Von Willebrand Factor, Red Cell Fragmentation, and Disease Activity in Systemic Lupus Erythematosus

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Abstract This study sought to determine whether the plasma levels of Von Willebrand factor (vWf) and the degree of red blood cell (RBC) fragmentation on peripheral smear correlate with disease activity in systemic lupus erythematosus (SLE). Forty consecutive patients who fulfilled the criteria for SLE were studied prospectively for 1 year. Patients were categorized according to the SLE Disease Activity Index (SLEDAI) as either active (>2) or inactive disease and followed up monthly (active) or quarterly (inactive). At each visit, patients were examined fully and had complete blood count, tests on antibodies to double-stranded DNA, C3, and C4 levels, and urinalysis. Citrated plasma was analyzed for vWf antigen by standard enzyme-linked immunosorbent assay. A Wright's stained blood smear was obtained and schistocytes were quantitated on blood smear. The number of schistocytes per 500 RBCs was determined and a schistocyte index (SI) was calculated. At baseline, vWf correlated with SLEDAI ($r=0.64$, $p<0.01$), SI correlated with SLEDAI ($r=0.62$, $p<0.01$), and vWf and SI correlated with each other ($r=0.41$, $p=0.01$). There was an inverse correlation between baseline C3 levels and vWf ($r=0.49$, $p=0.0013$) and C3 levels and SI ($r=0.40$, $p=0.01$). Over time, there was also a correlation of SLEDAI with vWf ($r=0.53$, $p=0.002$) and SI ($r=0.57$; $p=0.002$). The

relation of vWf with SI approached but did not reach statistical significance ($r=0.37$, $p=0.06$). We found that the plasma levels of vWf and the degree of RBC fragmentation correlate with lupus disease activity over time. Therefore, inflammation in SLE may be associated with endothelial injury.

Introduction

There is reason to believe that endothelial cell dysfunction is important in the pathogenesis of systemic lupus erythematosus (SLE). For example, microvasculopathy characterizes SLE organ pathology [1–3] and SLE has been associated with thrombotic thrombocytopenic purpura (TTP) [4–6]. More recently, it has been shown that patients with SLE are predisposed to accelerated atherosclerosis over and above the usual proatherogenic risk factors [7–9]. To this end, abnormal endothelial function can be measured in patients with SLE [10, 11].

Von Willebrand factor (vWf) is a circulating glycoprotein that is synthesized in endothelial cells and released into the plasma in increased amounts by activated or damaged endothelial cells [12–14]. Increased serum concentration of vWf has been shown to be a marker of endothelial dysfunction in human disease [15–17]. Specifically, elevated levels of vWf have been found in scleroderma, another autoimmune illness with significant vascular pathology [4, 18]. Another example involves patients with pulmonary hypertension where high levels of vWf or the presence of vWf multimers correlates with reduced survival [19, 22–24].

In the area of lupus research, new indicators of disease activity are needed in order to manage such patients because in some situations, such as lupus cerebritis, traditional measures of disease activity may be normal. In the work presented in this paper, we ask whether or not two novel

Level of Evidence: Level II: Development of diagnostic criteria on the basis of consecutive patients with reference to a gold standard.

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markers of endothelial dysfunction can serve as surrogate measures of disease activity. To this end, we conducted a prospective study to determine whether plasma levels of vWf along with assessment of the presence of schistocytes correlates with established clinical and laboratory markers of disease activity in SLE. Because of the known association of SLE with TTP [4–6], we were particularly interested in whether red blood cell (RBC) fragmentation, which also reflects microvascular damage, is found in active SLE.

Materials and methods

Forty consecutive patients who fulfilled the American College of Rheumatology criteria for SLE [20] and who consented to participate over a 12-month period were recruited from the Lupus Clinic at the George Washington University Medical Center. This represents approximately 30% of the SLE patients that were being followed up at that time. Patients were defined as having either inactive or active disease according to the SLE Disease Activity Index (SLEDAI) [21]. Patients with a SLEDAI >2 were defined as having active disease and were followed up monthly until inactive and then every 3 months for 12 months (an adjusted mean SLEDAI >2 is an important predictor of major outcome in SLE) [25]. Patients with inactive disease (SLEDAI ≤ 2) were followed up every 3 months for 12 months.

