Anti-carbamylated protein antibodies in rheumatoid arthritis

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1 Introduction

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Introduction

Rheumatoid arthritis, pre-rheumatoid arthritis stages and juvenile idiopathic arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease that principally affects synovial joints. RA is present in 0,5% to 1% of the global population. The incidence of RA is higher in women than in men and increases with age (1). RA can affect any joint but preferably small joints in hands and feet (2). The symptoms of RA include pain, swelling, stiffness, redness, warmth and can finally lead to loss of joint functions (2). The systemic symptoms of RA include fatigue, malaise, loss of appetite and muscle ache. Next to the joints RA can affect other organs such as skin, lungs, heart and blood vessels (3). RA can be classified using the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis (2010 ACR/EULAR criteria) (4). This is a quantitative system in which scores can be obtained from: joint involvement, serologic markers, inflammation markers and duration of symptoms (4).

Development of RA can be acute or can be preceded by pre-RA stages. Yet a uniform and precise definition of pre-RA stages does not exist. Pre-RA stages generally refer to patients who have clinical symptoms involving joints but not fulfill the diagnostic criteria of RA or other forms of arthritis (5). Arthralgia and undifferentiated arthritis (UA) are two common types of pre-RA stages. Arthralgia patients may have symptoms such as joint pain, psychological distress, muscle cramps, abnormal skin sensations, stiffness, loss of motor control, weakness, fatigue and sleeping difficulties but have no clinically apparent joint swelling/inflammation (6-8). UA patients, to the opposite, have clinically apparent joint swelling suggestive of inflammation (9). Patients in these two stages can progress to RA but also have high chance of remission (8,9). The likelihood of progression to RA for arthralgia and UA patients is partially predictable by clinical and serologic variables. The arthralgia and UA patients share the predictable variables: morning stiffness and the presence of anti-cyclic citrullinated peptide antibodies (10,11). The arthralgia patients specific prediction variables are: rheumatoid arthritis in a first degree family member, alcohol non-use, duration of symptoms <12 months, presence of intermittent symptoms, arthralgia in upper and lower extremities, visual analogue scale pain≥50, and history of swollen joints as reported by the patient (10). The UA patients specific prediction variables are: sex, age, localization of symptoms, the tender joint count, the swollen joint count, the C-reactive protein level, rheumatoid factor positivity, and magnetic resonance imaging (MRI)/ultra sound pattern (11-14). Since these associations apply at a group level additional biomarkers are needed that will allow a more personalized medicine approach in these pre-RA patients.

Arthritis can also occur in children and is referred to as juvenile idiopathic arthritis (JIA), the most common rheumatic disease in children (0,01%-0,4%) consisting of eight heterogeneous subgroups (15). A common feature of JIA is joint inflammation resulting in pain, loss of function, and morning stiffness (16). Unlike adult RA, JIA patients have higher chances of remission and a lower prevalence of autoantibodies (17,18). Rheumatoid factor (RF) positive polyarticular JIA is the subgroup which resembles most the clinical and immune-genetic characteristics of adult RA patients (15).

Autoantibodies in RA, pre-RA stage and JIA patients

RA patients are a heterogeneous group of patients with pronounced differences in disease activity and outcome. This heterogenous group can be subdivided by the presence of autoantibodies. Autoantibody positive and negative RA patients were found to have different genetic background, disease development processes and responses to treatments (2). Currently, RF and anti-citrullinated protein antibodies (ACPA) are two major autoantibody systems in RA patients. The identification of RF can be dated back to 1940, reported by Waaler et al (19). RF is a polyclonal antibody system which mainly recognizes the Fc part of IgG (20). RF is present in about 50%-90% of RA patients but can also be present in other rheumatic or non-rheumatic diseases (21). IgM-RF is the most frequently detected isotype in RA patients but IgG and IgA isotypes also exist (22). The possible functions of RF include helping immune complex (IC) formation and clearance as well as facilitating antigen presentation (23). RF was the most important diagnostic marker in RA before ACPA were discovered and it is still included in the newest 2010 ACR/EULAR RA classification criteria in the same way as ACPA (4). However, the clinical relevance of RF was shown to be limited. Given that IgM-RF can be detected in sera of blood bank donors who developed to RA several years later but not in those who did not develop to RA (24-26), a cross-sectional screening study detected only one RA patient in each 10 RF-positive subjects (27). The presence of IgM-RF predicts future development of RA in UA patients independent of ACPA (28). However, the presence of IgM-RF did not have such predictive value in arthralgia patients, no prognostic value for joint damage and for the chances of disease modifying anti-rheumatic drug (DMARD) free remission in early RA patients in the disease course independent of ACPA (7,29,30).

