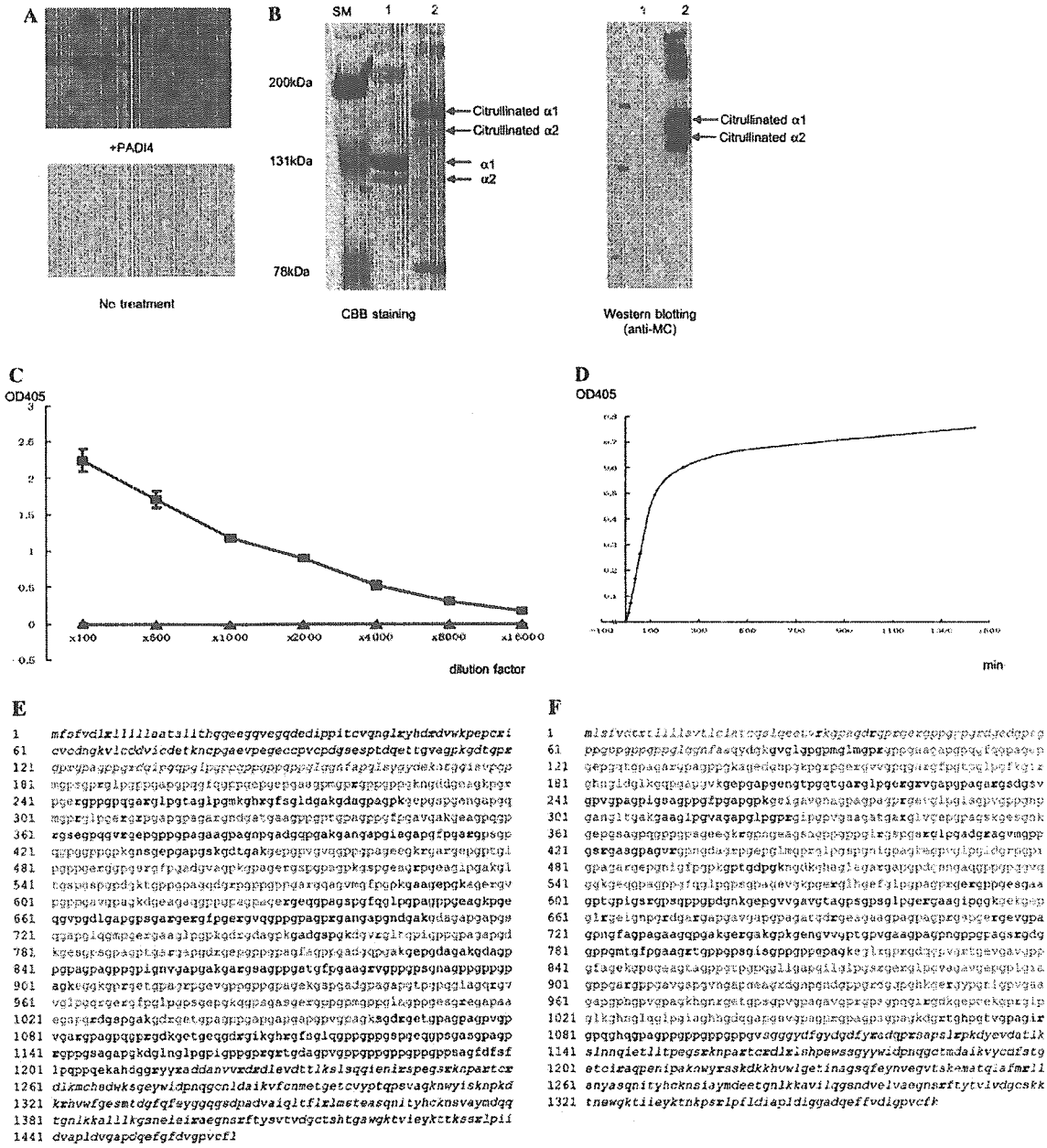

Fig. 1. Identification and confirmation of citrullinated CI modified by PADIs. (A) Secondary immunoscreening using λ-ZAP cDNA expression library, with detection by anti-MC (1:1000). Three selected positive clones were identical: CI. Citrullination of CI by PADI2 and PADI4. (B) Citrullination by PADI2 was confirmed using Western blotting (SM, size marker; lane 1, CI; lane 2, modified CI). (C) The citrullinated CI was detected by ELISA at each dilution rate of anti-MC. Square indicates reaction with PADI2 and triangle indicates reaction with enzyme reaction buffer. (D) Time course of citrullination of human CI by PADI. The reaction mixtures were incubated at 50 °C for 0, 10, 20, 40, 60, 120, 240, 480, and 1440 min, respectively. Anti-MC (1:1000) was used for detection of citrullination by ELISA. (E,F) Amino acid sequence of procollagens (NCBI database, NP_000079 and NP_000080). Procollagen type I α1 (E) and procollagen type I α2 (F) were digested by peptidase, producing mature collagen. Italic letters indicate digested peptide. Colored amino acid sequence indicates peptide fragments that were analyzed by LC/MS/MS. Red-colored residues are highly citrullinated arginine residues, and green-colored residues are arginine residues that are unlikely to be citrullinated. All arginines are shown in bold letters.

