Vascular endothelial growth factor (VEGF) is a key cytokine responsible for the spontaneous new blood vessel formation in the course of peripheral ischemia. It has repeatedly been observed in patients with critical leg ischemia that their clinical status does not reflect any effective local neovascularization processes as well as VEGF system up-regulation. Therefore, the aim of the present study was to compare the proangiogenic status, assessed as the serum VEGF concentration, in patients with mild, moderate, and severe peripheral ischemia and to analyze to what extent it is influenced by the therapy applied.

Serum VEGF level was evaluated by ELISA method in 31 patients with peripheral ischemia at different time points throughout the treatment. On Day 0 (before treatment), Day 2, and Day 7, VEGF concentration was significantly higher in subjects with critical leg ischemia (Group I) than in other groups (P<0.001). In Group I, VEGF decline was reported on Day 30 following radical surgery, while in a group of moderate disease treated by revascularization surgery a significant increase in serum VEGF concentration was observed (Day 7 and Day 30) (P=0.02). Serum cytokine level in the patients with mild ischemia (Group III) on pharmacotherapy was stable throughout the observation period. Interestingly, the increase in VEGF levels throughout the study period from Day 0 to Day 30 was significantly greater in unsuccessfully treated patients compared with subjects who positively responded to therapy or did not show any response at all. We conclude that mechanisms other than hypoxia might drive the observed up-regulation of VEGF production in peripheral ischemia.

Key words: limb ischemia, peripheral ischemia, VEGF
INTRODUCTION

Vascular endothelial growth factor (VEGF) is a key cytokine responsible for the spontaneous new blood vessels formation in the course of peripheral ischemia. Its biological effect is due to activation of highly specific tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (FLK-1/KDR), expressed by the endothelial and smooth muscles cells. Beyond that, VEGF is a multifunctional cytokine characterized by a strong proangiogenic effect, potent upregulation of vascular endothelial cells proliferation and differentiation, inhibition of vascular cells aging and apoptosis, and being responsible for increased permeability of peripheral blood vessels (2). VEGF is considered one of the most important factors regulating structural and functional integrity of the vasculature. Consequently, in the course of both physiological and pathological neovascularization (peripheral ischemia, cancer-related angiogenesis) VEGF production in the tissues involved increases considerably (1, 3, 4).

Local ischemia generates a strong biological signal for VEGF production (5). In ischemic regions, a high serum concentration of VEGF and increased expression of its specific receptors is observed. The level of soluble VEGF receptors in serum also is upregulated. As a result, tissue ischemia induces endothelial cells proliferation and intensive neovascularization (6). Increased density of VEGF receptors has even been found in atherosclerotic plaques and the expression of these receptors correlated with the number of newly formed invading vessels (7).

It has repeatedly been observed in patients with critical peripheral ischemia that their clinical status does not reflect any effective local neovascularization processes and VEGF system upregulation. Therefore, the aim of the present study was to compare the proangiogenic status, assessed as the serum VEGF concentration, in patients with mild, moderate, and severe peripheral ischemia and to analyze to what extent it is influenced by the therapy applied. Correlations with the basic morphological parameters: erythrocytes, leukocytes, trombocytes, and hemoglobin were also evaluated.

MATERIAL AND METHODS

The study was approved by a local Ethics Committee and each patient enrolled into the study gave signed consent to study procedures. Thirty one patients hospitalized due to peripheral ischemia of a lower limb participated in the study and were divided into three groups according to disease severity, as assessed by the Fontaine classification.

Group I consisted of 11 patients (F/M – 3/8) with critical leg ischemia and concomitant tissue necrosis (all in stage IV by Fontaine classification), treated radically with amputation of the lower leg. Group II consisted of 20 patients (F/M – 8/12) with moderate leg ischemia (12 in stage III and 8 in stage II by Fontaine classification) who underwent afterwards surgical revascularization. Group III contained 10 subjects (F/M – 5/5) with mild ischemia treated pharmacologically (all in stage I by Fontaine classification).
Blood samples were obtained four times throughout the study: in the morning before surgery (treatment) (Day 0), the following day (Day 2), a week (Day 7) and than a month (Day 30) later. Standard hematological analysis was performed to assess the absolute numbers of erythrocytes, leukocytes, and thrombocytes. Serum was isolated in a standard way by centrifugation, aliquoted, and stored at -70°C for further examination.

