J. Biochem. **144,** 393–398 (2008)
doi:10.1093/jb/mvn079

Citrullinated Fibrinogen Inhibits Thrombin-catalysed Fibrin Polymerization

Makiko Nakayama-Hamada[1,*], Akari Suzuki[2,†], Hidehiko Furukawa[1], Ryo Yamada[2,3] and Kazuhiko Yamamoto[2,4]

[1]Daiichi-Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710; [2]Laboratory for Rheumatic Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045; [3]Laboratory of Functional Genomics and Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639; and [4]Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Received April 2, 2008; accepted May 30, 2008; published online June 26, 2008

Citrullination is the post-translational modification of arginine residues by peptidylarginine deiminases (PADIs). Fibrinogen is one substrate of PADIs under physiological conditions. Fibrinogen is an important factor for blood coagulation and inducing inflammation. The citrullinated form of fibrinogen appears in rheumatoid arthritis synovial tissue together with the production of autoantibodies that target self-peptides containing citrulline. However, whether the function of fibrinogen changes after citrullination remains unclear. We found that citrullinated fibrinogen markedly impairs the function of thrombin-catalysed fibrin polymerization and also inhibits fibrin formation. Increased citrullinated fibrinogen might thus affect the balance between coagulation and fibrinolysis and alter antigenicity under physiological conditions. These data suggest that citrullination of proteins could physiologically change functions and subsequently generate pro-inflammatory conditions and autoimmune reactions.

Key words: autoantigen, citrullination, fibrinogen, fibrin formation, thrombin.

Abbreviations: Cit, citrulline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPA, fibrinopeptide A; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; PADI, peptidylarginine deiminase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; RA, rheumatoid arthritis; SDS, sodium dodecyl sulfate.

X-ray crystallography has revealed that fibrinogen is a large glycoprotein comprising two each of three non-identical polypeptide chains ($A\alpha_2B\beta_2\gamma_2$), forming a triglobular structure containing an E fragment and two D fragments (*1*, *2*). This triglobular structure is important for polymerization in either direction, the result being a two-molecule thick protofibril with a staggered half-molecule overlap. Thrombin specifically causes fibrinogen proteolysis, during which fibrinopeptides A and B are released from the N-terminal regions of the fibrinogen Aα and Bβ chains, respectively. Thrombin is a trypsin-like serine protease and the last protease in the clotting cascade to cleave soluble fibrinogen to insoluble fibrin. To start the clotting cascade, thrombin cleaves between residues Arg16 and Gly17 of the Aα chain and residues Arg14 and Gly15 of the Bβ chain to expose the polymerization sites in fibrinogen and the resultant monomers join together to form protofibrils (*3–8*). Extravascular coagulation frequently accompanies inflammation. The products of damaged cells stimulate endothelial cell retraction during inflammation, permitting fibrinogen and other plasma constituents to escape the vasculature (*9*).

Peptidylcitrullines in proteins are produced only through the post-translational modification of arginine residues catalysed by peptidylarginine deiminase (PADI), as no tRNA for citrulline is known. Five PADI isozymes with highly conserved peptide sequences have been identified in several mammals. The guanidino group of arginine is replaced by the ureido group during peptidylarginine conversion to peptidylcitrulline. This modification decreases the net positive charge of the protein, causes a loss of potential ionic bonds and interferes with H-bonds. Such changes lead to protein unfolding (*10*). Under physiological conditions, citrullination is involved in the maturation of keratin and myelin basic protein (MBP). Histone citrullination can antagonize transcriptional induction by regulating histone Arg methylation levels (*11*, *12*). However, the physiological roles of PADIs and their citrullinated protein products remain obscure.

Citrullination has recently become associated with rheumatoid arthritis (RA), as anti-citrullinated peptide (anti-CCP) autoantibodies are generated in patients with RA. The family of autoantibodies directed towards proteins containing modified citrulline (*13–15*) shows higher specificity and sensitivity for RA and seems likely to prove useful for the early diagnosis of RA in clinical practice (*16–19*). Furthermore, RA is closely associated with functional variants of the gene encoding PADI4 in various populations (*20–25*). These data suggest that

*These two authors contributed equally to this work
†To whom correspondence should be addressed. Tel: +81-45-503-9569, Fax: +81-45-503-9590, E-mail: akaris@src.riken.jp

citrullination of self-peptides leads to a break in tolerance during the very early stages of RA or is associated with onset of RA. Fibrin(ogen) is one of the citrullinated proteins recognized by anti-CCP in RA sera and citrullinated fibrins have been detected in the RA synovial membrane (*15*). We identified the sites of human fibrinogen that are citrullinated by human PADI2 and PADI4 using liquid chromatography with tandem mass spectrometry (LC/MS/MS) analysis (*26*). Arg16 of the Aα chain and Arg14 of the Bβ chain, both of which represent thrombin cleavage sites, were found to be converted to citrulline by both enzymes.

