Decreased Binding of Annexin V to Endothelial Cells
A Potential Mechanism in Atherothrombosis of Patients With Systemic Lupus Erythematosus

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Objective—The cause of the exceedingly high risk of atherothrombosis in systemic lupus erythematosus (SLE) is not clear but antiphospholipid antibodies (aPL) and potentially antithrombotic annexin V have been implicated.

Methods and Results—Twenty-six women (52±8.2 years) with SLE and a history of cardiovascular disease (CVD) (SLE cases) were compared with 26 women with SLE but no CVD (SLE controls) and 26 healthy women (population controls). Common carotid intima-media thickness (IMT) was determined by B-mode ultrasound as a surrogate measure of atherosclerosis. Annexin V binding to human umbilical vein endothelial cells (HUVECs) as determined by flow cytometry after 24-hour culture with plasma was decreased when plasma from SLE cases was used (SLE cases versus population controls: P<0.002; SLE cases versus SLE controls P=0.02). Antibodies against cardiolipin were among IgG antibodies causing decreased binding. There was a positive association between annexin V binding and IMT (R=0.73; P<0.001) among SLE cases. Immunohistochemical analysis revealed presence of annexin V in all human atherosclerotic plaques tested, especially at sites prone to rupture.

Conclusions—Decreased annexin V binding to endothelium caused by antibodies may represent a novel mechanism of atherothrombosis. We hypothesize that even though annexin V may promote plaque growth at some disease stages, it may also stabilize plaque. (Arterioscler Thromb Vasc Biol. 2005;25:198-203.)

Key Words: systemic lupus erythematosus ■ atherothrombosis ■ atherosclerosis ■ annexin V ■ antibodies

Atherosclerosis, the main cause of cardiovascular disease (CVD), has many characteristics of an inflammatory disease, including abundance of inflammatory cells and production of pro-inflammatory cytokines in lesions.1,2 The increased risk of morbidity and mortality caused by CVD in systemic lupus erythematosus (SLE) patients is an important clinical problem but could also shed light on the role of inflammation and autoimmunity in CVD, atherosclerosis, and thrombosis in general population, especially in women.

CVD in SLE is associated with traditional risk factors, such as dyslipidemia, and nontraditional risk factors, including increased oxidation of low-density lipoprotein, raised activity in tumor necrosis factor (TNF) system, systemic inflammation as determined by C-reactive protein, homocysteine, and antiphospholipid antibodies (aPL).3-6 aPL may cause the antiphospholipid antibody syndrome, common in SLE and characterized by recurrent pregnancy loss and recurrent thrombosis.7,8 aPL have also been implicated in CVD in the general population.9,10

Annexins comprise a group of evolutionary highly conserved proteins with calcium and membrane binding properties. They exert functions related to cell membrane properties, coagulation, and inflammation.11 Recently, annexin V has been implicated in antiphospholipid antibody syndrome because some aPL disrupt the annexin V antithrombotic shield in the placenta, predisposing to placental microthrombosis and recurrent miscarriage.12,13

We provide evidence that decreased binding of annexin V to endothelial cells may be an important mechanism in SLE-related CVD. Annexin V is abundant in atherosclerotic lesions and annexin V binding to endothelial cells positively associated with intima-media thickness (IMT). The implications of the findings are discussed.

Materials and Methods

Study Group

The study group, as reported previously,5 consisted of 26 women with SLE who were survivors of one or more manifestations of...
CVD, defined as thromboembolic stroke (n=15), myocardial infarction (n=7), angiina pectoris (n=9), or intermittent claudication (n=4); 26 age-matched women with SLE who had no clinical manifestations of CVD; and 26 age-matched healthy population-based women. Clinical characteristics of the 3 groups have been reported.5 All patients fulfilled the 1982 revised criteria of the American Rheumatism Association for SLE.14 The study was approved by the Ethics Committee of Karolinska Hospital. All participants gave informed consent before entering the study. The investigation included a written questionnaire, an interview, and physical examination by a rheumatologist. Routine, immunologic, and other laboratory measurements have been described.5,6

Carotid Ultrasound

The right and left carotid arteries were examined blinded with a duplex scanner (Acuson Sequoia, Mountain View, Calif) and the IMT was determined as described.5,6

Culture of Endothelial Cells and Flow Cytometry

Cryopreserved pooled human umbilical vascular endothelial cells (HUVECs) at passage 2 were purchased from Cascade Biologics, Inc (Portland, Ore). Cultures were maintained in EGM phenol red-free medium (Clonetics, San Diego, Calif), containing 2% of fetal bovine serum and supplements, at 37°C under humidified 5% CO2 conditions. All experiments were performed a passage 3 to 4. HUVECs were seeded at 2×10^4 cells/mL density on 12-well plates (NUNC Inc, Naperville, Ill) for flow cytometry; 1×10^5 cells per well per 100 μL on 96-well plates (AG, Trasadingen, Switzerland) for MTT assay; 8×10 cells/mL on 24-well plates (NUNC) for DNA fragmentation enzyme-linked immunosorbent assay (ELISA). HUVECs cultured under basal conditions or after 24 hours of serum-starvation (apoptotic) were prepared in quadruplicate as controls.

