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Unfavorably altered fibrin clot properties in patients with active rheumatoid arthritis

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ABSTRACT

Objective: Altered fibrin clot properties have been reported in cardiovascular diseases (CVD) and inflammatory states. Given increased prevalence of CVD in patients with rheumatoid arthritis (RA), we investigated whether fibrin characteristics are also altered in RA patients.
Patients and methods: We studied 46 consecutive RA patients versus 50 controls matched for age and gender. Ex vivo plasma clot permeability, turbidity, tissue-type plasminogen activator (tPA)-induced fibrinolysis, and scanning electron microscopy (SEM) images of clots were evaluated.
Results: Patients with RA had lower clot permeability, faster clot formation, higher maximum clot absorbancy indicating thicker fibrin fibers, maximum clot mass and prolonged fibrinolysis time than controls. Maximum rates of clot lysis were similar in both groups. SEM images showed formation of dense clots with many projections on fibrin fibers. Clot permeability inversely correlated with fibrinogen, tPA, plasminogen activator inhibitor-1 (PAI-1), CRP, platelet count, disease activity score (DAS28) and a marker of oxidative stress, 8-iso-prostaglandin $F_{2\alpha}$ (r from -0.44 to -0.79; all, $p<0.0001$). Similar positive associations were found for clot lysis time (r 0.44 to 0.69; all, $p<0.01$). Multiple regression analysis showed that fibrinogen was the only independent predictor of clot permeability ($R^2=0.87$, $p<0.0001$) and lysis time ($R^2=0.80$, $p<0.003$) in RA. Maximum D-dimer levels released from clots, maximum clot turbidity and the time of clot formation were predicted by PAI-1 (all, $p<0.05$).
Conclusion: We showed unfavorably altered plasma fibrin clot structure and function in RA, which might contribute to an increased risk of thrombotic events in this disease.

Introduction

Growing evidence indicates that chronic inflammatory disorders such as rheumatoid arthritis (RA) are associated with an increased risk for cardiovascular disease (CVD), including myocardial infarction and sudden cardiac death [1]. Conventional risk factors do not account for the premature development of CVD in RA. The pathogenesis of atherosclerosis and its thromboembolic complications in RA remains obscure. Interestingly, several similarities between inflammatory and immunological mechanisms in RA and atherosclerotic vascular disease have been reported [2,3], indicating the role of inflammatory cytokines, acute-phase reactants, oxidative stress, matrix metalloproteinases, leukocyte activation and altered Th1/Th2 lymphocyte balance.

Systemic inflammation of higher grade, typical of RA, leads not only to endothelial dysfunction, but also it can activate the coagulation cascade in a number of ways [4]. A systemic hypercoagulable state may predispose to a rapid thrombus formation [5]. Elevated plasma levels of fibrinogen, von Willebrand factor, thrombin generation markers, tissue plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1) and D-dimer have been demonstrated in RA patients [6,7]. This indicates that prothrombotic and antifibrinolytic mechanisms are involved in atherothrombosis in RA [8].

Acute coronary syndromes result largely from thrombus formation on the surface of ruptured or eroded atheromatosus plaque in the coronary artery and these thrombi contain substantial amounts of fibrin, the final product of blood coagulation [9]. There is evidence that patients with premature coronary atherothrombosis are characterized by the formation of abnormal fibrin clots, resistant to fibrinolysis with reduced permeability and increased stiffness [10–12]. Based on the data showing an adverse effect of increased fibrinogen and C-reactive protein (CRP) on fibrin clot properties [13,14], we hypothesized that in RA, a plasma fibrin clot displays unfavorable characteristics that might contribute to increased atherothrombotic risk in this population. The aim of our study was to investigate plasma fibrin clot properties and their determinants in RA patients.

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Patients and Methods

Patients

We enrolled 46 consecutive white patients with stable RA and 50 white control subjects, the hospital personnel and matched for age, sex, hypertension, blood cholesterol and glucose. The Bioethics Committee of the Jagiellonian University approved the study protocol and written consent was obtained from all participants. All patients fulfilled the American College of Rheumatology (ACR) criteria for RA [15]. The exclusion criteria were as follows: any acute illness, known cancer, hepatic or renal dysfunction (creatinine >120 μmol/l), diabetes mellitus, previous myocardial infarction, stroke or another acute vascular event, venous thrombembolism and current anticoagulant therapy. Patients taking disease modifying antirheumatic drugs (DMARDs) and biological treatment (blockers of TNFα and CD20 cells) were eligible.

Disease activity in RA patients was measured by the number of tender joints, number of swollen joints, assessment of the duration of morning stiffness, patient's global assessment of disease activity, physician's global assessment of disease activity and patient's assessment of pain using 100 mm visual analogue scales (VAS). Disease Activity Score (DAS 28) was calculated as described [16]. All RA patients performed Health Assessment Questionnaire (HAQ) [17].

Laboratory investigations

After an overnight fast blood was collected to determine serum glucose, creatinine, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) using routine laboratory techniques. Fibrinogen and CRP were measured by nephelometry (Dade Behring, Marburg, Germany). Venous blood samples for the fibrin clot analysis were taken into 0.13 mM trisodium citrate tubes (Becton Dickinson, Numbrecht, Germany) and centrifuged within 30 min at 2500 g for 20 min. Plasma was frozen at - 80 °C until analysis.

