Potential protein targets of the peptidylarginine deiminase 2 and peptidylarginine deiminase 4 enzymes in rheumatoid synovial tissue and its possible meaning

Martha Adriana Badillo-Soto1, Mayra Rodríguez-Rodríguez1, María Elena Pérez-Pérez1, Leonel Daza-Benitez2, Juan José Bollain-y-Goytia1, Miguel Angel Carrillo-Jiménez3, Esperanza Avalos-Díaz1, Rafael Herrera-Esparza1

Abstract

Objective: The molecular mechanism of citrullination involves the calcium-dependent peptidylarginine deiminase (PAD) family of enzymes. These enzymes induce a stereochemical modification of normal proteins and transform them into autoantigens, which in rheumatoid arthritis trigger a complex cascade of joint inflammatory events followed by chronic synovitis, pannus formation, and finally, cartilage destruction. By hypothesizing that PAD2 and PAD4 enzymes produce autoantigens, we investigated five possible synovial protein targets of PAD enzymes.

Material and Methods: We measured PAD2, PAD4, and citrullinated proteins in 10 rheumatoid and 10 osteoarthritis synovial biopsies and then assessed the post-translational modifications of fibrinogen, cytokeratin, tubulin, IgG, and vimentin proteins using a double-fluorescence assay with specific antibodies and an affinity-purified anti-citrullinated peptide (CCP) antibody. The degree of co-localization was analyzed, and statistical significance was determined by ANOVA, Fisher’s exact test, and regression analysis.

Results: The principal results of this study demonstrated that citrullinated proteins, such as fibrinogen, IgG, and other probed proteins, were targets of PAD2 and PAD4 activity in rheumatoid synovial biopsies, whereas osteoarthritis biopsies were negative for this enzyme (p<0.0001). An analysis of citrullination sites using the UniProtKB/Swiss-Prot data bank predicts that the secondary structure of the analyzed proteins displays most of the sites for citrullination; a discussion regarding its possible meaning in terms of pathogenesis is made.

Conclusion: Our results support the conclusion that the synovial citrullination of proteins is PAD2 and PAD4 dependent. Furthermore, there is a collection of candidate proteins that can be citrullinated.

Keywords: Peptidylarginine deiminase 2, peptidylarginine deiminase 4, rheumatoid arthritis, synovial membrane

Introduction

Peptidylarginine deiminase (PAD) belongs to a family of calcium-dependent enzymes that convert arginine residues of proteins into citrulline. These enzymes are expressed in most mammalian tissues, with the most common PAD2 enzyme widely distributed throughout human and murine tissues (1). This isozyme is responsible for the majority of PAD activity, and the importance of PAD2 has been demonstrated by the observation that all PAD activity is abrogated in the PAD2 knockout mouse (2). Protein citrullination is an important pathophysiological mechanism in rheumatoid arthritis (RA), which is supported by the evidence that patients with RA produce antibodies against modified cellular components known as antiperinuclear factor (APF). These autoantibodies are specific to RA, and their immunochemical specificity is directed against flaggrin. The APF antibody was identified using normal epithelial cells of the oral mucosa as the antigenic source and rheumatoid serum (3-5). The anti-citrullinated peptide (CCP) antibodies were identified many years later and are considered as early markers of RA (6-10). Therefore, the importance of citrullinated epitopes in rheumatoid pathogenesis has continued to increase, and the molecular significance of citrullination has been tested in vitro using various approaches. Because of these studies, it is known that fibrinogen, vimentin, enolase, collagen, and other proteins are post-translationally modified (11-14) through their arginine residues being deiminated and transformed into citrulline. This steric change modifies the electrostatic properties of peptides, resulting in an increase in immunogenicity and changes that intensify the interaction of target proteins with certain pockets of Class II major histocompatibility complex (MHC) molecules (DR4 0401). By this mechanism, a modified protein increases its probability of being presented in a restricted peptide repertoire as an autoantigen (15, 16).
Considering the fact that PAD2 and PAD4 isoforms are overexpressed in rheumatoid synovial tissue (17-20), it appears reasonable to infer that an antigenic stimulation may arise at a synovial level. Therefore, this study aimed to assess potential protein targets of PAD2 and PAD4 enzymes in rheumatoid synovial tissue.

Material and Methods

Rheumatoid arthritis and control synovial tissue

Tissue samples were obtained during joint replacement for prosthesis surgery, and 10 biopsies from patients with a mean age of 41.2±13.1 years and who met the American College of Rheumatology (ACR) criteria for RA classification (21) were analyzed. There were six females and four males with 10 years of evolution (range, 3–13 years), three with functional class (FC) III, five with II, one with I, and one was not defined by the ACR classification of functional status in RA (22). All patients were positive for rheumatoid factor and anti-CCP, were under treatment with a low dose of ≤5 mg prednisone, and received methotrexate with a mean dose of 12.5 mg (range, 7.5–17.5 mg). Additionally, 10 biopsies were obtained from patients with osteoarthritis (mean age, 54.2±7.1 years), wherein six patients were females and four males. They were included as controls, none of them had anti-CCP antibodies, and one was positive for rheumatoid factor at an irrelevant concentration; furthermore, these patients underwent knee surgery for osteoarthritis, all of them received analgesics and/or NSAIDs, and obesity was the accelerating factor for knee osteoarthritis (23, 24). In all cases, signed informed consent was obtained, and the bioethics committee at our institution approved this protocol.

