Original Article

Novel anti-citrullinated peptide autoantibodies identified by proteomics with *in vitro* citrullinated proteins in patients with rheumatoid arthritis

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Rheumatoid arthritis (RA) is immunologically characterized by generation of anti-citrullinated peptide antibodies (ACPAs). To promote understanding of ACPAs, we here identified novel ACPA antigens by proteomics using *in vitro* citrullination and investigated effects of citrullination on one of the ACPA antigens. Specifically, we citrullinated protein extracts from Jurkat cells *in vitro* and separated the citrullinated protein extracts as well as non-treated ones by 2-dimensional electrophoresis (2DE). By western blotting, we detected protein spots that reacted to RA serum samples only when they were citrullinated and identified them by mass spectrometry. On two of the identified proteins, adenosine deaminase (ADA) 1 and proliferation associated protein 2G4 (PA2G4), we confirmed the citrulline-dependent recognition by ACPAs using enzyme-linked immunosorbent assay (ELISA). Finally, we compared the activity of citrullinated ADA1 and non-treated one. As a result, we detected 8 candidate antigens for ACPAs, and identified 7 of them. In ELISA, 6 (24%) out of the tested 25 RA serum samples reacted to citrullinated ADA1, but none (0%) to non-treated ADA1 ($p = 0.022$). Similarly, 9 (36.0%) out of the same RA samples reacted to citrullinated PA2G4, but only 1 (4.0%) to non-treated PA2G4 ($p = 0.01$). In addition, we found that citrullination enhanced activity of ADA. In conclusion, we demonstrated novel autoantigens for ACPAs including ADA1 and PA2G4 and enhancement of the ADA activity by citrullination. The enhanced ADA activity may decrease anti-inflammatory adenosine and thus may hamper the pharmaceutical increase of adenosine by methotrexate in RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized histologically by severe synovitis and pannus formation in multiple joints. Immunologically, the most distinct feature of RA is generation of anti-citrullinated peptide autoantibodies (ACPAs)[1-3]. At present, ACPAs are usually tested by using the cyclic citrullinated peptide (CCP) for diagnosis of RA. In particular, the second and third generations of CCP, based on artificial peptides have great power to detect ACPAs[4, 5]. For the diagnosis of RA, the sensitivity and specificity of the ACPA tests were found to be significantly higher than those of rheumatoid factor (RF), a classical serological marker for RA[6]. Further, it was reported that ACPAs predicted the future development of RA and the severity of arthritis[7-9].

In addition to the diagnostic values of ACPAs, the immune reaction to the citrullinated peptides is highly expected to be involved in the pathophysiology of RA[1, 10]. Instantly, it was evidenced that the positive immune responses to citrullinated proteins were linked to the shared epitope contained by the major RA-susceptibility alleles like HLA-DRB1*0405[11, 12]. Also, citrullination possibly alters protein functions. For example, it was reported that citrullination of murine vimentin filaments disturbed assembly of the filaments[13]. Thereby, citrullination of autoantigens may contribute to the pathological mechanisms of RA by altering their functions. Considering these backgrounds, it would be important to understand the total profile of citrullinated peptides in RA and in fact they have been investigated for decades. Typical of the reported citrullinated autoantigens are fibrin (ogen)[14], vimentin[15], type II collagen[16], and α-enolase[17] and other various citrullinated autoantigens have been reported in RA as reviewed elsewhere[10]. We have also demonstrated by proteomics that citrullinated proteins and autoantigens overlapped each other considerably in synovial cells of patients with RA and that F-actin capping protein α-1 was a target of ACPAs[18]. However, the total profiles of the citrullinated proteins in RA remain to be elucidated. We here surveyed citrullinated autoantigens recognized by ACPAs using proteomics with an *in vitro* citrullination method, by which proteins that were recognized by ACPAs only in the citrullinated condition were identified. One of the identified autoantigens was adenosine deaminase (ADA) 1, which is known as a target of methotrexate (MTX), an anchor drug for RA[19]. Functionally, we found that citrullination up-regulated the activity of ADA. Our data would promote understanding of the roles of ACPAs and their antigens in the pathogenesis of RA.