The measurement of serum VEGF concentration was performed by a quantitative enzyme immunoassay technique (ELISA) using commercial kit (R&D) according to manufacturer recommendations. Optical density was measured at 450 nm using a spectrophotometric reader Elx800 (Bio-Tek Instruments, Winooski, VT, USA). Cytokine concentration was expressed as pg/ml. The serum VEGF concentration measured in a representative group of healthy volunteers (n=30) at the Department of Cell Biology of the Center of Oncology of the Maria-Sklodowska-Curie Memorial Institute in Warsaw, Poland was taken for comparison as a normal, control level. Data are presented as means ±SD. Statistical analysis was performed using a t-test for the comparison of mean values and the Pearson test for the analysis of correlations.

RESULTS

At Day 0, baseline before treatment, serum VEGF concentration in patients with critical leg ischemia (Group I) amounted to 982 ±810 pg/ml, which was significantly higher than those in both control subjects (220 ±209 pg/ml) and the other two groups of patients with moderate and mild ischemia (Group II - 349 ±325 pg/ml, Group III - 294 ±390 pg/ml) (P<0.001) (Fig. 1). A similar difference was observed on Day 2 and Day 7.

In Group I, there was a steadily increasing serum VEGF concentration in the period directly after radical surgery (lower limb amputation), which reached 1082 ±739 pg/ml on Day 7 and was followed by a considerable decline to 656 ±292 pg/ml on Day 30 (P=0.023 compared with Day 0).

By contrast, in Group II, whose patients underwent surgical revascularization, the serum VEGF significantly increased in the week after the surgery (baseline - 349 ±325 pg/ml vs. Day 7 - 621 ±469 pg/ml; P=0.023) and were increased till Day

![Fig. 1. VEGF serum concentration in peripheral ischemia patients with severe (Group I), moderate (Group II, and mild (Group III) disease, as assessed by ELISA method at 4 time points during the observation period.](image-url)
30 (627 ±506 pg/ml). Interestingly, no significant changes in serum VEGF concentration were observed on Day 1 after surgical treatment.

The serum VEGF concentration in Group III with mild ischemia on pharmacotherapy was stable, with a slight decreasing tendency, throughout the observation period (Fig. 1). It was significantly lower than those in the other two groups on Day 7 and Day 30 (baseline - 294 ±390 pg/ml vs. Day 7 - 249 ±295 pg/ml and Day 30 - 224 ±251 pg/ml; P=0.023).

VEGF concentration correlated with the blood leukocyte count, as observed on Day 0 (r=0.042; P<0.05), Day 7 (r=0.042; P<0.05), and Day 30 (r=0.034; P<0.05) (Fig. 2). No correlation between VEGF and other blood cells count was found.

Interestingly, unsatisfactory wound healing and poor wound neovascularization was noted in 28% of Group I patients, although the level of amputation was chosen as much peripherally as possible. In Group II, inadequate clinical effects after surgical revascularization were observed in 35% of the patients, while in the pharmacotherapy group such effects concerned 50% of the patients. Statistical analysis did not reveal any significant differences between the

![Fig. 2. VEGF serum concentration in peripheral ischemia patients stratified according to therapy outcome. Grey bars - unfavorable outcome, white bars - no effect, and black bars - positive effect.](image)

![Fig. 3. Changes in VEGF serum concentration throughout the treatment period in peripheral ischemia patients stratified according to the treatment outcome. Color code: grey bars: unfavourable therapy outcome, white bars: no effect, black bars: positive effect.](image)
baseline serum VEGF levels in patients with positive and negative treatment outcomes. However, a significantly higher serum VEGF concentration on Day 30 was observed in a subgroup with unfavorable treatment results compared with the subjects who positively responded to therapy or did not show any response at all (P=0.005) (Fig. 2). Similarly, the increases in VEGF levels throughout the study period from Day 0 to Day 30 and Day 2 to Day 30 were significantly greater in unsuccessfully treated patients than in the other subgroups (respectively P=0.010 and P=0.001) (Fig. 3).

