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The inhibition of antithrombin by peptidylarginine deiminase 4 may contribute to pathogenesis of rheumatoid arthritis

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Objective. **The gene for peptidylarginine deiminase 4 (*PADI4*) has been found to be closely associated with rheumatoid arthritis (RA). Peptidylarginine deiminase (PADI) catalyses the post-translational modification of peptidylarginine to citrulline, a reaction known as citrullination. PADI extracted from rabbit muscle has been reported to citrullinate antithrombin, a principal plasma inhibitor of thrombin. Thrombin is known to induce angiogenesis, fibrin formation and inflammation, the primary events of the RA joint. Here, we investigate whether human PADI4 can inhibit antithrombin by catalysing antithrombin citrullination and how the enzyme is involved in RA pathogenesis.**

Methods. **Antithrombin was incubated with recombinant PADI4 protein, and the inactivation of antithrombin was determined by reduction of its thrombin-inhibiting activity. Citrullination of antithrombin was detected by western blotting and enzyme-linked immunosorbent assay (ELISA). In addition, the citrullination level, activity and concentration of antithrombin in RA plasma were investigated by sandwich ELISA.**

Results. **Incubation of antithrombin with PADI4 resulted in loss of thrombin-inhibitory activity and in citrullination of antithrombin. RA plasma showed higher levels of citrullinated antithrombin than controls with non-arthritis disease and healthy individuals.**

Conclusion. **The results indicate that PADI4 could inactivate antithrombin through citrullination. The abnormal expression or activation of PADI4 in RA synovium is suggested to be responsible for the high level of citrullinated antithrombin in RA plasma. Local inhibition of antithrombin activity in RA synovium might lead to the excessive angiogenesis, fibrin deposition and inflammation of the tissue.**

KEY WORDS: Rheumatoid arthritis, Peptidylarginine deiminase 4 (PADI4), Antithrombin, Thrombin, Citrullination.

Peptidylarginine deiminase (PADI) catalyses citrullination, the post-translational modification of arginine residues in proteins to citrulline. To date, four PADI genes have been identified in the human genome (*PADI1–4*). These are located on chromosome 1p36, a region associated with susceptibility to rheumatoid arthritis (RA) [1]. Our large-scale genome-wide case–control study using single nucleotide polymorphisms (SNPs) has demonstrated that *PADI4* is closely associated with RA [2]. Hence, investigating the potential role of PADI4 in the predominant events of RA (excessive angiogenesis, fibrin formation and inflammation) might aid in understanding the nature of the disease. In 1997, Pike *et al*. found that PADI extracted from rabbit muscle could citrullinate antithrombin-α in the presence of heparin [3]. Antithrombin, a 52 kDa plasma-derived glycoprotein, is a principal plasma inhibitor of coagulation proteases, especially thrombin and Factor Xa. Thus, the conversion of arginine to citrulline catalysed by PADI could prevent the inhibition of antithrombin on thrombin. Because thrombin is a key mediator of angiogenesis, blood coagulation and inflammation [4, 5], it becomes essential for understanding RA pathogenesis to investigate whether human PADI4, like rabbit muscle PADI, can inactivate antithrombin. In this study, we tested the effect of PADI4 on antithrombin by *in vitro* incubation and by measuring the level of citrullination of antithrombin in the blood of patients with RA.

Methods and materials

Blood samples

Blood samples from RA patients ($n = 39$), healthy volunteers ($n = 14$) and patients with systemic lupus erythematosus (SLE) ($n = 11$) were drawn by standard venepuncture and collected with Monovette tubes containing 3.8% sodium citrate (Terumo, Japan). Following centrifugation at 1000 *g* for 30 min, the supernatant of anticoagulated plasma was collected and stored at −80°C until use. We obtained written informed consent to collect blood samples from patients and the healthy volunteers. The Ethics Committee of the Institute of Physical and Chemical Research gave ethical approval for the study.

