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Chronic venous insufficiency is associated with increased platelet and monocyte activation and aggregation

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Purpose: This study assessed whether the increased numbers of platelet-monocyte aggregates observed in patients with venous stasis ulceration (VSU) represent a response to dermal ulceration or if it is a condition associated with underlying chronic venous insufficiency (CVI). We also analyzed the expression of CD11b in patients with CVI to determine whether leukocyte activation, known to occur in VSU, is a precursor of or a response to ulceration.

Methods: Patients with varying classes of CVI (n = 24) and healthy control subjects (n = 15), whose status was documented by means of duplex scanning, stood upright and stationary for 10 minutes. Two aliquots of blood, drawn from a distal leg vein and an antecubital fossa vein, were incubated with either buffer or one of three platelet agonists. After fixation, these samples were further incubated with fluorescent-labeled monoclonal antibodies (f-MoAb) specific for CD14 (monocytes) and CD61 (platelets). The activated leukocyte assay was performed by incubating another aliquot of the blood samples with f-MoAb specific for CD11b and CD14. All samples were evaluated by means of flow cytometry.

Results: We observed significantly more platelet-monocyte aggregates throughout the circulation in patients with CVI than in control subjects (29% vs. 8%; $P < .0002$). Furthermore, patients with CVI formed significantly more of these aggregates in response to all platelet agonists than did control subjects. There were no significant differences between baseline numbers of aggregates or response to agonists in patients who had CVI with (n = 10) or without (n = 14) ulceration. Patients with CVI had more circulating platelet-neutrophil aggregates than control subjects (7.2% vs. 3.6%; $P = .05$). The addition of platelet agonists to the blood of patients with CVI resulted in more platelet-neutrophil aggregates than in control subjects. Monocyte CD11b expression was higher in patients with CVI than in control subjects (7.5 vs. 3.7; $P < .01$), with no differences noted in CD11b expression between patients with or without ulceration. Neutrophil CD11b expression was low and similar in control subjects and patients with CVI.

Conclusion: All classes of CVI are associated with significantly increased percentages of platelet-monocyte aggregates and increased percentages of platelet-neutrophil aggregates throughout the circulation. The presence of more of these aggregates and the increased propensity to form aggregates in the presence of platelet agonists in all classes of CVI suggests an underlying state of platelet activation and increased reactivity that is independent of the presence of ulceration. The increased expression of monocyte CD11b throughout the circulation in all classes of CVI suggests that although systemic monocyte activation occurs in CVI, its presence is independent of VSU as well. (J Vasc Surg 1999;30:844-53.)

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It is generally accepted that the fundamental derangement in patients with chronic venous insufficiency (CVI) is sustained venous hypertension, which results from valvular incompetence, outflow obstruction, calf muscle dysfunction, or a combination of these.[1] It is not yet known why a small subset of patients with CVI progresses to the development of stasis ulceration. At present, the most accepted hypothesis regarding the development of venous stasis ulceration (VSU) is the white cell trapping theory,[2] which postulates that activated leukocytes sequestered within the microcirculation of affected extremities release proteolytic enzymes with resultant endothelial damage, fibrin cuff deposition, and localized tissue ischemia and necrosis.

The role of activated leukocytes in the development of VSU has been studied by many authors, most of whom have concluded that monocytes are the leukocyte involved in the development the most severe clinical stage of CVI.[3-5] Despite these observations, the underlying stimulus for leukocyte activation has yet to be elucidated. In fact, some authors have questioned whether the ulcer itself might be responsible for the activation of leukocytes that has been observed in patients with VSU.[3]

Platelets, when activated, form aggregates with other platelets (homotypic aggregates), as well as with monocytes and neutrophils (heterotypic aggregates), but not lymphocytes.[6] Our laboratory has shown that patients with VSU have increased platelet-monocyte aggregation in systemic venous blood.[7] The primary objective of this study was to determine whether this increase in circulating platelet-monocyte aggregates is a result of CVI or if it is an inflammatory response to dermal ulceration. We also studied platelet-neutrophil aggregates in CVI to better understand the role, if any, these heterotypic aggregates might play in this disease.

The final part of our study was an evaluation of the degree and distribution of monocyte and neutrophil activation in patients with various degrees of CVI, as reflected by their surface expression of the endothelial adhesion molecule CD11b. We attempted to determine whether, like heterotypic aggregates, the presence of more of these cells in VSU is a response to or a precursor of active ulceration.

