Concise Report

Sensitivity and specificity of autoantibodies binding to citrullinated carboxyterminal telopeptides of types I and II collagens in an early arthritis series

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Objective. To assess the specificity and sensitivity of autoantibodies binding to citrullinated carboxyterminal telopeptides of types I and II collagens in an early arthritis series.

Methods. A cohort of 146 patients from the Kuopio 2000 Arthritis Survey having RA, AS, PsA, ReA, uSpA or undifferentiated arthritis were studied. Autoantibodies binding citrullinated types I and II carboxyterminal telopeptides were measured in two different inhibition ELISA assays. Sera from 135 adult persons were used as controls.

Results. In RA, the sensitivities were 0.83 with long type I telopeptide and 0.78 with long type II telopeptide and the respective specificities were 0.94 and 0.93, while the corresponding values in other inflammatory joint diseases were much lower. The likelihood ratio in RA increased with longer peptides from 4.20 to 14.06 for type I telopeptide and from 2.74 to 11.67 for type II telopeptide.

Conclusion. The antibody assay using long telopeptide from type I collagen was the most specific and sensitive method in every diagnostic category, although in the arthritides other than RA, binding was much less abundant and possibly citrulline-independent.

Key words: Anti-CCP antibodies, Autoantibodies, Citrullination, Early arthritis, RA, SpA, Telopeptide, Type I collagen, Type II collagen.

Introduction

Patient populations with early arthritis include patients with different autoimmune diseases of unknown aetiology, such as RA, AS, PsA, ReA, uSpA and undifferentiated arthritis (UA). Both genetic and environmental factors (especially smoking) have a role in the pathogenesis of these diseases [1]. There is no specific test available for classifying these diagnoses.

RF assay is the only laboratory test included in the classification criteria of RA [2], but only 70–80% of RA patients are positive for it [3, 4]. However, antiperinuclear factor (APF), anti-keratin antibody (AKA) and antibodies to several forms of cyclic citrullinated peptide (CCP) are more specific to RA than RF [3–5]. Anti-CCP assays have been developed to enable maximum diagnostic differentiation between patients with and without RA [5]. Initially, the anti-CCP assays were based on filaggrin proteins, while the current anti-CCP Mark2 seems to contain a mixture of artificial, non-physiological antigens [6]. However, to clarify the pathophysiological phenomena involved, natural or modified natural protein antigens should be studied [7]. The inhibition ELISA methods developed in our laboratory can be used to measure the autoantibodies binding specifically to citrullinated telopeptides of types I and II collagens on the sensitivity and specificity of such antibody assessments in adult patients with newly diagnosed different inflammatory joint diseases.

Patients and methods

Patients and controls

Serum samples were collected from 146 adult patients with previously undiagnosed arthritis included in the Kuopio 2000 Arthritis Survey, which was a population-based study of 76 000 adults [11]. At least one joint with peripheral synovitis or signs of an inflammatory disorder in the SI, glenohumeral or hip joints assessed by ultrasonography (US), scintigraphy or MRI had to be registered on the first visit. The patients were classified into the following subgroups: RA, AS, PsA, ReA, uSpA or UA. All patients gave their informed written consent. Patients with an established diagnosis or any other treatment except non-steroidal anti-inflammatory medication were excluded. RA was defined by the 1987 ACR classification criteria [2]. RF was measured by the Waaler–Rose method, and a dilution Χ1:80 (45–50 IU/l) was regarded as positive. The patient series included 18 patients (11 women and 7 men) with RA, 30 patients (16 women and 14 men) with SpAs, including AS, PsA, ReA and uSpA, and 98 patients (82 women and 16 men) with UA. The mean ages (± s.d.) of the aforementioned groups were 60 ± 13, 43 ± 12 and 48 ± 16 yrs, respectively. RF was positive in 67% and anti-CCP in 72% of the patients with RA, in 3 and 7% with SpA and in 14 and 9% with UA. The median delay [interquartile range (IQR)] from symptom onset to diagnosis was 6 (4–6) months in RA, 8 (3–27) months in SpAs and 4 (2–7) months in UA. The median joint count (IQR) and ESR (IQR) at diagnosis were 8 (6–9) and 22 (15–40) mm/h in RA, 2 (2–4) and 9 (5–17) mm/h in SpAs and 2 (1–3) and 10 (6–19) mm/h in UA, respectively.