Materials and Methods

1)Clinical samples

Serum samples were obtained from 25 patients with RA (20 women and 5 men, an average age of 59.7 years, range 33-81 years). The RA patients were diagnosed according to the 1987 classification criteria[20]. Serum samples obtained from age- and sex- matched healthy donors were used as a control (20 women and 5 men, an average age of 58.6 years, range 28-76 years). All the samples were obtained with informed consent and this study was approved by the local institutional ethics committee.

2)*In vitro* citrullination, 2-dimensional electrophoresis (2DE), and western blotting (WB)

Jurkat cells, suspended in a solution containing 50mM Tris-HCl pH7.4, 0.25% Tween20, were sonicated. To obtain protein extracts from the cells, supernatant was collected after centrifugation. A part of the extracted proteins was citrullinated *in vitro* in a solution (100mM Tris-HCl pH7.4, 10mM $CaCl_2$, 5mM DTT, and 20U/mg rabbit peptidyl arginine deiminase (PAD, Sigma-Aldrich Co, MO, USA). The citrullinated protein extracts and non-treated ones, resolved in a solution of 7M urea, 1.2% Destreak, 0.5% IPG buffer, 2.5% bromophenol blue, were subjected to 2DE as described previously[18]. Briefly, the protein samples were separated by isoelectric focusing (IEF) at 20℃ for 12 hours, using 11 cm Imobiline drystrip gels (pH range 3-11) and Ettan IPGphor (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Next, the separated proteins were further separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the gels were stained with a fluorescent dye of SYPRO Ruby® (Molecular Probes, Eugene, OR), and were used for protein transfer onto nitrocellulose membranes for the subsequent WB or used for protein identification by mass spectrometry. 100 μg and 500 μg of the extracts were used for WB and protein identification, respectively.

WB after 2DE was performed as described previously[18]. Briefly, the transferred proteins onto membranes were reacted with 1:200 diluted serum samples from the patients with RA. Bound antibodies were reacted with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Zymed Laboratories, San Francisco, CA) and were then visual-

ized with diaminobendzidine. Protein spots that reacted to the RA serum samples in 2DE-WB with citrullinated protein extracts but did not react to the same samples in 2DE-WB with non-treated protein extracts were selected as a target of ACPAs. In WB, peptidyl citrulline was detected by anti-modified citrulline (MC) antibodies (Upstate, Lake Placid, NY) as described previously[18].

3)Protein identification

Serum-reactive protein spots were recovered from the 2DE gels stained with SYPRO Ruby®, and were subjected to in-gel digestion with trypsin. Next, mass-to-charge ratios (m/z) of the digested proteins were measured using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany) as described previously[18]. Mass spectra of positively charged ions were recorded in the reflector mode. The flexControl, flexAnalysis, and Biotools software packages provided by the manufacturer were used for data processing. A list of the determined peptide masses were subjected to mass fingerprinting using MASCOT software program (Matrix Science, London, UK). MS/MS spectra obtained by the collision-induced dissociation in the TOF/TOF mode were also used for the protein database searching.

4)Preparation of proteins

Recombinant human (rh) ADA1 was prepared as a fusion protein with glutathione-S-transferase (GST). Specifically, cDNA for human ADA1 was amplified by PCR with two primers: 5'-tttgaattcatggcccagacgcccgcc (underline, the EcoRI recognition sequence) and 5'-tttctcgaggttctgccc tgcagagg (underline, the XhoI recognition sequence). Then the amplified cDNA for human ADA1 was inserted to pGEX-6P-1 using the EcoRI and XhoI recognition sites. By this construct, rhADA fused with GST and a histidine tag (rhADA1-GST) was produced in E. Coli, which was affinity-purified by HisTrap HP (GE healthcare), according to the manufacturer's instructions. Recombinant human (rh) proliferation associated protein 2G4 (PA2G4) with an N-terminal GST-tag (rhPA3G4-GST) was purchased (Abona Co., Taipei, Taiwan).

