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Angiogenic Activity of Sera from Extrinsic Allergic Alveolitis Patients in Relation to Clinical, Radiological, and Functional Pulmonary Changes

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Abstract Extrinsic allergic alveolitis (EAA) caused by inhaled organic environmental allergens can progress to a fibrotic end-stage lung disease. Neovascularization plays an important role in pathogenesis of pulmonary fibrosis. The aim of this study was to assess the effect of sera from EAA patients on the angiogenic capability of normal peripheral human mononuclear cells (MNC) in relation to the clinical, radiological, and functional changes. The study population consisted of 30 EAA patients and 16 healthy volunteers. Routine pulmonary function tests were undertaken using ERS standards. As an angiogenic test, leukocyte-induced angiogenesis assay according to Sidky and Auerbach was used. Compared with sera from healthy volunteers, sera from our EAA patients significantly stimulated angiogenesis ($P < 0.001$). However, sera from healthy donors also stimulated angiogenesis compared to PBS ($P < 0.001$). No correlation was found between serum angiogenic activity and clinical symptoms manifested by evaluated patients. A decrease in DLco and in lung compliance in EAA patients was observed but no significant correlation between pulmonary functional tests and serum angiogenic activity measured by the number of microvessels or an angiogenesis index was found. However, the proangiogenic effect of sera from EAA patients differed depending on the stage of the disease and was stronger in patients with fibrotic changes. The present study suggests that angiogenesis plays a role in the pathogenesis of EAA. It could be possible that the increase in the angiogenic activity of sera from EAA patients depends on the phase of the disease.

Keywords Angiogenesis · Hypersensitivity pneumonitis · Pulmonary function tests

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Introduction

Angiogenesis is essential for growth and tissue repair after injury. However, it may also contribute to the pathology of a number of human disorders, including neoplasia [1], atherosclerosis [2], and inflammatory diseases [3, 4]. Because the lung is composed of highly vascularized tissue, with finely organized and regulated microvascular beds, inflammation and hypoxia may initiate angiogenesis [5]. Neovascularization plays an important role in the pathogenesis of experimental and idiopathic pulmonary fibrosis [6, 7]. Extrinsic allergic alveolitis (EAA) describes a group of immunologically mediated lung disorders that are triggered by recurrent exposure to various organic environmental agents and can progress to disabling fibrotic end-stage lung disease [8]. Many causative agents have been identified in occupational dust or vapor, but most new cases arise from residential exposure to pet birds, contaminated indoor molds, and humidifiers [9]. EAA is characterized by an inflammatory lymphocytic alveolitis consisting of both $CD8^+$ and $CD4^+$ T cells, with a

predominance of IFN-γ-producing T cells resulting from a reduction in IL-10 production and an increase in high-affinity IL-12R [10]. However, it is still unclear what mechanism is involved in inflammatory cell recruitment and traffic throughout the lungs in patients with EAA. The overexpression of L-selectin and E-selectin on endothelial cells could contribute to this process [11], as E-selectin has been observed to play a role in neovascularization [12]. The data suggest the participation of endothelial cells in the pathogenesis of EAA. However, angiogenesis in EAA has not previously been explored. The aim of this study was to assess the effect of sera from EAA patients on the angiogenic capability of normal human MNC in relation to clinical, radiological, and functional changes.

Materials and Methods

Study Population

The study population consisted of 30 subacute or chronic EAA patients between 18 and 72 years old (46.9 ± 15.2 years), of whom 16 were women and 14 men; 21 had never smoked tobacco. The diagnosis of EAA was based on a range of findings, including clinical, radiological, functional, serological, bronchoalveolar lavage (BAL), and histopathological, and using the criteria established by Lacasse et al. [13]. Exposure to microorganisms and the detection of specific antibodies in their serum led to 13 of the patients being diagnosed with bird fancier's lung and an additional 5 with farmer's lung. In 3 cases, specific antibodies to *Aspergillus fumigatus* were found in the patients' serum. In 15 cases, the diagnosis of EAA was confirmed by histopathological examination following lung biopsy.

A questionnaire was developed to evaluate general symptoms (e.g., weakness, fever, and arthralgia) and pulmonary symptoms (coughing and breathlessness) in the study population. Three stages of breathlessness were identified and used in the classification (Table 1). Routine pulmonary function tests were also undertaken using European Respiratory Society (ERS) standards [14]. The lung function tests included vital capacity (VC); residual volume (RV); forced expiratory volume in one-second (FEV_1); maximal forced expiratory flow where 50% of the forced vital capacity (FVC) remains to be expired (MEF_{50}); total airway resistance (Rtot) measured by body plethysmography (MasterLab, Jaeger, Germany); static lung compliance (Cst); and single breath diffusing capacity of the lung for carbon monoxide (DLco). Values were expressed as a percentage of the predicted values calculated according to gender, height, and age, and using the European Community for Steel and Coal classification system [14].

