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Review

Citrullination under physiological and pathological conditions

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ABSTRACT

Citrullination, one of the enzymatic posttranslational modifications has become a hot topic of recent research as it is involved in various physiological and pathological processes. Antibodies against citrullinated proteins called anti-citrullinated protein antibodies, are the hallmark (diagnostic and prognostic factors) of rheumatoid arthritis, and are specific for the disease. Citrullination has an important role in the normal function of the immune system, skin keratinization, the insulation of neurons and the plasticity of the central nervous system including its essential role in gene regulation. Abnormal citrullination has been proposed to play a role in multiple sclerosis and Alzheimer's disease, and recent research has drawn attention to its implication in tumorigenesis. Still, it is unclear whether citrullination is the cause or the consequence of these pathological alterations. Here, we discuss crucial aspects of citrullination during both physiological and pathological conditions.

1. Introduction

Citrullination is involved in many physiological processes in the body from skin keratinization through gene regulation to immune functions, and also implicated in pathological processes such as in autoimmunity and tumorigenesis.

Citrullination, a recently discovered form of posttranslational modifications, has become the highlight of research due to its potential pathogenetic role in an autoimmune condition, RA. Antibodies produced against citrullinated proteins are referred to as ACPAs, which are major diagnostic and prognostic factors of RA, and also influence the age at disease onset [1].

2. Peptidylarginine deiminases (PADs)

Citrullination is catalyzed by PADs. Human PADs are calcium dependent enzymes, and convert peptidylarginine into peptidylcitrulline [2]. Due to the reaction, the protein loses its positive charge, and the subsequent conformational changes may promote the formation of new motifs for protein (un)binding, may generate neoepitopes, and possibly alter the function and half life of the modified proteins.

Five isoforms are distinguished with different tissue expression. PAD1 is expressed in the epidermis, while PAD2 is found in both muscle tissue and the CNS [2]. Citrullination by PAD2 is proposed to have a role in the pathogenesis of MS and Alzheimer's disease. PAD3 is localized in hair follicles, while PAD4 is mainly expressed by cells of the hematopoietic lineage [2]. PAD4 may also participate in gene regulation, and implicated in tumorigenesis [3]. Increased PAD2 and PAD4 expression has been observed in the RA synovium [4], accompanied by ACPAs. PAD6 is found in early embryos and ovaries [2], and is less investigated compared to PAD2 or PAD4.

Besides eukaryotes, PAD is only found in *P. gingivalis* among prokaryotes, and it may be one of the virulence factors of the bacterium. Recently, a possible connection between *P. gingivalis* and RA has been proposed [5].

3. Physiology

3.1. Skin physiology

During terminal differentiation of keratinocytes, several proteins are altered by cleavage, covalent cross-linking and posttranslational modifications (e.g. citrullination) that help to create a matrix that is resistant to insults (Fig. 1). CK (or simply keratin) is the main intermedier filament of keratinocytes building up keratin filaments in the skin, hair, and nail. Due to citrullination, the structure of CK is altered, which enables proteins to bind to it [6].

Abbreviations: ACPA, anti-citrullinated protein antibody; anti-CCP, anti-cyclic citrullinated peptide; AP-1, activator protein-1; CEP-1, citrullinated enolase peptide-1; cFb, citrullinated fibrinogen; CK, cytokeratin; CXCL, CXC ligand; CXCR, CXC receptor; CNS, central nervous system; DNA, desoxyreibonucleic acid; Fb, fibrinogen; HDAC, histone deacetylase; HLA, human leucocyte antigen; MBP, myelin basic protein; MS, multiple sclerosis; NET, neutrophil extracellular trap; NF-κB, nuclear factor κB; PAD, peptidylarginine deiminase; *P. gingivalis*, *Porphyromonas gingivalis*; PTPN22, protein thyrosine phosphatase, non-receptor type 22; RA, rheumatoid arthritis; SE, shared epitope; TNF, tumor necrosis factor.

