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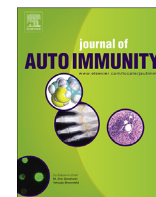

Identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis

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In 2011 a novel autoantibody system, anti-carbamylated protein (anti-CarP) antibodies, was described in rheumatoid arthritis (RA) patients. Anti-CarP antibody positivity associates with a more severe disease course, is observed years before disease onset, and may predict the development of RA in arthralgia patients. Although many clinical observations have been carried out, information on the antigenic targets of anti-CarP antibodies is limited. Most studies on anti-CarP antibodies utilize an ELISA-based assay with carbamylated fetal calf serum (Ca-FCS) as antigen, a complex mixture of proteins. Therefore, we analysed the molecular identity of proteins within Ca-FCS that are recognized by anti-CarP antibodies.

Ca-FCS was fractionated using ion exchange chromatography, selecting one of the fractions for further investigation. Using mass-spectrometry, carbamylated alpha-1-antitrypsin (Ca-A1AT) was identified as a potential antigenic target of anti-CarP antibodies in RA patients. A1AT contains several lysines on the protein surface that can readily be carbamylated.

A large proportion of the RA patients harbour antibodies that bind human Ca-A1AT in ELISA, indicating that Ca-A1AT is indeed an autoantigen for anti-CarP antibodies. Next to the Ca-A1AT protein, several homocitrulline-containing peptides of A1AT were recognized by RA sera. Moreover, we identified a carbamylated peptide of A1AT in the synovial fluid of an RA patient using mass spectrometry.

We conclude that Ca-A1AT is not only a target of anti-CarP antibodies but is also present in the synovial compartment, suggesting that Ca-A1AT recognized by anti-CarP antibodies in the joint may contribute to synovial inflammation in anti-CarP-positive RA.

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1. Introduction

Anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) are important serological markers in the diagnosis of rheumatoid arthritis (RA) [36,38] and are therefore part of the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) disease classification criteria for RA [4,5]. ACPA are mostly detected using anti-cyclic citrullinated peptide (CCP) antibody assays. In 2011, we described antibodies to carbamylated proteins (anti-CarP antibodies) in RA patients using an ELISA, based on carbamylated fetal calf serum (Ca-FCS) [29,31,34]. In contrast to citrullination, which is mediated by enzymes, carbamylation is a chemical reaction mediated by cyanate, converting lysine into a homocitrulline [35] which is very similar to citrulline (only one CH2 longer side chain) [33].

Importantly, anti-CarP antibodies have been detected in both

Abbreviations: Anti-CarP, anti-carbamylated protein; ACPA, anti-citrullinated peptide antibodies; AUC, Area under the curve; A1AT, alpha 1 anti-trypsin; Ca, Carbamylated; CCP, cyclic citrullinated peptide; ELISA, enzyme-linked immunosorbent assay; FCS, Fetal Calf Serum; RA, Rheumatoid arthritis; RF, Rheumatoid factor.

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ACPA-positive and ACPA-negative RA patients, indicating a potential diagnostic value of anti-CarP antibody testing [18,25,26,37]. A recent meta-analysis estimated the sensitivity, specificity and odds ratio of anti-CarP antibodies as 42% (95% CI 38–45), 96% (95% CI 95–97) and 17 (95% CI 12–24), respectively when comparing RA patients to healthy controls [22]. Also, using a large cohort of longitudinal samples, it was demonstrated that anti-CarP antibodies predict joint damage as assessed by total Sharp van der Heijde Score [29]. Additionally, anti-CarP antibodies are present in individuals that developed RA already many years prior to disease onset [8,14,30]. The presence of anti-CarP antibodies has a prognostic value in arthralgia regarding the future development of RA [31]. Similar to ACPA and RF, anti-CarP antibodies have been reported in first degree relatives of RA patients and could help to identify individuals that might develop RA [3]. Even though anti-CarP antibodies are predominantly found in RA patients, they can also present in other inflammatory conditions at considerably lower frequencies [7,9,32].

Studies have also shown that carbamylation can occur *in-vivo* [6,13]. However, little is known about the antigens that can be recognized by anti-CarP antibodies. Although it is well understood that Ca-FCS is recognized by anti-CarP antibodies, it is yet unknown which antigens within the complex mixture of Ca-FCS are targeted by anti-CarP antibodies. The identification of the molecular targets by anti-CarP antibodies in RA patients could aid in the etiological understanding of RA. Therefore, the goal of the current study was to characterize the protein(s) present in carbamylated FCS that are targeted by anti-CarP antibodies, where we, as a proof of concept, focus on one of the identified proteins.

