Degradation of neutrophil extracellular traps is decreased in patients with antiphospholipid syndrome

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Abstract

Objective

A decreased ability to degrade neutrophil extracellular traps (NETs) is seen in a subgroup of patients with systemic lupus erythematosus (SLE) and correlates with the presence of autoantibodies. Antiphospholipid syndrome (APS) can develop secondary to SLE or as a primary disease. In the current study we investigated the ability of sera from patients with APS to degrade NETs. The presence of antibodies against NETs and neutrophil remnants were also determined in the same patients.

Methods

In the study, 106 patients with APS (73 primary and 33 secondary), 76 patients with systemic sclerosis (SSc) and 77 healthy donors as control samples were included. NETs generated from neutrophils isolated from healthy volunteers were incubated with patient sera, followed by measurement of degraded NETs or deposited IgG.

Results

Sera of APS patients had a decreased ability to degrade NETs compared to healthy controls, with no difference between primary and secondary APS. Sera from SSc patients did not differ significantly from healthy controls in the ability to degrade NETs. A decreased degradation of NETs correlated weakly to increased levels of antibodies against NETs/ neutrophil remnants in patients with primary APS, but stronger in patients with secondary APS.

Conclusion

The ability to degrade NETs is decreased in a subgroup of patients with APS and is associated with antibodies against NETs and specific clinical manifestations in those patients.

Key words

neutrophils, extracellular space, antiphospholipid syndrome, autoantibodies, systemic sclerosis

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Introduction

Neutrophil extracellular traps (NETs) consist of chromatin covered with antimicrobial proteins (1). Formation of NETs is dependent on generation of reactive oxygen species (2) and constitutes a novel way for how neutrophils can help to fight infections. In serum, NETs are degraded by DNase-I (3). Interestingly, a subgroup of patients with systemic lupus erythematosus (SLE) fails to degrade NETs possibly due to autoantibodies (3, 4). The interest in NETs for involvement in SLE and other autoimmune diseases is growing rapidly (5, 6). Thus, we wanted to investigate if degradation of NETs was impaired in other diseases than SLE, which are also characterised by the presence of autoantibodies. In antiphospholipid syndrome (APS) autoantibodies are found mainly against phospholipids and cell surface proteins such as β2-glycoprotein 1 (β2-GP1) (7) and lead to an increased risk of thrombosis in the patient. APS can also develop secondary from other diseases, notably SLE. In the current study we investigated whether patients with APS degrade NETs and if any of the antibodies produced in APS could recognise NETs. Additionally, we investigated the NET-degrading abilities of patients with systemic sclerosis (SSc). Characteristic symptoms of SSc include excessive fibrosis, mainly in the skin, as well as microvascular injuries. The mechanisms are however not completely known (8). SSc occurs in two forms, limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc), depending on the distribution of skin fibrosis. Autoantibodies are also generated in SSc and an overlap with SLE is sometimes observed.

Material and methods

Patients and sera

Sera from patients with SSc and healthy controls were collected at Skåne University Hospital (Sweden). SSc patients fulfilled the American College of Rheumatology (ACR) criteria for SSc (9) and were classified as dcSSc or lcSSc on the grounds of skin involvement using the modified Rodnan skin score (10). None of the healthy controls suffered from any known diseases or received medication. Sera from patients with APS comprised part of the Serbian National Cohort Study (Serbia) (11) and were diagnosed according to the 2006 revised Sydney criteria for APS (12). The regional ethics committees in Belgrade and Lund approved the study and, in accordance with the Helsinki declaration, all patients gave their informed consent to participate in the study. The clinical manifestations and characteristics of patients and controls are summarised in Table I.

Laboratory tests

Sera from all patients with APS were analysed for levels of serum factors, such as C-reactive protein, by routine biochemistry. Full blood cell counts were determined and thrombosis was evaluated as previously described (11). Anti-cardiolipin, anti-β2-GP1 as well as anti-DNA antibodies, were measured by enzyme-linked immunosorbent assay (Binding Site). Antinuclear antibodies were determined by indirect immunofluorescence by using mouse liver and HEp-2 cell substrate.