MATERIALS AND METHODS

The study population consisted of 24 patients with CVI and 15 healthy individuals with normal findings on lower-extremity duplex examinations who served as control subjects. None of the patients enrolled in this study participated in our earlier work.[7] Within the group of patients with venous insufficiency, nine patients had active ulceration, and one patient had a recently healed ulcer. The remaining 14 patients had varying clinical degrees of venous insufficiency. In addition to their physical findings, 21 of the 24 patients had objective confirmation of venous insufficiency by means of duplex sonography scanning (Hewlett-Packard Sonos 5500). Patients were excluded when they had evidence of active infection or any associated inflammatory medical condition (eg, connective tissue disease, known malignancy). All study participants had palpable pedal pulses and no evidence of arterial insufficiency. The demographics of the study population are listed in Table I.

Informed consent was obtained from all study participants. The study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical Center.

All study participants were asked to stand upright and stationary for 10 minutes to induce lower-extremity venous hypertension. Blood was drawn into citrated tubes from the greater saphenous vein at the ankle (or a distal varix when active ulceration precluded such a venipuncture or the vein had been surgically removed) and an antecubital fossa vein. Immediately thereafter, 20 μL of this fresh, whole blood was incubated (22°C, 10 minutes) with 20 μL modified HEPES-Tyrode's (HT) buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4, 0.35% bovine serum albumin, 10 mmol/L HEPES, 5.5 mmol/L glucose; pH = 7.4), 15 μL of the peptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP) to inhibit fibrin polymerization and sample clotting[8] and 10 μL of either modified HT buffer (control) or a platelet agonist (epinephrine 10 μmol/L, ADP 0.5 μmol/L, or thrombin receptor activating peptide [TRAP] 5 μmol/L). Thrombin (1 U/mL) was added to a final sample as a positive control. Fixation with 1% formaldehyde in Hanks balanced saline solution (22°C, 10 minutes) was then performed. Samples were diluted 10-fold with distilled H_2O and incubated an additional 10 minutes to allow lysis of the erythrocytes. Another 0.5 mL HT buffer was added to the samples, followed by centrifugal washing for 5 minutes at 500 g. All the supernatant except 20 μL was discarded, and the remaining pellets were incubated (22°C, 20 minutes) with 5 μL each of an anti-CD14 monoclonal antibody conjugated with phycoerythrin (PE; Becton-Dickinson, Rutherford, NJ) and either an anti-CD61 monoclonal antibody (DAKO, Carpen-

Table I. Study population demographics

Characteristic	*All patients*	*Class 1 to 4*	*Class 5 to 6*	*Control subjects*
Age (mean ± SEM)	48.4 ± 14.4	43.9 ± 11.2	53.7 ± 16.5	34.2 ± 7.4
Men	12	5	7	9
Women	12	9	3	6
Hypertension	5	2	3	0
Diabetes mellitus	1	0	1	0
Cardiac disease	0	0	0	0
Tobacco use	4	2	2	2
Current use of nonsteroidal anti-inflammatory drugs	7	2	5	3

teria, Calif) or an isotype-matched mouse IgG control antibody, both of which were conjugated with fluorescein isothiocyanate (FITC; DAKO, Carpinteria, Calif). The samples were resuspended in 0.4 mL HT buffer and analyzed in an EPICS XL flow cytometer (Coulter, Miami, Fla). Monocytes were identified by means of CD14-positivity and their characteristic orthogonal light-scattering properties. The percentage of platelet-leukocyte aggregation was determined by means of surface binding of the platelet-specific CD61 antibody, within each subpopulation of leukocytes, above that of the mouse IgG control antibody.

For leukocyte CD11b analysis, 20 μL of fresh, whole blood was combined with 20 μL modified HT buffer and 5 μL each of anti-CD14 monoclonal antibody conjugated with PE and anti-CD11b monoclonal antibody conjugated with FITC. After 10 minutes of incubation at room temperature, the samples were fixed at 22°C for 10 minutes with 1% formaldehyde and 1.4X Hanks balanced saline solution (GIBCO, Grand Island, NY). Samples were diluted 10-fold with distilled H_2O and incubated an additional 10 minutes to allow lysis of the erythrocytes. Next, 0.5 mL HT buffer was added and the samples were washed by centrifugation for five minutes at 500 g. All the supernatant except 20 μL was discarded, and the remaining pellet was resuspended with 0.5 mL HT buffer. Samples were immediately analyzed in an EPICS XL flow cytometer.

The flow cytometer was equipped with a 500-mW argon laser (Cyonics, San Jose, Calif), operated at 15 mW and a wavelength of 488 nm. The fluorescence of FITC and PE were detected by using 525 nm and 575 nm band pass filters, respectively. Monocytes were identified based on their characteristic side light scatter and CD14-PE positivity. Neutrophils were identified by their relative low CD14 binding and their characteristic side light scatter. CD11b binding is expressed as linear fluorescent units.

