ANTI-PLASMINOGEN ANTIBODIES COMPROMISE FIBRINOLYSIS AND ASSOCIATE WITH RENAL HISTOLOGY IN ANCA-ASSOCIATED VASCULITIS

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ABSTRACT

Antibodies recognizing plasminogen, a key component of the fibrinolytic system, associate with venous thrombotic events in PR3-ANCA vasculitis. Here, we investigated the prevalence and function of anti-plasminogen antibodies in independent UK and Dutch cohorts of patients with ANCA-associated vasculitis (AAV).

We screened immunoglobulin isolated from patients (AAV-IgG) and healthy controls by ELISA. Eighteen of 74 (24%) UK and 10/38 (26%) Dutch patients with AAV had anti-plasminogen antibodies compared with 0/50 and 1/61 (2%) of controls. We detected anti-plasminogen antibodies in both PR3-ANCA- and MPO-ANCA-positive patients.

In addition, we identified anti-tissue plasminogen activator (tPA) antibodies in 13/74 (18%) patients, and these antibodies were more common among patients with anti-plasminogen antibodies (p = 0.011).

Eighteen of 74 AAV-IgG (but no control IgG) retarded fibrinolysis in vitro, and this associated with anti-plasminogen and/or anti-tPA antibody positivity. Only 4/18 AAV-IgG retarded fibrinolysis without harboring these antibodies; dual positive samples retarded fibrinolysis to the greatest extent.

Patients with anti-plasminogen antibodies had significantly higher percentages of glomeruli with fibrinoid necrosis (p < 0.05) and cellular crescents (p < 0.001) and had more severely reduced renal function than patients without these antibodies.

In conclusion, anti-plasminogen and anti-tPA antibodies occur in AAV and associate with functional inhibition of fibrinolysis in vitro. Seropositivity for anti-plasminogen antibodies correlates with hallmark renal histologic lesions and reduced renal function. Conceivably, therapies that enhance fibrinolysis might benefit a subset of AAV patients.
INTRODUCTION

The antineutrophil cytoplasmic antibody (ANCA)-associated small vessel vasculitides (AAV) are systemic disorders that may or may not incorporate a granulomatous component, especially in upper airways, and that may occasionally manifest as renal limited vasculitis. For the classical ANCA-types anti-proteinase 3 (PR3) and anti-myeloperoxidase (MPO), cumulative data from clinical and experimental studies strongly supports their pathogenic role.\textsuperscript{1,2}

Recently, it was reported that a subset of patients with PR3-ANCA harbor antibodies against human plasminogen.\textsuperscript{3} These anti-plasminogen antibodies recognize a structure within the protease domain and their presence correlated with venous thromboembolic events (VTEs), suggesting functional interference with coagulation and/or fibrinolysis.\textsuperscript{3} The relationship between anti-plasminogen antibodies and coagulation may also be directly relevant to glomerular injury and the evolution of fibrinoid necrosis, a hallmark lesion of AAV. In AAV, vascular injury is mediated by ANCA-induced neutrophil and monocyte activation\textsuperscript{4} and characterized by the influx of inflammatory cells with fibrinoid necrosis affecting glomeruli and occasionally small arteries. It is possible that fibrinoid necrosis is in fact an aberrant repair mechanism that follows vascular injury. This hypothesis would account for histologic and compositional similarities between fibrinoid necrosis and conventional fibrin clots\textsuperscript{5,6} and explain the importance of the fibrinolytic system in renal injury, as shown by animal models of rapidly progressive glomerulonephritis.\textsuperscript{7,8} Under these circumstances, down-regulation of fibrinolysis by anti-plasminogen antibodies in AAV patients would promote persistent or extensive fibrinoid necrosis.

In this study, we investigated the presence and functionality of anti-plasminogen antibodies in independent cohorts of United Kingdom (UK) and Dutch patients with AAV. We show that immunoglobulin from AAV patients retards fibrinolysis and that seropositivity for these antibodies correlates with reduced renal function and the presence of hallmark histopathologic renal lesions. We also detected antibodies against the partially homologous serine protease tissue plasminogen activator (tPA) in some patients.
METHODS

Study population

UK cohort

Stored frozen plasma exchange (PEX) fluid from 74 consenting AAV patients treated at University Hospital Birmingham was studied. All patients satisfied Chapel Hill Conference consensus criteria and received PEX for severe renal disease (serum creatinine ≥ 500 μmol/L or dialysis dependency) and/or pulmonary hemorrhage. Pulsed intravenous steroids were not routinely used, and the majority of patients started plasma exchange concurrently with oral corticosteroids. Cyclophosphamide (daily oral or pulsed intravenous) was initiated at the same time or immediately after corticosteroids and plasma exchange. Plasma exchange effluent was collected at the time of the first exchange.

ANCA positivity and specificity of paired serum from these patients were determined at the time of diagnosis by indirect immunofluorescence and ELISA respectively, at the Clinical Immunology Laboratory, University of Birmingham, Birmingham, UK. Twelve ANCA-negative patients with other renal diseases (anti-glomerular basement membrane [GBM] disease, n = 7; systemic lupus erythematosus, n = 3; myeloma, n = 1; antibody-mediated renal allograft rejection, n = 1) who had also undergone PEX were used as a disease control cohort. Laboratory personnel (n = 50) served as a healthy control cohort, and serum was separated from peripheral blood collected after the study started.