The present study investigated the functional effects of fibrinogen citrullination, revealing that thrombin-catalysed polymerization of fibrinogen is severely impaired by citrullination.

MATERIALS AND METHODS

Thrombin Cleavage of Synthetic Fibrinopeptides—The following peptide sequences were synthesized by Operon Biotechnologies (Germantown, MD, USA): those corresponding to fibrinogen Aα amino acid residues 7–25 (DFLAEGGGVRGPRVVERHQ, Fib_AαP) and Bβ-residues 6–24 (NEEGFFSARGHRPLDKKRE, Fib_BβP); those in which all arginine residues were substituted with citrulline (DFLAEGGGV –Cit- GP –Cit- VVE –Cit- HQ, Cit-Fib_AαP; NEEGFFSA –Cit- GH –Cit- PLDKK –Cit- E, Cit-Fib_BβP); and others in which only the target site of thrombin was citrullinated (DFLAEGGGV-Cit-GPRVVERHQ, 16Cit-Fib_AαP; NEEGFFSA-Cit-GHRPLDKKRE, 14Cit-Fib_BβP).

To create thrombin digests, a mixture containing 1 μl of thrombin (Sigma Aldrich, St Louis, MO, USA; 0.05 NIH units) and 1 mg/ml of peptide in 20 μl of 10 mM $CaCl_2$, 100 mM Tris/HCl (pH 7.4), 37.5 mM NaCl and 5 mM dithiothreitol (DTT) was incubated at 25°C for 0 or 60 min. Reactions were stopped with 2 μl of 0.5 M ethylenediaminetetra-acetic acid (EDTA) (pH 8.0). Samples were diluted with 30 μl of distilled water, separated by reverse-phase HPLC (Gilson, Middleton, WI, USA) using a Beta Basic-18 column (Thermo Fisher Scientific, Waltham, MA, USA) with a linear gradient comprising 0% solvent B (acetonitrile/0.1% trifluoroacetic acid) to 50% solvent B at a flow rate of 1 ml/min, then peptides were detected as absorbance at 214 nm (*27*). Inhibition assay was performed using a mixture of 0 or 0.5 mg/ml 16Cit-Fib_AαP and a dilution series of Fib_AαP. Reverse-phase HPLC was used for the detection of peaks, as described.

Expression and Purification of Human PADI4—His-tagged hPADI4 was expressed in bacteria BL21-SI (Invitrogen, Carlsbad, CA, USA) by 0.3 M NaCl induction for 3.5 h at 30°C. Fusion proteins were purified using a HiTrap column (GE Healthcare UK, Little Chalfont, Buckinghamshire, England) according to the instructions of the manufacturer. Human PADI4 (hPADI4) was extensively dialysed against hPADI4 dialysis buffer (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 200 μM DTT, 1 mM EDTA).

Thrombin-catalysed Polymerization of Native and Citrullinated Human Fibrinogen by hPADI4—Human PADI4 was mixed with reaction buffer comprising 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, 5 mM DTT and human fibrinogen (American Diagnostica, Stanford, CT, USA), then incubated for 3 h at 37°C. The degree of citrullination of human fibrinogen was determined colorimetrically (*28*). Native and citrullinated types of human fibrinogen were respectively purified using HiPrep26/60 Sephacryl S-300 and dialysed against 20 mM Tris–HCl (pH 7.4) and 150 mM NaCl, then polymerization was assayed as described (*29*). Briefly, 95 μl of native or citrullinated human fibrinogen in 20 mM Tris–HCl (pH 7.4), 150 mM NaCl and 1 mM $CaCl_2$ or 1 mM EDTA was added to each well. Thrombin (5 μl) thrombin was then added and turbidity was monitored every 20 s for 40 min at 350 nm and 25°C. The final concentrations were 0.15 mg/ml of native or citrullinated human fibrinogen and 0.05 U/ml of thrombin.

