Pitfalls in the detection of citrullination and carbamylation

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Review

Pitfalls in the detection of citrullination and carbamylation

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ABSTRACT

Carbamylation and citrullination are both post-translational modifications against which (auto)antibodies can be detected in sera of rheumatoid arthritis (RA) patients. Carbamylation is the chemical modification of a lysine into a homocitrulline, whereas citrullination is an enzymatic conversion of an arginine into a citrulline. It is difficult to distinguish between the two resulting amino acids due to similarities in structure. However, differentiation between citrulline and homocitrulline is important to understand the antigens that induce antibody production and to determine which modified antigens are present in target tissues.

We have observed in literature that conclusions are frequently drawn regarding the citrullination or carbamylation of proteins based on reagents that are not able to distinguish between these two modifications. Therefore, we have analyzed a wide spectrum of methods and describe here which method we consider most optimal to distinguish between citrulline and homocitrulline.

We have produced several carbamylated and citrullinated proteins and investigated the specificity of (commercial) antibodies by both ELISA and western blot. Furthermore, detection methods based on chemical modifications, such as the anti-modified citrulline-"Senshu" method and also mass spectrometry were investigated for their capacity to distinguish between carbamylation and citrullination.

We observed that some antibodies are able to distinguish between carbamylation and citrullination, but an overlap in reactivity is often present in the commercially available anti-citrulline antibodies. Finally, we conclude that the use of mass spectrometry is currently essential to differentiate between citrullinated and carbamylated proteins present in complex biological samples.

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Abbreviations: ACPA, anti-citrullinated protein antibodies; AMC, anti-modified citrulline; anti-CarP, anti-carbamylated protein; FCS, fetal calf serum; Fib, fibrinogen; HSA, human serum albumin; KOCN, potassium cyanate; MS, mass spectrometry; PAD, peptidyl arginine deiminase; RA, rheumatoid arthritis; rt, room temperature.

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1. Introduction

The presence of anti-citrullinated protein antibodies (ACPA) is a well-known phenomenon in rheumatoid arthritis (RA). These autoantibodies associate with disease development in RA patients [1,2] and were recently added to the classification criteria for RA [3,4]. ACPA target proteins that have undergone a post-translational modification, citrullination, which is the conversion of an arginine into a citrulline. This is an enzymatic reaction that is facilitated by peptidyl arginine deiminases (PAD) [5]. There are several PAD enzymes with the capacity to citrullinate proteins. These PAD enzymes are often present in the cell, in which they can citrullinate e.g. histones and play a role in gene regulation [6]. Under certain inflammatory conditions, PAD is thought to be released from cells, which may induce local extracellular citrullination [7]. Furthermore, protein citrullination occurs in different physiological processes as evidenced by the presence of several citrullinated proteins in the central nervous system or citrullinated forms of keratin and filaggrin in the skin [8,9].

When compared to ACPA, anti-carbamylated protein antibodies (anti-CarP antibodies) were discovered more recently [10]. Anti-CarP antibodies are increased in RA patients [10–14] and they associate with a more severe disease course as well [10,12]. However, anti-CarP antibodies are a different autoantibody system, with a limited cross-reactivity towards citrullinated respectively carbamylated proteins [15,16]. In human serum samples of RA patients, often both ACPA and anti-CarP antibodies are present. On the other hand, animal models, such as collagen-induced arthritis in mice or primates are characterized by the absence of ACPA, while anti-CarP antibodies can readily be detected [17,32]. Furthermore, anti-CarP antibodies have been detected in non-RA patients as well, although at lower percentages than in RA patients [13,18,19]. Anti-CarP antibodies target proteins that contain homocitrullines, also called carbamylated proteins. Carbamylation is a chemical reaction that is carried out in the presence of cyanate. Both PAD enzymes and cyanate can be increased upon chronic inflammation, which might therefore increase the formation of citrulline and homocitrulline residues. The increase in cyanate during inflammation can be due to the conversion of thiocyanate into cyanate by myeloperoxidase [20]. Furthermore, cyanate can be increased in renal failure, due to its equilibrium with urea [21–23]. Smoking is also thought to increase the presence of cyanate and therefore carbamylation [24].

