

# Differential regulation of endothelial cells proliferative and proangiogenic activity by sera from patients with lower limb ischemia

PAULINA JAGUŚ<sup>1</sup>, ROBERT PROCZKA<sup>2</sup>, JOANNA CHOROSTOWSKA-WYNIMKO<sup>1</sup>,  
MACIEJ MAŁECKI<sup>3</sup>, MAŁGORZATA POLAŃSKA<sup>2</sup>, MARTA KĘDZIOR<sup>1</sup>,  
ADRIANA ROŻY<sup>1</sup>, JERZY POLAŃSKI<sup>2</sup>

<sup>1</sup>Laboratory of Molecular Diagnostics and Immunology, National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland; <sup>2</sup>Department of General, Vascular and Oncological Surgery, Warsaw Medical University, Warsaw, Poland; <sup>3</sup>Department of Cell Biology, Centre of Oncology Warsaw, Poland

## Abstract

We have previously shown that in patients with peripheral ischemia serum concentration of vascular endothelial growth factor (VEGF) differs depending on the disease severity being the highest in subjects with critical leg ischemia (CLI), although no apparent neoangiogenic effect like collateral circulation was observed in their ischemic tissues. Therefore, the aim of present study was to assess the effective proangiogenic activity of sera from patients with peripheral ischemia. It was demonstrated that endothelial cells (HUVEC) incubated in medium enriched with 5% of sera from critical leg ischemia patients demonstrated greater proliferative activity than cells incubated with serum from healthy controls ( $p < 0.001$ ). Also, their ability to form new sprouts in vitro was significantly greater when cultured in medium enriched with serum from patients with CLI ( $1025.85 \pm 316.56$ ) in comparison to serum of moderate leg ischemia (MOD) ( $371.96 \pm 47.07$ ) ( $p < 0.001$ ) or controls. Thus, in patients with severe ischemia, VEGF is not only present in serum in considerably higher concentration than in healthy controls, but also is biologically active, able to evoke appropriate responses in tissues.

**Key words:** VEGF, HUVECs, critical lower limb ischemia.

(Centr Eur J Immunol 2009; 34 (4): 239-242)

## Introduction

The estimated incidence of critical limb ischemia (CLI) is 500 to 800 per million per year [1]. Most often, peripheral vascular disease is caused by atherosclerosis which leads to blood flow restriction. In 40% up to 60% patients diagnosed with CLI invasive treatment is required, including amputation in as many as 25% of them over the next year. Therefore, there is a pressing necessity to develop alternative treatment strategies. One of the options, a concept successfully applied in number of clinical studies, is therapeutic angiogenesis employing the VEGF encoding plasmids [2, 3].

Vascular endothelial growth factor (VEGF) is one of the growth factors playing key role in the pathogenesis of vascular diseases as it regulates cellular proliferation,

migration, differentiation and apoptosis orchestrating both physiological and pathological processes of new blood vessels formation [4]. It has been demonstrated in animal models first, that VEGF gene transfer into ischemic limbs induces angiogenesis and improves tissue perfusion [5]. Similarly, intramuscular injection of naked plasmid DNA encoding VEGF promoted angiogenesis in lower limbs of patients with critical ischemia [2, 4].

Our previous data showed that in patients with peripheral ischemia serum concentration of VEGF differs depending on the disease severity being the highest in subjects with critical leg ischemia [6]. It has been hypothesized however that VEGF biological activity might be uncertain in these patients as irrespective to its abnormally high serum concentration, no

apparent neoangiogenic effect was observed in their ischemic tissues. Therefore, the aim of present study was to assess the proangiogenic activity in sera of peripheral ischemia patients, by evaluating their impact on the proliferative and angiogenic activity of human endothelial cells.

## Material and Methods

Sera were obtained from five patients with critical leg ischemia (CLI), five patients with moderate leg ischemia (MOD), and from five control patients free from any immunological, inflammatory and cancer diseases.

Serum was isolated in a standard way by centrifugation, aliquoted, and stored at  $-70^{\circ}\text{C}$  for further examination.

### Cell Lines, Cell Culture Conditions

The human umbilical vein endothelial cells line (HUVEC) was purchased from Lonza's Clonetics. HUVECs were grown to confluency in EBM-2 MV growth medium in a humidified atmosphere at  $37^{\circ}\text{C}$ ,  $5\%\text{CO}_2$ . Afterwards cells were trypsinized in  $0.05\%$  trypsin and seeded into 96-well microplates at the density of 4 000 cells/well. HUVECs were grown for 24 h to the subconfluent state. Next fresh EBM-2 MV growth medium containing serum of patients or fetal calf serum (Gibco, UK) equal  $5\%$  of the culture medium volume, was added to triplicate wells. Treated cells were incubated for 72 hours at  $37^{\circ}\text{C}$ ,  $5\%\text{CO}_2$ .