**DISCUSSION**

It is well documented that a considerable number of growth factors, including VEGF, play a key role in the process of angiogenesis (1, 5). Neovascularization and formation of collaterals are important physiological defensive mechanisms induced by tissue hypoxia due to peripheral ischemia. Similarly, wound healing in the postoperative period is driven by the formation of new blood vessels. All those processes, to a considerable extent, involve and depend on VEGF system (VEGF - VEGF-R) activity (1). Therefore, an expected result of persistent tissue hypoxia would obviously be an increase in VEGF production.

As clearly presented in our study, serum VEGF concentration at baseline (before treatment) differed in patients with peripheral ischemia depending on the disease severity with the highest level in subjects with critical leg ischemia. Surprisingly, VEGF levels in moderate and mild disease were not as high and these levels did not differ significantly from those in controls. The distinction between the VEGF levels in severe, on the one side, and moderate and mild disease, on the other side, might be explained by different intensity and duration of ischemic episodes. A reasonable conclusion would be that the more severe the disease the more prolonged and severe incidents of tissue hypoxia. It should be noted, however, that VEGF levels in patients with mild and moderate disease were comparable, although the ischemia severity was not. Therefore, other than hypoxia mechanisms might drive the upregulation of VEGF production observed in severely ill patients. Apart from the primary upregulation of VEGF production by hypoxia, a high VEGF level in these patients might also be secondary to abnormal reactivity or expression of VEGF receptors or disrupted signal transduction from the receptor, all resulting in inadequate function of this cytokine and causing further intensification of its production. This hypothesis goes well along with clinical observations that in a considerable group of severely ill patients with very high serum VEGF concentrations no significant signs of neovascularization are observed (in clinical terms), while the disease rapidly progresses locally into the necrosis. Radical treatment (leg amputation), equating with removal of the main VEGF stimulus, did result in a decrease in its serum level. The VEGF level declined relatively slowly, still being considerably high on
Day 7 while a significant drop was found just on Day 30. Obviously, a decrease in serum VEGF was mostly caused by the amputation. However, as the VEGF half-life time is expressed in minutes, other mechanisms that were not as effectively terminated by the radical surgery, might also be responsible for the VEGF overproduction observed and more than a few days were necessary to terminate their activity. A similar disparity in VEGF serum levels following surgical treatment has been observed by other authors (8).

Likewise, the dynamics of VEGF serum concentration changes in Group II with moderate ischemia might be interpreted as a suggestion that other than hypoxia mechanisms drive this cytokine production. In the present study we found a considerable increase in VEGF following revascularization on Day 7 and Day 30, but not immediately after the treatment on Day 2. Although a temporary vessel clamping of the operated artery might cause low perfusion and temporary ischemia, it could not entirely explain the changes observed in the VEGF level. Moreover, one would expect that neovascularization treatment by improving tissue perfusion and decreasing local hypoxia should result in VEGF decline rather than increase.

In our opinion, attention should also be paid to our data demonstrating a relationship between higher VEGF production at the end of the observation period (Day 30) and unfavorable treatment outcome. That association might yet be another evidence that VEGF upregulation in peripheral ischemia is due not only to tissue hypoxia and/or surgical intervention.

It seems that a detailed molecular and functional analysis of both VEGF and its receptors, including their soluble forms, is necessary to further investigate the regulation of VEGF expression in peripheral ischemia. This is also important from the clinical standpoint, since new therapeutic methods including gene therapy with plasmid encoding vascular-endothelial growth factor are underway (9).

REFERENCES


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