Angiogenesis has been implicated in the pathogenesis of interstitial lung diseases. A correlation between serum angiogenic cytokines level of patients with idiopathic pulmonary fibrosis and radiographic manifestations or functional pulmonary changes has been described, but the role of angiogenesis in the pathogenesis of other interstitial lung diseases such as silicosis and pulmonary Langerhans cell histiocytosis remains unclear. The aim of the study was to examine the effect of sera from silicosis and pulmonary Langerhans cell histiocytosis patients on angiogenesis induced by human mononuclear cells (MNC) in relation to pulmonary function. The study population consisted of 12 patients with silicosis, 12 patients with pulmonary Langerhans cell histiocytosis (PLH), and 14 healthy volunteers. Spirometry, whole-body plethysmography, static lung compliance (Cst), and diffusing capacity of the lung for CO (DLco) were performed in all patients. As an angiogenic test, leukocyte induced angiogenesis assay according to Sidky and Auerbach was used. Sera from PLH patients exerted a significant inhibitory effect on angiogenesis (P<0.001). Sera from silicosis patients significantly (P<0.001) stimulated angiogenesis compared with sera from healthy donors. However, sera from healthy donors significantly stimulated the angiogenic activity of MNC compared with the control with PBS. The mean value of DLco was significantly lower in the group of patients with PLH compared with patients with silicosis (P<0.05). A significant correlation between angiogenesis index and DLco was observed (P<0.05). No significant correlation between the angiogenesis index and other functional parameters was found. Sera from interstitial lung diseases patients and healthy donors constitute a source of mediators modulating angiogenesis. Sera from silicosis patients stimulate neovascularization but sera from PLH patients exert an inhibitory effect on angiogenesis. A correlation between serum angiogenic activity and DLco was found.

Key words: angiogenesis, lung function, pulmonary Langerhans cell histiocytosis, silicosis
INTRODUCTION

Angiogenesis is a physiological process involving the growth of new blood vessels from the pre-existing vessels. This plays a major role in tumor growth and metastasis (1). Angiogenesis has also been implicated in the pathogenesis of interstitial lung diseases, such as idiopathic pulmonary fibrosis, sarcoidosis and the pulmonary manifestation of connective tissue diseases (2-7). Interstitial pulmonary fibrosis caused by the inhalation of inorganic dusts, such as silica particles, continues to be an important cause of interstitial respiratory disease (8). Silicosis is a chronic inflammatory disorder of the lung, resulting in fibrosis. Diffuse pulmonary fibrosis of any cause, including exposure to silica, appears to predispose to lung cancer (9, 10). Even though angiogenesis plays an important role in the pathogenesis of cancer and idiopathic fibrosis, the role of neovascularization in pneumoconiosis is unclear. Langerhans cell histiocytosis is characterized by a clonal proliferation and clustering of cells with a Langerhans cell-like phenotype at various sites within the body (11). Pulmonary Langerhans cell histiocytosis is a rare disorder of unknown etiology that occurs predominantly in young smokers and is characterized by focal Langerhans cell granulomas infiltration and destruction of distal bronchioles (11). Recently, Dina et al (12) have suggested that proangiogenic factors take part in the pathogenesis in Langerhans cell histiocytosis. The pathogenesis and the angiogenic processes of the disease remain poorly understood.

The aim of the study was to examine the effect of sera from silicosis and pulmonary Langerhans cell histiocytosis 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. The study population consisted of 12 patients with silicosis, 12 untreated patients with pulmonary Langerhans cell histiocytosis, and 14 healthy volunteers. In all cases, the diagnosis of PLH and silicosis was based on clinical, radiological and histopathological criteria. The population of silicosis patients consisted of 1 woman and 11 men aged 36-60 years (42.6 ±8.6), 8 of whom smoked tobacco. The population of PLH patients consisted of 4 women and 8 men aged 18-66 years (36.8 ±13.7), all current smokers. Fourteen healthy volunteers were recruited from medical staff as a control group. Eight women and 6 men aged 24-58 years (36.5 ±8.9), all non smokers, were evaluated. Blood samples were collected from all subjects, centrifuged and sera were placed in disposable tubes. All samples were stored at -80°C.