At each visit, patients were examined fully. Blood was tested for complete blood count and antibodies to double-stranded DNA, C3, and C4 levels, and urinalysis was performed for blood and protein in order to calculate the SLEDAI. Patients who had significant proteinuria on urinalysis underwent a 24-h urine collection for protein quantitation. A Wright's stained blood smear and a citrated plasma sample were obtained at each visit for further study. Plasma samples were coded, aliquotted, and frozen at -70°C until tested. A SLEDAI score was calculated based upon clinical disease activity and the results of the laboratory testing. The same investigator did all SLEDAI determinations.

Coded plasma was thawed and analyzed for vWf antigen by standard enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (Helena Laboratories). In essence, 100 μl of a 1:26 dilution of each patient sample was added in duplicate to 96-well microtiter plates coated with antibody specific for human vWf. Both kit controls and normal volunteer plasma were also added to each plate. Incubations and color development reactions were performed according to kit instructions. Results were expressed as percent normal vWf compared to pooled normal plasma. If duplicates varied by more than 20%, the ELISA on that sample was repeated. If the results were still discordant, then vWf was measured by rocket electroimmunoassay (Helena Laboratories) and that result was considered the true value.

Schistocytes were quantitated on blood smears at $\times 100$ magnification in microscopic fields where RBCs were not overlapping. To be counted as a schistocyte, the RBC was

definitely fragmented without overlying any other cell. The number of schistocytes per 500 RBCs was determined and the schistocyte index (SI; number of schistocytes per 100 RBCs) was calculated. The investigators read slides with hematology laboratory personnel to become familiar with the appearance and counting of schistocytes. Two investigators independently counted schistocytes on selected patients, and no significant differences in counts were found.

The baseline results were analyzed to determine if there was a correlation between SLEDAI and vWf and between SLEDAI and schistocytes. Because the data were not normally distributed, vWf and SI are expressed as the median with interquartile range (IQR) and the nonparametric Spearman rank correlation coefficient was used. The value of the correlation coefficient indicated the strength and direction of the association between SLEDAI and vWf and between SLEDAI and schistocytes. Additionally, we looked at the relative risks, 95% confidence intervals (95% CIs), and the chi-square test for the baseline data. We considered the SLEDAI score to be the dependent variable and both vWf and schistocytes to be the independent variables. The variable SLEDAI was grouped into active lupus (score >2) and inactive lupus (score ≤ 2), and the variables vWf and schistocytes were grouped according to their median, which was used as the cutoff point. The median for vWf was 136.2, resulting in two groups for vWf: those with vWf measurements >136.2 or ≤ 136.2 . Likewise, the median for the SI was 0.594, resulting in two groups for schistocytes: those with >0.594 or ≤ 0.594 .

Area under the curve (AUC) was used to determine the total area for vWf, schistocytes, and SLEDAI over the 1-year period for all patients repeatedly measured to take time into consideration. The formula for AUC was based on Tai's mathematical model where $\text{Area} = 1/2 \sum X_{i-1}(Y_{i-1} + Y_i)$, which gives the most accurate estimation of the total area under a curve (TAI). The total area was then used to determine if there was a correlation over time between SLEDAI and vWf and between SLEDAI and schistocytes. Once more, the Spearman rank correlation coefficient was used for this analysis.

Results

The demographics, clinical, and laboratory characteristics of the 40 enrolled patients were representative of our lupus population and those from other centers (Table 1) [26]. The majority were African American, which is characteristic of our lupus clinic population.