Anti-chemically modified citrulline antibodies (anti-MC) antibody was purchased from Millipore (Billerica, MA, USA). Anti-fibrinopeptide A antibody (anti-FPA) was obtained from Hytest (Turku, Finland). Anti-FPA recognizes FPA containing the fibrinogen Aα chain. Horseradish peroxidase-linked anti-rabbit immunoglobulin (Ig)G and anti-mouse IgG (GE Healthcare, UK) antibodies were used as secondary antibodies for western blotting.

SDS–PAGE and Western Blotting—Protein samples were resolved by 10% SDS–PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Chicago, IL, USA). Blotted membranes used for the detection of citrullinated proteins by hPADI4 were chemically modified before immunostaining according to the instructions provided with an anti-citrulline detection kit (Millipore, Billerica, MA, USA).

RESULTS AND DISCUSSION

Citrullinated Fibrinogen Peptides are Resistant to Thrombin Digestion—We previously indicated that PADI2 and PADI4 modify arginine residues of fibrinogen to form citrulline. Some of these modified residues are included in thrombin recognition sites. To investigate the effect of these modifications, we digested synthetic peptides that included the recognition sites with thrombin *in vitro* and then analysed the products by reverse-phase HPLC. We found that Cit-Fib_AαP and Cit-Fib_BβP, which are native forms modified by PADI enzymes, were resistant to thrombin digestion (Fig. 1A and B). In contrast, Fib_AαP and Fib_BβP were digested by thrombin and peaks corresponding to digested fragments were depicted as N and N′ (Fig. 1A and B; relative rates of cleavage for Fib_Aα and Fib_Bβ were 52.1 and 17.4%, respectively). We also found that 16Cit-Fib_AαP and 14Cit-Fib_BβP, which was the only arginine residue substituted with citrulline in the thrombin target site, were also resistant to thrombin digestion (Fig. 1A and B).

Fibrinogen Citrullination Inhibits Thrombin Cleavage—To understand how citrullinated fibrinogen impairs thrombin-catalysed polymerization, we investigated the release of FPA from citrullinated fibrinogen cleaved by thrombin. FPA release by thrombin of