Although there are similarities between the two autoantibody systems (ACPA and anti-CarP antibodies), they do target two different post-translational modifications. As the name of both residues already implies, the difference between a citrulline and a homocitrulline is small. We therefore often observe situations in which the distinction between a citrulline and a homocitrulline is difficult. However, it is important to distinguish between the two post-translational modifications in order to further investigate the role of ACPA and anti-CarP antibodies separately. Although both antibodies can be detected years before disease onset [2,12], it is at the moment not clear whether the RA autoantibody response is first initiated against a citrulline or a homocitrulline residue. In the future, it may therefore be important to gain a deeper understanding of these modifications, especially when further investigating the possible pathogenesis of RA.

Here, we describe the most specific methods to determine whether a modification is a citrulline or a homocitrulline. We show the that use of polyclonal and monoclonal antibodies for ELISA or western blot, are not always sufficiently specific to make formal conclusions on the nature of the modified proteins. Furthermore, we discuss the use of the chemical "Senshu" modification in different settings and conclude with the pitfalls in detecting citrulline and homocitrulline using mass spectrometry (MS). In the end, we conclude that antibodies or other (chemical) detection methods are suitable to determine whether an in vitro modification was successful, but that MS is required to distinguish between the two amino acids in more complex settings.

2. Description of different methods to measure carbamylation and citrullination

2.1. Antigens and modifications

For our analyses we have used citrullinated and carbamylated versions of three antigens, namely fetal calf serum (FCS, Bodinco), human serum albumin (HSA, Sigma) and human fibrinogen (Fib, Sigma). The non-modified counterparts served as controls. Carbamylation of proteins was carried out by incubating the protein in a final concentration of 1 M potassium cyanate (KOCN, Sigma) diluted in PBS. The protein concentration was between 2 mg/ml and 5 mg/ml, depending on the protein involved. This mixture was incubated overnight at 37 °C, after which the solution was dialyzed against PBS for 48 h at 4 °C. During this time, the PBS was refreshed at least 5 times. Citrullination of HSA, Fib and FCS was carried out by diluting the protein in 0.1 M Tris-HCL, 5 mM DTT and 10 mM CaCl2. For each mg of protein 10 units of PAD (Sigma) were added and the solution was incubated at 53 °C for 6 h. The presence of citrullination and or carbamylation was confirmed by both ELISA and MS.

2.2. Commercially available antibodies

Four commercially available anti-citrulline antibodies and two anti-homocitrulline antibodies were used in these experiments. The anti-citrulline antibodies were mouse monoclonal anti-citrulline antibody, clone F95 (Millipore, MABN328), rabbit polyclonal anti-citrulline antibody (Upstate, 07-377), rabbit polyclonal anti-citrulline antibody (Abcam, ab100932) and rabbit polyclonal anti-citrulline antibody (Abcam, ab6464). The anti-homocitrulline antibodies were goat polyclonal anti-homocitrulline antibody (Abcam, ab175576) and rabbit polyclonal anti-homocitrulline antibody (Cell biolabs, STA-078). As secondary antibodies, to detect binding of the antibodies described above, the following antibodies were used: goat anti-rabbit Ig-HRP (DAKO, P0214), goat anti-mouse Ig-HRP (DAKO, P0447) and rabbit anti-goat Ig-HRP (DAKO, P0449). Monoclonal antibody binding to modified proteins was detected with goat anti-mouse IgG2a-HRP (Southern biotech, 1080-05) or rabbit anti-human IgG-HRP (DAKO, P0214).

2.3. Monoclonal antibodies

The monoclonal antibody targeting anti-CarP antibodies was made previously [16]. Antibody-producing hybridomas were made by fusing

spleen cells of Ca-OVA immunized mice with SP2/0 myeloma cells using PEG1500 (Roche).

The monoclonal anti-citrulline antibody was made previously by culturing single B cells from an ACPA-positive RA patient, enriched via FACS sorting using a citrullinated fibrinogen peptide tetramer staining, for 14 days followed by screening of supernatants for production of antibodies reactive to citrullinated fibrinogen [25].Variable domain sequences were obtained from RACE PCR products, and synthetic DNA constructs cloned into a pcDNA3.1 vector for protein expression in Freestyle HEK293 cells.

