



National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

**Is protein citrullination by nanomaterials
a risk factor for inducing autoimmune
reactions?**

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R.J. Vandebriel



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Rapport in het kort

Sinds 2012 is bekend dat eiwitten in cellen of proefdieren op een bepaalde manier veranderen als zij aan nanodeeltjes worden blootgesteld. Dit proces heet citrullinerig. Onder bepaalde omstandigheden ontstaan antilichamen die gericht zijn tegen deze veranderde eiwitten. Omdat deze antilichamen zijn aangetroffen bij reumapatiënten wordt aangenomen dat er een verband bestaat tussen citrullinerig van eiwitten, de ontstane antistoffen en processen die leiden tot auto-immuunziekten zoals reuma. Vanuit die veronderstelling wordt gespeculeerd dat blootstelling aan nanomaterialen een risicofactor kan zijn voor het ontstaan van auto-immuunziekten zoals reuma. In een literatuuronderzoek door het RIVM is dit verband echter niet gevonden.

Er is namelijk nog nooit via metingen aangetoond dat antistoffen tegen gecitrullineerde eiwitten ontstaan na blootstelling aan nanomaterialen. Verder is alléén de aanwezigheid van antistoffen tegen gecitrullineerde eiwitten onvoldoende om artritis in proefdieren op te wekken. Dit literatuuronderzoek levert dus geen direct causaal verband tussen blootstelling aan nanomaterialen en reuma.

Om meer inzicht in deze complexe processen te krijgen worden enkele vervolgonderzoeken aanbevolen. Ten eerste gaat het daarbij om het meten van antistoffen tegen gecitrullineerde eiwitten in proefdieren die aan nanomaterialen zijn blootgesteld. Ten tweede is het van belang te onderzoeken welk effect nanomaterialen in een proefdiermodel voor reuma hebben op de ontwikkeling van gewrichtsontsteking. Wordt de artritis dan erger, treedt het eerder op of zijn er meer dieren die het krijgen?

Trefwoorden citrullinerig reuma nanodeeltje

Abstract

Since 2012 it is known that proteins in cells or laboratory animals change in a specific way if they are exposed to nanoparticles. This process is called citrullination. Under certain circumstances, antibodies directed against these altered proteins develop. Since these antibodies have been found in patients with rheumatoid arthritis (RA) it is assumed that there is a correlation between citrullination of proteins, the resulting antibodies, and processes that lead to auto-immune diseases such as RA. From this assumption it is speculated that exposure to nanomaterials is a risk factor for the development of autoimmune diseases such as RA. In a literature review by the RIVM, this link has however not been found.

Indeed, antibodies against citrullinated proteins have never been measured after exposure to nanomaterials. In addition, the mere presence of antibodies against citrullinated proteins is insufficient to induce arthritis in laboratory animals. Thus, these studies do not provide a direct causal link between exposure to nanomaterials and RA.

To gain more insight into these complex processes, some follow-up studies are recommended. Firstly, this involves the measurement of antibodies against citrullinated proteins in laboratory animals exposed to nanomaterials. Secondly, it is of importance to investigate the effect of the nano-materials in an animal model for RA on the development of joint inflammation. Is arthritis aggravated, does it develop earlier or are more animals affected?

Key words citrullination rheumatoid arthritis nanoparticles

Contents

Contents	5
Summary	6
1	Introduction 7
2	Protein citrullination 8
3	Anti-citrullinated protein antibodies 9
4	Protein citrullination and anti-citrullinated protein antibodies in animal models for arthritis 10
5	Role of autophagy in citrullination by nanomaterials 12
6	Discussion, conclusions, and suggestions for further research 13
6.1	Discussion and conclusions 13
6.2	Citrullination/PAD activity 13
6.3	ACPA 13
7	Acknowledgements 15
8	References 16

Summary

Exposure to engineered nanomaterials *in vitro* and *in vivo* has been shown to induce protein citrullination. Protein citrullination and the resulting appearance of anti-citrullinated protein antibodies (ACPA) are thought to be involved in processes leading to autoimmune diseases such as rheumatoid arthritis (RA). It was therefore speculated that exposure to engineered nanomaterials may be a risk factor for autoimmune diseases such as RA. If this were the case, there are potentially major consequences for hazard and risk assessment. We therefore performed a literature review with the aim of charting existing evidence for a causal relationship between nanomaterial exposure, protein citrullination, induction of ACPA, and autoimmune diseases.