The presence of ACPA and their prognostic value in RA patients were first reported around 50 years later than RF (31). ACPA bind to citrullinated epitopes of auto-antigens. The sensitivity of ACPA (~67%) was comparable to IgM-RF (~69%) but its specificity (~95%) was much higher than IgM-RF (~85%) (32). The presence and the level of ACPA contribute 2 points in the newly made 2010 ACR/EULAR RA classification criteria (4). The presence of ACPA in early RA patients is associated with worse disease development and a lower chance of reaching DMARD free remission (29,30). The presence of ACPA is also associated with a higher risk of cardiovascular disease and a higher mortality in RA patients (33,34).

In addition, the presence of ACPA is associated with more severe extra-articular manifestations in RA patients (35). ACPA positive UA and arthralgia patients have an increased chance of further developing to RA (7) while IgM-RF negative arthralgia patients almost do not develop to arthritis (36). ACPA are present in 24% of UA patients in the Leiden early arthritis cohort (EAC) and the positive predictive value for developing to RA is 67% (28). In addition, around 30% of RA patients developed ACPA before the appearance of any clinical symptom (24,25) The presence of ACPA before the onset of RA in asymptomatic blood donors is associated with the development of erosive RA (26).

Next to the presence of ACPA, several aspects of the ACPA responses, such as titer, isotype usage, epitope spreading and avidity (37-39), are associated with the clinical outcome of RA. An increased ACPA isotype usage was observed in RA patients compared to UA and healthy individuals who are also ACPA positive (37,40). High titer ACPA positive UA patients have a higher risk of developing to RA and a more acute development compared to low titer ACPA positive UA patients (38). The number of recognized citrullinated epitopes is positively associated with the risk of developing to RA and negatively associated with the chance of reaching DMARD free remission in ACPA positive arthralgia and UA patients (28,41,42). ACPA positive RA patients who have the lowest quartile avidity (binding strength to antigens) also have the most joint damage in their disease course (39). Above mentioned associations suggest that the autoantibody response is a developing process and it may trigger other symptoms/pathogenic effects of RA.

The autoantibody profile in JIA patients is quite different from adult RA patients. Antinuclear antibodies (ANA) have a lower prevalence and diagnostic value in RA patients compared to RF (43). But in JIA patients the presence of ANA is associated with a higher risk of uveitis and increased T and B cells infiltration (44,45). The prevalence of RF or ACPA in JIA patients is less than in adult RA patients and is predominantly confined in RF-positive polyarticular JIA subgroup (17,46). Like in adult RA, ACPA were confirmed as an independent risk factor associated with worse disease outcome in JIA patients (47,48).

In summary, both ACPA and RF are diagnostic markers for RA patients and predictive markers for future development of RA in pre-RA stage patients. ACPA are also a prognostic marker in RA patients.

Genetic predisposing and ACPA

As described before, RA patients can be divided into 2 distinct subsets which, at a group level, have different disease courses and genetic risk profiles based on ACPA positivity (49). This again suggests that the production of ACPA is a key intermediate step between the predisposing genetic risk and the development of RA. Eventhough the heritability of

ACPA negative and positive RA patients are both estimated to be about 66% (50), yet no predominant human leukocyte antigen (HLA) association and far less non-HLA risk single-nucleotide polymorphisms (SNPs) were found in association with ACPA negative patients (51-53). The most important genetic risk factors of ACPA positive RA reside in the Human Leukocyte Antigen (HLA) class II locus, more specifically the shared epitopes (SE) on HLA-DR beta 1 (HLA-DRβ1). SE consists of three homologous amino acid sequence variants in residues 70–74 of the HLA-DRβ chain: QKRAA, QRRAA and RRRAA (54). HLA SE alleles contribute 18% of the susceptibility of ACPA-positive RA patients (50). In non-HLA genes which have SNPs associated with higher risk of developing to ACPA-positive RA (52), many genes encode proteins which are potentially contributive to autoantibody production or immune complex activated pathways. Thus both HLA and non-HLA genetic risk factors of RA suggest the pathogenesis of ACPA positive RA patients is perhaps predominantly initiated via adaptive immunity.