2. Materials and methods

2.1. Patients and sera

We selected 80 ACPA-positive and 80 ACPA-negative RA patients from the Leiden Early Arthritis Clinic (EAC; Leiden, The Netherlands). Serum samples from disease controls were also derived from the EAC cohort; patients with gout (n = 51), psoriatic arthritis (n = 40), osteoarthritis (n = 40), sarcoidosis (n = 36) and spondylarthropaty (n = 40). In addition, serum samples were collected from 80 healthy controls from the Leiden area [29]. ACPA, RF and anti-CarP antibody status were acquired previously for the samples from RA patients and healthy controls [29]. Patient identity was not disclosed and the data were used anonymously in accordance with the Helsinki Declaration of human research ethics.

2.2. Carbamylation of antigens

Carbamylated proteins were produced by allowing the protein to react with potassium cyanate (KOCN, Sigma-Aldrich, St. Louis, MO) as previously described [29]. In brief, a 2 M solution of KOCN was prepared in PBS. Fetal calf serum (FCS, Bodinco, Alkmaar, the Netherlands) or Alpha-1-antitrypsin (A1AT, Lee Biosolutions, Maryland heights, USA) were mixed with the 2 M KOCN solution in a 1:1 volume-by-volume proportion. The mixed solution was incubated overnight at 37 °C to produce Ca-FCS and Ca-A1AT. Following the incubation period, the resulting carbamylated solution was dialyzed against PBS (2L) for 48 h, during which the PBS was refreshed at least 5 times. The *in-vitro* carbamylation of A1AT and FCS was confirmed by MS (data not shown).

2.3. Immunoassays

Anti-CarP antibodies were detected using Ca-FCS or Ca-A1AT as previously described [29]. In brief, unmodified FCS and Ca-FCS were coated overnight on NUNC MAXISORP® plates (Thermo Scientific, Waltham, MA). Following washing and blocking, the wells were incubated with serum samples obtained from human RA patients and healthy volunteers. Bound human IgG was detected either indirectly using rabbit anti-human IgG (Dako, Glostrup, Denmark), followed by HRP-labeled goat anti-rabbit IgG antibody (Dako), or directly using an HRP-labeled rabbit anti-human IgG (Dako). Following additional wash steps, HRP enzyme activity was measured using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). The cut-off for a positive response was chosen as the mean plus two times the standard deviation (SD) of the anti-CarP reactivity of healthy controls.

For epitope mapping studies using peptide fragments of A1AT, anti-biotinylated peptide ELISAs were carried out. NUNC MAXISORP® plates were coated overnight with streptavidin (Invitrogen) followed by incubation of 10 μg/ml of biotinylated peptide diluted in PBS with 0.1% BSA for a period of 1 h at room temperature. Serum samples were diluted 50× in PBS with 1% BSA and 0.05% Tween and also incubated for 1 h at 37 °C. Reactivity was detected using the HRP-labeled goat anti-rabbit IgG antibody for 1 h at 37 °C. Again, HRP enzyme reactivity was measured using ABTS.

2.4. Identification of A1AT by ion exchange fractionation and mass spectrometry

Ca-FCS was fractionated by ion-exchange HPLC (high-performance liquid chromatography) using a MonoQ column. HPLC fractions were analysed by measurement of absorption for their overall protein content using a plate reader as well as by ELISA used to test anti-CarP antibody reactivity (not normalized for protein content). Five fractions, covering the entire spectrum were selected for further ELISA experiments. Antibody reactivity against these fractions was determined in six serum samples, now coating equal concentrations. The samples included two serum samples with anti-CarP antibodies and no ACPA (CarP+/ACPA-), two serum samples with ACPA antibodies and no anti-CarP antibodies (CarP-/ACPA+), as well as two negative control serum samples from healthy volunteers. The fraction that showed highest reactivity in ELISA was run on an SDS-PAGE. Coomassie stained bands were excised and subjected to chymotryptic digestion and mass spectrometry (MS).

2.5. Synthetic overlapping peptides

Synthetic 21-mer peptides were synthesized based on the human A1AT sequence. Each lysine within the human A1AT sequence was identified and used as the basis for designing a peptide. On each side, the 10 flanking amino acids were added. If multiple lysines were present within 21 amino acids, multiple peptides were made. All peptides contained a biotin at the N-terminal end for ELISA testing. The peptides were synthesized as partially overlapping fragments, covering all lysines in the human A1AT sequence.

2.6. Modeling of A1AT

The known protein sequence of A1AT was entered into RasMol to generate a 3 dimensional model of the protein. RasMol (www.openrasmol.org) is a molecular graphics program intended for the visualisation of proteins, nucleic acids and small molecules. The program is aimed at display, teaching and generation of publication quality images. The program reads in a molecule coordinate file and interactively displays the molecule on the screen in a variety of colour schemes and molecule representations. All lysin residues were marked on the A1AT molecule and illustrations from two

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