Preparation of recombinant PADI4 protein

A complementary deoxyribonucleic acid (cDNA) containing the entire open reading frame of human PADI4 was inserted into a pQE-16 vector (Qiagen) to add a *C*-terminal His tag and

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expressed in *Escherichia coli* according to the manufacturer's instructions. Recombinant PADI4 protein was purified from *E. coli* by standard Ni-NTA affinity purification.

Determining antithrombin activity

Human antithrombin III (Sigma) at a final concentration of 150 μg/ml was incubated with various concentrations of recombinant PADI4 or rabbit peptidylarginine deiminase (Sigma) in working buffer (100 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.4). Heparin was not added to the reaction so as to simply observe the effect of PADI on antithrombin. The reaction proceeded for 24 h at 37°C and was then stopped with EDTA. Antithrombin III activity was quantified by measuring its thrombin-inhibiting activity, a method in routine use in clinical laboratories, using an Actichrome AT III kit (American Diagnostica, USA) as follows. After PADI treatment, antithrombin was incubated with thrombin reagent provided with the kit, then residual thrombin activity was determined by a thrombin-specific chromogenic reaction. The absorbance of the reaction was measured at a wavelength of 405 nm using a plate reader (Packard, USA). Hence, the level of active antithrombin was inversely proportional to the residual thrombin activity. The antithrombin activity of plasma was also measured by this method.

Determining citrullination in PADI4-treated antithrombin by enzyme-linked immunosorbent assay (ELISA)

Antithrombin (150 μg/ml) was incubated with 1 or 5 mM recombinant PADI4 as described above. Thereafter, the reaction was diluted 5-fold in phosphate-buffered saline (PBS) and coated onto polyvinylchloride (PVC) 96-well EIA/RIA microtitre plates (Costar) overnight at room temperature. The plates were washed with PBS containing 0.1% Tween 20 and then blocked with 5% non-fat dry milk for 1 h at room temperature. After washing with PBS-Tween, plates were incubated with 5000-fold diluted anticitrulline antibody (Biogenesis, USA) at room temperature. After another wash with PBS-Tween, alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 5000-fold in 0.05 M carbonate/bicarbonate buffer (pH 9.6) was added onto the plate at room temperature for 1 h. Following a wash with PBS, signals were developed by adding 1 mg/ml *p*-nitrophenyl phosphate (Sigma) in substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8), and then absorbance was measured at 405 nm.

Determining citrullination in PADI4-treated antithrombin by western blot

Antithrombin (150 μg/ml) was incubated with recombinant PADI4 (5 mM) as described above. The reaction mixture was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted onto a polyvinylidene difluoride membrane. Citrulline production in PADI4-treated antithrombin was detected with the Anti-Citrulline (Modified) Detection Kit (Upstate, USA) according to the manufacturer's instructions. The signal of the target protein was detected with the Protein Detector BCIP/NBT western blot kit (KPL, USA).

Determining the citrullination level of antithrombin by sandwich ELISA (double-antibody ELISA)

Antihuman antithrombin monoclonal antibody (American Diagnostica, USA) was diluted 25 000-fold in 0.05 M carbonate–bicarbonate buffer (pH 9.6) according to the manufacturer's instructions and coated onto PVC 96-well EIA/RIA microtitre plates (Costar) by overnight incubation at room temperature. After brief washing with PBS containing 0.1% Tween 20 (PBS-Tween), the plate was blocked with 5% non-fat dry milk for 1 h at room temperature. Plasma samples were diluted 4000-fold with PBS-Tween and added onto the plate, then incubated for 1 h at room temperature. After washing again with PBS-Tween, the plate was incubated with a 1/5000 dilution of anticitrulline antibody (Biogenesis, USA) for 1 h at room temperature. Rabbit anticitrulline antibody was labelled with alkaline phosphatase using the AP Labeling Kit (Roche) according to the manufacturer's instructions. Following a wash with PBS-Tween, the signal was developed by adding 1 mg/ml *p*-nitrophenyl phosphate in substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) (Sigma). The absorbance of the reaction was measured at 405 nm with a plate reader. Negative controls consisted of a series of wells with carbonate–bicarbonate buffer in place of the antihuman antithrombin antibody for each sample (plasma non-specific binding) and a well with PBS-Tween buffer in place of plasma (antibody non-specific binding). Data from the experimental samples were corrected by subtracting the sum of corresponding plasma non-specific binding and antibody non-specific binding.