5)Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously[18]. Each well of a multi-titer plate (Immulon; Thermo Labsystems, Franklin, MA) was coated with 10 μg/ml of the individual proteins in a carbonate buffer (50 mM sodium carbonate, pH 9.6), followed by blocking with phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.1% Tween 20. Serum samples diluted 1:200 were added to the wells and incubated for 90 minutes. Then the wells were washed 3 times in PBS with 0.1% Tween-20. Next, bound antibodies were reacted with HRP-conjugated goat anti-human IgG (Zymed, San Francisco, CA) for 1 hour at room temperature and then with o-phenylenediamine as a substrate and finally were quantitated using a micro-plate photometer at 492 nm. The reactivity of the serum samples to rhADA1-GST and rhPG2D4-GST was expressed using the arbitrary binding units, calculated according to the following formula: binding units (sample) = {OD sample/(mean OD control serum samples + 3SD control serum samples)} x 100); where OD is the optical density. According to this formula, 100 binding units were used as the cutoff point.

In addition, titers of anti-CCP antibodies in the patients were measured by MESACUP-2® as a clinical laboratory examination.

Measurement of ADA activity

Commercially available ADA1 extracted from human erythrocytes (Institute for Reference Materials and Mesurements, Geel, Belgium) and ADA extracted from bovine intestine (Roche Applied Bioscience) were used. They were citrullinated by PAD in vitro (Sigma-Aldrich) at 37 ℃ for 4 hours and then ADA activity was assayed by a commercially available assay kit (Diazyme Laboratories, CA, USA). In the case of human ADA1, the citrullinated ADA1 and non-treated one were dialyzed with a solution containing 100mM Tris-HCl (pH7.4), 10mM $CaCl_2$, 2.5mM oxidized glutathione, 2.5mM reduced glutathione, after which ADA activity was measured.

1)Statistical analysis

Fisher's exact probability test and Student's *t*-test were used to test statistical significance of the obtained data.

Results

1)Surveillance of citrullinated autoantigens by 2DE-WB

To identify novel ACPAs and their antigens in RA, we here conducted 2DE-WB differential screening using *in vitro* citrullinated Jurkat cell protein extracts and non-treated ones as antigen sources. Specifically, we first then citrullinated the protein extracts by PAD *in vitro*. As shown in

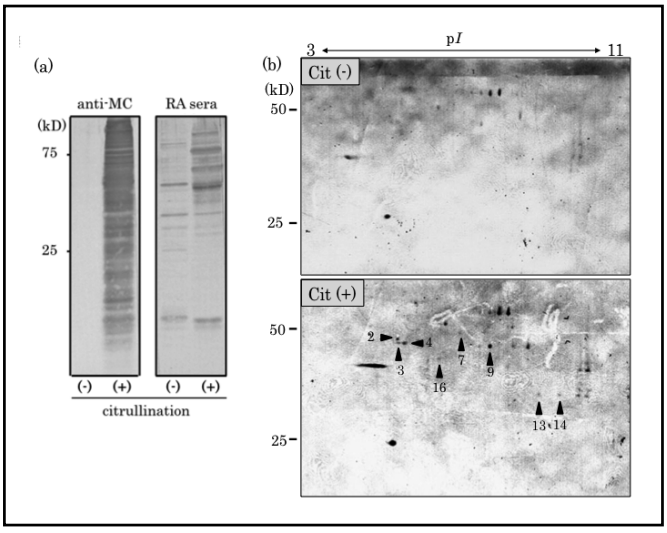

Fig.1 Protein citrullination and 2DE-WB screening of citrulline-dependent autoantigens

(a)Protein extracts from Jurkat cells, *in vitro*-citrullinated or non-treated, were separated by SDS-PAGE. Then the separated protein extracts were transferred onto nitrocellulose membranes and were reacted with anti-MC antibodies (left) and serum samples from patients with RA (right). A representative result is shown.