Environmental risk factors and ACPA

Beside genetic risk factors, also environmental risk factors such as cigarette smoking and infection are associated with a higher risk of developing to RA. Cigarette smoking is dose dependently associated with the susceptibility of RA and disease progression (55-57). It is also strongly associated with the combined presence of ACPA positivity and SE (55,56,58). Increased expression of peptidyl-arginine deiminase (PAD) induced by smoking is one hypothesis to explain the effect of smoking in RA patients and its association with ACPA positivity and SE (59). Periodontitis (PD), an infectious disease, is also associated with a higher incidence and severity of RA (60-62). Treatment of PD in established RA patients decreased the severity of RA (63-65). The presence of PD and the level of antibodies against P. gingivalis, a pathogen of PD, are associated with the presence of ACPA and RF-IgM (66,67). NETosis of neutrophils is another mechanism which can be triggered by the pathogens of PD (68). NETosis, a process in which the nuclear content of cells is extruded from the cell, will release intracellular PAD4, which offers another source for extracellular citrullination (69).

Pathological functions of ACPA

To explain the potential contribution of ACPA to the pathogenesis of RA, the functions of ACPA have been studied in several aspects. ACPA, citrullinated antigens and their immune complex (IC) were reported to induce the TNF-α production of several cell types including macrophage, monocyte, fibroblast-like synoviocytes and osteoclast precursors (70-72). TNF-α is a key cytokine in the pathogenesis of RA (73). Furthermore, ACPA stimulate osteoclastogenesis and osteoclast mediated bone erosion. In addition ACPA have been shown to activate the classical and alternative pathways of complement (74). ACPA may appear in mice with collagen induced arthritis without immunization of citrullinated

antigens depending on their genetic background (75). However, whether ACPA play a role in the progression of the disease in DBA1 and Balb/c mice is in debate (76-78).

Carbamylation

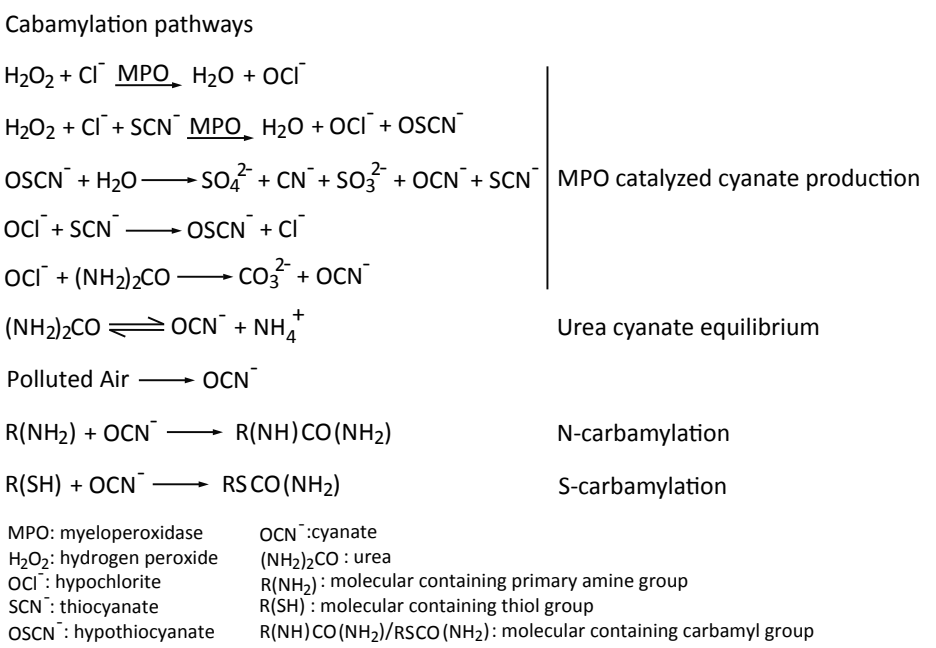
As ACPA are only present in about 67% of the RA patients and as part of the ACPA negative RA patients also have severe joint damage, there is a need for additional biomarkers to identify ACPA negative patients in need of a more aggressive intervention (79). In an attempt to identify additional biomarkers to be used for such identification we addressed the presence of antibodies directed against proteins modified by another form of post-translational modification, carbamylation. Homocitrulline is an amino acid with a high structural similarity to citrulline and therefore we hypothesized that anti-homocitrulline containing (carbamylated) protein antibodies may also be present in some RA patients and we tested this hypothesis following previous studies on carbamylation as described in detail below.

