Angiogenic activity of sera from interstitial lung diseases patients in relation to IL-6, IL-8, IL-12 and TNFα serum level

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Abstract

Neovascularisation is the principal vascular response in chronic inflammation. The role of angiogenesis in pathogenesis of interstitial lung diseases (ILD) is not clear.

The aim of the study was to examine the effect of sera from ILD patients on angiogenesis induced by human mononuclear cells (MNC) in relation to IL-6, IL-8, IL-12, and TNFα serum level. Serum samples were obtained from 68 patients with ILD (sarcoidosis – SAR 26 patients, avian fanciers' lung – AFP 13, idiopathic pulmonary fibrosis – IPF 13, histiocytosis – HIS 8, scleroderma – SCL 8) and from 14 healthy controls. In order to evaluate angiogenesis the Sidky and Auerbach leukocytes induced angiogenesis assay was performed. Cytokines in sera were evaluated by ELISA. Sera from AFL, SAR and IPF patients significantly stimulated angiogenic activity of MNC as compared with sera from healthy donors ($p<0.001$). However, sera from healthy subjects significantly stimulated angiogenic activity of MNC as compared with the control with PBS and with sera from HIS and SCL patients ($p<0.001$). The IL-12 serum level was significantly elevated in SAR patients compared with healthy controls and other groups. The TNFα serum level was significantly elevated in HIS and SAR patients compared with healthy control and SCL patients. The highest IL-8 serum level was observed in sera from IPF patients and the lowest one in AFL patients ($p<0.05$). We have not found any correlation between proangiogenic properties simultaneously determined in sera and IL-6, IL-8 and IL-12 serum level. However, we observed a correlation between serum TNFα level and the angiogenic activity of sera from ILD patients ($p<0.05$).

Sera from ILD patients and healthy people constitute the source of mediators modulating angiogenesis but the pattern of reaction is different in various diseases. Sera from SCL and HIS patients exert an inhibitory effect on angiogenesis while sera from AFL, SAR and IPF patients stimulate neovascularisation. TNFα as an important proinflammatory factor may stimulate angiogenesis in ILD.

Key words: *angiogenesis, interstitial lung diseases, IL-6, IL-8, IL-12, TNFα.*

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Introduction

Angiogenesis is a process involving the formation of new vessels from the pre-existing vasculature. It occurs in many physiological and pathological conditions [1]. Neovascularisation is essential for an embryonic development, mainly facilitating embryo implantation and placenta formation [2-4]. In adult life it occurs during endometrium regeneration and corpus luteum formation [5]. In physiological reactions angiogenesis contributes in wound healing [6] and sportsmen's myohypertrophy [7]. The process plays an important role in the pathogenesis of numerous diseases

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especially neoplasia [8], Crohn's diseases and psoriasis [9] etc. It is also closely associated with inflammation in several diseases including chronic inflammatory disorders (e.g. rheumatoid arthritis) [9]. It occurs with frequency in other conditions e.g. in diabetic retinopathy [10] and obesity [11]. Although neovascularisation is a principal vascular response in chronic hypoxia and chronic inflammation, the role of angiogenesis in chronic pulmonary inflammatory disorders such as sarcoidosis or hypersensitivity pneumonitis is not clear. However, microvascular changes were observed in lung specimens in ILD [12].

The angiogenetic process is controlled through the balance of two groups of modulators: stimulators and inhibitors. Many proangiogenic factors such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), tumor necrosis factor alpha (TNFα), prostaglandin E_2 (PGE_2), and vascular endothelial growth factor (VEGF) also demonstrate proinflammatory activity [13-15]. However, certain antiangiogenic factors as interleukin-10 (IL-10), interleukin-12 (IL-12), interferon alpha (INFα), interferon gamma (INFγ), transforming growth factor beta (TGFβ), 1,25OH-VitD_3, thrombospondin and angiostatin inhibit inflammation [13, 16]. The interstitial pneumonia is characterized by inflammation, cell proliferation and excessive extracellular matrix deposition. Several cytokines, including those involved in angiogenesis, have been implicated in ILD pathogenesis [17, 18].

The aim of the study was to asses the effect of sera from ILD patients on angiogenesis induced by MNC in relation to IL-6, IL-8, IL-12, and serum TNFα level.