The 2-tailed Student *t* test was used to compare results within and between groups, with statistical significance accepted for *P* values less than .05. Analysis of variance (ANOVA) was subsequently performed, with Bonferroni's *t* test, to test the null hypothesis among all three groups simultaneously. Again, significance was accepted for *P* values less than .05.

RESULTS

Patients with venous insufficiency, as a group, were older than the healthy control subjects (48.4 ± 14.4 years vs. 34.2 ± 7.4 years; $P < .01$). The older group had a higher incidence of arterial hypertension, but no other associated medical illnesses (Table I).

According to the classification of venous insufficiency, four patients were clinical class 1, six patients were class 2, two patients were class 3, two patients were class 4, one patient was class 5, and nine patients were class 6. All patients with venous insufficiency studied by means of duplex scanning had reflux rather than obstruction as their pathophysiologic dysfunction, with 19 of 21 patients having superficial reflux, 13 of 21 patients having deep system reflux, and 11 of 21 patients having both superficial and deep system reflux. Perforating veins were not consistently evaluated. The duplex records of three of the patients with CVI were not available for review.

Patients with CVI had significantly more platelet-monocyte aggregates in both the leg and arm samples than did the healthy control subjects ([mean ± SEM]: 28.8% ± 3.4% vs. 7.7% ± 0.8%, $P < .0001$). There were no significant differences between leg and arm values within either group. Furthermore, the addition of epinephrine, ADP, or TRAP resulted in the formation of significantly greater numbers of aggregates in patients with CVI than in control subjects; again, no differences were noted between arm and leg samples (Fig 1).

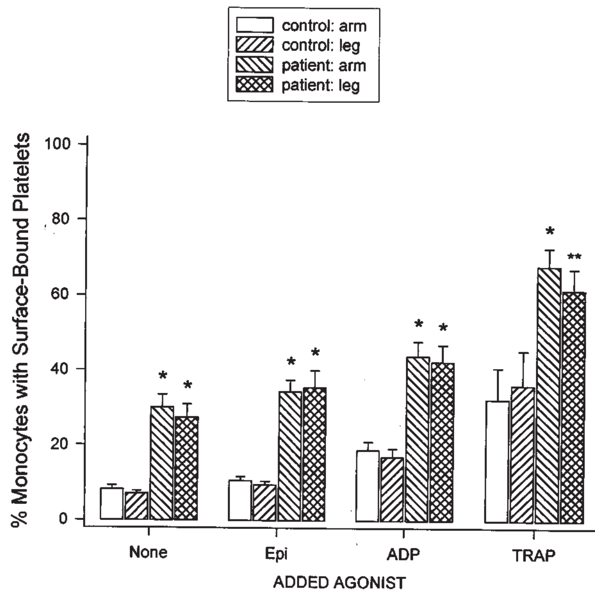

Fig 1. Percentage of circulating monocytes with surface-bound platelets (platelet-monocyte aggregates) at baseline (none) and with the addition of different platelet agonists. *Control,* healthy volunteer (n = 15); *Patient,* volunteer with any degree of chronic venous insufficiency (n = 24). $*P < .001$, patient vs. control subject. $**P < .02$, patient vs. control.

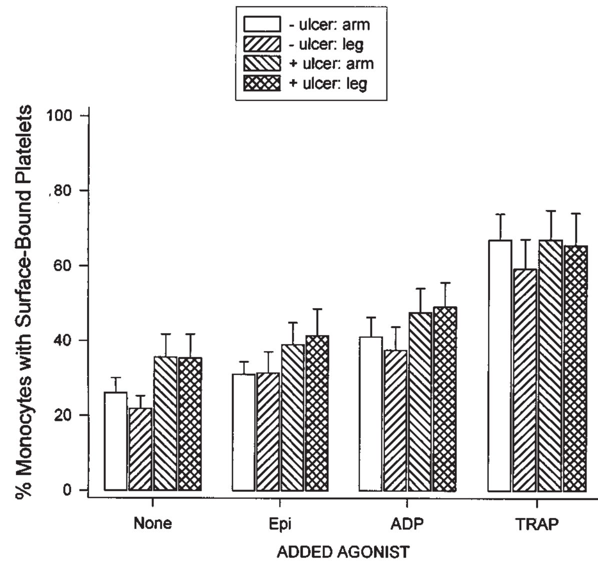

Fig 2. Percentage of circulating monocytes with surface-bound platelets (platelet-monocyte aggregates) at baseline (none) and with the addition of different platelet agonists. *– ulcer,* clinical classes 1 to 4 (n = 14); *+ ulcer,* clinical classes 5 to 6 (n = 10).

