

# The influence of sera from interstitial lung disease patients on human mononuclear cells production of VEGF and bFGF

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## Abstract

Angiogenesis is the process of new capillary formation from pre-existing vessels, occurring rarely under normal conditions. In contrast, there are many diseases, which are characterised by unrestricted new capillary growth. The process is under strict regulation determined by a dual, yet opposing balance of angiogenic and angiostatic factors that promote or inhibit neovascularization, respectively. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the most important direct proangiogenic factors. The aim of this study was to assess, *in vitro*, VEGF and bFGF release by mononuclear cells of healthy donors pre-incubated with sera from interstitial lung diseases patients. Human normal mononuclear cells after pre-incubation one hour at 37°C with sera from patients with sarcoidosis, extrinsic allergic alveolitis, systemic sclerosis or healthy donors were suspended in RPMI and cultured 24, 48 and 72 hours. Vascular endothelial growth factor and bFGF in cell supernatant and cell homogenate from culture were evaluated by sandwich enzyme-linked immunosorbent assay.

In cell culture supernatants and cell homogenates increase in VEGF concentration was observed on the three consecutive days. Concentration of bFGF was undetectable in the supernatant as well as in the homogenate of MNC pre-incubated with sera from patients, healthy donors or PBS in all examined groups. Significant ( $p < 0.001$ ) increase of VEGF level in the cell supernatant after 72 hours of culture compared to cell homogenate was observed. The results suggest that VEGF released by MNC pre-incubated with ILD patients sera may be partly responsible for the results obtained previously *in vivo* in the Sidky and Auerbach assay.

**Key words:** angiogenesis, VEGF, bFGF, interstitial lung diseases.

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## Introduction

Angiogenesis is a complex process depending on many stimulating and inhibiting factors. Under conditions of homeostasis, the rate of capillary endothelial cell turn-over is typically measured in months or years because in post-natal life, anti-angiogenic factors such as endostatin, angio-

statin, thrombospondin, inhibitors of metalloproteinases (MMPs) and platelet factor 4 dominate [1]. Neovascularization can be induced by many indirect and direct proangiogenic factors in response to diverse pathophysiologic stimuli. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the most impor-

tant direct pro-angiogenic factors [2]. The basic fibroblast growth factor was discovered as the first pro-angiogenic molecule [3]. This angiogenic mitogen can stimulate production of MMPs by endothelial cells [4]. Vascular endothelial growth factor is a specific endothelial cell mitogen that regulates endothelial cell differentiation, angiogenesis and maintenance of existing vessels [5]. It has been shown that VEGF and its receptor system is the fundamental regulator in the cell signalling angiogenesis. The levels of this growth factor in tumour cells can be significantly enhanced by hypoxia [6]. Hypoxia and inflammation are main causes of neovascularization in non-cancerous diseases [7]. Recently, the important role of angiogenesis in interstitial lung diseases (ILD) has been emphasized [8]. Previously, we have demonstrated, *in vivo*, with the Sidky and Auerbach animal model that sera from ILD patients constitute source of mediators participating in angiogenesis but the nature of these mediators is unknown [9-11]. However, correlation between angiogenic activity of sera from patients with ILD and serum tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) level was demonstrated [12-13]. The open question is if in examined model direct proangiogenic factors such as VEGF and bFGF are produced.

