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Research Article

Impaired contraction of blood clots as a novel prothrombotic mechanism in systemic lupus erythematosus

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The aim of this work was to examine a possible role of clot contraction/retraction in thrombotic complications of systemic lupus erythematosus (SLE). Using a novel automated method, we investigated kinetics of clot contraction in the blood of 51 SLE patients and 60 healthy donors. The functionality of platelets in the SLE patients was assessed using flow cytometry by expression of P-selectin and fibrinogen-binding capacity. The rate and degree of clot contraction were significantly reduced in SLE patients compared with healthy subjects, especially in the patients with higher blood levels of anti-dsDNA antibodies. The reduced platelet contractility correlated with partial refractoriness of platelets isolated from the blood of SLE patients to stimulation induced by the thrombin receptor activating peptide. To test if the anti-dsDNA autoantibodies cause continuous platelet activation, followed by exhaustion and dysfunction of the cells, we added purified exogenous anti-dsDNA autoantibodies from SLE patients to normal blood before clotting. In support of this hypothesis, the antibodies first enhanced clot contraction and then suppressed it in a time-dependent manner. Importantly, a direct correlation of clot contraction parameters with the disease severity suggests that the reduced compactness of intravascular clots and thrombi could be a pathogenic factor in SLE that may exaggerate the impaired blood flow at the site of thrombosis. In conclusion, autoantibodies in SLE can affect platelet contractility, resulting in reduced ability of clots and thrombi to shrink in volume, which increases vessel obstruction and may aggravate the course and outcomes of thrombotic complications in SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disorder, which involves inflammation of various organs and tissues, such as kidney, lungs, joints, and skin [1-3]. As a part of the complex multifactorial disorder, blood cells other than leukocytes that directly participate in normal and abnormal immune responses are deeply involved in the pathogenesis of SLE, including structural and functional changes of platelets and erythrocytes [2-7]. Hemostatic disorders also play an important role because SLE patients have an increased risk of thrombotic complications [8]. It has been shown that arterial and venous thrombosis contributes significantly to morbidity and mortality rates in SLE [1,9,10]. Blood coagulation abnormalities in SLE are revealed by hemostasis tests, such as elevated levels of protein C, fibrinogen, D-dimer, and an increased level of antithrombin III [11]. SLE is also associated with platelet activation, abnormal membrane structure of erythrocytes, alterations in the structure of fibrin network, and other pathological changes in blood and clot components [7,10,12]. The level of phosphorylated fibrinogen in SLE is significantly higher than in healthy donors and in non-SLE patients with venous thrombosis

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[10,13,14]. Phosphorylated fibrinogen is generated by activated platelets, leading to an increase of its coagulability and increased resistance of fibrin to fibrinolysis [12,15]. Importantly, platelet activation in SLE is probably caused by immune complexes that are abundant in the blood of SLE patients compared with healthy subjects [3,6] as well as by activation of the complement system [17]. Platelets are a well-known target for antiphospholipid antibodies [16] that activate platelets [7] and promote thrombotic complications in SLE patients [10].

One of the clinically important but relatively understudied stages of blood clotting *in vivo* is the volumetric shrinkage and remodeling of blood clots and thrombi known as clot contraction or retraction [18-20]. Clot contraction is driven by platelet non-muscle myosin IIa and actin that begin to interact when platelets are stimulated by activators, such as thrombin [21]. Activated platelets attach to fibrin fibers via the integrin receptor αIIbβ3 with participation of a plasma transglutaminase, factor XIIIa [22]. Because clot contraction depends strongly on the activity of platelets and fibrinogen level [20], procoagulant changes in the blood that occur in thrombotic disorders, such as ischemic stroke or venous thromboembolism, have been shown to alter the ability of clots to contract [19,23]. Because SLE patients are predisposed to thrombosis, it is likely that formation and maturation of clots in the blood of SLE patients is altered, which may have pathophysiological consequences and become clinically important.

In the present study, we revealed that clot remodeling or contraction is impaired in the blood of SLE patients due to platelet dysfunction and abnormal blood composition. The reduced contractile activity of platelets is a result of continuous hyperactivation induced by anti-dsDNA autoantibodies and immune complexes. A potential pathogenic importance of the impaired clot contraction in SLE was evaluated by correlating the parameters of clot contraction with clinical and laboratory manifestations of SLE. Reduced clot contraction may be a novel prothrombotic mechanism that affects the development, structure and properties of clots, and thrombi formed during chronic autoimmune inflammation.

Experimental

SLE patients and healthy subjects

The study was approved by the Ethical Committee of Kazan State Medical University and performed in accordance with the Declaration of Helsinki. Informed written consent was obtained from 51 patients with SLE. SLE was documented based on the criteria of the American College of Rheumatology. SLE patients were excluded from the present study if within 2 weeks of the time of examination they received anticoagulants, thrombolytic, or antiplatelet drugs. The SLE patients were divided into subgroups based on essential clinical and laboratory characteristics, such as a score of disease activity (SELENA-SLEDAI index), a level of anti-dsDNA antibodies, the existence of lupus nephritis and antiphospholipid syndrome, inflammation of skin or internal organs (Supplementary Table S1 online).

