Peripheral Vascular Disease
Rheologic Variables During Controlled Ischemia

Giovanni Ciuffetti, MD, Michele Mercuri, MD, Elmo Mannarino, MD, Martin K. Robinson, MB, BS, FRACP, Susan E. Lennie, MSc, and Gordon Lowe, DO, MD

The quantitative and qualitative behavior of hemorheologic factors both at rest and after treadmill exercise in 30 male patients with stage II peripheral vascular disease compared with 20 sex- and age-matched healthy controls have been studied. The aim of our study was to identify functional rheologic markers for peripheral vascular disease. At rest, whole blood viscosity (corrected for hematocrit at both high and low shear rates), fibrinogen levels (4.23±1.39 vs. 3.23±1.5), and white blood cell count (7.05±1.25 vs. 6.03±1.28) were significantly different between patients and controls. After treadmill exercise, white blood cell counts increased in both patients and controls, whereas only the filterability of mononuclear leukocytes showed a significant variation in the patient group (5.47±1.54 vs. 7.26±2.00, p<0.002). In this group, mononuclear filterability improved during the recovery period. The results suggest a relation between exercise-induced ischemia of the lower limb and mononuclear filterability in patients with peripheral vascular disease. Mononuclear filterability could be a functional rheologic marker for peripheral vascular disease. (Circulation 1989;80:348–352)

Peripheral vascular disease is characterized in rheologic terms by increases in whole blood and plasma viscosity and plasma fibrinogen levels. A normalization of these parameters is the target of some forms of therapy such as hemodilution, which is aimed at improving microvascular blood flow. However, hemodilution may be useful in only some patients, which suggests that other factors may be present that are important in modifying microvascular physiology. One such factor may be the white blood cells.

Recent studies on animals and humans have suggested that occlusion of nutritive capillaries by white blood cells could be a contributing factor of the “no-reflow phenomenon.”

Monitoring the behavior of the cellular contribution to both whole blood viscosity and filterability in patients with peripheral vascular disease under conditions of controlled ischemia could provide valuable information. Furthermore, separation and filtration of red blood cells from white blood cells, and polymorphonuclear from mononuclear leukocytes by the same method enable study of individual flow behavior for various subgroups of cells. This is technically feasible because routine hematologic techniques can separate blood into its cellular subpopulations, making it possible to study the potential flow resistance of blood in nutritive capillaries by filtering blood cell suspensions through polycarbonate filters with pores of similar diameter as these blood vessels (5 μm).

The identification of quantitative blood (blood cell count and fibrinogen levels) and qualitative (whole blood and plasma viscosity and blood cell filterability) rheologic markers for peripheral vascular disease was the aim of our study, which compared the hemorheologic pattern of 30 patients with stage II (Fontaine) peripheral vascular disease during treadmill exercise to 20 age-matched, healthy male controls.

Methods

Our study was conducted between May and December 1987 on 50 subjects recruited from the Institute of the II Internal Medicine, University of Perugia, Italy. Thirty male patients with peripheral vascular disease were matched for age, body mass index, and smoking habits with 20 healthy male controls (Table 1).

Our study was approved by the hospital ethics committee. Informed consent was obtained from each patient and control subject.

Inclusion criteria were male sex, age between 50 and 60 years, intermittent claudication of more than 500 m by treadmill walking test (Morgan LTD,
UK), clinical onset of disease of less than 3 months duration, and ankle/arm pressure ratio between 0.60 and 0.70 at rest as measured by Doppler ultrasound (Ultrasonomed Srl, Italy).

Exclusion criteria were rest pain, diabetes mellitus hyperlipidemia (serum cholesterol, >240 mg%; serum triglycerides, >160 mg%), hypertension (systolic, >160 mm Hg; diastolic, >95 mm Hg), the presence of vascular disease in other regions, serious medical or surgical disease, recent history of infection, previous vascular surgery, history of acute thromboembolic episodes, and chronic alcoholism.

When recruited into the study, both patients and controls were formally advised to interrupt any drug treatment 20 days before beginning the study. Because our selected patient group was not affected by serious illnesses other than peripheral vascular disease, only physical training and antiplatelet drug treatment were prescribed.