Material and methods

The study population consisted of 68 ILD patients who had not received the immunosuppressive treatment. According to the final diagnosis patients were divided into 5 groups: (i) 26 patients with SAR, (ii) 13 patients with AFL, (iii)13 with IPF, (iv) 8 patients with HIS, and (v) 8 patients with SCL with pulmonary manifestations. The diagnosis was based on clinical, radiological, immunological and histological criteria. All blood samples were obtained with consent of fully informed patients. Sera from 14 healthy volunteers were used as a control (group). All samples were stored at –80°C.

Serum cytokines were measured by sandwich enzyme-linked immunosorbent assay using commercially available kits (Quantikine™ human IL-6 and IL-8, R&D Systems, Inc. Minneapolis MN, USA, IL12+p40 and TNFα BIOSOURCE Europe SA) according to the manufacture's instructions.

Normal human peripheral blood mononuclear cells (MNC) derived from buffy-coat cells of healthy donors were prepared using a Histopaque 1077 (Sigma) and gradient technique at a room temperature for 20 min at 450 g, according to Boyum method [19]. MNC viability was assessed by trypan blue exclusion and found to be ≥98%.

Angiogenesis was quantified by the Sidky and Auerbach test (leukocyte induced angiogenesis assay) [20] in modification [21]. MNC were incubated in phosphate buffered saline (PBS) mixed with serum from patients and from healthy volunteers (25% of serum and 75% of PBS). As a control, MNC were preincubated in PBS only. Following an hour of incubation at 37°C with saturation of 5% CO_2, MNC were suspended in Parker liquid ($5x10^6$ cells/ml). Inbred 8 weeks old female BALB/c mice served as the recipients of normal MNC preincubated with sera or PBS. The mice, anaesthetised with 3.6% chloral hydrate, were injected intradermally with $5x10^5$ cells in Parker liquid supplemented with 0.01% trypan blue. Three mice constituted a group per each patient. Every mouse received 6 injections (3 into each side). Three days following the injection of MNC angiogenic reaction was quantified on the inner surface of the skin of each mouse. New blood vessels localized to the injection sites, trypan blue negative and contrasting with the pre-existing vasculature by a virtue of their tortuosity and divarications were counted under a dissection microscope (Nicon, magnification of 6x). The results are introduced as the mean number of new blood vessels grown in one group (18 injection points per patient). They are expressed as mean ±SD ($p<0.05$ is regarded as a statistical significance). Student's and Pearson's tests were used in statistical analysis (Statistica 6 for Windows).

Results

Sera from AFL, SAR and IPF patients significantly stimulated angiogenic activity of normal MNC compared with the sera from the healthy subjects measured by the mean number of new vessels following the injection of MNC ($p<0.001$) (table 1). The most important proangiogenic effect was observed following preincubation of MNC with sera from AFL patients (17.8±1.3). Weaker stimulation was induced by MNC preincubated with sera from SAR (16.2±0.97) and IPF patients (15.3±1.4) (figure 1). However, sera from the healthy donors significantly ($p<0.001$) stimulated the angiogenic activity of MNC (13.3±0.8) as compared with MNC preincubated with sera from HIS (11.1±0.5) and SCL patients (10.1±0.3). The differences between the mean numbers of new vessels in all groups were significant (table 1).

IL-12 serum level from 17 patients with SAR, 13 with AFL, 13 with IPF, 8 with SCL, 8 with HIS, and 10 healthy donors was measured. The serum level of IL-12 in SAR patients was significantly elevated (302±180 pg/ml, $p<0.001$) as compared with the control group (64±69 pg/ml) as well as with SCL patients (66±110 pg/ml, $p<0.01$) and AFL patients (72±89 pg/ml, $p<0.001$) (figure 2). An elevated IL-12 serum level was also observed in the group of IPF patients (246±285 pg/ml) but the differences in IL-12 level in other groups were not significant. In the sera of HIS patients IL-12 serum level was 147±211 pg/ml. No significant correlation has been established between IL-12

Table 1. Comparison of statistical differences between mean numbers of new vessels (±SD) formed after injection of normal MNC preincubated with sera from ILD patients, healthy donors and control with PBS