2.4. ELISA

Nunc Maxisorp plates (Thermo scientific) were coated with 10 μg/ml of the non-modified or modified antigens and incubated overnight at 4 °C. Plates were blocked with PBS with 1% bovine serum albumin (BSA, Sigma) for at least 6 h at 4 °C, followed by addition of the primary antibody in different concentrations, which were diluted in PBS, 1% BSA and 0.05% Tween20 (Sigma). The primary antibody was incubated overnight at 4 °C. The secondary antibody was incubated for 3.5 h at 4 °C. The presence of antibody-bound HRP was detected with ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)). In between the different steps, plates were washed 3× with PBS 0.05% Tween20.

2.5. Western blot

5 μg of (modified) FCS and 2 μg of (modified) HSA or fibrinogen was loaded on a 4%–15% gel (Bio-Rad) for gel electrophoresis. The Pageruler Plus Prestained protein ladder (Thermo Scientific, 26619) was used as a size comparison for the samples. Protein transfer was carried out using a Bio-Rad system and PVDF transfer packs (Bio-Rad, 170-4156) Membranes were blocked for 1 h with PBS, 3% skim milk powder (Sigma) and 0.05% Tween 20 at room temperature (rt). Primary antibodies were incubated for 2 h at rt and secondary antibodies were incubated for 1 h at rt. Washing steps were carried out with PBS 0.05% Tween20. The HRP signal was developed using ECL (GE healthcare, RPN2109).

2.6. Senshu method

The so-called anti-modified citrulline-"Senshu"-method was carried out as described [15,26]. The initial steps are similar as for the western blotting procedure. However, after protein transfer, the membrane is blocked in 0.1% ovalbumin for 15 min at rt and the membrane is fixed with 4% paraformaldehyde for 15 min at rt. After each of these two steps, milliQ is used for washing. Solution A, consisting of 0.25% FeCl3, 25% H2SO4 and 20% H3PO4 and solution B consisting of 0.5% butanedione monoxime, 0.25% antipyrine and 0.5 M acetic acid are both diluted in milliQ. The solutions are mixed 1:1 and incubated overnight at 37 °C with the membrane, while shaking. The blot is blocked with PBS, 5% skim milk powder and 0.05% Tween20 for 1 h at rt. Human anti-modified citrulline antibody (Modiquest, MQR2.601) is incubated at 1 μg/ml for 3 h at rt. As a secondary antibody, rabbit-anti-human IgG-HRP was used (DAKO, P0214). The "blocking buffer" is also used for the antibody dilutions. Washing steps were carried out with PBS 0.05% Tween20, unless mentioned otherwise. The HRP signal was detected using ECL (enhanced chemiluminescence, GE Healthcare).

3. Results

3.1. All tested commercial anti-citrulline antibodies also recognize carbamylated proteins in ELISA

We tested the reactivity of 6 commercially available antibodies, of which 2 are reported to be specific for homocitrulline and 4 are reported to be specific for citrulline, against three carbamylated, citrullinated or non-modified proteins in ELISA. The antigens used were FCS, HSA and Fib. The antibodies targeting homocitrullines display a binding pattern rather specific for carbamylated proteins, although low reactivity against citrullinated proteins is also observed for one of the antibodies (Fig. 1A).

Out of the 4 anti-citrulline antibodies, all 4 show, next to reactivity against citrullinated proteins, also extensive reactivity against carbamylated proteins (Fig. 1B). Often, the reactivity observed towards the carbamylated version of the protein is even higher than the reactivity towards the citrullinated version of the same protein. Reactivity to the non-modified protein was hardly present in all cases.

3.2. Antibodies can be specific for citrulline or homocitrulline in ELISA

Although we observed that many of the commercial antibodies are not specific for citrulline or homocitrulline, this does not directly implicate that antibodies in general cannot be specific for these post-translational modifications. Previously, we have shown for human antibodies in a polyclonal setting that antibody specificity for either carbamylation or citrullination does exist [15]. Here we also show this specificity for a monoclonal antibody response. We have previously acquired a human ACPA monoclonal antibody and generated a mouse anti-CarP monoclonal antibody, which we also tested in a similar ELISA setting as the commercial antibodies (Fig. 2). The results indicate that the ACPA monoclonal is very specific for citrullinated proteins, while the anti-CarP monoclonal also shows some reactivity towards the citrullinated proteins. This indicates that specificity towards citrulline or homocitrulline does exist, but is dependent on the antibodies that are used.

