Altered elastase—alpha1-antitrypsin balance in the blood of patients with chronic venous disease

M Budzyn-Napierala¹, M Iskra¹, Z Krasinski², W Turkiewicz², B Gryszczynska¹, M Kasprzak¹ and T Urbanek⁴

Abstract

Objectives: Although leukocyte elastase is suspected to be involved in the damage of vein wall during chronic venous disease, the equilibrium between this protease and its inhibitor, alpha1-antitrypsin, has not yet been evaluated. The aim of the present study was to determine the relationship between leukocyte elastase and alpha1-antitrypsin, in the blood of patients with chronic venous disease.

Patients and methods: The concentration and the activity of leukocyte elastase along with the activity of alpha1-antitrypsin were evaluated in the blood of 55 chronic venous disease patients. The results were compared with those obtained in 33 healthy age and sex-matched volunteers.

Results: A significant decrease in the leukocyte elastase activity that correlated with an increased alpha1-antitrypsin activity was observed in the serum of patients with mild clinical symptoms of chronic venous disease.

Conclusions: The results of the study did not confirm a hypothesis about an important role of proteolytic activity of leukocyte elastase in the vein wall injury mechanism. They show that the leukocyte elastase–alpha1-antitrypsin balance is rather shifted toward antiprotease activity, especially in an early stage of chronic venous disease.

Keywords

Chronic venous disease, varicose veins, elastase, alpha1-antitrypsin

Introduction

There is evidence that inflammatory process is one of the most important factors implicated in chronic venous disease (CVD) pathogenesis. It stimulates circulating leukocytes, which in response liberate various metabolites, contributing to pathological abnormalities observed in the vein wall.¹–³ Neutrophils isolated from blood of patients with CVD have an increased ability to release reactive oxygen species and serine proteases, such as elastase.⁴,⁵ Leukocyte elastase (LE) is a major proteinase responsible for extracellular proteolysis that is mediated by neutrophils. The activity of this enzyme is controlled by specific serine protease inhibitor, alpha1-antitrypsin (AAT). The relationship between LE and AAT levels has been examined extensively, since an imbalance between them is linked to local tissue injury and to many pathologies, including cancer, chronic obstructive lung disease, inflammation, and infectious diseases.⁶–⁸

Although LE is suspected to be involved in the proteolysis of vein wall components in CVD, the relationship between this protease and its inhibitor has not yet been determined. The purpose of the present study was to evaluate the LE–AAT balance by measuring activities of LE and its inhibitor in the blood of CVD patients.
Materials and methods

Patients

The group of patients consisted of 55 subjects (44 women and 11 men), aged 26–65 years (mean age: 44.59 ± 10.23) with primary varicose vein (VV), who underwent lower extremity VV excision. Preoperative, lower extremity venous color duplex ultrasound scanning was performed on all patients and both the superficial and the deep venous systems were studied. Venous reflux was defined as a flow in the inverted direction for a period longer than 0.5 s. Partial and complete venous obstruction were assessed by the degree of compressibility of venous walls, with normal defined as a complete compressibility. Superficial venous functional disease (SFD) and deep venous functional disease (DFD) were defined as a reflux on ultrasound or an abnormal compression in superficial and deep veins, respectively. In all cases, the patency of the deep vein system, as well as the lack of thrombotic changes, were confirmed. The patients with peripheral arterial occlusive disease (ankle-brachial index <0.9) or any other conditions that might result in leukocyte activation, such as diabetes, cancer, connective tissue disorders, or infection occurred within six weeks preceding the conducted procedures were not included in the study.

All patients had visible venous disease signs that corresponded to the Clinical Etiologic Anatomic Pathologic (CEAP) classification categories. There were 23 patients with VV (class 2), 13 patients with edema (class 3), 19 patients with pigmentation, lipodermatosclerosis, or atrophic blanche (class 4). Prior to a surgery, information on gender, age, body mass index (BMI), and disease duration was collected.