(b)Citrulline-dependent autoantigens were detected by 2DE-WB. The protein extracts from Jurkat cells, *in vitro*-citrullinated or non-treated, were separated by 2DE. Then the separated protein extracts were transferred onto nitrocellulose membranes and were reacted with serum samples from 5 patients with RA. A representative result from P1 is shown. The arrowheads indicate protein spots that reacted to two or more of the 5 serum samples in the 2DE-WB using the cutrullinated protein extracts but did not react to the samples in the 2DE-WB using non-treated protein extracts. Cit: citrullination, p*I*: isoelectric point

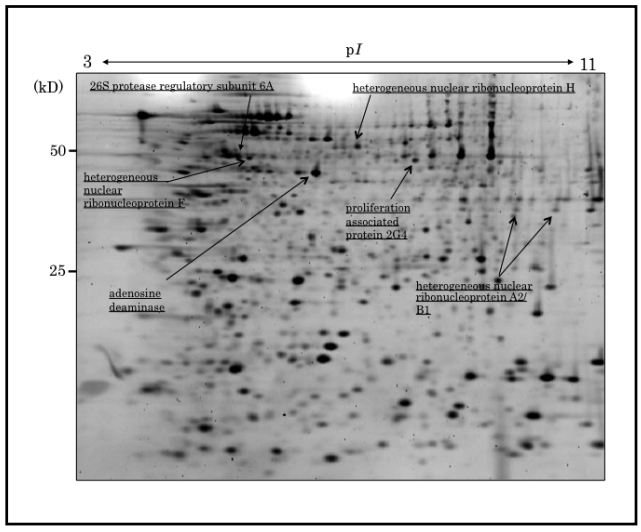

Fig.2 The positions of the identified 7 protein spots are indicated on a 2DE gel stained with SYPRO Ruby®.

The information on the identified proteins are summarized in Table 1.

the left panel of Figure 1(a), the number of protein bands reacted to the anti-MC antibody, which detected citrulline residues of peptides, was markedly increased by citrullination. This indicated that a considerable number of proteins in the extracts were citrullinated. Then we tested the reactivity of RA serum samples to the citrullinated and non-treated protein extracts by WB. As a result, we demonstrated that the number of RA serum-reactive protein bands was greatly increased by the citrullination, as shown in the right panel of Figure 1(a). This indicates that the *in vitro* citrullination is an effective way to detect ACPAs and their antigens in RA.

We then applied this method to 2DE-WB to detect and identify ACPAs and their antigens comprehensively. Specifically, we separated the citrullinated and non-treated Jurkat cell protein extracts by 2DE and transferred them onto membranes respectively, after which we tested the reactivity of the transferred proteins to 5 serum samples from patients with RA. We obtained multiple protein spots that reacted to the RA serum samples when we used citrullinated protein extracts in 2DE-WB. On the other hand, we obtained much fewer protein spots that reacted to the same serum samples when we used non-treated protein extracts in 2DE-WB. A representative result is shown in Figure 1(b). The protein spots that reacted to the serum samples only in the citrullinated condition were thought to be citrullinated autoantigens in RA, that is, targets of ACPAs.

2)Identification of the candidate autoantigens of ACPAs

From these candidate autoantigens of ACPAs, we selected 8 protein spots, which reacted to at least 2 out of the 5 RA serum samples, for protein identification by MALDI-TOF mass spectrometry (Spots 2, 3, 4, 7, 9, 13, 14, and 16 in the lower panel of Figure 1(b)). As a result, we successfully identified 7 out of the 8 protein spots. The profiles of the identified protein spots are summarized in Table 1 and the positions of them on a SYPRO Ruby®-stained 2DE gel are shown in Figure 2.

3)Antibody titers to the identified citrullinated autoantigens by ELISA

Among the 7 identified autoantigens, we confirmed citrulline-dependent antigenicity of them using ELISA, focusing

Table 1 A list of candidate citrullinated autoantigens

spot	pI (observed)	MW (observed)	reactivity to the RA sera 1	2	3	4	5	protein name	pI (theoretical)	MW (theoretical)	accession no.
2	5.5	48	+			+		26S protease regulatory subunit 6A	5.13	49.2	gi¦20532406
3	5.5	47	+	+				not determined	-	-	-
4	5.5	47	+	+				heterogeneous nuclear ribonucleoprotein F	5.38	45.6	gi¦1710628
7	6.0	51	+				+	heterogeneous nuclear ribonucleoprotein H	5.89	49.2	gi¦1710632
9	6.5	45	+				+	proliferation associated protein 2G4	6.13	43.8	gi¦13632817
13	7.8	33	+	+				heterogeneous nuclear ribonucleoprotein A2/B1	8.97	37.4	gi¦133257
14	8.5	33	+	+			+	heterogeneous nuclear ribonucleoprotein A2/B1	8.97	37.4	gi¦133257
16	5.7	43	+	+		+		adenosine deaminase	5.6	40.7	gi¦113339

The 8 protein spots that reacted to two or more of the 5 RA serum samples were subjected to protein identification by mass spectrometric analysis. MW: molecular weight, pI: isoelectric point.

