (407)*	100 (54)	376 (138)
Normal PFT	26	96 (173)	123 (300)	100 (50)	376 (160)
Altered PFT	19	358 (166)***	488 (477)***	100 (60)	372 (128)
Inactive sarcoidosis	28	81 (88)	132 (119)	90 (44)	372 (186)
Normal PFT	22	46 (76)	121 (121)	94 (46)	372 (250)
Altered PFT	6	1033 (60)	200 (88)	80 (30)	392 (144)
IPF	14	64 (34)	862 (1178)⁺	188 (89)	904 (799)⁺

Results are expressed as median pg·mL⁻¹ and interquartile range in parentheses. Results obtained in patients with sarcoidosis are expressed according to disease activity and to pulmonary function tests (PFT). *: p<0.05, significantly different from patients with inactive sarcoidosis; ***: p<0.01, significantly different from patients with active sarcoidosis and normal PFT; ⁺: p<0.05, significantly different from controls and sarcoid patients.

difference in TGF-β levels in BAL and AM supernatants was detected according to the radiographic stage of sarcoidosis (data not shown).

Relationship with pulmonary function tests

In BAL fluid, both active and total levels of TGF-β_1 were significantly higher in patients with altered PFT (median±IQR: 317±316 and 347±392 pg·mL^{-1}, respectively) than in patients with normal PFT (64±146 and 122±200 pg·mL^{-1}). TGF-β_1 level in AM supernatants was not significantly different according to lung function (data not shown).

Relationship with disease activity

In BAL fluid, both active and total levels of TGF-β_1 were significantly higher in active than in inactive sarcoidosis (table 2). The increased TGF-β_1 level in active sarcoidosis was mainly due to the increased level observed in patients with altered lung function, since TGF-β_1 levels were significantly increased in active sarcoidosis with altered lung function compared with active sarcoidosis with normal lung function (table 2). In addition, the TGF-β_1 level in BAL fluid was significantly related to altered PFT, as assessed by multiple regression analysis (p<0.01). The BAL lymphocyte percentage and TGF-β_1 in BAL were significantly correlated (r=0.45, p=0.006).

The TGF-β_1 level in AM supernatants was not significantly different between inactive sarcoidosis and active sarcoidosis. No correlation between TGF-β_1 levels in AM supernatants and BAL cells was observed.

Relationship with sarcoidosis treatment

The active TGF-β_1 level in BAL was significantly higher in patients treated with oral steroids (n=10) (379±308 pg·mL^{-1}) than in untreated patients (n=63) (177±189 pg·mL^{-1}). The total TGF-β_1 level in BAL was not different according to treatment. The TGF-β_1 level in AM supernatants was not significantly different according to treatment.

Immunohistochemistry

Immunoreactive TGF-β_1 was mainly detected within the non-necrotizing granulomas, predominantly in the epithelioid histiocytes, observed in lung specimens obtained from two untreated patients with pulmonary sarcoidosis (fig. 1A and B). Immunoreactive TGF-β_1 was detected in the bronchiolar epithelium of sarcoid patients (fig. 1C) in AM and in hyperplastic type II pneumocytes (fig. 1D). In lung biopsies obtained from IPF, immunoreactive TGF-β_1 was detected in alveolar epithelial cells, AM and in association with fibrous connective tissue bordering the epithelial cells (fig. 1E). Immunoreactive TGF-β_1 was only detected in some AM of healthy controls (fig. 1F). Immunostaining with the control nonimmune serum showed negative results (data not shown).

Discussion

This study demonstrated that TGF-β_1 levels in BAL and in AM supernatant were not significantly different between sarcoidosis and healthy subjects but were increased in IPF. Interestingly, the TGF-β_1 level in BAL fluid was significantly increased in sarcoid patients with altered pulmonary function.

Several studies have tried to find a correlation between in vitro cytokine secretion and lung function or disease activity in sarcoidosis [15, 16]. In a recent study, PRIOR et al. [17] did not demonstrate any significant correlation between the release of in vitro cytokines by BAL cells and the clinical indices comprising pulmonary function in sarcoidosis. In the present study, an increased level of TGF-β_1 was demonstrated in BAL fluid but not in AM supernatants from patients with altered lung function compared with patients with normal lung function. In agreement with these results, previous studies suggested that overproduction of TGF-β_1 leads to an increased risk of pulmonary fibrosis [18, 19]. Similarly, in other diseases with lung fibrosis, such as systemic autoimmune diseases, the transcription and secretion of TGF-β by bronchoalveolar mononuclear cells were increased [20]. This is important since TGF-β_1 may induce the synthesis of many extracellular matrix molecules and is a key cytokine in the development of tissue fibrosis by stimulating the synthesis of several extracellular matrix molecules [6].