When the patients with CVI were analyzed in groups either with (n = 10) or without (n = 14) ulceration, no differences were noted in the percentages of circulating aggregates at baseline or in the presence of added agonist. Furthermore, there were no significant differences in the percentages of heterotypic aggregates in either the arm or the leg specimens (Fig 2).

These observations were confirmed by means of Bonferroni's ANOVA. Specifically, both CVI groups had significantly more platelet-monocyte aggregates at baseline and on the addition of agonist than did control subjects. With the exception of the arm-blood specimens in patients who had CVI with ulceration at baseline, who had significantly more platelet-monocyte aggregates than the patients who had CVI without ulceration, there were no significant differences in numbers of platelet-monocyte aggregates in patients with or without ulceration in the presence or absence of platelet agonists.

Although patients with venous insufficiency had larger numbers of circulating platelet-neutrophil aggregates than did healthy control subjects, these values did not reach statistical significance (7.2 ± 1.4 vs. 3.6 ± 0.5; $P = .05$). Both epinephrine and TRAP lead to the formation of more (but not significantly more) platelet-neutrophil aggregates in patients with CVI than in control subjects, whereas ADP led to significantly more platelet-neutrophil aggregates in patients with CVI. As with platelet-monocyte aggregates, there were no significant differences between heterotypic-aggregate levels in the arm or leg samples of patients with CVI or control subjects (Fig 3). As with the platelet-monocyte aggregates, these observations were borne out when all groups were observed simultaneously by using Bonferroni's ANOVA.

Leukocyte CD11b values, recorded in arbitrary units, fell along a normal distribution curve when a logarithmic scale was used. Monocyte CD11b expression was significantly higher in both the leg and arm blood of all patients with venous insufficiency than in that of control subjects. Monocyte CD11b values were not different between arm and leg samples in either control subjects or patients with venous insufficiency. Furthermore, no differences were noted in monocyte CD11b values in the leg or arm samples of patients in the presence or absence of stasis ulceration (Figs 4 and 5). By means of ANOVA, however, the surface expression of monocyte CD11b was revealed to be statistically greater in patients who had CVI with ulceration than in control subjects, whereas the expression of CD11b on the monocytes of patients with CVI but with no ulceration was greater than in control subjects, but did not achieve statistical significance.

No significant differences in neutrophil CD11b

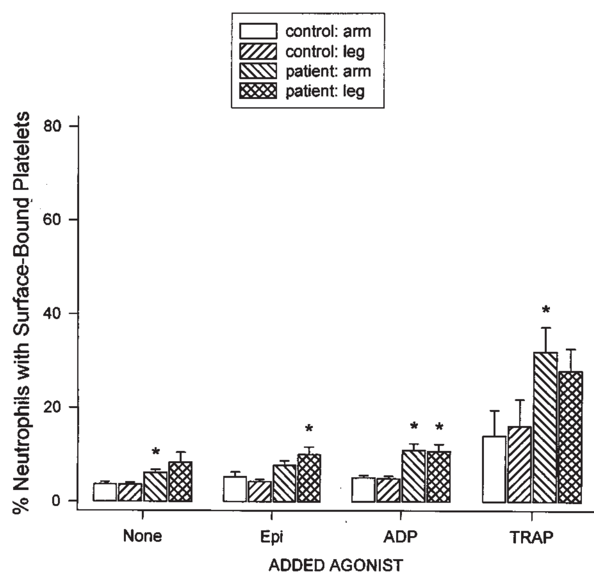

Fig 3. Percentage of circulating neutrophils with surface-bound platelets (platelet-neutrophil aggregates) at baseline (none) and with the addition of different platelet agonists. *Control,* healthy volunteer (n = 15); *Patient,* volunteer with any degree of chronic venous insufficiency (n = 24). $^*P < .04$, patient vs. control.

levels were noted in either the leg or arm blood between patients who had CVI with or without ulceration and control subjects, nor were there any significant differences in CD11b values within the venous insufficiency groups with or without ulceration (Figs 4 and 5). No statistically significant differences were noted when all three groups were evaluated simultaneously by means of ANOVA.