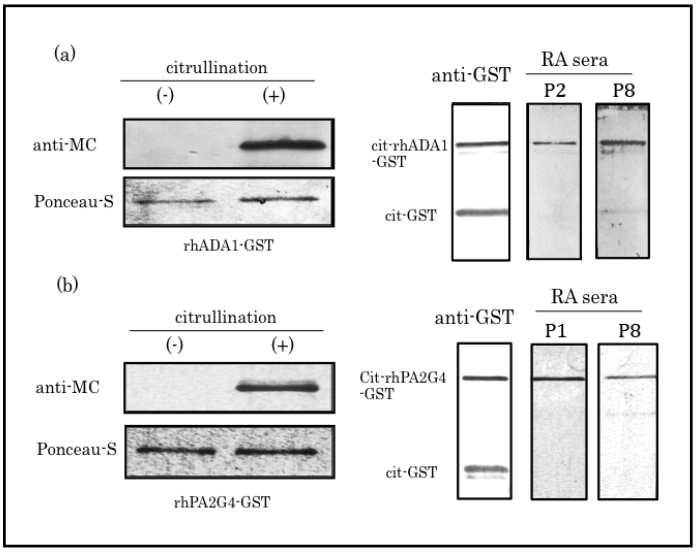

Fig.3 Preparation of citrullinated recombinant human ADA1 and PA2G4 proteins

(a)Recombinant human (rh) ADA1 prepared as a GST fusion protein was citrullinated *in vitro*, as positively detected by the anti-MC antibody by WB (left). The citrullinated rhADA1-GST and similarly citrullinated GST were incubated with the anti-citrullinated ADA1-positive serum samples in the 2DE-WB screening. The samples reacted to the citrullinated rhADA1 but not to the citrullinated GST. Results from P2 and P8 are shown (right).
(b)rhPA2G4 prepared as a GST fusion protein was citrullinated in vitro, as positively detected by the anti-MC antibody by WB (left). The citrullinated rhPA2G4-GST and citrullinated GST were incubated with the anti-citrullinated PA2G4-positive serum samples in the 2DE-WB screening. The samples reacted to the citrullinated rhPA2G4 but not to the citrullinated GST. Results from P1 and P8 are shown (right).

on Spot 16 of ADA1 and Spot 9 of PA2G4. It is because Spot 16 of ADA1 reacted to 3 (60%) out of the 5 RA serum samples in the screening (Table 1) and ADA1 is known to be one of the target molecules of MTX, an anchor drug of RA, and because autoantibodies to ADA1 and PA2G4 have not been reported until now to our knowledge.

To confirm their citrulline-dependent antigenicity, we used rhADA1 and rhPA2G4 with a glutathione-S-transferase tag (rhADA1-GST and rhPA2G4-GST, respectively). We citrullinated rhADA1-GST *in vitro* as shown in the left panel of Figure 3(a). Then we confirmed that the RA serum samples that reacted to the citrullinated ADA1 spot in 2DE-WB reacted to the ADA1 part not to the GST part of rhADA1-GST. Representative results from P2 and P8 samples in Table 1 are shown in the right panel of Figure 3(a). Similarly, we citrullinated rhPA2G4-GST as shown in the left panel of Figure 3(b) and then confirmed that the RA serum samples reacted to the PA2G4 part not to the GST part of rhPA2G4-GST. Representative results from P1 and P8 are shown in the right panel of Figure 3(b).

