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Autoantibodies against citrullinated serum albumin in patients with rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic, potentially debilitating, inflammatory disease that primarily affects synovial joints. While the etiology of RA remains incompletely understood, it is clear that the disease is autoimmune in nature. A hallmark of RA is that the specific epitopes on self-antigens that are targeted by the immune system are often modified by arginine deimination, also referred to as citrullination. In fact, anti-citrullinated protein autoantibodies (ACPA) at high enough titers are diagnostic of RA and appear to have many different targets. Here, we report that RA patients have IgG autoantibodies that react with human serum albumin (HSA) when it had been citrullinated by protein arginine deiminase (PAD) 4, but not by PAD2. Unmodified albumin was not recognized by autoantibodies. In a cohort of 79 RA patients, 38% had anti-citrullinated HSA (anti-cit-HSA) reactivity above the cut-off of the average plus two standard deviations in a healthy subject cohort (n = 16). The titers of these autoantibodies correlated with ACPA status and seropositivity. There was also a trend toward correlation with the presence of radiographic joint erosions, but this did not reach statistical significance. Finally, patients with anti-cit-HSA were more frequently treated with biologics and combination regimens than patients without these autoantibodies. We conclude that ACPA directed against citrullinated albumin exist in a subset of RA patients. Because of the abundance of albumin, its modification by citrullination, as well as autoantibodies binding to it, may have deleterious consequences for the health of affected RA patients.

1. Introduction

A hallmark of rheumatoid arthritis (RA) is the presence of anti-citrullinated protein antibodies (ACPA) in approximately 70% of patients [1] – a highly RA-specific feature [2,3] widely used in its diagnosis. The genetic association of RA with polymorphisms in the genes that encode two of the citrullinating enzymes, protein arginine deiminase (PAD) 2 and 4 [4–8], also supports the notion that this post-translational modification of self-proteins plays an important role in RA pathogenesis, perhaps by creating immunoreactive epitopes that drive an autoimmune response [9–11].

Several proteins that are recognized in their citrullinated form by ACPA have been identified, including histones [12], fibrinogen [13,14], vimentin [15], and α-enolase. In a proteomics approach, Tutturen and co-workers [16] identified over 100 citrullinated proteins in the synovial fluid of an RA patient, which included approximately equal numbers of intracellular proteins, such as neutrophil cytosol factor [17] and vimentin, and proteins of a predominantly extracellular location, such as immunoglobulins and complement proteins. An overlapping set of citrullinated proteins were identified by Wang et al. [18]. The repertoire of these citrullinated proteins suggests (but does not prove) that citrullination may occur in both intracellular and extracellular compartments.

Intracellular citrullination can occur when neutrophils are killed by cytotoxic lymphocytes using the pore-forming protein perforin, or by the membrane-attack complex of complement, both of which induce a large influx of calcium, which in turn triggers a large and acute citrullination reaction [19]. This reaction can be induced *ex vivo* in live neutrophils by many membranolytic stimuli that allow for influx of calcium in higher quantities than seen during receptor-mediated signaling [19,20]. Many of the >30 intracellular proteins that become citrullinated in this response [17] are also found in their citrullinated form in RA patients [16,18,21].

Extracellular citrullination, on the other hand, has been suggested to occur as a consequence of active externalization of PADs during NETosis

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[12] or more passively during other forms of neutrophil death and lysis [22], or through exposure and secretion of PADs from fully viable neutrophils [23]. The most obvious targets for extracellular citrullination are proteins that are not synthesized in immune cells, but typically in the liver, and that have important functions as soluble proteins in blood or extracellular fluid. In this paper, we report that one such protein, human serum albumin, can be citrullinated and targeted by citrullination-specific autoantibodies in patients with seropositive RA.

2. Materials and methods

2.1. RA patients

Sera from RA patients (n = 79) were from the UW Rheumatology Biorepository and kept at −20 °C until use. The patient characteristics and clinical and serological parameters of this cohort are summarized in Table 1. IRB approval for our study was obtained from the University of Washington ethics board (STUDY00006196) and informed written consent was obtained from all participants according to the Declaration of Helsinki.

