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BRIEF REPORT

Citrullination Within the Atherosclerotic Plaque: A Potential Target for the Anti–Citrullinated Protein Antibody Response in Rheumatoid Arthritis

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Objective. **To investigate whether citrullinated proteins within the atherosclerotic plaque can be targeted by anti–citrullinated protein antibodies (ACPAs), forming stimulatory immune complexes that propagate the progression of atherosclerosis.**

Methods. **Protein lysates prepared from atherosclerotic segments of human aorta were assessed for the presence of citrulline-modified proteins, and specifically citrullinated fibrinogen (Cit-fibrinogen), by immunoprecipitation and/or immunoblotting followed by mass spectrometry. Immunohistochemical analysis of coronary artery plaque was performed to determine the presence of citrullinated proteins and peptidylarginine deiminase type 4 (PAD-4). Serum levels of anti–cyclic citrullinated peptide (anti-CCP), anti–citrullinated vimentin (anti–Cit-vimentin), and anti–Cit-fibrinogen antibodies were measured in 134 women with seropositive rheumatoid arthritis; these subjects had previously been characterized for the presence of subclinical atherosclerosis, by electron beam computed tomography scanning.**

Results. **Western blot analysis of atherosclerotic plaque lysates demonstrated several citrullinated proteins, and the presence of Cit-fibrinogen was confirmed by immunoprecipitation and mass spectrometry. Immunohistochemical analysis showed colocalization of citrullinated proteins and PAD-4 within the coronary artery plaque. In age-adjusted regression models, antibodies targeting Cit-fibrinogen and Cit-vimentin, but not CCP-2, were associated with an increased aortic plaque burden.**

Conclusion. **Citrullinated proteins are prevalent within atherosclerotic plaques, and certain ACPAs are associated with the atherosclerotic burden. These observations suggest that targeting of citrullinated epitopes, specifically Cit-fibrinogen, within atherosclerotic plaques could provide a mechanism for the accelerated atherosclerosis observed in patients with RA.**

Patients with rheumatoid arthritis (RA) are at increased risk of cardiovascular disease, but this increased risk is not explained by traditional cardiac risk factors and generally is limited to patients with RA-associated autoantibodies such as rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPAs). We hypothesized that citrullinated epitopes within the atherosclerotic plaque may be targeted by RA-associated ACPAs to form immune complexes that are capable of perpetuating local plaque inflammation and progression.

MATERIALS AND METHODS

Atherosclerotic plaque tissue was obtained at the time of rapid autopsies of 5 white male subjects without clinical RA. All of the autopsy subjects were older than age 65 years, and all were free of human immunodeficiency virus, hepatitis B virus, hepatitis C virus, and malignancy. Tissue specimens were obtained under an open access protocol from the Stanford University Department of Pathology, and no further identifying information was available.

For proteomic analysis, specimens of grossly atherosclerotic tissue and adjacent tissue without obvious atherosclerosis were obtained from the anterior wall of the aortic arch

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(n = 2 subjects; data presented) or from carotid plaque (n = 3 subjects; data not shown) and snap frozen at −80°C. For immunohistochemical analysis, the right coronary artery was dissected free of the heart and cut axially into 1-cm segments. Several segments with grossly apparent atherosclerosis and adjacent segments that were free of obvious atherosclerosis were fixed in formalin and embedded in paraffin. IgG derived from patients with RA (RA IgG) was purified by protein G chromatography of plasma pooled from 3 male patients with RA known to be anti–cyclic citrullinated peptide 2 (anti–CCP-2), anti–citrullinated fibrinogen (anti–Cit-fibrinogen), and RF positive, as previously described (1).