3.3. Detection of carbamylated and citrullinated proteins in western blot shows similar specificity profiles as in ELISA

Besides ELISA, there are also other methods to detect carbamylated or citrullinated proteins. The anti-citrulline and anti-homocitrulline antibody that seemed to be most specific were selected and used for further experiments in western blot (Fig. 3a–b), using the same antigens as in ELISA. These data confirm that the anti-citrulline antibody is not specific for citrulline alone, while the anti-homocitrulline antibody does not seem to react with the citrullinated proteins.

Combined, these data therefore indicate that the antibodies that are thought to specifically target citrullinated proteins should be used with caution.

3.4. The Senshu method is also not able to distinguish between citrulline and homocitrulline residues

Another method to detect either citrulline or homocitrulline is the anti-modified citrulline (AMC)-Senshu method [26]. This method was initially used for the detection of citrullinated proteins. But more recent studies have indicated that next to citrulline also homocitrulline is modified in exactly the same way [15,27]. This method is based on the chemical modification of citrulline and homocitrulline residues, after which this additional chemical modification can be recognized by antibodies. This is also the case for the proteins that we modified (Fig. 3c), indicating that this method is indeed not specific. The chemical modification used for the Senshu method can also be used for 96 well-plate assays or immunohistochemistry staining (data not shown) [28].

3.5. Mass spectrometry

Methods to detect post-translational modifications with MS were described previously [16,29]. Distinguishing between a citrulline and a homocitrulline residue is straightforward because both residues are present at a different position within the protein (they are derived from either an arginine or a citrulline). Furthermore, homocitrulline differs from citrulline by 14 Da. We have recently used this approach for