IgG was purified from patient PEX fluid and healthy control serum by protein G affinity chromatography (GE Healthcare, Buckinghamshire, UK) and eluted with 0.1 M glycine (pH 2.7). Concentrations were determined by spectrophotometry. IgG samples were purified at the time of conducting this study, and results of the original ANCA testing were verified by retesting derived IgG samples in the Clinical Laboratory. AAV-IgG were also screened by ELISA for anti-cardiolipin antibodies using a kit available from the Binding Site (Birmingham, UK).

For separate assays using serial samples collected over time, serum from two AAV patients was available. For these experiments, sera from 127 healthy blood donors were used as the control population.
**Dutch cohort**

A total of 38 patients with AAV of whom serum or PEX fluid was available were recruited. The majority of patients (33/38) were seen in one nephrology unit (Meander Medical Center, Amersfoort, the Netherlands). All patients met the Chapel Hill criteria for diagnosis of AAV. The ANCA specificity of each patient was determined at Leiden University Medical Center using an ELISA kit from Wieslab (Lund, Sweden). IgG was isolated from patient sera/PEX using the Melon Gel IgG Purification Kit (Pierce Protein Research Products, Thermo Scientific). The integrity of isolated IgG was confirmed through SDS-PAGE and visualization through Coomassie blue staining.

Glomerular filtration rates were estimated (eGFR) at the time of serum/PEX (baseline) and at 1 year, using the four-variable Modification of Diet in Renal Disease equation (MDRD). To evaluate an independent predictive effect of anti-plasminogen antibodies on eGFR at 1 year follow-up, we corrected for the eGFR at baseline. The corrected eGFR at 1 year was defined as the difference between the observed eGFR at 1 year and its linear prediction on the basis of baseline eGFR.

Nineteen patients had a serum sample taken or underwent plasma exchange (PEX) at the same day a renal biopsy was performed. Two independent observers, who were unaware of the clinical data, reviewed all biopsies for glomerular lesions according to a previously designed scoring protocol. For each biopsy silver, Periodic acid-Schiff and Martius Scarlet Blue trichrome stainings were evaluated. The number of glomeruli with specific lesions was calculated as a percentage of the total number of glomeruli for each individual biopsy.

**ELISA**

High binding microtiter plates (Costar, Appleton Woods, Birmingham, UK) were coated overnight with 2.5 μg/ml plasminogen, plasmin (Cambridge Biosciences, Cambridge, UK), tPA (Merck Chemicals, Nottingham, UK) or 5 μg/ml recombinant alpha(IV)NC1 (purified from 293-F cells using the FreeStyle Expression System; Invitrogen, Paisley, UK) at 4°C. Plates were then blocked for 60 minutes at 25°C with Stabilcoat (Diarect AG, Freiburg, Germany) diluted to 50% with sterile water. After washing with buffer containing 1% Tris 1 M (pH 8.0), 2.5% NaCl 3 M and 0.5% Tween-20, test IgG was pipetted (5 μg/ml unless stated) in duplicates into appro-
priate microtiter wells and incubated at 25°C for 2 hours. Wells were washed and incubated (60 minutes at 25°C) with a 1:50,000 dilution of alkaline phosphatase-conjugated donkey anti-human IgG (Jackson ImmunoResearch, Cambridge, UK). All dilutions were performed using sample diluent (1% bovine serum albumin and 0.1% Tween-20). After washing, 4-nitrophenyl phosphate (Sigma-Aldrich, Gillingham, UK) was used as the substrate, and the 96-well microtiter plate was analyzed spectrophotometrically at 405 nm after 120 minutes. Each IgG was tested six times, and where data is expressed as a percentage of positive control, IgG from a separate cohort was used. In assays requiring the use of sera, a 1:100 dilution was used.

For competition assays, dose-response studies were performed to select the lowest concentration of IgG reactive on each antigen (1.25 μg/ml). IgG was preincubated (2 hours at 25°C) with increasing concentrations (0-1 μM) of native soluble plasminogen or plasminogen denatured by heating with 5% β-mercaptoethanol (5 minutes at 100°C). Before use, denatured plasminogen was diluted to the required concentration and residual levels of β-mercaptoethanol were calculated at 0.05%. The integrity of IgG after preincubation with denatured plasminogen was confirmed by SDS-PAGE and visualization through Coomassie blue staining. The subsequent ELISA was performed as described above. For separate assays, plasminogen denatured in this way was also used as the coat antigen.

For alternative competition assays, IgG was preincubated with tPA or plasmin that had been inactivated through mixing with Nα-tosyl-L-lysine chloromethyl ketone-HCl (TLCK, Sigma) according to a protocol previously described. Briefly, 12 μM plasmin was incubated with 6 mM TLCK in phosphate buffered saline (pH 7.4) for 100 minutes at 25°C. Using this method, the proteolytic activity of plasmin is reportedly inhibited in excess of 99%. The inactivation of plasmin was confirmed by SDS-PAGE and Coomassie blue staining after mixing inactivated plasmin with IgG under the conditions required for the ELISA.

**Immunoprecipitation Western**

Two micrograms of plasminogen was immunoprecipitated with 10 μg IgG and 100 μl of protein A (Sigma) and G sepharose (Pierce, Northumberland, UK) beads (50-μl bead volume) in nondenaturating lysis buffer. For control experiments, goat anti-plasminogen antibody (Abcam, Cambridge, UK) and irrelevant goat IgG (Bethyl
Laboratories, Cambridge, UK) were used. Equal volumes of immunoprecipitates were separated on a 10% SDS-PAGE gel, and the membrane was probed with rabbit anti-plasminogen antibody (Abcam) at a dilution of 1:1000 in 5% marvel/TBS-T overnight at 4°C. The membrane was incubated for 1 hour with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (GE Healthcare) diluted 1:10,000. Visualization of bound proteins was carried out using an enhanced chemiluminescence kit (GE Healthcare), and the image of each band was scanned with a densitometer using Genetools by Syngene (Cambridge, UK).

