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Coexistence of proangiogenic potential and increased MMP-9, TIMP-1, and TIMP-2 levels in the plasma of patients with critical limb ischemia*#

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The objective of this study was to assess the angiogenic potential expressed as a quotient of vascular endothelial growth factor A (VEGF-A), as an indicator of proangiogenic activity, and the circulating receptors (soluble VEGF receptor protein R1 (sVEGFR-1) and sVEGFR-2), as indicators of the effect of angiogenic inhibition, depending on the concentrations of matrix metalloproteinase 2 (MMP-2) and MMP-9 and their tissue inhibitor 1 (TIMP-1) and TIMP-2 in the plasma of patients with lower extremity artery disease (LEAD). These blood parameters in patients with intermittent claudication (IC) and critical limb ischemia (CLI) were compared for select clinical and biochemical features. Stimulation of angiogenesis in the plasma of individuals with LEAD was evident as indicated by the significant increase in VEGF-A concentration along with reduced inhibition depending on circulating receptors sVEGFR-1 and sVEGFR-2. Critical ischemia was associated with higher VEGF-A, MMP-9, TIMP-1, and TIMP-2 concentrations than in the case of IC.

The 77 patients with LEAD (average age (63.4±8.8) years) included 62 with IC and 15 with CLI. The exclusion criteria included other severe complications of arterial disease (unstable coronary heart disease, previous myocardial infraction, and/or stroke during the previous year), severe hypertension, chronic obstructive pulmonary disease, and tumor history. The control group consisted of 27 healthy volunteers (9 females, 18 males, average age (56±6) years) who were age- and sex-matched with the LEAD group. The patient population characteristics are shown in Table S1. The concentrations of VEGF-A, sVEGFR-1, sVEGFR-2, MMP-2, MMP-9, TIMP-1, and TIMP-2 were measured in plasma obtained from venous blood specimens using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, USA). The study was approved by the local Bioethics Commission (No. 509/2011) and the clinical research was carried out in accordance with the Helsinki Declaration. Appropriate statistical tests were performed (including the Mann-Whitney *U* test and the Spearman and/or Pearson correlation coefficient), assuming a value of $P<0.05$ as statistically significant.

Table 1 shows the values of investigated parameters in the test and control groups. Significantly higher levels of VEGF-A, MMP-9, and TIMP-2 and lower levels of sVEGFR-1, sVEGFR-2, MMP-2, and TIMP-1 were observed in the subjects suffering from

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LEAD. The sVEGFR-1/VEGF-A coefficient was 77.7% lower and the sVEGFR-2/VEGF-A coefficient was nearly 79.5% lower in patients than in the healthy counterparts. These findings suggested the considerably reduced inhibition of angiogenesis in the patients. As well, the concentration of the proangiogenic factor VEGF-A was nearly four times higher in the patients.

Table 2 displays the concentrations of substances analyzed in the subgroups of individuals with IC or CLI and in the control group. The difference in VEGF-A concentrations between the CLI and IC subgroups was statistically significant in favor of patients with CLI (P=0.02). The CLI subgroup revealed a VEGF-A concentration over six times higher than that of the control group. The VEGF-A level in the IC subgroup was 3.2 times higher than the level in healthy subjects. The sVEGFR-1/VEGF-A and sVEGFR-2/VEGF-A ratios were insignificantly higher in patients with IC compared to the patients with CLI, which suggested that the patients with CLI had the lowest inhibition of angiogenesis and the highest VEGF-A concentration.

In addition, significantly higher levels of MMP-9, TIMP-1, and TIMP-2 were observed in the CLI subgroup compared to the patients with IC (P=0.021, P=0.033, and P=0.045, respectively). The

Table 1 Investigated parameters for the test and control groups

Group	VEGF-A (pg/mL)	sVEGFR-1 (pg/mL)	sVEGFR-2 (pg/mL)	sVEGFR-1/ VEGF-A	sVEGFR-2/ VEGF-A
Test (LEAD, n=77)	83.90±66.70	119.44±42.66	9821.77±2718.80	2.31±2.34	193.46±175.38
Control (n=27)	17.60±7.44	145.86±62.50	14178.33±3646.93	10.34±7.55	946.01±482.42
P-value	**<0.001**	**0.04**	**0.02**	**<0.001**	**<0.001**
Group	MMP-2 (ng/mL)	MMP-9 (ng/mL)	TIMP-1 (ng/mL)	TIMP-2 (ng/mL)	
Test (LEAD, n=77)	3.03±2.27	8.60±7.11	393.88±342.34	12.69±5.12	
Control (n=27)	4.92±8.25	1.30±0.90	552.63±214.27	5.87±3.80	
P-value	**0.035**	**<0.001**	**0.013**	**<0.001**	

