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Citrullinated antigens as C1q-binding and monoclonal rheumatoid factor (mRF)-binding peptides in synovial fluids from rheumatoid arthritis patients

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ABSTRACT

Objective. *Previous studies have demonstrated that immune complexes (ICs) may be involved in the pathogenesis of rheumatoid arthritis (RA). However, autoantigens contained in rheumatoid ICs remain to be elucidated. In the present study, we investigated whether the peptides captured by C1q and monoclonal rheumatoid factor (mRF), presumably associated with ICs, were citrullinated in synovial fluids from patients with RA.*

Methods. *Sixteen rheumatoid arthritis synovial fluids (RASFs), 7 osteoarthritis synovial fluids (OASFs), and 20 sera from RA patients were used for experiments. ICs were measured using commercially available kits based on the C1q-binding (C1q-IC) and mRF-binding (mRF-IC) assays. Citrullination of the peptides captured by C1q and mRF was detected by anti-modified citrulline antibody (Senshu Ab) after chemical modification.*

Results. *There was a significant correlation between levels of citrullination of C1q-binding peptides and those of mRF-binding peptides in RASFs (r=0.77), both of which were significantly higher than those in OASFs. No citrullinated Ags captured by C1q and mRF were detected in sera from patients with RA.*

Conclusions. *We have demonstrated the presence of citrullinated Ags as C1q- and mRF-binding peptides in RASF. We suggest that citrullinated Ags may contribute to the pathogenesis of RA through IC formation in the joint.*

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by the chronic inflammation of joints, resulting in bone erosion and joint destruction. Although the etiology of RA remains to be elucidated, immune complexes (ICs) are considered to play an important role in its pathogenesis. Levels of intra-articular ICs were demonstrated to be higher than circulating ICs in RA, as measured by the C1q-binding assay and monoclonal rheumatoid factor-binding assay (1). The IC can activate the complement system and stimulate phagocytes via Fc and complement receptors within the joint, resulting in a release of mediators of inflammation including proinflammatory cytokines, metalloproteinases, and reactive oxygen intermediates in RA (2). However, the autoantigens contained in rheumatoid ICs remain to be elucidated.

Based on linkage disequilibrium and single nucleotide polymorphism (SNP) analysis, we previously identified an RA-susceptible haplotype in the gene encoding peptidylarginine deiminase (PADI) type 4 (3, 4). This gene is one of four known PADI genes that encode enzymes which change arginine into citrulline in peptides. Furthermore, peptidyl citrulline is targeted by anti-citrullinated peptide antibodies (ACPA), which are highly specific for RA (5-8). It is considered that citrullinated peptides originate in the synovium (9), and ACPA appear to be produced in the inflamed synovium in RA (10). Thus, it is possible that citrullinated peptides, which are targeted by ACPA, may form ICs in the joint and contribute to the initiation and persistence of rheumatoid synovitis.

Several citrullinated peptides, including fibrin(ogen) (11), vimentin (12), asporin, and the F-actin capping protein alpha-1 subunit (13), were reported in synovial tissues from patients with RA. Some of the citrullinated peptides were targeted by sera from RA patients, indicating that they may be involved in the pathogenesis of RA through acting as autoantigens. There have been several reports describing the citrullinated peptides contained in RASF. To date, fibrinogen (14), fibronectin (15), vimentin (15), and exosome-associated peptides, such as fibrinogen-related peptides and Sp-alpha (16), have been demonstrated to be citrullinated in RA. However, information regarding citrullinated peptides in association with rheumatoid ICs, which may be more relevant to the pathogenesis of RA, has been limited.

In the present study, we investigated whether the peptides captured by C1q and monoclonal rheumatoid factor, presumably the IC components, were citrullinated in synovial fluids from patients with RA. Based on the data obtained, we discuss the role of citrullinated antigens in the pathogenesis of RA.

Methods

Serum and synovial fluid samples

Sixteen rheumatoid arthritis synovial fluids (RASFs) and 7 osteoarthritis synovial fluids (OASFs) were collected in heparinised tubes at the time of therapeutic arthrocentesis, centrifuged at 450 *g* for 5 minutes to remove any debris, and the supernatants were collected. Sera were obtained from 12 patients with RA and 12 control subjects, after their informed consent was obtained.