**Fibrinolysis assays**

**UK AAV-IgG samples**

The functional effects of anti-plasminogen antibodies were determined in vitro using a slight modification of a method previously described. Clot formation was initiated by mixing 10 μM fibrinogen with 1 nM thrombin (both from Merck Chemicals). Immediately 100 μl was dispensed into each well of a 96-well microtiter plate. Increases in turbidity during clot formation were measured spectrophotometrically at 405 nm over 60 minutes (25°C). Sample diluent or IgG (final concentration 150 μg/ml) was simultaneously incubated with plasminogen (final concentration 10 μg/ml) for 60 minutes at 25°C prior to mixing with tPA (final concentration 10 nM). Clot dissolution was initiated by the addition of the IgG/plasminogen and tPA solution and measured by reductions in absorbance at 405 nm. All samples were loaded in duplicates and dilutions were performed in diluent containing 50 mM Tris-HCl, 150 mM NaCl and 5 mM CaCl$_2$ (pH 7.5).

In separate experiments IgG (final concentration 150 μg/ml) was incubated with plasmin (final concentration 50 nM) for 60 minutes on ice prior to loading onto the preformed fibrin clots. Each IgG was tested 4 times. During analysis, background absorbance and the time required for clot formation (60 minutes) were subtracted from the final values. Based on the clot lysis curves we chose to analyze the data when 50% fibrinolysis had occurred. The integrity of IgG premixed with plasmin on ice was confirmed through SDS-PAGE and visualization through Coomassie blue staining.

**Dutch AAV-IgG samples**

To determine the effect of anti-plasminogen antibodies on fibrinolysis in vitro, clot lysis experiments were performed. IgG isolated from patient sera/PEX was dialyzed
against triethanolamine (TEA) buffer (0.1% Tween, 50 mM TEA, 100 mM NaCl in MQ). Healthy control IgG (final concentration 0.4 mg/ml), patient IgG (final concentration 0.4 mg/ml) or buffer was preincubated with human Glu-plasminogen (final concentration 0.1 μM, Enzyme Research Laboratories) for 30 minutes at 31°C. After incubation, fibrinogen (final concentration 2.6 mg/ml, Sigma-Aldrich), Factor XIII (final concentration 2 U/ml, Fibrogammin, Behring) and tPA (final concentration 1.5 U/ml, kind gift of Dr. F. Havekate, Gaubius TNO, Leiden) were added, and subsequently, the mixture was added to a 96-well plate containing thrombin (final concentration 10 nM, Sigma-Aldrich) and CaCl₂ (final concentration 10 mM). Clot formation and dissolution were monitored by absorbance at 405 nm. Each reaction was performed in duplicate, and measurements took place overnight. In each experiment, the same healthy control IgG as well as a negative sample only containing TEA buffer were tested. Half-lysis times were calculated for each patient IgG sample and expressed as percentages of the healthy control sample.

**Statistical analysis**

A strict analysis criterion was used to determine whether patient samples reacted with plasminogen or other given antigens and also whether IgG was able to interfere with fibrinolysis. Any values exceeding the mean + 2 SD of the healthy control population were considered positive. These criteria had to be met on > 50% of the occasions tested. Differences between groups were analyzed using one-way ANOVA followed by Bonferroni or Dunnett’s post hoc tests.

A Mann-Whitney U test (two-tailed) was performed to assess differences in renal function and the percentage of glomerular lesions according to anti-plasminogen antibody subgroup. P ≤ 0.05 was considered significant.

All statistical tests were performed using Graphpad Prism (version 4; Graphpad, San Diego, CA) and the SPSS statistical software package for Windows (version 16.0; SPSS, Chicago, IL).
RESULTS

Identification of anti-plasminogen antibodies in AAV-IgG

Anti-plasminogen antibodies were identified in 18/74 (24.3%) UK AAV-IgG, compared with 0/50 healthy control IgG (HC-IgG) (**p < 0.001; Figure 1A). The prevalence of anti-plasminogen antibodies was 24.1% (7/29) in MPO-ANCA- and 24.4% (11/45) in PR3-ANCA-positive patients. Only 1/12 (8.3%) disease control IgG (DC-IgG) was positive for anti-plasminogen antibodies (anti-GBM disease without concurrent ANCA positivity).

To validate this observation, IgG from an independent cohort of Dutch AAV patients was screened: 10/38 (26.3%) harbored anti-plasminogen antibodies, compared with 1/61 (2%) HC-IgG (**p < 0.001). Anti-plasminogen antibodies were detected in three MPO-ANCA and six PR3-ANCA patients, as well as in one ANCA-negative patient.

In contrast, anti-plasmin antibodies were less common: only 5/74 (6.7%) UK AAV-IgG showed anti-plasmin reactivity compared with 1/50 (2%) HC-IgG (*p < 0.05) and 0/12 DC-IgG (Figure 1B). All 5 AAV-IgG positive for anti-plasmin antibodies were amongst the 18 AAV-IgG positive for antibodies against plasminogen. Anti-plasmin antibodies occurred in three MPO-ANCA and two PR3-ANCA patients. Furthermore, the three most reactive anti-plasmin IgG were also the top three most reactive with plasminogen. In the Dutch cohort, 2/38 (5.3%) AAV-IgG harbored anti-plasmin antibodies compared with 1/61 (2%) HC-IgG (p = 0.31). Both of these AAV-IgG samples were dual positive for anti-plasminogen and anti-plasmin antibodies.