Commercially available immunoenzymatic assays were used to determine t-PA, PAI-1, D-dimer (American Diagnostica, Greenwich, CT, USA), and 8-iso-prostaglandin $PGF_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), a marker of oxidative stress (Cayman Chemicals, Ann Arbor, MI, USA).

In the RA group serum IgM rheumatoid factor (RF) was assessed by quantitative nephelometry (Dade Behring, Marburg,Germany). A concentration of the IgM RF >15 IU/ml was considered positive. All laboratory tests listed below were performed by technicians unaware of the sample status.

Fibrin clot permeation

Fibrin clot permeation was assessed as described [13,18]. Briefly, plasma samples were recalcified with calcium chloride (final concentration, 20 mmol/L) and then 1 U/mL human thrombin (Sigma-Aldrich, St Louis MO, USA) was added to citrated plasma samples. After 2 h of incubation at room temperature, tubes containing the clots were connected via plastic tubing to a reservoir of a buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) and its volume flowing through the gels was measured within 60 min. A permeation coefficient (K_s) which indicates the surface of the gel allowing flow through a fibrin network, i.e. a pore size, was calculated from equation: $K_s = Q \times L \times \eta / t \times A \times \Delta p$, where Q is flow rate in time t, L is the length of a fibrin gel (13 mm), η is the viscosity of liquid (1/100 poise), A is the cross section area (0.049 cm^2), and Δp is a differential pressure in dyne/cm^2.

Clot turbidity

Plasma samples were diluted 1:1 with 0.05 mol/L Tris-HCl , 0.15 mol/L NaCl, pH 7.4 and addition of 1 U/mL human thrombin (Sigma-Aldrich, St Louis, MO, USA) and 15 mmol/L calcium chloride to plasma-initiated polymerization [19]. Absorbency was read at 405 nm for 15 min with a Perkin-Elmer Lambda 4B spectrophotometer (Molecular Devices Corp., Menlo Park, CA, USA). A lag phase of the turbidity curve which reflects the time required for fibrin protofibrils to grow to sufficient length to allow lateral aggregation to occur and maximum absorbency at plateau reached by all individuals (ΔAbs max), which reflects the number of protofibrils per fiber, were recorded. Each sample was analyzed twice.

Plasma clot lysis

Plasmin-mediated fibrinolysis was evaluated using two assays as described [19].

In assay 1, fibrin clots, formed as described above, were perfused with the same buffer containing 0.2 μmol/L recombinant tPA (rtPA) (Boerhinger Ingelheim, Ingelheim, Germany) The lysis rate was determined by measuring the concentration of D-dimers (American Diagnostica, Greenwich, CT, USA), a marker of fibrin degradation, in the effluent every 20 min. Maximum rates of increase in D-dimer levels and maximum D-dimer concentrations detected at 80 or 100 min were analyzed in each subject. The experiment was stopped after 80-120 min when the fibrin gel collapsed under the pressure.

In assay 2, 100 μL citrated plasma was diluted with 100 μL of a buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4), containing 20 mmol/L calcium chloride, 1 U/mL human thrombin (Sigma-Aldrich, St Louis, MO, USA,) and 14 μmol/L rtPA. Assembly kinetics were monitored by spectrophotometry at 405 nm in duplicate aliquots. The time required for a 50% decrease in clot turbidity ($t_{50\%}$) was chosen as a marker of the clot susceptibility to fibrinolysis.

Scanning electron microscopy (SEM)

Clots from randomly selected subjects (n = 10) with RA and controls (n = 10) were fixed by permeating them with a 2.5% glutaraldehyde solution for 2 hours, recovered from the tubes and further processed by dehydratation as described [13]. Samples were photographed digitally in five different areas with a Hitachi S-4700 SEM.

Statistical analysis

Data are expressed as mean values (±SD) or median (interquantile range) or otherwise stated. The Shapiro-Wilk test was used to determine normal distribution. Intergroup differences for continuous variables were assessed by the t test when normally distributed or the Mann-Whitney U test for variables of non-normal distribution. The Pearson or Spearmann rank correlation coefficients were calculated to test the associations between two variables with a normal or non-normal distribution, respectively. Multiple linear regression analysis (the forward stepwise method) was used to determine predictors of all fibrin parameters. The variables that entered the analysis were: age, disease duration, fibrinogen, CRP, DAS28, HAQ, tPA, PAI-1, and 8-iso-$PGF_{2\alpha}$ levels. A p-value <0.05 was considered statistically significant.

Results

A total of 46 consecutive RA patients were included in the final analysis. A mean disease duration was 8.1 years and DAS28 was 5.2 ± 1.1 indicating high activity of the disease. The most frequent mode of RA treatment in the patient group was monotherapy with methotrexate (Table 1). As shown in Table 2, both groups did not differ with regard to age, gender and most cardiovascular risk factors except lower percentage of cigarette smokers (4 [8.7%] vs. 17 [34%], p = 0.004) and higher HDL cholesterol in the RA group (1.53 [0.98 -