Citrullination is a post-transcriptional modification of the amino acid arginine to citrullin, with a concomitant charge loss. Citrullination is performed by the calcium-dependent enzyme "protein arginine deiminase" (PAD). Citrullination is a normal physiological process that occurs inside dying cells; these are usually ingested by macrophages. Thus, the immune system does not normally encounter citrullinated proteins. In situations of inadequate clearance, PAD and citrullinated proteins may leak from dying cells and be recognized by the immune system.

Citrullinated proteins can induce ACPA. Citrullinated fibrin (CF) is abundant in the joints of RA patients; immune complexes consisting of ACPA and CF are formed locally. These immune complexes can trigger macrophages to locally produce TNF- α , a key pro-inflammatory cytokine in RA, thereby aggravating disease. Moreover, ACPA are the serological hallmark of RA. Although these findings strongly suggest a role for ACPA in RA, their precise role in the disease process is still unknown. ACPA may play a causal role in disease onset or they may only be a proxy for the underlying process.

In experimental arthritis models the role of citrullinated proteins in induction or aggravation of disease is still inconclusive. It is likely that due to endogenous PAD a certain background level of citrullination is present. ACPA alone is insufficient for arthritis induction but they may play a role in aggravating disease.

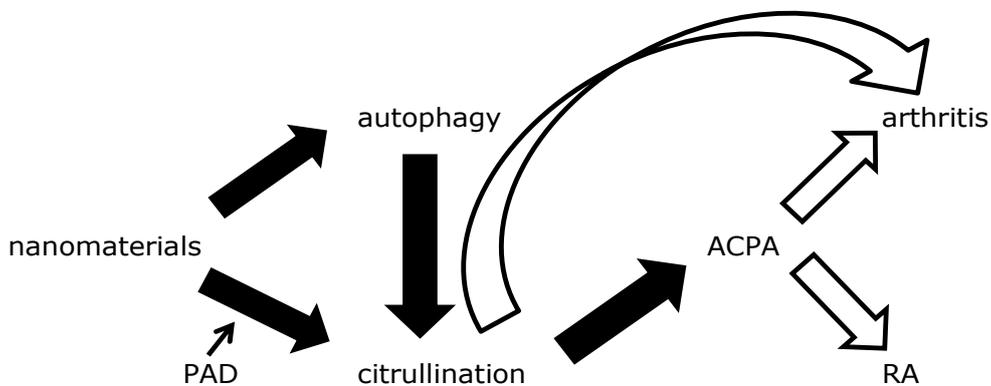
Autophagy plays an important role in citrullination. A wide range of nanomaterials has been shown to affect autophagy, suggesting that citrullination after nanomaterial exposure may be due (in part) to autophagy. It should be noted that autophagy is affected by various endogenous and exogenous stimuli and various diseases, suggesting that citrullination is a rather unspecific parameter to assess toxic effects of nanomaterials.

To gain more insight into these complex processes, some follow-up studies are recommended. Firstly, this involves the measurement of antibodies against citrullinated proteins in laboratory animals exposed to nanomaterials. Secondly, it is of importance to investigate the effect of the nano-materials in an animal model for RA on the development of joint inflammation. Is arthritis aggravated, does it develop earlier or are more animals affected? Additional studies that are recommended include establishing the role of autophagy in citrullination by nanomaterials, and establishing the role of reduced phagocytosis due to nanomaterial exposure in the induction of ACPA.