The control group consisted of 33 subjects (25 women and 8 men), aged 27–61 (mean age: 41.87 ± 6.99), all of whom were members of medical staff. They had no SFD or DFD on ultrasound, no VVs, edema, or trophic changes of the skin on physical examination and no reports of leg aching.

Sample collection

Blood samples were drawn preoperatively from the arms of CVD patients on the day of their operation. Blood samples were taken with subjects in the recumbent position after 10 min of rest. Samples were collected in EDTA anticoagulant and serum tubes. After 30 min, the tubes were centrifuged at 3000 r/min for 15 min. Serum and plasma samples were stored at temperature of −80 °C until all of the assays were performed. The study procedure was approved by the Bioethical Committee of the University of Medical Sciences in Poznan and an informed consent was obtained from all participants.

Laboratory analysis

Total plasma LE concentration and high-sensitive C-reactive protein (hsCRP) level were measured using enzyme-linked immunosorbent assay (Hycult Biotech, The Netherlands; DRG International, USA). LE activity was determined using succinyl-trialanin-p-nitroanilide as a substrate. The rate of substrate hydrolysis (the formation of p-nitroaniline) was measured at 410 nm. 1U was defined as an amount of the enzyme that catalyzes the conversion of 1 μM of substrate per minute. Unittest AAT chromogenic assay kit was used to evaluate the activity of AAT (Pathway Diagnostics, UK).

Statistical analysis

The statistical analysis was conducted using GraphPad Prism software 6.0 (GraphPad Software, San Diego, CA). The normality of quantitative variables was tested using the Kolmogorov–Smirnow or Shapiro–Wilk test. Any parameter not following the normal distribution was presented as a median and interquartile ranges, and analyzed using nonparametric Mann–Whitney test. Categorical data and proportions were compared using Chi-square or Fisher’s exact test, as appropriate. Normally distributed, continuous variables were presented as a mean and standard deviation and analyzed using the Student’s t-test. Multiple group comparisons were performed by one-way analysis of variance or Kruskal–Wallis test, respectively. The Pearson or Spearman correlation coefficient was used to test the strength of any association between different variables. In all cases, p value ≤0.05 was considered significant.

Results

No significant difference was found between CVD patients and the control group in terms of age, gender, or BMI (Table 1). The average white blood cell, red blood cell, and platelets counts were not significantly different between CVD patients and controls (Table 1). The value of LE activity was significantly decreased and AAT was statistically elevated in the whole group of patients with CVD, when compared with the control group (Table 2). Moreover, significant negative correlation between LE and its inhibitor activity was found (r = −0.426 p = 0.004) (Figure 1). No difference in LE and hsCRP concentration was observed between CVD patients and healthy subjects (Table 2). After classifying patients into appropriate groups according to CEAP classification, lower LE activity was reported in patients falling into C2 and C3 classes (Figure 2(a)). However, LE activity demonstrated a tendency to grow and reach a value comparable to that of healthy control in patients in C4 class (Figure
Tables 1 and 2. Parameters of LE–AAT balance in CVD patients.

**Table 1. Demographic characteristics and laboratory parameters of study groups.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 33)</th>
<th>CVD patients (n = 55)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42 ± 7</td>
<td>44 ± 10</td>
<td>0.140a</td>
</tr>
<tr>
<td>Gender F/M (n)</td>
<td>26/7</td>
<td>42/13</td>
<td>0.902b</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23 ± 2</td>
<td>25 ± 5</td>
<td>0.156a</td>
</tr>
<tr>
<td>WBC (10^3/L)</td>
<td>7.10 ± 2.15</td>
<td>7.85 ± 1.66</td>
<td>0.729a</td>
</tr>
<tr>
<td>RBC (10^12/L)</td>
<td>4.90 ± 0.40</td>
<td>4.76 ± 0.55</td>
<td>0.840a</td>
</tr>
<tr>
<td>PLT (10^9/L)</td>
<td>256 ± 76</td>
<td>262 ± 92</td>
<td>0.525a</td>
</tr>
</tbody>
</table>

*Results shown as mean ± standard deviation, unpaired t-test was used for comparison.
*bCategorical data, Fischer’s exact test was used for comparison.