After allowing 12 to 24 hours for attachment, cells were made quiescent in serum free medium (SFM) for at least 12 hours before treatment. Heparin-preserved plasma was added at concentration of 10% in SFM. After 24 hours, cells were harvested with Cell Dissociation Solution (Sigma-Aldrich) and carefully pooled with supernatants, to exclude selective loss of detached floating cells, centrifuged at 1200 rpm for 7 minutes. After resuspension in 100 μL of annexin V binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, and 2.5 mmol/L CaCl2: pH 7.4; Molecular Probes Inc), samples were stained with 2 μL of annexin V–fluorescein isothiocyanate (FITC) (R&D Systems Europe Ltd, Abingdon, UK) and incubated for 15 minutes on ice. Before acquisition, 1 mg/mL of propidium iodide (PI; Molecular Probes, Inc) was added. Analysis was performed on a FACSscan flow cytometer (BD Biosciences, San Jose, Calif) equipped with CellQuest software. For each sample, 10,000 events were collected. During acquisition, events smaller than 230 on linear FSC and SSC scales were excluded. The mean value of living annexin V+ cells cultured under basal conditions was set as 100%.

Apolipoprotein and Viability Measurements: Measurement of DNA Intercalosomal Splicing

Induction of apoptosis-associated DNA cleavage into intercalosomal fragments and their accumulation in cytoplasma was measured by commercially available ELISA (Roche Diagnostics GmbH, Mannheim, Germany). Cellular lysates were prepared according to the manufacturer’s instructions. The OD values were read after 30 minutes at 405 to 490 nm. The mean OD value of apoptotic control cells was set as 100%.

The MTT Assay

The reduction of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] salt was determined with Vybrant; Cell Proliferation Assay kit (Molecular Probes). Cells were labeled with 10 μL per well of MTT solution; after 4 hours, 100 μL per well of sodium dodecylsulfate-HCL (Mol Probes) was added. The absorbance was read at 562 nm. The mean OD value of HUVECs under basal conditions was set as 100%.

Determination of Annexin V Levels and Anti-Annexin IgG Antibodies

Levels of circulating annexin V and anti-annexin V IgG antibodies were determined by ELISA using high-sensitivity commercial kits (Hemochrom Diagnostica GmbH, Essen, Germany) according to manufacturer’s recommendations. The detection range for annexin V was indicated as 0.2 to 20 ng/mL.

Protein G Affinity Column Chromatography and Pre-absorption Experiment

Pooled sera with a high ability to inhibit annexin V binding to endothelium were 0.45-μm filtered and diluted with equal volumes of endothelial basal medium. The HiTrap Protein G HP, 1 mL column with binding capacity of 25 mg human IgG/mL gel from Amersham Biosciences (Upsala, Sweden), was used according to manufacturer’s instructions. The IgG fraction was obtained by eluting the column with 0.1 mol/L glycine–HCl (pH 2.7). For neutralization, 1 mol/L Tris–HCl (pH 9.0) was used. Effluates, eluate, and reconstituted samples were used for incubation with HUVECs on the day of separation at 1:10 dilution in SFM.

Sera (diluted in endothelial basal medium 1:10) were added to 96-well flat-bottom plates (Immulon 1B;Thermo Labsystems) coated with either 100 μg/mL of cardiolipin (AvantiPolar Lipids, Inc, Alabaster, Ala) in ethanol or an unrelated antigen, tetanus toxoid (Statens Serum Institute, Denmark), and incubated overnight at 4°C, leading to a 30% to 50% decrease in antibody levels against these antigens as determined by ELISA essentially as described.15