2.2. Proteins and antibodies

Bovine serum albumin (BSA), human serum albumin (HSA), and poly(Glu, Lys, Tyr; 6:3:1), 20–50 kDa, were from Millipore Sigma (Burlington, MA). Recombinant human PAD4 and PAD2 were from Cayman Chemicals (Ann Arbor, MI). Horse radish-peroxidase (HRP)-labeled anti-human immunoglobulin G (IgG) secondary antibody was from Invitrogen (Carlsbad, CA).

2.3. PAD4-modified western blotting

5 μg of BSA was resolved by gel electrophoresis and transferred to nitrocellulose membranes, washed in 20 mM Tris, pH 7.4, 150 mM NaCl, and blocked in 0.3 mg/ml poly(Glu, Lys, Tyr) in the same buffer overnight. After washing, the filter was incubated with 100 ng recombinant PAD4 in 20 mM Tris, pH 7.7, 150 mM NaCl, 5 mM CaCl2, 1 mM DTT for 1 h at 37 °C, washed several times in 20 mM Tris, pH 7.7, 150 mM NaCl, then immunoblotted with 1:100 diluted patient, or healthy control, serum, then developed by HRP-conjugated anti-human IgG and enhanced chemiluminescence.

Table 1
RA patient cohort characteristics.

Characteristic	Total (n = 79)	Female (n = 61)	Male (n = 18)
Age	52.6 ± 14.0	52.1 ± 14.3	54.6 ± 13.4
Ethnicity			
European	51	39	12
Hispanic	12	10	2
Asian	4	4	0
African	6	3	3
Native American	4	4	0
Native Hawaiian	1	1	0
Unknown/mixed	1	1	0
Laboratory measures			
RF-positive	56	44	12
RF-negative	23	18	5
ACPA-positive	59	46	13
ACPA-negative	20	15	5
C-reactive protein (CRP)	10.2 ± 15.9	10.9 ± 17.7	7.6 ± 5.8
Disease activity			
High (CDAI >22)	12	11	1
Moderate (CDAI >10–22)	15	14	1
Low (CDAI >2.8–10)	13	7	6
Remission (CDAI ≤2.8)	10	8	2
Current CDAI not recorded	27	19	8
Radiographic erosions	38	29	9
Current treatment			
NSAID/prednisone only	4	4	0
JAK inhibitor only	1	1	0
DMARD only	32	22	10
Biologic only	13	12	1
DMARD + biologic	29	22	7

2.4. ELISAs

96-well polystyrene plates were coated with 1 mg/ml BSA or HSA in 0.1 M carbonate, pH 9.6, buffer overnight, washed in phosphate-buffered saline-Tween, and blocked in 1% BSA or HSA in phosphate-buffered saline for 2 h. Patient serum was added at 0.5% in blocking buffer for overnight incubation at 4 °C, washed extensively and then incubated with 1:2000 dilution of HRP-conjugated anti-human IgG. The reaction was then washed, and developed with 3,3′,5,5′-tetramethylbenzidine (TMB), with the color reaction terminated with 2 N sulfuric acid, and the absorbance measured at 450 nm using a plate reader.

2.5. ELISA for citrullinated HSA

To induced the citrullination of albumin immobilized on ELISA plates, 300 ng of recombinant PAD4 was added in 20 mM Tris/HCl, pH 7.7, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM DTT and incubated at 37 °C for 1 h. After extensive washing, the plates were used for ELISA as described above. Controls included albumin wells without PAD4, but otherwise treated exactly the same way.

2.6. Statistical analysis

For non-paired sample sets with non-Gaussian distribution Mann-Whitney *U* test, and for paired sample sets Wilcoxon matched-pairs signed rank test, were used. The upper limit of 'normal' reactivity was defined as the average plus two standard deviations of the healthy control group. Values above this cut-off were termed 'positive'. GraphPad Prism was used for the analyses, which were considered statistically significant at $p < 0.05$.

3. Results

3.1. Recognition of citrullinated bovine albumin by RA autoantibodies

In the course of experiments to test RA patient sera for autoantibodies against another citrullinated protein, we found that BSA used in the ELISAs as a non-specific blocking protein was recognized by antibodies in some of the 8 RA patient samples tested on the plates treated with PAD4 (Fig. 1A), but not PAD2 (Fig. 1B). No reactivity was detected against unmodified BSA. The catalytic activities of the used preparations of PAD2 and PAD4 were verified using histone H3 as a substrate (not shown). To verify that the recognized protein indeed was citrullinated BSA, we ran 5 μg of BSA on a gel, transferred it to nitrocellulose, blocked the filter with a protein that cannot be citrullinated, the 20–50 kDa poly(Glu, Lys, Tyr), and incubated the filter with recombinant PAD4. After washing, the filter was immunoblotted with serum from one of the reactive patients, followed by a secondary anti-human IgG antibody. As shown in Fig. 1C, BSA was indeed detected.