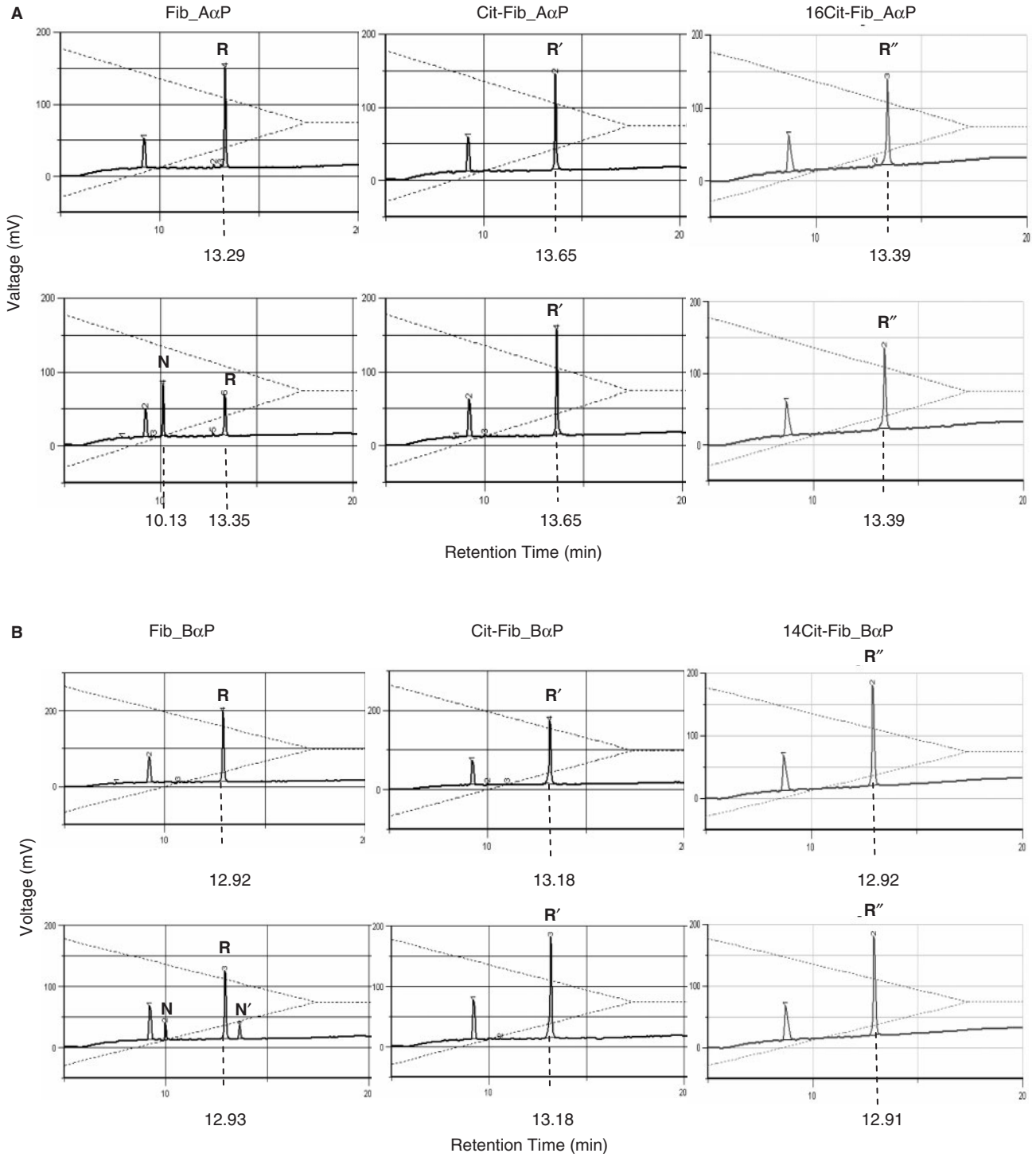

Fig. 1. **Reverse-phase HPLC separation of peptides released during thrombin digestion of fibrinogen peptides.** Thrombin digestion of peptide Fib_AαP and the variants Cit-Fib_AαP with 16, 19 and 23 Arg-to-Cit substitutions are indicated as R and R′, respectively. R″ is 16 Cit-Fib_AαP with 16 Arg-to-Cit substitution. The digested peptide is indicated as N. (B) Thrombin digestion of peptide Fib_BβP and variants Cit-Fib_BβP with 14, 17 and 23 Arg-to-Cit substitutions are indicated as R and R′, respectively. R″ is 14Cit-Fib_BβP with 14 Arg-to-Cit substitution. The digested peptides are indicated as N and N'. Thrombin digests were obtained after 0 or 60 min incubations at 37°C (upper, 0 min; lower, 60 min).

citrullinated fibrinogen was found to be significantly impaired, while native fibrinogen released FPA in the presence of thrombin. The γ dimer (γ–γ) was detectable only upon thrombin cleavage of native fibrinogen in the presence of 1 mM $CaCl_2$ (Fig. 2). These data support the notion that the citrullination of cleavage sites by thrombin in fibrinogen impairs polymerization by preventing the generation of thrombin digests.

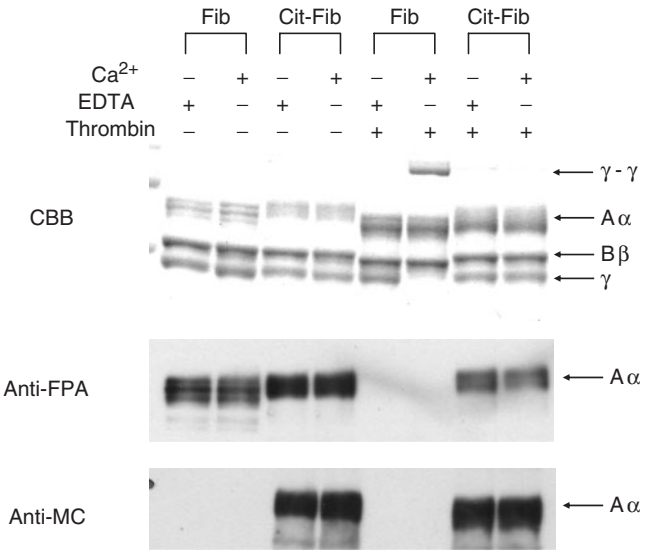

Fig. 2. **Immunoblots of thrombin digests of native and citrullinated human fibrinogen.** Fibrinogen (Fib: native, citrullinated fibrinogen) was incubated with thrombin and 1 mM $CaCl_2$ or 1 mM EDTA at 37°C for 30 min. Untreated fibrinogen and thrombin digests of fibrinogen were resolved by electrophoresis on 10% polyacrylamide/SDS gels. Formation of γ dimer (γ–γ) upon thrombin digestion was detected by Coomassie Brilliant Blue (CBB) staining. Western blotting detected the release of FPA from fibrinogen citrullination the fibrinogen Aα chain.