Immunohistochemical Staining of Human Atherosclerotic Plaques

Immunostaining was performed on advanced carotid plaques obtained from 11 patients undergoing endarterectomy, characterized previously.2 In addition, we obtained one atherosclerotic plaque from femoral artery of an SLE case patient who underwent operation because of clinical symptoms. As a control, unrelated macroscopically healthy mesenteric artery was used. The cryostat sections were fixed for 20 minutes in 2% formaldehyde in phosphate-buffered saline (Merek, Darmstadt, Germany) at 4°C and stored at −70°C. After blocking of endogenous peroxidase, sections were incubated overnight with monoclonal anti-annexin V antibody (Alexis Biochemicals, Corp, Lausen, Switzerland) of mouse type IgG3, anti-CD68 (DakoCytomation, Glostrup, Denmark), or anti-CD31 (Monosan, Uden, the Netherlands) both of mouse type IgG1, irrelevant mouse IgG2a, or rabbit IgG (Serotec Ltd, Oxford, UK) and incubated with either 100 μg/mL of cardiolipin (AvantiPolar Lipids, Inc, Alabaster, Ala) in ethanol or an unrelated antigen, tetanus toxoid (Statens Serum Institute, Denmark), and incubated overnight at 4°C, leading to a 30% to 50% decrease in antibody levels against these antigens as determined by ELISA essentially as described.15

Statistical Methods

The statistics were computed using Stat View software (SAS Institute AB, Göteborg, Sweden). Skewed continuous variables were logarithmically transformed. Study groups were compared using ANOVA for continuous variables, with paired t test as post hoc analysis. Correlation coefficients were calculated using simple regression or, for not normally distributed variables, Spearman rank correlation. The significance level was set at P<0.05.

Results

Clinical, Metabolic, and Other Laboratory Characteristics

Basic clinical and metabolic characteristics have previously been reported.5 As reported, SLE cases also had elevated...
very-low-density lipoprotein and decreased high-density lipoprotein cholesterol concentrations, raised acute phase reactants including sedimentation rate, alfa-1-antitrypsin, C-reactive protein, and orosomucoid, and elevated lupus anticoagulants and homocysteine levels as compared with SLE controls and population controls. Blood pressure, cumulative or present smoking, and prevalence of diabetes mellitus did not differ significantly between the 3 groups. SLE cases had taken a higher cumulative prednisolone dose ($P < 0.05$), but there was no significant difference in present prednisolone dosage as compared with SLE controls. Therapy with lipid-lowering agents (statins in all cases), antihypertensives, low-dose aspirin, warfarin, and azathioprine were more common among SLE cases.

SLE cases had significantly higher levels of circulating annexin V as compared with SLE controls (3.6 ± 3.0 versus 2.3 ± 1.6 ng/mL; ±SD, $P = 0.03$) and population controls (3.6 ± 3.0 versus 1.84 ± 1.3 ng/mL; ±SD $P = 0.004$). Annexin V levels in supernatants after culture with plasma from the individuals tested did not show any significant differences between SLE cases versus SLE controls versus PC (1.1 ± 0.6 versus 1.5 ± 1.2 versus 1.3 ± 1.2 ng/mL; ±SD).

**Effect of Plasma on Annexin V Binding to HUVECs and on Cell Toxicity Measurements**

The frequency of annexin V$^+$ HUVECs was determined as percentage of annexin V$^+$/PI$^-$ cells on a bivariate dot plot or percentage of annexin V$^+$ cells on a histogram. Annexin V binding to HUVECs incubated with plasma from SLE cases was significantly lower than SLE controls and population controls (Figure 1). There were no differences in annexin V binding when serum was used instead of heparin plasma (data not shown). Simultaneous evaluation of plasma membrane integrity with the vital dye—PI has shown no differences in PI$^{bright+}$ cells, indicating no cytotoxic effect of plasma on endothelium (data not shown). There was no significant difference in HUVEC capability to reduce MTT component (SLE cases: 130.1 ± 16.7; SLE controls: 126.6 ± 16.2; PC: 131.3 ± 15.7; % of control; mean ±SD). Induction of apoptosis-specific DNA fragments was not observed to be different between 3 groups (SLE cases: 1.35 ± 0.29; SLE controls: 1.25 ± 0.3; PC: 1.35 ± 0.42; % of control; median ± interquartile range)

**Associations Between Annexin V Binding and Clinical and Laboratory Measurements**

There was a positive significant association among SLE cases between annexin V binding to endothelium and atherosclerosis, as measured by IMT (Figure 2A) and plaque presence. (Figure 2B) However, this was not the case among SLE or population controls. As reported earlier, activity in the TNF system was raised among SLE cases and associated with dyslipidemia in this group. Exclusively among SLE cases, annexin V binding was negatively associated with TNFR2 ($r = 0.50$, $P = 0.002$) and trend-wise negatively with TNFR1 ($r = 0.34$, $P = 0.08$), but there were no significant associations with TNF. There were no significant associations between annexin V binding and inflammatory markers (C-reactive protein, erythrocyte sedimentation rate, etc), SLAM, SLICC, lupus anticoagulants or aCL, lipid measurements (high-density lipoprotein, low-density lipoprotein, triglycerides), plasma glucose, insulin levels, or estimated insulin resistance (data not shown).