3.2. IgG autoantibodies against citrullinated human albumin in RA patients

To determine if these anti-citrullinated BSA autoantibodies recognize citrullinated HSA, which is 76% identical to BSA, we repeated the ELISA with HSA, with or without PAD4 treatment, and sera from a larger RA cohort (n = 79). As shown in Fig. 2, RA patient sera contained a range of different titers of IgG autoantibodies reactive with PAD4-treated HSA, but not untreated HSA. The difference was statistically highly significant ($p < 0.0001$) and also significantly different ($p = 0.0005$) from healthy controls (n = 16), which had low background titers similar to the RA

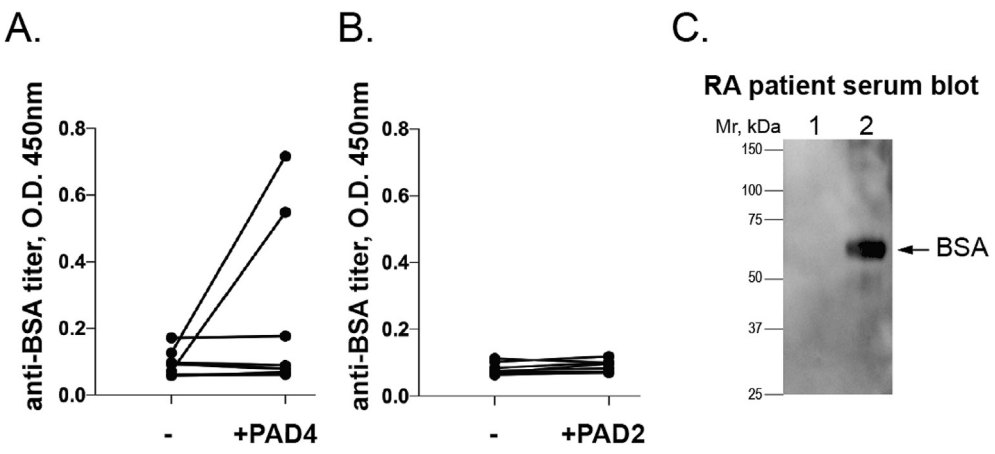

Fig. 1. IgG autoantibodies from a test cohort of RA patients recognize BSA treated with PAD4, but not with PAD2. A, ELISA with 8 RA patient sera in BSA-coated wells treated with buffer alone (−) or with PAD4 (+PAD4) for 1 h at 37 °C and then washed extensively. **B,** same experiment in BSA-coated wells treated with buffer alone (−) or with PAD2 (+PAD2). **C,** immunoblot with RA patient serum of a nitrocellulose filter with no protein in lane 1 and 5 μg of BSA in lane 2, blocked in excess poly-(Lys, Glu, Tyr), treated with PAD4 for 1 h at 37 °C and then washed extensively.

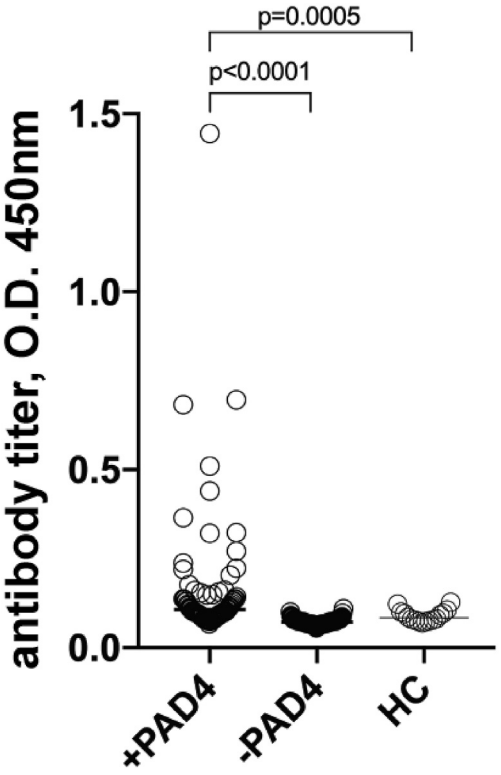

Fig. 2. IgG autoantibodies against citrullinated HSA in the full cohort of RA patients. Detection by ELISA of anti-cit-HSA reactivity (+PAD4) in the RA patient cohort (n = 79) compared to anti-unmodified HSA (-PAD4) or the control group (HC)(n = 16). Statistical significance was calculated using Mann-Whitney *U* test.