Data are expressed as mean±standard deviation (SD). The difference is considered to be significant at P<0.05 (in bold)

Table 2 Parameters in the subgroups of patients with IC and CLI, and in control individuals

Group	VEGF-A (pg/mL)	sVEGFR-1 (pg/mL)	sVEGFR-2 (pg/mL)	sVEGFR-1/VEGF-A	sVEGFR-2/VEGF-A
Test (LEAD, n=77)					
IC (n=62)[a]	73.10±47.40	114.75±43.27	9732.57±2637.34	2.45±2.54	208.46±188.54
CLI (n=15)[b]	128.90±108.27	138.83±34.92	10190.43±3104.85	1.74±1.11	131.44±83.48
Control (n=27)[c]	17.60±7.44	145.86±62.50	14178.33±3646.93	10.34±7.55	946.01±482.42
P-value					
a vs. b	**0.02**	NS	NS	NS	NS
a vs. c	**<0.001**	**0.03**	NS	**<0.001**	**<0.001**
b vs. c	**<0.001**	NS	NS	**<0.001**	**<0.001**
Group	MMP-2 (ng/mL)	MMP-9 (ng/mL)	TIMP-1 (ng/mL)	TIMP-2 (ng/mL)	
Test (LEAD, n=77)					
IC (n=62)[a]	3.10±2.21	7.79±6.28	358.55±239.95	12.20±5.22	
CLI (n=15)[b]	2.76±2.56	11.92±9.38	539.93±597.46	14.71±4.31	
Control (n=27)[c]	4.92±8.25	1.30±0.90	552.63±214.27	5.87±3.80	
P-value					
a vs. b	NS	**0.021**	**0.033**	**0.045**	
a vs. c	NS (0.054)	**<0.001**	**<0.001**	**<0.001**	
b vs. c	NS	**<0.001**	NS	**<0.001**	

Data are expressed as mean±standard deviation (SD). The difference is considered to be significant at P<0.05 (in bold). NS: not significant

aforementioned parameters were significantly higher in both subgroups than in healthy subjects.

The correlations regarding the concentrations of the MMP-2, MMP-9, TIMP-1, and TIMP-2 were analyzed depending on age, body mass index (BMI), absolute claudication distance, ankle-brachial index (ABI), number of pack-years (Table 3), and the levels of VEGF-A, sVEGFR-1, and sVEGFR-2 (Table 4).

Table 3 Correlation analysis 1

Parameter	MMP-2	MMP-9	TIMP-1	TIMP-2
Age	NS	NS	NS	NS
Number of pack-years	NS	NS	R=−0.26 P=0.02	NS
IC distance	NS	R=−0.27 P=0.02	NS	NS
ABI	NS	NS	NS	NS
BMI	NS	NS	NS	NS

IC: intermittent claudication; ABI: ankle-brachial index; BMI: body mass index; NS: not significant

Table 4 Correlation analysis 2

Parameter	MMP-2	MMP-9	TIMP-1	TIMP-2
MMP-2		R=0.26 P=0.02	R=−0.25 P=0.02	NS
MMP-9	R=0.26 P=0.02		NS	NS
TIMP-1	R=−0.25 P=0.02	NS		NS
TIMP-2	NS	NS	NS	
VEGF-A	NS	NS	NS	NS
sVEGFR-1	R=0.23 P=0.04	NS	R=−0.24 P=0.037	NS
sVEGFR-2	NS	NS	NS	NS

NS: not significant

A negative correlation was identified between MMP-9 concentrations and the absolute claudication distance (R=−0.27; P=0.02), and the number of pack-years and TIMP-1 (R=−0.26; P=0.02).

In addition, a positive relationship was observed between the levels of MMP-2 and MMP-9 (R=0.26; P=0.02) and sVEGFR-1 (R=0.23; P=0.04). Negative correlations were evident between MMP-2 and TIMP-1 (R=−0.25; P=0.02) and between TIMP-1 and sVEGFR-1 (R=−0.24; P=0.037).