Pathways that induce carbamylation of proteins

Carbamylation: cyanate reacting to primary amino or thiol groups

Carbamylation is a post-translational modification in which cyanate reacts to primary amino or thiol groups. The reaction of cyanate to either amino groups or thiol groups is specified as N-carbamylation or S-carbamylation. Beside the N-terminus of all proteins, the amino acids lysine, arginine and cysteine contain side chains that can react with cyanate (80,81). However, since carbamylation on side chains of cysteine and arginine, the N-terminus of proteins and free amino acids is rarely reported we therefore here refer to carbamylation as cyanate reacting on peptidyl-lysine without further specification. Urea is a source of cyanate in all individuals and is present in body fluids in equilibrium with ammonium cyanate (Fig. 1). The equilibrium ratio between cyanate and urea has been suggested to be around 1 to 500.000 (82). Despite the low concentration of cyanate, trace amount of carbamylation can be detected in healthy individuals (83,84). As expected, elevated carbamylation was extensively reported among patients with renal dysfunction and elevated blood urea nitrogen (BUN) levels (85-87).

Figure 1. Pathways involved in in vivo cyanate generation



Inflammation increases the level of cyanate

In addition to renal insufficiency, inflammation is another factor which can stimulate the degree of carbamylation. Wang and Holzer et al. demonstrated that inflammation can enhance carbamylation via a mechanism which depends on myeloperoxidase (MPO) (88,89). MPO is mainly stored in granules of neutrophils (90) and it can generate cyanate using hydrogen peroxide and thiocyanate as substrates. Thiocyanate, derived from e.g. food or smoke exposure (88), can be oxidized by hydrogen peroxide with the help of MPO, resulting in the formation of hypothiocyanate (84) which decomposes to cyanate and other ions (91). In addition MPO can also catalyze the reactions between hydrogen peroxide and chloride (90) that via a series of reactions leads to increased levels of cyanate (Fig. 1) (89). The marked increased levels of MPO in inflammation (92) therefore stimulates the formation of cyanate. These findings indicate that MPO released from neutrophils can further increase the level of carbamylation during inflammation.

Direct inhalation of cyanate

Cyanate (~200 parts-per-trillion volume) can also be directly inhaled from urban air. A five times higher concentration, one parts-per-billion volume of cyanate in inhaled breath, can

already generate an aqueous solution of 100 μM (93). This concentration is equal to or higher than the effective dose of cyanate which is able to cause notable effects in several in vitro studies (94-96). Cyanate in air can be derived from various sources such as biomass burning, coal burning, biofuel usage, cooking, tobacco usage and wild fire. Even in the absence of inflammation the direct exposure to air borne cyanate can be sufficient to generate low levels of carbamylation.

Lysine carbamyltransferase converts free amino acid lysine to homocitrulline

Beside above mentioned mechanisms, carbamylation on free amino acids can also be catalyzed by the enzyme lysine carbamyltransferase. This enzyme converts the free amino acid lysine and carbamyl phosphate to homocitrulline (97,98). Whether this enzyme is able to catalyze the reaction between carbamyl phosphate and peptidyl-lysine has, to the best of my knowledge, not been studied. Carbamyl phosphate injected in rats caused extensive carbamylation (99). Thus leaking of carbamyl phosphate synthetase or lysine carbamyltransferase due to apoptosis or necrosis of cells might potentially be a currently unexplored source of introducing carbamylation. At this stage, protein carbamylation mediated by enzymes seems unlikely but clearly requires further investigation.