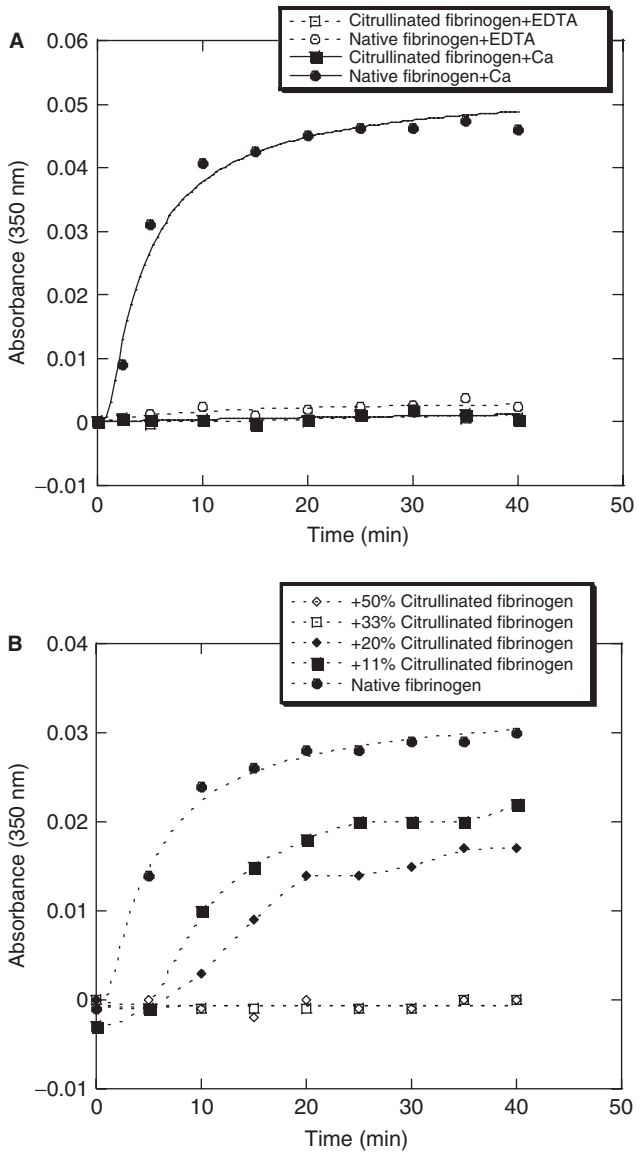

Fig. 3. **Polymerization profiles of native and citrullinated human fibrinogen induced by thrombin.** (A) Polymerization was initiated by adding thrombin at time 0 (0.05 U/ml) to purified native (filled square or open square) and citrullinated (filled circle or open circle) fibrinogen (0.15 mg/ml) with 1 mM $CaCl_2$ or 1 mM EDTA. Polymer formation was measured as changes in turbidity at 350 nm over time. (B) Polymerization profiles of native human fibrinogen with or without increasing ratios of citrullinated human fibrinogen. Final concentrations of human fibrinogen in each preparation were identical.

To identify the effect of citrullinating human fibrinogen on thrombin-catalysed fibrin polymerization, we monitored the thrombin-catalysed polymerization of native and citrullinated human fibrinogen (Fig. 3A). In the presence of Ca^{2+}, thrombin catalysed the polymerization of native fibrinogen, whereas that of citrullinated human fibrinogen was significantly impaired. Thrombin did not appear to catalyse the polymerization of either native or citrullinated human fibrinogen in the presence of EDTA (Fig. 3A). Furthermore, the presence of citrullinated fibrinogen inhibited the polymerization of native fibrinogen in a dose-dependent manner (Fig. 3B). These results suggest that citrullinated fibrinogen is resistant to fibrin monomer polymerization. To determine the type of inhibition produced by citrullinated fibrinogen, we used synthetic peptides that citrullinated the digestion site as inhibitors for inhibition experiments of the thrombin reaction. The pattern of the inhibition indicates that both V_{max} and K_m were decreased in the presence of inhibitor (Fig. 4). Citrullinated fibrinogen thus appears to represent an uncompetitive inhibitor of thrombin reaction.