Functional tests

In all patients, lung function tests were performed with the use of MasterLab Jaeger equipment (Hochberg, Germany). Whole body plethysmography, static lung compliance, and single breath diffusing capacity of the lung for carbon monoxide were carried out according to the ERS standards
Values were expressed as a percentage of predicted values calculated according to sex, height, and age, using the European Community for Steel and Coal Classification (13).

**Angiogenesis test**

A cutaneous angiogenesis assay was conducted according to the method of Sidky and Auerbach, with modification (14, 15). Briefly, study involved 2-month old female inbred Balb/c mice weighting ca 20 g each. Normal human peripheral blood mononuclear cells from healthy volunteers were obtained using a gradient technique, as previously described (15, 16). First, MNC were incubated for one hour at 37°C with saturation of 5% CO₂ in phosphate buffered saline (PBS) with serum from patients or from healthy volunteers (1/4 of serum and 3/4 of PBS). As a control, MNC were preincubated in PBS only. Then, MNC suspended in Parker liquid (5x10⁶ cells/ml) with 0.1% of trypan blue were injected intradermally to the mice (six injections per mice and 3 mice for each tested patient) anaesthetized with 3.6% chloral hydrate (POCH, Poland). After 3 days, the mice were sacrificed with a lethal dose of Morbital (Biowet, Poland). All new blood vessels were identified and counted by a dissection microscope at magnification 6x (Nikon, Japan). In all cases identification, based on the previously described criteria, was performed by one expert (14, 15).

**Statistical analysis**

Angiogenic activity was assessed with the use of an angiogenesis index (AI), which is a ratio of the mean number of new vessels created after the injection of MNC preincubated with sera of the examined patients or healthy donors divided by the mean number of new blood vessels after the injection of MNC preincubated with PBS only. 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 PLH patients exerted an inhibitory effect on angiogenesis (Fig. 1A). The mean number of vessels after the injection of MNC preincubated with sera from PLH (10.0 ±0.6) was significantly lower than the number of vessels created after the injection of MNC preincubated with sera from healthy donors (14.1 ±0.8) (P<0.001). The same relation was observed when PLH patients sera effect was compared with PBS (12.6 ±1.1) (P<0.001). However, sera from silicosis patients significantly stimulated angiogenesis compared with sera from healthy donors (P<0.001) (Fig. 1A). The mean number of new vessels formed after the injection of MNC preincubated with silicosis patients sera (15.7 ±0.3) was significantly higher than after the injection of MNC preincubated with sera from healthy subjects or with PBS (P<0.001). Sera from healthy volunteers also exerted a stimulating effect on angiogenesis compared with PBS (P<0.001). When the results were expressed as the angiogenic index similar relations were observed (Fig. 1B). The highest angiogenesis index was seen in the group of patients with silicosis (1.3 ±0.1), lower in the healthy control (1.1 ±0.1), and the lowest in the group of patients with PLH (0.9 ±0.1).
The mean value of DLco was significantly lower (P<0.05) in the group of patients with PLH (69.9 ±17.5%) compared with silicosis patients (86.4 ±20.9). The mean value of VC (76 ±23.8%) and Cst (74.3 ±20.6) in the group of patients with silicosis was lower than in the group of patients with PHL (86.3 ±14.1% and 80.8 ±17.8% respectively), but the differences were not significant (Fig. 2). A significant (P<0.05) correlation was observed between the angiogenesis index and DLco (Fig. 3). No correlation between the angiogenesis index and other functional parameters was found (Fig. 3).

DISCUSSION

A critical importance of tumor angiogenesis in the development and metastatic spread of tumors is proved (17). Neovascularization has been implicated in the pathogenesis of fibrotic lung diseases, such as bleomycin-induced pulmonary fibrosis, idiopathic pulmonary fibrosis, and cryptogenic organizing pneumonia (18-20). Microcirculation responds to chronic stimuli either by producing more capillaries or by dilating the existing ones (21). The inflammatory and fibrotic

![Fig. 1](image-url)
Fig. 2. Distribution of spirometric and plethysmographic results for patients with pulmonary fibrosis: A - VC, B - FEV\textsubscript{1}, C - DL\textsubscript{co}, and D - Cst. The mean values are indicated by horizontal bars; significant differences between the groups are indicated.