Standard AMBER method posteroanterior and lateral chest radiographs were taken of all patients and radiological changes were used as a basis for classifying the study population as follows: Group 1, those with no changes or with small nodular or reticular changes (14 cases), and Group 2, those with advanced fibrotic changes (16 cases). Blood samples were taken from patients before treatment with corticosteroids or cytotoxic agents was started. As a control, sera from 16 healthy, nonsmoking volunteers were used (10 women and 6 men, mean age = 34.5 ± 8.58, range = 20-58 years). The study protocol was approved by the local ethics committee and informed consent was obtained from each participant.

Mononuclear Cells (MNC)

We prepared normal human peripheral blood MNC, derived from the healthy volunteers' buffy-coat cells, using Histopaque 1077 (Sigma, St. Louis, MO) and the gradient technique of 20 min at 500 g at room temperature. This yielded an MNC preparation containing 10-15% monocytes and 85-90% lymphocytes based on morphologic criteria and MPO staining. MNC viability was assessed by Trypan Blue exclusion and was found to be 98% or better.

Animals

Animal handling in all the experiments conformed to Polish legal requirements for the protection of animals and to U.S. National Institutes of Health (NIH) standards. The local ethical commission for experimenting on animals approved all procedures involved in this study. The study was performed on 8-10-week-old inbred female Balb/c mice, with a body mass of 20-25 g, bred from our laboratory's colony. The animals were fed a standard diet and tap water ad libitum.

Angiogenesis Assay

We used the leukocyte-induced angiogenesis assay described by Sidky and Auerbach [15] with some

Table 1 Classification of patients by stage of breathlessness

Stage of breathlessness	Description	No. of patients
1	No dyspnea	2
2	Moderate dyspnea	13
3	Severe or very severe dyspnea at rest	15

modifications [16]. For the experimental group, multiple 0.05 ml samples of 2×10^5 MNC, preincubated for 60 min at 37°C in phosphate-buffered saline (PBS) and supplemented with 25% of serum from EAA patients, were intradermally injected into partly shaved, narcotized mice (using a ratio of three mice for every patient). We used two MNC control groups: Group 1 was preincubated in PBS and supplemented with 25% of serum taken from healthy donors, and Group 2 was preincubated in PBS only. After 72 h the mice were killed with a lethal dose of Morbital (Biowet, Poland). We then counted the newly formed blood vessels found on the inner surface of each mouse's skin using a microscope (Nikon, Japan) at 6× magnification and the criteria of Sidky and Auerbach [15].

Statistical Analysis

Statistical evaluation of the results was performed using Student's t test and the Pearson test (Statistica 6 for Windows, StatSoft, Inc., Tulsa, OK). The data are presented as the mean ± SD (standard deviation) and $P < 0.05$ was regarded as statistically significant.

Results

Compared with sera from healthy volunteers, sera from our EAA patients significantly stimulated angiogenesis ($P < 0.001$). However, sera from healthy donors also stimulated angiogenesis compared to PBS ($P < 0.001$). The mean number of microvessels formed after the injection of MNC preincubated with sera from EAA patients was 17.53 ± 1.57, while it was 11.9 ± 0.9 and 13.41 ± 0.74 for those injected with MNC preincubated only with PBS and for those injected with MNC preincubated with sera from healthy donors, respectively (Fig. 1).

The majority of our patients manifested a cough ($n = 27$) and general symptoms ($n = 21$). However, in the patients evaluated we found no correlation between serum angiogenic activity and the presence of a cough or general symptoms. The difference between the numbers of microvessels created after the injection of MNC preincubated with sera from those EAA patients with moderate dyspnea (15 patients) and those with severe dyspnea (15 cases) was not significant (Fig. 2a). The proangiogenic effect of sera from EAA patients differed depending on the radiological stage of the disease (Fig. 2b). As for radiological changes, the number of microvessels created after the injection of MNC preincubated with sera from patients with fibrotic radiological changes was significantly higher ($P < 0.05$) than that created after injection of MNC preincubated with sera from patients without or with only small nodular/reticular radiological changes.