**Effect of IgG Depletion and Antibodies Against Cardiolipin on Annexin V Binding**

Depletion of IgG isotype of immunoglobulins from sera resulted in up to 2.7-fold increase in median fluorescence...
intensity of annexin V binding as compared with that of the complete serum (Figure 3A). Reconstitution of IgG fraction to the depleted sera decreased the median fluorescence intensity comparable to that of complete sera (Figure 3A). When sera with high capacity to induce decreased annexin V binding was pre-incubated with cardiolipin, median fluorescence intensity of annexin V binding increased (Figure 3B). In contrast, unrelated antibodies against tetanus toxoid did not influence annexin V binding to HUVECs (Figure 3B).

**Annexin V Staining in Atherosclerotic Plaques**

Immunohistochemical analysis revealed regions staining for annexin V in all 12 examined lesions, including 1 from an SLE patient (Figure 4L and 4M). Four areas were intensively stained: (1) cell (macrophage)-rich regions (Figure 4B and 4C); (2) small vessels of the shoulder region (Figure 4F); (3) endothelial luminal surface (Figure 4H); and (4) atheromatous core (Figure 4J). No staining was observed in a healthy mesenteric artery (Figure 4A).

**Discussion**

The cause of clinically significant cardiovascular events is not mainly atherosclerosis per se, but rupture of unstable plaques, leading to superimposed thrombosis and occlusion of the vascular lumen, as in MI and stroke. Animal models for atherosclerosis have increased our knowledge about underlying mechanisms in atherogenesis, but plaque rupture and CVD do not develop in animals as they do in humans. Human studies of SLE-related CVD could therefore give insight into the involvement of inflammation and autoimmunity in atherothrombosis in general and into its being an important clinical problem.

One major finding in this report is that binding of annexin V to endothelial cells is significantly decreased when endothelial cells are cultured in the presence of plasma from patients with SLE and CVD as compared with SLE patients with no history of CVD and with controls.

Annexin V can form 2-dimensional crystals on lipid bilayers containing negatively charged phospholipids, such as phosphatidylserine, and it has been suggested that this shield...
could have antithrombotic properties.\textsuperscript{18} Patients with anti- phospholipid antibody syndrome are prone to miscarriage, which is often caused by microthrombosis of placenta. Annexin V binding to and covering trophoblast surface from the interaction with coagulation factors could be disrupted by some aPL bound to the thrombogenic phospholipids, thus exposing a strongly procoagulative surface.\textsuperscript{18} Our findings suggest that decreased annexin V binding may be an underlying cause also of atherothrombosis in large arteries in SLE.

We also demonstrate that the inhibitory capacity of plasma from SLE cases to a large extent resides in the IgG fraction and that antibodies against cardiolipin, the most commonly measured antiphospholipid antibody, have the capacity to inhibit annexin V binding. This is in line with previous studies indicating that antibodies against phospholipids could disrupt the antithrombotic annexin V binding.\textsuperscript{12,19}

Our data indicate that annexin V levels are raised in plasma from SLE cases, which could depend on displacement of annexin V and/or raised production, although relatively little is known about the role of circulating annexin V. There were no significant differences between levels of annexin V in supernatants from HUVECs cultured with serum from the different groups, and the amounts of annexin V detected were low.

We excluded the possibility that the effect of plasma in this study of annexin V binding only reflects different degrees of apoptosis induced by the plasma by determining apoptosis and cell death using 3 different methods: DNA splicing to evaluate apoptosis-specific fragments, MTT assay to evaluate function of cellular reductases, and PI staining to evaluate membrane permeability.

Cultured HUVECs are extensively used as an in vitro model of vascular endothelial cells, although recent evidence indicates that there may be phenotypic variation in endothelial cells from different locations.\textsuperscript{20} However, annexin V binding is commonly used for studies on many different cell types, and previous observations have demonstrated a similar mechanism for thrombosis using cells also from the microvascular system (placenta).\textsuperscript{18} We therefore believe HUVECs are still useful as a model for endothelial cells in this context, although further studies using coronary or carotid endothelial cells are of interest.

