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MODULATORY EFFECT OF SERA FROM PATIENTS WITH VARIOUS TYPES OF PULMONARY FIBROSIS ON MONONUCLEAR CELL-INDUCED ANGIOGENESIS IN RELATION TO PULMONARY FUNCTION

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Angiogenesis plays an important role in the pathogenesis of idiopathic pulmonary fibrosis. Pulmonary fibrosis occurs also in many diseases, such as other types of interstitial pneumonias or drug-induced pulmonary fibrosis. The aim of the study was to examine the effect of sera from patients with various types of pulmonary fibrosis on angiogenesis induced by human mononuclear cells (MNC) in relation to lung functions. The study population consisted of 32 patients with idiopathic pulmonary fibrosis (IPF), 11 patients with drug-induced pulmonary fibrosis (DIPF), 6 with cryptogenic organizing pneumonia (COP), and 20 healthy volunteers. An animal model of leukocyte-induced angiogenesis assay was used as an angiogenic test. Spirometry, whole-body plethysmography, static lung compliance (Cst), and diffusing capacity of the lung for CO (DLco) were performed in all patients. Sera from IPF and COP patients significantly stimulated angiogenic activity of MNC, compared with sera from healthy donors and from DIPF patients ($P<0.001$). However, sera from healthy donors and DIPF significantly stimulated angiogenic activity of MNC compared with the control group with PBS ($P<0.001$). In all groups, a decrease in the mean value of Cst and DLco was observed, but no significant correlation between VC, FEV_1, DLco, Cst, and angiogenic activity of sera from examined patients was found. Sera obtained from patients with pulmonary fibrosis constitute a source of mediators modulating angiogenesis, but the pattern of reaction is different in various diseases. The strongest reaction is observed in IPF and the weakest one in DIPF. The angiogenic activity of sera did not correlate with the pulmonary function of patients with pulmonary fibrosis.

Key words: *angiogenesis, cryptogenic organizing pneumonia, drug-induced pulmonary fibrosis, idiopathic pulmonary fibrosis, lung function tests*

INTRODUCTION

Idiopathic interstitial pneumonias are a heterogeneous group of diffuse parenchymal lung diseases (1). Idiopathic pulmonary fibrosis is the most common idiopathic interstitial pneumonia. This is a progressive lung disease of unknown etiology characterized by fibroblast proliferation and extracellular matrix remodeling, which results in irreversible distortion of the lung's architecture (1). International multidisciplinary consensus classification of the idiopathic interstitial pneumonias distinguishes six other forms, such as acute interstitial pneumonia, nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia, respiratory bronchiolitis - associated interstitial lung disease and lymphoid interstitial pneumonia (2). Cryptogenic organizing pneumonia (COP) is a fibrous pulmonary disorder in which a new fibromyxoid connective tissue is formed in distal air spaces. In COP this tissue is subject to a complete reversal, but in usual interstitial pneumonia (UIP/IPF) it participates in the remodeling of pulmonary interstitium (3). Pulmonary fibrosis is also observed in the course of interstitial lung diseases (ILD) with known etiology, such as drug-induced pulmonary fibrosis, radiotherapy, pneumoconiosis, extrinsic allergic alveolitis, and in disorders of unknown etiology, such as sarcoidosis and collagen vascular disease - associated interstitial pneumonia (4-6). Although the pathogenetic mechanisms and etiology in ILD are diverse, they all can lead to an identical end-stage fibrotic pulmonary scar. The drug-induced chronic lung damage does not resemble IPF (7). Pulmonary function tests including vital capacity (VC), Cst, and DLco reflect a degree of pulmonary fibrosis (8). Aberrant vascular remodeling is a central hallmark of the development and progression of IPF (9-11), but the role of angiogenesis in various fibrotic pulmonary diseases remains to be determined.

The aim of the study was to evaluate the effect of sera from various types of pulmonary fibrosis on angiogenesis induced by human mononuclear cells in relation to pulmonary function.