was no difference in the level of anti-huCI antibodies among RA patients, non-RA patients, and normal controls (RA versus normal individuals, $p = 0.87$; non-RA versus normal individuals, $p = 0.88$; Student's t test; Fig. 3A). Five of 117 sera (4%) from RA patients, 1 of 46 sera from non-RA patients (2%), and 1 of 37 sera (3%) from healthy controls were positive for anti-huCI antibodies.

Table 1
Summary of Western blotting using antisera from RA patients and normal controls

	Citrullinated CI	Non-citrullinated CI
RA patients ID		
RA_1	+	+
RA_2		
RA_3	+	
RA_5	+	
RA_6	+	+
RA_7	+	
RA_8		
RA_9	+	
RA_10	+	
RA_11		
	7/10 (70%)	2/10 (20%)
Healthy controls ID		
HC_2		
HC_3		
HC_4		
HC_5		
HC_11		+
	0/5 (0%)	1/5 (20%)

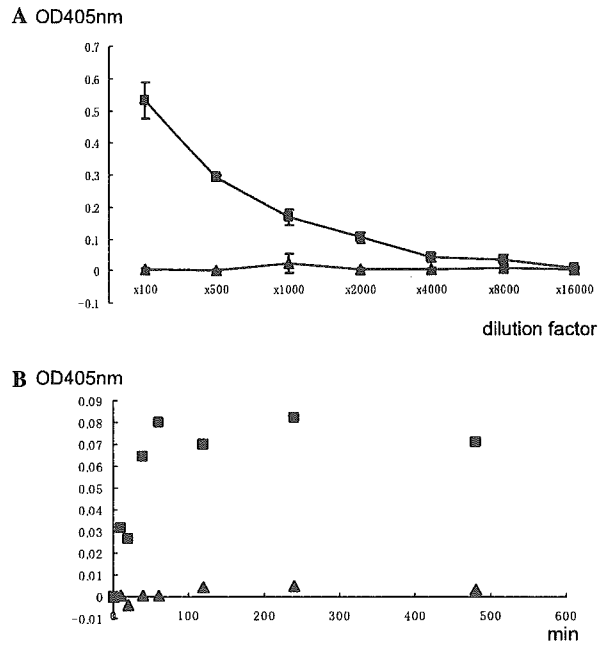

Fig. 2. Detection of citrullinated CI in sera of a RA patient and a normal control. (A) Citrullinated huCI modified by rPADI2 was detected by ELISA, using antisera from a RA patient (square) and a normal control (triangle). (B) Time course was confirmed using serum from the patient in (A).

Also, we measured the relative levels of anti-citrullinated collagen antibodies in RA sera, non-RA sera, and healthy control sera (Figs. 3C and D). The relative levels of anti-citrullinated huCI were significantly higher for RA patients than for normal controls or non-RA patients ($p = 0.000026$, $p = 0.0011$, Student's t test; Fig. 3C). Thirty-eight of 117 sera (32%) from RA patients, none of 37 sera from non-RA patients (0%), and 1 of 47 sera from normal controls (2%) were positive for anti-citrullinated huCI antibodies. In contrast, there was no significant difference in levels of anti-citrullinated huCII between RA patients and either normal controls or non-RA patients. Thirty-seven of 56 sera (66%) from RA patients, 7 of 13 sera from non-RA patients (54%), and none of 9 sera from normal controls (0%) were positive for anti-citrullinated huCII antibodies. In addition, no correlation between age and both of the level of anti-citrullinated CI ($R^2 = 0.0037$) and anti-citrullinated CII ($R^2 = 0.022$) was observed.

We investigated correlation between the relative levels of anti-citrullinated collagen antibodies and anti-CCP antibodies (Figs. 4B and D). Correlation between the level of anti-CCP and anti-citrullinated huCI antibodies was higher than the correlation between anti-CCP and anti-citrullinated huCII antibodies. We also compared the level of anti-CCP with that of anti-collagen antibody. Weak correlation or no correlation was observed between anti-CCP and both anti-huCII and anti-huCI (Figs. 4A and C). Furthermore, we found that anti-citrullinated huCI weakly correlated with anti-citrullinated huCII (Fig. 4E). We hypothesize that some of the anti-citrullinated huCI antibody cross-reacted with citrullinated huCII.