We next measured antibody titers to the citrullinated and non-treated rhADA1-GST and rhPA2G4-GST by ELISA, using serum samples from 25 RA patients and 25 healthy donors. As a result, 6 (24.0%) out of the 25 RA serum samples recognized the citrullinated rhADA1-GST, while none of the 25 healthy donor samples recognized the

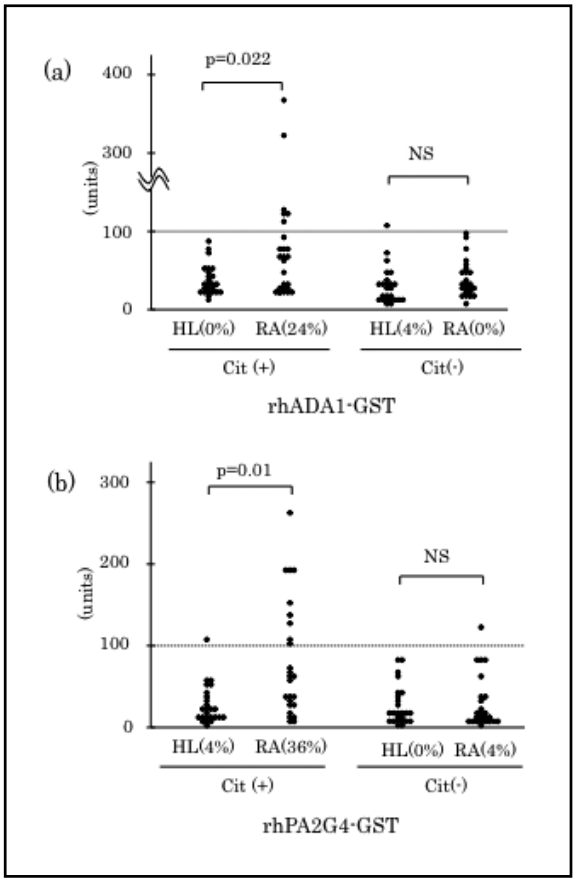

Fig.4 Reactivity of the citrullinated ADA1 and PA2G4 measured by ELISA

Autoantibodies to the citrullinated and non-treated rhADA1 (a) and rhPA2G4 (b) were measured by ELISA using serum samples from 25 patients with RA and 25 healthy donors. The binding units of 100 were defined as the cut-off point, as described in the materials and methods section. The percentages of serum samples with positive titers are described in the parentheses.

Cit-: citrullinated, NS: not significant.

citrullinated rhADA1-GST as shown in Figure 4(a)-right. The positive ratio was significantly higher in the RA group than in the healthy group (p= 0.022). On the other hand, none of the 25 RA samples and only 1 (4.0%) of the 25 healthy donor samples reacted to the non-treated rhADA1-GST (Figure 4(a)-left). We thus confirmed that the RA serum samples recognized ADA1 only in the citrullinated condition. We obtained similar results in the case of PA2G4. Nine (36.0%) out of the same 25 RA samples reacted to the citrullinated rhPA2G4-GST, while only one (4.0%) of the 25 healthy donor samples reacted to the citrullinated rhPA2G4-GST (p= 0.01, Figure 4(b)-right). On the other hand, only 1 (4.0%) of the 25 RA samples and none of the

Table 2 Relations between the presence of antibodies to citrullinated ADA1 and that of antibodies to citrullinated PA2G4

		anti-citrullinated ADA1	
		+	-
anti-citrullinated PA2G4	+	5	4
	-	1	15
p = 0.012			

Table 3 Relations between the presence of antibodies to citrullinated ADA1 and that of anti-CCP antibodies

		anti-CCP	
		+	-
anti-citrullinated ADA1	+	6	0
	-	14	5
p = 0.289			

Table 4 Relations between the presence of antibodies to citrullinated PA2G4 and that of anti-CCP antibodies

		anti-CCP	
		+	-
anti-citrullinated PA2G4	+	9	0
	-	11	5
p = 0.123			

25 healthy donor samples reacted to the non-treated rhADA1-GST (Figure 4(a)-left). We thus confirmed that the RA serum samples recognized PA2G4 only in the citrullinated condition. These data demonstrated that ADA1 and PA2G4 were citrulline-dependent autoantigens in RA.

