How is autoimmunity against citrullinated proteins regulated?
Cantaert, T.

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CHAPTER 6

Citrullination in extra-articular manifestations of rheumatoid arthritis

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Abstract

Objectives

Anti-citrullinated protein antibodies have been detected with high specificity in serum of patients with rheumatoid arthritis (RA) and citrullination of proteins may play a key role in the pathogenesis of RA. We therefore investigated the presence of citrullination in two extra-articular manifestations of RA, interstitial pneumonia (IP) and rheumatoid nodules.

Patients and methods

Open-lung biopsy specimens from patients with RA-associated IP (n=18), idiopathic IP (n=20) and controls (n=10) as well as specimens of rheumatoid nodules from 26 patients were examined. All sections were incubated with an anti-modified citrulline antibody. Blinded scoring of stained sections and analysis of results by stratification according to demographic and clinical characteristics was performed.

Results

Presence of citrulline could be detected in 8 lung specimens of patients with RA associated IP (44%) and 9 patients with idiopathic IP (46%). Conversely, lung tissue from control patients showed weak extracellular citrullination in only 2 cases (20% of patients). Citrullination did not show any significant associations with demographic or clinical characteristics such as age, gender, smoking habits, disease severity, histologic subtype, degree of inflammation or steroid use. Rheumatoid nodules were citrulline positive in a majority of cases (70%).

Conclusion

Citrullination is present in extra-articular manifestations of RA such as IP and nodules. In contrast to the high specificity of anti-citrulline antibodies in RA, citrullination is not restricted to RA but can also be observed in idiopathic IP. Whether citrullination significantly contributes to the initiation or perpetuation of autoimmunity or merely reflects ongoing inflammation remains to be clarified.
Introduction

Anti-citrullinated protein antibodies (ACPA) have been detected with high specificity in serum and synovial fluid of patients with rheumatoid arthritis (RA) (1,2). Several observations have indicated that ACPA positive patients with RA may develop a more severe disease than those without ACPA (3). The presence of their targets in the inflamed synovium, some of which appear to be RA-specific, suggests a key role for citrullinated proteins in the pathogenesis of RA (4-6). Indeed, synovial citrullinated proteins may be one of the major autoantigens driving the local immune response as suggested by the local production of ACPA in the joint (7) and the direct association between RA-specific synovial citrullinated proteins and local and systemic ACPA levels (8). While extra-articular disease, including interstitial lung disease, is a major contributor to the morbidity and premature mortality of RA (9), it remains unknown if citrullinated proteins are present at extra-articular sites of RA and if they could contribute to the local disease process by a citrullinated protein-ACPA immune conflict.

Therefore, we undertook an investigation to determine whether citrullination can be detected in lung tissue from patients affected by RA-associated interstitial pneumonia (IP). We further examined whether citrullination is specific for RA-associated IP by comparing our findings to idiopathic disease and normal lung tissue. In order to clarify whether citrullination would be restricted to RA lung tissue, we also evaluated rheumatoid nodules as another form of extra-articular disease for evidence of citrullination.

Methods

Selection of tissue samples

Paraffin-embedded open lung hematoxylin and eosin (H&E) stained biopsy specimens from patients with RA-associated IP, idiopathic IP and control lung were reviewed by a participating pathologists (JM) blinded to the clinical diagnoses in order to assign a histologic subclassification according to the international consensus statement of the American Thoracic Society (ATS) and the European Respiratory Society (ERS) (10). Paraffin-embedded specimens of subcutaneous rheumatoid nodules from 26 patients with RA were also included in our analysis. All patients classified as having RA met the 1987 American College of Rheumatology criteria for diagnosis of RA (11).
The medical records from all patients and controls were reviewed for demographic information, disease duration, pulmonary function testing, smoking habits, and medications at the time of biopsy. Lung specimens were also examined from DBA1 male mice (n=6) with severe collagen-induced arthritis (arthritis score 8-10) induced by immunisation with 100µg collagen in complete Freud’s adjuvant, followed by a second boost at day 21 in incomplete Freud’s adjuvant and sacrificed at day 120. Four DBA1 positive naïve male mice served as controls. The study was approved by the Mayo Clinic Institutional Review Board.