NET-degradation and IgG deposition

Freshly isolated neutrophils from healthy volunteers were induced to produce NETs as previously described (4). After induction, degradation of NETs by 10% patient or control sera was quantified using PicoGreen (Invitrogen) as previously (4). To reduce intra-assay variability, degradation of NETs was normalised to the mean of an internal control consisting of pooled normal human serum. To determine IgG deposition 2% patient or control sera in PBS with 4 mM EDTA were added to NETs. IgG deposition was quantified using goat anti-human IgG Alexa Fluor 647 (Invitrogen) and NETs were stained using Sytox green (Invitrogen) as previously (4). IgG deposition was calculated as a ratio of IgG to NETs signal. IgG deposition was also confirmed using a LSM 510 Meta confocal microscope (Zeiss).

Statistical analysis

Statistical significance of differences was tested for multiple groups using

Table I. Characteristics and distribution of clinical manifestations in the different patient groups included in the study.

Patient characteristics or clinical manifestation	Primary APS	Secondary APS	SSc	Controls
Number of patients (n)	73	33	76	77
Age; median (range)	42 (21-79)	52 (27-80)	50 (18-77)	42 (19-77)
Females	62 (85%)	29 (88%)	58 (76%)	62 (81%)
Smoking	28 (38%)	8 (24%)	NA*	NA
Disease duration in years; median (range)	5 (0.1-23)	9 (0.5-28)	1 (0.2-3)	NA
Fetal losses	59 (81%)	21 (64%)	NA	NA
Presence of anti-CL* IgG	27 (37%)	23 (70%)	NA	NA
Presence of anti-CL IgM	51 (70%)	24 (73%)	NA	NA
Presence of anti- β2-GP1* IgG	24 (33%)	19 (58%)	NA	NA
Presence of anti- β2-GP1 IgM	35 (47%)	23 (70%)	NA	NA
Elevated C-reactive protein	14 (29%)	8 (24%)	NA	NA
Presence of anti-DNA	0 (0%)	12 (37%)	NA	NA
Presence of anti-nuclear antibodies	13 (18%)	29 (88%)	57 (75%)	NA
Presence of lupus anticoagulant	38 (53%)	13 (39%)	NA	NA
Presence of thrombosis	36 (49%)	21 (64%)	NA	NA
dcSSc	NA	NA	38 (50%)	NA

*CL: cardiolipin; β2-GP1: β2 glycoprotein 1; NA: not available.

Kruskal-Wallis test with Dunn's multiple comparisons post-test and correlation of two parameters was calculated according to Spearman, both in Prism5 (GraphPad). For comparison of two groups with continuous data Wilcoxons test was used and for nominal data Pearsons χ^2 test was used, both in JMP 7 (SAS). Manifestations only present in one positive patient were excluded.

Results

The ability of serum from patients with primary and secondary APS to degrade NETs was determined and compared to healthy controls. Sera from a cohort of patients with lcSSc or dcSSc were also evaluated (Fig. 1A). A cut-off was set below 3 SDs from the mean of the healthy controls and defined low-