Effects of carbamylation in (patho)physiology

Consequences of carbamylation have been reported to occur at the protein, cellular and systemic level. Decreased activity upon carbamylation has been reported for several enzymes and hormones, (100-105). Altered binding affinity to target ligands upon carbamylation has also been reported on hemoglobin A and human serum albumin (106-109). Other reported effects of carbamylation on proteins include changing their polymerization ability (collagen and actin), sensitivity to proteinases (collagen and glutamate dehydrogenase) and antibody antigen binding avidity (blood group specific glycoprotein) (110-112).

Not surprisingly, carbamylation of proteins and small molecules has an impact on normal cellular functions. Exposure to relatively high concentrations of cyanate is cytotoxic, which has been reported for e.g. human erythrocytes (114,115). Lower levels of carbamylation may also change cellular functions in several ways as described below. For example, in vitro incubation with cyanate dose-dependently decreased protein synthesis of rat bone marrow cells (108), insulin secretion of pancreatic β cells (95), the respiration rate of rat mitochondria (116) and ROS production of human neutrophils (117). Next to a role of cyanate on cellular functions also the interaction with carbamylated proteins has an impact on cellular functions. For example, carbamylated BSA increased collagen production of mesangial cells (118-120) and adhesion of monocytes onto carbamylated collagen was significantly enhanced (121).

As a consequence of the effects on protein and cellular functions, carbamylation may also trigger systemic effects. Cyanate is one of the agents which have been used to treat sickle cell anemia patients (122,123). However, neurotoxicity and cataract were observed as side effects. More than half of sickle cell anemia patients who received cyanate treatment developed nerve conduction abnormalities (124). Similar to neurotoxicity, cataract was also reported as a side effect in sickle cell disease patients treated with cyanate (125).

As mentioned above, patients suffering from uremia have increased levels of carbamylation due to high level of urea (126-128). The quantity of carbamylation is also associated with other renal function markers such as creatinine, cystatin C and estimated glomerular filtration rate (129-131). Since the degree of carbamylation is associated with these other markers of renal function, it is not easy to judge the independent contribution of carbamylation to kidney dysfunction. Two recent findings regarding the prognostic value of carbamylated proteins in end stage renal disease (ESRD) may suggest that carbamylation is an independent risk factor in the progression of renal dysfunction (131). ESRD is the last stage of chronic kidney disease (CKD) in which kidney failure is permanent and medical interventions (dialysis or kidney transplantation) are compulsory for patients to survive. Baseline percentage of carbamylated Lys549 on albumin appeared to be the risk factor with the highest hazard ratio for mortality in two independent ESRD cohorts after correcting for all other known risk factors (132). This was recently independently confirmed (131).

Similar to CKD, increased levels of carbamylation are also observed in cardiovascular disease (CVD) which is associated with adverse clinical events. Increased carbamylation levels of plasma protein were found in patients with atherosclerotic CVD and systolic heart failure compared to healthy controls and was associated with developing adverse clinical events of these patients even after correcting other risk factors (88,130). The degree of carbamylation on high density lipoprotein (HDL) in the lesions of atherosclerosis patients is also correlated with the severity of the lesions and MPO mediated oxidative stress (133).

Induction of antibody responses against carbamylated proteins

Post-translationally modified proteins have been described to have the capacity to break immunological tolerance and induce autoantibody responses (134-136). The notion that this can also occur in the setting of carbamylation was initiated by Steinbrecher et al. who reported immunization experiments with carbamylated proteins (137). This hypothesis was also supported by the presence of anti-CarP antibodies as described for animal models previously. Both rabbits (138) and mice (139,140) were shown to be able to develop antibody responses against homocitrulline containing antigens upon immunization with peptides containing homocitrulline or carbamylated proteins. Besides, the presence of antibody reactivity against carbamylated proteins was suggested in a small RA cohort.(140) Following

these previous studies, we aimed to investigate the presence of anti-CarP antibodies in RA, pre-RA stages and JIA patients and whether they have similar diagnostic, predictive and prognostic value in these patients as ACPA and RF.

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