Discussion

Citrullinating enzymes [35] and autoantibodies that specifically recognize peptidylcitrulline, including anti-keratin antibody [26], anti-filaggrin autoantibodies [10], anti-Sa [19], and anti-CCP [11,14,29], are associated with RA. Citrullination of self-peptides is strongly suspected to be pathogenic in RA. However, self-peptides that are citrullinated have not been found to be pathologically linked to RA. Because anti-citrullinated antibodies are thought to be locally produced in RA synovium [17,22], we adopted RA synovial tissue as a source of pathologic citrullinated self-peptides. Among five PADI isoforms in humans, PADI2 and PADI4 are present in synovial fluid as well as synovial fluid mononuclear cells [36]. Citrullinated proteins have been detected not only in nuclear and intracellular areas, but also in amorphous deposits and extracellular matrix in RA synovial tissue [18]. Therefore, we widely targeted proteins expressed in synovium including intra- and extracellular proteins, regardless of their expression level. To identify substrates of PADIs as candidate autoantigens, we immunoscreened an expression cDNA phage library of RA synoviocytes. Our method allows

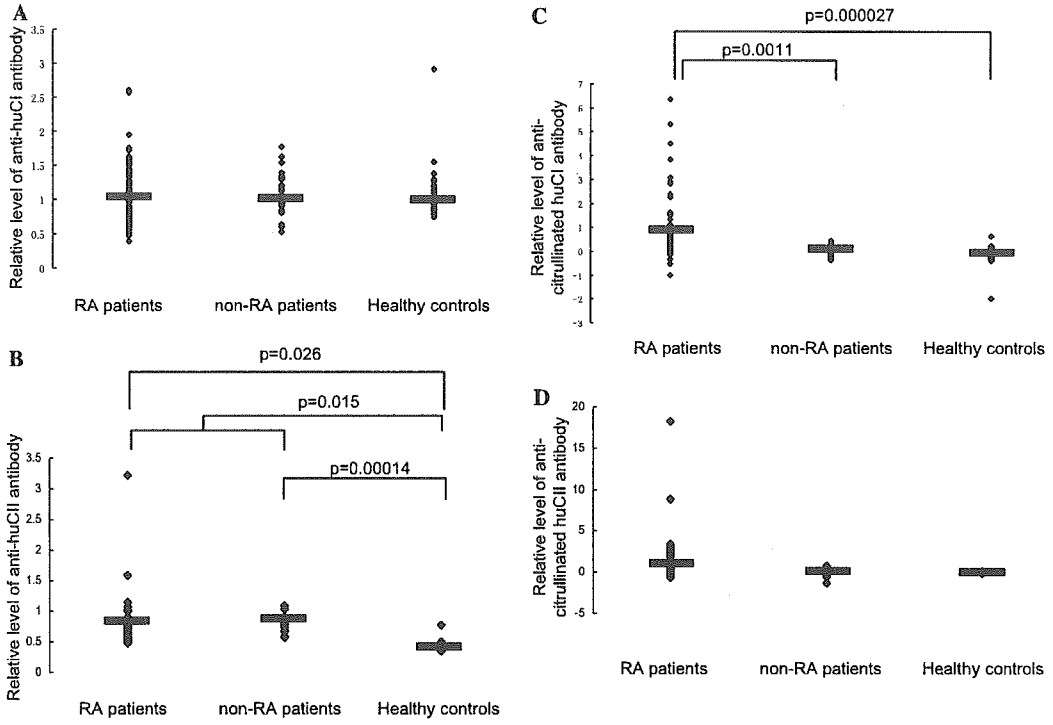

Fig. 3. Measurement of anti-collagen antibodies and anti-citrullinated collagen antibodies in RA patient sera and normal control sera. After coating, citrullination was produced by incubation with PADI2, followed by testing of the titers of anti-huCI antibodies and anti-citrullinated huCI antibodies (A,C, 117 RA sera, 37 non-RA patient sera, and 47 normal control sera). We also tested the titers of anti-huCII antibodies and anti-citrullinated huCII antibodies (B,D, 56 RA sera, 13 non-RA patient sera, and 9 normal control sera). The mean + 2 SD of healthy control values (A, >1.71; B, >0.72; C, >0.58; D, >0.12, respectively) was positive.

detection of insoluble proteins (including extracellular matrix proteins) as well as soluble proteins. This method also detects target proteins expressed at low levels, and is superior to other methods such as 2D-PAGE. One of several clones we identified was huCI peptides, which we examined in conjunction with other collagens known to be relevant to RA [9]. We confirmed citrullination of huCI by Western blotting, ELISA, and LC/MS/MS. We identified many citrullinated sites in huCI.