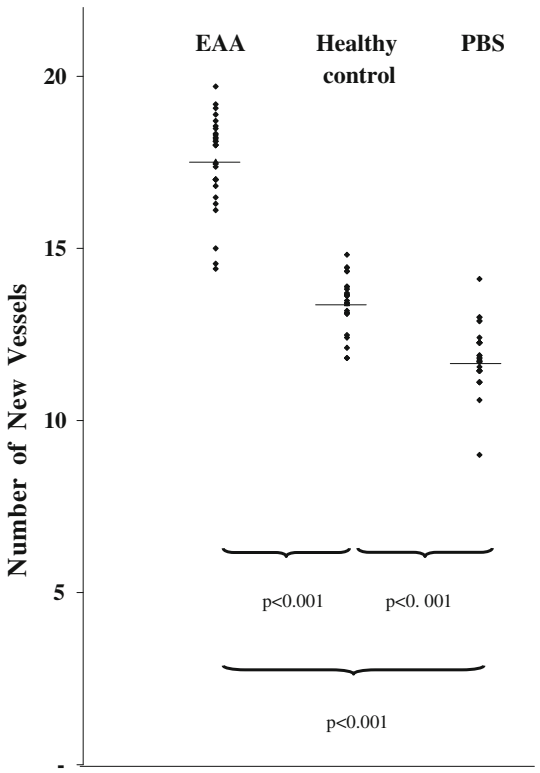

Fig. 1 Number of microvessels created after injection of MNC preincubated in sera from EAA, from healthy donors or PBS

The functional lung data of the study patients are given in Table 2. The percentage of DLco showed a slight decrease (76.7 ± 25.9%). However, 14 patients presented abnormal results. Pulmonary function testing revealed the most important decrease in Cst (63.4 ± 29.2%), yet 20 patients still had a result below a lower limit of the predictive value. We found no significant correlation between pulmonary functional tests and the number of microvessels (Fig. 3).

Discussion

EAA is an immunologically induced interstitial pulmonary disease that can trigger granulomatous inflammation in the lung [8]. The connection between chronic inflammation leading to granuloma formation and angiogenesis has been shown [17]. Similar granulomatous inflammation is observed in sarcoidosis [18]. Many papers have demonstrated that neovascularization plays a part in the pathogenesis of sarcoidosis [16, 19–22], but few have described the involvement of proangiogenic factors in EAA [11, 23]. Navarro et al. [23] observed an increase in vascular endothelial growth factor (VEGF) serum levels and a significant decrease in BALF in EAA patients compared to

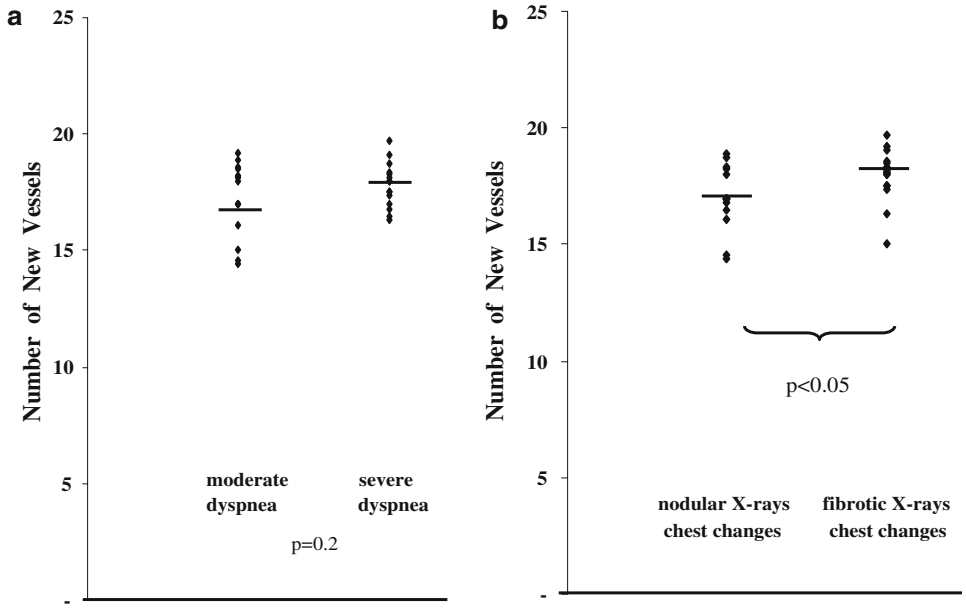

Fig. 2 **a** Number of microvessels in relation to dyspnea. EAA patients with moderate dyspnea ($n = 15$) and with severe dyspnea ($n = 15$). **b** Number of microvessels in relation to radiological stage of the disease. Nodular pulmonary radiological changes or without radiological changes ($n = 14$) and fibrotic pulmonary radiological changes ($n = 16$). The mean value for groups is indicated by *horizontal bars*; significant differences between the groups are indicated

Table 2 Lung function tests of examined patients

Parameters	Mean value ± SD (% of predicted value)
VC	81 ± 28.3
FEV_1	79 ± 25.3
FEV_1%VC	99 ± 14.8
MEF_{50}	82 ± 43.2
Rtot	103 ± 60.9
RV	92 ± 36.5
Cst	63 ± 29.2
DLco	77 ± 25.9

healthy controls. An increase in VEGF expression in sarcoid granulomas and alveolar macrophages has also been shown [20]. VEGF is an essential factor in regulating the neovascularization process and in stimulating the degradation of extracellular matrix proteins by metalloproteinases (MMPs). The MMPs involved in the remodeling of the extracellular matrix—collagenase-2 (MMP-8) and gelatinase B (MMP-9)—may play a role in the pathogenesis of EAA [24]. Navarro et al. also suggested that upregulation of the endothelial cell adhesion molecules L-selectin and E-selectin during the development of EAA may contribute to the increased traffic of lung inflammatory cells [11].