The blood of SLE patients was analyzed against a control group of blood samples from 60 healthy subjects with informed written consent. Healthy donors were included in or excluded from the present study based on a standard of blood donation eligibility (see Supplementary Material). The control group matched the SLE patients by gender composition and average age (Supplementary Table S2 online).

Blood collection and processing

Peripheral blood was drawn following the approved standard procedure of blood drawing. The first blood sample was collected into a container with a clotting activator silicate and allowed to clot for 20–30 min at 37°C, then it was centrifuged for 10 min at 2000 ***g*** to obtain serum, which was used for biochemical and immunological tests as well as for purification of anti-DNA antibodies. The second blood sample was drawn into vacuum containers with 3.8% trisodium citrate at 9:1 v/v and used directly for the clot contraction assay within 4 h. Another sample of citrated blood was centrifuged at 1500 ***g*** for 10 min to obtain platelet-poor plasma (PPP) for coagulation tests. Blood was also stabilized with EDTA (1.6 mg/ml final concentration) for hematological analysis.

Optical registration of the blood clot contraction kinetics

The process of clot contraction was followed using an original method of optical tracking *in vitro* [19]. Clot size dynamics were tracked by measuring light scattering over time followed by computational processing of the serial images using a Thrombodynamics Analyser System (HemaCore, Moscow, Russia) (Supplementary Figure S1). Citrated blood was activated with 1 U/ml human thrombin (Sigma-Aldrich, U.S.A., cat. # T8885) and 2 mM $CaCl_2$ (both final concentrations). The activated blood sample was transferred into a 12 × 7 × 1-mm plastic cuvette precoated with a thin layer of 4% (v/v) Triton X-100 in 0.15 M NaCl or phosphate-buffered saline to prevent fibrin sticking to inner walls of the cuvette, which was placed in the thermostatic chamber of a Thrombodynamics Analyzer System at 37°C. Images of the contracting clot were taken automatically every 15 s for 20 min to register the changes in clot

size. The collected serial clot images were analyzed computationally to extract the following parameters of clot contraction: the extent of contraction (calculated as $[(S_0 - S_t)/S_0] \times 100$, where S_0 is the initial clot size and S_t is the final clot size at the end point $t = 20$ min), lag time (time from the addition of thrombin until the clots reaches 95% of its initial size), the average contraction velocity (average of first derivative at each time point), and the area over the kinetic curve that reflects roughly the amount of mechanical work on clot compression performed by the contracting platelets.

Purification and characterization of anti-dsDNA antibodies

Polyclonal anti-dsDNA antibodies were chromatographically purified from pooled samples of serum of SLE patients by a chromatographic system Akta Avant 25 (GE Healthcare, Sweden) based on the affinity of antibodies toward Protein G and dsDNA. Serum of SLE patients diluted seven times with phosphate-buffered saline (PBS) was passed through the column of Protein G-Sepharose at room temperature. The column was washed with PBS and the adsorbed IgG was eluted with 100 mM glycine-HCl buffer, pH 2.3. The eluted IgG was immediately neutralized with 1 M Tris-NaOH, pH 9.8 at a 20:1 volume ratio. The IgG sample was preheated in a water bath at 56°C for 45 min to destroy any nuclease activity in the suspension and passed through the column of dsDNA-cellulose at room temperature. The column was washed with the binding buffer (50 mM NaCl, 20 mM Tris, 2 mM EDTA, pH 7.4) and the adsorbed anti-dsDNA antibodies were eluted with a high ionic strength buffer (1 M NaCl, 20 mM Tris, 2 mM EDTA, pH 7.4). The anti-DNA antibodies-containing fraction was collected and used alongside with the negative control, i.e. the IgG fraction that was not absorbed on the dsDNA column. The protein-containing fractions were pooled, concentrated, and transferred into PBS by dialysis. The antibodies were characterized by agarose gel electrophoresis (Figure 1A) and SDS/PAGE (Figure 1), showing they were not contaminated with DNA and were highly pure. The purified anti-dsDNA antibodies in PBS were aliquoted and stored at −80°C before use.

Characterizing DNA-binding activity of purified anti-dsDNA antibodies by ELISA

To confirm the affinity of purified anti-dsDNA antibodies to dsDNA, a standard enzyme-linked immunosorbent assay (ELISA) was used (Figure 1C). Calf-thymus dsDNA (Serva, Germany) was absorbed on a 96-well plate followed by the sequential incubation with the purified anti-dsDNA Abs and then with anti-human-IgG monoclonal antibodies conjugated with horseradish peroxidase (Sorbent LLC, Russia). 3,3′,5,5′-Tetramethylbenzidine (TMB) (Life Technologies, U.S.A.) was added as a chromogenic substrate followed by addition of sulfuric acid to stop the reaction. Results of ELISA were read by an automated microplate Reader Stat Fax 2100 (Awareness Technology, U.S.A.) at $\lambda = 450$ nm.