DISCUSSION

Platelet-leukocyte aggregates have been described in a variety of clinical situations, including stable coronary artery disease,[9] unstable angina,[10] and after the passage of blood through extracorporeal circuits, including cardiopulmonary bypass grafting[11] and hemodialysis.[12] It was Peyton et al who first showed that these platelet-monocyte aggregates are present in increased amounts in patients with VSU.[7] We have expanded this observation to show that all clinical classes of venous insufficiency are associated with similar, significantly increased levels of circulating platelet-monocyte aggregates. Platelet-neutrophil aggregates are also increased throughout the circulation in patients with CVI in amounts that approach, but do not achieve, statistical significance. Although the small number of patients in each clinical class of venous insufficiency increases the possibility of a type-2 error, we conclude that platelets are activated to a similar degree in all clinical classes of CVI and, as shown elsewhere,[13] these activated platelets have an increased affinity for monocytes (relative to neutrophils). Restated in clinical terms, the severity of CVI is not predictive of the level of circulating heterotypic aggregates. Furthermore, the presence of venous ulceration does not cause platelet activation and heterotypic-aggregate formation. Finally, these heterotypic aggregates are not sequestered in the affected limbs of patients with CVI.

The present study has also provided evidence of enhanced platelet reactivity in patients with CVI. Three platelet agonists, epinephrine, ADP, and TRAP, each of which stimulates platelets by different degrees and via different receptors,[9] led to the formation of progressively more platelet-monocyte aggregates in patients with venous insufficiency than in control subjects. This enhanced responsiveness was observed to a similar degree in patients who had CVI with and without ulceration, eliminating the ulcer as the source of this excess reactivity. As a part of our experiment (results not shown), we stimulated every sample with thrombin, the most potent platelet agonist,[8] as a positive control. We observed a similar, almost complete (more than 95%) platelet-monocyte–aggregate formation in all patients with CVI as well as in controls, indicating that the platelets in control subjects were capable of complete activation. This supports our theory that enhanced platelet responsiveness to submaximal physiologic stimulation in CVI, as reflected by heterotypic-aggregate formation, represents a state of platelet hyperreactivity. The cellular mechanisms responsible for priming these platelets remain unknown.

The role of heterotypic aggregates in the pathogenesis of CVI remains under investigation. It is plausible that circulating heterotypic aggregates are complexes that are capable of injuring venous endothelium and valves, leading to the development of venous valvular dysfunction. Leukocytes, when activated, produce reactive oxygen species, chemoattractant (eg, IL-8), hydrolases, arachidonic acid metabolites, and platelet-activating factor.[14] It is known that activated platelets, through P-selectin, induce monocyte tissue factor (a co-factor for factor VII),[15] chemotactic protein-1, and the cytokines interleukin-1β and tumor necrosis factor-α.[14,16,17] Interleukin-1 and tumor necrosis factor-α both act on endothelial cells to increase leukocyte adhesion.[18,19]

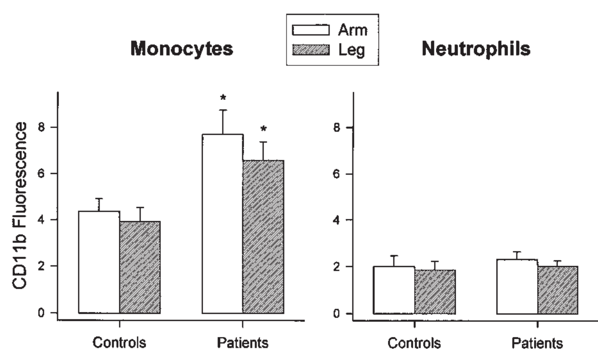

Fig 4. Relative CD11b fluorescence of monocytes and neutrophils in control subjects (n = 15) and patients (n = 24). **P* < .01, patient vs. control.

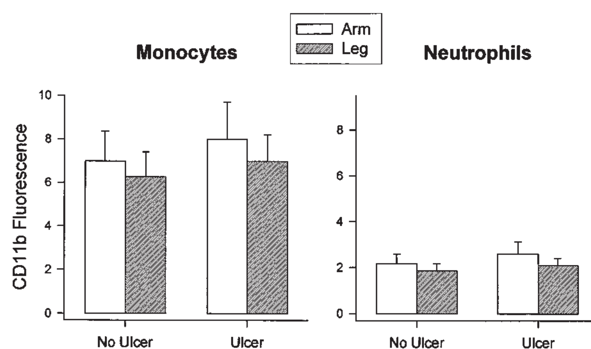

Fig 5. Relative CD11b fluorescence of monocytes and neutrophils in patients who have chronic venous insufficiency with (n = 10) and without ulceration (n = 14).