The vWf and SI assessments correlated with traditional measures of disease activity. SLEDAI scores varied from 0 to 16 with a mean of 4.78 and median of 2. Eighteen patients (45%) had a SLEDAI >2 (SLEDAI scores=4–16). The plasma vWf concentrations (% normal) varied from 10.89 to 345.18, median 136.2 (IQR=103.6). SI varied from 0 to 5.6, median 0.59. (IQR=0.95). At baseline, vWf correlated with SLEDAI (Spearman $r=0.64$, $p<0.01$), SI correlated with SLEDAI (Spearman $r=0.62$, $p<0.01$), and

Table 1 The baseline clinical and demographic characteristics of the SLE cohort (40 patients)

Age (median years/range)	37/20–53
Gender (% female)	87.5
Race (%)	
African American	72.5
Asian	12.5
Caucasian	15
Disease duration (median years/range)	8.76/1–26
Organ involvement by ACR criteria, baseline (%)	
Neurological	0
Discoid rash	5
Hematological	17.5
Renal	27.5
Oral ulcers	10
Serositis	5
Malar rash	5
Photosensitivity	0
Arthritis	5
No. of ACR criteria met (mean/median)	6/6
Autoantibodies	
ANA	100
Anti-RNP	73
Anti-dsDNA	53
Anti-Sm	40
Anti-SSA/Ro	37
Anti-SSB/La	17
Anticardiolipin (IgG)	28
Anticardiolipin (IgM)	17
Medication at baseline	
Prednisone	80
Hydroxychloroquine	58
Azathioprine	10
Cyclophosphamide	15
Mycophenolate mofetil	15
Methotrexate	7.5

vWf and SI correlated modestly with each other (Spearman $r=0.41$, $p=0.01$) (Table 2). Also, at baseline, active patients had a mean SLEDAI of 9 with a median vWf of 190.7 (IQR=95.8) and a median SI of 1.18 (IQR=2.7). Inactive patients had a mean SLEDAI of 1 with a median vWf of 106.7 (IQR=56.6) and a median SI of 0.36 (IQR=0.6). The baseline relationships of SLEDAI, vWf, and SI are shown in Figs. 1 and 2. There was an inverse correlation between baseline C3 levels and vWf (Spearman $r=-0.49$, $p=0.0013$) and baseline C3 levels and SI (Spearman $r=-0.40$, $p=0.01$).

The AUC calculations for cumulative analysis of follow-up visits also correlated with the SLEDAI. The AUC method was utilized to help determine the predictive strength of associations found on Spearman analysis. Eight patients came for only one visit. Therefore, cumulative results and AUC calculations were done on 32 patients.

Table 2 The Spearman rank correlations between SLEDAI and measures of endothelial dysfunction

	SLEDAI	
	Correlation (r)	Significance (p)
vWF	0.64	<0.01
SI	0.62	<0.01

vWF Von Willebrand factor, SI schistocyte index

Fig. 1. A scatterplot of SLEDAI and vWf plasma values at study entry ($r=0.64$)

The AUC results for SLEDAI, vWf, and SI showed a moderate correlation of SLEDAI with vWf ($r=0.53$, $p=0.002$) and with SI ($r=0.57$, $p=0.002$). The relation of vWf with SI approached, but did not reach, statistical significance ($r=0.37$, $p=0.06$). The data were also analyzed in all patients using an averaging method: by taking the measurements for SLEDAI, vWf, and SI for each patient and dividing by the number of measurements (visits). The results were similar to those using AUC methodology demonstrating moderate correlations of SLEDAI with vWf (Spearman $r=0.60$, $p=0.0001$), with SI (Spearman $r=0.57$, $p=0.001$), and weaker but significant correlation of vWf with SI (Spearman $r=0.48$, $p=0.01$).

The relative risk of active lupus with a vWf >136.2 (median result) was 3.87 (95%CI=1.54, 9.72). The relative risk of active lupus with an SI >0.594 (median result) was 2.60 (95%CI=1.14, 5.92).