Flow cytometry of isolated platelets

Citrated venous blood from SLE patients or healthy subjects was spun at 200 ***g*** for 10 min to obtain platelet-rich plasma (PRP), which was gel-filtrated through a Sepharose 2B column (Sigma-Aldrich, U.S.A.) to collect platelets in the void volume. Isolated platelets were analyzed for expression of P-selectin and the active integrin αIIbβ3 (GPIIb/IIIa) before and after activation of cells by thrombin receptor activating peptide (TRAP-6) (Bachem Americas Inc., U.S.A.). Platelets were activated with 50 μM TRAP-6 (Ser-Phe-Leu-Leu-Arg-Asn) for 3 min at room temperature. A total of 400,000 quiescent or activated platelets in 50 μl of Tyrode's buffer (135 mM NaCl, 5.4 mM KCl, 1,0 mM MgCl, 5.6 mM Glucose, 10 mM HEPES, 0.2 mM Na_2HPO_4, pH 7.4) were incubated for 10 min at room temperature in the dark with 1.5 μl anti-human CD62P R-phycoerythrin-labeled murine antibodies (BD Biosciences, U.S.A.) for measurement of P-selectin expression or with Alexa fluor 488-labeled human fibrinogen (5 μg/ml final concentration) (ThermoFisher Scientific, U.S.A.) to assess the expression of active αIIbβ3. Flow cytometry was performed using a FacsCalibur flow cytometer connected to a computer equipped with BD CellQuest™ software (BD Biosciences, U.S.A.). Platelets were gated based on the size and granularity using Light Forward Scatter (LFS) and Light Side Scatter (LSS) channels, and 5,000 cells were counted in each sample. Two channels with green and yellow filters were used to detect platelets bearing Alexa fluor 488-labeled fibrinogen and anti-CD62P PE-labeled antibodies respectively. The data were analyzed using FlowJo X program. A total of 11 preparations of isolated platelets from SLE patients and 10 from healthy donors were analyzed with flow cytometry.

Coagulation, hematological, biochemical, and immunological tests

All the SLE patients underwent routine cell count, hemostasis, hematological, biochemical, and immunochemical tests. Citrated blood plasma was applied to the automated coagulometer system ACL TOP 500 (Beckman Coulter,

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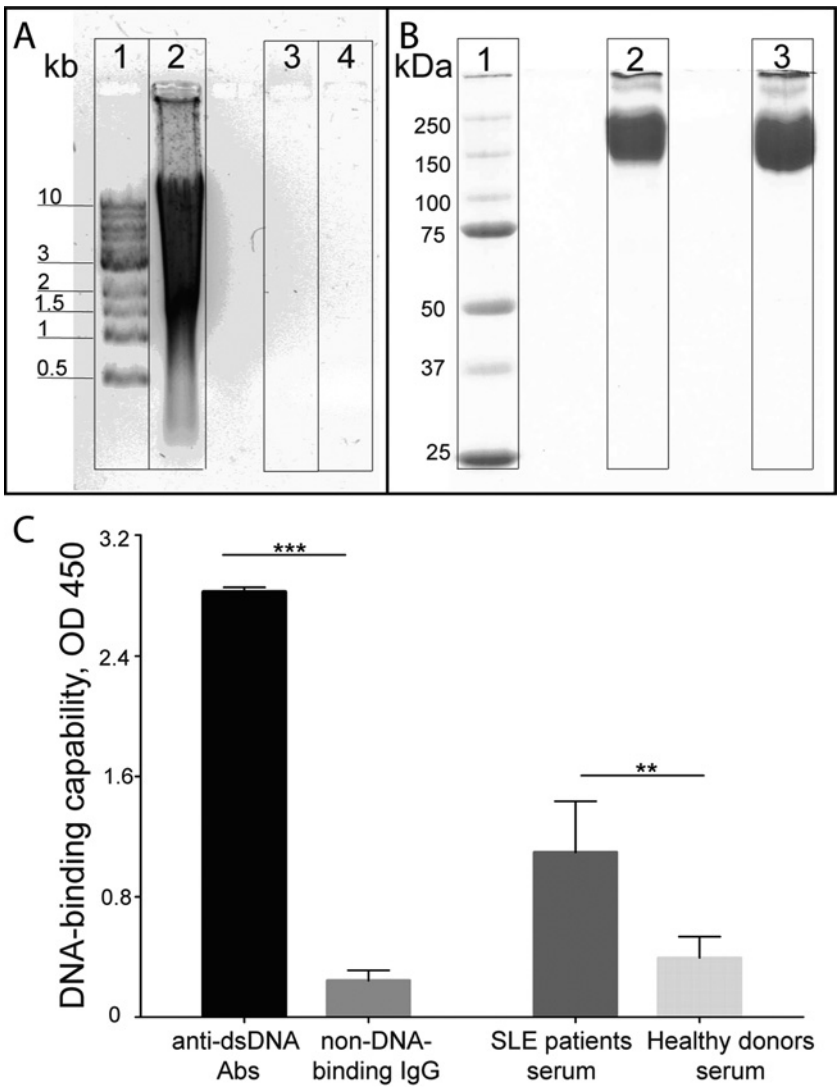

Figure 1. Purity and DNA-binding activity of purified anti-dsDNA antibodies

(**A**) Agarose gel electrophoresis showing no DNA contamination in the preparation of purified anti-DNA Abs; Lane 1: DNA markers, Lane 2: calf thymus DNA (10 μg), Lane 3: anti-DNA antibodies (10 μg), Lane 4: IgG (10 μg). (**B**) SDS/PAGE; Lane 1: molecular weight markers, Lane 2: non-dsDNA-binding IgG (20 μg per lane), Lane 3: purified anti-dsDNA antibodies (20 μg per lane). (**C**) ELISA assay of dsDNA-binding activity of purified anti-dsDNA antibodies versus equimolar amount of non-dsDNA-binding IgG and pooled serum of SLE patients (n=10) versus pooled serum of healthy donors (n=10); $^{**}P<0.05$, $^{***}P<0.001$ (Student's t-test).