We found that citrullinated fibrinogen inhibited thrombin reaction and was a non-competitive inhibitor. By definition, non-competitive inhibitors bind to the complex of enzyme and substrate. Citrullinated fibrinogen thus seems likely to affect the complex of fibrinogen and thrombin and inhibit fibrin formation by thrombin.

The present findings indicate that an increase of citrullinated fibrinogen would result in impaired fibrin polymerization and would thus influence haemostasis under physiological conditions. Since fibrinogen can induce chemokine production (*30*), an imbalance between coagulation and fibrinolysis caused by citrullinated fibrinogen might induce chemokine production in synovial cells and affect the development of RA.

An increase in citrullinated fibrinogen might also influence antigenicity. Indeed, anti-citrullinated protein antibody can detect citrullinated fibrin(ogen) in RA sera (*15*). Furthermore, fibrinogen in mice acquires antigenicity through citrullination (*31*). Conformational changes caused by the loss of electrical charge and physiological function due to citrullination might thus affect the

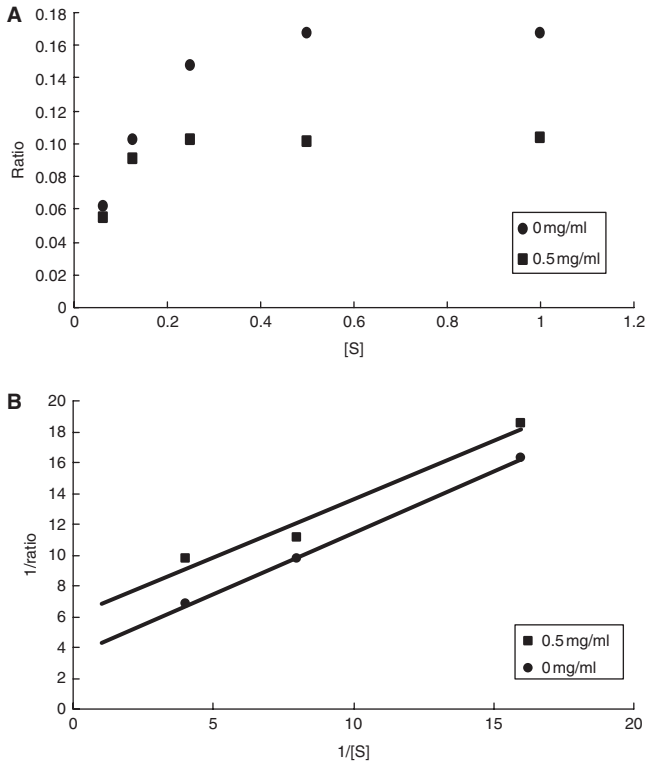

Fig. 4. **Effect of citrullinated fibrinogen peptides on thrombin digestion.** (A) The synthetic peptide Fib_AαP digested by thrombin (0.01 U) with (filled square) or without (filled circle) citrullinated peptide, 16 Cit-Fib_AαP. The *x*- and *y*-axis indicate concentration of substrate, Fib_AαP, and ratio of digested peptide to substrate, respectively. (B) Lineweaver–Burk plot of the thrombin reaction of Fib_AαP with or without 16 Cit-Fib_AαP.

antigenicity of citrullinated proteins and be associated with the reduced tolerance found in RA.

CONCLUDING REMARKS

We have provided evidence that fibrinogen citrullination impairs thrombin-catalysed fibrin polymerization. In fact, citrullinated fibrinogen and synthetic fibrinopeptides corresponding to citrullinated fibrinogen alter the fibrinopeptide production induced by thrombin, suggesting that citrullination alters fibrinogen and results in defective thrombin proteolysis. Proteins citrullinated by PADIs appear to play various roles in physiological and pathological phenomena. Citrullination in molecular disposition could affect the establishment of autoimmune reactions toward citrullinated fibrinogen in RA, although the effects of citrullination on antigenicity itself would contribute more to autoimmune reactions (*31*). Further investigation of the physiological and pathological functions of citrullination may significantly improve the understanding of the pathogenesis of RA and contribute to the molecular biology and immunology of pathological conditions associated with the coagulation cascade.