The 135 controls (87 women and 48 men) were either apparently healthy persons or persons who had no rheumatic disorders but possibly had other chronic diseases, such as hypertension or hypercholesterolaemia, but no serious inflammatory diseases or malignancies. Their mean age was 57 ± 16 yrs.
Serum samples were taken at the time of the diagnosis and stored at −20°C. This study was approved by the Ethical Committee of Kuopio University Hospital. The study was performed according to the Declaration of Helsinki.

**Immuoassays**

Autoantibodies binding citrullinated carboxytelopeptides of type I or II collagen were measured in ELISA assay without and with inhibition [10]. Short type I telopeptide (EKA HDG GRY YCitA) or type II telopeptide (EKG PDP LQY MCitA) or long antigens (SAGF DFS FLE PPQ EKA HDG GRY YCitA or GIDM SAF AGL GPR EKG PDP LQY MCitA) were biotinylated and inhibited with soluble identical non-biotinylated peptides. All the sera were tested under standard conditions, and the final concentrations of the inhibiting peptides were 200 μg/ml. For each serum, the differences in binding with and without inhibition were calculated and compared with those of control sera.

Anti-CCP ELISA (the anti-CCP Mark2 assay) was carried out according to the procedure described by the manufacturer (Euro-Diagnostica, Malmö, Sweden). All sera, calibrators and controls were measured in duplicate. The cut-off limit for a positive result in the assay was 25 U/ml. Patients with a result of 25–50 U/ml were strongly positive and were retested.

**Statistical analyses**

The results were expressed as mean or median, s.d. or IQR. The comparisons of continuous data were carried out using Kruskall–Wallis test. Receiver operating characteristic (ROC) curves were constructed to determine the cut-off points of types I and II citrullinated short and long peptides that differentiate patients with inflammatory disease from controls, with bias-corrected and accelerated bootstrap CIs. Permutation-type test of equality of the areas under the curve, the algorithm suggested by the Delong et al. [12], was used in an analysis of different peptides. Sensitivity, specificity and positive likelihood ratio as well as their 95% CIs were calculated for each peptide in the different diagnostic categories.

**Results**

The inhibition of autoantibody binding was much stronger in RA patients than in patients with other arthritides (Fig. 1). The area under the curve (AUC), sensitivity and specificity in the different diagnostic groups are shown in Table 1. The length of the telopeptide affected sensitivity and specificity. Specificity increased from 0.81 (short) to 0.94 (long) for type I collagen and from 0.70 to 0.93 for type II collagen. The likelihood ratio in RA also increased from 4.20 to 14.06 for type I telopeptides and from 2.74 to 11.67 for type II telopeptides (Table 1).

The other arthritides studied (Fig. 1) also showed some binding to these telopeptides, but the magnitude of inhibition was much smaller than in RA. Table 1 demonstrates that AUC was close to 0.5 for the short forms of both telopeptides. Only the long type I telopeptide and, to a lesser extent, the long type II telopeptide showed an increase of AUC.

Inhibitions using citrullinated long type I telopeptides were the most specific and sensitive tests in every diagnostic category. However, in detailed inhibition analysis (data not shown), the binding was not due to citrullination but to other amino acids near the carboxyterminus. To ensure the validity of the inhibition ELISA results, background and controls with three different concentrations of IgG antibodies against the citrullinated C-telopeptides of types I and II collagens were measured. The coefficients of variation were 1.4–10.5% for the different peptides.

Disease activity assessed with ESR (P = 0.005) and joint count (P < 0.001) was highest in RA. In RA 12/13, in UA all nine and in SpAs both the patients with positive anti-CCP had results above the cut-off levels of citrullinated long type I collagen. Of the 24 patients with anti-CCP antibodies, 19 were or had been smokers. Of these individuals, 12 patients (63%) had been smoking for 15–60 yrs. All the seven men with RA were heavy smokers.