The cell source of TGF-β_1 in the lung remains controversial. Contradictory results are found in the literature. SANTANA et al. [21] reported that TGF-β was widely distributed throughout the normal rat lung and was observed in bronchial epithelial cells, endothelial cells, alveolar epithelial cells and alveolar macrophages. In contrast, KHALIL et al. [22] showed that TGF-β_1 was not expressed by AM, epithelial cells or extracellular matrix in normal rat lung. In the normal human lung, a high intensity of staining for TGF-β was seen in bronchial epithelial cells and smooth muscle cells [14]. A faint staining was present in AM and in association with the extracellular matrix, whereas TGF-β was not detectable in endothelial cells, fibroblasts or alveolar epithelial cells [14]. KHALIL et al. [23] demonstrated that TGF-β_1 was prominently expressed by epithelial cells and AM in lung areas with advanced pulmonary fibrosis and was also found to be associated with the extracellular matrix [24].

In sarcoidosis, the increased TGF-β_1 level in BAL fluid is probably due, firstly, to an increased production by granulomas, and, secondly, to an increased secretion by inflammatory cells present in alveolar spaces. TGF-β_1 was detected within the non-necrotizing granulomas, especially in the epithelioid histiocytes. This finding is in keeping with the study of LIMPER et al. [25], which showed that the epithelioid histiocytes comprising non-necrotizing granulomas of pulmonary sarcoidosis contained abundant TGF-β_1. Inflammatory cells present in the lower respiratory tract might also be responsible for overproduction of TGF-β. It is unlikely that AM were responsible for the overproduction of TGF-β_1 in sarcoid patients with altered lung function since no difference in TGF-β_1 levels in AM supernatants was observed according to lung function. In addition, no correlation between TGF-β_1 levels in BAL fluid and total number of AM was observed. LIMPER et al. [25] also demonstrated that the distribution of TGF-β_1 in other regions
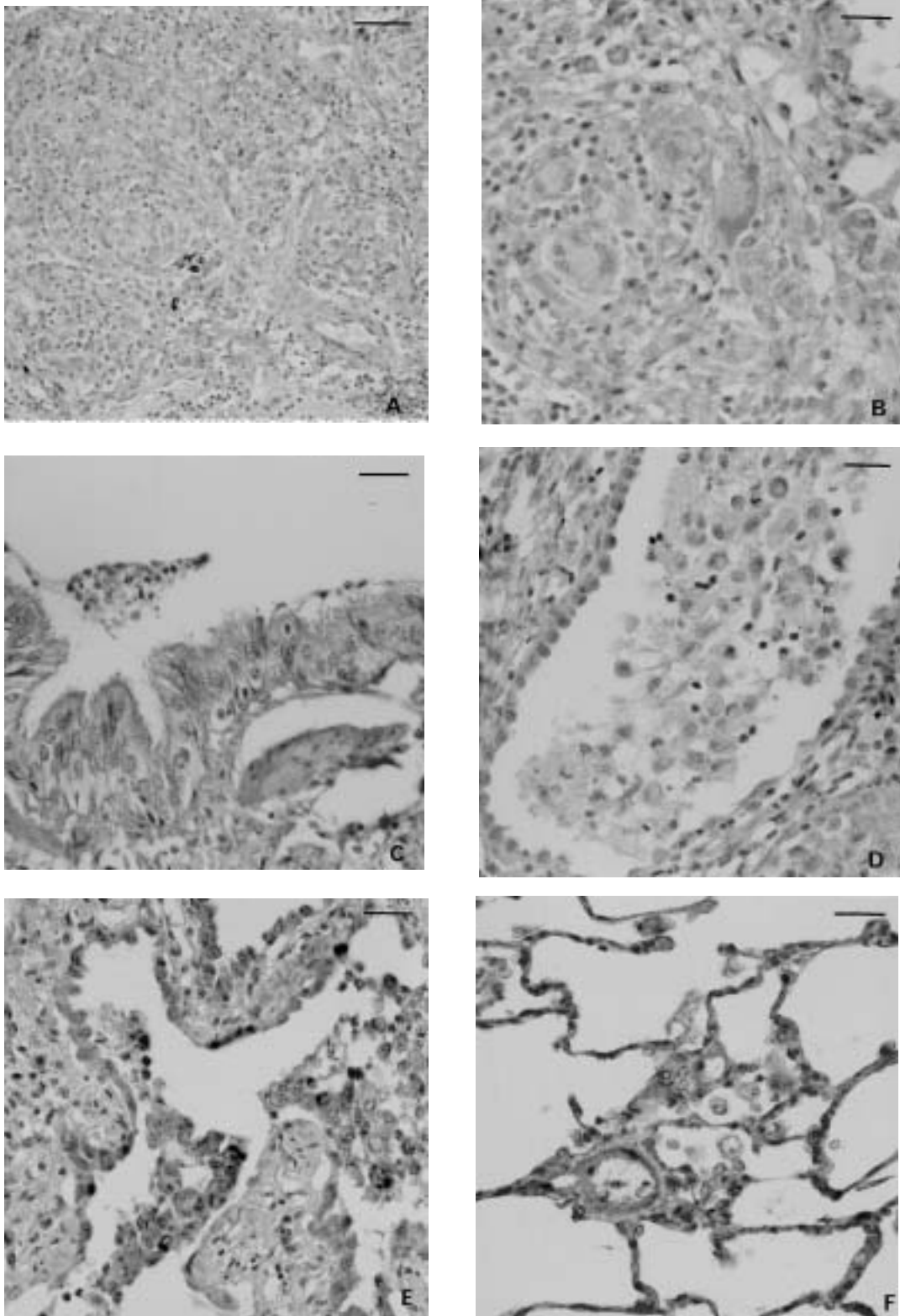
Fig. 1. – Immunohistochemical localization of transforming growth factor (TGF)-β_1 in lung tissue. Lung biopsies obtained from patients with sarcoidosis were examined to determine the localization of TGF-β_1. A-D) representative sections of lung sections from patients with sarcoidosis: A) TGF-β_1 was detected predominantly within the non-necrotizing granuloma; B) TGF-β_1 was localized predominantly in the epithelioid histiocytes comprising non-necrotizing granuloma; C) bronchiolar epithelium was positive for TGF-β_1 expression; D) TGF-β_1 staining was detected in alveolar macrophages (AM) and hyperplastic type II pneumocytes. E) representative section of lung section from an untreated patient with idiopathic pulmonary fibrosis. TGF-β_1 antibody is seen in alveolar epithelial cells lining cystic spaces and in AM, and is also distributed in association with fibrous connective tissue bordering the epithelial cells. F) representative section of lung section from a control. TGF-β_1 was localized in some AM. (Internal scale bar: A=50 μm, B–F=25 μm.)