Purification and labeling of affinity-purified anti-CCP antibodies

An anti-CCP serum sample obtained from a patient with RA and who displayed high anti-CCP antibodies according to ELISA (25) was used to purify high-affinity antibodies in the following manner: serum was incubated in a commercial ELISA microwell plate (Axis-Shield Diagnostics Ltd.; Dundee, Scotland), and the anti-CCP-positive antibodies were then eluted from the plates after a 2-h incubation with purified human IgG (precipitated from normal human sera with ammonium sulfate and IgG was purified using HiTrap protein G HP columns) to neutralize the presence of possible rheumatoid factor activity in the synovial tissues. After three washes, the tissues were incubated for 1 h with the monoclonal antibody or affinity-purified human autoantibodies diluted in 10% fetal calf serum/PBS (anti-citrulline antibody, cat. 231246, Calbiochem, Darmstadt, Germany; 1:100 dilution), anti-PAD2 antibody (PAS-19474, Pierce Antibody products, Thermo Fisher Scientific Inc.; Rockford, IL, USA; 1:30 dilution), and anti-PAD4 (Pierce PAS-12236; 1:30 dilution). After multiple washes with PBS, the slides were then incubated for 1 h with HRP-labeled goat anti-human IgG. After additional washes, a color reaction was induced using 3,3′-diaminobenzidine–0.06% H2O2 (Sigma; San Louis, MO, USA), and the reaction was stopped with 2 N sulfuric acid. All assays were performed in duplicates. In the case of the HRP-labeled anti-CCP, the immunohistochemistry was direct and did not rely on the use of a secondary antibody.

Double-fluorescence labeling assays

To assess the presence of citrullinated protein in the synovial tissue, we co-localized the possible target proteins using the following steps. Citrullinated proteins were tagged as green through a 120-min incubation with an unlabeled and affinity-purified anti-CCP antibody, followed by a 120-min incubation with a FITC-labeled secondary antibody (ab4220 or ab68881, Abcam; Cambridge, MA, USA). After each incubation, the slides were washed three times with PBS. The target protein was tagged as red by overnight incubation with specific antibodies against fibrinogen (Abcam ab666).

Table 1. PAD enzymes and citrullinated protein expression in the synovial tissues of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) as controls

<table>
<thead>
<tr>
<th>Biopsies</th>
<th>PAD2 positive</th>
<th>PAD4 positive</th>
<th>CP positive</th>
<th>Citrulline positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA (n=10)</td>
<td>10 (100%)</td>
<td>7 (70%)</td>
<td>10 (100%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>OA (n=10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.0001</td>
<td>&lt;0.003</td>
<td>&lt;0.0001</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

CP: citrullinated proteins; PAD: peptidylarginine deiminase; RA: rheumatoid arthritis; OA: osteoarthritis

Fisher’s exact test (two-tailed).
cytokeratin (Sigma K8.13, Prod. C6909), IgG, vimentin (Cell Signaling D2H3), and β-tubulin (Abcam ab21057), followed by a 120-min incubation with a rabbit anti-human Texas Red-labeled antibody (IgG-TR: sc-3920, Santa Cruz Biotechnology; Paso Robles, CA, USA). Finally, the slides were counterstained with DAPI, mounted, and evaluated under a fluorescence microscope using the appropriate filters for the dyes.

Statistical analyses
Data were processed using the GraphPad Prism software (GraphPad Software Inc.; CA, USA) for the non-parametric assays, such as ANOVA and Fisher’s exact test. Regression analyses were performed using Pearson’s test. A p value <0.05 was considered statistically significant.

Results
PAD2 and PAD4 expression exclusive to rheumatoid synovial tissues
PAD2 and/or PAD4 enzymes were expressed in the synovial tissues of all patients with RA (100%). In sharp contrast, all of the OA controls tested negative for these enzymes, and the differences between both the groups were significant (p<0.0001). In rheumatoid synovial tissue, PAD2 was expressed in the 10 biopsies of RA with a strong signal by immunohistochemistry assays; however, PAD4 expression was faint, and it was present in seven synovial biopsies. Notably, PAD expression was detectable at a range of levels, particularly at the synovial border and in some biopsies along the villi. Moreover, PAD expression was particularly prominent along the inflammatory infiltrates as well as in specific cells, such as fibroblast-like synoviocytes (Figure 1 and Table 1).