To evaluate the correlation between ACPAs and subclinical atherosclerosis, the levels of anti–Cit-fibrinogen, anti–citrullinated vimentin (anti–Cit-vimentin), and anti–CCP-2 antibodies (second-generation CCP peptides linked to Bio-Plex beads, kindly provided by Bio-Rad) were assessed by a bead-based immunoassay (2) of plasma from a cohort of 134 RF-positive female patients with RA (disease duration ≥2 years) who had no history of clinical cardiovascular disease. All of these patients had undergone electron beam computed tomography (EBCT) scans for quantitation of coronary artery and aortic calcium as surrogate measures of atherosclerotic burden and cardiovascular risk, as well as extensive assessment of traditional cardiovascular risk factors, as previously described (3). All specimens were obtained with informed consent under protocols approved by institutional review boards at Stanford University and/or the University of Pittsburgh.

Immunohistochemical analysis. Paraffin-embedded human right coronary artery specimens were stained with hematoxylin and eosin to identify atherosclerotic plaques. Immunohistochemical analysis was performed with rabbit anticitrulline antibodies (Millipore) to identify the presence of citrullinated proteins as well as rabbit anti-human peptidylarginine deiminase type 4 (PAD-4) antibodies, in order to localize the presence of PAD-4 (Dako).

One- and 2-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Specimens (~2.0 × 1.5 cm) of tissue from the grossly atheromatous portion of the aortic arch and an adjacent matching specimen free of gross atherosclerosis were pulverized over liquid nitrogen and homogenized in tissue lysis buffer (Bio-Rad) containing protease inhibitors. After centrifugation, the protein content was measured, and equal amounts of protein were run by PAGE, transferred to nitrocellulose, and subjected to acid modification of citrulline residues. Next, citrullinated proteins were identified using anti–modified citrulline antibody according to the manufacturer's instructions (Millipore).