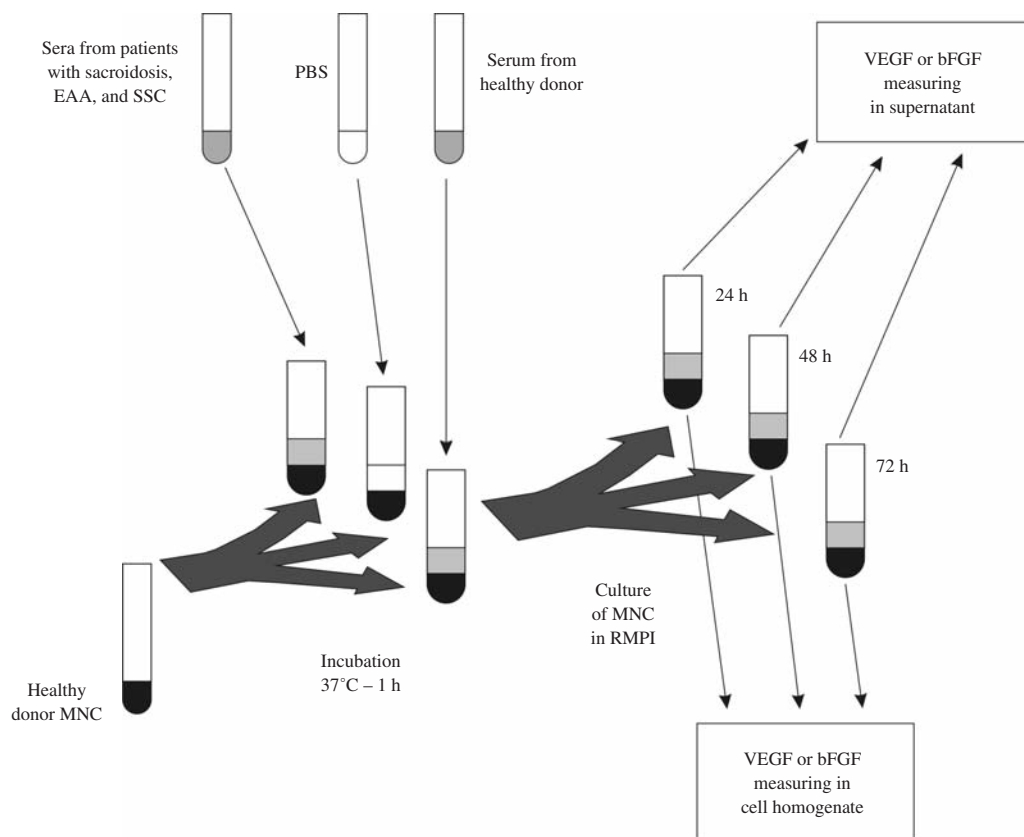
The aim of the study was to assess, *in vitro*, VEGF and bFGF release by mononuclear cells of healthy donors pre-

incubated with sera from ILD patients such as sarcoidosis, extrinsic allergic alveolitis and systemic sclerosis with pulmonary manifestations.

## Material and methods

### Patients

The protocol was approved by a local Ethics Committee and informed consent was obtained from every subject. In the study, sera from 5 patients with sarcoidosis, 5 patients with extrinsic allergic alveolitis (EAA), 5 patients with systemic sclerosis (SSC) and 5 healthy volunteers were used. The diagnosis of sarcoidosis was based on clinical and radiological symptoms and confirmed by histological criteria according to ATS/ERS/WASOG standards [14]. The diagnosis of EAA was based on clinical, radiological, functional, serological, BAL and histological findings according to Lacasse *et al.* criteria [15]. All patients fulfilled the diagnostic criteria of American Rheumatism Association for SSC [16]. All cases with SSC demonstrated pulmonary manifestation by high resolution computed tomography of the thorax. For control purpose we used sera from 5 healthy volunteers recruited from our medical staff. The scheme of the study is presented in Figure 1.



**Fig. 1.** Scheme of the experiment

### Isolation of normal human peripheral blood mononuclear cells

Normal human peripheral blood mononuclear cells (MNC) derived from buffy-coat cells of healthy donors (Warsaw Central Blood Bank) were prepared using a gradient technique according to the Boyum method [17]. The samples were diluted 1 : 1 with a phosphate buffered saline (PBS), layered over Histopaque 1077 (Sigma) and centrifuged at room temperature for 10 min at 500 G. The MNC were suspended in culture medium RPMI 1640 (Biomed, Lublin) in a concentration of  $5 \times 10^6$  cells/ml.

### Preincubation of MNC with sera

MNC were suspended in RMPI with supplementation of 25% of pooled sera from 5 patients with sarcoidosis or pooled sera from 5 EAA patients, or from 5 SSC patients or from 5 healthy donors in concentration  $10^7$  cells/ml and incubated one hour at 37°C in a saturation of 5% CO<sub>2</sub>.