Immunoprecipitation Western blotting corroborated the existence of anti-plasminogen antibodies in AAV-IgG (Figure 1C). Three AAV-IgG that were positive for anti-plasminogen antibodies by ELISA effectively immunoprecipitated plasminogen in contrast to two HC-IgG that did not immunoprecipitate plasminogen.
Figure 1. Identification of anti-plasminogen and anti-plasmin antibodies in AAV patients. (A) Representative plasminogen ELISA for UK AAV patients. ***p < 0.001 AAV IgG compared to healthy control IgG. (B) Representative plasmin ELISA for UK AAV patients. *p < 0.05 AAV IgG compared to healthy control IgG. (C) Presence of anti-plasminogen antibodies was confirmed by IP Western blot with quantitative densitometry. Plasminogen was immunoprecipitated with (lane 1) goat anti-plasminogen positive control, (2) species specific IgG negative control, (3-5) patient IgG, (6 & 7) healthy control IgG, (8) plasminogen control.
**Specificity of antibody binding to plasminogen**

Competitive inhibition assays also supported a specific interaction between AAV-IgG and plasminogen (Figure 2 A, solid bars). Premixing anti-plasminogen antibody-positive UK AAV-IgG (n = 4) samples with soluble plasminogen inhibited binding to plasminogen used as the coated antigen in a concentration-dependent manner. Inhibition was 60.1% ± 0.87% using soluble plasminogen at 1 µM (**p < 0.01), which is comparable to the inhibition obtained by premixing affinity-purified polyclonal rabbit antibody with 1 µM soluble plasminogen (70.1% ± 5.2%). In contrast, premixing with 1 µM denatured plasminogen inhibited AAV-IgG binding to native plasminogen by only 17.93% ± 0.99% (Figure 2 A, open bars; **p < 0.01). Similarly, binding of anti-plasminogen antibody-positive Dutch AAV-IgG (n = 4) to coated plasminogen was inhibited by 58.8% ± 1.59% (**p < 0.01) after preincubation with soluble plasminogen, and 11.4% ± 1.07% (*p < 0.05) with denatured plasminogen.

When denatured plasminogen acted as coat antigen, 0/74 UK and 1/38 Dutch AAV-IgG exhibited significant binding (Figure 2 B). In contrast, denatured plasminogen was recognized by the affinity-purified polyclonal rabbit antibody compared with negative control rabbit antibody (***p < 0.001). This implies that conformational epitopes predominate in AAV-IgG, whereas the rabbit anti-plasminogen antibody contains some dominant linear epitopes.

As a negative control for AAV-IgG, reactivity towards an unrelated autoantigen was also examined. Only 1/18 (5.5%) anti-plasminogen antibody-positive UK AAV-IgG bound to recombinant alpha3(IV)NC1 compared with 1/50 (2%) HC-IgG. No anti-plasminogen antibody-positive Dutch AAV-IgG reacted with alpha3(IV)NC1. In contrast 7/7 IgG from patients with anti-GBM disease bound alpha3(IV)NC1 as anticipated (***p < 0.001).
Figure 2. Specificity of antibody binding to plasminogen. (A) IgG (1.25 μg/ml) from four UK patients harboring antibodies against plasminogen was premixed with increasing concentrations of native (solid bars) or denatured (open bars) soluble plasminogen for two hours prior to the plasminogen assay. Data are means ± SEM (n = 3). *p < 0.05, **p < 0.01 compared to uninhibited IgG. (B) UK AAV-IgG binding to native (▲) and denatured plasminogen (●). ***p < 0.001 AAV-IgG compared to healthy control IgG binding to native plasminogen.
Binding properties of AAV-IgG to plasminogen, plasmin and tissue plasminogen activator

Previously, anti-cardiolipin antibodies (aCLs) that cross-react with the catalytic domains of several serine proteases involved in coagulation/fibrinolysis have been reported in patients with anti-phospholipid syndrome (APS). However, 73/74 UK AAV-IgG and 11/12 DC-IgG samples were negative for aCLs.

Meanwhile, significant binding to tissue plasminogen activator (tPA) was prevalent among AAV-IgG. Thirteen of 74 (17.6%) UK AAV-IgG were positive for anti-tPA antibodies compared with 1/50 (2%) HC-IgG (*p < 0.05) (Figure 3 A). The prevalence of anti-tPA antibodies was 20.7% (6/29) and 15.6% (7/45) in MPO-ANCA and PR3-ANCA patients. One DC-IgG was positive for anti-tPA antibodies, this patient with anti-GBM disease also expressed antibodies reactive with plasminogen. Examining the relationship between anti-plasminogen and anti-tPA antibodies in the UK AAV cohort, 7/18 (39%) anti-plasminogen antibody-positive patients harbored anti-tPA antibodies versus 6/56 (11%) anti-plasminogen-negative patients (p = 0.011, Fishers’ exact test). Four of the dual anti-plasminogen and anti-tPA antibody-positive patients were also positive for antibodies against plasmin. Four of 38 (10.5%) Dutch AAV-IgG also tested positive for anti-tPA antibodies.

The specificity of IgG interactions with tPA was confirmed by competition assays (Figure 3 B). Premixing IgG from one anti-tPA antibody-positive UK AAV patient with soluble tPA inhibited binding to coated tPA (**p < 0.01). Inhibition was 85.2 ± 6.3% using soluble tPA at 1 µM.