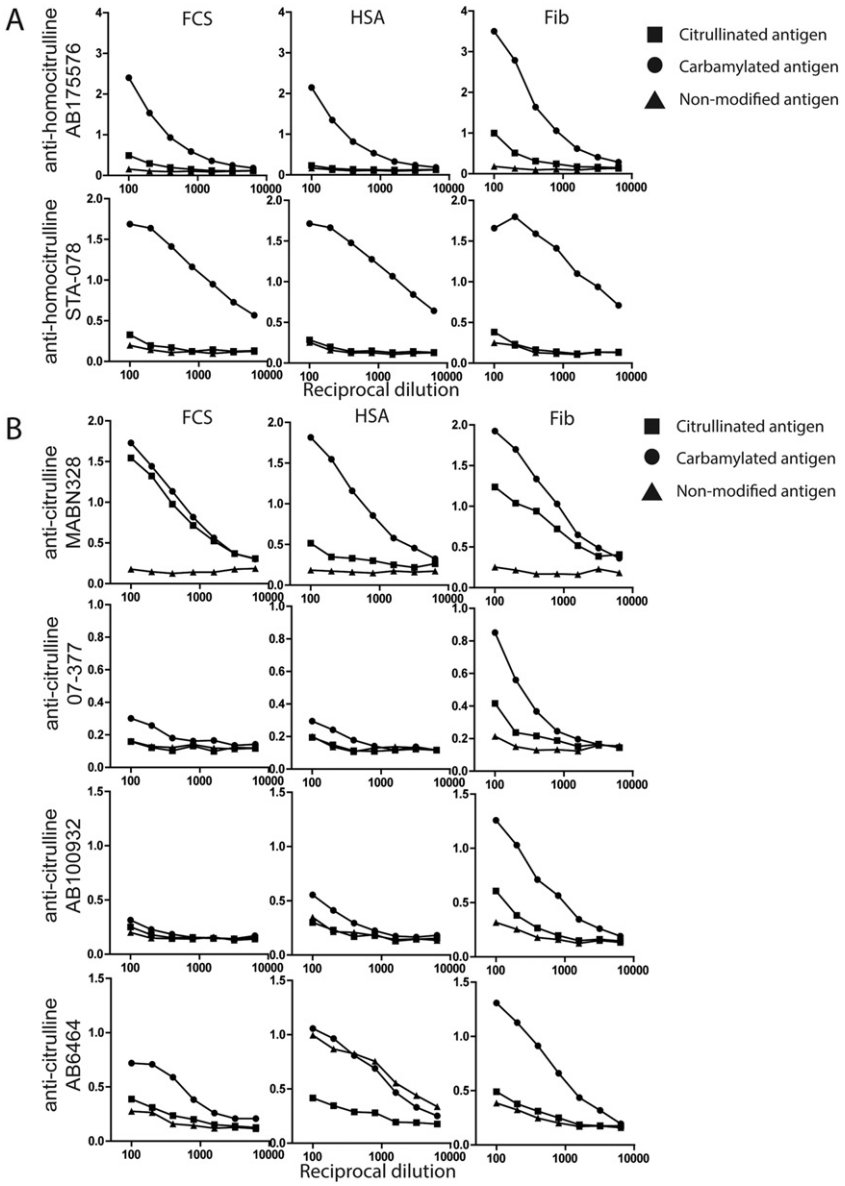

Fig. 1. Reactivity of commercial antibodies against three carbamylated and citrullinated protein. Antibody reactivity of 2 anti-homocitrulline antibodies (A) and 4 anti-citrulline antibodies (B) against carbamylated (circle), citrullinated (square) and non-modified (triangle) proteins was determined by ELISA. The numbers on the Y-axis represent the absorbance values measured at 415 nm, while the x-axis shows reciprocal dilution. FCS: fetal calf serum, HSA: human serum albumin, Fib: fibrinogen.

the identification of carbamylated alpha-1 antitrypsin and albumin in human samples [16,29]. However, confident determination of citrullination (ΔMCit-Arg = +1 Da), is prone to false positive assignments using automated standard proteomic workflows. A number of complicating factors (of which some may also apply for the identification of homocitrulline residues) are briefly discussed below.

Citrullination is a sub stoichiometric post-translational modification. Therefore, low abundant peptides have to be analyzed in a full proteome background. Because of their low abundance the spectral quality can be poor. In addition, the low abundance also leads to more interferences during precursor ion isolation, i.e. co-isolation of other species, which leads to mixed or contaminated MS/MS spectra that are more difficult to interpret (and more prone to wrong assignments). An extra complication is that the deamidation of an asparagine residue (Δm = +1 Da) is rather common and has the same mass change. Therefore, deamidation has to be included as a variable modification during the matching of the recorded tandem mass spectra with the protein database. If deamidation is not included the software can wrongly fit the tandem mass spectrum to include e.g. a citrulline residue at an arginine position. However, the inclusion of two variable modifications, both leading to a +1 Da mass shift, is also a further complication. A citrulline may also be assigned wrongly at an arginine position due to inadvertent calling and selection of the second isotope of the precursor isotope pattern as the precursor mass. Finally, conversion of an arginine into a citrulline makes that particular site insensitive to trypsin cleavage, resulting in longer peptides, which generally yield less complete fragment ion series as well. Of note, C-terminally citrullinated tryptic peptides (protein C-terminal residue excluded), regularly 'identified' by the database matching process, are a clear illustration of false positive assignments.

In spite of the above, citrulline residues can be confidently assigned if performed with great caution from the tandem mass spectra, e.g. as performed before [30] in which citrullinated fibrinogen peptides in SF were identified. Using a similar approach we identified the same citrullinated peptides (data not shown). The best solution to correct assignment of tandem mass spectra is the comparison of the tentatively identified sequences and their synthetic counterparts. It unequivocally establishes the correctness of the tentatively assigned peptide sequence.

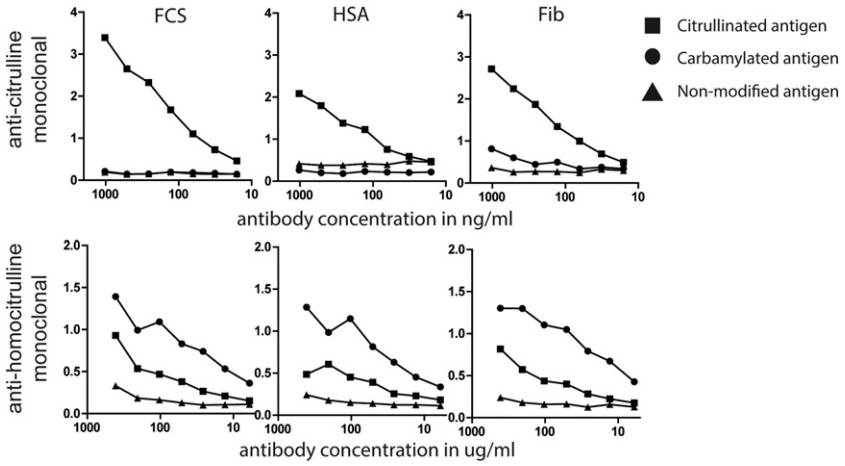

Fig. 2. Reactivity of two monoclonal antibodies against three carbamylated and citrullinated proteins. Antibody reactivity of one human anti-citrulline monoclonal and one mouse anti-homocitrulline monoclonal against carbamylated (circle), citrullinated (square) and non-modified (triangle) proteins was determined by ELISA. The numbers on the x-axis represent the concentration of the monoclonal antibody. The numbers on the Y-axis represent the absorbance values measured at 415 nm. FCS: fetal calf serum, HSA: human serum albumin, Fib: fibrinogen.