Blood samples, anticoagulated with dry dipotassium edetate (EDTA 1.5 mg/ml), were taken from an arm vein of each patient after an overnight fast, immediately before treadmill test (at rest), after standard exercise (>10 minutes walking, 2 km/hr at a constant gradient of 12°), and when the ankle/arm pressure ratio had recovered its initial value (recovery). Recovery time, claudication time (duration of pain free exercise), and walking time (maximum duration of exercise after claudication) were the endpoints of individual treadmill tests.

The hemorheologic parameters determined were hematocrit by microcentrifugation (DDR) and 20,000 rpm for 5 minutes, whole blood viscosity at corrected hematocrit8 by rotational viscometer (Haake Rotovisco RV 20/CV 100, Haake, FRG) using shear rates of 94.5/sec and 0.945/sec at 37°C, plasma viscosity by the same viscometer at a shear rate of 300/sec at 37°C, plasma fibrinogen by radial immunodiffusion9 (M Partigen plates, Behringwerke, FRG), and leukocyte count by automated hematology analyzer (Toa Medical Electronics Co LTD, Japan).

The separation and filtration of blood cells were performed as follows.

**Separation Procedure**

Polymorphonuclear and mononuclear leukocytes were separated with a Ficoll-Hypaque (Pharmacia, Sweden, and Sterling Research Ltd, UK, respectively) density gradient (1.114 g/ml and 1.022 g/ml) after centrifugation (TJ & R, Beckman, FRG). Ficoll is a synthetic, high-molecular-weight polymer. It is nontoxic to cells, may exert a protective influence on them, and has proved particularly useful for separating leukocytes from whole blood.10 Recently Nash et al11 have shown that exposure of leukocytes to density separation media does not affect the filterability of the cells. Tackur et al12 have shown that Ficoll allowed a low degree of erythrocyte contamination, while the percentage of cell viability was 99%.

The cell subpopulations were washed and resuspended in phosphate-buffered saline (pH 7.4, 290 mosm/kg) containing 0.5% bovine serum albumin (Sigma, UK) at a concentration of 1,000,000 cells/ml13; we have previously found that addition of a small amount of bovine serum albumin (0.5%) reduced cell activation (G.D.O. Lowe, unpublished material), presumably by reducing cell adhesion to foreign surfaces. In fact, an appropriate protein solution, such as bovine serum albumin in the case of the mammalian cells, ensures that any glass and syringe surfaces have a similar zeta potential to the cells.10 Furthermore, in this way it is possible to look at cell filterability free of any interaction with plasma proteins.

Red blood cells were separated after centrifugation at 3,000 g for 10 minutes. The plasma buffy coat and upper 10% of packed red blood cells were aspirated and discarded. Red blood cells from the middle part of the column were suspended in phosphate-buffered saline at a 10% hematocrit. Each sample of cell suspension was diluted 1:10 in 2% glacial acetic acid to which a few grains of crystal violet stain (Sigma, UK) has been added. Leukocyte contamination was then checked in a Neubauer counting chamber (Gellenkamp, UK) with a light microscope.

**Filtration Procedure**

Each suspension sample was filtered by a constant-flow syringe pump (Vickers, UK) (1.5 ml/min) for 6 minutes, and the pressure rise was monitored by a pressure transducer (Bell and Howell, UK) linked to an amplifier (Gaeltech, UK) and chart recorder (Logos SpA, Italy). Filterability was expressed as the final filtration pressure generated by the cell suspension relative to the buffer previously filtered through the same filter.13

A single batch (no. 5414C32) of nucleopore membranes (Nucleopore Corporation, Pleasanton, California) with pore diameter of 5 μm was used for the filtrations throughout the study. Filtrations were performed at 25±1°C. All measurements were completed within 3 hours of venipuncture.