In the present study, we identified huCI peptide as a candidate substrate of citrullinated autoantigens by immunoscreening and found that anti-citrullinated huCI peptide antibody was specific to RA patients. huCI is one of the collagens that function as structural proteins, all of which have a characteristic triple helix structure with cyclic glycine and a high content of proline and hydroxyproline in their amino acid sequence. Among the collagens, CII has been the most studied, and there is evidence that it plays a pathologic role in RA. CII is major collagen in cartilage, and immunization with CII induces arthritis in mice and rats [6]. Bovine CII is also highly antigenic in transgenic mice that express HLA-DR1(*0101) and (*0401), which are associated with susceptibility to RA [5]. Anti-CII antibodies were observed in both RA (IgG, 41–72.4%) and non-RA (e.g., osteoarthritis and infective arthritis) (IgG, 36–88%) sera [3,31]. In the present study, anti-huCII antibody was also detected (41%) in RA patients. Compared to CII, there have been few reports indicating that CI plays a pathologic role in RA, although CI is widely expressed in bone, tendon, vascular tissues, synovial tissue, and various other tissues. In the present study, we observed no autoantibody recognizing non-citrullinated huCI in RA or control sera. In RA subjects, we observed that a marked increase in autoantibody positivity was associated with citrullination of huCI, but not with citrullination of huCII.

Although the present data indicate that anti-citrullinated huCI antibody is an RA-specific autoantibody, there are several issues that remain unresolved. First, collagen molecules form a triple helix with post-translational modification and their tertiary structure is believed to be a determinant of epitopes [13,15,20], although epitopes of anti-CCP antibodies are modified peptides. Second, it is not known how peptidylcitrullination alters antigenicity and breaks immunologic tolerance. The present findings, obtained by peptide-based immunoscreening and confirmation of recognition of citrullinated acid-extracted huCI molecules, provide a basis for further investigation to clarify the mechanisms of the roles of anti-citrullinated peptide antibodies in RA.