of lung parenchyma was similar in the bronchiolar epithelium and AM in sarcoid patients and controls. TGF-β_1 was also detected by immunohistochemistry in hyperplastic type II pneumocytes, as previously demonstrated in inflammatory lung disorders such as silicosis [26] and pulmonary eosinophilic granulomas [27].

An additional potential source of TGF-β_1 in sarcoidosis may be the T-cell [28], given the location of TGF-β in the granulomas and the correlation between BAL lymphocytes and TGF-β. There was also an increased level of TGF-β_1 in BAL fluid from patients treated with oral steroids. Because dexamethasone has been shown to increase TGF-β production by T-lymphocytes in a dose-dependent fashion [28], the increased level of TGF-β_1 in BAL fluid from treated patients may reflect an increased production by alveolar lymphocytes.

The role of TGF-β in sarcoidosis is unclear, since it has both immunosuppressive and profibrotic properties [6, 7]. In addition, the biological functions of TGF-β are dose dependent: at low levels, it induces the production of platelet-derived growth factor (PDGF), which promotes fibroblast growth; at higher levels, it downregulates the expression of PDGF receptors, which blocks the autocrine PDGF loop or, possibly, directly inhibits fibroblast proliferation [29]. TGF-β also has immunosuppressing properties and has been identified as a potent inhibitor of interleukin-2-mediated effects on T-cells. In a recent controversial study, ZISSEL et al. [30] hypothesized that TGF-β would be a regulator of the inflammatory process in sarcoidosis, since the patients with sarcoidosis had increased TGF-β levels in culture of BAL cells that showed a spontaneous remission within 6 months [30]. The present findings may app-ear inconsistent with this study since no differences were seen in the TGF-β_1 level in AM supernatants in any subgroup of patients (normal or altered PFT, active or inac-tive disease). However, the present study and the previous study are not strictly comparable. Firstly, in the previous study [30] the evaluation of the TGF-β_1 level was per-formed in supernatants of BAL cell culture (including lym-phocytes) but not in AM supernatants. Secondly, in the present study TGF-β was evaluated in BAL fluid, which seems to be a better reflection of the whole alveolar cell population. Thirdly, in the study by ZISSEL et al. [30], the TGF-β level was evaluated with a cell assay (inhibition of proliferation of CCL 64 cells), while the present study used an ELISA system, which analysed more specifically the TGF-β_1 iso-form. Lastly, they also observed elevated levels of TGF-β in BAL cell culture from patients receiving corticosteroid treatment, who probably had altered lung function.

In conclusion, our data underline the fact that sarcoid patients with altered lung function have a marked overpro-duction of transforming growth factor-β_1. Since this factor initiates and terminates tissue repair, its sustained produc-tion might underlie the development of tissue fibrosis, which could explain the altered lung function observed in sarcoidosis.

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