

The expression of PADI4 in synovium of rheumatoid arthritis

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Received: 11 August 2008 / Accepted: 14 January 2009 / Published online: 8 February 2009
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Abstract PADI4 that catalyzes the conversion of peptidylarginine to citrulline is associated with rheumatoid arthritis in some populations. The current study investigated the expressions of PADI4 in synovial fluid of RA ($n = 73$), osteoarthritis (OA, $n = 96$) and ankylosing spondylitis (AS, $n = 32$) using ELISA and western blotting following immuno-precipitation ($n = 6$ for each diseases). The study also compared the mRNA level of PADI4 in the synovial membrane of RA with the levels in the samples of OA and AS ($n = 6$ for each diseases) using real time PCR. ELISA detected a higher level of PADI4 in SF of RA than in samples of OA and AS ($P = 0.0001$). The level of PADI4 was significantly correlated with the level of rheumatic factor ($P = 0.015$), but not with anti cyclic citrullinated peptide antibody (anti-CCP) in the RA fluids. Western blotting confirmed the expression of PADI4 in SF of RA. Quantitative PCR measured higher transcription of PADI4 in the synovial membrane of RA than in the samples of OA and AS. The results confirmed increased expression of PADI4 in synovium of RA.

Keywords Rheumatoid arthritis · PADI4 · Synovial fluid · Synovial membrane · RF · Anti-CCP

Introduction

Peptidylarginine deiminase (PAD) post-translationally converts peptidylarginine to citrulline in a process known as citrullination [1]. Many studies have suggested an important role for PAD in the pathogenesis of rheumatoid arthritis (RA), an autoimmune disease with chronic inflammation in joint tissues [2]. Recent genetic studies have found that PADI4, one member of the PAD gene family located on chromosome 1p36, can confer susceptibility to RA among the Japanese, Korean and German populations [3–5]. There have been some studies about the expression of PADI4 in synovial tissues. Vossenaar et al. identified the expression of PADI4 mRNA and protein in polymorphonuclear neutrophils infiltrating the synovial tissue of collagen-induced arthritis (CIA) mouse [6]. Lundberg et al. detected PADI4 and citrullinated protein in inflamed joint tissues of CIA rats [7]. We previously detected extensive expression of PADI4 in RA synovial membranes, and identified the enzyme in T cells, B cells, macrophages, neutrophils, fibroblast-like cells and endothelial cells of the tissue [8]. In the current study, we continued to investigate the expression of PADI4 in the synovial fluid (SF) of RA. To better understand the relationship between PADI4 levels and disease activity, we also measured citrullinated peptide antibodies (anti-CCP) and rheumatoid factor (RF) in the SF samples, as well as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), serum procalcitonin (PCT), and white blood cell count (WBC) in the blood samples that were paired with the fluid samples. In addition, we quantitatively compared mRNA levels of PADI4 in synovial membranes of

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RA with tissues from osteoarthritis (OA) and ankylosing spondylitis (AS) as controls.

Materials and methods

Collection of synovial fluid and membranes

Synovial fluid samples were collected from patients with RA ($n = 73$), osteoarthritis (OA, $n = 96$) and ankylosing spondylitis (AS, $n = 32$). The samples were aspirated from the inflamed joints of patients with arthritis, centrifuged at 5,000g for 10 min to remove debris and stored at -80°C until using. All RA patients, including 29 males and 44 females (21–73 years; mean 47), had disease durations of 3–10 years and varying severities of joint inflammation.

Synovial membranes were collected during arthroplasty from patients with RA ($n = 6$), OA ($n = 6$) and AS ($n = 6$). The patients who donated synovial membranes of RA had disease durations of 3–10 years and were classified as having erosive RA (Larsen class IV–V). They had high levels of CRP (30–100 mg/l, mean 24 mg/l), anti-CCP (300–3,000 U/ml) and RF (160–2,560 U/ml). All RA, OA and AS patients met the American College of Rheumatology Classification Criteria [9, 10]. In addition, two synovial tissues were collected during amputation surgery from the health with traffic accidents.

Written consent was obtained from all patients who donated the synovial samples. Ethics Committee of Shandong Academy of Medical Sciences approved this study.