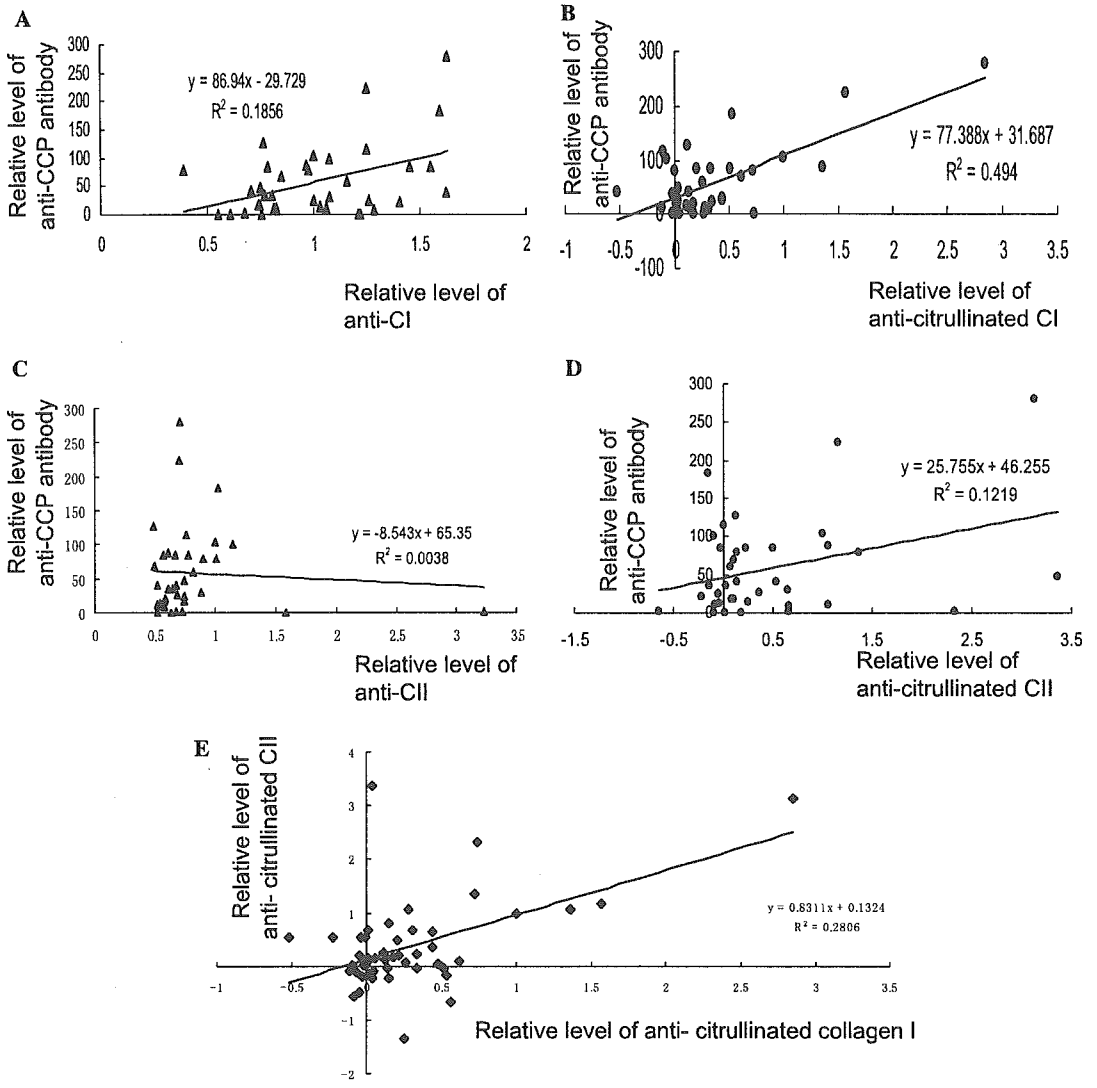

Fig. 4. Comparison of titers of anti-CCP and relative levels of anti-citrullinated collagen in RA sera. Comparison of (A) anti-huCI and (B) anti-citrullinated huCI levels with anti-CCP levels in RA sera. Comparison of (C) anti-huCII and (D) anti-citrullinated huCII levels with anti-CCP levels in RA sera. (E) Comparison of anti-citrullinated huCI levels with anti-citrullinated huCII levels in RA sera. There were no significant differences in any of these comparisons for any of the 37 RA samples. Regression line and correlation coefficient (R^2) are shown.

The present sensitivity and specificity of anti-citrullinated huCI antibody were 32% and 99%, respectively, and they correlated strongly with those of anti-CCP. The specificity of anti-citrullinated huCI antibodies was nearly equal to that of anti-CCP antibody, but the sensitivity of anti-citrullinated huCI antibodies was significantly less than that of anti-CCP antibody. Although almost all subjects who were positive for anti-CCP antibody were also positive for anti-citrullinated huCI antibody, a few were positive for anti-citrullinated huCI antibody but not for anti-CCP antibody. Because anti-CCP recognizes a mixture of synthetic peptides containing citrulline, and because huCI molecules contain multiple arginine residues that are citrullinated, it appears likely that epitopes of anti-CCP antibodies comprise the majority of those of anti-citrullinated huCI, but not all of them.

In conclusion, we found that huCI is a substrate of PADIs and that citrullinated huCI strongly correlates with RA. However, the present results indicate that CI can become an autoantigen via citrullination by PADIs, and citrullination as post-translational modification appears to be an important factor in RA. In addition, the present results suggest that anti-citrullinated collagen antibodies comprise a subclass of anti-CCP. To produce autoantigens in RA patients, PADIs must modify their substrates, but the mechanisms of this modification are unclear. However, PADIs are also clearly present in

the extracellular region [36]. Also, PADIs may be activated in the extracellular region, because the calcium ion concentration is sufficiently higher in the extracellular region than in the cytoplasm or intracellular region [21]. We speculate that autoantibodies for citrullinated collagens react or cross-react with other citrullinated proteins that are locally produced at the site of rheumatoid inflammation of synovial tissue. We believe that anti-citrullinated huCI plays important roles in the development of RA. More study of the mechanisms of citrullination in vivo may provide findings that are applicable to RA therapy.

Acknowledgments

We thank R. Kawaida, M. Ohsaka, and all the other members of the Laboratory for Rheumatic Diseases for their advice and technical assistance; M. Nakayama-Hamada, H. Furukawa (Sankyo Co., Ltd.), and Dr. A. Ishigami (Department of Molecular Pathology, Tokyo Metropolitan Institute of Gerontology) for useful technical advice; and many members of the SNP Research Center for helpful comments and assistance with various aspects of this study. This work was supported by a grant from the Japanese Millennium Project.

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