This study focused on identifying the relationships between angiogenesis factors (VEGF-A, sVEGFR-1, sVEGFR-2) and MMPs (MMP-2 and MMP-9), and their inhibitors (TIMP-1 and TIMP-2), based on the evaluation of their concentrations in patients with IC and CLI. This objective was hard to meet due to the presence of many other factors involved in angiogenesis and arteriosclerosis (Rundhaug, 2005; Bogaczewicz et al., 2006; Liu et al., 2006).

VEGF-A is a recognized and undisputed promoter of the multi-stage process of angiogenesis (Jazwa et al., 2016). Soluble receptors types 1 and 2 inhibit the activity of VEGF-A by bonding the factor, which circulates as a biologically inactive complex and significantly reduces VEGF-A availability to the stationary receptor VEGFR2 on the endothelial cell surface. The association of VEGF-A and VEGFR2 is necessary for VEGF-A activation.

The plasma VEGF-A concentration was nearly four-times higher in patients with LEAD than in their healthy counterparts. This difference differentiated patients with CLI from those with IC based on the statistically significantly higher concentrations in the CLI group. The findings are consistent with published results (Findley et al., 2008; Stehr et al., 2010).

Furthermore, our findings indicated the coexistence of elevated levels of serum VEGF-A in patients with CLI and statistically significantly higher levels of MMP-9, TIMP-1, and TIMP-2.

Elevated concentrations of MMP-9 and MMP-2 in patients with LEAD were significantly higher than the levels in the healthy subjects. Similar results were observed by Signorelli et al. (2016). Similarly, as in our studies, when comparing patients with IC (n=36) and CLI (n=43), Tayebjee et al. (2005) recorded significantly higher levels of MMP-9 (including TIMP-1) in patients with CLI compared to those with IC. Furthermore, higher MMP-9 expression (complementary DNA) was noted in patients with CLI compared with their healthy counterparts. Expression of MMP-2 was reduced and no differences were found within TIMP-1 or TIMP-2 expression (Baum et al., 2007). Of note, the present data revealed a negative correlation between the MMP-9 concentration and the absolute claudication distance, which suggests that higher MMP-9 levels correspond with shorter painless distances.

Among the MMPs, MMP-9 is most strongly related to instability and atherosclerotic plaque rupture (Hobeika et al., 2008). A mouse model was used to prove that the elevated level of MMP-9 leads to increased plaque hemorrhage and plaque rupture. In humans, however, higher serum MMP-9 concentrations

were observed in patients with unstable coronary heart disease. These concentrations correlated with clinical signs of instability and plaque rupture in coronary and cerebral vessels. The serum concentrations of MMP-2 and MMP-9 were higher in patients with hypertensive than in the control group (Rajzer et al., 2017). Likewise, the values of plasma MMP-9 were higher in patients with type 2 diabetes mellitus (Vitlianova et al., 2015).

With regard to the physiological circumstances, the level of MMPs is adjusted by inhibition that depends on TIMPs and by α-2-macroglobuline (a plasma proteinase inhibitor with broad inhibitory specificity). The inhibition of the activity of TIMPs relies on the irreversible bonding with the catalytic domain of MMPs. TIMPs inhibit the activity of MMPs and promote proliferation. The presence of TIMP-1 was determined in fibroblast nucleons and it was demonstrated that TIMP-2 can inhibit endothelial proliferation induced by basic fibroblast growth factor (Lipka and Boratyński, 2008). TIMP-1 and TIMP-2 have antiapoptotic properties (Hrabec et al., 2007; Groblewska et al., 2011; Fink and Boratyński, 2012).

Adjustment processes are disrupted in tissues exposed to ischemia and hypoxia. The coexistence of reduced TIMP levels and an increased activity of MMPs has been frequently observed in pathological circumstances (Kugler, 1999; Herman et al., 2001). Presently, elevated levels of TIMP-1 and decreased levels of TIMP-2 were evident in the patients with LEAD compared to their healthy counterparts. The CLI subgroup of patients displayed high MMP-9 concentration and significantly elevated TIMP-1 and TIMP-2 levels in comparison with the IC subgroup. Changes in the proportion of MMPs-TIMPs and their significance in the development of severe complications of LEAD, such as myocardial infarction and reperfusion, have been investigated (Lalu et al., 2005). Patients with acute myocardial infarction and unstable angina pectoris displayed higher MMP-2 levels compared with patients with stable angina and healthy control subjects (Wu et al., 2016). Presently, complications of severe arterial disease were an exclusion criterion.