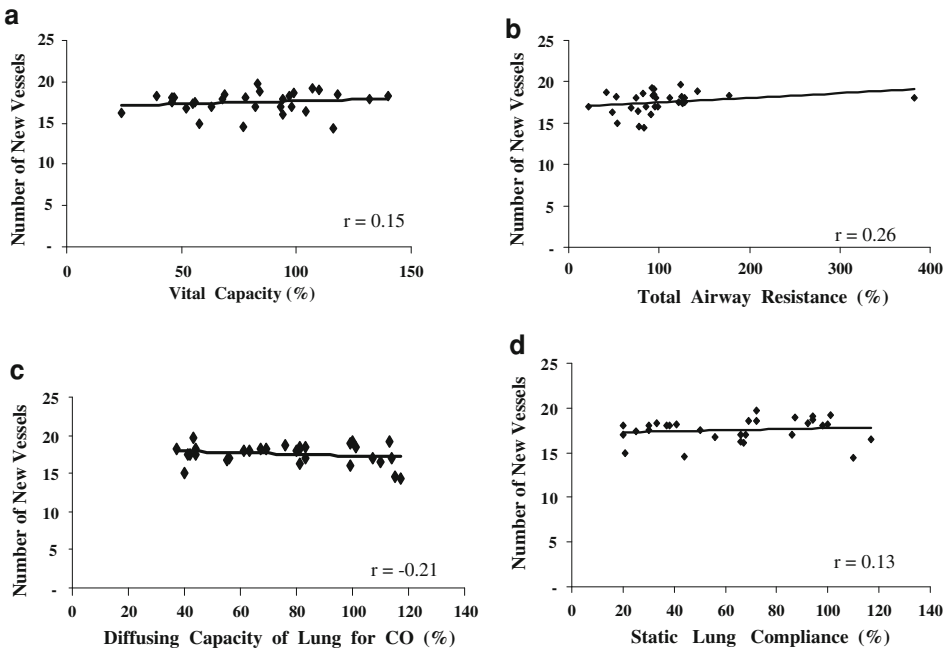

Fig. 3 Correlations between number of microvessels created after injection of MNC preincubated with sera from EAA patients and **a** vital capacity, **b** total airway resistance, **c** diffusing capacity of the lung for CO, **d** static lung capacity. (*r*, Pearson's coefficient)

E-selectin was identified as a marker of angiogenic activity of the endothelium [25].

Hypoxia also strongly stimulates neovascularization in many disorders [26]. Hypoxia is a common feature of fibrotic interstitial lung diseases. Renzoni et al. [27] have demonstrated vascular remodeling in both idiopathic pulmonary fibrosis and fibrosing alveolitis associated with systemic sclerosis. It is also well documented that angiogenic chemokines are elevated in both animal tissues and in specimens from patients with idiopathic pulmonary fibrosis, and one would expect that those mediators may promote angiogenesis in inflamed lungs [6, 28, 29]. Pulmonary fibrosis is associated with a poor prognosis in patients with EAA [30]. However, it is not clear what role neovascularization plays in chronic EAA with fibrotic pulmonary changes.

It seems possible that the increased angiogenic activity of sera from EAA patients is related to the stage of the disease. A correlation between serum VEGF levels and the high-resolution computed tomography (HRCT) fibrosis score has been observed in idiopathic pulmonary fibrosis (IPF) [31]. Simler et al. [31] described a negative relationship between serum VEGF levels and the changes in FVC after 6 months of observation. Our findings do not confirm any correlation between serum angiogenic activity and functional changes such as VC, Raw, Cst, and DLco. Other research has described the existence of neovascularization in patients with IPF, drug-induced pulmonary fibrosis, sarcoidosis, and connective tissue diseases with pulmonary manifestation. However, its role in EAA, which is one of the most common interstitial lung diseases, is not clear. An earlier study concluded that the angiogenic activity of sera from EAA patients was stronger than that of sera from sarcoidosis and IPF patients [32]. Therefore, further research on the role of neovascularization in EAA is needed.

Conclusions

Sera from EAA patients are a source of mediators participating in angiogenesis. The angiogenic activity of sera from EAA patients is related to fibrotic radiological changes.

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