Immunohistochemical staining

Citrullination was detected using an anti-modified citrulline antibody (Upstate, New York, USA) which allows the detection of all citrulline residues after chemical modification independently of their amino acid context (4,5). After post-fixation in formaldehyde/glutaraldehyde and antigen retrieval in sodium citrate, the citrulline residues were derivatised as previously described (4;5). Sections were then incubated overnight with the anti-modified citrulline antibody, followed by a two step peroxidase staining. Non-derivatised sections were used as negative control and normal human skin as positive control. The sections were blinded and scored by three independent observers.

In order to evaluate a potential association of the presence of inflammatory cells and citrullination, we performed a quantitative analysis of CD1, CD3 and CD20 positive cells in our tissue samples. Immunohistochemical staining of these cells was performed as reported previously (12). The following mouse monoclonal antibodies were used: anti-CD20 (clone L26, dilution 1/60, DAKO Cytomation), anti CD1a (clone O10 dilution 1/50 DAKO Cytomation), and anti CD3 (clone PS1 dilution 1/100 DAKO Cytomation). The sections were counterstained with Modified Schmidt’s Hematoxylin. Each slide was scanned using the Bacus Laboratories Inc. Slide Scanner (BLISS; Bacus Laboratories, Inc.). Computer assisted analysis was performed by one of the investigators (SA) who was blinded to the diagnosis, using the IHCScore software (Bacus Laboratories, Inc). As shown previously, comparison of stained tissue areas reflect the ratios calculated on the basis of actual lymphocyte counts (12).
Analysis and data display

Proportions of samples with a positive anti-citrulline staining were calculated for RA-associated interstitial pneumonia, idiopathic interstitial pneumonia, and control tissue and were compared between disease groups using Fisher’s exact test. To examine for potential associations between patient characteristics and citrulline positivity, results were stratified according to demographic characteristics such as age, gender, smoking status, and steroid use, and according to disease characteristics such as forced vital capacity (FVC) as a measure of disease severity (13), histologic subtype, and degree of inflammatory infiltration. In each disease group, these data were compared between the citrulline positive and the citrulline negative group using Fisher’s exact test or Mann Whitney U test as appropriate. A p value <0.05 was regarded as statistically significant. All calculations were performed using StatsDirect statistical Software (www.statsdirect.com).

Results

Patient Characteristics

Lung tissue specimens from 18 patients diagnosed with RA and associated interstitial pneumonia could be retrieved from our archive, classified as either non-specific interstitial pneumonia (NSIP) type (n=10) or usual interstitial pneumonia (UIP) type (n=8). This group of patients with RA consisted of 10 women and 8 men with a mean (±SD) age of 62.5 ± 10.0 years and a mean disease duration of 8.3 (±7.8) years. There were 20 patients with idiopathic disease (8 with UIP; 12 with NSIP). The mean age of these 10 men and 10 women was 58.6 (±11.4) years at the time of lung biopsy. Control biopsies were from 10 patients (6 men and 4 women) with lung carcinoma, who had a mean age of 56.8 (±15.9); tissue specimens used for analysis were from uninvolved tissue areas as confirmed by histologic assessment.

Citrullination in RA-associated interstitial pneumonia

Staining with the anti-modified citrulline antibody, which recognizes citrulline after chemical modification independently of the surrounding amino acid context, indicated the presence of citrulline in the lung tissue of 8 patients with RA-associated interstitial pneumonia. As illustrated in Figure 1, citrullination was located intracellularly in mononuclear cells in the vast majority of cases. Citrulline-positive
cells were predominantly found in subpleural tissue with sometimes also staining of interalveolar tissue. The staining was more pronounced in areas of inflammatory infiltrates. No clear spatial relation to areas of fibrosis could be detected.

Specificity of citrullination for RA interstitial pneumonia

In order to assess if this citrullination was specific for RA-associated interstitial pneumonia, we compared this with lung tissue from patients with idiopathic interstitial pneumonia and control patients. Whereas anti-citrulline staining was observed in 44% of the RA lung samples, lung tissue from normal controls showed presence of weak extracellular citrullination in only 2 cases (20%). Conversely, pulmonary citrullination was detected in 9 of 20 patients with idiopathic interstitial pneumonia (45%) and was not significantly different from patients with RA-associated pneumonia. Further subanalysis in function of the staining pattern showed that intracellular staining was seen in 39% of the RA samples versus 35% in idiopathic interstitial pneumonia. Extracellular staining accompanied intracellular staining in 2 patients with RA-associated pneumonia (11%) and 1 patient with idiopathic pneumonia (10%). Exclusive extracellular staining was observed in 1 patient with RA-associated and 2 patients with idiopathic disease (see table1). Thus, citrullination occurs commonly in RA as well as idiopathic interstitial pneumonia. In order to investigate if diseased lung tissue is the only extra-articular site of RA characterized by citrullination, we also stained rheumatoid nodules from 26...
patients. As shown in Table 1, intracellular staining was seen in 5 of these specimens (19%), and was accompanied by extracellular staining in 3 patients (12%). Exclusive extracellular staining, which occurred mostly in necrotic areas (Figure 2) was seen in 13 patients (50%).