### Culture of pre-incubated MNC

After pre-incubation with pooled sera from patients or healthy donors, MNC were washed with PBS and suspended in RMPI 1640 in concentration  $2 \times 10^6$  cells/ml, enriched with inactivated in 56°C 10% fetal bovine serum (FBS), L-glutamine 2 mM/ml and antibiotics: penicillin (1000 U/ml) and streptomycin (10 µg/ml). MNC cultures were established in microplates (four repetitions of each MNC sample) and cultivated for 24, 48 and 72 hours in humidified atmosphere at 37°C, 5% CO<sub>2</sub> (ASSAB, Sweden). After culture, supernatants and cell sediments were separated and frozen at -70°C.

### Measurement of VEGF and bFGF concentration

Thawed cells or supernatant from culture was supplemented with 1% fetal calf serum for stabilization of VEGF or bFGF. Before measuring the cytokines concentration, cells were homogenized with an ultrasonic disrupter Vir-Sonic (Virtis) for 2 minutes at a frequency of 22.5 KHz. Human VEGF and bFGF in cell supernatant and cells homogenate were evaluated by sandwich enzyme-linked immunosorbent assay using commercially available kits (R&D Systems, Inc. Minneapolis MN, USA) according to the manufacturer's instructions. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotec Instruments, Inc. USA). Cytokine concentration was expressed as pg/ml.

### Statistical analysis

The data were presented as mean  $\pm$  SD and  $p < 0.05$  was regarded as statistical significance. Test t was used for statistical analysis (Statistica 6 for Windows).

## Results

The results are graphically presented on the Figures 2-5.

