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ORIGINAL ARTICLE

Expression of Peptidylarginine Deiminase 4 and Protein Tyrosine Phosphatase Nonreceptor Type 22 in the Synovium of Collagen-Induced Arthritis Rats[△]

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Key words: peptidylarginine deiminase 4; protein tyrosine phosphatase nonreceptor type 22; rheumatoid arthritis; rat model

Objective To study the expression level of peptidylarginine deiminase 4 (PADI4) and protein tyrosine phosphatase nonreceptor type 22 (PTPN22) in the synovium of rat model of collagen-induced arthritis, and to explore their possible therapeutic role in rheumatoid arthritis.

Methods Thirty-two female Wistar rats weighing 100 ± 20 g were randomly assigned into 3-week collagen-induced arthritis (CIA) model group ($n=8$), 4-week CIA model group ($n=8$), 6-week CIA model group ($n=8$), and the control group ($n=8$). The body weight changes of each group were recorded. The expression levels of PADI4 and PTPN22 were detected and compared by the methods of immunohistochemical staining and Western blot.

Results Arthritis of rat began to form 14 days after sensitization and the joint swelling reached peak at 28 days. The weights of the rats slowly grew both in CIA model groups and the control group. Immunohistochemical staining results showed that the positive expression of PADI4 and PTPN22 was mainly located in cartilage peripheral mononuclear cells, the cytoplasm of infiltrated cells, and bone marrow cavity. There were significant differences in the optical density of PADI4 and PTPN22 among CIA model groups and the control group (PADI4, 0.2898 ± 0.012 , 0.2982 ± 0.022 , 0.2974 ± 0.031 , 0.2530 ± 0.013 in 3-week CIA model, 4-week CIA model, 6-week CIA model and control groups; PTPN22, 0.2723 ± 0.004 , 0.2781 ± 0.010 , 0.2767 ± 0.008 , 0.2422 ± 0.019 ; all $P < 0.05$). The expression bands of PADI4 were observed in Western blot 3 weeks after initial immunization, the thickest in the 4th week, and decreased in the 6th week. The expression bands of PTPN22 were observed at all the time points, with no obvious time-dependent trend.

Conclusions PADI4 and PTPN22 are obviously correlated with CIA in rat model. PADI4 is expressed at early stage of the disease, while the expression of PTPN22 sustains throughout the course.

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PEPTIDYLARGININE deiminase 4 (PADI4) is a posttranslational modification enzyme, which can catalyze the transformation from arginine residues into citrulline residues in the presence of calcium. The citrullinated proteins tend to have an altered molecular conformation, resulting in changes in the biochemical activity. Serum PADI4 level is significantly elevated in rheumatoid arthritis (RA) patients, and the anti-PADI4 autoantibody is produced in the body. PADI4 also convert various proteins into corallines, inducing autoimmune response, which contributes to the progression of RA.¹⁻⁴

As a susceptibility gene associated with autoimmune disease, single nucleotide polymorphisms in protein tyrosine phosphatase nonreceptor type 22 (PTPN22) plays an important role in autoimmune diseases.⁵ This study was designed to introduce the function of PADI4 and PTPN22, and further explore their relationship with the pathogenesis of RA. In the present study, the expression levels of PADI4 and PTPN22 in rats of collagen-induced arthritis (CIA) were measured and their correlation with CIA explored, so as to provide new theoretical evidences for the clinical diagnosis of RA.

MATERIALS AND METHODS

Materials

Forty female Wistar rats weighing 100 ± 20 g were provided by the animal center of Shengjing Hospital of China Medical University. All the animals were adaptively bred for 1 week before experiment. Type II chicken collagen (CCII) and Complete freund's adjuvant (CFA) were purchased from Sigma-Aldrich (San Francisco, CA, USA). Rabbit anti-rat PADI4 monoclonal antibody and rabbit anti-rat PTPN22 monoclonal antibody were purchased from Novus Biologicals (Littleton, CO, USA) and ProteinSimple (San Francisco, CA, USA) respectively.