**Table 2. Parameters of LE–AAT balance in CVD patients.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 33)</th>
<th>CVD patients (n = 55)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE concentration (ng/ml)</td>
<td>44 (26–85)</td>
<td>53 (17–88)</td>
<td>0.975c</td>
</tr>
<tr>
<td>LE activity (U/L)</td>
<td>0.736 (0.447–1.253)</td>
<td>0.587 (0.258–0.935)</td>
<td>0.028c</td>
</tr>
<tr>
<td>AAT activity (U/ml)</td>
<td>0.805 ± 0.205</td>
<td>1.056 ± 0.508</td>
<td>0.019b</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>1.63 (0.17–2.40)</td>
<td>1.18 (0.27–2.98)</td>
<td>0.723e</td>
</tr>
</tbody>
</table>

*Results shown as median and interquartile range, Mann–Whitney test was used for comparison.
*aCVD patients versus control, p ≤ 0.05.
*bResults shown as mean ± standard deviation, unpaired t-test was used for comparison.
*cResults shown as median and interquartile range, Mann–Whitney test was used for comparison.

**Discussion**

LE is a glycoprotein consisting of a single polypeptide chain of 218 amino acids that belongs to the largest class of mammalian serine proteinases characterized by the presence of serine residue in their active site. The release of LE by an activated neutrophils occurs during inflammation and allow these cells to reach and eliminate foreign pathogenic agents. Host tissues are protected from unregulated proteolysis catalyzed by LE and other serine proteases by the activity of AAT. In human serum a critical balance occurs, favoring an excess of AAT over LE and allowing 90% of proteases to be inhibited.
Figure 2. Parameters of LE–AAT balance in CVD patients classified according to disease severity. Box and whisker plots in (a, c, and d) show median (central line), upper and lower quartiles (box), and range excluding outliers (whiskers). Data were analyzed using Mann–Whitney tests. The results in (b) are presented as mean and standard deviation and compared using t-student test. *C2 versus control, p ≤ 0.05; **C3 versus control, p ≤ 0.05; ***C4 versus control, p ≤ 0.05.
to exist as an inactive LE–AAT complex. However, some genetic or, more commonly, environmental factors can break the homeostatic LE–AAT equilibrium. There is a number of diseases in which an LE–AAT imbalance has already been shown to play a significant causative role.

It is suggested that neutrophils of CVD patients undergo activation and degranulation, which is accompanied by the liberation of proteases, including LE. Massive release of LE could be one of the factors promoting disruption of the LE–AAT balance toward an excessive amount of free circulated LE. Although LE is assumed to take part in proteolytic degradation of the vein wall during CVD, the relationship between the protease and its inhibitor has not yet been examined.

There is little evidence to indicate that CVD is associated with an increased level of LE in the venous wall, blood, or other body fluid. Only Shields et al. demonstrated an increased concentration of LE in the plasma of patients with mild as well as severe symptoms of venous disease. Our studies are contrary to these findings and show that LE concentration in the plasma of CVD patients does not differ significantly in comparison with healthy volunteers. The lack of difference in LE concentration in blood of CVD patients may result from their young age (not exceeding 50 years old), confirming an observation made previously by Bujan et al. in the tissues of patients with venous disease.

<table>
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<th>Table 3. Comparison between CVD patients categorized into quartiles according to LE activity.</th>
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<tr>
<td>LE activity (U/L)</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>%C4 (n)</td>
</tr>
<tr>
<td>Disease duration (years)</td>
</tr>
<tr>
<td>AAT activity (U/ml)</td>
</tr>
<tr>
<td>LE concentration (ng/ml)</td>
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<tr>
<td>hsCRP (mg/L)</td>
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</table>

Note: CVD, chronic venous disease; LE, leukocyte elastase; AAT, alpha1-antitrypsin; hsCRP, high-sensitive C-reactive protein; BMI, body mass index.