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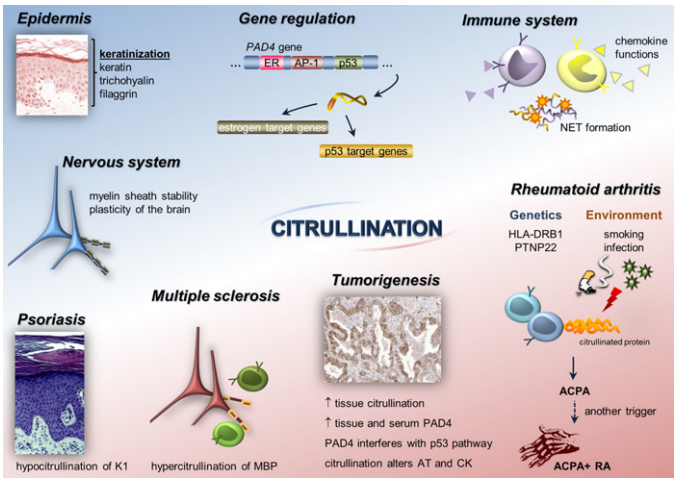

Fig. 1. During keratinization several proteins become citrullinated to create a protective matrix in the skin. Citrullination of myelin basic protein (MBP) is important in the function of the myelin sheath, as well as in the plasticity of the central nervous system (CNS). PAD4 is involved in gene regulation: its gene contains binding sites for estrogen receptor (ER), AP-1, and p53. PAD4 reciprocally influences the expression of estrogen and p53 target genes. Physiological citrullination alters the function of chemokines, and participates in antibacterial NET formation. Pathologically, hypocitrullination is seen in psoriasis, while hypercitrullination is found in multiple sclerosis. Citrullination is also implicated in tumorigenesis as increased tissue citrullination and PAD4 expression are detected in cancer patients. It is hypothesized that PAD4 interferes with the p53 pathway, and citrullination alters antithrombin (AT) and cytokeratin (CK) activity, all of which may contribute to abnormal cell function. The interaction of genetics and environment plays a role in the pathogenesis of anti-citrullinated protein antibody (ACPA) positive rheumatoid arthritis (RA). Aspecific citrullination triggered by e.g. smoking or infection may lead to ACPA formation if lymphocytes of susceptible individuals (with shared epitope [SE] containing human leucocyte antigen [HLA] molecules and/or general autoimmunity marker protein thyrosine phosphatase, non-receptor type 22 [PTPN22]) recognize citrullinated epitopes. If a further environmental trigger affects the body/joint, synovitis of autoimmune nature may be evoked.

Citrullination of another skin protein, filaggrin, facilitates its cleavage by proteases into smaller units [6]. The modified filaggrin can bundle keratins into a three dimensional structure. It is noteworthy that RA-specific ACPAs can (cross)-react to filaggrin, however, it seems that filaggrin is not an RA-specific autoantigen. Serre et al. have shown that deiminated forms of fibrin (α- and β-chains) deposited in the rheumatoid synovial membrane are the major target of these antifilaggrin antibodies, which are highly reactive to the Aα- and Bβ-chains of human Fb only after deimination of the molecules by PAD [7,8].

3.2. Central nervous system

In the CNS mostly PAD2 is expressed, mainly by oligodendrocytes, astrocytes and microglial cells, and the enzyme citrullinates MBP and glial fibrillary acidic protein, among others [6]. It is suggested that citrullination of MBP is essential for the plasticity of the CNS in young age as the ratio of citrullinated MBP and total MBP changes rapidly after postnatal life (Fig. 1) [6].

3.3. Gene regulation

Gene regulation is the major focus of epigenetic research. It is fine tuned by posttranslational modifications of histones, and coordinated by counteracting enzymes such as histone acetyltransferases, HDAC, protein arginine methyltransferases, and PADs.

Among PADs only the PAD4 isoform comprises a nuclear transfer signal, which enables the enzyme to participate in gene regulation [2]. PAD4 can citrullinate arginine and methylarginine of histone H3 and H4, thus, disabling histone methylation on these amino acids [9].

Table 1
Role of PAD4 in gene regulation.

p53 pathway
p53 transactivates PAD4 through intronic p53-binding site [10]
Silencing of PAD4/p53 inhibites DNA damage induced protein citrullination [10]
PAD4 interferes the function of nucleophosmin, essential for ribosome biogenesis and thus cell homeostasis and growth [10]
Ectopic expression of PAD4 inhibites tumor cell growth, and silencing of PAD4 attenuates p53-mediated growth-suppression [10]
Overexpression of PAD4 induces apoptosis through upregulating p53 and p21 [11]
PAD4 functions as a p53 corepressor of p53 target genes [12,13], and HDAC2 can interact with PAD4 to repress gene expression [14]
PAD4 citrullinates ING4, and disrupts its interaction with p53 thus reducing the transcriptional activity of p53 [15]
Estrogen pathway
PAD4 expression is activated by 17β-estradiol via estrogen responsive element and AP-1 binding on the PAD4 promoter [16]
PAD4 and HDAC1 associate cyclically to the pS2 promoter to reduce gene expression in the presence of estradiol [17]