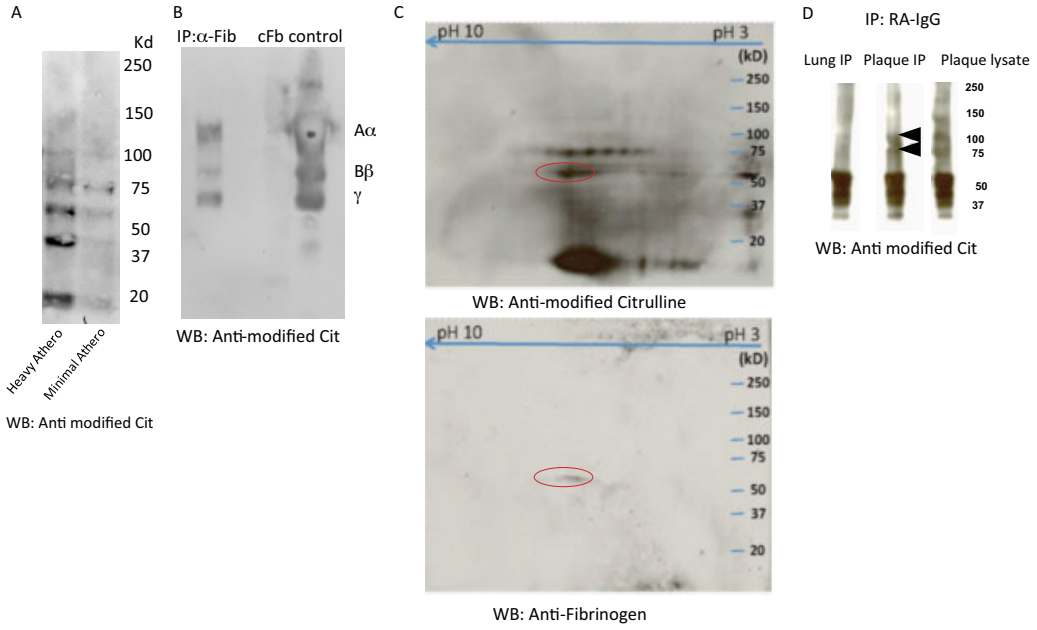

Figure 1. Presence of citrullinated (cit) proteins, including citrullinated fibrinogen (cFb), in the atherosclerotic (athero) plaque. **A,** Anti–modified citrulline blot showing the presence of multiple citrullinated proteins within the atherosclerotic plaque. Plaque lysates were generated from areas of extensive major atherosclerosis and areas of microscopic but gross atherosclerosis and evaluated for the presence of citrullinated proteins by Western blotting (WB). **B,** Anti–modified citrulline staining of fibrinogen immunoprecipitated from plaque lysates. Immunoprecipitation (IP) was performed on plaque lysates using anti-human fibrinogen antibody, and the results were evaluated by Western blotting to assess for the presence of citrullination. **C,** Presence of citrullinated proteins, as determined by 2-dimensional polyacrylamide gel electrophoresis on atherosclerotic plaque lysates and Western blot analysis. The antifibrinogen immunoprecipitation product was analyzed by mass spectrometry. **D,** Immunoprecipitation of citrullinated proteins. Immunoprecipitation was performed on plaque lysates using IgG from anti–citrullinated protein antibody–positive patients with rheumatoid arthritis (RA), and the results were evaluated by Western blotting to assess for the presence of citrullination.

Atherosclerotic plaque lysates were additionally analyzed by 2-D PAGE (11-cm pH 3–10 nonlinear gradient strip), transferred to nitrocellulose, and subjected to acid modification; citrullinated proteins were identified as described above. The blot was stripped and reprobed with a rabbit polyclonal antifibrinogen antibody (Dako), which previously was demonstrated to recognize both native and citrullinated fibrinogen (1).

Immunoprecipitation assay. Atherosclerotic plaque lysates were incubated with either polyclonal rabbit anti-human fibrinogen antibody or RA IgG purified from a pool of RA patient–derived plasma (n = 3) that had previously been demonstrated to be reactive with both anti–CCP-2 and anti–Cit-fibrinogen by enzyme-linked immunosorbent assay (1). Briefly, antibodies were incubated with protein A beads and washed, and the bead–antibody conjugates were incubated with plaque lysates overnight at 4°C. The beads were washed, subjected to 1-D PAGE and Western blotting, as described above, or directly digested with trypsin before mass spectrometry analysis, as previously described (4).

Autoantibody assays. ACPAs targeting CCP-2 peptides, Cit-fibrinogen protein, and Cit-vimentin protein were measured with a custom bead-based immunoassay using a Bio-Plex platform, as previously described (2).

Statistical analysis. Statistical analysis was limited to the seropositive population, as defined by the presence of RF as identified by nephelometry (n = 134). We conducted unadjusted and multivariable regression analyses with the level (in relative fluorescence units) of each autoantibody as the explanatory variable and the aortic calcium score as the response variable. Because the distribution of coronary artery calcium scores in these asymptomatic patients was skewed due to the fact that a large proportion (56%) had no measurable calcified plaque, aortic calcium scores were chosen as the outcome measure. The correlation between aortic calcium scores and the development of clinical coronary heart disease has been well established (5). Additionally, the presence of aortic calcium may be an earlier predictor of clinical coronary heart disease among younger women (5) and thus may better represent our entire female RA population (median age 58 years; interquartile range 51–65 years).

We performed median (quantile) regression analysis, because this methodology does not require a distributional assumption or equal variances. By modeling the median as opposed to the mean, the analysis was less influenced by outliers compared with ordinary least squares regression analysis and thus allowed evaluation of the association of ACPAs with vascular calcium while minimizing the effect of outlying vascular calcium values. Multivariate models were constructed as follows: model 1, unadjusted; model 2, age adjusted; model 3, adjusted for age, pack-years of smoking, fasting low-density lipoprotein (LDL):high-density lipoprotein (HDL) cholesterol ratio, body mass index, systolic blood pressure (BP), serum glucose, and RA disease duration. Notably, only age and pack-years of smoking contributed significantly to the adjusted models. The addition of medication use (including prednisone) as well as the erythrocyte sedimentation rate as a surrogate for disease activity were highly correlated with other variables but did not contribute significantly to the model.