Animal groups and experiment procedures

Except for 8 rats in which CIA model establishment was failed, the other rats were randomly assigned into 3-week CIA model group ($n=8$), 4-week CIA model group ($n=8$), 6-week CIA model group ($n=8$), and the control group ($n=8$).

The process of CIA model establishment was as follows: sufficiently emulsifying 0.1 mol/L CCII dissolved in glacial acetic acid and mixed with CFA of equal volume to reach a final concentration of 0.05 g/L. After the rats were anesthetized with chloral hydrate (0.3 ml/100 g), prepared CCII solution was injected into multiple sites on the back and tail root of the rats, and the total volume was 1 ml. One week later, the same approach was repeated with the same dose. The same amount of normal saline was applied in the

control group.

Samples collection and embedment

The rats were sacrificed at the end of the experiment, and the metatarsophalangeal joints of left feet were collected. The samples were fixed with 10% paraformaldehyde fixative, and decalcified in decal solution (hydrochloric acid and formic acid mixture). After the decalcification was confirmed by pin detection, the joints were dehydrated, embedded, and sliced. Bilateral knee joint synovial tissues were simultaneously separated, and immediately put into -80°C freezer for future use.

Observational index

General situation: the color and lustre of body hair, active state, defecation, and changes in diet of the rats were observed. The weights of the rats were measured every week.

Arthritis index (AI): the degrees of joint damage were evaluated based on joint swelling, color, and joint activity record once every 4 days, 9 times in total. Scores ranged from 0 (no swelling), 1 (slightly swollen little toe), 2 (swollen toe joints and toes), 3 (swollen foot below ankle joint), to 4 (swollen foot including ankle joint). Accumulated scores of all 4 extremities were AI, which was within the range of 0-16.

Immunohistochemical staining

Metatarsophalangeal joint specimens were fixed with 10% neutral formalin solution, embedded in paraffin, sliced into 4- μm sections, and stained with streptavidin peroxidase conjugate. After dewaxing by dimethyl benzene, dehydration by gradient ethanol and hot repair of tissue antigens, primary antibodies were added and the specimens were incubated in 4°C refrigerator overnight (1:150 rabbit anti-PADI4 antibody, 1:50 rabbit anti-PTPN22 antibody). A horseradish peroxidase-conjugated secondary antibody was added and incubated for 20 minutes in room temperature. The sections were washed with phosphate buffered saline for 5 minutes (three times) after each step. After stained with 3, 3'-diaminobenzidine and re-dyed with hematoxylin, the specimens were observed under microscope and those showing cell membranes or cytoplasm stained with brown or brown-yellow were considered to be positive. Positive cell numbers for PADI4 and PTPN22 were counted in 10 random and non-overlapping visual fields ($\times 400$) in 3 slides for each sample.

Western blot

Knee joint synovial tissues were collected from CIA Wistar rats at different stages and applied to the Western blot. Lysis buffer was added into the thawed specimens recovered from -80°C , the specimens were pulverized with Ultrasonic

Cell Disruptor (Sonics VCX150, Sonics & Materials, Newtown, CT, USA) and then the supernatant was collected. After the protein concentration was determined with bicinchoninic acid Protein Assay Kit (Beyotime Institute of Biotechnology, China), the final concentration of each sample was adjusted to 40 µg/L, 20 µl of each was loaded for sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE). The voltage was 80 V initially, and switched to 120 V for another 2 hours. Membrane transfer was done after the electrophoresis with the condition being 100 V and 2 hours. Polyvinylidene fluoride membrane was immersed into 5% skim milk, added with primary antibodies (1:1000 rabbit anti-PADI4, 1:500 rabbit anti-PTPN22, and 1:15000 mouse anti-GAPDH), and incubated at 4°C overnight. After rewarming for 30 minutes, secondary antibody was added (1:5000 donkey anti-rabbit/mouse IgG conjugated with horseradish peroxidase). After washed with tris-buffered saline with tween (TBST), the membrane was incubated under room temperature for 2 hours, washed with TBST, detected with electrochemiluminescence, exposed, developed, watered, and fixed.