Determining antithrombin concentration

The concentration of antithrombin in the blood was assessed by sandwich ELISA using the Matched-Pair Antibody Set for ELISA of Human Antithrombin Antigen (Affinity Biologicals, Canada) according to the manufacturer's instructions.

Data analysis

All data were collected from triplicate independent tests. The concentration, activity and citrullination level of antithrombin in blood are expressed as the median and range. Differences between groups were statistically assessed by the Mann–Whitney U-test. A level of $P < 0.05$ was considered significant.

Results

To test whether antithrombin is citrullinated by PADI4, recombinant human PADI4 was incubated with antithrombin *in vitro*. After the incubation, the thrombin-inhibitory activity of antithrombin was significantly decreased compared with the control without PADI4 (Fig. 1a). The decrease was dose-dependent, and antithrombin activity completely disappeared after incubation with high PADI4 concentrations (1–2.5 mM). A similar result was obtained with commercially available rabbit muscle PADI (Fig. 1b). The citrulline production of the above reactions was analysed by ELISA. Incubation with human PADI4 protein or with rabbit muscle PADI considerably increased content of the citrullinated antithrombin (Figs 2a and b). The PADI4-treated antithrombin was also analysed by western blotting. Anticitrulline antibody detected a single 52 kDa band, which corresponds to the molecular weight of antithrombin (Fig. 3). These results indicate that PADI4 inhibits antithrombin through arginine citrullination.

Next, we investigated plasma for its citrullination level, concentration and activity of antithrombin. The content of citrullinated antithrombin, as assessed by absorbance at 405 nm after sandwich ELISA, was considerably higher in the plasma of RA patients (0.483 ± 0.039) than in the control groups: 11 patients with SLE (0.221 ± 0.038; $P < 0.0003$) and 14 healthy individuals (0.238 ± 0.016; $P < 0.0002$) (Fig. 4). In addition, a small increase in antithrombin activity and concentration were observed in the same blood samples for the RA group and SLE plasma (Fig. 4).

Discussion

Pike *et al.* [3] found that rabbit muscle PADI citrullinated antithrombin-α in the presence of heparin. They further found