Mean numbers of new vessels ±SD	**AFL** n=13	**IPF** n=13	**SAR** n=26	**HIS** n=8	**SCL** n=8	**Healthy control** n=14	**PBS** n=14
AFL 17.79±1.34	**XXXX**	p=0.003	p=0.00001	p<0.00001	p<0.00001	p<0.00001	p<0.00001
IPF 16.19±0.97	p=0.003	**XXXX**	p=0.055	p<0.00001	p<0.00001	p<0.00001	p<0.00001
SAR 15.29±1.43	p=0.00001	p=0.055	**XXXX**	p<0.00001	p<0.00001	p=0.00003	p<0.00001
HIS 11.08±0.52	p<0.00001	p<0.00001	p<0.00001	**XXXX**	p=0.001	p=0.000002	p=0.009
SCL 10.13±0.32	p<0.00001	p<0.00001	p<0.00001	p=0.001	**XXXX**	p<0.00001	p<0.00001
Healthy control 13.27±0.82	p<0.00001	p=0.00001	p=0.00003	p<0.00001	p<0.00001	**XXXX**	p=0.001
PBS 12.08±0.84	p<0.00001	p<0.00001	p<0.00001	p=0.009	p<0.00001	p=0.001	**XXXX**

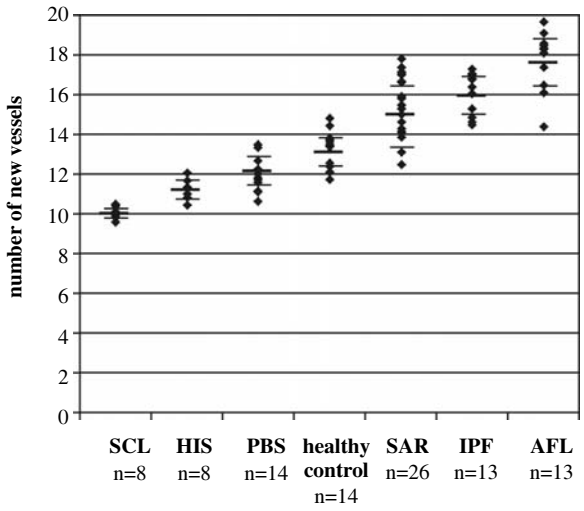

Fig. 1. Mean number of new vessels formed after injection of MNC preincubated with sera from patients with ILD, healthy donors and control with PBS. The mean value and ±SD are indicated by horizontal bars (n – number of examined patients)

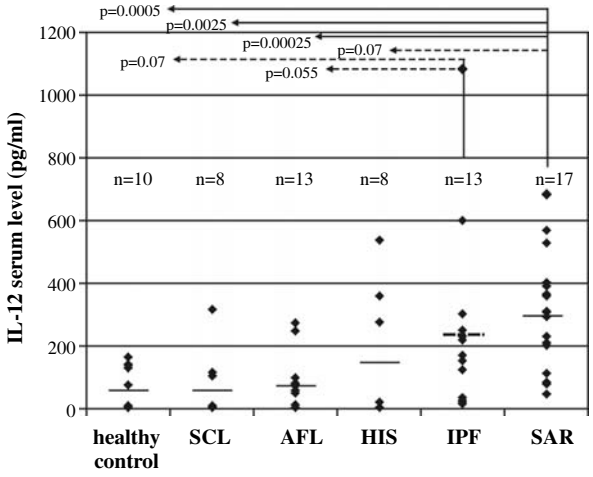

Fig. 2. IL-12 serum level from patients with interstitial lung diseases and healthy control (n – number of sera, —— – mean serum level in examined group, ←— – significant differences, ←- - – quasi significant differences)

serum level and the number of new vessels formed, following injections of MNC preincubated with the sera from ILD patients and from healthy controls (figure 3).

Serum TNFα level was measured in sera from 10 patients with SAR, 10 with IPF, 10 with AFL, 8 with SCL, 6 with HIS, and from 8 healthy donors. The serum level of TNFα was significantly elevated in HIS patients (28±3.9 pg/ml) as compared not only with the healthy controls (20.7±3.6 pg/ml, p<0.005, figure 4), but also with SCL patients (22.7±2.9 pg/ml, p<0.01) and IPF patients (22.4±3.9 pg/ml, p<0.05). Also, in the SAR group the TNFα serum level (27±5 pg/ml) was significantly elevated compared with the healthy controls (p<0.01) and IPF patients (p<0.05). The difference between serum TNFα level from patients with