Several studies have confirmed a reciprocal action of PAD4 and p53 (Table 1): PAD4 is recruited to p53-targeted gene promoters, while p53 can regulate the expression of PAD4. p53 becomes activated in response to multiple stress signals, and results in cell growth arrest or apoptosis. Tanikawa et al. have shown that p53 transactivated PAD4 through an intronic p53-binding site [10]. Due to DNA damage, several proteins became citrullinated, but the silencing of PAD4 or p53 significantly inhibited their citrullination. This indicates that protein citrullination is regulated in a p53/PAD4-dependent manner [10]. PAD4 also interfered the function of the histone chaperone protein nucleophosmin, essential for ribosome biogenesis and thus cell homeostasis and growth [10]. Ectopic expression of PAD4 inhibited tumor cell growth, and concordantly, the silencing of PAD4 attenuated p53-mediated growth-suppression [10]. In line with this, the overexpression of PAD4 induced apoptosis in HL-60 and Jurkat cells upregulating p53 and p21 [11].

In contrast, Yao et al. [12] and Li et al. [13] have found that PAD4 functions as a p53 corepressor and counteracts arginine methylation of the promoter of p53 target genes (tumor suppressors OKL38 and p21) (Table 1): inhibition or depletion of PAD4 elevated their expression leading to apoptosis in a p53-dependent way. Li et al. have also reported that HDAC2 can interact with PAD4 to repress gene expression of p53 target genes, and after DNA damage PAD4 and HDAC2 dissociated from these promoters [14]. PAD4 also citrullinates the p53-binding domain of a tumor suppressor protein called inhibitor of growth 4 (ING4), and disrupts the interaction between ING4 and p53 [15]. Normally, ING4 can enhance the transcriptional activity of p53, while citrullination seems to counteract this process. The results of these studies are quite controversal, but show that complicated negative and positive feedback loops coordinate mutually the p53-PAD4 system and cell homeostasis, and PAD4 may function differentially in various cell types.

Estrogens also seem to regulate PAD4 (Table 1): Dong et al. have reported that PAD4 expression is activated by 17β-estradiol: on the one hand, the complex of estrogen receptor and estrogen binds to the estrogen responsive element of PAD4 promoter to induce expression; on the other hand, estrogen-estrogen receptor complex enhances AP-1 to bind to PAD4 promoter and to increase its expression [16]. Denis et al. have provided evidence for a negative feedback: PAD4 and HDAC1 associate cyclically with the estrogen-regulated tumor suppressor pS2 promoter to promote gene suppression in the presence of estradiol [17].

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Table 2
Role of citrullination in immune functions.

Innate immune responses
PAD4 catalyzed histone hypercitrullination is essential in NET formation [19]
PAD2 interacts with inhibitor κB kinase, and suppresses NF-κB activity in macrophages after lipopolysaccharide stimulation [20]
Chemokines
Citrullinated CXCL8 has reduced affinity to glycosaminoglycans, is resistant to thrombin/plasmin-dependent cleavage, is unable to attract neutrophils to the peritoneum, however, can more efficiently recruit neutrophils into the blood circulation [21]
Citrullinated CXCL12 has reduced effects through CXCR4 [22]
Citrullinated CXCL10 and CXCL11 have decreased chemoattracting and signaling capacity through CXCR3 [23]
Effects of cytokines
TNF induces the translocation of PAD4 to the nucleus [24]

In conclusion, these studies show that citrullination is one of the essential regulatory mechanisms of epigenetics, and PAD4 functions are quite diverse encompassing the p53 and estrogen pathways (Fig. 1).