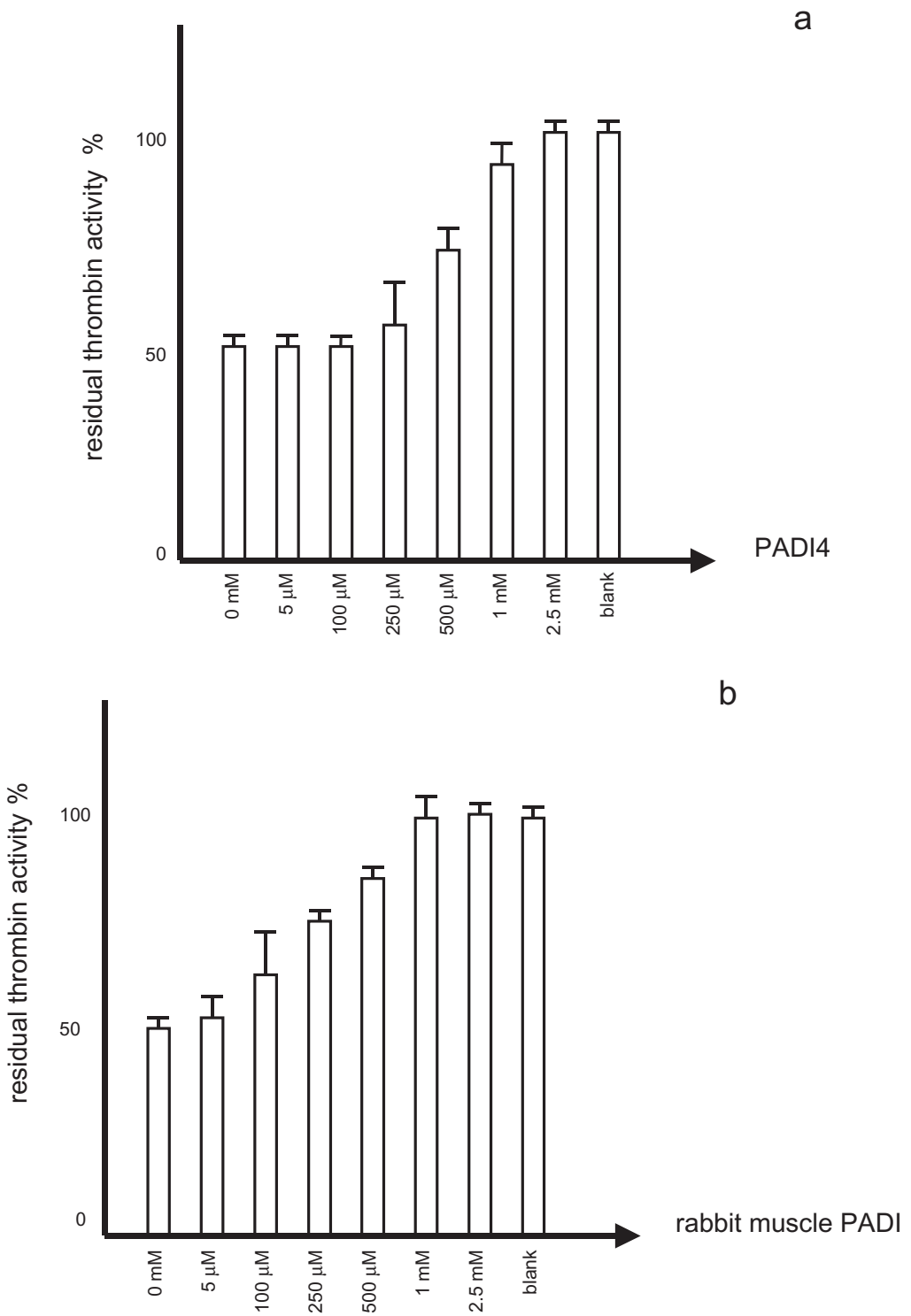

FIG. 1. Effect of human PADI4 and rabbit muscle PADI on antithrombin. Human antithrombin was incubated with (a) His-tagged recombinant PADI4 or (b) rabbit muscle PADI at different concentrations. The residual antithrombin activity was determined by measuring inhibition of thrombin-specific chromogenic absorbance. Reactions with buffer to replace antithrombin and PADI served as controls. Here, thrombin activity of the control was defined as 100% and activities of other samples were normalized to this control. Data given are mean ± S.E.M.

the P1 arginine on the reactive site loop to be the only amino acid of antithrombin accessible to citrullination. In the current study, we provide evidence that human PADI4 protein can citrullinate antithrombin and inactivate its thrombin-inhibiting ability. Also, we found that PADI4 can catalyse antithrombin citrullination in the absence of heparin, though the efficiency of citrullination may be reduced.

Thrombin is a multifunctional protease. It can activate haemostasis and coagulation through the cleavage of fibrinogen, inducing formation of fibrin clots. Excessive fibrin deposition is a