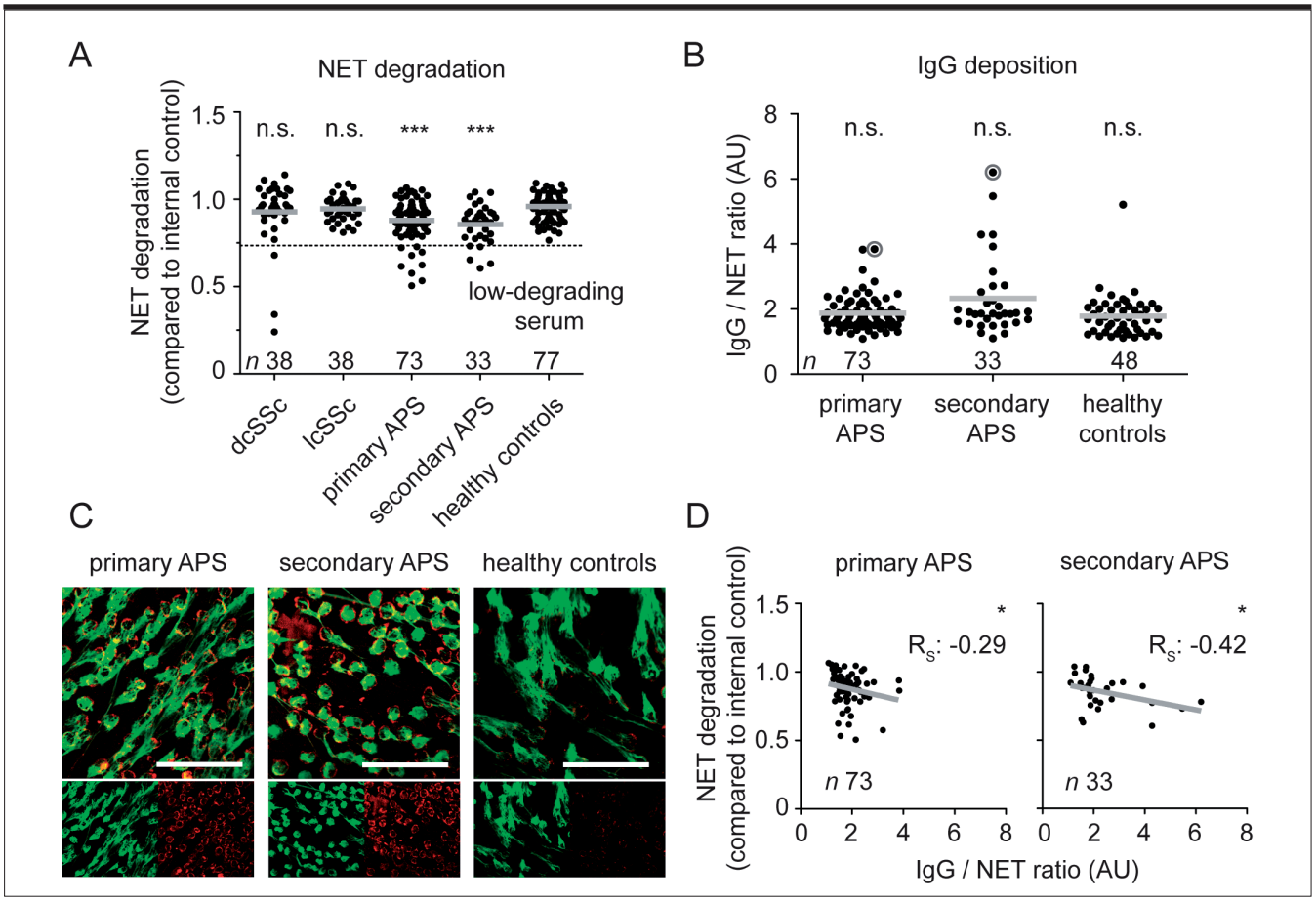

Fig. 1. Sera of patients with APS have a decreased ability to degrade NETs.
A. Degradation of NETs, for patients with dcSSc, lcSSc, primary APS and secondary APS compared to healthy controls. Sera from patients with primary and secondary APS showed decreased degrading abilities compared to sera from healthy controls. **B**. Deposition of IgG on NETs from patient sera with primary or secondary APS compared to healthy controls. None of the patient groups had significantly increased IgG deposition on NETs compared to the healthy controls. Dark grey circles indicate samples displayed as confocal images where NETs are visualised in green and IgG deposition in red, scale bar indicates 100 μm (**C**). **D**. Correlation of NET-degradation and presence of antibodies against NETs was determined. Weak correlation was found in patients with primary APS whereas in patients with secondary APS stronger correlation was observed. Correlation coefficient is displayed as R_S, significance of differences or of correlation is displayed as; ***, $p<0.001$; *, $p<0.05$; n.s., not significant.

Table II. Distribution of clinical manifestations among patients with low and normal NET degrading ability.

