Plasma Matrix Metalloproteinase-9 as a Marker of Blood Stasis in Varicose Veins

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Background—Possible intermediate circulating markers linking blood stasis to vein remodeling were explored in patients with varicose veins in the lower limbs.

Methods and Results—Blood was sampled at rest (supine position) and after a stasis of 30 minutes both in the varicose vein (limbs hanging down) and in the brachial vein (arm hanging down) as a paired control. Several endothelial and leukocyte markers were measured in plasma with the use of ELISA kits. Angiotensin-converting enzyme activity was determined by use of a specific substrate. Matrix metalloproteinases (MMPs) 9 and 2 were evaluated with the use of gelatin zymography. No markers were significantly modified after 30 minutes of blood stasis in the brachial vein. After 30 minutes of blood stasis in the varicose vein, oxygen partial pressure decreased ($P_{O_2}$). Although thrombomodulin, von Willebrand factor, vascular endothelial growth factor, and MMP-2 were not modified in these conditions, the proteins released by proteolysis from the endothelial membrane intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and angiotensin-converting enzyme were increased ($P<0.01$). After blood stasis in varicose veins, the leukocyte markers lactoferrin, myeloperoxidase, and interleukin-8 were not modified, whereas L-selectin shed from leukocytes increased ($P<0.05$), and a major increase in pro-MMP-9, which is released from tertiary granules during polymorphonuclear activation, was observed ($P=0.0001$).

Conclusions—The marked increase in plasma pro-MMP-9 activity provides evidence of polymorphonuclear activation and granule release in the varicose vein in response to postural blood stasis. Similarly, detection in the plasma of membrane proteins shed from the endothelium or leukocytes provides evidence of pericellular proteolysis. (Circulation. 2002;106:535-538.)

Key Words: cell adhesion molecules ■ angiotensin-converting enzyme ■ metalloproteinases ■ plasma ■ hypoxia

Varicose veins are common in developed countries and are related to postural conditions leading to prolonged blood stasis in the legs and to genetic background predisposing to venous incompetence. The pathophysiology of the disease is relatively unexplored. Beyond the functional description of blood backflow induced by postural stimulus in varicose veins and the measurement of venous dilatation by ultrasonic scanning, the exploration of intermediate biological markers between blood stasis and vein remodeling remains limited. Michiels et al used in vitro and ex vivo approaches and have suggested a coherent pathophysiological hypothesis of varicosis development involving polymorphonuclear (PMN) leukocyte adhesion to endothelial cells activated by hypoxia. Similarly, previous clinical investigations demonstrated the involvement of leukocyte activation in chronic venous ulcers.

Therefore, in view of this hypothesis, the objective of the present clinical investigation was to further evaluate intermediate biological markers of these interactions induced by blood stasis in varicose veins. For this purpose, oxygen partial pressure ($P_{O_2}$) and 12 biological markers were measured in blood sampled from varicose veins and paired brachial veins (as control), before and after induction of postural blood stasis. The selected markers are secreted proteins or solubilized membrane proteins from endothelial cells or leukocytes.

Methods

Patients

Characteristics of the 22 patients available for clinical investigation are summarized in Table 1. Patients were rated C3 (n=12) or C4 (n=10) according to the CEAP classification. Valvar incompetence of the sapheno-femoral junction was verified by Duplex ultrasonic scanning in all patients. Pregnant women or those at risk of pregnancy were excluded. All drugs were prohibited. All patients gave their informed written consent, and the experimental design was approved by a relevant French ethics committee (Comités Consultatifs de Protection des Personnes se prêtant à des Recherches Biomédicales [CCPPRB]).