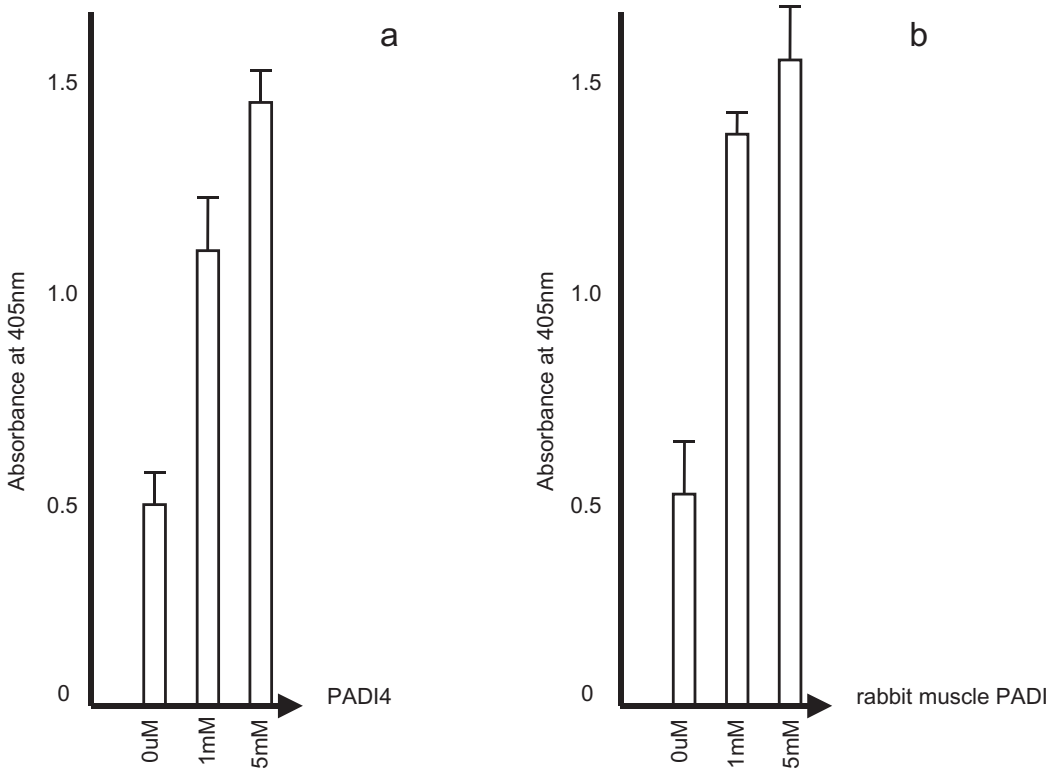

FIG. 2. Detecting antithrombin citrullination with ELISA. Following incubation of antithrombin with human PADI4 (a) or rabbit muscle PADI (b) at various concentrations, citrulline production was detected by ELISA. Data given are mean ± S.E.M.

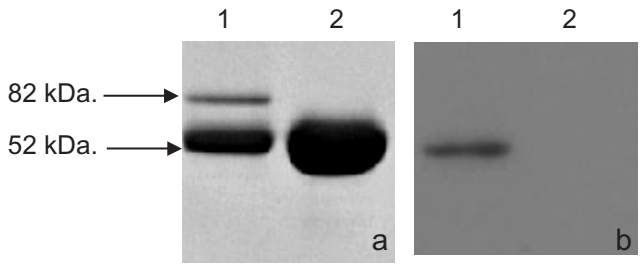

FIG. 3. Detecting antithrombin citrullination by western blotting. Antithrombin was incubated with or without PADI4 and then analysed by western blot. (a) SDS-PAGE of the reactions; protein samples were stained with Coomassie brilliant blue. The arrows indicate the position of PADI4 recombinant protein (82 kDa) and antithrombin (52 kDa). (b) The protein blot probed with anticitrulline antibody. Lane 1, antithrombin incubated with PADI4; lane 2, antithrombin incubated without PADI4.

prominent feature of RA that contributes to chronic inflammation and progressive tissue abnormality of the arthritic synovium [6–8]. By a clotting-independent pathway, thrombin also stimulates angiogenesis by up-regulation of expression of vascular endothelial growth factor receptor (VEGFR) [9–12]; increasing neovascularization is another primary phenomenon of RA synovium [13–15]. In addition, thrombin plays an important pro-inflammatory role. Activated Factor Xa and thrombin are able to trigger inflammation by elevating production of pro-inflammatory mediators such as prostacyclin [5, 16]. Varisco *et al.* [17] reported that hirudin, a thrombin inhibitor, could reduce joint inflammation in murine antigen-induced arthritis. It is well known that thrombin activity is regulated by antithrombin. Once bound to heparin pentasaccharide, antithrombin can make a complex with thrombin to prevent the thrombin protease cascade. Hence, antithrombin has anti-inflammatory properties and can balance angiogenesis and coagulation.