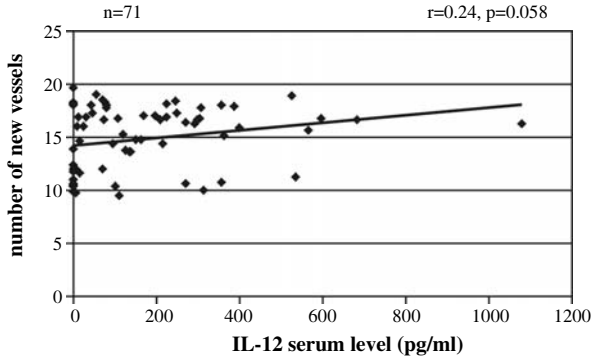

Fig. 3. Correlation between IL-12 serum level and number of vessels created after injection of normal MNC preincubated with sera from ILD patients or healthy donors (r – Pearson's coefficient; n – number of patients)

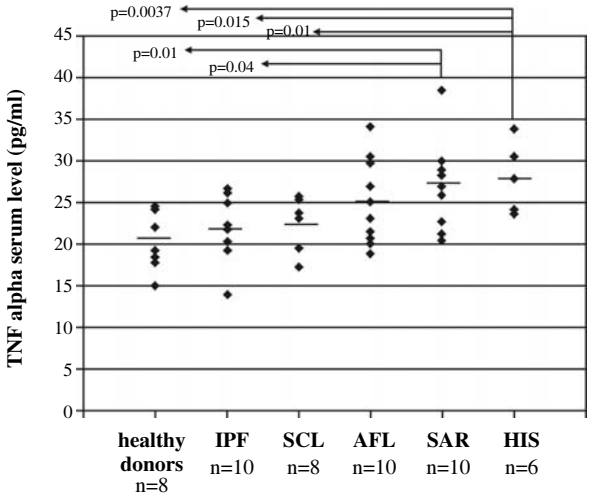

Fig. 4. TNF alpha serum level from patients with ILD and healthy control (⟵ – significant differences, —— – mean serum level in examined group, n – number of sera)

SAR and SCL was not significant (p=0.052), and similarly, between the serum TNFα level from AFL patients (25.1±5.1 pg/ml) and the healthy donors (p=0.06). The TNFα level in the sera from IPF and SCL patients was similar to its level in the sera from the control group. A significant correlation between the serum TNFα level and the number of new vessels created after injection of MNC preincubated with the sera of the studied patients was observed (r=0.29, p<0.05, figure 5a). In contrast, in the group of 46 patients with SAR, IPF, AFL, SCL and healthy controls (without HIS patients) the correlation between the serum TNFα level and the number of new vessels appeared stronger (Pearson coefficient r=0.39, p<0.01, figure 5b).

The IL-6 serum level was measured in sera from 43 patients (25 with SAR, 10 with AFL, and 8 with IPF). The highest IL-6 level was observed in the sera from IPF patients (33.7±7.9 pg/ml) and the lowest in the sera from AFL patients (26.8±6.7 pg/ml), but the differences between these groups were not significant (p<0.056, figure 6). In the SAR patients the mean serum level of IL-6 was 30.8±13.5 pg/ml. We have not found any correlation between the IL-6 serum level and the number of new vessels formed following the injection of MNC preincubated with the sera from ILD patients (figure 7a).

The IL-8 serum level was measured in sera from 42 patients (25 with SAR, 9 with AFL, and 8 with IPF). The highest IL-8 level was observed in sera from AFL patients (872±129 pg/ml), lower in those from IPF patients (741±120 pg/l) and the lowest in SAR patients (699±234 pg/l). The differences between the IL-8 serum level from AFL patients and SAR patients were significant (p<0.05, figure 8). We have not found any correlation between the IL-8 serum level and the number of new vessels created after injection of MNC preincubated with the sera from ILD patients (figure 7b). However, the IL6 and IL8 serum levels correlated with each other (r=0.46, p<0.01, figure 9).

Discussion

The role of neovascularisation in pathogenesis of ILD is not clear. Microvascular changes have been observed in specimens of lung parenchyma in sarcoidosis and IPF [12, 22]. Keane et al. demonstrated an increase of the angiogenic activity in the lung tissue of IPF and experimental fibrosis [23]. An increased angiogenesis-inducing ability of activated alveolar macrophages was found in bronchoalveolar lavage (BAL) specimens from sarcoidosis patients [24].