Statistical analysis

Statistical analyses were performed using SPSS19.0 software

(SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as means±SD. The means of multiple samples were compared using a one-way analysis of variance (ANOVA). *P*<0.05 was considered statistically significant.

RESULTS

General condition of the arthritis in each group

Fourteen days after sensitization, the skin of feet and ankles of the rats in CIA model groups began to turn red and mildly swollen, and arthritis began to form. On day 28 after sensitization, the joint swelling reached the peak. The weights of the rats grew slowly in all the groups (Table 1).

Immunohistochemical staining results

Immunohistochemical staining results showed that the distribution of PADI4 and PTPN22 in the bone tissue and synovial tissue were basically the same. Positive expression was mainly located in cartilage peripheral mononuclear cells, the cytoplasm of infiltrated cells, and bone marrow cavity. The number of positive cells in CIA model groups appeared higher than that in the control group, and the staining was heavier than in the control group as well (Fig. 1).

Table 1. The weight changes of each group at different stages (g)[§]

Group	0 d	7 d	14 d	21 d	28 d	42 d
Control group (n=8)	111.00± 5.10	155.50± 3.11	183.25± 6.13	197.50± 6.36	251.00± 5.19	257.01± 3.89
CIA model groups (n=24)*	112.17±11.16	152.33±12.39	169.00±13.80	190.00±22.63	207.00±33.94	247.50±42.97

§: Plus-minus values are means±SD.

CIA: collagen-induced arthritis.

*The CIA model groups included 3-week group, 4-week group, and 6-week group (each n=8), which were sacrificed at 21 d, 28 d, and 42 d respectively.

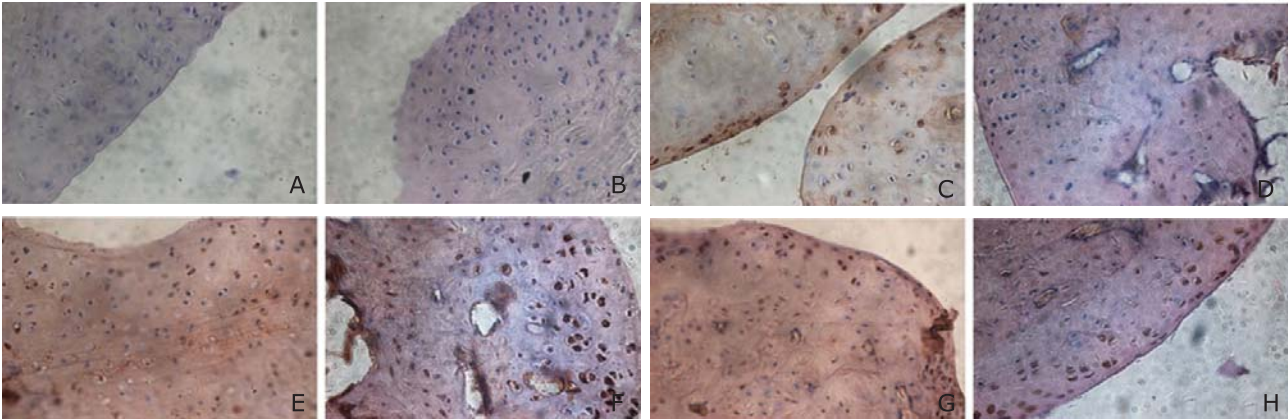


Figure 1. Immunohistochemical staining of peptidylarginine deiminase 4 (PADI4) and protein tyrosine phosphatase nonreceptor type 22 (PTPN22). (SABC ×400)

A. PADI4 in the control group; B. PTPN22 in the control group; C. PADI4 in the 3-week CIA model group; D. PTPN22 in the 3-week CIA model group; E. PADI4 in the 4-week CIA model group; F. PTPN22 in the 4-week CIA model group; G. PADI4 in the 6-week CIA model group; H. PTPN22 in the 6-week CIA model group.