Cross-inhibition studies were performed to characterize relative affinities of UK AAV-IgG for plasminogen, plasmin and tPA. All 5 IgG dual positive for anti-plasminogen and anti-plasmin antibodies as well as the 13 anti-plasminogen-only reactive IgG were tested. Soluble plasminogen (1 µM) inhibited binding of the dual positive AAV-IgG to coated plasminogen by 59.75 ± 1.37% and to coated plasmin by 48.76 ± 0.57% (**p < 0.01, Table 1). Although TLCK-inactivated plasmin significantly inhibited binding of dual positive AAV-IgG to coated plasmin by 43.35 ± 1% (**p < 0.01), binding to plasminogen was only reduced by 24.39 ± 1% (**p < 0.01). Pretreating IgG that was positive for only anti-plasminogen antibodies had a similar effect: soluble plasminogen inhibited binding to coated plasminogen by 61.37 ± 0.63%,
whereas soluble plasmin inhibited binding to plasminogen by 23.49 ± 0.54% (Table 1).

Soluble tPA (1 μM) effectively inhibited binding of anti-tPA antibody-positive AAV-IgG to coated tPA (**p < 0.01). Similarly, soluble tPA reduced binding of anti-tPA and anti-plasminogen dual positive AAV-IgG to coated plasminogen by 63.29 ± 0.91% (**p < 0.01; Table 1). In contrast, soluble plasminogen was less efficient in reducing dual positive IgG binding to tPA (47.23 ± 0.58%; **p < 0.01). Collectively, these data suggest that AAV-IgG from the patients tested in these experiments bound to the fibrinolysis-associated serine proteases with the differing relative affinities: tPA > plasminogen > plasmin.

**Table 1.** Binding properties of UK AAV-IgG to plasminogen, plasmin and tPA

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<th>AAV-IgG</th>
<th>PLG (coat) and PLG (soluble)</th>
<th>PLM (coat) and PLM (soluble)</th>
<th>PLM (coat) and PLG (soluble)</th>
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IgG was preincubated with 1 μM soluble plasminogen (PLG), plasmin (PLM) or tissue plasminogen activator (tPA) for 2 hours at 25°C prior to loading onto coat antigen as indicated. Data shown are mean percentage inhibition in IgG binding to the coat antigen ± SEM (n = 4). NA, not applicable.
Figure 3. Identification of anti-tissue plasminogen activator antibodies in AAV patients. (A) Representative ELISA from UK AAV patients. *p < 0.05 AAV IgG compared with healthy control IgG. (B) Preincubation with increasing concentrations of soluble tPA inhibited IgG binding. **p < 0.01 compared with uninhibited IgG.
Anti-plasminogen antibodies in AAV-IgG compromise fibrinolysis

UK AAV-IgG also retarded in vitro fibrinolysis compared with HC-IgG (Figure 4 A). IgG were classified as significantly retarding fibrinolysis if they extended time to 50% fibrinolysis by more than the mean + 2 SD effect of the HC-IgG cohort. Accordingly, 18/74 (24.3%) UK AAV-IgG delayed fibrinolysis (***p < 0.001; Figure 4 B). Four fibrinolysis-retarding IgG were from MPO-ANCA- and 14 from PR3-ANCA-positive IgG samples. None of the HC or DC-IgG significantly delayed fibrinolysis. A total of 11/18 (61%) UK AAV-IgG positive for anti-plasminogen antibodies significantly retarded clot lysis versus 7/56 (12.5%) of anti-plasminogen-negative patient IgG (p = 0.0001, Fisher’s exact test). Furthermore, 7/13 (54%) anti-tPA-positive UK AAV-IgG retarded clot lysis compared with 11/61 (18%) anti-tPA-negative IgG (p = 0.01, Fisher’s exact test). Five out of 18 fibrinolysis retarding AAV-IgG were positive for both anti-plasminogen and anti-tPA antibodies. Only 4/18 IgG that significantly retarded fibrinolysis were negative for antibodies against plasminogen or tPA.

The dual presence of anti-plasminogen and anti-tPA antibodies in UK AAV-IgG caused the greatest inhibition, retarding lysis by 12.8 ± 3.4 minutes. Anti-plasminogen antibodies alone were shown to delay fibrinolysis by 5.19 ± 2.8 minutes, whereas anti-tPA antibodies alone reduced fibrinolysis by 1.73 ± 1.5 minutes (Figure 4 C).

When UK AAV-IgG was mixed with plasmin and loaded onto a preformed fibrin clot, only 5/74 (6.7%) IgG delayed fibrinolysis compared with none of the HC or DC-IgG (p = 0.09; Figure 4 D). Four of the 5 (80%) AAV-IgG positive for anti-plasmin antibodies retarded plasmin-mediated clot lysis compared with 1/69 (1.4%) antibody-negative patients (p = 0.0003, Fisher’s exact test). These IgG were in fact also positive for anti-plasminogen and anti-tPA antibodies. No IgG degradation was observed when IgG was preincubated with plasmin (data not shown).

Confirming results obtained with UK AAV-IgG, Dutch anti-plasminogen antibody-positive AAV-IgG delayed in vitro fibrinolysis significantly compared with anti-plasminogen antibody-negative patient IgG and HC-IgG (Figures 4E & 4F).
Figure 4. Anti-plasminogen antibodies in AAV patient IgG compromise fibrinolysis. (A) Fibrin clot formation was stimulated by mixing thrombin with fibrinogen and monitored spectrophotometrically. No clot was formed in the absence of thrombin (●). Sample diluent (■), healthy control IgG (▲) or UK AAV-IgG (▲) were incubated with plasminogen (plg) before the addition of tissue plasminogen activator (tPA). The tPA containing solution was immediately loaded onto the fibrin clot. Fibrinolysis was measured by monitoring the reduction in absorbance at 405 nm. Plasminogen alone did not initiate fibrinolysis (x) whereas AAV-IgG retarded fibrinolysis compared to sample diluent and control IgG (▲). (B) Time to 50% fibrinolysis was retarded by 18/74 (24.3%) UK patient-IgG compared to none of the healthy or disease controls. ***p < 0.001 AAV-IgG compared to healthy control IgG.
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HC, healthy control; PLG, plasminogen; tPA, tissue plasminogen activator.

