Anti-carbamylated protein antibodies in rheumatoid arthritis

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Discussion

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In this thesis we report the presence of anti-carbamylated protein (anti-CarP) antibodies in a subset of patients suffering from established rheumatoid arthritis (RA) (chapter 2),(1) juvenile idiopathic arthritis (JIA) (chapter 5) (2) or one of the conditions preceding RA, such as undifferentiated arthritis (UA) (chapter 7), arthralgia (chapter 4) (3) or asymptomatic period before the onset of clinical symptoms of RA (chapter 6).(4) The time points when anti-CarP antibodies first appear in asymptomatic individuals are comparable to that of anti-citrullinated protein antibodies (ACPA) but significantly earlier than rheumatoid factor (RF) (chapter 6).(4) In arthralgia patients, the presence of anti-CarP antibodies is independently associated with the future development of RA (chapter 4).(3) In ACPA-negative RA patients, the presence of anti-CarP antibodies is associated with more severe joint damage in the disease course (chapter 2).(1) Overall these studies highlight anti-CarP antibodies as a novel and interesting autoantibody system with potentially important implications in the diagnosis and prognosis of RA. Regarding this series of studies, several issues are worthwhile to be discussed.

The specificity and reproducibility of the anti-CarP antibody ELISAs

Since the anti-CarP antibody ELISAs were the major technique which we applied in chapter 2-7,(1-5) its reproducibility is crucial for the validity of our conclusions. Therefore, we performed several internal control experiments to judge their reproducibility. The intra-assay and inter-assay variability of anti-carbamylated fetal calf serum (anti-Ca-FCS) and anti-carbamylated fibrinogen (anti-Ca-Fib) ELISAs were both around 10% and 15%. In repeated measurements, the chances for negative samples becoming positive and vice versa, were generally around 5% in both anti-Ca-FCS and anti-Ca-Fib ELISA's. The samples of which anti-CarP antibody levels are around the cut-off line have relatively high chance to change their status. These data suggests a certain degree of variation in anti-CarP ELISAs. However, we have also observed that integrating data of repeated measurements of anti-CarP antibody ELISAs increased the effect size of all our reported clinical associations (chapter 2,4).(1,3) This observation suggests that random variation might contribute to a great part of all variation of our assays.

Furthermore, recently we saw independent replications of our two findings (chapter 2, 6). These replications include the presence of anti-CarP antibodies in both ACPA positive and ACPA negative RA patients (6) and their association with joint damage after corrected for the presence of ACPA (chapter 2),(7-10) the presence of anti-CarP antibodies before the appearance of RA symptoms in asymptomatic individuals (chapter 6). With the help of these replications, our major conclusions have already been reproduced in at least two

independent cohorts. Therefore we are convinced that the possibility that our previous published conclusions are chance findings is very low.

When generating the first version of the anti-CarP antibody ELISA we choose to use Ca-FCS as the antigen because FCS contains a wide mix of proteins and is readily available and relatively cheap. Carbamylated human serum is a potentially better source of antigens but the interference of human IgG in the ELISA cannot be easily avoided. Later we identified carbamylated human fibrinogen as another antigen for anti-CarP antibodies, which is our first identified human protein antigen of anti-CarP antibodies.

Since homocitrulline structurally highly resembles citrulline, it is possible that anti-CarP antibodies would be cross-reactive to citrullinated antigens. Here we carefully discussed this possibility with respect to our previous results. When analysing the RA patients from the Leiden early arthritis cohort, we observed that 9.4% of the patients are anti-CarP antibody positive (defined as either anti-Ca-FCS or anti-Ca-Fib antibody positive) and ACPA negative (defined as anti-cyclic citrullinated peptide 2 (CCP2) antibody negative), 9.6% are ACPA positive and anti-CarP antibody negative, 37.3% are double negative and 43.7% are double positive. In this double positive group we observed patients whose anti-CarP antibodies and ACPA totally do not cross-react to the other type of antigens and patients whose anti-CarP antibodies and ACPA are cross-reactive to the other type of antigens to a certain degree (chapter 3).(5) In a small double positive RA cohort, we used a CCP2 peptide bound column to deplete anti-CCP2 antibodies in patients' serum samples. After depletion, the median percentage of remaining anti-Ca-FCS antibodies in these samples is 70% (interquartile range, IQR: 47%-87%) (chapter 3).(5) The remaining anti-Ca-Fib antibody in serum samples is lower (median: 27%, IQR: 18%-58%). This higher cross-reactivity of anti-Ca-Fib antibodies might be explained by the hypothesis that some B cell receptors (BCR) and/or T cell receptors (TCR) are able to recognize both citrullinated and carbamylated epitopes/peptides of fibrinogen.