1 Introduction

In 2012 and 2013 the group of Prof Volkov showed that in vitro and in vivo exposure to engineered nanomaterials induced protein citrullination (Mohamed et al., 2012,2013). Protein citrullination and the resulting appearance of anti-citrullinated protein antibodies (ACPA) are thought to be involved in processes leading to autoimmune diseases such as rheumatoid arthritis (RA). It was therefore speculated by the authors that exposure to engineered nanomaterials may be a risk factor for autoimmune diseases such as RA. If this were the case, there are potentially major consequences for hazard and risk assessment of engineered nanomaterials. We therefore performed a literature review with the aim of charting existing evidence for a causal relationship between nanomaterial exposure, protein citrullination, induction of ACPA, and autoimmune diseases. Knowledge gaps and suggestions for possible follow-up studies are presented. Figure 1 shows the interrelationship between the items mentioned in this report.

Figure 1. Interrelationship between the items mentioned in this report. Closed arrow: causal relationship identified; open arrow: causal relationship not identified.



2 Protein citrullination

Citrullination is a post-transcriptional modification of the amino acid arginine to citrullin, with a concomitant charge loss (reviewed by Bicker & Thompson, 2013; Willemze et al., 2012; van Venrooij et al., 2011). Citrullination is performed by the calcium (Ca^{++})-dependent enzyme "protein arginine deiminase" (PAD). This enzyme is inactive at normal intracellular [Ca^{++}] concentrations; during programmed cell death (apoptosis) a rise in [Ca^{++}] concentration causes PAD activation. In addition, PAD may be released from the dying cell and become activated due to the higher extracellular [Ca^{++}] concentration.

Citrullination is a normal physiological process that occurs inside many dying cells of the body; dying cells are usually ingested by macrophages and other cells active in clearance. Thus, the immune system does not normally encounter citrullinated proteins. In situations of inadequate clearance, PAD and citrullinated proteins may leak from the dying cells and be recognized by the immune system. The proteins citrullinated by PAD are, amongst others, fibrin, collagen, vimentin, α -enolase, and EBNA-1.

3 Anti-citrullinated protein antibodies

Citrullinated peptides or proteins can induce anti-citrullinated protein antibodies (ACPA). In the joints of RA patients citrullinated fibrin (CF) is abundant and immune complexes consisting of ACPA and CF are formed locally. ACPA levels are elevated in synovial fluid. In ACPA-positive patients these CF-containing immune complexes circulate. ACPA can activate complement, while CF-containing immune complexes can trigger through Fc γ R2 binding human macrophages to locally produce TNF- α , a key pro-inflammatory cytokine in RA thereby aggravating disease (Clavel et al., 2008). Moreover, IgE ACPA and mast cells play a role in RA pathogenesis (Schuerwegh et al., 2010).

ACPA are the serological hallmark of RA; since 2010 they are included in the classification criteria for RA. Importantly, because of their strong association with RA and their presence years before onset of the disease, ACPA are suspect of causing or at least participating in the disease.

RA patients can be sub grouped according to their ACPA levels, with differing genetic and environmental risk factors. ACPA-positive RA patients, that amount to 50-70% of all RA patients, experience a more persistent and destructive disease. Therapeutic effects of DMARDs, such as of methotrexate and rituximab, also differ between the two subgroups.

These findings strongly suggest a role for ACPA in RA. Their precise role in the disease process is, however, still unknown; ACPA may play a causal role in disease onset or they may only be a proxy for the underlying process.

Experimental arthritis models have been used in order to establish whether ACPA play a causal role in arthritis development and severity (see below).

4 Protein citrullination and anti-citrullinated protein antibodies in animal models for arthritis

The animal model for RA that is used most widely is the collagen-induced arthritis model. In this model, arthritis is induced by a combination of collagen type II (CII) and Freund's Complete Adjuvant (Bevaart et al., 2010). Another model for RA that is used regularly is the rat adjuvant arthritis model (Waksman, 2002).

Hill et al. (2008) reported that arthritis was induced with citrullinated but not native fibrinogen in DR4-IE transgenic mice (DR4 is RA-associated HLA class II). In rather contrast, Ho et al. (2010) observed that arthritis could be induced with native fibrinogen.