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Heparinized blood was immediately analyzed for $P_{VO_2}$ by use of an automatic radiometer (Copenhagen ABL520). von Willebrand factor and thrombomodulin were measured in citrated plasma by immunoassay (Diagnostica Stago). Soluble intercellular adhesion molecule (sICAM)-1, soluble vascular cell adhesion molecule (sVCAM)-1, lactoferrin, myeloperoxidase, interleukin-8, vascular endothelial growth factor (VEGF), and L-selectin were measured in EDTA plasma by use of immunoassays (R&D Systems). ACE activity was quantified by fluorometric assay. MMP-9 and -2 activities were measured in 1 µL of citrated plasma by gelatin zymography.

### Experimental Design
The patients fasted for $\geq$2 hours before the investigation. After the patient had remained in the supine position with the arms alongside the body for 15 minutes, blood samples were taken from the brachial and the varicose veins (time $t=15$). Patients then let one arm hang down for 30 minutes, after which a blood sample was taken from the brachial vein ($t=45$, blood stasis in control veins). After an additional 15 minutes in the supine position with arms alongside the body, the patient was then made to sit for 30 minutes with the legs hanging down (no skeletal muscle contraction). The arm was maintained horizontally on a support. At the end of this phase, blood samples were taken from both the varicose and the brachial veins ($t=90$, blood stasis in varicose veins).

### Biological Assays
Heparinized blood was immediately analyzed for $P_{VO_2}$ by use of an automatic radiometer (Copenhagen ABL520). von Willebrand factor and thrombomodulin were measured in citrated plasma by immunoassay (Diagnostica Stago). Soluble intercellular adhesion molecule (sICAM)-1, soluble vascular cell adhesion molecule (sVCAM)-1, lactoferrin, myeloperoxidase, interleukin-8, vascular endothelial growth factor (VEGF), and L-selectin were measured in EDTA plasma by use of immunoassays (R&D Systems). ACE activity was quantified by fluorometric assay. MMP-9 and -2 activities were measured in 1 µL of citrated plasma by gelatin zymography.

### Statistical Methods
Results are expressed as mean±SD. Statistical analysis was performed with the use of the Wilcoxon’s test for matched pairs.

### Results

#### $P_{VO_2}$
The $P_{VO_2}$ was significantly higher in varicose than in brachial venous blood after 15 minutes of rest ($t=15$, $P<0.01$, Table 2). Postural stasis induced no variation in $P_{VO_2}$ in the brachial vein ($t=45$, $P=0.93$) but induced a significant decrease in $P_{VO_2}$ in the varicose vein ($t=90$, $P<0.01$). Moreover, the stasis-induced decrease in $P_{VO_2}$ in the varicose vein was associated with a significant systemic decrease in the brachial vein ($t=90$, $P<0.03$).

#### Endothelial Markers
There was no significant variation in the plasma levels of von Willebrand factor, thrombomodulin, VEGF, and pro-MMP-2 in either normal or varicose veins after 30 minutes of blood stasis (Table 2).

ICAM-1 and VCAM-1, as well as ACE, are proteins shed from the endothelium into the blood by proteolysis. Plasma sICAM-1 was not different in varicose and brachial veins at rest ($t=15$, $P=0.74$) and was not influenced by postural blood stasis in the brachial vein ($P=0.11$), but was increased after stasis in the varicose vein ($P=0.0001$). Blood stasis in the varicose vein did not influence systemic sICAM-1 ($P=0.15$).

### Table 2: Measurement of Endothelial and Leukocyte Factors in Brachial (Control) and Varicose Veins at Rest and After 30 Minutes of Postural Blood Stasis