Enzyme-linked immunosorbent assay (ELISA)

The level of PADI4 in all of SF samples was measured by ELISA. One milliliter of the fluid samples was diluted 50-fold in 0.05 M carbonate–bicarbonate buffer (pH 9.6). Two hundred microliter of diluted fluid was coated onto 96-well microplates (Costar, USA) by overnight incubation at 4°C temperature. After three brief washes with PBS (8 g NaCl, 0.2 g KCl, 1.15 g NaHPO_4 and 0.2 g KH_2PO_4 /l, pH 7.4–7.6) containing 0.1% Tween 20 (PBST), the plate was blocked with 5% non-fat dry milk for 1 h at room temperature. The anti-PADI4 antibody was diluted 4,000-fold with PBST and added onto the plate, then incubated for 2 h at room temperature. The antibody was prepared by immunizing rabbits using synthetic oligo-peptide (FGDSCYPSND-SRQMH) that was unique for the amino acid sequence of PADI4 but not for other PAD members. After three washes with PBST, the plate was incubated with a 15,000-fold dilution of anti-rabbit IgG alkaline phosphatase-conjugated antibody (catalog number: A3687, Sigma, USA) for 30 min at room temperature. Following three washes with PBST, the signal was developed by adding the Alkline Phospha-

tase Yellow (pNPP) liquid Substrate System for ELISA (catalog number: A3469, Sigma). The absorbance of the reaction was measured at 405 nm with a plate reader (Synergy HT, Bio-Tek, USA).

The immuno-specificity of the anti-PADI4 antibody had been identified in previous studies [8–12]. To confirm the specificity of anti-PADI4 in ELISA, the antibody was incubated with the synthetic oligopeptide at 37°C for 2 h and then added to the plate in place of the anti-PADI4 antibody without immuno-absorbance during the reaction. In addition, a series of various concentrations of anti-PADI4 was incubated with a series of dilutions of SF for this experiment to observe dose-dependence effects and to find the best experimental condition.

Measurement of anti-CCP and RF in synovial fluid, and other inflammation indicators in the paired blood

Anti-CCP levels in all of SF samples were measured by ELISA using the ImmunoScan RA Anti-CCP Test Kit (Euro-Diagnostica AB, Sweden) according to the instructions of the manufacturer. One milliliter of fluid sample was diluted 50 times with dilution buffer (provided by manufacturer) before the measurement. The wells coated with cyclic citrullinated peptides were incubated with 100 μl of the diluted fluid for 1 h at room temperature. The wells were then washed to remove unbound antibodies and other components with wash solution (provided by manufacturer). An alkaline phosphatase conjugated antibody to human IgG was added to the wells for the second incubation for 30 min at room temperature. After a further washing step, detection of specific antibodies was obtained by incubation with substrate solution for 30 min at room temperature. The amount of bound antibodies correlates to the color intensity and was measured in terms of absorbance (optical density, OD). The absorbance was then calculated against a calibrator curve and the results were given in arbitrary units.

Rheumatic factor in the SF samples was measured by immunonephelometry using the N Latex RF kit (Dade Behring, USA), which specifically detects IgM-type RF. The clinic laboratory of hospitals cooperating with us measured fluid RF and ESR, PCT, WBC, CRP and RF in the blood samples that were paired with the fluid samples. The measurement was conducted by regular ways. WBC was determined by an automatic cell counter. Serum CRP concentration was measured by the immunonephelometric method. PCT was measured by Sandwich ELISA method with Human Procalcitonin ELISA Kit (USCNlife, USA). ESR was measured by the Westergren method. Rheumatic factor was measured by immunonephelometry using the N Latex RF kit (Dade Behring, USA), which specifically detects IgM-type RF.

Data of the above ELISA were collected from three independent tests. Statistical analyses were performed

using SPSS (version 11.0). The levels of PADI4 in the fluid samples were expressed as median and SEM. Differences between groups were statistically assessed by the Mann–Whitney *U* test. The correlations among expressions of PADI4, RF and anti-CCP in the fluid samples as well as their relations with levels of ESR, PCT, WBC, CRP and RF in the paired bloods were analyzed by Spearman's rank correlation. A level of $P < 0.05$ was considered significant.