MATERIAL AND METHODS

Patients

The protocol was approved by a local Ethical Committee and all subjects gave informed consent. The study population consisted of 49 patients with pulmonary fibrosis. According to their final diagnosis, patients were divided into 3 groups: 32 patients with IPF, 11 patients with DIPF, and 6 with COP. Diagnoses of IPF, COP, and DIPF were based on clinical, radiological, and histopathological findings, according to the ATS/ERS standards (1-2). The population of IPF patients consisted of 14 women and 18 men aged 37-73 (60.7 ±11.5 years), 23 of whom had never smoked tobacco. The population group of DIPF patients consisted of 4 women and 7 men aged 39-77 (63.2 ±11.2 years), 10 of whom had never smoked tobacco. Fibrosis was induced in 6 patients by amiodarone and in one each by nitrofurantoin, colchicine, methotrexate, cyclophosphamide, and

gold salts. The population group of COP patients consisted of 3 women and 3 men aged 44-68 (59.3 ±9.1 years), 5 of whom had never smoked tobacco. Sera from 20 healthy, never smoking volunteers were used as a control. All samples were stored at -80°C.

Lung function tests

In all cases, spirometry, whole body plethysmography, static lung compliance, and single-breath diffusing capacity of the lung for carbon monoxide tests were performed by the experienced staff according to ERS standards (12), using MasterLab Jaeger equipment (Hochberg, Germany). Reference values according to ERS guidelines were applied (12).

Angiogenesis test

The study was performed in 8-10 week old inbred female Balb/c mice. Mononuclear cells were isolated from the peripheral blood samples of healthy volunteers using a Histopaque 1077 (Sigma Chemicals, St. Louis, MA) gradient technique according to the Boyum method (13), with our own modification (14). As an angiogenic test, a leukocyte-induced angiogenesis assay (LIA) in animal model according to Sidky and Auerbach was used (15), with modification described previously (14). Briefly, 6 samples of 0.05 ml volume containing 2.5 x 10^5 MNC were injected intradermally into mice anesthetized with 3.6% chloral hydrate (*Fig. 1*). Three mice were used for each examined

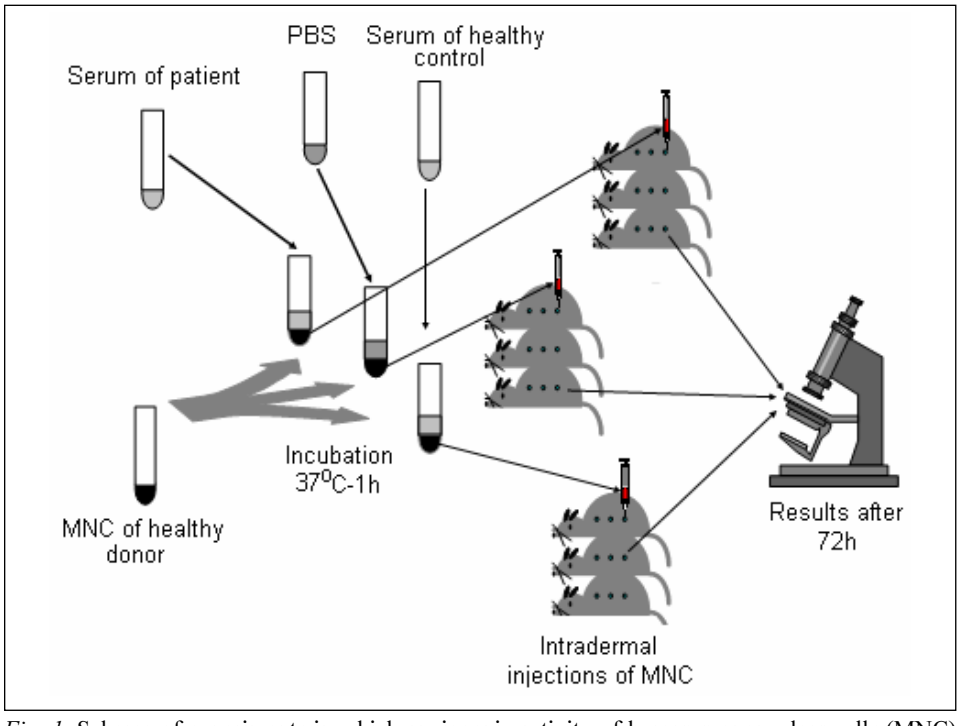

Fig. 1. Scheme of experiments in which angiogenic activity of human mononuclear cells (MNC) was evaluated after their preincubation with patients or healthy volunteers' sera and with PBS without sera as a control.

patient. After 72 h the mice were sacrificed with a lethal dose of Morbital and their skin was dissected from the underling tissue. All newly formed blood vessels were identified on the inner skin surface and counted by dissection microscope, at magnification 6x, in 1/3 of the central area of the microscopic field. The identification was based on the criteria proposed by Sidky and Auerbach (15). New blood vessels connected with the injection sites (visible by trypan blue) and contrasting with the background vasculature by virtue of their tortuosity and divarication were counted.