Discussion

This is a small prospective study of 40 patients but has yielded quite striking results. We have found a moderate but significant and temporally durable correlation of lupus activity with plasma vWf and SI. A vWf result of >136.2 carried a relative risk of 3.87 for active SLE whereas an SI >0.59 carried a relative risk of 2.60 for active SLE. Furthermore, vWf and SI showed an inverse correlation with C3 levels, another laboratory marker of active SLE. This supports our hypothesis that markers of endothelial injury correlate with active SLE as has been also shown by

Fig. 2. A scatterplot of SLEDAI and SI values at study entry ($r=0.62$)

a recent study demonstrating circulating endothelial cells in patients with active lupus [2]. These results suggest that endothelial dysfunction (ED) is a significant concomitant feature of active SLE. Although schistocyte counts have not been validated as test for ED, these results along with the general correlation of vWf with SI suggest that RBC fragmentation reflects ED in a way similar to vWf. Thus, it is likely that the higher vWf levels found in active SLE are related to endothelial injury, possibly immune-mediated either through specific antibodies or through immune complexes. Although we did not measure antibodies to endothelial cells, which have been reported in SLE [27–29], the correlation of high vWf and SI to low C3 levels is suggestive that immune processes and possibly immune complexes are involved in the endothelial cell injury. A recent study has shown elevated vWf levels in SLE compared to controls, but there was no correlation between vWf and SLEDAI or between vWf and antibodies to endothelial cells. Moreover, the range SLEDAI results are not given and the study was cross-sectional and not prospective in design [27].

There is increasing evidence that SLE is associated with accelerated atherosclerosis with the potential clinical consequences of myocardial infarction and stroke [30–33]. In fact, premature atherosclerotic disease is a major cause of mortality in SLE [30–33]. While lupus patients, especially those with nephritis and those requiring prolonged corticosteroid therapy, have known general risk factors that lead to the development of atherosclerosis such as hypertension, hyperglycemia, and hyperlipidemia, these are not enough to account for the overall risk seen in SLE [9]. It appears that lupus per se, or the medications used to treat it, endow a proatherogenic risk and that this increased risk may be due endothelial injury. In that light, our finding of the correlation of vWf levels to disease activity, not only at one reading at baseline but also with multiple readings over the period of 1 year, supports the concept that lupus activity over time could lead to cumulative damaging effects on the endothelium, which in turn could contribute to the development of atherosclerosis. Flow-mediated vasodilation of the brachial artery has been found to be abnormal in conditions with known atherosclerotic risk such as hypertension [10]. Recent studies have detected abnormalities in some SLE patients as well [11]. It would be of interest to study whether or not these abnormalities of endothelial function correlate with high vWf levels in SLE. We propose, on the basis of our findings, that a large prospective study of atherosclerotic risk in SLE also include measurements of plasma vWf over time.

The major limitation of this study is the small number of patients followed up prospectively for only 1 year. This limited our ability to analyze patient subgroups according to specific disease clinical and laboratory features as well as treatments. Specifically, larger numbers would enable stratification based upon not only disease activity but disease severity (or damage) as well. Another limitation involves that lack of corroborating assessment endothelial dysfunction with measures of both vascular reactivity and of circulating endothelial cells.

While these results are too preliminary to establish that measurement of vWf levels is a useful biomarker for lupus activity, it is suggestive enough that it is worthy of further study. If vWf levels truly correlate with lupus disease activity and if they are true markers of endothelial injury in SLE, then their usefulness as biomarkers may be twofold—as a laboratory correlate of active or inactive SLE and as a prognostic indicator of future atherogenesis. Larger prospective studies could address such questions as: are elevated vWf levels seen in patients with some types of organ involvement and not others; does a therapeutic clinical response correlate with a drop of vWf to normal or near normal levels; does a rise in vWf predate a clinical flare; do vWf levels correlate with flow-mediated brachial artery values; and does treatment result in a normalization of both the vWf levels and the flow-mediated brachial artery dilation values? We propose that vWf levels be considered for future studies of biomarkers in SLE.

In summary, we have found that the plasma levels of vWf and the quantity of RBC fragmentation individually correlate with lupus disease activity as measured by SLEDAI and by C3 levels. This correlation is true with multiple measurements over a period of 1 year. We propose that these results reflect endothelial injury, which is a critical element in lupus pathophysiology. We suggest that plasma vWf be studied as a biomarker for lupus disease activity as well as for predisposition to atherosclerotic disease.

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