### Neutral red assay

The neutral red (NR) assay was performed according to the method of Borenfreund and Purner (Sigma-Aldrich, Poland) [6]. Neutral red solution ( $500\ \mu\text{g/ml}$ ) was added to

**Table 1.** Proliferative activity of HUVEC cells cultured in medium enriched with  $5\%$  serum from individual patients with critical leg ischemia (CLI), moderate leg ischemia (MOD) and control group as assessed by the neutral red assay. Results were presented as a percentage ratio of absorbance in cultures with investigated serum versus cultures with  $5\%$  fetal calf serum

Patients	HUVECs proliferative activity (%)		
	Control ( $\bar{x} \pm \text{SD}$ )	MOD ( $\bar{x} \pm \text{SD}$ )	CLI ( $\bar{x} \pm \text{SD}$ )
1	$65.35 \pm 1.55$	$87.74 \pm 4.79$	$98.67 \pm 1.57$
2	$61.93 \pm 1.35$	$91.23 \pm 5.61$	$99.03 \pm 1.61$
3	$87.39 \pm 4.14$	$88.70 \pm 5.83$	$92.19 \pm 2.32$
4	$86.80 \pm 2.44$	$91.14 \pm 1.16$	$96.82 \pm 2.99$
5	$83.88 \pm 1.17$	$90.87 \pm 2.5$	$97.87 \pm 3.31$
MEAN	$77.07 \pm 12.39$	$89.94 \pm 1.61$	$96.92 \pm 2.77$
		* $p < 0.001$	* $p < 0.001$ ** $p < 0.001$

\* comparison versus control; \*\* comparison versus MOD patients.

each well in an amount equal to  $10\%$  of the culture medium volume ( $10\ \mu\text{l}$  to  $100\ \mu\text{l}$ ) and the microplate was incubated at  $37^{\circ}\text{C}$ ,  $5\%\ \text{CO}_2$  for 2 hours. Afterwards the medium was removed and cells were rinsed with fixative. Next  $100\ \mu\text{l}$  of solubilization solution was added to each well equal to the original volume of culture medium. Absorbances were read at the  $540\ \text{nm}$  on a microplate reader. Results were presented as an index – a percentage ratio of absorbance in cultures with investigated serum versus cultures with  $5\%$  fetal calf serum.

### Sprout Formation Assay

HUVECs were grown to confluency in EBM-2 MV growth medium. Subsequently cells were trypsinized and seeded onto  $0.5\%$  agarose coated culture dishes. After 2 h of incubation at  $37^{\circ}\text{C}$ , HUVEC cells formed aggregates which were incubated for 30 min at room temperature. The old-medium supernatant was decanted, and HUVEC aggregates were suspended in 5 ml of fresh EBM-2 MV growth medium without serum and expanding agent.

Three-dimensional fibrin gels were prepared by mixing:  $960\ \mu\text{l}$  of human fibrinogen (type III,  $60\%$  of protein clottable;  $2.50\ \text{mg/ml}$  concentration in RPMI 1640),  $60\ \mu\text{l}$  of HUVEC aggregate suspension, and  $25\ \mu\text{l}$  of human thrombin ( $25\ \text{units/ml}$  concentration in RPMI 1640) in 12-well culture plates. The mixture was mixed and incubated for about 5-10 min at  $37^{\circ}\text{C}$  in order to gel formation. Next  $1\ \text{ml}$  EBM- 2 MV growth medium containing serum from patients equal  $5\%$  of the culture medium volume, added over the fibrin gel. After 3 days of cell incubation, cultures were fixed in situ for 24 h with  $1\ \text{ml}$  of  $10\%$  formalin solution and photographed under a phase-contrast microscope.

### Image Processing and Analysis

The measurement of sprout length was performed by a ImageJ program. The length of a given sprout was defined in pixels. Because the sprouts are three-dimensional objects, several subsequent two-dimensional images of one aggregate were analyzed. An average value of the length of one aggregate was obtained. Finally, an average value of sprouts length for each patients serum was determined. We measured six different aggregates for each patients.

### Statistical analysis

Data were presented as a mean ( $\bar{x}$ )  $\pm$  standard deviation (SD). Statistical analysis was performed using one-way ANOVA test for mean values comparison. A value of  $p < 0.05$  was considered significant.