Synovial fluid levels of anti-cyclic citrullinated peptide antibodies (anti-CCP Ab) were determined by enzyme-linked immunosorbent assay (ELISA) using DIASTAT anti-CCP kit (MBL Medical & Biological Laboratories Co., Nagoya, Japan)

Measurement of ICs

ICs were measured by ELISA using commercially available kits based on the C1q-binding assay (C1q-IC, TFB Inc., Tokyo, Japan) and monoclonal rheumatoid factor (mRF) assay (mRF-IC, Nissui Pharmaceutical, Tokyo, Japan), according to the manufacturers' instructions. In brief, for ELISA detection of C1q-IC, diluted serum (1:100) or synovial fluid (1:50) was incubated in a microtiter plate well in duplicate, which was pre-coated with human C1q. After washing, the bound ICs were detected by horseradish peroxidase (HRPO)-labeled rabbit anti-human IgG antibody and an enzyme substrate. The absorbance at a wavelength of 450 nm (A450) was read. A microplate pre-coated with murine monoclonal rheumatoid factor was used for mRF-IC measurement.

Sandwich ELISA for detection of citrullination of C1q- and mRF-captured immune complex

For the detection of peptidyl-citrulline, an anti-citrulline (modified) detection kit (Upstate, Chicago, IL, USA) was used. Sera (1:100) and synovial fluids (1:50) were applied to wells of IC ELISA kit microplates, and the wells were incubated for 3 hours at room temperature. After washing three times with phosphate-buffered saline (PBS), the wells were incubated with 1% glutaraldehyde/PBS for 1 hour, followed by treatment with 0.2 M Tris-Cl, pH 7.8 for 30 minutes.

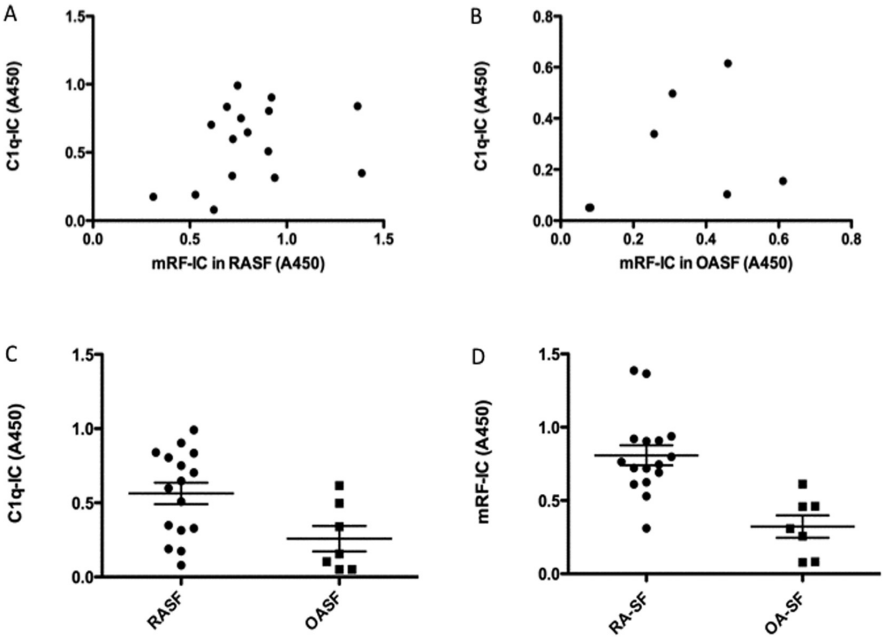

Fig. 1. Immune complex (IC) levels in synovial fluids from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).
Levels of C1q-IC and mRF-IC, which were measured by ELISA and expressed as optical density values read at 450 nm (A450), are shown in synovial fluids from RA patients (RASF) (**A**) and patients with OA (OASF) (**B**). In Figures 1**C** and 1**D**, levels of mRF-IC and C1q-IC were compared between RASF and OASF. The top bar shows the 75 percentile, the bottom the 25 percentile, and the middle bar the median A450.