Our previous study and others indicated that PADI4 is expressed mainly among various haematopoietic tissues and their lineage cells [1, 2, 18]. We have observed extensive expression of PADI4 in T cells, B cells, macrophages, fibroblast-like cells and capillary endothelial cell as well as strong citrullination in RA synovium, but not in various other tissues [19]. Because inflammation of RA synovium is mainly formed from abnormally proliferating macrophages and largely infiltrated lymphocytes, RA synovial tissue has a high abundance of PADI4 [18, 19]. Moreover, we found that PADI4 SNPs associated with RA susceptibility confer a longer messenger ribonucleic acid (mRNA) half-life than non-susceptibility alleles, implying that mRNAs of the RA-susceptibility haplotype may accumulate in RA synovium [2]. Hence, extremely high expression of PADI4 and its catalysing activity may be an important feature of the RA synovium [18, 19]. In the current study, we observed significantly higher citrullination of antithrombin in RA plasma compared with the controls. Because our study confirmed that PADI4 could inhibit antithrombin *in vitro* by citrullination, we strongly suggest that excessive expression and/or activation of PADI4 enzyme in RA synovium is responsible for abnormal production of citrullinated antithrombin. We postulate the following mechanism for the involvement of the PADI4 enzyme in RA pathogenesis: unknown factors such as genetic variants or the tissue microenvironment up-regulate PADI4 expression in the RA synovium, and then the excess PADI4 activity largely blocks the ability of antithrombin to inhibit thrombin by arginine citrullination. The resulting unregulated thrombin activity locally disrupts thrombin-mediated physiological processes and stimulates excessive new capillary formation, abnormal fibrin deposition and extensive inflammation in RA synovial tissue.

The high coagulation activity in RA blood and synovial fluid has been reported by other groups. Jones *et al.* [20] found that