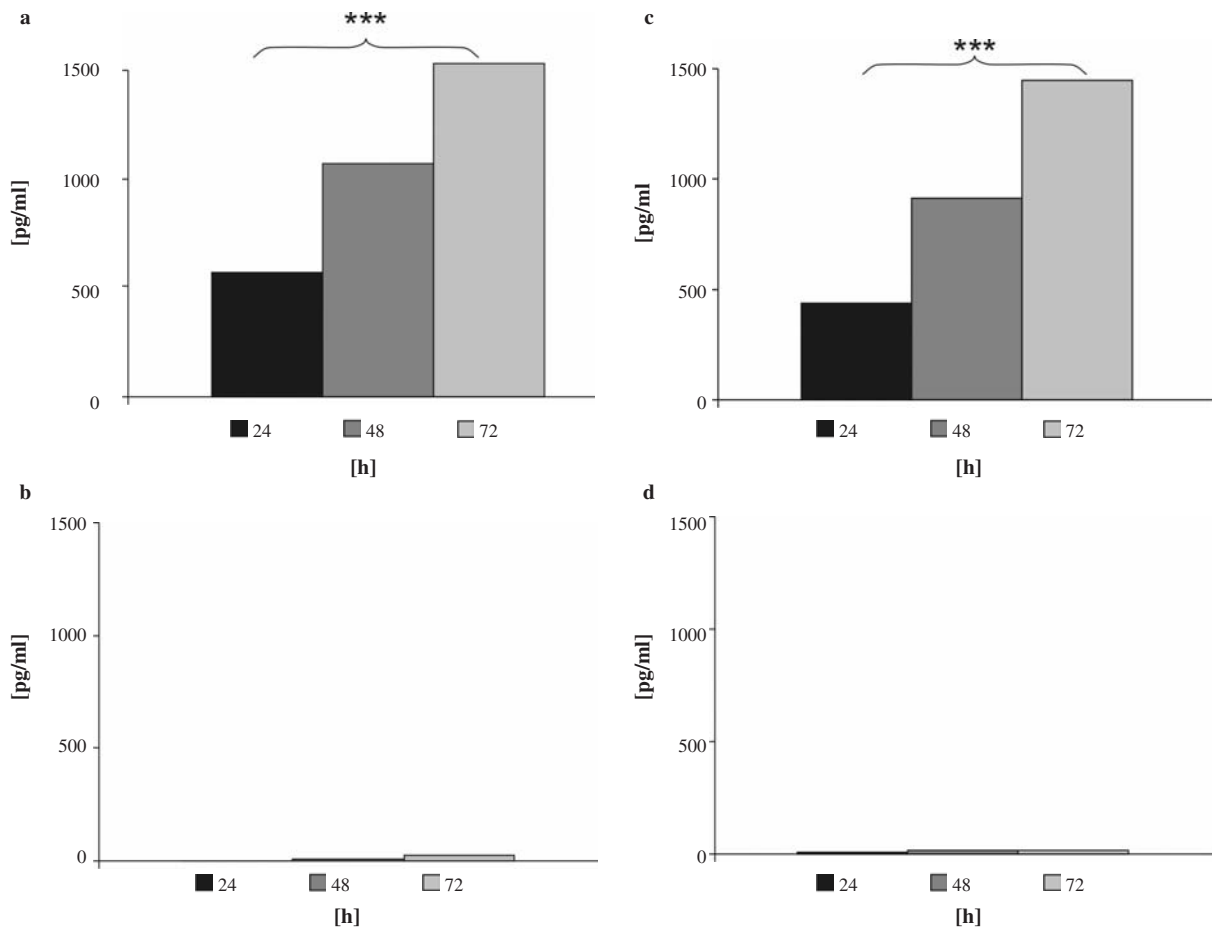
Vascular endothelial growth factor concentration in cell supernatants of MNC cultures collected after 72 hours was significantly ( $p < 0.001$ ) higher compared to concentration after 24 hours (respectively  $1445 \pm 223$  pg/ml vs.  $441 \pm 48$  pg/ml for MNC pre-incubated with sera from sarcoidosis patients,  $1531 \pm 336$  pg/ml vs.  $564 \pm 72$  pg/ml for MNC pre-incubated with sera from EAA patients,  $1382 \pm 342$  pg/ml vs.  $325 \pm 84$  pg/ml for MNC pre-incubated with sera from SSC patients,  $772 \pm 103$  pg/ml vs. 0 pg/ml for MNC pre-incubated with sera from healthy volunteers, and  $179 \pm 26$  pg/ml vs. 0 pg/ml for MNC pre-incubated with PBS). VEGF level in supernatant of 72 hours cultures of MNC pre-incubated with sera from ILD in all cases was significantly higher than VEGF content of corresponding cell homogenate (respectively  $1531 \pm 336$  pg/ml vs.  $29 \pm 3.1$  pg/ml after pre-incubation of MNC with sera from EAA patients,  $1382 \pm 342$  pg/ml vs.  $25 \pm 2.1$  pg/ml for SSC patients,  $1445 \pm 223$  pg/ml vs.  $21 \pm 1.6$  pg/ml for sarcoidosis patients,  $772 \pm 103$  pg/ml vs.  $26 \pm 1.2$  pg/ml for healthy donors, and  $179 \pm 26$  pg/ml vs.  $5 \pm 1.2$  pg/ml for PBS control).

Concentration of bFGF was undetectable in supernatant as well as in homogenate of MNC pre-incubated with sera from patients, healthy donors or PBS in all examined groups.