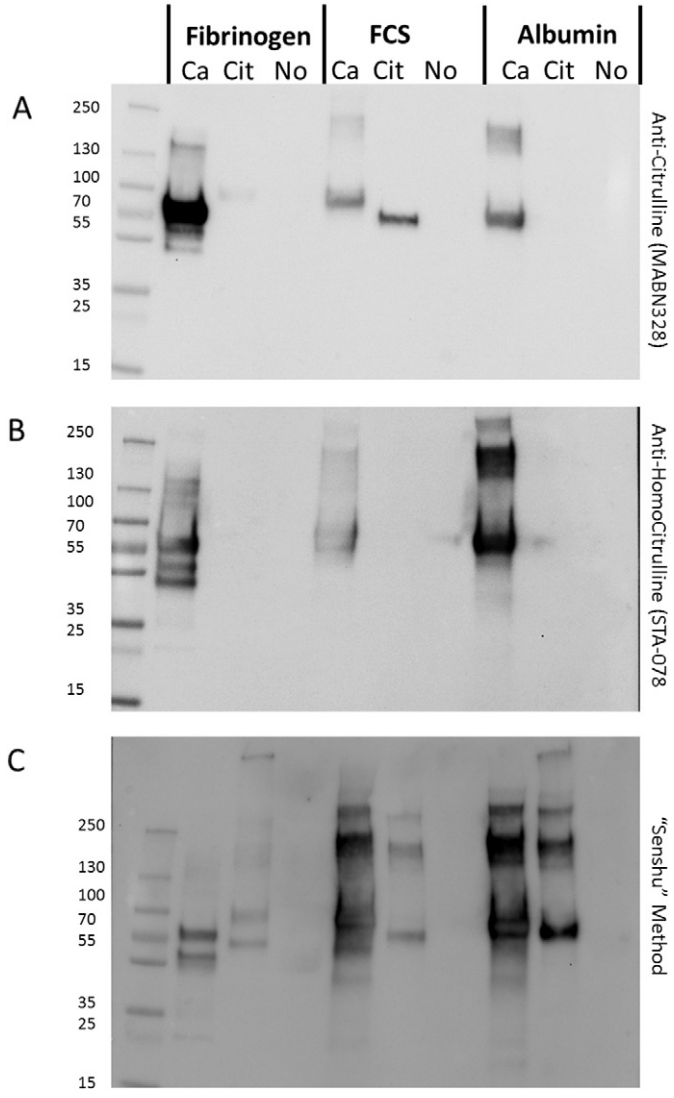

Fig. 3. Reactivity of two commercially available antibodies in western blot and a senshu on the same proteins. Antibody reactivity of anti-citrulline antibody (Millipore MABN328) (A) and anti-homocitrulline antibody (Cell biolabs, STA-078) (B) against carbamylated, citrullinated and non-modified proteins was determined by western blot. The same proteins were also used to carry out a Senshu, a chemical detection method for carbamylation and citrullination (C).

In full proteomics experiments, a high number of identifications might prevent a one-by-one comparison strategy because of costs involved. However, if investments are made in follow-up work, checking the correct identifications with synthetic peptides is strongly recommended.