reactivity with untreated HSA. It should also be noted that the RA reactivity with PAD4-treated HSA covered a broad range from high to as low as controls. Using the average plus 2 standard deviations in the healthy control group as a cut-off, 30 (38%) of the 79 RA patients were positive for citrullinated HSA (cit-HSA), but none of them for unmodified HSA.

3.3. Anti-cit-HSA autoantibodies correlate with patient ACPA status and seropositivity

Next we analyzed the data set for correlations between anti-cit-HSA titers and clinical and serological characteristics of the patients. Anti-cit-HSA reactivity was higher in RA patients who also were ACPA-positive, albeit not reaching statistical significance ($p = 0.0576$) (Fig. 3A), and seropositive ($p = 0.0176$) (Fig. 3B). There was also a trend towards a correlation with the presence of joint erosions (Fig. 3C) and extent of therapy, but neither of these reached statistical significance. Nevertheless, it is clear that the patients with the highest anti-cit-HSA titers had joint erosions and received combination treatments and biologics for their disease.

4. Discussion

Our discovery of IgG autoantibodies that react with citrullinated HSA in 38% of the tested RA patients adds a new autoantigen to the list of citrullinated proteins targeted by ACPA. We are aware of only one previous publication documenting citrullination of HSA, namely a proteomics study by Tutturen and co-workers [16], who identified over 100 citrullinated proteins in the synovial fluid of an RA patient. Among these was HSA with 15 arginines (residues 105, 122, 138, 141, 168, 169, 242, 246, 281, 434, 452, 469, 508, 509, and 545) deiminated to citrulline. Eleven of these arginine residues are also present in BSA. Since we obtained very similar autoantibody reactivities against human and bovine albumin upon citrullination by PAD4, we assume that the conserved arginines (residues 105, 122, 168, 169, 242, 281, 434, 452, 469, 508, and 509) include at least some of the citrullinated residue(s) recognized by patient autoantibodies.

Since albumin is the most abundant protein in circulation with a normal concentration range of 35–55 mg/ml, its citrullination could well have serious detrimental consequences for the patient. First, it is possible that the loss of positive charge on the deiminated arginine(s) alter the overall isoelectric point of HSA, which may affect its properties in maintaining the oncotic pressure of the extracellular fluid and/or its ability to bind and transport biomolecules, such as vitamins, metabolites, and drugs, in plasma. The binding of autoantibodies to citrullinated epitopes on HSA may also create immune complexes, particularly if several autoantibodies can bind simultaneously, which may activate Fcγ receptors on immune cells and thereby promote inflammation. These questions will require further investigations.

RA is a systemic autoimmune disease, and though its primary target is the synovial joints, extraarticular manifestations can occur in long-standing or severe disease. It has been reported that citrullinated proteins are present in the lung tissue of RA patients with rheumatoid lung disease [24]. ACPA positivity, especially at high titers, has also been reported as an independent risk factor for cardiovascular disease in RA patients [25]. Given the finding that the most abundant protein, albumin, can be citrullinated in some RA patients, the presence of anti-cit-HSA and its overall correlation with ACPA seropositivity and clinical measures of disease activity have potentially important clinical implications. The