After washing, a mixture of reagents A and B, contained in the kit, were applied, and the wells were incubated overnight at 37°C. After washes, the wells were blocked with 5% bovine serum albumin (BSA)/PBS with 0.05% Tween 20 (PBST) for 30 minutes, and subsequently incubated with 1:2,500 anti-modified citrulline (Senshu) antibody in 2.5% BSA/PBST for 3 hours at 37°C. Bound antibody was detected with HRPO-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG (Biosource, Camarillo, CA, USA) by 2-hour incubation at 37°C at a dilution of 1:50,000. Bound antibodies were detected by incubation with substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB)(Sure Blue Reserve, Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for 30 minutes at 37°C, the reaction was stopped by the addition of TMB stop solution (Kirkegaard & Perry Laboratories), and the absorbance at a wavelength of 450 nm (A450) was read.

Results

ICs in synovial fluid

We first measured ICs in synovial fluids based on C1q- and mRF-binding assays, in which ICs, captured by immobilised C1q or mRF, were subsequently detected by anti-human IgG polyclonal antibodies. As shown in Fig.1, levels of C1q-IC and mRF-IC, which were expressed as A450 values, were not correlated with each other in RASFs (r=0.388, p=0.137). Both C1q-IC and mRF-IC were significantly higher in RASFs than in OASFs, with p-values of 0.011 and less than 0.01, respectively.

Citrullinated antigens associated with ICs in RASFs

We examined levels of IC citrullination captured by immobilised C1q or mRF using the sandwich ELISA and a microplate, which was precoated with C1q or mRF, contained in the IC ELISA kit in combination with anti-modified citrulline (Senshu) antibody.

Levels of citrullination associated with C1q-IC and those with mRF-IC, which were expressed as A450 values, were significantly correlated each other in RASF (r=0.769, p=0.0005). However, no correlation was observed in OASF (r=0.315, p=0.498). Furthermore, levels

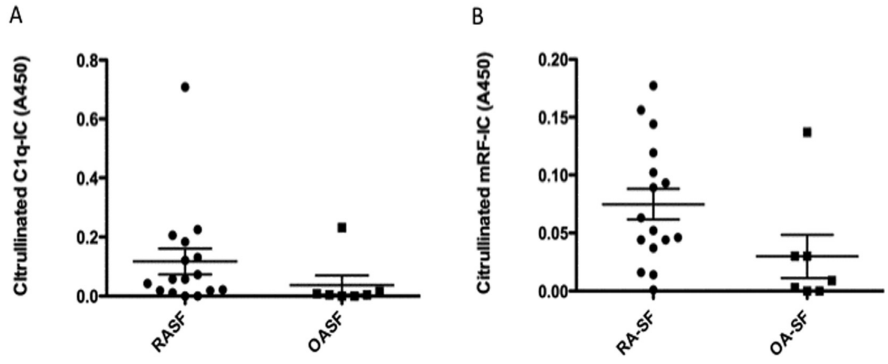

Fig. 2. Levels of citrullination of C1q-binding and monoclonal rheumatoid factor (mRF)-binding peptides in synovial fluids from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).
Levels of citrullination of C1q- (**A**) and mRF-binding (**B**) peptides were measured by ELISA using anti-modified citrulline antibodies in conjunction with C1q-IC and mRF-IC ELISA kits, and were expressed as optical density values read at 450 nm (A450). The top bar shows the 75 percentile, the bottom the 25 percentile, and the middle bar the median A450.

of citrullination associated with C1q-IC (Fig. 2A) and those with mRF-IC (Fig. 2B) were significantly higher in RASF than OASF, with a *p*-value of 0.024 and less than 0.01, respectively. In contrast, no citrullinated Ags associated with ICs were detected in sera from patients with RA and control individuals (data not shown).

Subsequently, we measured the level of anti-CCP Ab in 15 RASFs (one RASF sample from an anti-CCP positive RA patient was not available for this assay) and examined its association with those of citrullination associated with C1q-IC and mRF-IC. All the RASFs tested were considered to be positive for anti-CCP Ab, the titers of which exceeded the serum cutoff value described in the kit. The correlationship between synovial fluid levels of anti-CCP Ab and those of citrullination associated with C1q-IC and mRF-IC was not significant with the Spearman's correlation coefficient of 0.40 (p=0.141) and 0.39 (p=0.149), respectively.