The results of the optical density of cells in bone tissue positive for immunohistochemical staining of PADI4 and PTPN22 showed significant differences among the 3-week CIA model group, 4-week CIA model group, 6-week CIA model group, and the control group (all $P<0.05$, Table 2). 3 weeks after initial immunization, the positive expression of PADI4 was found almost in the cellula. In the 4th week, the expression was apparently elevated, soon after which decreased along with the progression of the disease (Table 2). While there was some positive expression of PTPN22 after the initial immunization, the time trend was not obvious as in PADI4.

Western blot result

3 weeks after initial immunization, expression bands of PADI4 were visible by Western blot. The bands were thickest in the 4th week, followed by a decrease of expression, but still positive, till the 6th week. The expression bands of PTPN22 were visible after initial immunization, but no such time trend was identified (Fig. 2).

DISCUSSION

RA is a chronic systemic autoimmune disease characterized by articular synovitis, but the etiology has yet to be elucidated. Both environmental factors and genetic factors play joint roles in the pathogenesis and development of the disease. The majority of RA cases present with a progressive development. Early diagnosis and active treatment is the precondition of prevention and control of RA, but the differential diagnosis of RA and other self-limited diseases is difficult. A definite diagnosis is usually months or even years delayed, leading to irreversible joint deformity and stiffness, high disability rate, and overall severely compromised quality of life. The study of susceptible genes contributes to the elucidation of the underlying mechanism and could be a valid target for RA diagnosis, treatment, as well as the prediction of prognosis. Anti-cyclic citrullinated peptide antibody (anti-CCP) is one of the early diagnostic indicator with the highest specificity and detectable prior to the presentation of clinical symptoms, which makes it a valuable

Table 2. The immunohistochemical staining optical density (OD) values of PADI4 and PTPN22 positive cells in bone tissue[§]

Group	n	PADI4	PTPN22	P1	P2
Control group	8	0.2530±0.013	0.2422±0.019		
3-week CIA model group	8	0.2898±0.012	0.2723±0.004	0.048	0.000
4-week CIA model group	8	0.2982±0.022	0.2781±0.010	0.001	0.000
6-week CIA model group	8	0.2974±0.031	0.2767±0.008	0.002	0.000

§: Plus-minus values are means±SD.
P1: the P value of PADI4 OD value compared with that in the control group; P2: the P value of PTPN22 OD value compared with that in the control group.

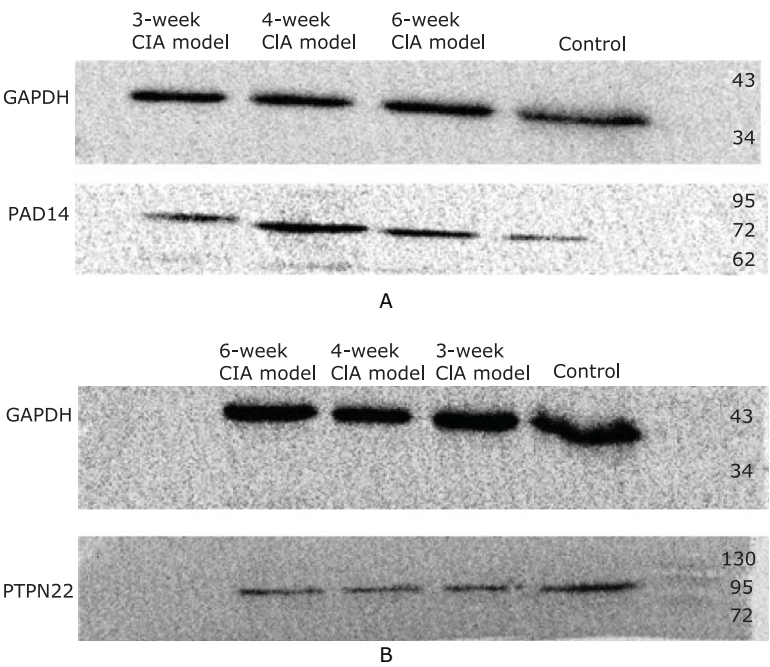


Figure 2. Western blot results of PADI4 (A) and PTPN22 (B) in different groups.

