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-l 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)-labelled 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. For the detection of peptidyl-citrulline, the wells were incubated with 1% glutaraldehyde/PBS for 3 hours at 37°C, the reaction was stopped by the addition of TMB stop solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for 30 minutes at 37°C, and the absorbance was measured at 650 nm (A650) and B, contained in the kit, were applied, followed by treatment with 0.2 M Tris-Cl, pH 7.8 for 30 minutes, and subsequent incubation with 1:50,000 anti-modified citrulline (Senshu) antibody. As shown in Fig.1, levels of C1q-IC and mRF-IC were significantly higher in RASF than in OASF, with correlation values read at 450 nm (A450), are shown in synovial fluids from RA patients (RASF) (A) and patients with OA (OASF) (B). In Figures 1C and 1D, 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.

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 OASF, 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...
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 correlation between synovial fluid levels of anti-CCP Ab and that of citrullination associated with C1q-IC and mRF-IC was not 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

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