U.S.A.) for activated partial thromboplastin time (aPTT), prothrombin time, thrombin time, International Normalization Ratio (INR), concentration of fibrinogen (Clauss method), and D-dimer concentration. Hematological tests were performed in an automated cell counter system ABX Micros 60 (Horiba, Japan) to retrieve red blood cell, leukocyte, and platelet counts. The following biochemical tests were performed by analyzing serum in the automated analyzing system Cobas Integra 400 plus (Cobas, Switzerland): total serum protein, serum albumin concentration, serum protein fractions, total bilirubin, creatinine, glucose, aspartate transaminase, alanine transaminase, sodium ion, potassium ion, and cholesterol. Serum antibodies (IgG, IgM, and IgA) and circulating immune complexes were analyzed in the automated system Freedom EVO clinical (TECAN, Germany). Concentration of anti-dsDNA antibodies and anticardiolipin antibodies was determined using the ELISA analyzer Alegria (Orgentec, Germany). The results of laboratory tests are presented in Supplementary Table S3 online.

Table 1 Clot contraction in the blood of SLE patients and healthy donors (mean ± SD)

	SLE patients (*n*=51)	Healthy subjects (*n*=60)
	Parameters of clot contraction	
Extent of contraction (%)	37 ± 2***	49 ± 1
Lag time (s)	200 ± 18***	106 ± 7
Average velocity (%/s × 10^{-3})	29 ± 2***	41 ± 1
AOC (a.u.)	264 ± 15***	375 ± 7
	Phase rate constants	
Phase 1 (1/s)	0.02 ± 0.01	0.03 ± 0.01
Phase 2 (%/s)	–0.04 ± 0.01*	–0.07 ± 0.01
Phase 3 (1/s)	0.001 ± 0.001*	0.002 ± 0.001
	Phase duration	
Phase 1 (s)	80 ± 5	75 ± 5
Phase 2 (s)	225 ± 15	210 ± 10
Phase 3 (s)	900 ± 30	925 ± 15

*$P<0.05$; ***$P<0.001$ (Mann–Whitney *U* test)

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, U.S.A.). A nonparametric Mann–Whitney *U* test was used to determine the statistical significance between averages at a 95% confidence level in the clot contraction parameters between healthy donors and SLE patients, and between clinical subgroups. In the model experiments, including those with a small sample size, a Student's *t*-test was used after confirmation of a large effect size and a statistical power to avoid a Type I error [24]. In our observations, the effect sizes of all the data were large (>1.3) and the statistical power was approximately 80% or higher (Supplementary Table S4), justifying the use of the Student's *t*-test [25].

Results

Clot contraction in the blood of SLE patients

Contraction of clots formed from the blood of SLE patients occurred significantly slower and to a lesser extent than contraction of clots formed from the blood of healthy subjects, despite the fact that blood clotting and clot contraction in all the *in vitro* assays were triggered with thrombin and calcium ions under standard conditions (Table 1, Supplementary Figure S2 online). Specifically, the average velocity of clot contraction, the extent of contraction, and the area over the kinetic curve were all reduced approximately 1.5-fold and the lag period was prolonged nearly 2-fold in clots from the SLE patients compared with controls. It has been previously shown that clot contraction of the blood of healthy subjects occurs in 3 phases: initiation of contraction (phase 1), linear contraction (phase 2), and mechanical stabilization (phase 3) [19]. Regression analysis conducted on the average kinetics curves (Figure 2A) indicated that the rate constants of phase 2 and phase 3 in SLE patients were reduced by almost 1.5-fold (Figure 2B, Table 1), indicating the impairment of clot contraction in SLE that occurred mainly at the stages of linear contraction and mechanical stabilization of the clots. Duration of all three phases was not different in SLE patients from those of healthy donors (Table 1).

Analysis of platelet functionality in SLE patients

Since platelets are the source of contractile forces generated during clot contraction, any changes in platelet functionality could be crucial for clot contraction. To test if platelet dysfunction is responsible for the impaired clot contraction observed in SLE, we compared the functional status of platelets isolated from SLE patients and healthy subjects and their responsiveness to activation. Thrombin receptor-activating peptide (Ser-Phe-Leu-Leu-Arg-Asn or TRAP-6) was used as a platelet activator to mimic the effect of thrombin on PAR receptors. Platelet functionality was assessed using flow cytometry by the ability of cells to express P-selectin and the active integrin αIIbβ3 capable of binding fibrinogen. The quiescent platelets from SLE patients exposed to fluorescently labeled anti-P-selectin antibodies and fibrinogen followed by the cytometric assay were found to be insignificantly different from the platelets in healthy donors by the initial background activity (Table 2). However, after TRAP-induced activation, platelets from SLE patients were much less responsive compared with the cells from healthy donors (Table 2). The most likely cause of the platelet partial refractoriness to the stimulus is their chronic activation that might be induced by various pathological blood