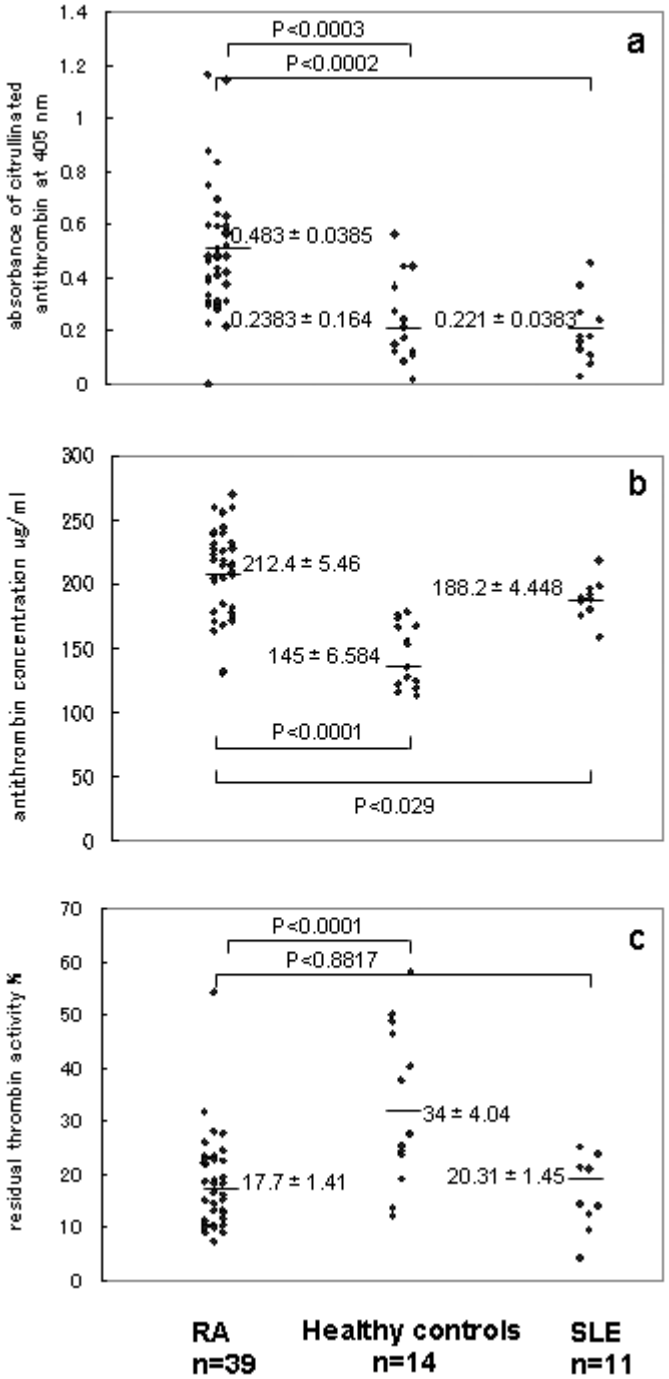

FIG. 4. Scatter plots showing the citrulline level, activity and concentration of plasma antithrombin. (a) The contents of citrullinated antithrombin by sandwich ELISA quantified by absorbance at 405 nm. (b) The concentrations of plasma antithrombin. (c) The antithrombin activities of plasma by measuring reduction of thrombin-specific chromogenic absorbance. Thrombin activity of the negative control (using reaction buffer to replace plasma) was defined as 100%, and activities of other samples were normalized to the control.

antithrombin activity in synovial fluid was lower in RA patients than in osteoarthritis (OA) patients and healthy controls. Ohba [21] and So *et al*. [22] detected elevated thrombin activity in RA synovial fluid. These data are consistent with our observation of significantly citrullinated antithrombin in RA blood. Although some groups have proposed overproduction of thrombin or inactivation of antithrombin by metalloproteinases and neutrophil elastase in RA synovium [20–24], the presence of citrullinated antithrombin clearly demonstrates that citrullination of antithrombin at least partially leads to the elevated thrombin activity in RA synovium. Subsequently, thrombin-mediated RA pathogenesis worsens specifically in joint tissue due to inactivation of antithrombin by PADI4.

We detected a small increase of antithrombin concentration and activity in the plasma of SLE controls. The changed antithrombin level had been reported in SLE plasma by another group [25]. A similar result was also found in OA blood samples by Jones *et al*. [20]. However, we did not detect elevation of citrullinated antithrombin in the non-arthritis plasma. This result reveals that increased citrullination of antithrombin is specific to RA, not a general feature of disease states, reinforcing the specific association of the PADI4 gene and its citrullination function with RA pathogenesis.

We also found increased antithrombin concentration and activity in RA plasma, as Jones *et al*. observed [20]. Many factors can influence antithrombin production and activity under physiological conditions [26]. Although the mechanism of regulation of antithrombin synthesis is still unclear, we believe that the elevated concentration of antithrombin in RA blood may result from a feedback mechanism to compensate for the inactivation of antithrombin in the synovium. The small increase of antithrombin activity in RA blood is possibly caused by the increased antithrombin production which consequently maintains the normal physiological function of other organs. In contrast, the antithrombin activity of RA synovial fluid was much lower than in the controls [20–22], indicating the local inactivation of antithrombin by PADI4 in the synovium. We did not check the level of thrombin–antithrombin III complex (TAT) in RA blood. So *et al*. detected a higher level of TAT in synovial fluid and plasma in RA [22]. Thus, we postulate that citrullination of antithrombin does not affect formation of TAT, although citrullinated antithrombin loses inhibitory activity to thrombin.

In summary, we found inactivation of antithrombin by PADI4 and a high level of citrullinated antithrombin in RA plasma. Note, however, that PADI has many potential substrates, and many kinds of citrullinated protein such as fibrin and vimentin have been found in RA synovial tissue [1, 18, 19, 27]. Because the excessive fibrin deposit in synovium is formed with progress of RA, the citrullination of antithrombin by PADI4 is suggested to be a possible mechanism for triggering the initiation of RA pathogenesis.

The authors have declared no conflicts of interest.

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