CD11b is the alpha subunit of the $beta_2$ integrin surface heterodimer Mac-1 that is expressed on circulating leukocytes. Mac-1 is a member of the larger beta integrin family of leukocyte adhesion receptors, which have a common beta subunit (CD18) and a distinct alpha subunit (CD11a, CD11b, and CD11c).[20] These receptors promote adhesion of monocytes and neutrophils to endothelial cells, extracellular matrix, and components of the complement cascade.[14] Binding of CD18/CD11b to its complementary endothelial receptors intercellular adhesion molecule (ICAM-1) and endothelial leukocyte adhesion molecule (ELAM-1)[18,21] permits subsequent diapedesis through endothelial cells.[22,23] CD11b levels are expressed constitutively at relatively low levels on circulating quiescent leukocytes.[24] With leukocyte activation, surface levels of CD11b increase markedly.[14,25]

The beta subunit of the Mac-1 receptor has other functions in addition to intracellular adhesion. In fact, neutrophil CD11b is believed to be a predominantly proinflammatory receptor, active in the oxidative burst necessary for cell killing. Monocyte CD11b, however, is believed to have a procoagulant, rather than inflammatory, role.[20]

Patients with lipodermatosclerosis and VSU have significantly more monocytes in the skin of their affected extremities than patients with less clinically severe venous insufficiency.[26] The endothelial cells of patients with clinically advanced venous disease have increased expression of monocyte, but not neutrophil, adhesion molecules,[26,27] further implicating monocytes as the leukocyte responsible for the pathogenesis of clinically advanced CVI. Finally, the role of monocytes in the genesis of primary venous dysfunction by valve destruction[28] has been clearly identified. Our results further support the involvement of activated monocytes in venous insufficiency.

We observed that patients with CVI had increased levels of circulating activated monocytes, as reflected by increased surface expression of CD11b. The differences were significant between control subjects and patients with ulceration and were not significant within CVI groups (with or without ulceration) or between control subjects and patients who had CVI without ulceration. Our data suggest that monocyte activation cannot be explained by active stasis ulceration or experimentally induced venous hypertension. Although the presence of activated monocytes in the circulation of patients with CVI precedes the development of stasis ulceration, their presence is not predictive of disease stage or potential for disease progression.

As with the heterotypic aggregates, not all of the activated monocytes in patients with CVI were sequestered in the lower extremities. In fact, no differences were noted in monocyte CD11b surface expression in arm or leg samples. The role of these circulating, activated monocytes in the pathogenesis of CVI warrants further investigation.

Neutrophil CD11b levels were no different between control subjects and patients with venous insufficiency. Although it is possible that all activated neutrophils were bound in the microcirculation of limbs with CVI, the obvious discrepancy between circulating activated monocytes and leukocytes makes this an unlikely explanation. The absence of neutrophil activation in CVI has been noted by other authors,[3] and, despite their previously mentioned proinflammatory role, it is much more likely that activated neutrophils do not assume a significant role of the pathogenesis of CVI.

Whether venous dysfunction occurs as a result of

obstruction (secondary) or unknown cause (primary), ambulatory venous hypertension results. All the patients in our study had experimentally induced venous hypertension, yet this was not an adequate stimulus to induce platelet or monocyte activation in patients without reflux. Clearly, reflux plays a role in the development of ambulatory venous hypertension. It may also play a role in platelet and monocyte activation.

We did not study patients with acute venous obstruction that was a cause of venous insufficiency, although two patients did give a remote history of deep vein thrombosis. The patterns of venous reflux we observed are consistent with the observations of others: its presence correlates with venous disease; it may exist without obvious venous disease; and, in general, superficial system reflux is more prevalent than deep system reflux.[29] Furthermore, superficial reflux contributes to the development of venous ulceration at least as often as deep system reflux.[30] Whether circulating heterotypic aggregates, activated monocytes, or both contribute to the development of reflux, or vice versa, with subsequent variable progression of venous disease, remains to be shown.

In summary, we have demonstrated that CVI is associated with increased platelet and monocyte activation and aggregation throughout the circulation. Increased platelet-neutrophil aggregation also occurs, although we found no evidence of increased neutrophil activation (as reflected by surface CD11b expression). The stimulus for platelet and monocyte activation and the explanation for platelet hyperreactivity are, as yet, unknown. What also remains to be shown is the degree to which these heterotypic aggregates communicate with each other and with the endothelium.

Because platelets bind monocytes and neutrophils by the same receptors,[31,32] it is difficult to attribute the differential activation of monocytes and neutrophils, as reflected by surface CD11b expression, in CVI solely to the binding of activated platelets, unless different signals are sent by activated platelets to monocytes and neutrophils or the response of these cells to activated platelets is different. Such a study is beyond the scope of this work.