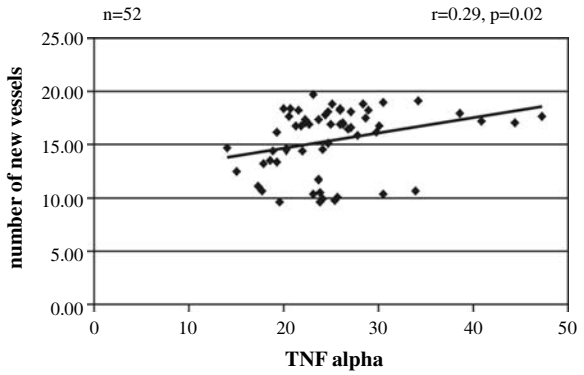

Fig. 5a. Correlation between number of vessels created after injection of normal MNC preincubated with sera from ILD patients or healthy donors and TNF alpha serum level (r – Pearson's coefficient; n – number of patients)

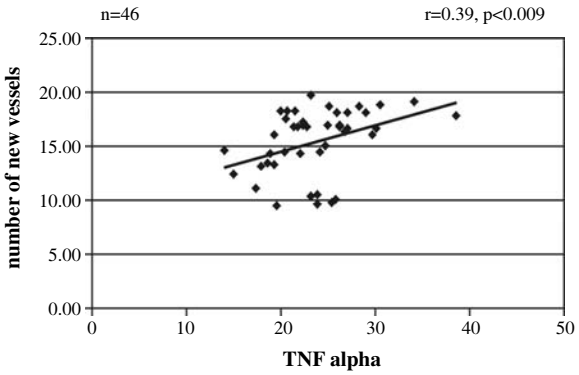

Fig. 5b. Correlation between number of vessels created after injection of normal MNC preincubated with sera from SAR, IPF, AFL, SCL patients or healthy control and TNF alpha serum level (r – Pearson's coefficient; n – number of patients)

Moreover, proinflammatory and antiinflammatory chemokines modulating angiogenesis were found in sera from ILD patients [18]. Generally, the role of angiogenesis in pathogenesis of ILD requires further research. Our results showed that the sera from ILD patients constitute a source of mediators modulating angiogenesis. Serum effect varies depending on the disease. The sera from AFL, IPF and SAR patients demonstrate proangiogenic activity. The most pronounced proangiogenic effect is exerted by the sera from AFL patients. Interestingly, no information on angiogenesis in hypersensitivity pneumonitis has been found. In contrast to AFL, IPF and SAR patients, sera from SCL patients with pulmonary manifestations and from HIS patients exert an inhibitory effect on angiogenesis as compared with the healthy controls and the control with PBS. Previously, Majewski et al. demonstrated the sera from patients with acrosclerosis and diffuse scleroderma modify angiogenic capability of normal MNC. The effect decreased or disappeared in severe and chronic cases [25]. We have not come across any research papers on angiogenesis in histiocytosis.

The results suggest that the angiogenesis may play a role in the pathogenesis of chronic inflammation and fibrosis in ILD. Chronic inflammation is accompanied by neovasularisation [9, 15]. An inflammatory state can promote angiogenesis, and in turn, angiogenesis can facilitate chronic inflammation. Macrophages and lymphocytes which constitute a cellular infiltrate secrete both angiogenic and inflammatory mediators such as the examined IL-6, IL-8, and TNFα [9]. Most of the agents produced during the inflammation process are direct or indirect promoters of angiogenesis. They act by altering the balance between angiogenesis inhibition and stimulation promoting the production of some more directly acting inducers such as bFGF or VEGF which influence endothelial cells proliferation and migration [15].

IL-6 is a multifunctional cytokine presenting biological activities. They involve: activation of B and T cells, production of acute-phase reactants and production of immunoglobulins [26]. *In vivo* IL-6 production is dominated by cells of macrophage/monocytes lineage [26]. IL-6 induces