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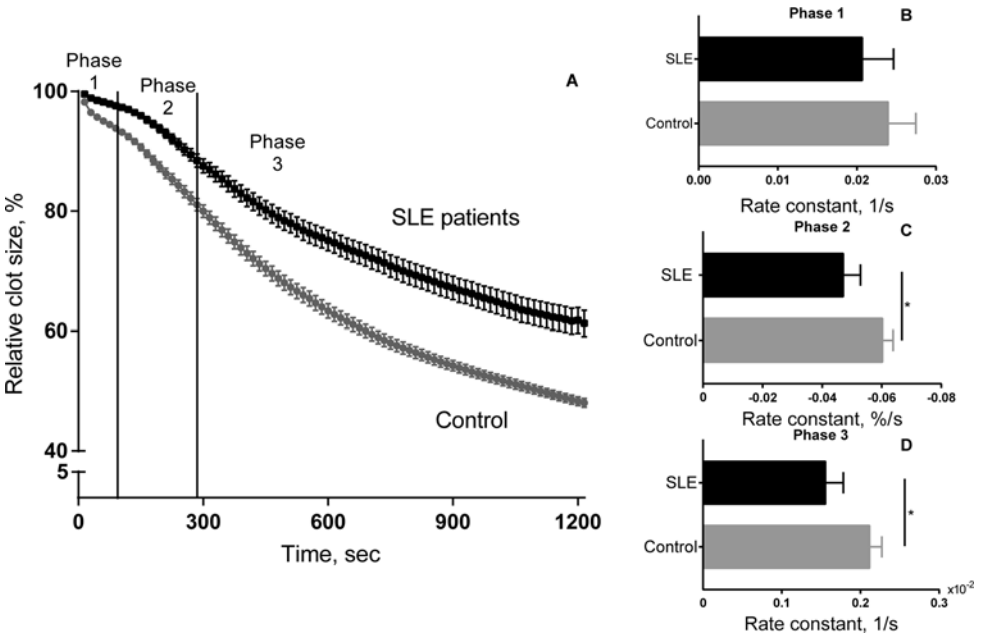

Figure 2. Contraction of blood clots in patients with SLE and healthy donors

Averaged kinetic curves of contraction of clots formed in the blood of SLE patients (n=51) and healthy subjects (n=60) (**A**). Optical tracking was used to measure the relative changes in clot size during 20 min with 15-s intervals. Phase analysis of the kinetic curves shown in (A): rate constants of Phase 1 (**B**), Phase 2 (**C**), and Phase 3 (**D**). Data are shown as a mean ± SEM; *P<0.05 (Mann–Whitney U test).

Table 2 Fraction of platelets (%) isolated from the blood of SLE patients and healthy donors that express P-selectin and bind fibrinogen before (quiescent platelets) and after stimulation with TRAP-6 assessed by flow cytometry

	P-selectin expression		Fibrinogen-binding capacity	
	Quiescent platelets	TRAP-activated	Quiescent platelets	TRAP-activated
SLE patients (n=11)	2.5 ± 1.1	33.4 ± 8.7*	2.9 ± 0.7	26.2 ± 4.2†
Healthy donors (n=8)	1.5 ± 0.8	72.7 ± 4.4*	1.5 ± 0.9	47.5 ± 5.4†

*,†Statistically significant difference (P<0.05, Mann–Whitney U test) between the cells from SLE patients and healthy donors.

components, including autoantibodies. However, the extent of the continuous latent platelet activation may be not enough to cause degranulation (assessed by expression of P-selecting) and αIIbβ3 activation (Table 2).

Association of clot contraction parameters with changes in blood composition

To see if the changes in clot contraction could be attributed to variations in blood composition, we correlated clot contraction with some hemostatic, immunological, and biochemical parameters in the SLE patients (Supplementary Table S3 online). The level of fibrinogen, an acute-phase protein, was considerably higher in the blood of SLE patients than in healthy donors, and fibrinogen has been shown to hamper clot contraction in a dose-dependent manner [19]. An increase in leukocyte count, mainly monocytes, may result in tissue factor expression in activated cells, leading to enhanced thrombin generation via the extrinsic pathway. A shortened activated partial thromboplastin time (aPTT) confirmed hypercoagulability that is probably associated with enhanced thrombin generation that cause continuous platelet activation followed by platelet dysfunction, including impaired contractility, as has been found in ischemic stroke [20]. Immunological parameters showed a significantly higher level of autoantibodies (anti-dsDNA and anticardiolipin) and a lower concentration of complement in the blood of SLE patients. In addition, the SLE patients had a considerably lower concentration of protein, higher urea and lower level of creatinine in their blood, which reflect impaired kidney function in the SLE patients.