We did observe a significant age difference between control subjects and patients with CVI. Age has been shown to be an independent risk factor for the development of VSU.[33] It is, therefore, not surprising that the patients in our CVI group were older. What remains to be answered is whether patient age affects platelet and monocyte activation.

Although the presence of platelet-leukocyte aggregates throughout the circulation may simply be as a nonspecific marker of venous valvular reflux, the possibility that platelet-leukocyte aggregates represent a fundamental derangement in CVI is an intriguing one. Still, if circulating platelet-leukocyte aggregates do play a role in the development and progression of CVI, it remains to be shown why similar levels of circulating aggregates exist in patients with very different degrees of clinical disease severity and why these aggregates are present in similar percentages throughout the circulation, whereas the clinical manifestations of the disease are most prevalent in the legs. Identification of the stimulus (or stimuli) for platelet activation and the cause for increased platelet responsiveness to agonists and identification of any proinflammatory or procoagulant signals unique to platelet-leukocyte aggregates in CVI may further our understanding of the development and progression of venous insufficiency and potentially open new avenues in the prevention and treatment of this prevalent and frequently disabling disorder.

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DISCUSSION

Dr Peter J. Pappas (Worcester, Mass). This presentation by Dr Powell represents the second investigation on the relationship between platelet monocyte aggregation and chronic venous insufficiency by the University of Massachusetts group. In their first investigation, they reported an increase in platelet-monocyte aggregates in patients with venous ulceration, compared with healthy control subjects. This presentation tries to determine if this observation is caused by disease factors associated with chronic venous insufficiency or the inflammatory reaction caused by the presence of the venous ulcer. After reviewing the manuscript and listening to this presentation, I am not sure whether they answered the question.

The current study separated patients into three groups. Group one consisted of healthy patients (n = 15), as defined by means of the absence of reflux on a duplex examination. Group two consisted of patients who had chronic venous insufficiency with a CEAP class of 1 through 4 (n = 14), and group three consisted of patients with active ulcers (n = 9 and n = 1, respectively). The authors state that all the patients with chronic venous insufficiency had increased platelet-monocyte aggregates at rest and with agonist stimulation, compared with control subjects. They also noted an increase in aggregates in blood samples taken from the antecubital arm veins and increased CD11b expression on circulating monocytes. However, no differences among the CVI groups were identified.

I have several serious concerns with these conclusions, based on how the statistical analyses were performed, and with the scientific methods. Based on these concerns, I have several questions for the authors.

The authors used flow cytometry as a means of determining the percentage of cells that demonstrated platelet-monocyte binding and linear intensity units as means of quantifying the number of CD11b receptors on mono-

cytes and neutrophils. Anyone who has ever used flow cytometry knows that the Achilles' heel of this technique is measurement variability and reproducibility. For example, linear intensity varies based on the amount of time the antibody has been bound before measurement and differences in fluorescence between antibody lots. To normalize for this expected variability, two methods are normally used. You can calibrate the flow cytometer against a bead with a known intensity and adjust the cytometer according to the standard. The second method is developing a logarithmic regression curve with several beads of known intensity and then measuring your samples on the developed standard curve. No mention of this normalization or standardization technique was presented in the methods section of the manuscript. These normalization techniques are mandatory to minimize the day-to-day variability inherent with these types of measurements. Similarly, did you measure your total leukocyte count in each patient and normalize your aggregation measurements to the total number of platelets, monocytes, and neutrophils per patient? I believe the answers to these questions are critical. If no standardization process was used, then the accuracy of your measurements is in doubt.

My second question relates to how you performed your statistical analyses. In the current study, the authors had three study groups, as previously mentioned. The patients with class 1 through 4 disease were crucial to answering the central question: Is increased platelet monocyte aggregation caused by chronic venous insufficiency or inflammatory processes associated with venous ulceration? To answer this question, the patients with chronic venous insufficiency and ulcers need to be compared with healthy control subjects. This is typically done by performing an analysis of variance and a nonparametric post-hoc test, such as the Student *t* test, when the analysis of variance is positive.

Instead, the authors combined all the patients with chronic venous insufficiency, compared them with control subjects, and proceeded to use a two-tail *t* test. They clearly had enough patients in each group to do a subgroup analysis, so my second question for the authors is have you done this subgroup analysis? Are there significant differences between classes 1 through 4 patients and control subjects?

My third question relates to the three patients with clinical chronic venous insufficiency and normal findings on duplex examinations. Did these patients really have chronic venous insufficiency? Since the development of our venous clinic at the New Jersey Medical School, I have examined several patients who had normal findings on duplex examinations and clinical evidence of chronic venous insufficiency. Most of these patients have calf muscle pump dysfunction, as demonstrated by means of air plethysmography. The remainder have dermatologic diseases. So, what class disease did these three patients have? If you eliminate them from your analysis, do your results change?