Statistical analysis was performed using parametric (one-way analysis of variance) and nonparametric (Wilcoxon ranked sum) tests. Single and multiple regression analyses were also used.14

### Table 1. Age, Number of Cigarettes Smoked Per Day, and Body Mass Index in 30 Patients With Peripheral Vascular Disease (Group 1) and 20 Matched Controls (Group 2)

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>55.59±2.79</td>
<td>54.53±2.56</td>
</tr>
<tr>
<td>Cigarettes (n)</td>
<td>18.00±4.84</td>
<td>19.6±4.30</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>23.71±2.28</td>
<td>22.7±2.01</td>
</tr>
</tbody>
</table>

Values given are expressed as mean±SD.
Table 2. Results of the Main Rheologic Variables in Patients With Peripheral Vascular Disease (Group 1) and Controls (Group 2)

<table>
<thead>
<tr>
<th></th>
<th>At rest</th>
<th>Exercise</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
<td>Group 1</td>
</tr>
<tr>
<td>White blood cell count (10^9/l)</td>
<td>7.05±1.52</td>
<td>6.03±1.28*</td>
<td>8.18±1.64†</td>
</tr>
<tr>
<td>Red blood cell filterability</td>
<td>2.90±0.73</td>
<td>2.99±0.72</td>
<td>3.03±0.83</td>
</tr>
<tr>
<td>Polymorphonuclear filterability</td>
<td>4.13±0.80</td>
<td>4.19±0.63</td>
<td>4.04±0.68</td>
</tr>
<tr>
<td>Mononuclear filterability</td>
<td>5.47±1.54</td>
<td>5.55±1.23</td>
<td>7.26±2.00‡§</td>
</tr>
<tr>
<td>Whole blood viscosity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(94.5/sec)</td>
<td>4.81±0.29</td>
<td>4.59±0.31*</td>
<td>4.88±0.35</td>
</tr>
<tr>
<td>(0.945/sec)</td>
<td>17.31±3.27</td>
<td>15.33±2.37</td>
<td></td>
</tr>
<tr>
<td>Plasma viscosity (300/sec)</td>
<td>1.44±0.09</td>
<td>1.34±0.06†</td>
<td>1.47±9.09</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>4.23±1.39</td>
<td>3.23±1.50</td>
<td></td>
</tr>
</tbody>
</table>

Values given are expressed as mean±SD.
Filterability, pressure ratio of cell suspension to buffer after 6 minutes filtration.
Intragroup analysis: †p<0.02; ‡p<0.002.
Intergroup analysis: *p<0.05; †p<0.02; ‡p<0.002.

Results

Quality controls were satisfactory for the separation and filtration procedures of blood cell subpopulations. Morphologic examination on slides of cell suspensions showed that purity and viability (trypan blue exclusion) were more than 97% and 95%, respectively, for the cellular subpopulations under study. No significant differences in the percentage of "active" cells (defined as the presence of pseudopodia or cytoplasmatic irregularities) in any of the cell subpopulations were found after filtration.

Reproducibility was confirmed by five successive filtrations of cell suspensions giving mean coefficients of variance. At the fifth filtration, the mean was 4.2% for mononuclear, 4.6% for polymorphonuclear, and 2.4% for the red blood cells.

Table 2 reports the results obtained.

Statistical analysis of the results revealed significant differences between the two groups at rest (peripheral vascular disease, group 1; control, group 2). Differences were observed in whole blood viscosity at corrected hematocrit at both high (p<0.02) and low (p<0.05) shear rates, in plasma viscosity (p<0.001), in fibrinogen levels (p<0.05), and in the leukocyte count (p<0.02). These differences persisted after treadmill exercise and recovery, in whole blood viscosity at corrected hematocrit at low shear rates (p<0.002), in plasma viscosity (p<0.001), and in fibrinogen levels (p<0.001). At high shear rates, whole blood viscosity, at corrected hematocrit, maintained this significant difference only after treadmill exercise (p<0.02), whereas the leukocyte count varied only during recovery (p<0.02).

However, intragroup statistical analysis revealed a significant difference in the leukocyte count after treadmill exercise both in the controls (p<0.02) and patients (p<0.02). Moreover, in the latter group, the filterability of the mononuclear leukocyte subpopulation was significantly impaired (p<0.002).