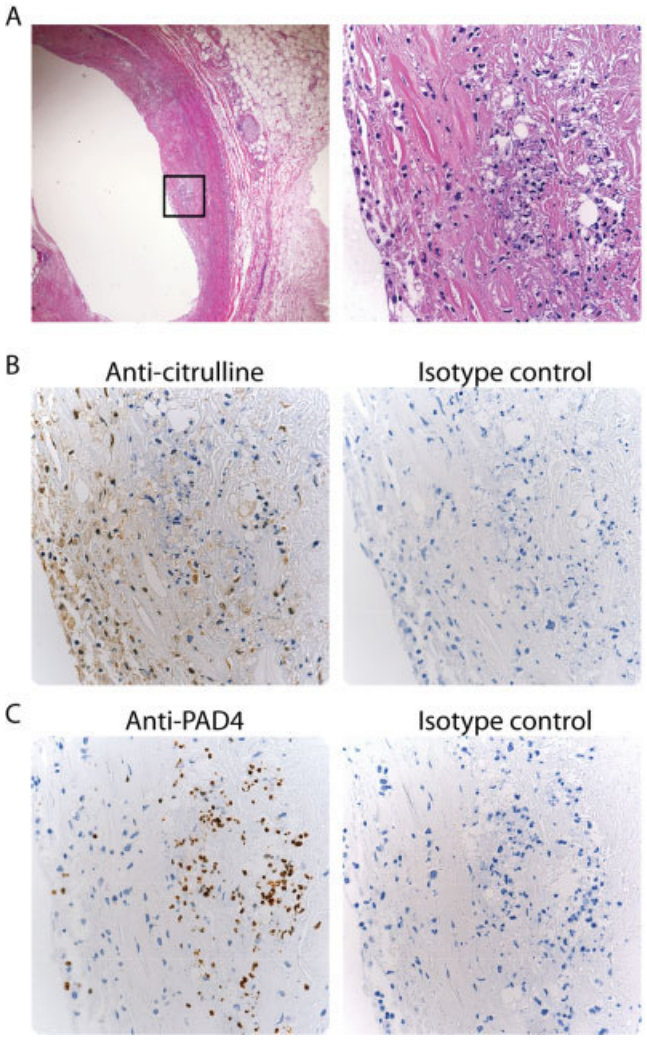

Figure 2. Colocalization of citrullinated proteins and peptidylarginine deiminase type 4 (PAD-4) antibody in the atherosclerotic plaque. **A,** Hematoxylin and eosin staining of a representative atherosclerotic plaque within the right coronary artery. A higher-magnification view of the boxed area (left) is also shown (right). **B** and **C,** Atherosclerotic plaque tissue stained for the presence of citrullinated proteins (**B**) and PAD-4 (**C**). Original magnification × 40 in **A** (right); × 200 in **A** (left), **B,** and **C**.

RESULTS

Presence of citrullinated proteins within the atherosclerotic plaque. Figure 1A shows an anti–modified citrulline blot demonstrating the presence of multiple citrullinated proteins within the atherosclerotic plaque. Although a similar citrullination profile was observed after loading of identical masses of protein, increased citrullination was visualized in areas with heavy atherosclerosis compared with areas with minimal atherosclerosis. As shown in Figure 1B, anti–modified citrulline

Table 1. Association of ACPAs with the aortic calcium burden in RF-positive patients with RA, determined using multivariable regression analysis*