Immunoprecipitation and western blot analysis

Two hundred micrograms of SF samples from RA ($n = 6$), OA ($n = 6$) and AS ($n = 6$) patients were homogenized in Cell Lysis Solution (Sigma) and centrifuged at 16,000g for 5 min at 4°C. The supernatant was collected and the protein concentrations were determined using the BCA protein assay kit (Pierce, USA). Immunoprecipitation (IP) was performed using a Protein G IP kit (Sigma) according to the manufacturer's instructions. Briefly, an equal amount of lysate from each sample was incubated with the PADI4 antibody in the presence of Protease Inhibitor Cocktail (Sigma) overnight at 4°C. Protein G beads provided with the kit were added to the mixtures and incubated for 2 h at 4°C. After a thorough wash, purified PADI4 was eluted with 1X Laemmli sample buffer (Sigma). Ten microliters of the IP samples were separated by SDS-PAGE and then transferred onto a PVDF membrane using Mini-PROTEAN 3 (Biorad, USA). The blots were probed with the monoclonal anti-PADI4 antibody made from a recombinant fragment using the residues 2-111 of human PADI4 (catalogue number: ab57167, abcam, USA), and then hybridized using sheep anti-mouse IgG conjugated to alkaline phosphatase (Sigma). Immunosignals were visualized with the Protein Detector BCIP/NBT western blot kit (KPL, USA) according to manufacturer's instructions.

Real time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) from synovial membranes of RA ($n = 6$), OA ($n = 6$) and AS ($n = 6$) patients. The concentration of total RNA was determined with a spectrophotometer. The cDNA was prepared with 1 µg of total RNA from each sample by the random hexamer method of reverse transcription using the PrimeScript™ RT-PCR Kit (TaKaRa) according to the manufacturer's protocol. The PADI4-specific oligonucleotide primers included forward (5'-GACTTCAACGGGCTC ATTC-3') and reverse (5'-GGACCGATGACTCGTTT GA-3') primers, which were designed according to the mRNA sequence in Genbank (NM012387). Beta-actin, an endogenously expressed housekeeping gene, was used as a reference with forward (5'-TGGCACCCAGCACAATGA A-3') and reverse (5'-CTAAGTCATAGTCCGCCTAGAA

GCA-3') primers. Real time PCR reactions were performed in 20 µl final volumes in capillary tubes in a LightCycler instrument 2.0 (Roche Diagnostic, Germany). Reaction mixtures contained 2 µl of LightCycler FastStart DNA mastermix for SYBR Green I (Roche Diagnostic), 0.5 µM each primer, 4 mM MgCl₂ and 2 µl of template cDNA. PCR was run using the following protocol: initial activation of polymerase at 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Negative controls contained all the elements of the reaction mixture except template DNA. PCR reactions for each sample were done in triplicate. Relative quantification of PADI4 mRNA expression was calculated by the comparative Ct method described by the manufacturer after confirming that PADI4 and beta actin cDNAs were amplified with the same efficiency. The relative quantification value of the target, normalized to an endogenous control and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (fold), where $\Delta Ct = Ct$ of target gene - Ct of endogenous control gene, and $\Delta\Delta Ct = \Delta Ct$ of samples for target gene - ΔCt of the calibrator for the target gene.

The two synovial membranes were pooled for RNA extraction. The transcription level of PADI4 in the normal synovial tissues was used as calibrator to standardize the expression levels of PADI4 in the synovial tissues of RA, OA and AS. Primer efficiency was determined by serially diluting a standard RT reaction product. PCR efficiency was automatically calculated according to the dilution curve by the instrument software. Specificity of primers was determined by both gel electrophoresis and melting curve analysis. The levels of PADI4 were expressed as median and range. The differences between groups were statistically assessed by the Mann–Whitney *U* test. A level of $P < 0.05$ was considered significant.

Results

Using ELISA, we investigated levels of PADI4, RF and anti-CCP in SF samples. Compared with samples of OA and AS, PADI4 was significantly elevated in SF of RA ($P = 0.0001$). The contents of PADI4 were not considerably altered in the samples of OA and AS. The anti-PADI4 antibody following immuno-absorbance with the oligo-peptide could not detect significant immuno-signals in SF samples, indicating the specificity of the antibody for the ELISA. Similar to other studies, the levels of RF and anti-CCP were markedly higher in SF of RA than in the samples of OA and AS. Figure 1 shows the results of ELISA. Significant correlations were detected between expressions of PADI4 and IgM-RF ($P = 0.015$) in SF of RA. However, the PADI4 level was not significantly correlated with the anti-CCP level in the RA fluids. In addition, the levels of PADI4 and RF in the RA samples were not correlated with levels of