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Brachial (Control) Vein</th>
<th>Varicose Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 (At Rest)</td>
<td>45 (After 30 min Blood Stasis)</td>
</tr>
<tr>
<td>$P_{VO_2}$, kPa</td>
<td>4.63±1.36</td>
<td>4.70±1.42</td>
</tr>
<tr>
<td><strong>Endothelial markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Von Willebrand factor, %</td>
<td>121.3±63.9</td>
<td>111.1±34.2</td>
</tr>
<tr>
<td>Thrombomodulin, ng/mL</td>
<td>27.1±7.9</td>
<td>27.1±6.6</td>
</tr>
<tr>
<td>sICAM-1, ng/mL</td>
<td>201±148</td>
<td>212±47</td>
</tr>
<tr>
<td>sVCAM-1, ng/mL</td>
<td>468±111</td>
<td>478±120</td>
</tr>
<tr>
<td>ACE, UI/mL</td>
<td>26.2±8.2</td>
<td>27.6±11.0</td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>44.3±60.9</td>
<td>33.6±14.1</td>
</tr>
<tr>
<td>Pro-MMP-2, DU/µL</td>
<td>117 600±24 100</td>
<td>121 000±24 500</td>
</tr>
<tr>
<td><strong>Leukocyte markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactoferrin, ng/mL</td>
<td>321±380</td>
<td>261±131</td>
</tr>
<tr>
<td>Myeloperoxidase, ng/mL</td>
<td>23.7±17.0</td>
<td>26.8±18.6</td>
</tr>
<tr>
<td>Interleukin-8, pg/mL</td>
<td>16.8±8.0</td>
<td>17.5±7.8</td>
</tr>
<tr>
<td>L-selectin, ng/mL</td>
<td>934±312</td>
<td>952±320</td>
</tr>
</tbody>
</table>

$^*P<0.05$, $^\ddagger P<0.01$, varicose vein compared with brachial vein at rest.

$^\ddagger P<0.05$, $^\ddagger\ddagger P<0.01$, $^\ddagger\ddagger\ddagger P<0.001$, effect of stasis in varicose vein.

$^\ddagger P<0.05$, $^\ddagger\ddagger P<0.01$, effect in brachial vein of stasis in varicose vein.
Plasma sVCAM-1 was not different in the varicose and brachial veins at rest \((P=0.4)\) and was not influenced by postural blood stasis in the brachial vein \((P=0.15)\), but was increased after stasis in the varicose vein \((P=0.002)\). Blood stasis in the varicose vein influenced sVCAM-1 levels in the brachial vein \((P=0.05)\). The variations of plasma sVCAM-1 in the varicose vein negatively correlated with variations of \(P\text{O}_2\) \((r=0.50, \ P<0.05)\).

Plasma ACE levels were not significantly different in the varicose and brachial veins at rest \((P=0.73)\) and were not influenced by blood stasis in the brachial vein \((P=0.09)\), whereas they increased in the varicose vein \((P=0.002)\). Blood stasis in the varicose vein influenced systemic ACE activity \((P=0.005)\).

Although blood stasis in the varicose vein induced significant increases in the local concentrations of sICAM-1, sVCAM-1, and ACE, the mean variations were rather small \((+9.8\%, +9.6\%, \text{ and } +11.7\%, \text{ respectively})\).

**Leukocyte Markers**

Plasma concentrations of lactoferrin, myeloperoxidase, and interleukin-8 were not influenced by blood stasis either in the brachial vein or in the varicose vein (Table 2).

Plasma soluble L-selectin (sL-selectin) did not differ between brachial and varicose veins at rest and was not significantly influenced by stasis in the brachial vein \((P=0.18)\), but it increased after stasis in the varicose vein \((P<0.02)\). Stasis in the varicose vein did not influence systemic sL-selectin concentration \((P=0.85)\).

No active MMP-9 was detected in plasma with the use of zymography. Pro-MMP-9 activity was the most sensitive marker of blood stasis in the pathological vein (Figure). Plasma pro-MMP-9 activity was much higher in the varicose vein than in the brachial vein at rest \((P=0.0002)\). In response to postural stasis, plasma pro-MMP-9 activity was not influenced in the brachial vein \((P=0.14)\) but increased in the varicose vein \((\text{mean variation: } +51\%, \ P=0.0001)\). Postural stasis in the varicose vein significantly influenced systemic plasma pro-MMP-9 activity in the brachial vein \((P<0.03)\). The activity of pro-MMP-9–lipocalin complexes, detected at a low level in plasma, was similar in brachial and varicose veins at rest; after 30 minutes of blood stasis, it was not modified in the brachial vein but increased \((\text{mean variation: } +28\%, \ P=0.02; \text{ Figure})\) in the varicose vein.