	SD of antibody concentration	Increase in aortic calcium score per SD increase in antibody concentration	95% CI	*P*
Model 1				
Citrullinated vimentin	6,292.6	224.1	45.1, 403.2	0.015
Citrullinated fibrinogen	1,116.3	586.7	469.7, 703.8	<0.001
CCP-2†	8,624.1	109.8	−42.2, 261.9	0.159
Model 2				
Citrullinated vimentin	6,292.6	235.0	19.5, 450.5	0.034
Citrullinated fibrinogen	1,116.3	474.7	313.2, 636.1	<0.001
CCP-2†	8,624.1	130.9	−76.3, 338.2	0.218
Model 3				
Citrullinated vimentin	6,292.6	108.8	−95.2, 312.8	0.298
Citrullinated fibrinogen	1,116.3	383.6	193.4, 573.7	<0.001
CCP-2†	8,624.1	117.6	−127.9, 368.1	0.350

* Model 1 = unadjusted; model 2 = age adjusted; model 3 = adjusted for age, pack-years of smoking, disease duration, systolic blood pressure, fasting low-density lipoprotein:high-density lipoprotein cholesterol ratio, and fasting glucose. ACPAs = anti–citrullinated protein antibodies; RF = rheumatoid factor; RA = rheumatoid arthritis; 95% CI = 95% confidence interval; CCP-2 = cyclic citrullinated peptide 2.
† Linked to Bio-Plex beads.

staining of fibrinogen immunoprecipitated from plaque lysates confirmed citrullination of fibrinogen within the plaque. Human atherosclerotic plaque lysate was separated by 2-D PAGE; Western blot analysis was performed to evaluate the presence of citrullinated proteins, and >12 such citrullinated proteins were identified (Figure 1C).

The membrane was stripped and reprobed with an antifibrinogen (α-chain specific) antibody, identifying a single protein spot colocalizing with an area of anti-citrulline signal intensity. These studies confirmed that Cit-fibrinogen was one of several citrullinated proteins within the atherosclerotic plaque. Finally, the antifibrinogen immunoprecipitation product (Figure 1B) was analyzed by mass spectrometry, and citrullinated peptides were directly identified, confirming citrullination of amino acids derived from fibrinogen α-chain and β-chain.

Colocalization of citrullinated plaque proteins with PAD-4. Human coronary artery plaque tissue specimens were stained with antibodies to citrullinated proteins as well as antibodies to human PAD-4. Figure 2A shows hematoxylin and eosin staining of an atherosclerotic plaque, and Figures 2B and C demonstrate the presence of citrullinated proteins and PAD-4, respectively, within the plaque. Although citrullinated proteins and PAD-4 colocalized, citrullinated proteins appeared scattered and were predominantly extracellular, while PAD-4 appeared to be primarily intracellular or in punctate regions, suggestive of localized cellular leakage.

Recognition of Cit-fibrinogen from within atherosclerotic plaques by RA patient–derived ACPAs. To confirm that Cit-fibrinogen from within atherosclerotic plaques is immunoreactive with ACPAs, we subjected plaque lysates to immunoprecipitation with IgG derived from ACPA-positive patients with RA. Immune complexes were evaluated by Western blot analysis with anti–modified citrulline antibodies or were directly analyzed by mass spectroscopy. Figure 1D shows the precipitation of citrullinated proteins consistent with fibrinogen α-chain and β-chain from plaque lysate but not healthy lung lysate (containing nonatherosclerotic pulmonary artery). Mass spectroscopic analysis of immunoprecipitation products identified 4 confirmed proteins including fibrinogen α-, β-, and γ-chains as well as the complement component C1q (2) (additional information is available from the corresponding author).

When peptides were interrogated for posttranslational modifications, we identified a prominent citrullinated peptide from fibrinogen β-chain, which, of note, was identified by immunoprecipitation from RA synovium in 2 previous studies (4,6) (additional information is available from the corresponding author). These data confirmed the potential for RA IgG to target Cit-fibrinogen within the atherosclerotic plaque.