standard in the diagnosis of early, atypical, rheumatoid factor (RF) negative RA, and it is positively correlated with the severity of RA. PADI is the key enzyme catalyzing the transformation from arginine residues into citrulline residues, and its polymorphism becomes the hot spot in the study of the susceptibility and mechanism of RA.⁶⁻⁸

PTPN22 is the gene encoding lymphoid protein tyrosine phosphatase (LYP). Plenty of evidences have showed that LYP plays a key role in maintaining cellular immune homeostasis, and such allelic mutation will increase the risk of autoimmune diseases. Some researchers have discovered that only RF positive RA is associated with PTPN22R620W, with homozygous 1858 T/T being focused upon. Other researchers have also reported that the mutations in PTPN22 gene are associated with some autoimmune diseases such as RA and systemic lupus erythematosus (SLE), and unanimously indicated PTPN22 is one of susceptible genes for RA and SLE,⁹⁻¹² but the results from different races or different regional populations are not always consistent, indicating such genetic association may have something to do with the race difference. One study in British population demonstrated that PTPN22 SNP is not only associated with the pathogenesis of RA, but also its severity. Similar association has also been identified in the patients of juvenile idiopathic RA, which provides a new method for the diagnosis, treatment and mechanism study of this juvenile RA, the cause of which is known.¹³⁻²¹ Although we did not draw similar conclusion in Chinese population, PTPN22 is a well-known factor that is closely associated with autoimmune diseases. The scientists are working on the association between single nucleotide polymorphisms in PTPN22 and autoimmune diseases, as well as the specific mechanism of LYP in T cell signaling pathways (LYP was recently found to play an important role in β cell signaling pathway).²²

CIA was discovered by Trentham et al²³ in 1977, being similar to human RA regarding clinical manifestations, joint pathology, cellular and humoral immunity, which makes it a relatively ideal RA model and it has been widely used in the pathogenesis research and drug evaluation of the disease. This research showed that at early stage of CIA pathogenesis, i.e. 3 weeks after the first immunization, PADI4 and PTPN22 was positively expressed, earlier than the appearance of clinical symptom, i.e. joint swelling, followed by gradual increase of PADI4 expression, which reached its peak on fourth week after the first immunization, slightly decreased thereafter, but maintained a relatively high level of expression till the end of the experiment in sixth week. Whereas no obvious time dependence of PTPN22 expression was noted. It could be

concluded that in the process of CIA pathogenesis, PADI4 and PTPN22 were both positively expressed, but their time courses differed, PADI4 was positive at early stage of the disease, while the expression of PTPN22 was sustained all through the course, and no time-specific pattern was observed.

This study needs to be improved in some aspects: it has been reported that PADI4, PTPN22 and anti-CCP antibody and RF factor are correlated, but nothing has been done to investigate regional or gender difference in that correlation. Secondly, a larger sample size can provide more accurate and convincing results.

Anti-PADI4 antibody is a new antibody with relative high RA specificity, possesses considerable diagnostic significance in the diagnosis of RF negative, AKA negative and anti-CCP antibody negative RA, and may help with the early diagnosis of RA. It could be speculated that the inhibition of the expression of PADI4 and PTPN22 gene is expected to be applied in the treatment of RA, but the replication, translation and transcription of protein are a complicated process, and RA pathogenesis is mediated by many pathways and their synergistical or inhibitory interactions. Large-scale animal experiments and clinical trials are therefore warranted to confirm and test the hypothesis that the inhibition of PADI4 and PTPN22 gene expression can be used in the treatment of RA.

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