Statistical analysis

For standardization of the value of the angiogenesis test, the results were expressed as an angiogenesis index (AI):

$$AI = \frac{\text{The mean number of new blood vessels created after injection of MNC preincubated with serum of patients (or PBS)}}{\text{The mean number of new blood vessels created after injection of MNC preincubated with serum of healthy donors}}$$

The data were presented as means ±SD and $P<0.05$ was regarded as statistical significance. Student's *t* and Pearson's tests were used for statistical analysis (Statistica 6 for Windows).

RESULTS

Sera from IPF and COP patients significantly stimulated angiogenesis compared with those from healthy subjects (*Fig. 2A*). The mean number of new vessels formed after injection of MNC preincubated with sera from IPF patients was 16.2 ±0.9 and was significantly higher than the 15.4 ±0.9 after injection of MNC preincubated with COP patients ($P<0.05$). Sera from IPF and COP significantly stimulated angiogenesis compared with those from DIPF patients ($P<0.001$). However, compared with PBS, sera from healthy donors also exerted a stimulating effect on angiogenesis ($P<0.001$). The mean number of vessels after the injection of MNC preincubated with sera from a healthy control (13.7 ±0.7) did not statistically differ from the number of vessels created after the injection of MNC preincubated with sera from DIPF patients (13.2 ±1.3). Sera from DIPF patients significantly stimulated angiogenesis compared with PBS ($P<0.05$). The results expressed as an angiogenesis index are presented in *Fig. 2B*. The highest angiogenesis index was in the group of patients with IPF (1.2 ±0.1) and the lowest one was in the group of patients with DIPF (1.0 ±0.1) ($P<0.001$).

The mean value of VC in all groups of patients with pulmonary fibrosis was within a normal range and no significant differences were observed between the examined groups (*Fig. 3A*). The mean DLco value decreased in the group of patients with IPF (59.0 ±17.6%) and DIPF (57.0 ±19.8). The mean DLco in the group of COP patients was higher (75.0 ±22.7%), which was not significant in relation to the IPF patient ($P=0.067$) (*Fig. 3B*). In all groups, static compliance was lower than the predictive value (*Fig. 3C*). The lowest mean value of Cst was

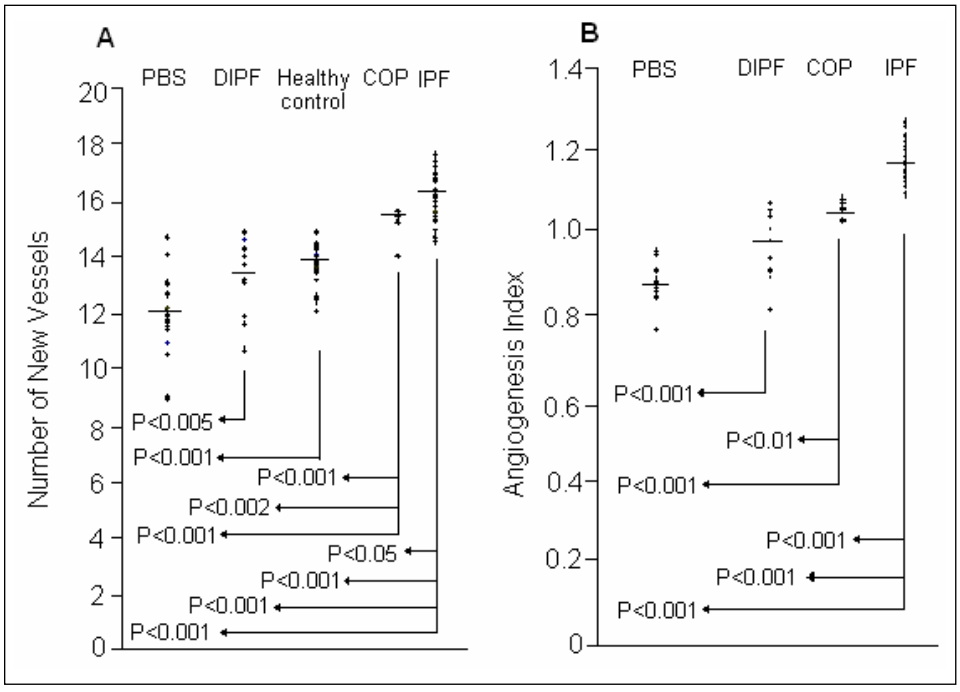

Fig. 2. A - Number of new vessels formed after injection of MNC preincubated in sera from IPF (n=32), DIPF (n=11), COP patients (n=6), and from healthy donors (n=20) or PBS (n=20); B - Angiogenesis index after preincubation of MNC in sera from IPF, DIPF, and COP patients compared with the angiogenesis index for PBS. The mean values are indicated by horizontal bars, significant differences between the groups are indicated.