Discussion

ICs containing rheumatoid factor and other autoantibodies may play an important role in the pathogenesis of RA. However, the antigenic components of ICs remain to be elucidated. Recently, several newly characterised autoantibodies have been described that appear to be specific for RA (17). Of particular interest are autoantibodies to citrullinated proteins including anti-CCP (cyclic citrullinated peptides) antibodies, which are highly specific for RA. It is considered that citrullination, mediated by PADIs, is a crucial step for changing a nonimmunogenic into an autoimmunogenic protein in RA (4). Several citrullinated peptides have been reported in rheumatoid synovial tissues (11-13) and in rheumatoid synovial fluids (14-16). However, the citrullinated autoantigen that is pathogonomic of RA remains to be clarified. In the present study, we aimed to explore whether there exist citrullinated antigens that may constitute rheumatoid ICs in synovial fluids.

We first demonstrated that levels of C1q-IC and mRF-IC were significantly higher in RASF than those of OASF. Although previous studies have demonstrated that several cartilage proteins were targeted by sera from OA patients, the data suggest that the overall intensity of autoimmunity is much greater in RA in terms of IC formation in the joint.

We subsequently demonstrated that the peptides in RASF, which were captured by mRF and C1q, were citrullinated *in vivo* by ELISA using anti-modified citrulline antibodies. Rheumatoid factor is an autoantibody that interacts with the Fc portion of complexed IgG, to which C1q can also be bound. Since immunoglobulins and complement fragments have not been reported to be citrullinated *in vivo*, we consider that the citrullinated peptides captured by mRF were antigenic components of mRF-IC. Regarding C1q-IC in RA, we have demonstrated the presence of citrullinated peptides in RASF that were captured by C1q. Zao *et al*. recently demonstrated that citrullinated fibrinogen is present as a component of circulating C1q-IC in one-half of anti-CCP-positive RA patients, and that ICs isolated from pannus tissue also contained citrullinated fibrinogen (18). Their findings were in favour of the possibility that the citrullinated peptides, as demonstrated in the present study as C1q-binding peptides in RASF, can also be antigenic components of C1q-IC. The relationship between the level of anti-CCP Ab and that of citrullination associated with ICs in RASF was not statistically significant. It is possible that anti-CCP Ab contained in ICs may not be detected by anti-CCP ELISA.

On the other hand, several peptides have been reported that can interact directly with C1q, including retroviral peptides (19), adiponectin(20), fibronectin (21), amyloid beta-peptide (22), alpha2beta1 integrin (23), C-reactive protein (24), and Tamm-Horsfall protein (25). Therefore, it cannot be precluded that such C1q-binding peptides were present and citrullinated in RASF. Further studies to characterise the citrullinated peptides captured by C1q and mRF employing a proteomic approach are warranted and currently underway.

In conclusion, we have demonstrated the presence of citrullination in association with C1q- and mRF-binding peptides in RASF. The data suggest that citrullinated antigens may contribute to the pathogenesis of RA through IC formation in the joint. Further studies to identify the antigenic components of rheumatoid ICs as citrullinated peptides would be useful to further our understanding of the pathogenesis of RA.

References

1. ROBERTS-THOMSON PJ, NEOH SH, BRADLEY J, MILAZZO SC: Circulating and intra-articular immune complexes in rheumatoid arthritis: a comparative study of the C1q binding and monoclonal rheumatoid factor assays. *Ann Rheum Dis* 1980; 39: 438-44.
2. WEISSMANN G: Pathogenesis of rheumatoid arthritis. *J Clin Rheumatol* 2004; 10 (Suppl): S26-31.
3. SUZUKI A, YAMADA R, CHANG X *et al*.: Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003; 34: 395-402.
4. YAMADA R, SUZUKI A, CHANG X, YAMA-