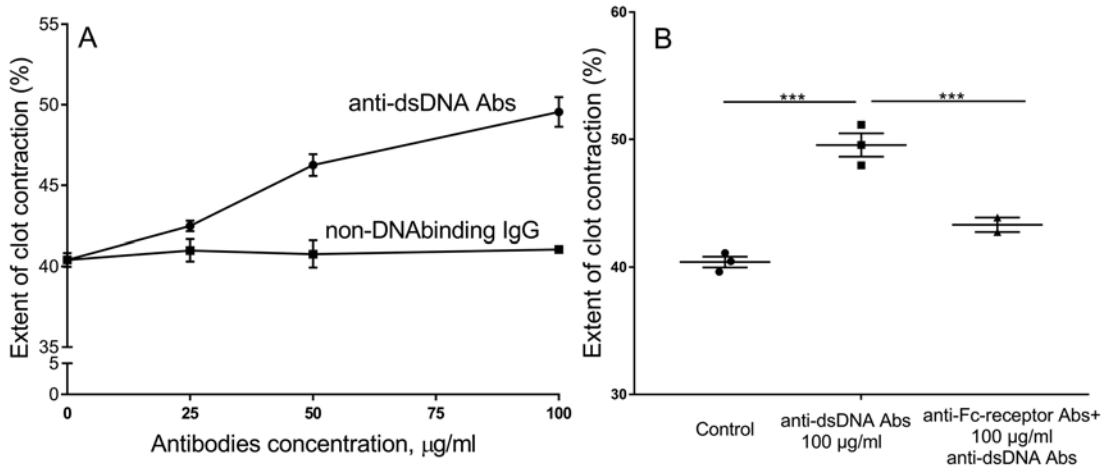

Figure 3. Changes of the parameters of blood clot contraction shortly after addition of exogenous anti-dsDNA antibodies to normal blood

(**A**) An immediate dose-dependent effect of purified anti-dsDNA antibodies on the extent of clot contraction in healthy donors (n=3). (**B**) The average degree of thrombin-induced clot contraction in healthy donors' blood, preincubated with anti-dsDNA antibodies (100 μg/ml) alone (central bar) and in the presence of a monoclonal antibody IV.3 against the *Fc*-receptor, compared with control without antibodies (left bar) (n=3); ***P<0.001 (Student's t-test).

Clot contraction is modulated by anti-dsDNA antibodies

This relationship between clot contraction and the level of anti-dsDNA antibodies suggests a mechanistic link between the immune inflammation and clot contraction. Anti-dsDNA antibodies were found in the blood of SLE patients at a level that was about 22-fold higher than in healthy donors (Supplementary Table S3 online). Moreover, clots formed in the blood of patients with a level of anti-dsDNA antibodies higher than 100 IU/ml contracted significantly slower and to a lesser extent compared to those with a level of anti-dsDNA antibodies below 100 IU/ml, while in both groups contraction ability of clots was still remarkably reduced compared with the healthy donors (Supplementary Figure S3 online). Therefore, platelet function alterations in SLE are probably caused by effects of these autoantibodies and their immune complexes that can potentially activate platelets via Fc-receptors and probably exhaust them [9].

To mimic the effect of autoantibodies on platelets in SLE, the rate and degree of clot contraction in the blood of healthy donors were measured in the absence and presence of exogenous purified anti-dsDNA antibodies isolated from the blood of SLE patients. If clot contraction was registered immediately after addition of anti-dsDNA antibodies to blood samples, the rate and degree of clot contraction were remarkably higher than in the absence of the antibodies or in the control with addition of non-DNA binding IgG (Figure 3A). This dose-dependent short-term contraction-promoting effect on platelets was extinguished by preincubating blood samples with an anti-Fc-receptor monoclonal antibody IV.3 that brought the average velocity and extent of clot contraction back to the baseline (Figure 3B). To mimic the effect of continuous chronic activation of platelets by anti-dsDNA in SLE patients on platelet functionality, we incubated the blood of healthy donors with anti-dsDNA for several hours and then measured the time-dependent contraction kinetics. Unlike the immediate effect, prolonged incubation of blood samples with anti-dsDNA Abs reduced the rate and degree of clot contraction, compared with contraction in the presence of control with DNA-non-binding IgG (Figure 4). Taken together, these results indicate that the anti-dsDNA autoantibodies induce Fc-receptor-mediated continuous hyperactivation of platelets followed by their exhaustion and dysfunction, including impaired contractility.

Clot contraction and severity of SLE

The SLE Disease Activity Index (SLEDAI) is one of the most common parameters to characterize the severity of SLE; it ranges from 0 to 105, with a score between 3 and 12 corresponding to mild and moderate SLE and SLEDAI>12 corresponding to severe or highly active SLE. We found that SLE patients with SLEDAI>12 had a significantly reduced extent of clot contraction and average velocity, compared to those with SLEDAI<12 (Figure 5). Importantly, the extent of clot contraction in SLE patients with SLEDAI score higher than 12 was significantly suppressed, while clot contraction in SLE patients with a SLEDAI score between 3 and 12 did not differ from that of healthy donors.

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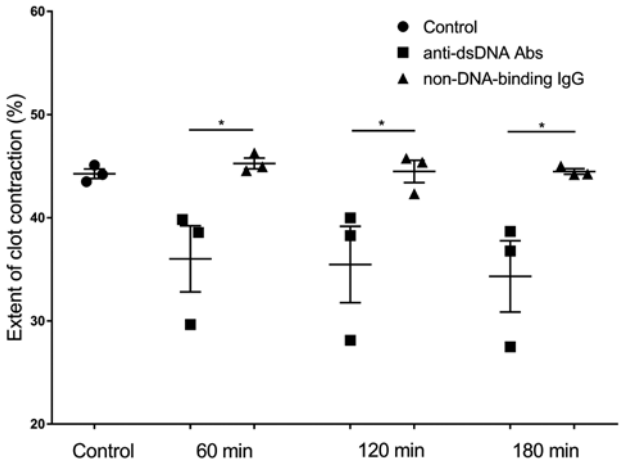

Figure 4. Delayed effects of exogenous anti-dsDNA antibodies on the contraction of blood clots in normal blood

Time-dependent effects of purified anti-dsDNA antibodies on contraction of clots in healthy donors' blood (n=3) after prolonged (1–3 h) incubation with the blood contrasted with the non-DNA-binding IgG and contraction at the zero time point without any antibodies; *P<0.05 (Student's t-test).