**Figure 4.** Anti-plasminogen antibodies in AAV patient IgG compromise fibrinolysis continued. (C) Average change in 50% clot lysis times (UK patients). (D) When IgG was premixed with plasmin on ice before loading onto a fibrin clot, 5/74 (6.7%) UK AAV-IgG retarded fibrinolysis (2 from MPO-ANCA and 3 from PR3-ANCA patients).
Figure 4. Anti-plasminogen antibodies in AAV patient IgG compromise fibrinolysis continued. (E) In separate experiments, Dutch AAV-IgG samples were preincubated with plasminogen and then mixed with fibrinogen, Factor XIII and tPA. This mixture was subsequently added to thrombin and CaCl₂. Fibrin clot formation and lysis, monitored by change in absorbance at 405 nm, are shown for 2 anti-plasminogen antibody-positive AAV-IgG and one healthy control. Arrows indicate 50% lysis times. (F) Half lysis times are given as percentage of a negative control with AAV-IgG grouped by anti-plasminogen antibody status (mean and 95% confidence intervals).
**Transient nature of anti-plasminogen antibodies**

The UK AAV-IgG were all obtained during acute disease. Longitudinal testing of these patients was not possible but, intriguingly, serial samples from two other AAV patients suggested that anti-plasminogen antibodies may disappear with treatment (Figure 5). However, in the Dutch cohort, one patient tested positive for anti-plasminogen antibodies while being in clinical remission. These observations merit further examination.

![Graph A](image1.png)

**Figure 5.** Transient nature of anti-plasminogen and anti-tissue plasminogen activator antibodies. Serial serum samples (diluted 1:100) from two patients collected over 52 weeks were screened for antibodies against plasminogen (A) and tissue plasminogen activator (B). Antibody titers were correlated with disease activity using the Birmingham Vasculitis Activity Scoring (BVAS) system. Data are means ± SEM (n = 3). The dotted line is the positive cut-off representing the mean + 2 standard deviations of the healthy controls. *p < 0.05 compared to the antibody titer at presentation.
Seropositivity for anti-plasminogen antibodies correlates to reduced renal function

Serum creatinine measurements made at the same time as serum samples were collected for 36/38 Dutch patients. The 10 patients who tested positive for anti-plasminogen antibodies had a significantly higher serum creatinine and a lower estimated GFR (eGFR; four-variable MDRD equation\textsuperscript{10,11}) compared with patients who tested negative for anti-plasminogen antibodies (Table 2).

At 12-month follow-up, patients with anti-plasminogen antibodies in their initial serum samples still had a higher serum creatinine, and a lower eGFR, compared with patients who tested negative for anti-plasminogen antibodies, whereas there was no difference in corrected eGFR at 12 months, suggesting that the effect of differences in baseline eGFR persisted.

Table 2. Clinical characteristics of Dutch cohort by anti-plasminogen antibody status

<table>
<thead>
<tr>
<th>Dutch Cohort (n = 38)</th>
<th>Anti-PLG Antibodies (n = 10)</th>
<th>No Anti-PLG Antibodies (n = 28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD) (years)</td>
<td>68 ± 5</td>
<td>62 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>male</td>
<td>7 (70%)</td>
<td>14 (50%)</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>3 (30%)</td>
<td>14 (50%)</td>
<td></td>
</tr>
<tr>
<td>ANCA</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>PR3</td>
<td>6 (60%)</td>
<td>19 (68%)</td>
<td></td>
</tr>
<tr>
<td>MPO</td>
<td>3 (30%)</td>
<td>8 (29%)</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>1 (10%)</td>
<td>1 (-1%)</td>
<td></td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>WG</td>
<td>6 (60%)</td>
<td>25 (89%)</td>
<td></td>
</tr>
<tr>
<td>MPA</td>
<td>1 (10%)</td>
<td>2 (7%)</td>
<td></td>
</tr>
<tr>
<td>RLV</td>
<td>3 (30%)</td>
<td>1 (4%)</td>
<td></td>
</tr>
<tr>
<td>ENT</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>yes</td>
<td>4 (40%)</td>
<td>16 (57%)</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>6 (60%)</td>
<td>9 (32%)</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td></td>
<td>3 (11%)</td>
<td></td>
</tr>
</tbody>
</table>