Finally, my last question relates to the significance of platelet monocyte aggregates. As was pointed out in the manuscript, platelet-monocyte aggregates form in patients undergoing coronary artery bypass grafting with the pump oxygenator. This leads to bleeding problems in these patients, and as a result, many centers now sequester the patients' platelets before they go on bypass and retransfuse them at the completion of the case, which results in fewer bleeding problems. So, what is the significance of platelet-monocyte aggregation in patients with chronic venous insufficiency? Is it involved in the pathogenesis of chronic venous insufficiency, or are your observations just phenomenology?

I enjoyed reading the manuscript. I find the concept of platelet monocyte interactions interesting and encourage the authors to continue their investigations. Thank you.

Dr Craig C. Powell. Thank you, Dr Pappas. A critical and objective interpretation of presented work is necessary to either validate that work or expose it as potentially flawed, and I hope to be able to do the former by answering your questions.

The Center for Platelet Function Studies at the University of Massachusetts Medical Center, which is headed by Drs Michelson, Rohrer, and Furman, has a reputation that has been established by meticulous attention to detail and reproducibility of results. Our XL flow cytometer is calibrated daily for alignment by using DNA check beads, the former of the two methods of validation that you asked about. We also undertake daily cleansing and monthly maintenance of the machine, and any drift that is identified in bead fluorescence necessitates any setting adjustments. Because of the meticulous attention to detail on the machine, setting adjustments are rarely needed, so daily validation with flow beads was performed.

Using our flow cytometer, we measured the percentage of circulating subgroups of each of the leukocyte populations: monocytes, neutrophils, and lymphocytes. Although we did not record actual numbers, we found that the percentages within each subgroup were about the same: 55% to 60% neutrophils, 5% to 8% monocytes, and the rest lymphocytes. Our results were reported as a percentage of these circulating cells with bound platelets, so the absolute values of bound aggregates were not as important as the percentage of circulating cells.

We did use a two-tail *t* test, and we evaluated two groups at a time, because we were trying to prove whether the increased significant numbers of circulating heterotypic aggregates in venous insufficiency were a response to ulceration or if it was present in all groups. In the manuscript, I just presented classes 1 to 4 versus 5 and 6 and found no significant difference. Your question is still a good one: What about the controls versus classes 1 to 4? Although it is not presented in the manuscript, I do have that information, and there is a significant difference, with a *P* value of less than 10^4 for circulating heterotypic aggregates in the lesser clinical classes versus healthy control patients.

You asked about three patients who had normal find-

ings with duplex examinations. Actually, three of the 24 patients with venous insufficiency did not have duplex results that I could find in our vascular laboratory, so I was unable to comment on the degree of reflux that they had. None of the patients with venous insufficiency had normal findings on their duplex scans, however.

Finally, with regard to the significance of this process, that is obviously the next question. We set out only to discover whether this increased platelet activation, which is what the significance of heterotypic aggregates really is, is present in all stages of venous insufficiency. The next question obviously becomes, do they play a role or are they a nonspecific marker? We know that P-selectin, PSGL1, and CD11b are all integrins. They are cell surface adhesion molecules that are not only involved in cell binding, but also in cell-cell signaling, and it is quite probable, although it will require much more work on the cellular level, that different messages are being sent by these circulating aggregates that may play a very important role in the development and progression of the disease.

Dr Kevin Burnand (London, England). Have you looked at the instance of earlier thrombosis in your patients with chronic venous insufficiency and have you also looked at the thrombophilia status of all your patients with chronic venous insufficiency? Because it is attractive to think that this might be egg, rather than chicken. The final group that would be very interesting to look at would be patients who have had a venous thrombosis and to differentiate those patients who develop skin changes from those who do not in a period that might give you an answer as to whether this is important etiologically.

Dr Powell. Thank you. We did look at the background of these patients with regard to the etiology of their venous insufficiency. Of our 24 patients, only two had a known history of deep vein thrombosis, the rest had reflux, which they knew about. And again, a lot of patients probably have venous obstruction and they don't know about it. CD11b, an integrin, has been shown to be involved in cell-cell signaling. As mentioned in the manuscript, monocyte CD11b (but not neutrophil CD 11b) has been associated with a prothrombotic state. The relevance of increased circulating CD11b may be the presence of an as-yet incompletely identified prothrombotic state. Patients may have microthrombosis and not know about it. These patients are showing up in our offices years later with venous insufficiency of various degrees.