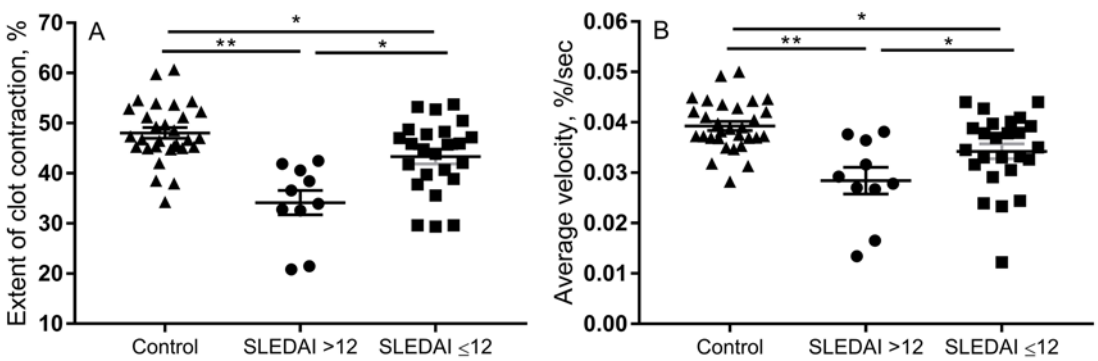

Figure 5. Contraction of blood clots and the disease severity

Parameters of clot contraction in the blood of SLE patients with SLEDAI scores >12 (n=7) and ≤12 (n=22): the extent of clot contraction (**A**) and the average velocity (**B**). Data are shown as a mean ± SEM; *P<0.05; **P<0.01 (Mann–Whitney U test).

Discussion

SLE patients have an increased risk of thrombotic disorders, which are among the main causes of death in SLE [1]. Despite the remarkable clinical significance, the mechanistic relationship between blood clotting disorders and autoimmune inflammation in SLE remains largely unclear. The process of clot contraction or volumetric shrinkage is one of the least studied stages of blood coagulation. Despite the potential clinical significance of normal versus impaired clot contraction, systematic studies of the relationship between clot contraction and various pathological conditions have not been performed until recently [19,20]. This work is the first study of blood clot contraction in SLE patients.

The main finding of the present study is that contraction of clots formed in the blood of SLE patients occurred at a significantly slower rate and the degree of clot shrinkage was significantly smaller compared with those from the blood of healthy donors. Moreover, the average velocity and the extent of clot contraction in the SLE patients correlated directly with the higher levels of anti-dsDNA Abs in the blood and higher severity of the disease (SLEDAI score). The reduction of clot contraction in SLE patients might be explained by a combination of several mechanisms. Since clot contraction is driven by the contractile force generated by the actomyosin machinery of platelets and propagated through platelet–fibrin interactions [21,26], any disturbances in platelet count and functionality may lead to changes in clot contraction. Our results with flow cytometry of isolated platelets confirmed that platelets from the blood of SLE

patients were less responsive to a chemical stimulus because the expression of P-selectin and the binding of fibrinogen after TRAP-induced cell activation were significantly reduced compared with normal cells.

A likely cause for platelet dysfunction in SLE is continuous chronic hyperactivation of cells [16,20], resulting in metabolic and energetic exhaustion. Among pathogenic factors that can potentially induce platelet activation in SLE are abundant autoantibodies against dsDNA. This presumption was corroborated by the results of our model experiments where the clot contraction assay was performed in the absence and presence of purified anti-dsDNA antibodies that showed a quick dose-dependent enhancing effect on clot contraction. This effect was mediated via the Fc-receptor, as would be predicted if the antibodies formed immune complexes on or near the platelet surface [9,16]. Since anti-dsDNA Abs and immune complexes are abundant in the blood of SLE patients, platelets are very likely to be exhausted by this persistent immune stimulation, leading to reduced contraction of the myosin-actin machinery as a part of platelet dysfunction. Indeed, unlike the immediate contraction-stimulating effect, the anti-dsDNA Abs significantly reduced clot contraction in a time-dependent manner when the incubation time was prolonged for hours (Figure 4). Therefore, autoantibodies, including anti-dsDNA antibodies, are a pathogenic factor that can reduce clot contraction via chronic platelet hyperactivation followed by defective contractility. SLE is associated with an increase in white blood cell counts. Some of them, such as lymphocytes, monocytes and neutrophils, are powerful platelet stimulators [27-29] and they can contribute to continuous platelet activation in SLE, thus exaggerating their exhaustive dysfunction.