**Association of pulmonary citrullination with demographic characteristics**

Considering the presence of citrullination in some but not all lung samples of both RA-associated and idiopathic interstitial pneumonia, we next analyzed if important demographic features may influence this process. As shown in table 2, there were no significant differences in age and gender between citrulline-positive and citrulline-negative patients in both the RA and the control group. Because smoking may lead to subclinical or clinical lung inflammation and may thereby trigger citrullination, we also investigated the relation between pulmonary citrullination and smoking habits. In both RA and idiopathic interstitial pneumonia, there was no difference in smoking habits between citrulline-positive and the citrulline-negative patients. Strikingly, intracellular citrullination was not observed in normal lung samples although all these tissues were obtained in smokers. Finally, the presence of citrullination was not dependent on steroid use by the patients.

<table>
<thead>
<tr>
<th>Citrulline Staining Pattern</th>
<th>RA associated IP (n=18)</th>
<th>Idiopathic IP (n=20)</th>
<th>Control Lungs (n=10)</th>
<th>Rheumatoid nodules (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only extracellular citrulline positive</td>
<td>1 (5%)</td>
<td>2 (10%)</td>
<td>2 (20%)</td>
<td>13 (50%)</td>
</tr>
<tr>
<td>Only intracellular citrulline positive</td>
<td>5 (28%)</td>
<td>6 (30%)</td>
<td>0 (0%)</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>Intra- and extracellular citrulline positive</td>
<td>2 (11%)</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>Overall citrulline positive</td>
<td>8 (44%)</td>
<td>9 (45%)</td>
<td>2 (20%)</td>
<td>18 (70%)</td>
</tr>
</tbody>
</table>

Table 1: Citrulline staining pattern in RA associated interstitial pneumonia (IP), idiopathic IP, control lungs and rheumatoid nodules. RA=rheumatoid arthritis; IP=interstitial pneumonia; p values were obtained using a Fisher’s exact test.
Association of pulmonary citrullination with disease characteristics

To further assess the relationship between citrullination and the type or degree of local inflammation of the lung, we assessed the histologic subtype, the degree of inflammatory infiltration, and the FVC in citrulline-positive and citrulline-negative patients in both RA-associated and idiopathic interstitial pneumonia. As shown in table 2, there were no differences in NSIP versus UIP in function of the presence or absence of citrullination. Moreover, although positive staining was essentially seen in areas with a high degree of inflammatory infiltration, the global degree of inflammation as assessed by the infiltration with CD3+ T lymphocytes, CD20+ B lymphocytes, and CD1a+ antigen-presenting cells was not significantly different between citrulline-positive and citrulline-negative patients in RA as well as idiopathic interstitial pneumonia (table 3). Finally, local disease severity as assessed by the FVC was also not different between citrulline-positive and citrulline-negative patients.