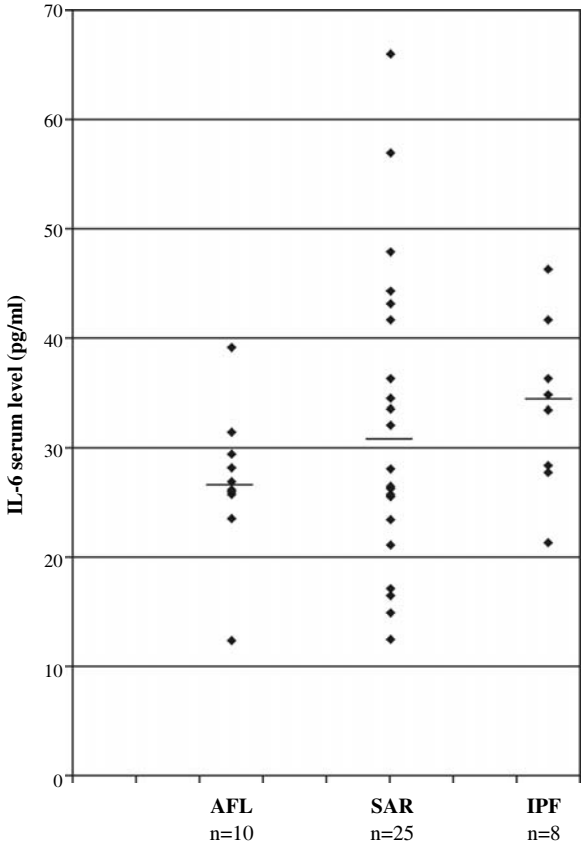

Fig. 6. IL-6 serum level from IPF, AFL and SAR patients (— – mean serum level in examined group, n – number of patients)

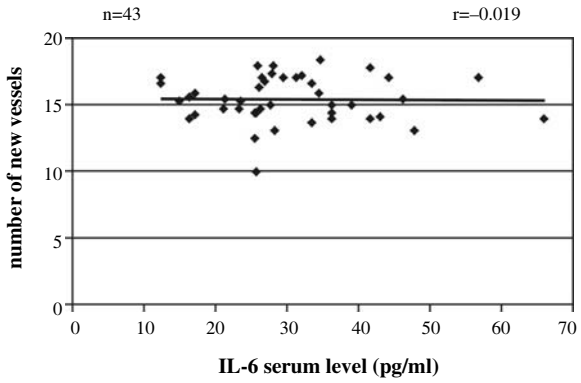

Fig. 7a. Correlation between IL-6 serum level and number of new vessels formed after injection of normal MNC preincubated with sera from SAR, IPF, and AFL patients (r – Pearson's coefficient; n – number of patients)

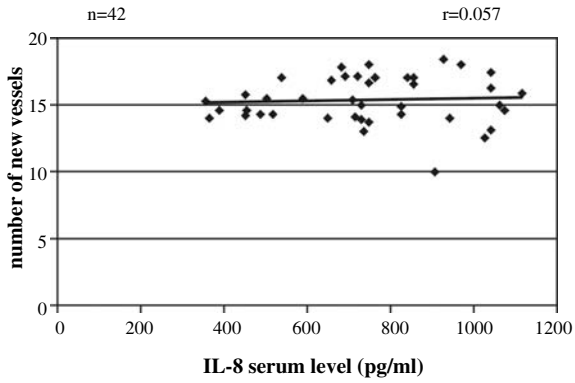

Fig. 7b. Correlation between IL-8 serum level and number of new vessels formed after injection of MNC preincubated with sera from SAR, IPF, AFL patients

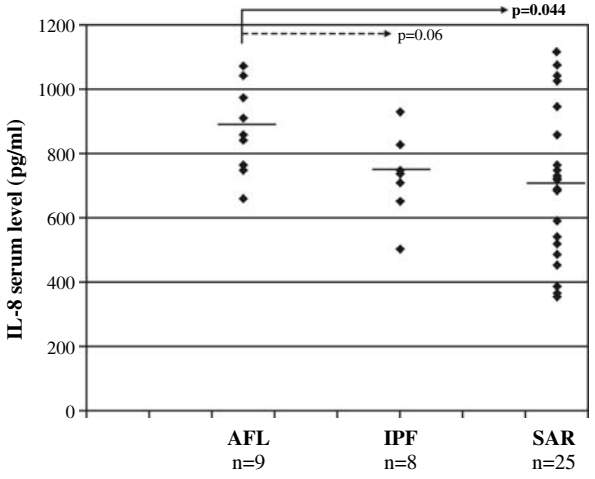

Fig. 8. IL-8 serum level from patients with IPF, AFL and SAR (n – number of sera, ⟵ – significant differences, ⟵ – quasi significant differences, — – mean serum level in examined group)