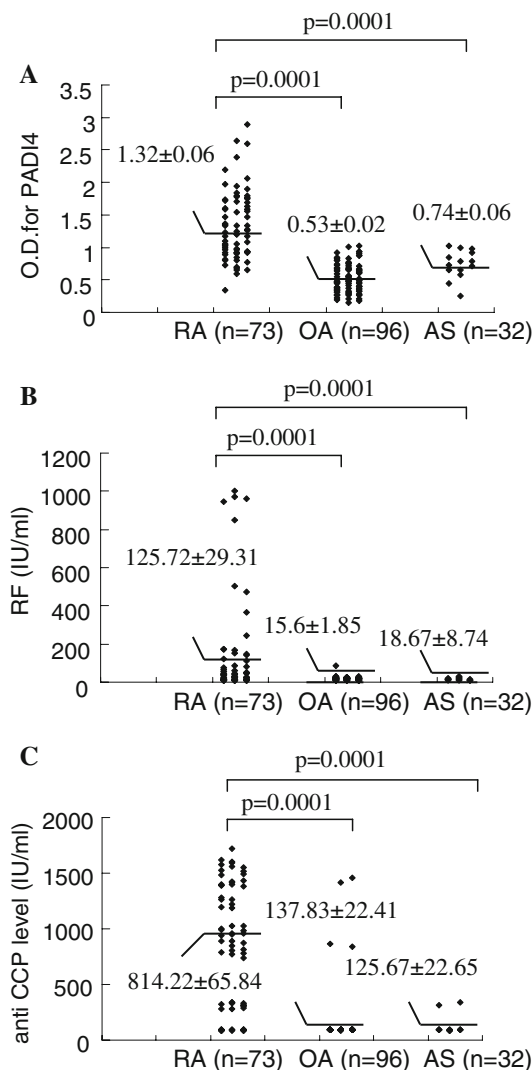


Fig. 1 Scatter plots showing the levels of PADI4 (a), RF (b) and anti-CCP (c) measured by ELISA. The levels are expressed as mean \pm SEM

WBC, ESR, PCT, CRP and RF in the paired sera, but the synovial anti-CCP level was significantly correlated with serum CRP ($P = 0.02$) and serum RF ($P = 0.045$). There were no significant correlations among expression levels of PADI4, RF and anti-CCP in the fluid samples from patients with OA and AS. We repeated this experiment using the monoclonal antibody made by abcam and observed results similar to the ELISA.

By western blotting following IP, the PADI4 antibody detected a band of 67 kDa in SF of RA, OA and AS. The density of the immuno-signal in the SF of RA appeared stronger than that in samples of OA and AS. All samples showed similar expressions. The results of western blotting are shown in Fig. 2. Although the same amounts of total protein were processed using the same protocol, it was possible that protein samples get lost during IP. Thus, the density of the immuno-signals could not accurately represent

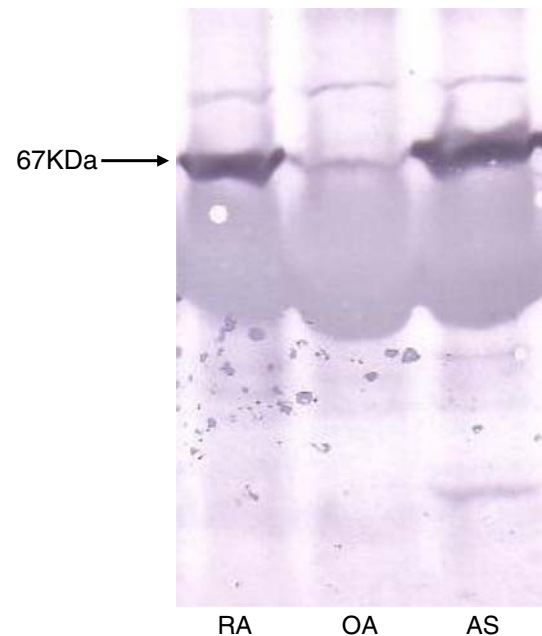


Fig. 2 Western blot analysis of PADI4 expression in synovial fluid. Anti-PADI4 antibody detected a band of 67 kDa in extracts of RA (lane 1), OA (lane 2) and AS (lane 3). The arrow indicates the molecular weight of the PADI4 protein

the expression level of PADI4 in SF samples. However, the result of the western blotting confirmed the expression of PADI4 in SF and supported the results of ELISA.

With beta-actin as a reference, real time PCR detected increased expression of PADI4 in the synovial membrane of RA, which was three times higher in the samples of RA than the expression in the samples of OA ($P = 0.002$), and significantly higher than in the sample of AS ($P = 0.03$). This result corresponded to the results of the ELISA. The results of real time PCR are shown in Fig. 3. PCR efficiency is 0.99. A single peak emerged at 88°C of the melting curve, and one band was seen on the gel of electrophoresis, indicating specificity of the PCR primers.