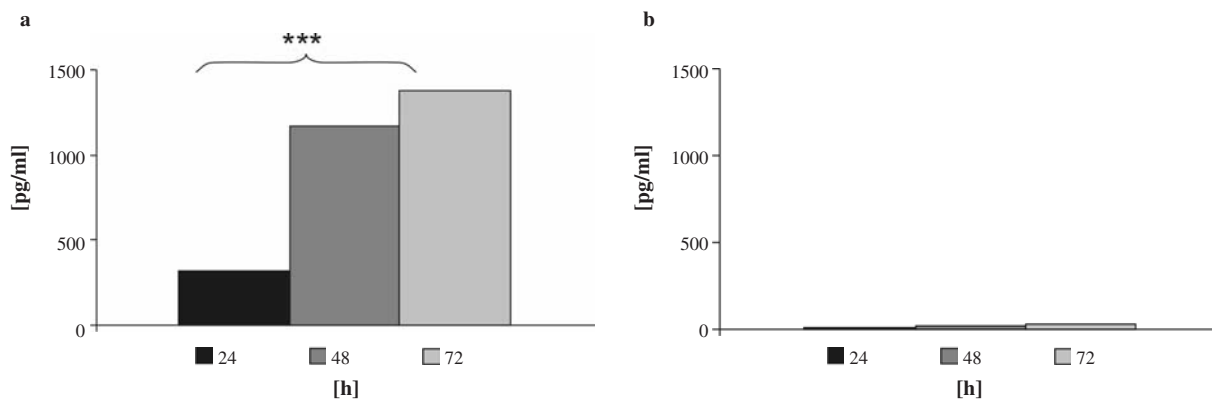
## Discussion

Our results demonstrate, that MNC pre-incubated with sera from some ILD patients produce during 3-day *in vitro* culture increasing amounts of VEGF. Increase of VEGF level in the epithelial lining fluid on 4th day patients with ARDS was showed [19]. However, this increase was associated with resolution of lung injury.

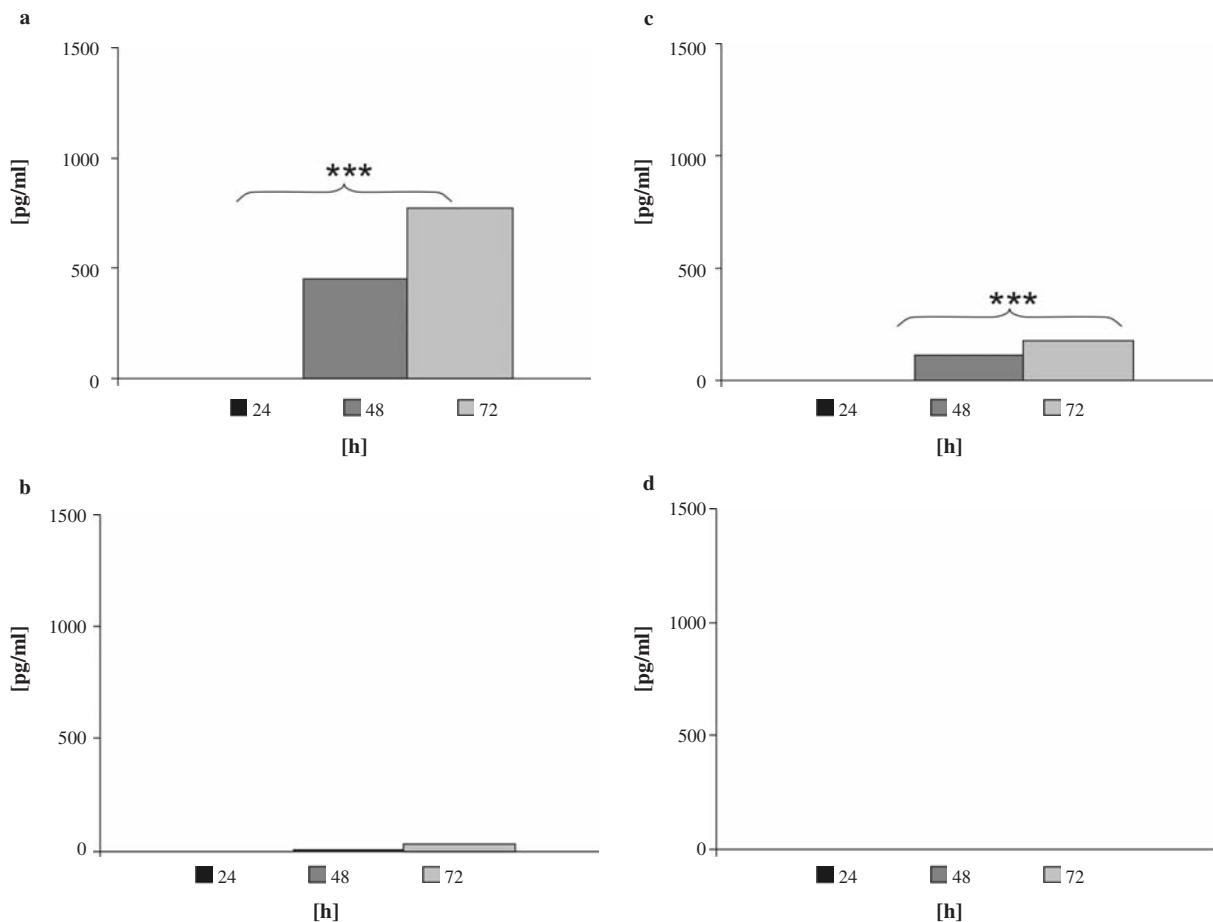
Significantly higher level of VEGF in supernatant from cultures of MNC than in cell homogenate from these cultures was demonstrated. Vascular endothelial growth factor and its receptors are the most important factors regulating process of angiogenesis [20, 21]. Vascular endothelial growth factor stimulates migration and proliferation of endothelial cells, increases vascular permeability and modulates coagulation system [5]. This factor stimulates NO release by endothelium cells, provokes dilatation of vessels [22] and protects endothelium against apoptosis [23]. Vascular endothelial growth factor is produced by tumour cells, macrophages and other immunological cells [24]. It is abundantly expressed in the normal lung, where may be released by numerous cell types found around the airspaces, including alveolar type 2 cells, alveolar macrophages and polymorphonuclear neutrophils. Many different factors stimulate production of VEGF, but hypoxia is one of the most important. Hypoxia-inducible factor (HIF) provokes transcription of gene for VEGF [25]. Hyperoxia decreases the expression of VEGF and its receptors in rat