Anti-CarP antibodies in mice

Even though ACPA are considered to be a highly successful serological marker in RA patients, their importance in mouse arthritis models is far less clear. Appearance of ACPA without immunization with citrullinated antigens was reported in the collagen induced arthritis (CIA) model of DBA1 mice (12-14) but this observation is in debate.(15) A recent study suggested that the appearance of ACPA in CIA mice depends on their genetic background.(14) Appearance of ACPA following immunization of citrullinated antigens was reported in several mouse strains in the presence or the absence of arthritis.(16-18) However, whether ACPA can induce or exacerbate symptoms of arthritis on DBA1 and Balb/c mice is also in debate.(13,17,18)

Following our studies on the presence of anti-CarP antibodies in RA patients, our team has studied the presence and the role of anti-CarP antibodies in arthritis mouse models. Using sera of mice with CIA we observed the presence of anti-CarP antibodies, but not anti-CCP2 antibodies, in the majority of DBA1 and some of the C57BL/6 mice. The onset of CIA was preceded by an increase of anti-CarP antibody levels. This observation is in line with the data that in asymptomatic blood bank donors there is a clear rise in the levels of anti-CarP antibodies prior to the clinical onset of RA (chapter 6).(4)

As only part of the C57BL/6 mice developed CIA in this setting this allowed us to analyse the relationship between the presence of anti-CarP antibodies and clinical manifestation of arthritis. The appearance of anti-CarP antibodies in C57BL/6 mice was not limited to the mice which developed arthritis. This observation is in line with the observation that we can detect anti-CarP antibodies in the healthy population as well as in individuals prior to the onset of clinical symptoms (chapter 4,6).(3,4)

As a control DBA mice were injected with CFA only instead of the combination of CFA and CII and some of them also developed anti-CarP antibodies. However, the speed and magnitude of anti-CarP antibody responses were clearly associated with the presence of arthritis. In addition the presence of anti-CarP antibodies in the mice immunized with CFA only was largely limited to mice with significant tail damage and systemic inflammation as a consequence of the immunization procedure.

The immunization of DBA1 mice with carbamylated ovalbumin and CFA also invoked a strong anti-CarP antibody response which is cross-reactive to other carbamylated antigens. Currently our team is generating mouse monoclonal anti-CarP antibodies to study the role of anti-CarP antibodies in arthritis and to identify more antigens of this auto-antibody system.

In summary, the hypothesis that ACPA/autoantibodies play a key role in the pathogenesis of RA still awaits solid and repeatable animal data. The presence of anti-CarP antibodies may offer an alternative and potentially more powerful tool to study the generation and contribution of autoantibodies in murine models of arthritis.

Autoantibodies in the pathogenesis of RA

Despite many years of intensive research, the pathogenesis of RA remains to be elusive. Generation of ACPA is believed to be a hall mark in the pathogenesis.(19) Genetic predisposing and environmental factors, as elaborated in the introduction, are suggested to contribute to the break of tolerance and the generation of autoantibodies. However, asymptomatic individuals can harbor autoantibodies without developing to RA for many years, which suggests that only the presence of autoantibody is insufficient to trigger the onset

of RA. Currently, it is unclear if a second environmental stimulus is compulsory for the onset of symptoms, since the autoantibody-antigen system may develop, as the recognition of fine-specificities, isotype usage and avidity of autoantibodies increase over time. The symptoms of joints may appear after sufficient amount of modified proteins have accumulated on e.g. matrix molecules. Detailed analysis on the presence of citrullinated and carbamylated proteins in the inflamed joints of mice suffering from CIA and in samples from human RA patients is currently underway. Preliminary data clearly indicated that in both mice and human beings, there are carbamylated proteins in the inflamed joints.(20) After binding to ACPA and/or anti-CarP antibodies, the immune complexes can initiate inflammation via complement activation and/or attraction of leucocytes and ultimately lead to chronic inflammation and bone erosion. Based on this hypothesis, ACPA and anti-CarP antibodies may lead to tissue destruction through the same pathways, both in orchestra with RF. As we have shown in chapter 6,(4) either ACPA or anti-CarP antibodies may appear earlier in asymptomatic blood bank donors who developed to RA later. The epitope-spreading across these two types of auto-antigens may occur no matter which autoantibody system appeared first. Whether or not ACPA and anti-CarP antibody responses are initiated in the joints is not known but unlikely. The presence of anti-CarP antibodies implies that the citrullination is not indispensible in the pathogenesis of autoantibody positive RA. Taking the risk factors, such as smoking and chronic inflammation into account, one could speculate that the lungs and or other sites of chronic inflammation may actually be the places where the tolerance against these post-translationally modified proteins is broken. But the elucidation of these processes awaits experimental proof.