Another intriguing finding in this study is that annexin V binding was strongly and positively associated with atherosclerosis as measured by IMT and plaque presence among SLE cases. We previously demonstrated that SLE cases had slightly but significantly increased IMT.\textsuperscript{5} Recent publications also support the notion that SLE patients have increased atherosclerosis.\textsuperscript{21,22} It may thus seem paradoxical that SLE patients with a history of CVD have antibodies that apparently inhibit annexin V binding and that atherosclerosis is associated with annexin V binding. It should be noted, however, that these patients are in an advanced stage of disease, with a history of severe manifest CVD. Likewise, all immunostained plaques were from patients with manifest CVD. It is possible than annexin V only contributes to growth of atherosclerotic plaques at a late stage of disease, in which apoptosis, fissures, and microthrombi, as well as endothelial cell activation, are common features of the plaque, leading to annexin V binding to the exposed surfaces.

Also, other factors like estrogen could inhibit atherosclerosis but still increase the risk of CVD, with one suggested explanation being an adverse effect on advanced plaques.\textsuperscript{23} Apoptosis may also prevent atherosclerosis by reducing the cellular content and neointimal thickening of the plaque. However, increased apoptosis may render the plaques more vulnerable, especially at the shoulder region where the fibrous cap is thin.\textsuperscript{24} Antibodies against oxidized low-density lipoprotein may also have dual roles because they may be protective at earlier disease stages but pathogenic at later stages.\textsuperscript{5,15}

Atherosclerosis per se could be regarded as a response to injury mechanism,\textsuperscript{25} and it is thus possible that annexin V could contribute both to protection against atherothrombosis and to increased atherosclerosis as determined by IMT at a late stage of disease.

Annexin V is widely used as a tool in apoptosis research, but its physiological role has not been studied extensively in relation to vascular biology. The pattern of immunostaining with annexin V, which we present here, is in line with our findings showing strong correlation between annexin V binding to endothelium and IMT in SLE cases. Also, the intensively stained small vessels in the shoulder region of the plaque might indicate predisposition to microthrombosis and shoulder region destabilization leading to rupture and acute events. Of note is the fact that 11 plaques used for immunostaining are from the general population, indicating that annexin V binding is of importance per se and not only in SLE.

We have recently suggested that raised activity of the TNF-α system is strongly associated and may contribute to dyslipidemia, CVD, and disease activity in general in SLE.\textsuperscript{6,26} In the present study, an association between soluble tumor necrotic factor receptors and decreased annexin V binding is reported. Circulating TNF receptors are shed by the endothelium and may thus reflect endothelial activation and/or dysfunction.\textsuperscript{27} Decreased annexin V binding and an activated endothelium may act in concert in these patients, promoting thrombotic events.

Another putative mechanism through which annexin V binding may be related to CVD is by interfering with phospholipase A\textsubscript{2} (PLA\textsubscript{2}), an inflammatory enzyme pivotal in the prostaglandin synthesis, and in generation of inflammatory phospholipids, like platelet-activating factor and lyso-phosphatidylcholine.\textsuperscript{28,29} Much of the pro-inflammatory and immune stimulatory effects of oxidized low-density lipoprotein are caused by these phospholipids. Furthermore, PLA\textsubscript{2} is present in atherosclerotic lesions.\textsuperscript{30,31} Annexin V can inhibit PLA\textsubscript{2} both in its cytosolic and soluble forms,\textsuperscript{32} and an intriguing possibility is therefore that inhibition of annexin V binding caused by a factor in plasma from CVD patients may increase PLA\textsubscript{2} activity, leading to raised production of inflammatory lipids and risk of atherothrombosis. Likewise, annexin V binds to proteoglycans in the arterial wall, which is also the case with modified low-density lipoprotein, which may be an important early step in atherogenesis.\textsuperscript{33} If low-density lipoprotein binding to the artery wall is inhibited by annexin V, this could represent yet another mechanism by which annexin V could decrease the risk of atherothrombosis.
In a previous elegant study, it was demonstrated that arterial thrombosis could be inhibited by recombinant annexin V in a rabbit carotid artery injury model. Our findings add further support to the notion that annexin V has anti-thrombotic properties. If annexin V binding could be restored, eg, by giving antibodies neutralizing the annexin V-inhibiting antibodies, this could represent a novel therapeutic principle against atherothrombosis. Likewise, annexin V per se or components thereof may be used as a therapeutic agent that could decrease the risk of plaque rupture and atherothrombosis by covering exposed procoagulant surfaces in the plaque.

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References
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