We next investigated relations between the presence of antibodies to citrullinated ADA1 and that of antibodies to citrullinated PA2G4. As shown in Table 2, the anti-citrullinated ADA1 antibody-positive patients tended to possess anti-citrullinated PA2G4 antibodies with statistical significance (p= 0.012). However, a part of the tested serum samples were positive for only one of the two antibodies. This indicates that the antigenicity of the citrullinated ADA1 and citrullinated PA2G4 is different from each other.

Furthermore, we investigated relations between the presence of anti-citrullinated ADA1 antibodies and that of anti-CCP antibodies and between the presence of anti-citrullinated PA2G4 antibodies and that of anti-CCP antibodies. As shown in Tables 3 and 4, no specific relation was ob-

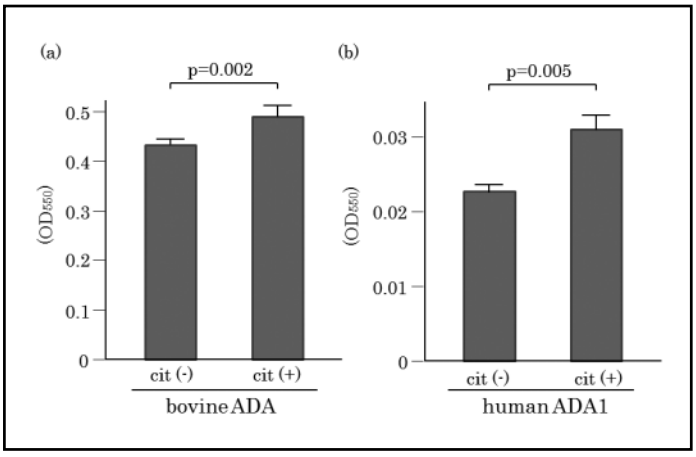

Fig.5 Effects of citrullination on the ADA activity
Bovine intestine-derived ADA, citrullinated or incubated in the same solution without PAD as a negative control, was subjected to the ADA activity assay (a). Human erythrocyte-derived ADA1, similarly citrullinated or incubated without PAD as a negative control, were refolded by dialysis and then were subjected to the ADA activity assay (b).

served between the presence of anti-CCP antibodies and that of anti-citrullinated ADA1 or PA2G4 antibodies. Of note, all the samples that were positive for either anti-citrullinated ADA1 or PA2G4 antibodies were also positive for anti-CCP antibodies.

4)Effects of citrullination on the ADA activity

ADA decreases the adenosine level by converting adenosine to inosine, while MTX releases endogenous anti-inflammatory adenosine as a pharmaceutical action, as reviewed elsewhere[19, 21]. Thereby, citrullination of ADA may affect the responses of RA patients to MTX if citrullination alters the ADA activity. We thus investigated whether citrullination affected the activity of ADA using ADA extracted from bovine intestine and ADA1 extracted from human erythrocytes. As a result, we found that citrullination increased the activity of the bovine ADA with statistical significance as shown in Figure 5(a). In the case of the human ADA1, since reducing agents contained in the reagent of PAD and also in its buffer abolished the ADA activity in our preliminary experiments (data not shown), we measured the activity of human ADA1 after refolding by dialysis. As a result, the citrullinated human ADA1 showed significantly higher activity than non-treated human ADA1 (Figure 5(b)), similarly as the case of bovine ADA. From these two results, we concluded that citrullination increased the activity of ADA.

Discussion

Citrullinated autoantigens, that is, targets of ACPAs, are of great interest in the investigation of the pathology of RA. To identify citrullinated autoantigens, it would be one way to detect proteins that are citrullinated and also recognized by RA sera in clinical samples like synovial tissue from patients with RA, as we previously reported[18]. However, the profile of citrullinated autoantigens would be different from patient to patient, which may hamper efficient screening. Thereby, we here used an alternative way, screening of enzymatically citrullinated protein extracts as an antigen source[17]. The method possibly detects novel citrullinated autoantigens, even though it is unclear whether the *in vitro* citrullination reflects *in vivo* citrullination occurred in patients with RA from the viewpoints of degrees and sites of citrullination.

As shown in Figure 1(a), the PAD treatment clearly increased citrullinated proteins, which would be a useful source of citrullinated autoantigens. Here we used Jurkat cells as the first try, however, other cell types such as synoviocytes and chondrocytes can be also used. Such studies may reveal additional citrullinated autoantigens and should be tried in the future.