We cannot readily detect autoantibodies in RA patients against glycated, carbonylated and tobacco smoke treated FCS (unpublished data). Interestingly, anti carbamylated low density protein (anti-Ca-LDL) antibodies were found cross-reactive to malondialdehyde (MDA)/malondialdehyde acetaldehyde (MAA) modified and carbonylated LDL in healthy individuals.(21,22) MDA and MAA modified proteins belong to advanced oxidation protein products which occur during lipid peroxidation.(18) MDA and MAA modifications occur on lysine, the same as carbamylation.(18) Previously we have also detected the presence of anti-Ca-LDL antibodies (unpublished data) in RA patients. It would be interesting to study if anti MDA/MAA modified/carbonylated LDL antibodies may appear in RA patients independent of anti-Ca-LDL antibodies.

The future of anti-CarP antibodies

The characteristics of anti-CarP antibodies, in comparison with ACPA and IgM-RF, are listed in table 1. In summary, the specificity and sensitivity of anti-Ca-FCS and anti-Ca-Fib antibodies in RA patients are slightly lower than ACPA and RF-IgM (chapter 7). Similar to ACPA, anti-CarP antibodies have prognostic value in RA patients and predictive value in pre-

RA stage patients (chapter 2,4).(1,3) Recently together with a Japanese group we confirmed the presence of anti-CarP antibodies in the Japanese population (not published), suggesting the presence of anti-CarP antibodies is not limited to the Caucasian population. Currently we are collaborating with INOVA diagnostics to develop the second generation anti-CarP ELISA which may have better sensitivity, specificity and reproducibility compared to the assays we are currently using. If the new generation assay can considerably improve these aspects of anti-CarP ELISA, the anti-CarP antibodies might have the value to be included in the next version of RA classification criteria.

Table 1 Comparison of ACPA, anti-CarP antibodies, RF in our studies

	IgM-RF	ACPA[1]	Anti-CarP antibodies[2]
Sensitivity in RA[3,4]	59%	54%	44%
Specificity in RA[3,4]	91%	96%	89%
Isotype switch[3,5]	yes	yes	yes
Sensitivity in UA[3,6]	28%	25%	30%
Presence in Arthralgia[7]	yes	yes	yes
Presence before the onset[8]	yes	yes	yes
Presence in JIA[9]	yes	yes	yes
Predict joint damage[3,5]	no	yes	yes
Predict arthralgia to RA[4,7]	no	yes	yes
Predict UA to RA[3,6]	yes	yes	no
Predict DMARD free remission[3,5,10]	no	yes	no

[1]defined as anti-CCP2 antibodies
[2]defined as positive for either anti-Ca-FCS or anti-Ca-Fib antibodies
[3]in LUMC early arthritis cohort
[4]established RA patients according to 1987 ACR RA criteria
[5]studied in RA population
[6]defined as UA patients according to 1987 ACR RA criteria in early diagnosis
[7]in arthralgia cohort from Reade institute
[8]in asymptomatic blood donor cohort from Reade institute
[9]in a combination of JIA cohorts as described in the paper "Anti-carbamylated protein (anti-CarP) antibodies are present in sera of Juvenile Idiopathic Arthritis (JIA) patients"
[10]not published

It is known that ACPA positive RA patients respond better to B cell depletion therapy and their level is associated with treatment effect.(19) ACPA positive UA patients, but not ACPA negative patients, respond to disease modifying antirheumatic drugs (DMARDs).(25) Yet, the relationship between the anti-CarP antibody status and the responses to treatments in RA/UA patients has not been studied. Since the occurrence of anti-CarP antibodies is

supposed to be the same as ACPA, they may be associated with the treatment effects of B cell depletion therapy in RA patients and DMARDs in UA patients as well.

ACPA positive RA patients have rather different genetic risk loci compared to ACPA negative RA patients.(26) They are also suspected having different pathogenesis. Although anti-CarP positive and ACPA positive RA patients may have different HLA class II susceptibility, we assume anti-CarP antibodies positive/ACPA negative RA patients should share similar non-HLA genetic risk loci as ACPA positive patients. After excluding anti-CarP antibody positive patients from ACPA negative patients, it may be easier to find out the true genetic risk factors of autoantibody negative RA patients and unravel their pathogenesis.

Conclusions

In conclusion, we discovered the presence of a new autoantibody system in RA and pre-RA stage patients. This new autoantibody system, anti-CarP antibodies, may contribute to the prediction and prognosis of RA. The discovery of anti-CarP antibody also expanded the profile of autoantibody systems in RA, changing the concept that ACPA are unique in RA. The presence of anti-CarP antibodies in CIA mice might also offer a new study tool to investigate the role of autoantibody in the pathogenesis of RA.

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