4. Discussion

Here we describe that many of the commercially available antibodies are often not suitable for the distinction between carbamylation and citrullination in both ELISA and western blot. Especially the tested anti-citrulline antibodies, recognize both citrulline and homocitrulline. Changing experimental conditions, such as buffers and incubation times, might improve the specificity of the antibodies. However, most other protocols that we applied for some of the anti-citrulline antibodies resulted in a reduced specificity and/or sensitivity (data not shown). Changes in experimental set-up may however influence the observed specificity of these antibodies. We also show that the use of a monoclonal antibody can result in increased specificity, indicating that antibodies can be more specific for citrulline. Furthermore, the commercial antibodies targeting carbamylation seemed to be more specific than the commercial anti-citrulline antibodies. For all of the tested polyclonal antibodies, a small molecule or peptide, sometimes coupled to KLH was used for animal immunization. Often, affinity purification was carried out (not for AB100932 and AB6464) as well, but cross-reactivity still seems to be present, especially for the anti-citrulline antibodies. Using stricter protocols and negative selection for the unwanted reactivity may result in improved specificity. However, it cannot be excluded that these antibodies recognize other, (non-)modified proteins when applied to a complex mixture of proteins. Therefore the use of these antibodies to identify the presence of one of these two modifications in complex biological samples is in our opinion very limited. Determination of the success of an in vitro modification, such as carbamylation or citrullination on the other hand, would be a very useful application for which these antibodies can be used, when the non-modified protein is taken along as a negative control.

The use of chemical modifications, such as the Senshu method was shown before to be insensitive for the distinction between citrulline and homocitrulline [15,27]. Here we confirm these data and show that although this method is very efficient in identification of the post-translational modifications, discrimination is not possible. Another method, using a rhodamine-phenylglyoxal probe also recognizes both citrulline and homocitrulline residues [31].

Finally, we have discussed that MS is a very effective method to identify the presence of a citrulline or a homocitrulline. However,

identification of citrullination requires additional attention to avoid false-positive assignments.

Even though the detection of carbamylation or citrullination with antibodies or chemical methods may not be most optimal, these methods have often been used in the past, when the focus in the RA-field was more on ACPA and not yet on anti-CarP antibodies. The findings that most of the methods used here are also able to detect carbamylation do not completely invalidate these previous studies. However, it is important to retrospectively evaluate these studies which may result in a different view on the relative role of citrullinated versus carbamylated proteins in health and disease.

5. Conclusion

To summarize, the use of commercial antibodies or chemical modification at this point in time, is not optimal to distinguish between carbamylation and citrullination. MS on the other hand, is a very effective method for making this distinction, at least when all pitfalls are avoided successfully.

Take-home messages

- Many commercially available antibodies do not distinguish between citrulline and homocitrulline.
- Chemical modifications such as the “Senshu method”, do not distinguish between citrulline and homocitrulline.
- Mass spectrometry is the best method to detect the presence of citrullination and carbamylation.

Conflict of interest

PAvV, REM and LAT are listed as inventors in a patent regarding the detection of anti-CarP antibodies for RA. The other authors declare that they do not have a conflict of interest.