Table continues on next page. ENT, ear-nose-throat disease; MPA, microscopic polyangiitis; MPO, myeloperoxidase; PR3, proteinase 3; RLV, renal-limited vasculitis; WG, Wegener’s granulomatosis.
<table>
<thead>
<tr>
<th>Dutch Cohort (n = 38)</th>
<th>Anti-PLG Antibodies (n = 10)</th>
<th>No Anti-PLG Antibodies (n = 28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal disease</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>yes</td>
<td>9 (90%)</td>
<td>20 (~71%)</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>1 (10%)</td>
<td>6 (~21%)</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>2 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung disease</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>yes</td>
<td>4 (40%)</td>
<td>12 (43%)</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>6 (60%)</td>
<td>13 (46%)</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>3 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVT</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>yes</td>
<td>1 (10%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>9 (90%)</td>
<td>24 (86%)</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>4 (14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical state</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>new, active</td>
<td>8 (80%)</td>
<td>19 (68%)</td>
<td></td>
</tr>
<tr>
<td>remission</td>
<td>1 (10%)</td>
<td>2 (7%)</td>
<td></td>
</tr>
<tr>
<td>relapse</td>
<td>1 (10%)</td>
<td>4 (14%)</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>3 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entry creatinine(^a)</td>
<td>503.6 ± 325.2</td>
<td>147.7 ± 139.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MDRD entry(^a)</td>
<td>20.4 ± 19.4</td>
<td>59.7 ± 28.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Creatinine 12m(^a)</td>
<td>172.9 ± 79.7</td>
<td>106.6 ± 31.3</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>MDRD 12m(^a)</td>
<td>42.4 ± 20.0</td>
<td>61.9 ± 17.1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Corrected MDRD 12m(^a)</td>
<td>2.1 ± 14.2</td>
<td>-1.1 ± 11.3</td>
<td>NS</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>death within 12m</td>
<td>1 (10%)</td>
<td>5 (18%)</td>
<td></td>
</tr>
<tr>
<td>alive at 12m</td>
<td>9 (90%)</td>
<td>17 (61%)</td>
<td></td>
</tr>
<tr>
<td>lost to follow-up</td>
<td>6 (21%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Mean ± SD is given. DVT, deep venous thrombosis; MDRD, glomerular filtration rate estimated according to the Modification of Diet in Renal Disease equation; 12m, at 12-month follow-up.
Anti-plasminogen antibodies are correlated with the extent of hallmark renal histologic lesions

For 19 Dutch patients tested for anti-plasminogen antibodies, contemporaneous renal biopsies were available. The average number of glomeruli per biopsy was 18 (range 6-35).

Seven of 19 biopsied patients were positive for anti-plasminogen antibodies: renal biopsies from these 7 patients showed higher percentages of glomeruli with fibrinoid necrosis (p < 0.05) and cellular crescents (p < 0.001; Table 3). No difference in globally sclerosed glomeruli or fibrous crescents was found.

These results demonstrate that patients with anti-plasminogen antibodies show more active renal lesions than patients without anti-plasminogen antibodies.

Table 3. Anti-plasminogen antibodies and renal histology

<table>
<thead>
<tr>
<th>Renal Histology Cohort</th>
<th>Anti-PLG Antibodies (n = 7)</th>
<th>No Anti-PLG Antibodies (n = 12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomeruli with fibrinoid necrosis</td>
<td>21 ± 18</td>
<td>5 ± 7</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Glomeruli with crescents</td>
<td>52 ± 21</td>
<td>20 ± 17</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Glomeruli with cellular crescents</td>
<td>37 ± 23</td>
<td>9 ± 7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Glomeruli with fibrous crescents</td>
<td>15 ± 15</td>
<td>11 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>Glomeruli with global sclerosis</td>
<td>13</td>
<td>13 ± 11</td>
<td>NS</td>
</tr>
</tbody>
</table>

The percentage of glomeruli (mean ± SD) showing hallmark renal lesions of ANCA-associated glomerulonephritis is reported in biopsies of anti-plasminogen antibody (anti-PLG)-negative patients compared with anti-plasminogen antibody-positive patients. NS, not significant.
DISCUSSION

We identified anti-plasminogen antibodies in approximately 25% of patients in two independent AAV cohorts. Furthermore, anti-tPA antibodies were detected in 17.6% of AAV patients from one cohort, and the majority of AAV-IgG that inhibited in vitro fibrinolysis harbored either anti-plasminogen and/or anti-tPA antibodies. Importantly, there was also a correlation between anti-plasminogen antibodies and the proportion of glomeruli exhibiting fibrinoid necrosis and cellular crescents.

Antibodies that react with plasminogen/plasmin and other serine proteases of the coagulation/fibrinolysis cascade were previously detected in patients with autoimmune disease, particularly as a subspecies of aCL antibodies. Notably, high affinity monoclonal anti-plasmin antibodies isolated from patients with antiphospholipid syndrome (APS) bind plasminogen, thrombin, factor X and activated protein C with considerably lower affinity. Typically, these autoantibodies appear to cross-react with epitopes within the catalytic domains of serine proteases and conformational epitopes in β2 glycoprotein 1.

In contrast, IgG derived from AAV patients preferentially bind plasminogen with infrequent binding to plasmin. Cross-inhibition studies also suggest lower affinity binding to plasmin than plasminogen in dual positive samples, and aCL antibodies did not account for anti-plasminogen antibodies in AAV patients. We examined total IgG rather than affinity-purified IgG, but collectively, the data suggest that anti-plasminogen antibodies in AAV are likely to be distinct from related antibodies in other autoimmune diseases.

The epitope(s) recognized by anti-plasminogen antibodies in AAV are not yet fully defined. A lack of reactivity towards denatured plasminogen suggests conformational epitope(s). The failure of most anti-plasminogen-positive AAV-IgG to recognize plasmin suggests that major epitopes are not preserved in the active serine protease. Anti-plasminogen antibodies isolated from PR3-ANCA vasculitis patients in a North American cohort seem to recognize an epitope within the protease domain, but again these antibodies did not bind to plasmin nor thrombin for reasons that are unclear.
The detection of anti-tPA antibodies in AAV is intriguing. Tissue plasminogen activator is a serine protease that exhibits an overall 35% shared identity with plasminogen. In addition to homologous protease domains, tPA and plasminogen contain kringle domains. Anti-tPA antibodies characterized in two APS patients bound to its protease domain but did not cross-react with plasminogen or thrombin. AAV-IgG which display dual reactivity against plasminogen and tPA may comprise distinct immunoglobulins which bind exclusively to one or other serine protease or alternatively, cross-reactive immunoglobulins recognizing common epitopes. The cross-inhibition studies favor the existence of cross-reactive immunoglobulins in at least some AAV patients. The previously proposed core epitope for anti-plasminogen antibodies isolated from PR3-ANCA patients is a ‘PHxQ’ motif, whereas alignment of plasminogen and tPA reveals that of these 3 residues, only glutamic acid (Q) is conserved.