*Results shown as mean ± standard deviation, one-way analysis of variance test was used for comparison.

*Results shown as median and interquartile range, Kruskall–Wallis test was used for comparison.

*Categorical data, Fischer’s exact test was used for comparison.

p ≤ 0.05

<table>
<thead>
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<th>Table 4. Comparison between CVD patients categorized into quartiles according to AAT activity.</th>
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<tr>
<td>AAT activity (U/mL)</td>
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<tr>
<td>---------------------</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>BMI (kg/m²)</td>
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*Results shown as mean ± standard deviation, one-way analysis of variance test was used for comparison.

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p ≤ 0.05
The evaluation of LE concentration has provided no information about free and unbound forms of the enzyme, capable of degrading protein component of vein wall. The LE burden was assessed by determining activity of free LE in the serum of CVD patients. A decrease in LE activity was found in CVD patients, especially those with mild clinical symptoms of CVD, representing C2 and C3 classes of CEAP classification. These results contradict those of Stvrtinova et al., who reported an increase in LE activity in the serum of patients with VVs in clinical stages 2, 3, and 4 according to CEAP. However, there are some factors that may explain these conflicting results. The number of patients enrolled in author’s study was lower and their average age was higher (50.8, range 32–68), which may influence LE activity in a positive manner.

Some investigators postulate that an elevation in proteolytic activity can lead to a matrix degradation and cause the weakening and dilution of venous wall, resulting in the VVs development. A number of studies support this concept and demonstrate an increased enzymatic activity of leukocyte-derived proteases, including LE, within the walls of VVs or in wound fluids. However, some authors obtained completely opposite results. Gandhi et al. did not report any rise in the elastase activity in the segments of greater saphenous vein of patients with primary VVs. No differences were found for gelatinolytic and caseinolytic activities. In conclusion, authors excluded enzymatic matrix degradation as an essential component in the formation of venous varicosities. Weckroth et al. detected low activity of LE and cathepsin G in leg ulcer exudate samples. Furthermore, most of collagenase found in these exudates were not neutrophil type but probably derived from other cells such as monocytes–macrophages, keratinocytes, or endothelial cells. This finding indicates that not polymorphonuclear neutrophils but rather mononuclear cells may be the main source of proteinases in venous disease.

Most studies focus on the changes that occur in the affected legs, as the major site of the disease process. However, obtaining appropriate controls for this study might be difficult. Some authors have chosen veins harvested for bypass surgery, as the controls. Davies et al. demonstrated that these veins exhibit many pathological features, which cast the doubts on the aspect of use them as an appropriate sample for comparison.

If the blood is taken from the legs of CVD patients, then the blood obtained from the arms of the same patients act as controls. This procedure is also controversial since there have been shown to the legs but may spread throughout the body. Elevated levels of prothrombotic markers were found in the venous blood as well as in the peripheral blood of CVD patients. Similarly, an increased regional and systemic oxidative stress were observed. Tisato et al. evaluated cytokines–chemokines in paired blood samples, collected both from the forearm vein and VVs. The comparison between the levels measured in paired samples belonging to the same patient revealed the identity between the systemic and VV blood samples for most (15 out of 18) cytokines–chemokines. This analysis shows that the systemic profiles of some biomarkers may match the local profile with great accuracy.

Our study also demonstrates that low elastase activity could occur not only locally, in the superficial veins of legs, but also in the blood of CVD patients. The decrease in LE activity was associated with an increase in its inhibitor activity, especially in the mild clinical stage of CVD. This observation suggests that LE–AAT balance may be shifted in favor of the antiprotease, especially in the early stage of the disease development.