3.4. Immune functions

The cells of the hematopoietic lineage (especially, monocytes and granulocytes) express PAD4, suggesting that citrullination has a key role in the physiological function of the above cells. One of the neutrophil defense mechanisms is the trapping and killing of bacteria by forming highly decondensed chromatin structures, termed NETs (Table 2) [18]. Histone hypercitrullination catalyzed by PAD4 is essential in this process, and PAD4 knock out mice are more susceptible to bacterial infections in the lack of NET formation [19]. Results obtained from studies on macrophage cell lines suggest that PAD2 interacts with inhibitor κB kinase, and suppresses NF-κB activity after lipopolysaccharide stimulation, indicating PAD2 involvement in the innate immune defense [20].

Naturally occurring citrullinated chemokines have been shown to be less potent than the arginine-containing variants (Table 2) [21]. Citrullinated CXCL8 (IL-8) has reduced affinity to glycosaminoglycans (heparin), is resistant to thrombin- or plasmin-dependent cleavage into a more potent CXCL8 fragment, and is unable to attract neutrophils to the peritoneum. In contrast, citrullination of CXCL8 significantly increases the chemokine's ability to recruit neutrophils into the blood circulation from the bone marrow, and impairs its clearance from the circulation maintaining serum leukocyte level [22]. Modification of CXCL12 by citrullination severely reduces its CXCR4-mediated biological effects, while maximally citrullinated CXCL12 is inactive [23]. Citrullination reduces the chemoattractive and signaling capacity of CXCL10 and CXCL11 on CXCR3 and impaires T cell activation.

Cytokines also influence PAD activity (Table 2): TNF treatment induces the translocation of PAD4 from the cytosol to the nucleus in oligodendroglial cell lines [24]. Transgenic mice overexpressing TNF have increased levels of citrullinated histones and elevated nuclear PAD4.

These examples disclose a complex function of PADs with mainly anti-inflammatory and antibacterial effects (Fig. 1).

4. Pathology

4.1. Psoriasis

Psoriasis is a chronic immune-mediated disease, characterized by red scaly plaques on the skin, and sometimes accompanied by arthritis. In the psoriatic hyperproliferative epidermis, decreased keratin 1 (CK1) deimination has been reported [6] (Fig. 1). It is however intriguing that a small percent of psoriatic patients with arthritis have anti-CCP antibodies and their affected joints often show a polyarthritic pattern similar to RA [25]. These data suggest that skin lesions and joint inflammation may be evoked by different underlying mechanisms.

4.2. Multiple sclerosis

MS is an autoimmune demyelinating disease of the CNS with multifactorial etiology. Moscarello et al. have proposed on the basis of their results that myelin damage in MS white matter results from a failure to maintain the myelin sheath due to abnormally enhanced citrullination of MBP (Fig. 1) [26]. The cause of hypercitrullination may be increased PAD2 and PAD4 expression [24] and/or hypomethylation of the PAD2 promoter [27]. In PAD2-knockout mice CNS citrullination is diminished and demyelination is not seen [28], whilst in a transgenic mouse line containing multiple copies of the PAD2 cDNA, increased severity of clinical symptoms of MS is observed in line with increased PAD2 expression and activity and MBP citrullination [29]. Transgenic mice overexpressing TNF have increased levels of citrullinated histones and elevated nuclear PAD4 in the CNS before demyelination [24]. Citrullinated peptide fragments from MBP for example elicit a Th1-polarized response of T cells isolated from MS patients [30].

However, it is unclear whether abnormal citrullination is a pathogenetic factor or an accompanying phenomenon in MS.

It is puzzling that despite the increased citrullination of MBP and the autoimmune nature of the disease, ACPAs are not found [31]. This may reveal that the constellation of specific factors (genetics and environment) besides the presence of citrullination are required for ACPA generation and ACPA positive RA development. It is also supported by our results: we found increased citrullination in lung cancer tissues, and probably the immune system was confronted with these citrullinated antigens due to the increased cell necrosis in the tumor but despite this, no autoantibody (ACPA) production was seen [32].

4.3. Tumorigenesis

There is a body of evidence that PAD4 and citrullination play a role in tumorigenesis.

Chang and Han have found high tissue expression of PAD4 and citrullination in various malignant tumors, but not in benign tumors or non-tumourous tissues [3]. They have also reported elevated serum PAD4 and citrullinated antithrombin levels in patients with various malignancies [33]. The serum levels were associated with tumor markers, and considerably dropped after tumor excision therapy.