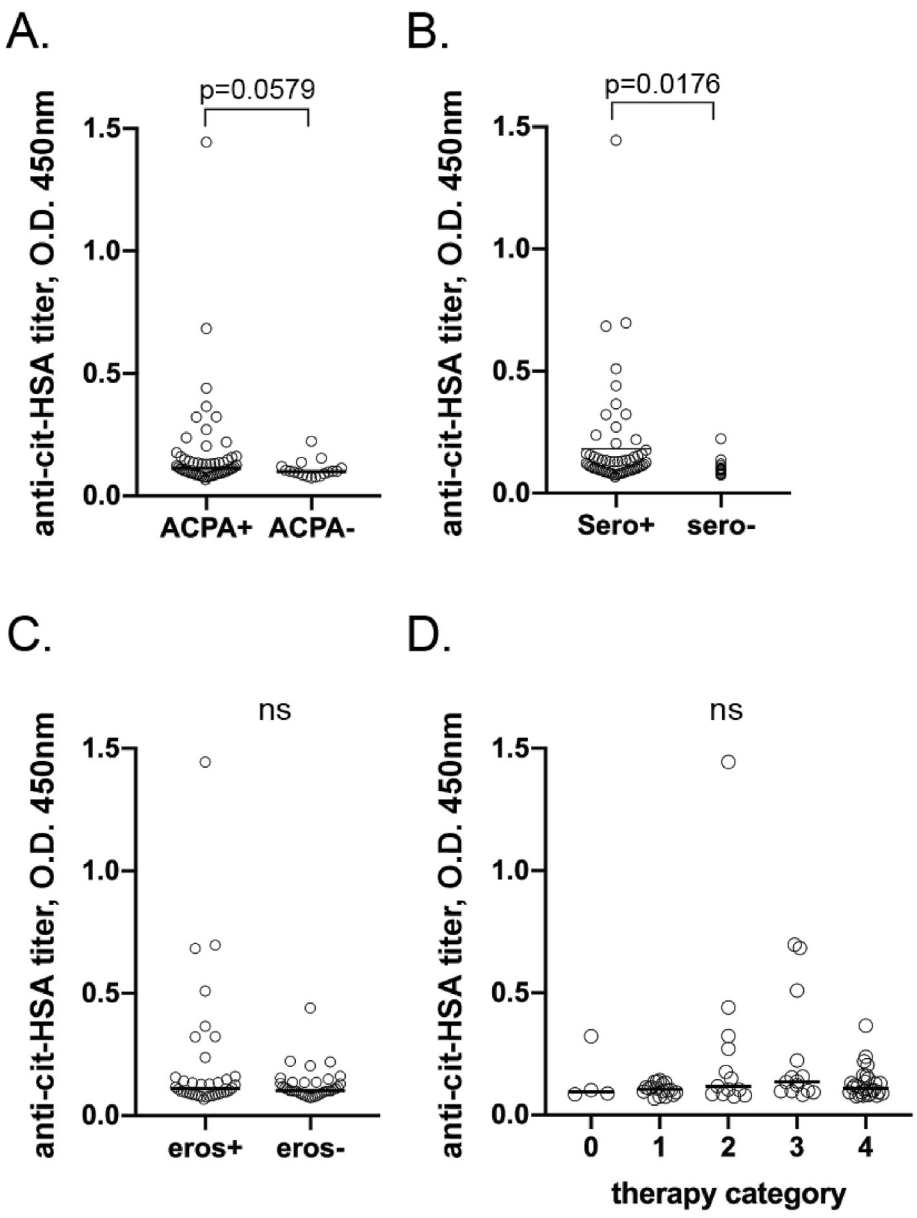

Fig. 3. Correlation between anti-cit-HSA autoantibodies with laboratory and clinical measures. A, anti-cit-HSA reactivity in RA patients with (n = 59) or without (n = 20) ACPA. **B,** anti-cit-HSA reactivity in RA patients who are seropositive (sero+) or seronegative (sero-). **C,** anti-cit-HSA reactivity in RA patients grouped by the presence (Eros+) or absence (Eros-) of radiographic joint erosions. **D,** anti-cit-HSA reactivity in RA patients by treatment regimen: 0 = none or anti-inflammatory alone, 1 = methotrexate alone, 2 = combination of DMARDs; 3 = biologic alone, and 4 = combination of DMARD and biologic. Statistical significance was calculated using Mann-Whitney *U* test; ns = not significant.

presence of anti-cit-HSA, especially at high titers, could indicate the existence of a subset of RA patients at risk of progressing to more severe disease with extraarticular manifestations which may warrant additional diagnostic and therapeutic interventions. These questions will also require further investigations.

Declaration of competing interests

T.M. has received consultant fees from Cugene, Glysantis, Kiniksa, and MiroBio, and research funding from Gilead Sciences.

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