Discussion

We and others have previously detected expression of PADI4 and citrullinated protein in RA synovial membrane [5, 8, 13]. In the current study, we detected increased expression of PADI4 in SF of RA. We also detected high transcription of PADI4 in the synovial membrane of RA. These results suggest an increased expression of PADI4 in synovium of RA, which correspond to the finding that PADI4 is specifically associated with RA in some populations.

The current study detected high levels of anti-CCP and RF in SF of RA and revealed a significant correlation between PADI4 level and IgM-RF level. Caspi et al. had

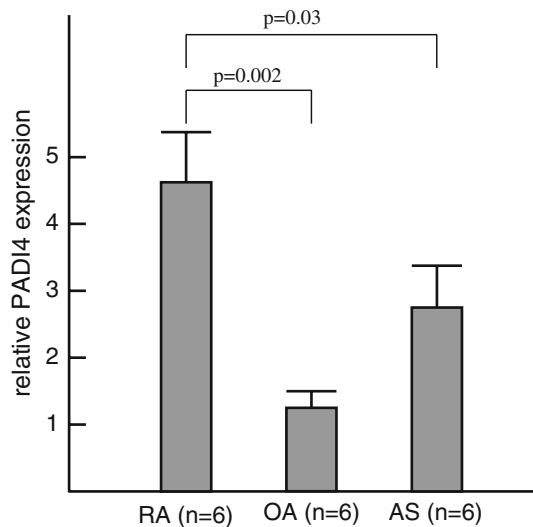


Fig. 3 Real time PCR determining the transcriptional levels of PADI4 in synovial membranes of RA (lane 1), OA (lane 2) and AS (lane 3). The cDNA templates used in the reaction were extracted from the same amount of total RNA for each sample. The levels of PADI4 were normalized to levels of beta-actin. The data represent the mean \pm SEM of three separate experiments

reported increased levels of anti-CCP and RF in SF of RA in comparison with samples of psoriatic arthritis and OA [14]. RF and anti-CCP are widely used to diagnose RA in clinical practice and the blood levels of RF and anti-CCP were reported to be associated with joint erosions and deformities of RA patients [15, 16]. Arnason et al. observed a close correlation between RF isotype levels in simultaneously drawn serum and SF samples [17]. The increased levels of PADI4 as well as the significant correlation with RF in SF of RA suggested that the expression of PADI4 could be related to clinical performance of RA, although further study of the relation between synovial PADI4 levels and X-ray joint deformity data is needed.

The current study could not detect a significant association between PADI4 and anti-CCP levels in RA SF. Greiner et al. reported that cycle citrullinated protein (CCP) is not the physiological target of the autoantibodies [18]. By measurement of RF and anti-CCP in serum of patients with infliximab treatment, De Rycke et al. found that RF and anti-CCP are independent autoantibody systems in RA [19]. Thus, it is possible that PADI4 level is related with RF level but not with anti-CCP level. In addition, del Val del Amo et al. reported that RA patients with higher level of anti-CCP antibody had elevated values of CRP in serum and more severe radiological damage, and there was a significant correlation between anti-CCP levels and CRP value in serum of the patients [16]. Their result is corresponding to our finding about significant correlation of fluid anti-CCP to serum CRP for RA.

The current study detected some expression of PADI4 in SF and synovial membranes of OA and AS. Vossenaar

et al. suggested that citrullination of synovial antigens may be a general process that occurs during joint inflammation in arthritic mice and humans [6]. However, we found that a significant correlation between expression levels of PADI4 and RF was only detectable in the SF of RA, but not in the samples of AS and OA, implying that PADI4 contributes to pathogenesis of RA by a different mechanism.

Summarily, we detected increased expression of PADI4 in SF of RA, and found that the levels of PADI4 were significantly correlated with the levels of RF in the samples. We also detected increased transcription of PADI4 in RA synovial membranes. These results correspond to the finding that PADI4 is specifically associated with RA in some populations.

Acknowledgments The present study was supported by the National Natural Science Foundation of China (NTFC) (30671949), the Shandong Science and Technology Research Program (2007GG20002001, 2006GG2202050), the Shandong Scientific Instrument Equipment Promotion Transformation Project (2006GG1108097-41, 2007GG2TC02050) and the Leading Project of Medical Science of Shandong.

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