	Primary APS			Secondary APS		
Manifestation	Low	Normal	*p*	Low	Normal	*p*
Rheumatoid factor	3 (33%)	2 (3%)	**0.0008***	0 (0%)	7 (25%)	0.2078
Ro antibodies	2 (22%)	1 (2%)	**0.0041**	1 (20%)	13 (46%)	0.2707
Anti-nuclear antibodies	1 (11%)	12 (19%)	0.5749	3 (60%)	26 (93%)	**0.0381**
Arthalgias	4 (44%)	9 (14%)	**0.0257**	3 (60%)	16 (57%)	0.9052
Cardiac vegetations	0 (0%)	4 (6%)	0.4405	0 (0%)	14 (50%)	**0.0372**

*Numbers in bold indicate significant difference in distribution according to Pearsons χ^2 test.

degrading sera. Sera from 9 (12%) of patients with primary and 5 (15%) with secondary APS were low-degrading. Among patients with dcSSc, 3 patients (8%) had decreased ability to degrade NETs whereas no patient with lcSSc failed to degrade NETs.

Patients with APS develop antibodies against cellular constituents (7). Therefore, levels of antibodies specific for NETs and neutrophil remnants were measured in the patient sera (Fig. 1B). No statistically significant difference could be observed. However, some patients with secondary APS clearly displayed increased levels of autoantibodies against NETs and neutrophil remnants, as did one healthy control. Deposited IgG on NETs was confirmed by confocal microscopy (Fig. 1C). As judged by confocal images, autoantibodies from primary APS patients appeared to be directed mainly against the neutrophil remnants, whereas autoantibodies from secondary APS patients seemed to have antibodies also against NET structures. APS patients positive for anti-DNA antibodies further showed higher titers of anti-NET antibodies (data not shown).

A correlation between decreased NET-degradation and elevated levels of antibodies against NETs has previously been established in SLE (3). Therefore we evaluated whether a similar correlation could be observed in patients with APS. For patients with primary APS only, a weak but statistically significant correlation was observed. For patients with secondary APS a slightly stronger and statistically significant correlation was observed (Fig. 1D).

We further investigated if a decreased NET-degradation in APS is associated with any clinical manifestations (Table II). In primary APS, a decreased degradation of NETs was associated with presence of Ro-antibodies, rheumatoid factor as well as with arthralgias. In secondary APS, a decreased degradation was inversely associated with anti-nuclear antibodies and cardiac vegetations.

Discussion

A decreased ability to degrade NETs is important for the pathogenesis of SLE (3, 4). In the current study we found that sera from 13% of patients with APS showed a decreased ability to degrade NETs. In general, the reduction of NETs-degrading abilities was less pronounced for APS patients compared to SLE (3, 4). Patients with lcSSc had normal ability to degrade NETs while a limited number of dcSSc patients displayed a decreased ability to degrade NETs.

In the APS patients, a decreased degradation of NETs did not correlate with presence of autoantibodies, against β2-GP1, cardiolipin or lupus anticoagulant (data not shown). These autoantibodies seem not to be a consequence or cause of failed NET-degradation. However, some patients with APS generated antibodies that are able to recognise NETs or neutrophil remnants. These patients were often also positive for anti-nuclear antibodies as well as antibodies against DNA. These antibodies could potentially affect the degradation of NETs as shown for SLE (3, 4). The majority of patients with secondary APS suffered from SLE as the primary condition. This may explain the stronger association of NET-degradation and NET-specific antibody titers in these individuals.

It has been previously implied that NETs are important in both thrombosis (13) and foetal losses (14). However, in the current study, no significant associations between the above mentioned manifestations and degradation of NETs were observed. Thrombosis and foetal losses were recorded as historic events and the ability to degrade NETs at the event could not be determined. Associations could hence not be ruled out. In SLE patients, the ability to degrade NETs changed with disease activity (4). Longitudinal studies could elucidate if similar relation appears also in APS. The extent of NET-generation in APS may also be of interest since neutrophils appear to generate NETs more easily when exposed to inflammatory environment (15).

In this study we have shown that a subset of patients with APS presented a decreased ability to degrade NETs. In secondary APS, this was associated with elevated titers of antibodies against NETs or neutrophil remnants. This indicates different mechanisms for the decreased ability to degrade NETs in primary and secondary APS, and show necessities of further investigation.

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