How might the observations in the present study be reconciled with previous findings in the North American AAV cohort? If the development of anti-plasminogen autoantibodies is driven by exposure to exogenous antigens such as microbial proteins, divergent immunological memory in patients from distinct geographic areas could influence autoantibody development. Notably, anti-plasminogen antibodies in North American AAV patients are also anti-complementary proteinase 3 (anti-cPR3) antibodies. A range of microbial proteins demonstrate homology to cPR3 and might initiate an immune response accounting for anti-cPR3 antibodies that cross-react with plasminogen in a cohort-specific manner. We did not examine anti-cPR3 antibodies in European patients, but the occurrence of anti-plasminogen antibodies in both PR3-ANCA- and MPO-ANCA-positive AAV patients indicates differences from the North American cohort.

This study showed the capacity of anti-plasminogen and anti-tPA antibodies to delay fibrinolysis, which supports the hypothesis that they have pathogenic significance. Studies in four distinct AAV patient cohorts have identified an increased incidence of VTEs, which does not seem to be attributable to conventional prothrombotic risk factors. Meanwhile, anti-plasminogen antibodies correlated with VTEs in PR3-ANCA patients. Although one of the Dutch patients with particularly high titers of anti-plasminogen antibodies had a history of deep venous thrombosis, we do not have comprehensive data on VTEs in the cohorts. Nevertheless, we hypothesized that
anti-plasminogen antibodies might also influence glomerular pathology through a disturbance of fibrinolysis in the microvasculature.

In AAV, vascular injury is mediated by ANCA-induced neutrophil and monocyte activation and characterized histopathologically by an influx of inflammatory cells with fibrinoid necrosis in small vessel walls. In the renal biopsy, fibrinoid necrosis is apparent in glomeruli. Although fibrinoid necrosis is a ubiquitous feature of ANCA-associated vascular injury, little is known about its pathogenesis. Meanwhile, there are striking parallels in composition between fibrinoid necrosis and the fibrin clot in vascular repair. Both lesions consist primarily of fibrin, platelets, leucocytes and extracellular matrix molecules such as fibronectin. Conceivably, fibrinoid necrosis may therefore originate from a flaw in the physiological vascular repair mechanism wherein the fibrin clot fails to be removed after its scaffold function for angiogenesis is completed. This disturbance may be initiated or accelerated by anti-plasminogen antibodies.

To this end, we scored histopathologic lesions in patients with biopsy-proven ANCA-associated glomerulonephritis, and found that the occurrence of anti-plasminogen antibodies was associated with higher percentages of glomeruli with fibrinoid necrosis and cellular crescents, accompanied by more severely reduced renal function.

Fibrinoid necrosis is often encountered in glomeruli with cellular crescents, and there are indications that fibrin is an important mediator of extracapillary proliferation. Fibrinogen leakage through damaged glomerular basement membranes could potentially trigger the formation of crescents. Several experimental models support a role of coagulation, and fibrin in particular, in the formation of fibrinoid necrosis and crescents. Experimental anti-GBM glomerulonephritis has a more severe course in tPA and plasminogen knockout mice, with more fibrin deposition, tuft necrosis and crescents, in addition to more severe renal failure. Other knockout models demonstrate that fibrinogen deficiency is protective in experimental crescentic glomerulonephritis.

The role of glomerular fibrin deposition in crescent formation is underlined by experimental data that showed that defibrinating agents substantially prevented crescent
formation and renal failure. \textsuperscript{27-29} In AAV, large studies on the efficacy of anti-coagulant therapies have not been performed, but case reports suggest that heparin and defibrotide can be therapeutically beneficial. \textsuperscript{30-34} Similarly, systemic plasminogen concentrates or recombinant tPA could be of interest. Recombinant tPA treatment reduced fibrin deposition and crescent formation and improved renal function in experimental crescentic glomerulonephritis, \textsuperscript{35} whereas streptokinase reduced glomerular fibrin deposition and preserved renal function when used early after disease onset. \textsuperscript{36}

In conclusion, approximately 25\% of AAV patients harbor anti-plasminogen antibodies. Anti-plasminogen antibodies, particularly in combination with anti-tPA antibodies, delay fibrinolysis \textit{in vitro} and are related to hallmark renal histologic lesions that are closely associated with disturbances in coagulation. Therapies aiming at enhancing or replacing fibrinolytic activity may be of benefit in AAV patients with anti-plasminogen and anti-tPA antibodies.

ACKNOWLEDGEMENTS

We thank Dr. Matthew Morgan for permitting the use of the serial serum samples and providing clinical information relating to these patients. We also thank Dr. Gloria Preston (University of North Carolina, Chapel Hill, NC) for providing IgG from an anti-plasminogen antibody-positive patient for use as a positive control while setting up the ELISA. Additionally, we thank Timothy Plant for screening patient IgG for anti-cardiolipin antibodies. We thank Dr. J. Eikenboom (Department of Hematology, LUMC, Leiden) for kindly providing fibrogammin and Dr. F. Havekate (Gaubius TNO, Leiden) for providing tPA. Furthermore, we thank A. Bakker for performing the ANCA ELISAs.

DISCLOSURES

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