**Discussion**

Franzeck et al\(^8\) previously reported a significant decrease in transcutaneous \(\text{PO}_2\) in ulcers because of chronic venous disease. Taccoen et al\(^9\) measured partial oxygen tension during surgery and showed a nonsignificant increase in \(\text{PO}_2\) in the lumen of varicose veins at rest as compared with controls. However, these investigations were always performed in patients in the supine position, in the absence of posture-induced blood stasis. The higher \(\text{PO}_2\) observed here in the varicose compared with the brachial vein was detected in the recumbent position. This increase could be because of the opening of micro-arteriovenous shunts at rest in response to chronic blood stasis. Thirty minutes of blood stasis induced a highly significant decrease of \(\text{PO}_2\) in the varicose vein with no significant change in the brachial vein. The exact role of \(\text{PO}_2\) variations in the varicose vein remains to be evaluated by further investigations.

The von Willebrand factor, stored in the Weibel-Palade granules of endothelial cells, and thrombomodulin, an endothelial membrane glycoprotein cleavable by neutrophil elastase, are released into the circulation in response to several agonists.\(^{10,11}\) These 2 parameters were not modified by the postural stimulus. Probably, the degrees of leukocyte activation and elastase release from azurophil granules in response to postural blood stasis in varicose veins were too low to induce significant thrombomodulin shedding from the endothelium. Neither VEGF, expression of which is regulated at the transcriptional level by hypoxia, nor latent MMP-2, which is constitutively expressed and secreted by the vascular cells,\(^{12}\) was modified by acute postural stasis.

In contrast, significant variations of sICAM-1 and sVCAM-1 on stasis were observed. They have already been used as markers of venous hypertension.\(^6\) ACE was also significantly influenced in situ in the varicose vein and at distance in the brachial vein in response to blood stasis in the varicose vein. Therefore, as with sVCAM-1, ACE activity could be a systemic marker of blood stasis–induced endothelial protein shedding in the varicose vein.

The leukocytic markers lactoferrin, myeloperoxidase, and interleukin-8 were not modified after stasis, probably because
the release of these markers requires a higher level of leukocyte activation. In contrast, sL-selectin significantly increased in the varicose vein in response to postural stimulus, but this increase was limited and did not modify the systemic concentration. Therefore sL-selectin, shed from neutrophils, seems to be less sensitive to postural stimulus than sVCAM-1 shed from endothelial cells during neutrophil-endothelial cell interactions.

Pro-MMP-9 was the most sensitive marker in our investigation. Pro-MMP-9 and pro-MMP-9–lipocalin complexes are stored in tertiary and secondary PMN granules which are the first to degranulate with the lowest levels of stimulation. Therefore, a variation in plasma pro-MMP-9 activity could be a very sensitive intermediate marker of PMN activation in vivo. The increase in plasma pro-MMP-9 further evidenced leukocyte involvement and activation in response to posture-induced blood stasis in varicose veins.

In conclusion, the present clinical investigation demonstrated that, in situ, the acute proteolytic release of membrane proteins from cells into the blood provides valid intermediate markers of the effects of posture-induced blood stasis in varicose veins. However, the response of the leg vein to postural stasis in nondiseased subjects requires further investigation. Nevertheless, an acute change in pro-MMP-9 activity appears to be a powerful and sensitive marker of blood stasis, providing direct evidence of PMN activation in varicose veins. Such markers could be useful tools for evaluating the efficiency of molecules and compounds able to limit leukocyte–endothelial cell interactions during blood stasis.

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