observed in the IPF group (44.0 ±15.9%) and the highest one in the COP group (64.0 ±28.8%). The difference was significant (P<0.02). No significant correlation between VC, FEV_1, DLco, Cst, and the number of new vessels or angiogenesis index was found (*Fig. 4*).

DISCUSSION

Angiogenesis is characterized by neoformation of blood vessels in many physiological and pathological processes, such as the growth of malignant solid tumors, chronic inflammation, and a formation of inflammatory granulation tissue during wound healing (16). IPF is a typical interstitial pulmonary disease characterized by chronic inflammation. Recently, pulmonary fibrosis has been proposed to result from abnormal wound healing in the lung in response to injury to the alveolar epithelium (17). Parallels have been drawn between the biology of IPF and cancer (18). The existence of neovascularization in IPF was originally

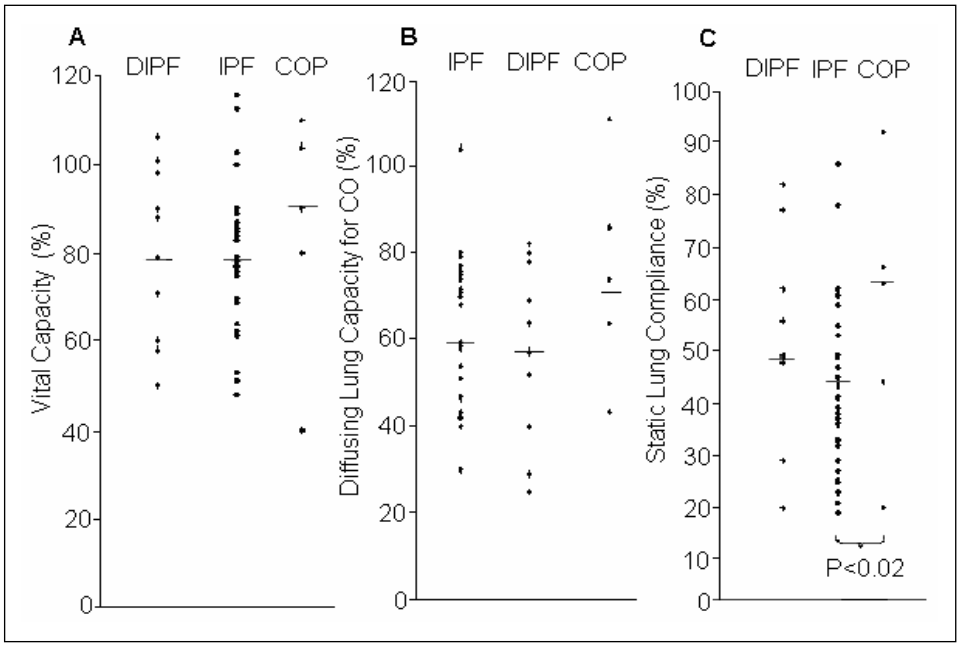

Fig. 3. Distribution of lung function results for patients with pulmonary fibrosis: A - VC, B - DLco, and C - Cst. The mean values are indicated by horizontal bars, significant differences between the groups are indicated.