Discussion

Whole blood filterability is a factor that may affect blood flow characteristics in the microcirculation.16 Measurement of whole blood filterability may be influenced by other rheologic variables including blood cell filterability and plasma viscosity, which in turn are influenced by macroglobulins such as fibrinogen.17,18 Determination of the relative contributions of individual rheologic factors has prime importance, particularly during acute ischemia, when low perfusion pressure increases the intrinsic resistance of microvascular flow.8

Impaired blood filterability in patients with peripheral vascular disease was initially attributed to a decrease in red blood cell deformability.19,20 However, recent studies have emphasized the limited contribution of red blood cells to whole blood filterability. Whole blood filterability is, in fact, influenced mainly by factors other than erythrocytes, such as the plasma fibrinogen concentration and the number of contaminating leukocytes.21,22

Our results, obtained using a specific method to filter an erythrocyte suspension unaffected by leukocyte contamination, confirmed that red blood cell filterability in peripheral vascular disease was similar to that of healthy subjects at rest, after treadmill exercise, and in the recovery period. Hematocrit in the peripheral vascular disease group was not significantly different from the control group. On the other hand, our results confirmed fibrinogen levels to be a predictor for blood viscosity and a probable marker for atherosclerosis.23,24 In the peripheral vascular disease group at rest, the fibrinogen level, whole blood viscosity, and plasma viscosity were significantly different from that of the healthy controls. This concurs with results obtained in studies on other chronic occlusive arterial diseases.24,26 We would, however, like to emphasize that no significant variations were observed in fibrinogen levels during ischemia or in the recovery period. These
remained constant throughout the study in both controls and patients.

The principal aim of our study, however, was to see whether an increase in the number of white blood cells, suggested to be a risk factor for vascular disease\textsuperscript{3,16} and a related stress-induced phenomenon in atherosclerotic patients during treadmill exercise\textsuperscript{27} could be correlated to ischemia. Our results indicate an increase in the leukocyte count can only be considered as a nonspecific marker for atherosclerosis.\textsuperscript{16} The number of white blood cells was, in fact, significantly higher in the peripheral vascular disease group at rest, but this difference disappeared during exercise. It is likely that the increase in the number of leukocytes observed in both groups during exercise was the physiologic expression of the "hematologic stress syndrome."\textsuperscript{728}

On the other hand, the leukocyte filterability pattern and, in particular, the behavior pattern of its subpopulation were extremely interesting and well worth further study. Our results indicate that during rest, white blood cell subpopulation filterability in both patients and controls is similar. This suggests that white blood cell filterability is not affected by atherosclerotic disease per se. However, during exercise, significant alterations occur only in the rheologic properties of the mononuclear subpopulation. Furthermore, these alterations are limited to patients with peripheral vascular disease. This appears to confirm the hypothesis that the mononuclear subpopulation can be considered a specific, dynamic, and functional marker for peripheral vascular disease–related ischemia. The constant and significant increase in filterability that proceeded hand-in-hand with the functional recovery appears to provide further evidence in support of this hypothesis. Indeed, it has long been known that white blood cells condition the microvascular flow in narrow vessels due to their high internal viscosity and their adheresiveness when activated.\textsuperscript{17} Their lack of deformability and their adheresiveness render them prime candidates for causing capillary occlusion and the "no-reflow phenomenon."\textsuperscript{34–7,29}

Once impacted, white blood cells may cause tissue damage by release of toxic enzymes and oxygen-derived products when activated by ischemic conditions.\textsuperscript{3} It has been suggested that this phenomenon may play a pathogenetic role in circulatory shock\textsuperscript{7} and may even condition the size of the "ischemic penumbra."\textsuperscript{17}

The difference observed between mononuclear and polymorphonuclear filterability rates emphasizes the need to study the filterability of white blood cell subpopulations separately. In fact, recent investigations into the mononuclear subpopulation seem to indicate that its reduced filterability is due to monocytes rather than lymphocytes (G. Ciuffetti, unpublished material).

In summary, our present findings indicate that red blood cell filterability is normal in patients with peripheral vascular disease both at rest and under ischemic conditions. While increased plasma and whole blood viscosity due to hyperfibrinogenemia are capable of reducing microvascular flow in peripheral vascular disease, it has been shown that they had no effect on performance during physical exercise. The only rheologic variable modified in the presence of ischemia was the mononuclear filterability. Mononuclear filterability could be a specific marker for ischemia in peripheral vascular disease.

Acknowledgments

The authors express gratitude to Geraldine Boyd, Dott Litt, and Karla Essick for their help in preparing the manuscript.

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KEY WORDS • peripheral vasculature • exercise • ischemia • blood cells • rheology
Peripheral vascular disease. Rheologic variables during controlled ischemia.
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Circulation. 1989;80:348-352
doi: 10.1161/01.CIR.80.2.348
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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