Pulmonary citrullination in collagen-induced arthritis

To further investigate whether there was an association between peripheral arthritis and subclinical pulmonary inflammation with or without local citrullination, we next investigated lung tissue in the most commonly used mouse model of RA: collagen induced arthritis in DBA1 mice. In the lung tissues of non-immunized control mice (n=4) without arthritis, there were neither histologic alterations on H&E staining nor positive staining with the anti-citrulline antibody. Similarly, the DBA1 mice immunized with collagen and developing severe arthritis (n=6) displayed no signs of histologic inflammation or citrullination in their lungs.
### Table 2: Citrullination in RA associated interstitial pneumonia, idiopathic interstitial pneumonia and normal lungs, stratified by demographic and clinical characteristics. RA=rheumatoid arthritis; IP=interstitial pneumonia; FVC=forced vital capacity; UIP=usual interstitial pneumonia; NSIP=non-specific interstitial pneumonia. Information not available for every patient; p values were obtained using a Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Stratum</th>
<th>RA associated IP</th>
<th>Idiopathic IP</th>
<th>Control Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrull +</td>
<td>Citrull -</td>
<td>p</td>
</tr>
<tr>
<td>Total samples</td>
<td>8 (44%)</td>
<td>10 (56%)</td>
<td>9 (45%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>5 (63%)</td>
<td>3 (27%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Women</td>
<td>3 (30%)</td>
<td>7 (70%)</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis&lt;65</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>0.66</td>
</tr>
<tr>
<td>&gt;65</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>3 (60%)</td>
<td>2 (40%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Ever smokers</td>
<td>5 (45%)</td>
<td>6 (55%)</td>
<td>3 (38%)</td>
</tr>
<tr>
<td>Steroid use&lt;6&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (38%)</td>
<td>5 (62%)</td>
<td>0.62</td>
</tr>
<tr>
<td>No</td>
<td>5 (63%)</td>
<td>3 (37%)</td>
<td>5 (45%)</td>
</tr>
<tr>
<td>Disease severity&lt;6&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC&gt;70</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
<td>0.99</td>
</tr>
<tr>
<td>FVC&lt;70</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>Histology subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UIP</td>
<td>4 (50%)</td>
<td>4 (50%)</td>
<td>0.99</td>
</tr>
<tr>
<td>NSIP</td>
<td>4 (40%)</td>
<td>6 (60%)</td>
<td>6 (50%)</td>
</tr>
</tbody>
</table>

### Discussion

Protein modification by citrullination may represent an important step in bypassing immunotolerance (14) or enhance autoimmune reactivity (15). In RA, the high specificity of ACPA (1,2) and the presence of distinctive citrullinated proteins in the synovial tissue (6) raised the hypothesis that the immune response to citrullinated autoantigens may play a specific role in the disease process. Considering this hypothesis and the fact that extra-articular disease is also associated with increased...
RA severity, we investigated here the presence of citrullination in RA-associated interstitial pneumonia and a potential contribution of citrullination to the local disease process.

According to the results of our study, citrullination is not only present in synovial tissue of patients with RA but can also occur in extra-articular tissue affected by the disease. Almost half of our patients with RA-associated interstitial pneumonia showed evidence of pulmonary citrullination, which is similar to the proportion of RA patients with synovial citrullination observed in previous studies (6). It is however unclear if indeed only a subset of the patients depict citrullination in their affected lungs or if this process occurs more commonly but is variable over time in an individual patient. Besides evidence of sporadic extracellular positive staining, citrullination in both RA-associated as well as idiopathic lung disease (but not control tissue) was especially located inside mononuclear cells. This intracellular location appears especially interesting in the light of recent insights in the role of cellular location of antigens and the positive and negative selection of autoreactive B cells: while extracellular presence of self-antigens usually leads to B cell tolerance, intracellular location may lead to exaggerated positive B cell selection and predispose for autoantibody production (16). In this context, our findings provide a solid base to proceed with biochemical characterization of citrullinated proteins from fresh RA lung tissue and to compare this with the citrullinated proteins present in synovium.

Independently of the exact biochemical nature of these proteins, the demonstration of citrullination in RA-associated interstitial pneumonia raises the question if this
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process is related to the disease pathogenesis. Although ACPA are highly specific for RA, citrullination was not restricted to RA-associated interstitial pneumonia and could also be detected in idiopathic disease. These findings are consistent with previous observations that citrullination of proteins such as fibrin is apparent in different types of synovial tissue inflammation and is not specific for RA (4;17). Along the same line, citrullination was not only found in RA-associated interstitial pneumonia but also at other sites of extra-articular disease such as rheumatoid nodules. Because we used an antibody that recognizes all citrullinated proteins after chemical modification independently of their amino acid context, our observation does not exclude the presence of distinctive citrullinated epitopes which are specific for RA-associated IP. In fact, Baeten et al. detected RA specific synovial citrullinated proteins using a polyclonal and a monoclonal anti-citrulline antibody, which recognizes not only the citrulline residue but also the surrounding amino acids (6). Unfortunately, assay with these antibodies, which have only been successfully used on frozen tissue sections, is not technically possible using paraffin-embedded lung tissue specimens. Taken together, our data confirm that citrullination appears to occur in a variety of pathological conditions but the question remains open if citrullination of some well-defined proteins is specific for RA and which of the citrullinated proteins are really pathophysiologically relevant for the humoral autoimmune response (18).