Correlation of ACPA levels with subclinical atherosclerosis in patients with established RA. Finally, we evaluated the association between ACPA levels and the presence of subclinical atherosclerosis as measured by aortic calcium scores, which were quantitated using EBCT. Table 1 shows the unadjusted and adjusted

associations of ACPA levels with aortic calcium scores. In the age-adjusted analysis, the levels of anti–Cit-fibrinogen ($P < 0.001$) and anti–Cit-vimentin ($P = 0.034$) were associated with greater subclinical atherosclerosis as measured by the aortic calcium score. Notably, bead-based anti–CCP-2 titers were not associated with aortic calcium scores in the unadjusted model ($P = 0.159$) or the age-adjusted model ($P = 0.218$).

In regression models adjusted for age, pack-years of smoking, RA disease duration, systolic BP, fasting glucose, and fasting LDL:HDL ratio, anti–Cit-fibrinogen antibody was the only ACPA that remained significantly associated ($P = 0.0001$), with an estimated increase in the aortic calcium score of 383 units for each SD increase in the level of anti–Cit-fibrinogen antibody. These observations further support the potential role of ACPAs, specifically anti–Cit-fibrinogen antibodies, as drivers of RA-associated atherosclerosis.

DISCUSSION

In this study, we demonstrate the presence of citrullinated proteins including Cit-fibrinogen within atherosclerotic plaques, show the ability of RA patient–derived ACPAs to target these proteins, and identify an association between plasma levels of anti–Cit-fibrinogen antibodies and subclinical atherosclerosis. Previous studies have identified the inflammatory potential of Cit-fibrinogen–containing immune complexes (1,7); therefore, these observations support our hypothesis that citrullinated epitopes within the atherosclerotic plaque may be targeted by RA-associated ACPAs, thus forming immune complexes capable of locally perpetuating plaque inflammation and progression.

The process of citrullination is inflammation dependent (8), and we now demonstrate the presence of citrullination within the inflamed atherosclerotic plaque. Our observations confirm the previous demonstration of extensive fibrinogen deposition, with diffuse distribution throughout the atherosclerotic plaque (9), and further demonstrate that at least a portion of this protein is citrullinated. Additionally, the presence of inflammation and PAD enzymes supports the hypothesis regarding in situ generation of Cit-fibrinogen within the atherosclerotic plaque. Furthermore, the ability of RA IgG to precipitate Cit-fibrinogen demonstrates the potential for RA-associated autoantibodies to directly target citrullinated proteins within the plaque.

Limitations of this study include the lack of direct demonstration of ACPA immune complexes in plaque tissue due to the unavailability of rapid autopsy specimens from subjects with seropositive RA. However, the ability of RA patient–derived ACPAs to directly immunoprecipitate citrullinated proteins from the plaque tissue of non-RA subjects supports the potential for direct immunoreactivity of ACPAs with citrullinated antigens within the plaque. Additionally, our epidemiologic studies associating the ACPA level with the calcified plaque burden provide only indirect evidence of a role for ACPAs in the development/propagation of atherosclerosis. Similarly, although epidemiologic studies have suggested that the accelerated atherosclerosis observed in RA is mainly limited to the seropositive population (10,11), seropositive RA is tightly linked to the presence of the HLA–DRB1 shared epitope, which, in association with increased systemic inflammation, has itself been linked to the presence of subclinical cardiovascular disease in RA (12). Thus, unmeasured genetic factors as well as systemic and local inflammatory mediators could contribute to or confound the observed association. However, we propose that this observation could also further support a direct role for RA-associated autoantibodies in the local progression of the atherosclerotic process.

In conclusion, we confirm the presence of citrullinated proteins (most prominently Cit-fibrinogen) within the atherosclerotic plaque and demonstrate the ability of RA-associated autoantibodies to target this protein in atherosclerotic plaque tissue. These results provide the foundation for future studies investigating the role of ACPAs as a contributor to the accelerated atherosclerosis observed in patients with RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sokolove had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sokolove, Wasko, Robinson.
Acquisition of data. Sokolove, Brennan, Sharpe, Kao, Lepus, Wasko.
Analysis and interpretation of data. Sokolove, Sharpe, Lahey, Kao, Krishnan, Edmundowicz, Wasko, Robinson.

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