A high concentration of fibrinogen in the blood of SLE patients might be an additional cause of reduced clot contraction due to an increased elasticity of a dense fibrin network, comprising the mechanical scaffold of clots and thrombi [19,20]. Moreover, recent studies have revealed an alteration in the ultrastructure of fibrin network in the blood of SLE patients [30] which, in combination with hyperfibrinogenemia and fibrinogen phosphorylation [10,12], might also contribute to the increased elasticity and reduced deformability of the fibrin network, resulting in the impaired clot contraction. It is likely that rigidity and decreased deformability of the erythrocyte membrane resulting from lipid peroxidation associated with SLE also reduces the extent of clot contraction [31]. These and other findings suggest that the impaired clot contraction in SLE is a complex and multifactorial phenomenon due to multiple pathological alterations of blood composition that can perturb platelet function as well as the structure and mechanical properties of clots and thrombi.

What are potential mechanistic implications of the impairment of clot contraction in SLE? There is emerging indirect evidence that extent and average velocity of contraction of clots and thrombi may be an important pathogenic factor that affects the local blood hydrodynamics and the course and outcomes of thrombosis [20,32,33]. The significance of reduced contraction of intravascular clots and thrombi becomes even more clear when it is converted to the degree of vessel obstruction and its local hydrodynamic perturbations. Thrombotic obstruction increases the vascular resistance as described by Poiseuille's law, which says that resistance is inversely related to the radius to the fourth power of the vessel diameter. Therefore, if the diameter of a vascular segment is reduced by one-half, the resistance or the volumetric blood flow within that narrowed segment increases by 16-fold, which is a dramatic effect relative to the change in degree of vessel obstruction modulated by clot shrinkage or contraction. Moreover, there is a strong inverse correlation between the degree of arterial blockage and flow shear rate, which is a strong modulator of platelet function and thrombus formation and growth [34-36].

Although the reduced contractility of *in vitro* clots in the blood of SLE patients and its relation to platelet dysfunction seem to be well documented in the present study, the pathophysiological relevance of these findings remains uncertain. Whether or not modulation of platelet contractility has any pathogenic importance for the likelihood, course and outcomes of thrombosis, needs direct *in vivo* observations of reduced or enhanced contraction of intravascular thrombi with respect to variation of the local blood flow, embolization, fibrinolytic resistance etc. Although the hypothesis looks attractive, it has to be proven that reduced clot contraction in SLE might serve as a prothrombotic mechanism that exaggerates the disturbance of blood flow caused by uncontracted obstructive thrombi. In addition, mechanisms of the impaired clot contraction could be various and not limited to the effects of anti-DNA–autoantibodies on platelets. These and many other uncertainties dictate further investigations of this novel and potentially significant phenomenon.

In conclusion, our results show that contraction of clots formed from the blood of SLE patients is significantly impaired. This defect appears to be largely due to platelet dysfunction that is a result of their continuous chronic activation by autoantibodies and perhaps leukocytes, followed by metabolic exhaustion/refractoriness of platelets and failure of contractility. If the reduced ability of intravascular clots and thrombi to shrink indeed exaggerates vessel obstruction, this may be an important pathogenic mechanism that promotes disturbance of blood flow in thrombosis and worsens outcomes of thrombotic states. In addition, a clot contraction assay for SLE patients might potentially serve as an additional test for disease severity and a tool for early diagnosis and prognosis of thrombotic

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complications. Therefore, our results revealed a strong relationship between clot contraction and the pathogenesis and clinical features of SLE, which makes it worth further investigation as a new avenue to develop prophylaxis, diagnosis, and treatment of thrombotic complications of SLE.

Clinical perspectives

- Despite the fact that systemic lupus erythematosus remains one of the hardest diseases to treat and thrombotic complications are among the most usual causes of death in systemic lupus patients, the link between autoimmune activities and thrombosis remains unknown.
- This work shows that in systemic lupus erythematosus the ability of blood clots to shrink in volume (clot contraction) is significantly reduced due to platelet dysfunction and refractoriness because of continuous hyperactivation of platelets by anti-dsDNA autoantibodies.
- These findings provide a foundation for a new concept of modulating the blood flow at the site of thrombotic occlusion by variations in clot size as a result of contractile forces generated by activated platelets attached to fibrin. The reduced clot contractility that leads to increased thrombotic vessel obstruction reveals a possible mechanism of how autoimmunity effects blood clotting and thrombosis, pointing out a role of impaired clot contraction in the pathogenesis of systemic lupus erythematosus. Platelet-driven clot contraction might be a target for developing new methods of prophylaxis, diagnosis, and treatment of thrombotic complications.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

J.W.W. and R.I.L. designed the research; G.L.M., A.D.P., and I.A.A. performed experiments; A.D.P., G.L.M., I.A.A., T.B.S., A.N.M., J.W.W., and R.I.L. analyzed the data; A.D.P., G.L.M., I.A.A., J.W.W., and R.I.L. wrote the paper.

Abbreviations

aPTT, activated partial thromboplastin time; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglubulin; INR, International Normalization Ratio; LFS, Light Forward Scatter; LSS, Light Side Scatter; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SDS/PAGE, sodium dodecyl/sulfate polyacrylamide gel electrophoresis; SELENA, Safety of Estrogens in Lupus National Assessment study; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; TMB, 3,3′,5,5′-Tetramethylbenzidine; TRAP, thrombin receptor activating peptide.

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