Arthritis was induced in mice using citrullinated but not native CII (Thiele et al., 2012). Importantly, in this study arthritis was induced without the use of adjuvant. In an earlier study, citrullinated CII resulted in a higher incidence and earlier arthritis onset compared to native CII (Lundberg et al., 2005). Here, clinical signs preceded the presence of citrullinated proteins and PAD4. Possibly, detection of citrullination and PAD4 positivity are less sensitive parameters than arthritis, explaining their later appearance.

ACPA can develop upon immunization with native CII; they enhanced tissue injury in CII-induced arthritis (Kuhn et al., 2006; Uysal et al., 2009). It should be noted that native proteins can be citrullinated in vivo by mouse PADs; blocking endogenous PAD reduced the severity of CII-induced arthritis and the level of anti-CII antibodies (Willis et al., 2011). Transfer of a mouse T-cell line specific for citrullinated fibrinogen to mice with CII-induced arthritis, aggravated disease and resulted in increased pathogenic autoantibody levels to mouse CII (Cordova et al., 2013). Duplan et al. (2006), on the other hand, reported that ACPA raised by citrullinated fibrinogen did not modulate adjuvant arthritis in rats. Importantly, ACPA alone are insufficient to induce disease in rodents (Rubin & Sonderstrup, 2004; Duplan et al., 2006; Cantaert et al., 2013). Immunization with citrullinated fibrinogen induced ACPA but not arthritis (Cantaert et al., 2013). Moreover, citrullinated CII did not modulate the course of disease compared to native CII (Cantaert et al., 2013).

In conclusion, the role of citrullinated proteins in arthritis induction or aggravation is still inconclusive. It is likely that due to endogenous PAD a certain background level of citrullination is present and this may depend on the mouse strain and the protein that is citrullinated. ACPA alone is insufficient for arthritis induction but they may play a role in aggravating disease.

The inconclusive role of citrullinated proteins and especially ACPA in arthritis does not, however, preclude a study of effects of nanomaterial exposure on arthritis. The model used by Thiele et al. (2012) seems to be the model of choice since (1) it does not require adjuvant to induce arthritis; therefore this model better reflects disease development in humans than other models which require the use of adjuvant, and (2) arthritis is induced by citrullinated but not native CII suggesting that in this model citrullination plays a causative role in arthritis development. After nanomaterial exposure, not only arthritis and citrullination but also ACPA levels should be measured. In Mohamed et al. (2012) citrullination was shown in vivo 7 days, 28 days, 2 months, and 6 months after single pharyngeal aspiration of 40 µg SWCNT per mouse. In humans, at an early stage of developing RA antibody titers are low and peptide reactivity is limited, while towards disease onset, antibody titers and peptide reactivity rise. For this reason, the time period for ACPA to be present may be

significant. Next to single MWSCNT exposure, ACPA development upon repeated exposure should also be investigated. Moreover, different dosages and other nanomaterials besides MWCNT should be tested.

5 Role of autophagy in citrullination by nanomaterials

In an important study, Ireland et al. (2006) showed that immunization of hen egg-white lysozyme (HEL) transgenic mice with HEL resulted in presentation of citrullinated as well as native HEL peptides. Moreover, T-cells specific for citrullinated HEL peptides could be identified. Dendritic cells and macrophages presented both citrullinated and native peptides; both cell types harbour a constitutive level of autophagy. Blocking autophagy blocked the presentation of citrullinated peptides but left the presentation of native peptides intact (Ireland & Unanue, 2011). B-cells (a third type of antigen-presenting cell) do not harbour constitutive autophagy but this activity can be induced by B-cell receptor (BCR) engagement. Only after BCR engagement did B-cells present citrullinated peptides. Thus, autophagy plays an important role in citrullination. A wide range of nanomaterials has been shown to affect autophagy (Stern et al., 2012). First, this may suggest that the observations by Mohamed et al. (2012,2013) may be due (in part) to autophagy. This was not studied or discussed by the authors. Second, it should be noted that autophagy is influenced by various endogenous and exogenous stimuli and various diseases (Ravikumar et al., 2010). This may suggest that citrullination is a rather unspecific parameter to assess toxic effects of nanomaterials.