**Discussion**

We have previously reported that both citrullinated telopeptide antigens derived from types I and II collagens have nearly similar sequences, —Y-Y-Cit-A and —Y-M-Cit-A, respectively [10]. As expected, both antibodies behave similarly in RA (Table 1). Soluble citrullinated telopeptide antigens can also inhibit the binding in both types I and II telopeptide ELISA assays [10]. However, in other diseases studied here (SpAs and UA, Table 1), only the long telopeptide from type I collagen showed reasonable binding. Most likely, the differences between the short and long forms of type I telopeptide are not due to citrulline, since both have the same sequence in the vicinity of citrulline. The further experiments performed showed that, in SpAs and UA, the binding of the long I telopeptide was due to the sequence not containing citrulline.

In spite of the fact that the newest version of the anti-CCP assay (Mark2) was developed to enable maximal differentiation of RA patients and controls, there are some cases (e.g. patients having type I autoimmune hepatitis) in which positive binding is due to citrulline-independent reactivity [13]. In this study, high
levels of anti-CCP antibodies as well as antibodies to citrullinated long telopeptides of type I collagen occurred in the same patients independent of the final diagnosis.

Type I collagen is abundant in soft tissues like synovium and tendon, and it is also the major collagen of mineralized bone, whereas type II collagen is the most abundant collagen in hyaline cartilage. Because RA patients had a more active disease with polyarthritis and higher ESR than SpA and UA patients, the active citrulline-dependent binding of both types I and II collagens in RA may reflect a more serious, irreversible disease, whereas the non-citrullinated antigen binding of type I collagen might reflect less-destructive disease without cartilage damage.

This is the first cohort of RA patients in which the sensitivities and specificities of citrullinated telopeptide assays have been analysed. Although the number of patients in this study was rather small, the sensitivities were 0.83 (type I telopeptide) and 0.78 (type II telopeptide) and the respective specificities 0.94 and 0.93. For comparison, the mean (± s.d.) sensitivity for anti-CCP2 assay [14] was 0.68 (± 0.15) in 58 articles published in 1999–2006, and the specificity in differentiating RA from other rheumatic diseases was strikingly high, 0.96 (± 0.03). In a recent publication comparing six different citrullinated protein/peptide antibody assays, the AUC ranged from 0.851 to 0.884 [15], which is comparable with that found here for the telopeptide assays (0.93 and 0.84, respectively). The positivity likelihood ratio in antibodies to citrullinated protein antigens assays ranged from 6.8 to 21.0 [13], which is similar to that found in this study (14.06 and 11.67, respectively).

The autoimmuneity of citrullinated proteins in different arthritides is not fully understood. However, it seems that the citrullinated proteins are associated with inflammation, and citrullination of self-proteins or peptides is suspected to be pathogenic in RA. Smoking is the only environmental factor that has been verified, when occurring in association with a shared epitope, to increase the risk of developing RA [16, 17]. Positive staining for citrullinated proteins was recorded in cells obtained from broncho-alveolar lavage fluid and pulmonary cells with inflammation of smokers [1]. There is a need, indeed, to identify potentially erosive diseases among arthritides. Anti-CCP antibodies precede the development of RA, and their presence has been reported to predict erosive disease [3, 5]. Also, antibodies to citrullinated telopeptide antigens are found before the onset of the disease, and they have a synergistic effect with anti-CCP antibodies in predicting the risk of seropositive RA [8]. However, the function of citrullination of collagens is not understood. In this study, we introduce telopeptide assays as a new potential test to promote the absolutely necessary search for new data in this field.