Recently, we have investigated the connection of lung cancer and ACPAs in relation to smoking, and found significantly elevated serum PAD4 levels in smoker lung cancer patients, compared to non-smoker healthy controls [32]. On the tissue level, both PAD4 expression and citrullination distinguished the tumors from the non-tumor tissue without any preference according to smoking history. The tissue expression of PAD4 and citrullination correlated with CK7 expression (a known tumor marker of lung cancer) suggesting that CK7 might have been citrullinated. In accordance with our data, Chang and Han reported that CK was possibly citrullinated in their tumor samples.

The molecular mechanism behind these observations are not unambiguous:

- PAD4 and p53 interact with each other (see Section 3.3). In line with this, a PAD4 inhibitor has been shown to display cytotoxic effects on various cancerous cell lines [34];

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- Omary et al. have found that the posttranslational modifications of CK alter the physical and chemical properties of CK [35], thus, citrullinated CK found in cancerous tissues [3,32] may also interfere cell homeostasis;
- citrullination of antithrombin abolishes its activity to inhibit thrombin [36]. Studies have shown that the increased thrombin activity may promote angiogenesis and tumour cell invasion.

According to our recent knowledge, citrullination may promote tumorigenesis through PAD4's interference with p53 pathway (ING4, repression of p53 target genes), attenuating the activity of antithrombin, and altering CK functions. The results on tumor cell lines treated with PAD4 inhibitors suggest that targeting the enzyme may be a potent future anti-cancer strategy in combination with other chemotherapeutics (e.g. HDAC inhibitors) (Fig. 1).

4.4. Rheumatoid arthritis

RA is an autoimmune disease accompanied by chronic progressive polyarthritis. The disease affects about 0.5-1% of the adult population. Both genetic and environmental factors play a role in its pathogenesis.

4.5. Genetics and environment

Two third of the genetics is associated with the presence of specific *HLA*-alleles, more precisely *HLA-DRB1 (*1001, *0401, *0404* etc.*)* alleles encoding the SE, a common, RA-risk motif on the HLA molecule [37]. The other significant genetic predisposition is a specific polymorphism of *PTPN22* that increases RA-risk in SE carriers [38].

Various environmental factors are suggested to have an influence on disease development: the most established one is smoking, and others involve infections, coffeine intake, and exposure to silica, while alcohol seems to have a protective role [39,40].

4.6. Smoking

Several epidemiological studies have confirmed that smoking is a major environmental risk factor in ACPA positive RA [41], especially in those carrying one or two copies of the SE epitope [42]. It is suggested that smoking, citrullination, PAD, and ACPA formation are associated, which is confirmed by the following observations. Higher anti-CCP titers are associated with tobacco smoking in RA [43]. In the bronchoalveolar lavage of healthy smokers, citrullination and PAD2 expression are increased, compared to non-smokers [44]. We previously showed that serum PAD4 levels were significantly elevated in smoker lung cancer patients, compared to non-smoker healthy controls [32]. It is suggested that smoking enhances citrullination, however, how smoking acts on PADs remains to be clarified. Free radicals in cigarette smoke may activate redox-sensitive transcription factors such as AP-1 [41], which may induce PAD expression via binding to the PAD4 promoter [16]. Various cell damage signals can activate p53 expression, and smoke contains hundreds of noxious compounds that may increase PAD4 expression via the p53 pathway [10]. In line with this, the interaction of smoking and HLA SE alleles does not influence autoimmunity toward specific citrullinated antigens, but rather predisposes for ACPA development, suggesting that smoking evokes non-specific citrullination [45].

It is hypothesized that smoke enhanced citrullination and subsequent ACPA formation is evoked in the lung [42], however a recent report may contradict this by showing the lack of association between the development of RA and preonset respiratory dysfunction [46]. It should be emphasized though that citrullination and modulation of the immune system by smoke may not necessarily lead to measurable (i.e. macroscopic) alterations in the lung.