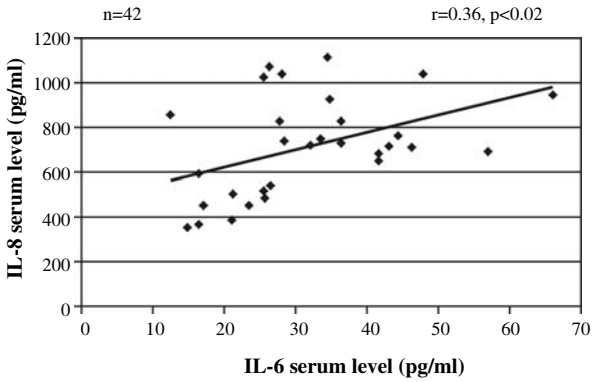

Fig. 9. Correlation between IL-6 and IL-8 serum level from ILD patients (r – Pearson's coefficient, n – number of patients)

endothelial cells activation and vascular smooth muscle cells proliferation and migration [27]. Although this cytokine has been implicated in angiogenesis, *in vitro* it has demonstrated both stimulatory and inhibitory effects on the endothelial cell growth [9, 28]. IL-6 has been reported to be involved not only in the development of inflammatory disorders such as sarcoidosis and rheumatoid arthritis but also in diabetes mellitus and osteoporosis [26, 29-31]. IL-8 is secreted by activated monocytes, neutrophiles, endothelial cells, fibroblasts, and lung epithelial cells [32, 33]. Various studies have implicated IL-8 as pro--inflammatory mediator stimulating neutrophil accumulation, chemotaxis and degranulation [33, 34]. However, it is not only chemotactic but also mitogenic for endothelial cells [9]. Aberrant increases in IL-8 caused by psoriatic keratinocytes have been said to be crucial in their ability to produce an angiogenic response [35]. IL-8 is implicated in the pathogenesis of lung damage and remodelling, especially in IPF [36]. This cytokine regulates angiogenesis, a key component of the fibrotic response in IPF [23]. An increase of angiogenic activity has been attributed to an imbalance between proangiogenic chemokines (as IL-8) and antiangiogenic CXC chemokines (IP-10) [23]. The correlation between IL-6 and IL-8 serum level in IPF, AFL and sarcoidosis patients is particularly interesting. It confirms the role of the cytokines in pathogenesis of ILD.

IL-12 plays a crucial role in controlling the development of Th_1 immune response and stimulates the proliferation and lytic activity of the activated lung T cells and natural killer cells [37]. It is a heterodimeric molecule composed of the p35 and p40 subunits. The data have indicated IL-12 involvement in the development of the lung granulomas typical for sarcoidosis and tuberculosis [38]. In our experiments IL-12 serum level in SAR patients was significantly higher as compared with other ILD groups containing a dominant fibrotic component. IL-12 strongly inhibits tumoral neovascularisation [39]. Induction of INFγ by IL-12 appears to play a decisive role in the antiangiogenic effects of IL-12 [40]. However, we did not establish any negative correlation between IL-12 serum level and the number of new vessels created after injection of MNC preincubated with the sera from ILD patients.

TNFα is a proinflammatory, pleiotropic and heparin-binding cytokine secreted mainly by monocytes and macrophages [41]. The cytokine stimulates the synthesis of IL-1, GMCSF and ICAM-1 by endothelial cells. It is well established that there exists a strong link between the overexpression of TNFα and the development of IPF [42]. The importance of TNFα in the pathogenesis of sarcoidosis remains uncertain [43]. It is known that TNFα stimulates angiogenesis in many models [44-45]. TNFα is involved in promoting angiogenesis *in vivo* while inhibiting endothelial cell proliferation *in vitro* [15]. We demonstrated a strong correlation between serum TNFα level and the number of new vessels created after injection of MNC preincubated with the sera from ILD patients. TNFα has not only proinflammatory but also proangiogenic properties. The results of this study suggest that angiogenesis plays a role in the pathogenesis of ILD. This raises a question whether hypoxia may be responsible for vascular cell proliferation and angiogenesis in interstitial lung diseases [46]. From all the studied cytokines in ILD patients, TNFα seems to exert the most important proangiogenic effect.

Conclusions

Our findings indicate that sera from ILD patients and from healthy people constitute a source of mediators modulating angiogenesis but the pattern of reaction varies in different diseases. Sera from SCL and HIS patients exert an inhibitory effect on angiogenesis while sera from AFL,

SAR and IPF patients stimulate neovascularisation. TNFα as an important proinflammatory factor may stimulate angiogenesis in ILD.

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