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References

[1] Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 2003;48:2741–9.
[2] Willemze A, Trouw LA, Toes RE, Huizinga TW. The influence of ACPA status and characteristics on the course of RA. Nat Rev Rheumatol 2012;8:144–52.
[3] Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham 3rd CO, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis 2010;69:1580–8.
[4] Damoiseaux J, Andrade LE, Fritzler MJ, Shoenfeld Y. Autoantibodies 2015: from diagnostic biomarkers toward prediction, prognosis and prevention. Autoimmun Rev 2015;14:555–63.
[5] Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays 2003;25:1106–18.
[6] Christophorou MA, Castelo-Branco G, Halley-Stott RP, Oliveira CS, Loos R, Radzisheuskaya A, et al. Citrullination regulates pluripotency and histone H1 binding to chromatin. Nature 2014;507:104–8.
[7] Romero V, Fert-Bober J, Nigrovic PA, Darrah E, Haque UJ, Lee DM, et al. Immune-mediated pore-forming pathways induce cellular hypercitrullination and generate citrullinated autoantigens in rheumatoid arthritis. Sci Transl Med 2013;5:209ra150.
[8] Bradford CM, Ramos I, Cross AK, Haddock G, McQuaid S, Nicholas AP, et al. Localisation of citrullinated proteins in normal appearing white matter and lesions in the central nervous system in multiple sclerosis. J Neuroimmunol 2014;273:85–95.
[9] Rogers G, Winter B, McLaughlan C, Powell B, Nesci T. Peptidylarginine deiminase of the hair follicle: characterization, localization, and function in keratinizing tissues. J Invest Dermatol 1997;108:700–7.
[10] Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A 2011;108:17372–7.
[11] Jiang X, Trouw LA, van Wesemael TJ, Shi J, Bengtsson C, Kallberg H, et al. Anti-CarP antibodies in two large cohorts of patients with rheumatoid arthritis and their relationship to genetic risk factors, cigarette smoking and other autoantibodies. Ann Rheum Dis 2014;73:1761–8.
[12] Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther 2015;17:25.
[13] Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, McCormick JK, et al. Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. J Rheumatol 2014;41:270–9.
[14] Turunen S, Hannonen P, Koivula MK, Risteli L, Risteli J. Separate and overlapping specificities in rheumatoid arthritis antibodies binding to citrulline- and homocitrulline-containing peptides related to type I and II collagen telopeptides. Arthritis Res Ther 2015;17:2.
[15] Shi J, Willemze A, Janssen GM, van Veelen PA, Drijfhout JW, Cerami A, et al. Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the ‘AMC-Senshu’ method. Ann Rheum Dis 2013;72:148–50.
[16] Dekkers JS, Verheul MK, Stoop JN, Liu B, Ioan-Facsinay A, van Veelen PA, et al. Breach of autoreactive B cell tolerance by post-translationally modified proteins. Ann Rheum Dis 2017;76:1449–57.
[17] Stoop JN, Liu BS, Shi J, Jansen DT, Hegen M, Huizinga TW, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One 2014;9:e102163.
[18] Shi J, van Steenbergen HW, van Nies JA, Levarht EW, Huizinga TW, van der Helm-van Mil AH, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther 2015;17:339.
[19] Chimenti MS, Triggianese P, Nuccetelli M, Terracciano C, Crisanti A, Guarino MD, et al. Auto-reactions, autoimmunity and psoriatic arthritis. Autoimmun Rev 2015;14:1142–6.
[20] Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, et al. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. Nat Med 2007;13:1176–84.
[21] Hagel P, Gerding JJ, Fieggen W, Bloemendal H. Cyanate formation in solutions of urea. I. Calculation of cyanate concentrations at different temperature and pH. Biochim Biophys Acta 1971;243:366–73.
[22] Shi J, van Veelen PA, Mahler M, Janssen GM, Drijfhout JW, Huizinga TW, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev 2014;13:225–30.
[23] Kalim S, Karumanchi SA, Thadhani RI, Berg AH. Protein carbamylation in kidney disease: pathogenesis and clinical implications. Am J Kidney Dis 2014;64:793–803.
[24] Roberts JM, Veres PR, Cochran AK, Warneke C, Burling IR, Yokelson RJ, et al. Isocyanic acid in the atmosphere and its possible link to smoke-related health effects. Proc Natl Acad Sci U S A 2011;108:8966–71.
[25] van de Stadt LA, van Schouwenburg PA, Bryde S, Kruithof S, van Schaardenburg D, Hamann D, et al. Monoclonal anti-citrullinated protein antibodies selected on citrullinated fibrinogen have distinct targets with different cross-reactivity patterns. Rheumatology (Oxford) 2013;52:631–5.
[26] Senshu T, Sato T, Inoue T, Akiyama K, Asaga H. Detection of citrulline residues in deiminated proteins on polyvinylidene difluoride membrane. Anal Biochem 1992;203:94–100.
[27] Turunen S, Koivula MK, Risteli L, Risteli J. Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. Arthritis Rheum 2010;62:3345–52.
[28] Knipp M, Vasak M. A colorimetric 96-well microtiter plate assay for the determination of enzymatically formed citrulline. Anal Biochem 2000;286:257–64.
[29] Verheul MK, Yee A, Seaman A, Janssen GM, van Veelen PA, Drijfhout JW, et al. Identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis. J Autoimmun 2017;80:77–84.
[30] Raijmakers R, van Beers JJ, El-Azzouny M, Visser NF, Bozic B, Pruijn GJ, et al. Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. Arthritis Res Ther 2012;14:R114.
[31] Bicker KL, Subramanian V, Chumanevich AA, Hofseth LJ, Thompson PR. Seeing citrulline: development of a phenylglyoxal-based probe to visualize protein citrullination. J Am Chem Soc 2012;134:17015–8.
[32] Verheul MK, Vierboom MPM, 't Hart BA, Toes REM, Trouw LA. Anti-carbamylated protein antibodies precede disease onset in monkeys with collagen-induced arthritis. Arthritis Res Ther 2017;19(1):246.