High concentration of active AAT molecules may be a physiological response to an excessive liberation of LE by neutrophils, which protects organism from the uncontrolled proteolysis of host tissues. However, this mechanism was not confirmed by the present study, because as it has already been mentioned, no difference in the plasma LE concentration between CVD patients and healthy volunteers was observed.

It is well known that AAT is an acute phase reactant produced by the liver in response to various stimuli, including inflammatory mediators. Elevated levels of interleukin-6 or tumor necrosis factor α observed in the CVD pathogenesis are probably associated with an acute phase response that stimulates hepatocyte secretion of AAT. It seems that in CVD development, AAT may be a better marker of inflammation than hsCRP, for which any rise in the blood of CVD patients was not observed. The fact that CRP failed to reflect inflammation accompanying CVD was also demonstrated by other authors. Yasim et al. did not show any change in CRP concentration in the blood of patients with primary VVs. However in the same study, an increased level of another marker of inflammation—interleukin 12 was reported. No raise in CRP concentration was noticed, even in those patients with the most serious symptoms of CVD, characterized by the presence of active or healed venous ulcers.

The imbalance between LE and AAT resembles the relationship between matrix metalloproteinases (MMPs) and their inhibitors tissue inhibitor of metalloproteinases (TIMPs), described elsewhere by other authors. When the proteolytic MMP/TIMP equilibrium, which may participate in the remodeling of the venous wall was investigated, a higher TIMP-I...
concentration and lower MMP-2 level and activity were found in VVs, compared to control veins. Consequently, the TIMP-1/MMP-2 ratio was 3.6-fold higher in VVs compared to control veins.4 It is well known that LE is an activator of many MMPs,4,4 so its decreased activity, noted in the present study, may be an important factor escalating a disruption in MMP-TIMP equilibrium in favor of antiprotease activity.

Extracellular material (ECM) is a major constituent of the vessel wall crucial for its functioning and integrity. Previous studies have shown that VVs are characterized by an elevated content of collagen, proteoglycans, and laminins.45–47 The ECM deposition may cause a breakup of a regular cellular pattern of the vein wall with a separation and interruption of muscle bundles and a significant intimal thickening. Both local and systemic deficiency in proteolytic activity could account for these biochemical, histological, and ultrastructural changes. An elevated inhibitor may favor the accumulation of matrix components, like collagen, by suppressing proteases. However, low LE activity detected in the blood of CVD patients makes it difficult to explain the observations of a fragmented elastin and a decreased elastin content in VVs.48 Perhaps elastin is destroyed by mechanisms other than an increased LE activity, or the LE activity is only transiently elevated and then returns to the baseline or even lower values by the action of AAT.

The role of LE in destruction of vein wall is still not understood because of the conflicting results obtained by different authors. Differences between studies may be due to differences in the factors such as age, gender, and clinical stage of the disease. However, an increased LE activity is usually demonstrated in samples obtained from patients with leg ulcers,21,22 which are characteristics for an advanced stage of the disease. The present work did not include patients with the most severe symptoms of CVD, which could explain the inconsistency with some previous studies. To explain the role of LE–AAT balance in the severe stage of the disease, patients falling into C5 and C6 classes should be taken into consideration in further studies.

We are aware about the limitation of this pilot study mainly due to the low number of patients. However, the present work calls attention to important aspect of disrupted LE–AAT balance in the pathogenesis of CVD, that has not yet been evaluated. These preliminary observations are therefore the proof of concept for future clinical studies involving a larger cohort of patients with the aim of identifying factors participating in CVD development.

In conclusion, the data collected in the present study demonstrate that a decreased LE activity observed in patients with mild symptoms of CVD may result from the fact that the enzyme is largely complexed with its inhibitor. This finding suggests that LE-mediated proteolysis of the vein wall does not occur in the early stages of CVD development.

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Conflict of interest
None declared.

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