By the comparison of the RA serum reactivity between the citrullinated and non-treated protein extracts, we detected 8 citrulline-dependent autoantigens, 7 of which were identified. Further, we demonstrated that two of the 7 identified proteins really possessed citrulline-dependent autoantigenicity to RA serum samples by ELISA using recombinant proteins. One of the two citrullination-dependent autoantigens was ADA1.

Biochemically, ADA converts adenosine to inosine, in other words, ADA decreases adenosine levels. Adenosine is a molecule of particular interest in RA, since one of

the anti-inflammatory mechanisms of MTX has been reported to increase endogenous anti-inflammatory adenosine[19, 21] In adenosine A2a- and A3- deficient mice, MTX did not inhibit acute inflammation[22]. Furthermore, adenosine receptor antagonism reduced anti-inflammatory effects of MTX in animal models[22]. These data indicate the significance of adenosine in the therapeutic functions of MTX and in the pathophysilology of RA. Interestingly, high ADA activity was observed in synovial fluid of patients with RA[23]. In accordance with the report, the MTX treatment was shown to decrease ADA activity in patients with RA[24]. Our data indicates that citrullination of ADA1 increases its enzymatic activity. Thus citrullination of ADA1 would decrease the endogenous adenosine levels, in turn, may hamper the anti-inflamatory effects of MTX. The relation among citrullination of ADA1, ACPAs to citrullinated ADA1, and the responses to MTX should be investigated in patients with RA in the future.

In the measurement of the human ADA1 activity, we encountered a difficulty. The citrullinating enzyme of PAD did not work efficiently without reducing agents. In fact, the reagent of PAD and its buffer contained reducing agents. On the other hand, the ADA activity was abolished by the reducing agents in our preliminary experiments (data not shown) and as previously suggested[25]. Thereby we here refolded the human ADA1 by dialysis after citrullination with reducing agents and then measured the activity. The results from experiments using the bovine ADA and the human ADA1 were similar to each other: the citrullination up-regulated the activity of ADA. It is needed to investigate whether this up-regulation occurs in patients with RA in the future.

We here investigated PA2G4 as well as ADA1. PA2G4, also termed Ebp1, was initially identified as a protein binding to the Erb-3 receptor[26-28]. PA2G4 was reported to be a transcription regulator, affecting cell growth, proliferation, differentiation, and survival[29, 30]. Recently, up-regulation of PA2G4 was reported in melanoma cells[31]. Thereby, PA2G4 may be involved in the massive proliferation of synovial cells in patients with RA. However, no report on PA2G4 has been available in the context of RA until now. Roles of PA2G4, citrullinated PA2G4, and ACPAs to PA2G4 in RA should be investigated in the future.

From the analysis of relations among the anti-citrullinated ADA1 antibodies, anti-citrullinated PA2G4 antibodies, and anti-CCP antibodies, we found that the antigenicity of the citrullinated ADA1 and PA2G4 would be different, since a part of the tested serum samples possessed antibodies to only one of the two antigens, citrullinated ADA1 and PA2G4. All the 10 serum samples that were positive for either anti-citrullinated ADA1 or PA2G4 antibodies were also positive for anti-CCP antibodies. Thereby, the antigenicity of CCP may contain that of the citrullinated ADA1 and PA2G4. Further studies would be needed to elucidate this point.

In addition, other identified citrullinated proteins by the 2DE-WB screening included heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP-A2/B1). Antibodies to hnRNP-A2 are also known as anti-RA33 antibodies, which have been reported to have a diagnostic value for RA[32, 33]. Our data indicate that citrullinated hnRNP-A2/B1 would react to RA sera more strongly than non-treated one. Roles of citrullination in the serum recognition of hnRNP-A2/B1 in RA should be investigated.

In conclusion, we here reported novel citrullinated autoantigens in RA using *in vitro* citrullination and differential 2DE-WB. As ADA1 increased its enzymatic activity by citrullination, biochemical roles of citrullination as well as immunological ones should be investigated in the pathology of RA.

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Disclosure

All authors have no conflict of interest.

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