We are grateful to Dr Tetsuji Sawada (Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo), Dr Yuta Kochi, (Laboratory for Autoimmune Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research), Dr Akira Shinagawa, Dr Takafumi Kohama (Sankyo, Tokyo, Japan) and the staff of the Laboratory for Autoimmune Diseases for helpful advice and excellent technical assistance.

REFERENCES

1. Spraggon, G., Everse, S.J., and Doolittle, R.F. (1997) Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. *Nature* **389**, 455–462
2. Everse, S.J., Spraggon, G., and Doolittle, R.F. (1998) A three-dimensional consideration of variant human fibrinogens. *Thromb. Haemost.* **80**, 1–9
3. Ferry, J.D. and Morrison, P.R. (1947) Preparation and properties of serum and plasma proteins, VII: the conversion of human fibrinogen to fibrin under various condition. *J. Am. Crem. Soc.* **69**, 388–400
4. Blomback, B., Hessel, B., Hogg, D., and Therkildsen, L. (1978) A two-step fibrinogen–fibrin transition in blood coagulation. *Nature* **275**, 501–505
5. Hurlet-Jensen, A., Cummins, H.Z., Nossel, H.L., and Liu, C.Y. (1982) Fibrin polymerization and release of fibrinopeptide B by thrombin. *Thromb. Res.* **27**, 419–427
6. Ruf, W., Bender, A., Lane, D.A., Preissner, K.T., Selmayr, E., and Muller-Berghaus, G. (1988) Thrombin-induced fibrinopeptide B release from normal and variant fibrinogens: influence of inhibitors of fibrin polymerization. *Biochim. Biophys. Acta* **965**, 169–175
7. Gorkun, O.V., Veklich, Y.I., Medved, L.V., Henschen, A.H., and Weisel, J.W. (1994) Role of the alpha C domains of fibrin in clot formation. *Biochemistry* **33**, 6986–6997
8. Weisel, J.W. (1986) Fibrin assembly. Lateral aggregation and the role of the two pairs of fibrinopeptides. *Biophys. J.* **50**, 1079–1093
9. Dvorak, H.N., Senger, D.R., Dvorak, A.M., Harvey, V.S., and McDonagh, J. (1985) Regulation of extravascular coagulation by microvascular permeability. *Science* **227**, 1059–1061
10. Tarcsa, E., Marekov, L.N., Mei, G., Melino, G., Lee, S.C., and Steinert, P.M. (1996) Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. *J. Biol. Chem.* **271**, 30709–30716
11. Wang, Y., Wysocka, J., Sayegh, J., Lee, Y.-H., Perlin, J.R., Leonelli, L., Sonbuchner, L.S., McDonald, C.H., Cook, R.G., Dou, Y., Roeder, R.G., Clarke, S., Stallcup, M.R., Allis, C.D., and Coonrod, S.A. (2004) Human PAD4 regulates histone arginine methylation levels via demethylimination. *Science* **306**, 279–283
12. Cuthbert, G.L., Daujat, S., Snowden, A.W., Erdjument-Bromage, H., Hagiwara, T., Yamada, M., Schneider, R., Gregory, P.D., Tempst, P., Bannister, A.J., and Kouzarides, T. (2004) Histone deimination antagonizes arginine methylation. *Cell* **118**, 545–553
13. Schellekens, G.A., de Jong, B.A., van den Hoogen, F.H., van de Putte, L.B., and van Venrooij, W.J. (1998) Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest.* **101**, 273–281
14. Girbal-Neuhauser, E., Durieux, J.J., Arnaud, M., Dalbon, P., Sebbag, M., Vincent, C., Simon, M., Senshu, T., Masson-Bessiere, C., Jolivet-Reynaud, C., Jolivet, M., and Serre, G. (1999) The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues. *J. Immunol.* **162**, 594