## Results

Cell proliferative activity as assessed by the neutral red test shown noticeable differences between proliferation index of endothelial cells cultured with serum from patients with CLI (critical leg ischemia) and MOD (moderate leg ischemia)

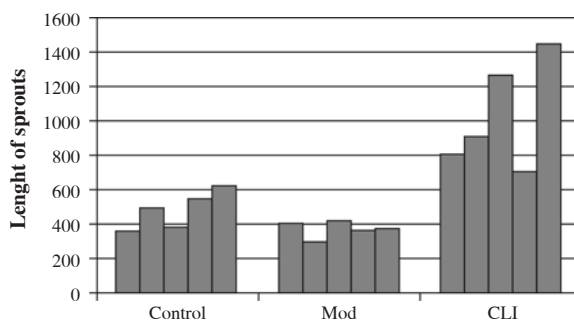
as well as healthy controls with no apparent immunological, inflammatory and cancer diseases). HUVECs incubated in medium enriched with 5% of sera from CLI and MOD patients demonstrated greater proliferative activity than cells incubated with serum from healthy controls ( $p < 0.001$ ). Significant differences were observed also between HUVECs incubated with serum from CLI and MOD patients (Table 1).

Similarly, HUVECs proangiogenic activity observed in the *in vitro* sprout formation assay as the average sprout length, was significantly greater for cells cultured in medium enriched with serum from patients with critical leg ischemia (CLI) ( $1025.85 \pm 316.56$ ) in comparison to those incubated in medium containing serum of moderate leg ischemia (MOD) ( $371.96 \pm 47.07$ ) ( $p < 0.001$ ) or controls free from immunological, inflammatory and neoplastic diseases (CON) ( $482.34 \pm 111.28$ ) ( $p < 0.001$ ) (Fig. 1).

## Discussion

It is commonly accepted that VEGF is the most important physiological and pathological mediator of angiogenesis, strongly induced by many stimuli including hypoxia and tissue disruption therefore fundamental for pathomechanism of critical lower limb ischemia [1, 7]. It has been demonstrated however that high VEGF serum concentration observed in severely ischemic patients is not necessarily followed by appropriate proangiogenic response in their hypoxic tissues and subsequent formation of collateral circulation in lower limbs [4]. One of the possible explanations to this fact might be an inadequate biological activity of VEGF produced in ischemic tissues of these patients. Therefore, the present study was conducted to examine this hypothesis and assess the effect of sera from patients of different, severe or moderate vascular insufficiency and healthy controls on the key endothelial cells functions: proliferation and ability to form new blood vessels. Accordingly, neutral red test and *in vitro* sprout formation assay were employed, demonstrating considerable differences in the effect exerted by sera from severe ischemic patients versus moderate and healthy ones. Both, proliferative and proangiogenic activity was higher for HUVECs cultured with sera from patients with the critical lower limb ischemia providing therefore evidence for adequately preserved VEGF function in this group. Thus, in patients with severe ischemia, VEGF is not only present in serum in considerably higher concentration than in healthy controls, but also is biologically active, able to evoke appropriate responses in tissues.

Therefore, it might be worth considering that the lack of noticeable response to the high peripheral concentrations of VEGF i.e. the development of additional vascular network – collateral circulation clinically observed in patients with severe ischemic disease might be perhaps due to the abnormal functional activity of specific VEGF receptors or alternatively increased activity of angiogenesis inhibitors. Tuomisto et al. who utilized a DNA array of 8400 genes to analyze the



**Fig. 1.** Proangiogenic activity assessed for HUVEC cells incubated in serum from CLI, MOD patients and controls. Bars represent average result for each individual serum tested. Sprouts length was determined in pixels.

ischemia-induced angiogenesis in human skeletal muscle demonstrated that in parallel to the hypoxia-inducible transcription factors HIF-1 $\alpha$  and HIF-2 $\alpha$ , their downstream target VEGF and VEGFR-2, as well as the inflammatory and cell-death promoting TNF- $\alpha$  pathway, anti-angiogenic factors, like interferons or TIMPs (TIMP-3) were also induced [8]. Similarly, some authors suggested that VEGFR expression in chronic lower limb ischemia is attenuated, while others demonstrated quite opposite effect [7-9]. Further studies are planned to examine that phenomenon.

## Acknowledgments

This study was supported by grant nr 2 P05C 017 30 of Ministry of Science and Higher Education, Republic of Poland.

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