6 Discussion, conclusions, and suggestions for further research

6.1 Discussion and conclusions

The studies by Mohamed et al. (2012,2013) have shown that nanomaterial exposure results in protein citrullination. Since citrullinated proteins induce ACPA and these antibodies may play a role in the development of RA the authors speculated that exposure to engineered nanomaterials may be a risk factor for autoimmune diseases such as RA. If this were the case, there are potentially major consequences for hazard and risk assessment of engineered nanomaterials.

Autoimmunity is not routinely tested in hazard and risk assessment of chemicals and safety assessment of drugs. Still, several chemicals and drugs have been shown to be able to induce this type of adverse effect (IPCS, 2006; Putman et al., 2003).

According to the OECD, their test guidelines are suitable for safety testing of nanomaterials. The OECD has produced guidance documents which specifically address the dosing of nanomaterials (1,2). The OECD is active in preparatory steps towards safety testing for nanomaterials (3).

For nanomaterials it is currently not clear which toxic endpoints should be tested in addition to (or possibly (in part) instead of) the ones set in OECD guidelines and EU legislation. In an animal model that is generally accepted as being predictive for human asthma, TiO₂ and SiO₂ nanoparticles have been shown to exert adjuvant activity, meaning that these nanoparticles are able to induce or aggravate allergic (asthmatic) reactions. These findings point towards a potential hazard, and it should be put on the agenda whether this endpoint should be included for predictive testing. In case of protein citrullination by nanomaterials we are not at that point yet, as the adverse effect (autoimmunity) resulting from citrullination has not been established yet (and is the topic of the present report). Both allergy and autoimmunity present serious and irreversible health risks, especially for sensitive individuals. It is therefore important to much better understand the consequences of protein citrullination for autoimmunity and possible autoimmune diseases.

SiO₂, SWCNT, and carbon black (CB) nanoparticles have been shown to induce citrullination (Mohamed et al., 2012,2013). SiO₂ exposure may result from nutrition, while SWCNT and CB exposure often occurs at the workplace. encountered by occupational exposure. The domains of nutrition and workplace are important for the Netherlands Food and Consumer Product Safety Authority.

6.2 Citrullination/PAD activity

From the studies described in this report it may be suggested that increased [Ca⁺⁺], either due to apoptosis in general or more specifically after nanomaterial exposure, induce or increase PAD activity resulting in protein citrullination.

The role of autophagy in citrullination by nanomaterials has not been investigated up to now. This mechanism may be investigated e.g. by blocking autophagy in in vitro and in vivo models of nanomaterial-induced citrullination.

6.3 ACPA

A mechanism resulting in ACPA may be reduced phagocytic activity of macrophages. In situations of excessive apoptosis this may result in a spill over

of apoptotic cells, priming the immune system for citrullinated proteins. To our knowledge, such a mechanism has not been described after nanomaterial exposure.

Therefore, a role for reduced phagocytosis due to nanomaterial exposure in the induction of ACPA may be experimentally addressed. It is likely that this can only be investigated in vivo.

Whether ACPA influence the incidence, time of onset, and severity in experimental arthritis is subject of debate. The arthritis model recently developed by Thiele et al. (2012) has an important advantage over traditional arthritis models that it does not employ adjuvant, making it more reminiscent of human disease (RA). In this model citrullinated but not native CII induced arthritis, suggesting that citrullination in fact plays a causative role in arthritis.

This model seems most suited to evaluate a role for nanomaterials in arthritis incidence, time of onset and severity.

Whether or not ACPA play a role in the development of arthritis and RA, Mohammed et al. (2012,2013) did not investigate the presence of ACPA in vivo after nanomaterial exposure.

We propose to measure ACPA in nanomaterial exposed animals.

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