**Fig. 2.** Vascular endothelial growth factor level in supernatant (a) and cell homogenate (b) from culture of human MNC preincubated with sera from EAA patients; VEGF level in supernatant (c) and cell homogenate (d) from cultures of MNC preincubated with sera from sarcoidosis patients



**Fig. 3.** Vascular endothelial growth factor level in supernatant (a) and cell homogenate (b) from cultures of MNC preincubated with sera from SSC patients



**Fig. 4.** Vascular endothelial growth factor level in supernatant (a) and cell homogenate (b) from culture of human MNC preincubated with sera from healthy donors patients; VEGF level in supernatant (c) and cell homogenate (d) from culture of human MNC preincubated with PBS

lungs [26]. The role of VEGF in early phases of angiogenesis was demonstrated [27]. Our results confirm early participation of VEGF in angiogenesis. However, we did not observe significant differences between VEGF level in supernatant from cultures of MNC pre-incubated with sera from different ILD patients. Previously, we demonstrated that sera from sarcoidosis and EAA patients significantly stimulated and from Systemic sclerosis inhibited neovascularization measured by Sidky and Auerbach assay [11, 12]. Systemic sclerosis was characterized by hypovascularity subunit expression [28]. However, concentration of VEGF was increasing in the serum of SSC patients in the earliest stages of the disease [29]. Mackiewicz [30] suggests that the imbalanced expression of VEGF and its vascular receptors, in part due to insufficient local production of VEGF, which was low compared to VEGFR expression, is responsible for angiogenesis fail in SSC. The data confirm that mechanism of angiogenesis is complicated and depends on many factors.

The second angiogenic factor, bFGF, was not released by MNC pre-incubated with sera from ILD patients and prob-

ably was not important in the induction of early steps of MNC-induced angiogenesis in Sidky-Auerbach model (*in vivo*). Basic fibroblast growth factor joins in the process of neovascularization latter than VEGF and plasmin mobilizes bFGF from extracellular matrix [30]. In presented assay of MNC produced VEGF, however, lack *in vitro* of extracellular matrix and clotting factors such as plasmin can explain lack of bFGF in this study. The further studies to explore the role of angiogenesis in interstitial lung diseases are needed.

## Conclusion

The results suggest that VEGF released by MNC preincubated with ILD patients sera may be partly responsible for the results obtained previously *in vivo* in the Sidky and Auerbach assay.

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

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