In order to evaluate whether certain clinical characteristics predispose individual patients with RA-associated or idiopathic lung disease to citrullination in the lung tissue, we performed a stratified analysis on several demographic and clinical variables. Although the limited power of our analysis has to be acknowledged, we were unable to detect any strong association between the presence of citrullination and age, gender, or use of steroids in both patient cohorts. Moreover, there was no association with smoking status in the RA-associated and idiopathic lung disease and normal lung tissue obtained from smokers did not show intracellular citrullination. These data do thus not confirm the recent data that intracellular citrullination in the lungs may be induced by smoking, as suggested by the positive immunostaining for citrullinated proteins recorded in BAL cells from smokers but not from non-smokers. However, this issue certainly deserves further confirmatory studies considering the major gene-environment interaction between smoking and HLR-DR shared epitope genes in ACPA positive RA patients.

We have previously shown a significant increase of infiltrating T cells (12) and B cells (19) in RA-associated IP as compared with idiopathic IP. In the present staining, a statistically significant increase of infiltrating cells in RA-associated IP versus idiopathic IP was only observed when comparing citrulline-positive lung tissue. This suggests that although citrullination may occur only in a subset of RA-associated IP, it could play an important role in dysregulated immune responses and lymphocyte
Although a recent study indicated a small increase of the immunogenicity of collagen after citrullination in collagen induced arthritis (15), the exact role of citrullination in synovial as well as extra-articular inflammation is unclear. Interestingly, intracellular citrullination in our tissue samples was spatially associated with areas of inflammatory infiltrates, independently of the histologic subtype of interstitial pneumonia. This could simply be due to the higher cellularity in these areas, but also be a sign of an enhanced immunostimulation in areas with citrullination of potential antigens. At the same time, more than half of our RA-associated IP tissue samples had no detectable citrullinated proteins even in the presence of strong inflammation. In addition, the quantification of CD1a, CD3 and C20 positive cells in citrulline positive versus negative tissue specimens revealed no significant differences. It is as yet uncertain why citrullination takes place in only some inflamed tissues independently of the amount of infiltrating cells. Still, when present, citrullination appears to be spatially associated with areas of inflammation. It is also unclear if this citrullination may enhance the local disease process since we could not detect any difference in FVC between patients with and without citrullination in the lungs. Because it is unlikely that this question can be fully addressed in human RA and since inflammation-related citrullination has been demonstrated in synovium in experimental arthritis models (15 and 20), we also assessed pulmonary citrullination in CIA. Although subclinical pulmonary interstitial disease may be present in a majority of patients with early RA (21), we did not detect any abnormalities including presence of citrullinated proteins in the lung tissues of mice with collagen induced arthritis. Apparently, this mouse model differs significantly from human disease and these mice do not develop pulmonary disease as an extra-articular disease manifestation of RA.

Due to the retrospective nature of our study, we were unable to evaluate the presence of ACPA in our cohort. Therefore, we determined ACPA titers in a separate, consecutive sample of 17 patients recently diagnosed with idiopathic IP (10 UIP, 6 NSIP, 1 desquamative interstitial pneumonia). None of these patients was ACPA positive. Conversely, in a sample of 6 patients with RA-associated IP, all individuals showed high titers of anti-CCP antibodies (unpublished data). This observation makes it unlikely that pulmonary citrullination as such is sufficient to drive the autoimmune response but does not exclude that it contributes to the local pathology in ACPA positive RA patients. In conclusion, our study indicated clearly that citrullination occurs in RA-associated interstitial pneumonia but also demonstrated similar findings in idiopathic lung disease. This strengthens the concept that citrullination itself is not specific for RA but rather is associated with the inflammatory process in certain patients. However, the detection of citrullination independently of the amino acid context
cannot exclude a specificity of distinctive citrullinated proteins for RA-associated disease as previously indicated in synovium. The absence of relation with features such as smoking status, with local disease severity, and with serum ACPA leaves the question open as to what factors lead to pulmonary citrullination and whether citrullination of pulmonary proteins contributes to the initiation, perpetuation or acceleration of autoimmunity or merely reflects ongoing inflammation without a significant pathophysiologic role in the disease process.

Acknowledgements

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References