The role of MMPs in the breakdown of extracellular matrix proteins and, more importantly, MMPs, by the degradation of the extracellular matrix, can release different substances including growth factors. MMP substrates can be also found on the surface of many types of cells.

A positive activation-modification loop has been observed between MMPs, growth factors, and cytokines (Chase and Newby, 2003). However, presently no significant correlations were apparent between the key proangiogenic factor (VEGF-A) and MMP-2 and/or MMP-9 concentrations, although the concentrations of VEGF-A and MMP-9 were significantly higher in patients with more advanced artery disease, i.e. in the CLI subgroup. When digesting the components of the extracellular matrix, MMPs reveal a range of proangiogenic and anti-angiogenic factors (Zawierucha et al., 2012). This may be evident as the positive correlation between MMP-2 and sVEGFR-1 concentrations that we observed.

The correlations between MMP-2, MMP-9, and angiogenesis parameters in patients with CLI are difficult to clearly explain, since MMPs participate in the development of artery disease, especially in the progression of the atherosclerotic plaque and a compensation response to ischemia and hypoxia that includes angio- and arteriogenesis (Hobeika et al., 2008; Busti et al., 2010). This can be reflected in high gene expression at the mRNA level that has been observed for MMPs (MMP-9) and VEGF-A in subjects with CLI using popliteal artery samples collected during amputation (Baczynska et al., 2016).

The importance of identifying other MMPs (e.g. MMP-1 and MMP-8) has been emphasized in the stratification of patients with CLI concerning their amputation risk when surgical revascularization is done (de Caridi et al., 2016). Since arterial disease is an important concern globally, pathogenetic events that occur in ischemic tissues and the body's compensatory response to ischemia are areas that warrant study.

A limitation of this study is the relatively small sample size of patients with CLI and different numbers of patients with IC and CLI. Examinations of a larger group of patients are underway. The present findings should be considered preliminary.

The activation of angiogenesis that was dependent on significant proangiogenic activity of VEGF-A and reduced inhibition of angiogenesis by receptors sVEGFR-1 and sVEGFR-2 (which are natural angiogenic inhibitors) was observed in patients with

LEAD. The proangiogenic activity of VEGF-A was significantly higher in the subjects with CLI than in patients with chronic ischemia. In subjects with CLI, high concentrations of VEGF-A were accompanied by elevated levels of MMP-9, TIMP-1, and TIMP-2, which confirms the interaction of proangiogenic factors and MMPs.

Contributors

Radosław WIECZÓR and Danuta ROŚĆ performed the experimental research and data analysis, wrote and edited the manuscript. Anna Maria WIECZÓR performed the data analysis and wrote the paper. Arleta KULWAS collected and analyzed the data. Danuta ROŚĆ, Grzegorz PULKOWSKI, and Jacek BUDZYŃSKI contributed to the study design. All authors read and approved the final manuscript and, therefore, had full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Radosław WIECZÓR, Anna Maria WIECZÓR, Arleta KULWAS, Grzegorz PULKOWSKI, Jacek BUDZYŃSKI, and Danuta ROŚĆ declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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List of electronic supplementary materials

Table S1 Characteristics of the study group

中文概要

题　目：严重肢体缺血患者血浆中促血管生成潜力与血浆MMP-9、TIMP-1和TIMP-2水平升高的相关性研究

概　要：本研究通过比较间歇性跛行（IC）和严重肢体缺血（CLI）患者的血液参数，选择合适的临床和生化特指标，以评估促血管生成的潜力和抑制血管生成的作用。结果表明，通过刺激下肢动脉疾病（LEAD）病人血浆中的血管生成，内皮生长因子 A（VEGF-A）浓度会显著增加，同时依赖于循环受体 sVEGFR-1 和 sVEGFR-2 的抑制也会显著减少。与 IC 病人相比，CLI 病人具有较高的 VEGF-A、金属蛋白酶 9（MMP-9）、金属蛋白酶组织抑制因子 1（TIMP-1）和 TIMP-2 浓度。

关键词：严重肢体缺血；间歇性跛行；血管内皮生长因子；金属蛋白酶