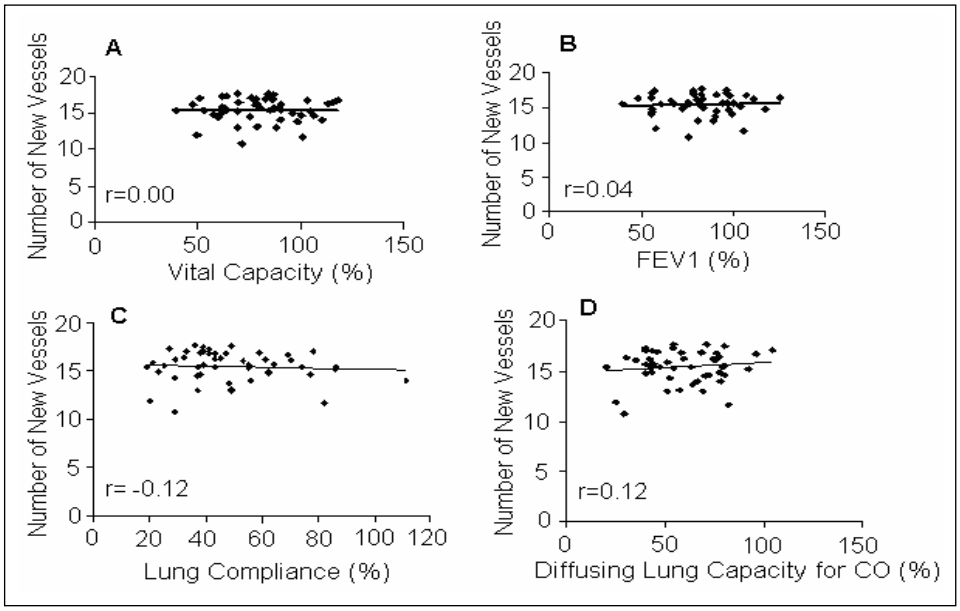

Fig. 4. Correlations between VC (A), FEV_1 (B), Cst (C), and DLco (D), and the number of new vessels (r - Pearson's coefficient).

identified by Turner-Warwick in 1963 (19), but only Keane *et al* (20, 21) have demonstrated an increased angiogenic activity in the lung tissue of IPF and experimental drug-induced pulmonary fibrosis. Our present results demonstrated significant differences between serum angiogenic activity in patients with IPF and DIPF, but similar functional disturbances. Patients with DIPF presented radiological and functional fibrotic changes without continuous progression typical of IPF. The animals with intensive angiogenesis in experimental bleomycin-induced pulmonary fibrosis presented an active phase of the process induced by the drug. In all our cases of DIPF, the harmful treatment was stopped after diagnosis and patients were examined in an inactive phase of the disease. Perhaps, it explains why serum angiogenic activity in patients with DIPF was similar to the activity of sera from healthy controls.

Koyama *et al* (22) and Meyer *et al* (23) have reported a decreased expression of a vascular endothelial cell growth factor (VEGF) in IPF. Renzoni *et al* (24), after examination of interstitial vascularity in fibrosing alveolitis, have suggested that angiogenesis may occur in earlier stages of the development of pulmonary fibrosis, because of the reduction in total vascular area and vascular density in IPF patients. Burdick *et al* (25) have demonstrated that angiogenic activity in the lungs of patients with IPF was significantly decreased. However, Simler *et al* (26) have described a correlation between the serum VEGF level and HRCT fibrosis score, and a negative relation to the change in FVC after 6 months. In our study, no correlation was found between serum angiogenic activity and functional changes connected with pulmonary fibrosis such as VC, FEV_1 Cst, and DLco. No differences in serum angiogenic activity were observed between patients with advanced and moderate functional pulmonary changes. The results of the present study indicate that serum angiogenic activity does not depend on the functional or radiological changes, but relates to the phase and activity of the fibrotic process.

Angiogenesis also participates in the pathogenesis of COP. Angiogenic chemokines and factors such as CXCR3, VEGF, and bFGF are highly expressed in lesions in COP (27, 28). Presented results showed high angiogenic activity of serum from patients with COP. Previously it has been reported that capillarization is less frequent in fibroblastic foci of IPF than in the intraluminal fibromyxoid lesions COP (3). In the present study, the angiogenic activity of sera from IPF patients was higher than that of sera from COP patients.

Recently, Tachihara *et al* (29) have demonstrated in NSIP and IPF patients a decrease in expression of VEGF mRNA in the alveolar septa, which was associated with a reduction in the number of capillary tubes *via* endothelial cell apoptosis, and which possibly results in alveolar remodeling. Nakayama *et al* (30) have observed that serum levels of ENA-78 and BALF levels of IP10 in NSIP patients were significantly higher than in patients with IPF and in controls. Therefore, further research on neovascularization in pulmonary fibrosis seems necessary. The fundamental question remains: ‘Are there too many or too few new vessels in pulmonary fibrosis?’

We conclude that sera of patients with pulmonary fibrosis constitute the source of mediators modulating angiogenesis, but the pattern of reaction is different in various diseases. The strongest angiogenic reaction is observed in IPF and the weakest one in DIPF. The angiogenic activity of sera did not correlate with pulmonary function of patients with pulmonary fibrosis.

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Conflicts of interest: No conflict of interest was declared with relation to this work.

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