PAD enzymes citrullinate synovial proteins
This study also aimed to assess whether PAD2 enzyme could convert the residues of target proteins into citrulline in situ. Current results suggest that PAD2 and PAD4 are functional enzymes because they induced post-translation modifications of target proteins as suggested by the immunohistochemical tag induced by the anti-CCP antibody. A subset of the biopsies exhibited a widespread distribution of post-translationally modified proteins that correlated well with citrullinated areas that were identified by anti-citrulline antibodies. Therefore, the presence of these three immunoreagents (PAD2/PAD4, CP, and citrulline) suggests that the citrullination process depends on PAD activity, which was developed in the following locations: within villi, fibroblast-like cells, and inflammatory infiltrates (Table 2).
Protein candidates for citrullination
To address which proteins are the targets of PAD activity, we screened a group of five proteins using a double-fluorescence assay and were able to demonstrate a good degree of co-localization between candidate proteins tagged in red and citrullinated protein domains tagged in green or vice versa. Such a PAD activity induces an in situ protein citrullination that produces a yellow color, and a possible catalytic reaction was expressed as an r value. The image analysis yields the following values: IgG, 0.993±0.0; fibrinogen, 0.846±0.06; vimentin, 0.771±0.01; β-tubulin, 0.806±0.05; and cytokeratin, 0.990±0.04. The results of this assay suggested that a variety of proteins suitable for citrullination are potential targets of PAD2 and PAD4 enzymes.

Various attempts to disclose the possible targets of PAD activity have been proposed (28), and on the basis of recent data, we know that PAD activity may exhibit a range of substrate specificities. For example, PAD2 citrullinates β/γ-actin, whereas PAD4 citrullinates the H3 histone. Additionally, functional PAD2 and PAD4 isoforms are predominantly active in rheumatoid synovial tissue (29-31). Furthermore, the in vitro assays demonstrated that fibrinogen, α-enolase, and other PAD-modified proteins become autoantigens, and patients with RA produce autoantibodies against citrullinated peptides. Notably, some of these citrullinated peptides can be detected in vivo in synovial fluid (30). Considering the aforementioned results, we assumed that the citrullination process occurs in rheumatoid synovial tissue. It is important to note that all these works suggest that the citrullination process is developed in the synovial tissue or synovial fluid, and proteins, such as fibrinogen or vimentin, are stereochemically modified and become potential sources of autoantigens (32). The main limitation of this study and other studies is the limited number of samples. Another limitation is the lack of association between synovial citrullinated proteins and HLA-DRB1 (shared MHC epitope) as reported by Verpoort et al. (33); finally, another limitation is that most of the studies indirectly demonstrated citrullination by an anti-CCP antibody rather than directly elucidating the changes in protein structure induced by citrulline that are demonstrated by magnetic resonance spectroscopy (32-34).

These results confirmed synovial PAD activity, suggesting that this catalytic mechanism is important in the pathophysiology of RA. Addi-

Discussion
This study aimed to identify the possible protein targets of PAD activity in rheumatoid synovial tissue. To accomplish this goal, a double-fluorescence approach was used to detect the possible protein targets of PAD2 and PAD4 enzymes in the synovial tissues of patients with RA. The primary results of this study can be summarized as follows: 1) PAD2 and PAD4 were exclusively detected in rheumatoid synovial tissue and 2) to demonstrate whether synovial PADS were functional, an anti-CCP antibody was purified from rheumatoid serum and then used in immunohistochemistry and in a double-fluorescence assay to co-localize candidate substrates for PAD, including fibrinogen, IgG, β-tubulin, vimentin, and cytokeratin. The results of this assay suggested that a variety of proteins suitable for citrullination are potential targets of PAD2 and PAD4 enzymes.
Badillo-Soto et al. PAD2 and PAD4 enzymes in synovial tissue

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - ABS, MRR, MEPP, MABS, RHE; Collection and/or Processing - LDB, M.A.C.J, MRR, MEPP; Materials and Data - JJBG, Supervision - EAD, Winter and Critical Review - RHE, EAD.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This project was financially supported by PROMEP UAZ-CAS Autoinmunidad.

References


Ethics Committee Approval: Ethics Committee approval was received for this study from participating institutions.

Financial Disclosure: This project was financially supported by PROMEP UAZ-CAS Autoinmunidad.

References


27. Avrameas S, Ternynck T. Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry 1971; 8: 1175-9. [CrossRef]


35. De Ceuleneer M, Van Steendam K, Dhaenens M, Deforce D. Proteomics 2012; 12: 752-60. [CrossRef]


37. Libich DS, Harauz G. Backbone dynamics of the 18.5 kDa isoform of myelin basic protein reveals transient alpha-helices and a calmodulin-binding site. Biophys J 2008; 94: 4847-66. [CrossRef]


41. Söderlin MK, Petersson IF, Bergman S, Svensson B. BARFOT study group. Smoking at onset of rheumatoid arthritis (RA) and its effect on disease activity and functional status: experiences from BARFOT, a long-term observational study on early RA. Scand J Rheumatol 2011; 40: 249-55. [CrossRef]