15. Masson-Bessiere, C., Sebbag, M., Girbal-Neuhauser, E., Nogueira, L., Vincent, C., Senshu, T., and Serre, G. (2001) The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *J. Immunol.* **166**, 4177–4184
16. Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., and Luthra, H.S. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **31**, 315–324
17. Jansen, A.L., van der Horst-Bruinsma, I., van Schaardenburg, D., van de Stadt, R.J., de Koning, M.H., and Dijkmans, B.A. (2002) Rheumatoid factor and antibodies to cyclic citrullinated Peptide differentiate rheumatoid arthritis from undifferentiated polyarthritis in patients with early arthritis. *J. Rheumatol.* **29**, 2074–2076
18. Meyer, O., Labarre, C., Dougados, M., Goupille, P., Cantagrel, A., Dubois, A., Nicaise-Roland, P., Sibilia, J., and Combe, B. (2003) Anticitrullinated protein/peptide antibody assays in early rheumatoid arthritis for predicting five year radiographic damage. *Ann. Rheum. Dis.* **62**, 120–126
19. Suzuki, K., Sawada, T., Murakami, A., Matsui, T., Tohma, S., Nakazono, K., Takemura, M., Takasaki, Y., Mimori, T., and Yamamoto, K. (2003) High diagnostic performance of ELISA detection of antibodies to citrullinated antigens in rheumatoid arthritis. *Scand. J. Rheumatol.* **32**, 197–204
20. Kang, C.P., Lee, H.S., Ju, H., Cho, H., Kang, C., and Bae, S. C. (2006) A functional haplotype of the PADI4 gene associated with increased rheumatoid arthritis susceptibility in Koreans. *Arthritis Rheum.* **54**, 90–96
21. Suzuki, A., Yamada, R., Chang, X., Tokuhiro, S., Sawada, T., Suzuki, M., Nagasaki, M., Nakayama-Hamada, M., Kawaida, R., Ono, M., Ohtsuki, M., Furukawa, H., Yoshino, S., Yukioka, M., Tohma, S., Matsubara, T., Wakitani, S., Teshima, R., Nishioka, Y., Sekine, A., Iida, A., Takahashi, A., Tsunoda, T., Nakamura, Y., and Yamamoto, K. (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet.* **34**, 395–402
22. Ikari, K., Kuwahara, M., Nakamura, T., Momohara, S., Hara, M., Yamanaka, H., Tomatsu, T., and Kamatani, N. (2005) Association between PADI4 and rheumatoid arthritis: a replication study. *Arthritis Rheum.* **52**, 3054–3057
23. Iwamoto, T., Ikari, K., Nakamura, T., Kuwahara, M., Toyama, Y., Tomatsu, T., Momohara, S., and Kamatani, N. (2006) Association between PADI4 and rheumatoid arthritis: a meta-analysis. *Rheumatology* **45**, 804–807
24. Worthington, J. and John, S. (2003) Association of PADI4 and rheumatoid arthritis: a successful multidisciplinary approach. *Trends Mol. Med.* **9**, 405–407
25. Hoppe, B., Haupl, T., Gruber, R., Kiesewetter, H., Burmester, G.R., Salama, A., and Dorner, T. (2006) Detailed analysis of the variability of peptidylarginine deiminase type 4 in German patients with rheumatoid arthritis: a case-control study. *Arthritis Res. Ther.* **8**, R34
26. Nakayama-Hamada, M., Suzuki, A., Kubota, K., Takazawa, T., Ohsaka, M., Kawaida, R., Ono, M., Kasuya, A., Furukawa, H., Yamada, R., and Yamamoto, K. (2005) Comparison of enzymatic properties between hPADI2 and hPADI4. *Biochem. Biophys. Res. Commun.* **327**, 192–200
27. Brennan, S.O., Hammonds, B., and George, P.M. (1995) Aberrant hepatic processing causes removal of activation peptide and primary polymerisation site from fibrinogen Canterbury (A alpha 20 Val –> Asp). *J. Clin. Invest.* **96**, 2854–2858
28. Knipp, M. and Vasak, M. (2000) A colorimetric 96-well microtiter plate assay for the determination of enzymatically formed citrulline. *Anal. Biochem.* **286**, 257–264
29. Moen, J.L., Gorkun, O.V., Weisel, J.W., and Lord, S.T. (2003) Recombinant BbetaArg14His fibrinogen implies participation of N-terminus of Bbeta chain in desA fibrin polymerization. *Blood* **102**, 2466–2471
30. Liu, X. and Piela-Smith, T.H. (2000) Fibrin(ogen)-induced expression of ICAM-1 and chemokines in human synovial fibroblasts. *J. Immunol.* **165**, 5255–5261
31. Hida, S., Miura, N.N., Adachi, Y., and Ohno, N. (2004) Influence of arginine deimination on antigenicity of fibrinogen. *J. Autoimmun.* **23**, 141–150