MOTO K: Peptidylarginine deiminase type 4: identification of a rheumatoid arthritis-susceptible gene. *Trends Mol Med* 2003; 9: 503-8.
5. NIKOLAISEN C, REKVIG OP, NOSSENT HC: Diagnostic impact of contemporary biomarker assays for rheumatoid arthritis. S*cand J Rheumatol* 2007; 36: 97-100.
6. SNIJDERS GF, BROEDER AA, BEVERS K, JEURISSEN ME, VAN EERD JE, VAN DEN HOOGEN FH: Measurement characteristics of a new rapid anti-CCP2 test compared to the anti-CCP2 ELISA. *Scand J Rheumatol* 2008; 37: 151-4.
7. XUE Y, ZHANG J, CHEN YM, GUAN M, ZHENG SG, ZOU HJ: The HLA-DRB1 shared epitope is not associated with antibodies against cyclic citrullinated peptide in Chinese patients with rheumatoid arthritis. *Scand J Rheumatol* 2008; 37: 183-7.
8. NISHIMURA K, SUGIYAMA D, KOGATA Y *et al*.: Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Ann Intern Med* 2007; 146: 797-808.
9. VOSSENAAR ER, NIJENHUIS S, HELSEN MM *et al*.: Citrullination of synovial proteins in murine models of rheumatoid arthritis. *Arthritis Rheum* 2003; 48: 2489-500.
10. CASPI D, ANOUK M, GOLAN I *et al*.: Synovial fluid levels of anti-cyclic citrullinated peptide antibodies and IgA rheumatoid factor in rheumatoid arthritis, psoriatic arthritis, and osteoarthritis. *Arthritis Rheum* 2006; 55: 53-6.
11. MASSON-BESSIERE C, SEBBAG M, GIRBAL-NEUHAUSER E *et al*.: The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *J Immunol* 2001; 166: 4177-84.
12. TILLEMAN K, VAN STEENDAM K, CANTAERT T, DE KEYSER F, ELEWAUT D, DEFORCE D: Synovial detection and autoantibody reactivity of processed citrullinated isoforms of vimentin in inflammatory arthritides. *Rheumatology* (Oxford) 2008; 47: 597-604.
13. MATSUO K, XIANG Y, NAKAMURA H *et al*.: Identification of novel citrullinated autoantigens of synovium in rheumatoid arthritis using a proteomic approach. *Arthritis Res Ther* 2006; 8: R175.
14. TAKIZAWA Y, SUZUKI A, SAWADA T *et al*.: Citrullinated fibrinogen detected as a soluble citrullinated autoantigen in rheumatoid arthritis synovial fluids. *Ann Rheum Dis* 2006; 65: 1013-20.
15. TABUSHI Y, NAKANISHI T, TAKEUCHI T *et al*.: Detection of citrullinated proteins in synovial fluids derived from patients with rheumatoid arthritis by proteomics-based analysis. *Ann Clin Biochem* 2008; 45(Pt 4): 413-7.
16. SKRINER KAPRJGRB K: Association of citrullinated proteins with synovial exosomes. *Arthritis Rheum* 2006; 54: 3809-3814.
17. NELL VP, MACHOLD KP, STAMM TA *et al*.: Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis. *Ann Rheum Dis* 2005; 64: 1731-6.
18. ZHAO X, OKEKE NL, SHARPE O *et al*.: Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. *Arthritis Res Ther* 2008; 10: R94.
19. SPEAR GT, JIANG HX, SULLIVAN BL, GEWURZ H, LANDAY AL, LINT TF: Direct binding of complement component C1q to human immunodeficiency virus (HIV) and human T lymphotrophic virus-I (HTLV-I) coinfected cells. *AIDS Res Hum Retroviruses* 1991; 7: 579-85.
20. PEAKE PW, SHEN Y, WALTHER A, CHARLESWORTH JA: Adiponectin binds C1q and activates the classical pathway of complement. *Biochem Biophys Res Commun* 2008; 367: 560-5.
21. SORVILLO J, GIGLI I, PEARLSTEIN E: Fibronectin binding to complement subcomponent C1q. Localization of their respective binding sites. *Biochem J* 1985; 226: 207-15.
22. WEBSTER S, BONNELL B, ROGERS J: Charge-based binding of complement component C1q to the Alzheimer amyloid beta-peptide. *Am J Pathol* 1997; 150: 1531-6.
23. EDELSON BT, STRICKER TP, LI Z *et al*.: Novel collectin/C1q receptor mediates mast cell activation and innate immunity. *Blood* 2006; 107: 143-50.
24. AGRAWAL A, VOLANAKIS JE: Probing the C1q-binding site on human C-reactive protein by site-directed mutagenesis. *J Immunol* 1994; 152: 5404-10.
25. RHODES DC: Binding of Tamm-Horsfall protein to complement 1q and complement 1, including influence of hydrogen-ion concentration. *Immunol Cell Biol* 2002; 80: 558-66.