Fig. 3. Correlations between angiogenic activity in sera of PHL and silicosis patients examined and VC (A), FEV\textsubscript{1} (B), DL\textsubscript{co} (C), and Cst (D); r - Pearson's coefficient.
processes contribute to the pathogenesis of silicosis (22). A number of cohort and case-controlled studies consistently suggest increased incidence of lung cancer in individuals with occupational silica exposure (23). The mechanism of mineral-induced pulmonary fibrosis appears to be similar to that involved in IPF or a bleomycin model of fibrosis (22). However, no data on angiogenesis in pneumoconiosis was found. Silica stimulates production of oxidants, chemokines, and cytokines, such as platelet-derived growth factor (PDGF), transforming growth factors (TGF), insulin growth factor (IGF-1), tumor necrosis factor-alpha (TNF-α), and interleukin 8 (IL-8), all of which also participate in a process of angiogenesis (22). Presented data suggest that neovascularization may be implicated in the pathogenesis of silicosis which is an inflammatory and fibrotic disorder associated with malignancy.

Data about neovascularization in PLH are also very scarce. The main pro-angiogenic factor, the vascular endothelial growth factor (VEGF), was expressed in disease lesions in the majority of patients with multifocal Langerhans cell histiocytosis (12). Serum VEGF level was elevated in children with Langerhans cell histiocytosis and declined after efficient treatment (24). The histiocytosis patient had a good response to treatment with thalidomide which significantly inhibits angiogenesis (25). Recently, Senechal et al (26) have demonstrated that Langerhans cell histiocytosis lesions constituted a site of active inflammation, tissue remodeling, and neo-angiogenesis. The majority of proliferating cells were the endothelial cells, fibroblasts, and polyclonal T lymphocytes. Examination of histological slides also clearly indicates that PLH granulomas are connected with extensive neo-angiogenesis and tissue remodeling. Both IL-1α and TNF-α are amply expressed in PLH lesions (26). TNF-α can recruit leukocytes and promote angiogenesis and fibroblast proliferation in wound healing, and similar activities may take place in the PLH lesions (27). In contrast to these results, we observed that the sera from PLH patients inhibited angiogenesis. However, angiogenesis was demonstrated in children with multifocal Langerhans cell histiocytosis (LCH). We presented exclusively cases of pulmonary form of the disease. In adults, pulmonary involvement of Langerhans cell histiocytosis usually occurs as a single-system disease and is characterized by focal Langerhans cell granulomas infiltrating and destroying of distal bronchioles. PLH resembles bronchiolitis rather than a diffuse infiltrating lung disease. Acute disseminated LCH (Letterer-Siwe disease) is a severe multisystem disease that predominantly affects young children and carries a poor prognosis. Multifocal LCH is seen mainly in older children and adolescents (Hand-Schuller-Christian syndrome or multifocal eosinophilic granuloma) and runs a variable, but usually more favorable, course. A single-system disease (eosinophilic granuloma and primary pulmonary histiocytosis) is characterized by the involvement of a single organ (bone, lungs or skin) and usually follows a benign course, and can regress spontaneously. Although certain stages of the disease process that leads to
pulmonary Langerhans’ cell histiocytosis, are beginning to come to light, further work is needed to explain the different aspects of the Langerhans’ cell granuloma (11). Perhaps angiogenesis only takes part in multifocal LCH, characterized by a clonal proliferation of cells, and the pathogenic differences between PLH and multifocal LCH partly results from various level of angiogenic activity in these diseases. Recently, Simler et al (28) have reported that serum VEGF level of patients with IPF negatively relates to the change in FVC (28). We also observed a negative trend, however, insignificant (r=-0.26), between VC and angiogenic activity of sera from examined patients. On the other hand, the correlation between DLco and serum angiogenic activity was demonstrated. It is an extremely interesting observation, because the diffusing capacity of the lung depends not only on ventilation and thickness of the alveolar-vascular barrier but also on the vascularity of alveolus. Our results demonstrated a relation between the diffusing capacity of the lung and serum angiogenic activity in patients with interstitial lung diseases. Compared with silicosis patients DLco in PLH patients was lower. VC and Cst were lower in silicosis patients. It may be possible that emphysema contributes to the cystic appearance of advanced lesions in PLH patients. This may account for the observed functional results.

In conclusion, sera from silicosis patients stimulate neovascularization, but sera from PLH patients exert an inhibitory effect on angiogenesis. The angiogenic activity of sera from silicosis and PHL patients correlate to DLco. However, further studies to explore the role of angiogenesis in PLH and pneumoconiosis are needed.

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