References

Table 1. AUC of types I and II short and long peptides with sensitivity, specificity and likelihood ratio in each diagnostic category

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Measurement AUC (95% CI)*</th>
<th>Cut-off point</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>LR+ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td></td>
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<tr>
<td>Short type I collagen</td>
<td>0.82 (0.67, 0.94)</td>
<td>≥11</td>
<td>0.78 (0.52, 0.94)</td>
<td>0.81 (0.74, 0.87)</td>
<td>4.20 (2.63, 6.36)</td>
</tr>
<tr>
<td>Short type II collagen</td>
<td>0.79 (0.62, 0.92)</td>
<td>≥10</td>
<td>0.83 (0.69, 0.96)</td>
<td>0.70 (0.61, 0.77)</td>
<td>2.74 (1.87, 3.74)</td>
</tr>
<tr>
<td>Long type I collagen</td>
<td>0.93 (0.85, 0.98)</td>
<td>≥26</td>
<td>0.83 (0.59, 0.94)</td>
<td>0.94 (0.89, 0.97)</td>
<td>14.06 (7.04, 23.18)</td>
</tr>
<tr>
<td>Long type II collagen</td>
<td>0.84 (0.69, 0.96)</td>
<td>≥20</td>
<td>0.78 (0.52, 0.94)</td>
<td>0.93 (0.88, 0.97)</td>
<td>11.67 (5.93, 22.72)</td>
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<tr>
<td>SpA</td>
<td></td>
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<td></td>
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<tr>
<td>Short type I collagen</td>
<td>0.54 (0.42, 0.65)</td>
<td>≥6</td>
<td>0.73 (0.54, 0.88)</td>
<td>0.39 (0.31, 0.48)</td>
<td>1.21 (∼1.51)</td>
</tr>
<tr>
<td>Short type II collagen</td>
<td>0.57 (0.44, 0.68)</td>
<td>≥11</td>
<td>0.40 (0.23, 0.59)</td>
<td>0.79 (0.71, 0.86)</td>
<td>1.93 (1.08, 3.22)</td>
</tr>
<tr>
<td>Long type I collagen</td>
<td>0.83 (0.74, 0.89)</td>
<td>≥11</td>
<td>0.90 (0.73, 0.98)</td>
<td>0.74 (0.66, 0.81)</td>
<td>3.47 (2.54, 4.75)</td>
</tr>
<tr>
<td>Long type II collagen</td>
<td>0.65 (0.55, 0.75)</td>
<td>≥10</td>
<td>0.80 (0.61, 0.92)</td>
<td>0.49 (0.40, 0.54)</td>
<td>1.57 (1.18, 1.96)</td>
</tr>
<tr>
<td>UA</td>
<td></td>
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<tr>
<td>Short type I collagen</td>
<td>0.57 (0.50, 0.65)</td>
<td>≥7</td>
<td>0.64 (0.73, 0.74)</td>
<td>0.39 (0.31, 0.48)</td>
<td>1.21 (∼1.51)</td>
</tr>
<tr>
<td>Short type II collagen</td>
<td>0.62 (0.54, 0.69)</td>
<td>≥11</td>
<td>0.42 (0.32, 0.52)</td>
<td>0.82 (0.75, 0.88)</td>
<td>2.35 (1.54, 3.63)</td>
</tr>
<tr>
<td>Long type I collagen</td>
<td>0.83 (0.77, 0.88)</td>
<td>≥13</td>
<td>0.84 (0.75, 0.90)</td>
<td>0.80 (0.72, 0.86)</td>
<td>4.18 (3.00, 5.98)</td>
</tr>
<tr>
<td>Long type II collagen</td>
<td>0.72 (0.66, 0.78)</td>
<td>≥11</td>
<td>0.78 (0.68, 0.85)</td>
<td>0.50 (0.50, 0.68)</td>
<td>1.90 (1.52, 2.41)</td>
</tr>
</tbody>
</table>

*95% CI: obtained by bias-corrected bootstrapping (5000 replications). LR: likelihood ratio.
17 Linn-Rasker SP, van der Helm-van Mil AH, Van Gaalen FA et al. Smoking is a risk factor for anti-CCP antibodies only in RA patients that carry HLA-DRB1 shared epitope alleles. Ann Rheum Dis 2006;65:366–71.