4.7. Porphyromonas gingivalis

Recent research has drawn a parallel between periodontitis and RA on the basis of their etiology [5]. Periodontitis similarly to RA is a chronic progressive inflammation that leads to bone resorption in the oral cavity. The major causative agent of the disease is *P. gingivalis*, especially in genetically predisposed individuals (HLA-DRB1*04 alleles). According to epidemiological studies, RA seems to be more common among patients with periodontal disease [5], and RA patients have a higher frequency of advanced periodontal disease [5]. *P. gingivalis* antibody levels correlate with anti-CCP antibody titers [47]. Wegner et al. have reported that Porphyromonas PAD rapidly citrullinates both bacterial and host peptides (Fb and α-enolase) [48]. The immunodominant epitope (CEP-1) is 100% identical with the corresponding region of *P. gingivalis* enolase, and antibodies to CEP-1 (found in 40–60% of RA patients) react with both human and *P. gingivalis* enolase [49]. Moreover, citrullinated α-enolase is found in RA synovial fluid [50]. These data suggest the potential role of molecular mimicry (enolase) in the pathogenesis of RA. The virulence factors of *P. gingivalis* may provoke the host's immune responses against bacterial citrullinated α-enolase in an inflamed environment, which may lead to autoimmune cross-reactivity against human enolase under genetic predisposition (SE carriers). To support this, genetically susceptible (DR4-IE) mice immunized with human recombinant α-enolase or *P. gingivalis* enolase – either citrullinated or uncitrullinated – rapidly developed arthritis with formation of autoantibodies against human citrullinated and unmodified enolase (CEP-1 as well) [51].

4.8. Citrullinated proteins

Citrullination generates "altered-self" epitopes that may be presented only when both key arginine residues of proteins are converted by PAD enzymes, and they may be recognized by HLA molecules (under genetic predisposition). James et al. have shown that citrullinated sequences preferentially bind to HLA-DR*1001, and T cell clones proliferate only in response to citrullinated peptides [52]. In line with this, vimentin 66–77 binds to SE alleles only when residue 70 is changed to citrulline [53].

Several decades ago Dumonde and Glynn established an animal model of RA by inducing chronic synovitis via the intra-articular injection of fibrin into rabbits previously sensitized to this protein [54]. Foulquier et al. have reported that in synovial tissues of RA patients, PAD-2 and PAD-4 expression was seen within or in the vicinity of citrullinated fibrin deposits, and correlated with the intensity of inflammation [4]. Antibodies to cFb are found in two third of RA patients [55], and circulating immune complexes containing cFb are present in one-half of anti-CCP positive RA patients [56]. These immune complexes colocalise with complement C3 in the rheumatoid synovium [56]. All these data suggest a potential arthritogenic role of cFb generated by local PADs. Hill et al. have reported that transgenic mice (for human HLA-DRB1*0401) immunized with human cFb developed arthritis but none of those animals immunized with the unmodified Fb [57]. Importantly, wild-type mice immunized with either form of Fb did not develop arthritis, nor did mice (wild-type and transgenic) immunized with murine unmodified Fb or cFb. Interestingly, immunization of both rats with complete Freund's adjuvant-emulsified autologous cFb and wild-type mice with human cFb together with adjuvant breaks tolerance, and results in an antibody response, however, this does not elicit arthritis [58,59]. Similarly, the citrullination of the ubiquitous self-antigen, rat serum albumin can break tolerance but without arthritis, and can increase the arthritogenicity of type II collagen

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[58]. These data indicate the importance of both human MHC II genotype (SE alleles) and citrullination of human antigen (e.g. Fb) in mediating autoimmune arthritis.

PAD inhibitors are beneficial in animal models of RA, suggesting that citrullination and PAD activity may play a role in the pathogenesis/course of RA [60]. The most potent PAD inhibitor Cl-amidine reduces both synovial citrullination and disease activity in collagen induced arthritis but not in collagen antibody induced arthritis. This indicates that its efficacy is due to its ability to inhibit deimination (e.g. during gene regulation and immune functions) and to reduce epitope spreading [61]. As Cl-amidine-action is calcium dependent, it may potentially inhibit PAD4 in its activated state at sites of inflammation. Another drug candidate in RA is the HDAC inhibitor trichostatin A, which may exert its anti-inflammatory action through the mutual interaction of PAD4 and HDAC on gene regulation [14,17,62].

In conclusion, a subclinical inflammation (infection) or smoking may induce citrullination of proteins. In combination with a specific genetic context, immune tolerance may be broken, and ACPAs may be generated together with the activation of autoreactive T cells. If a further environmental trigger afflicts the body/joint, synovitis of autoimmune nature may be evoked (Fig. 1).

5. Conclusion

Even if the significance of citrullination has been recognized in various pathological conditions, we should be cautious declaring it directly either beneficial or detrimental. Rather we should consider it as a posttranslational regulatory mechanism in several physiological functions of the body that may turn into pathology (or accompany pathology as a bystander process).

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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