HHS Public Access

Author manuscript

Curr Drug Targets. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Curr Drug Targets. 2015 ; 16(7): 700–710.

Protein Arginine Deiminases and Associated Citrullination: Physiological Functions and Diseases Associated with Dysregulation

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Abstract

Human proteins are subjected to more than 200 known post-translational modifications (PTMs) (e.g., phosphorylation, glycosylation, ubiquitination, S-nitrosylation, methylation, N-acetylation, and citrullination) and these PTMs can alter protein structure and function with consequent effects on the multitude of pathways necessary for maintaining the physiological homeostasis. When dysregulated, however, the enzymes that catalyze these PTMs can impact the genesis of countless diseases. In this review, we will focus on protein citrullination, a PTM catalyzed by the Protein Arginine Deiminase (PAD) family of enzymes. Specifically, we will describe the roles of the PADs in both normal human physiology and disease. The development of PAD inhibitors and their efficacy in a variety of autoimmune disorders and cancer will also be discussed.

Keywords

Apoptosis; autoimmune disease; citrullination; gene regulation; inflammatory disease; protein arginine deiminases

1. INTRODUCTION

Posttranslational modifications (PTMs) can have consequential effects on protein structure and function. One such PTM, citrullination, is catalyzed by the Protein Arginine Deiminase (PAD) family of enzymes [1]. This enzyme family consists of 5 isozymes (PAD1–4 and 6) [2–4] that have individual tissue specificity and target substrates. PADs rely on increased concentrations of calcium to citrullinate peptidyl-arginine [5]. Under physiological conditions in cells, PADs are not normally active until stimulated with calcium. Once stimulated, these enzymes citrullinate a number of structural proteins (e.g., vimentin,

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

filaggrin, and keratin) [6, 7] and proteins involved in the regulation of gene transcription (e.g., histones H1, H2A, H3 and H4) [8–10]. Since PADs play functional roles in key cellular processes, their regulation is necessary. There have been several inhibitory compounds developed to study PAD activity. From the parent structures of Cl-amidine and F-amidine, second generation compounds have been synthesized with increased isozyme specificity, *in vivo* stability, and bioactivity [11–13]. To date, Cl-amidine is the most widely used pan-PAD inhibitor, while Cl4-amidine and F4-amidine are the most potent PAD3 specific inhibitors [13]. Many of these compounds are being tested as therapies and preventatives for numerous diseases where PADs are known to become dys-regulated such as diseases of the nervous system, skin, joints, immune system, and colon [14–17]. Overall, based on PADs' role in physiological and pathological functions (such as gene regulation and immune response), the field of PAD regulation is gaining traction as a promising target for the treatment and prevention of autoimmune and inflammatory diseases linked to abnormal PAD activity.

2. THE PROTEIN ARGININE DEIMINASE FAMILY OF ENZYMES

2.1. Citrullination

First described in 1958 by Rogers and Simmonds [1], protein citrullination is the process of converting peptidyl-arginine to peptidyl-citrulline. Since citrulline is a non-genetically encoded amino acid, citrullination occurs post-translationally [18], and because it is a hydrolytic reaction, it results in a 1Da decrease in mass [19, 20]. As a result, the strongly basic positively charged side chain of arginine is hydrolyzed by water to form the neutral urea (Fig. 1). This shift in charge can affect protein-protein interactions, hydrogen bond formation, protein structure, and, in some cases, cause denaturation [13, 21]. There is a wide range of cytoplasmic, nucleic, membrane, and mitochondrial proteins that can be citrullinated [22]. This review will discuss physiologically targeted protein substrates and citrullination in diseases.

2.2. PADs

The PADs were first described in 1977 as the enzymes responsible for this PTM [23] and it is now known that these enzymes are cysteine hydrolases. Citrullination proceeds via nucleophilic attack of a critical active site cysteine on the substrate guanidinium, resulting in the formation of a tetrahedral intermediate that ultimately collapses to form an acyl-enzyme intermediate that is subsequently hydrolyzed to form citrulline (Fig. 1) [13, 19, 24]. While the substrate scope of the PADs remains poorly defined, it is known that these enzymes will not citrullinate free arginines or even methylated arginine in the context of a peptide or protein [18, 19, 25, 26].

2.3. PAD Distribution and Structure

There are 5 PAD isozymes found in humans: PAD1, PAD2, PAD3, PAD4, and PAD6 [2–4]. Of these isozymes, PAD6 is the only PAD for which no *in vitro* activity has been detected [27]. The PAD isozymes have unique tissue localization and overlapping substrates. PADs 1 and 3 are both distributed in the epidermis and hair follicles [28], with PAD1 also localizing in the uterus [2, 29]. PADs 2 and 4 have widespread protein distributions. For instance,

PAD2 can be located in the central nervous system (CNS), spleen, skeletal muscle, and leukocytes [30–33]. PAD4 is found in inflammatory cells (macrophages and neutrophils), mammary gland cells, and tumors [16, 34, 35]. PAD6 is localized to eggs, ovary, and the early embryo [4, 35, 36].

Since the crystal structure for PAD4 has been determined, more is known about this isozyme. All mammalian PAD isozymes share 70–95% homology in their amino acid sequence and are approximately 663 amino acids long with a molecular weight of ~74 kDa (Fig. 2) [18, 24, 37, 38]. The PADs have two immunoglobulin-like subdomains at the N-terminus (aa 1–300) and a highly conserved C-terminal domain (aa 301–663) that includes the active site of the enzyme [24]. PAD4 also contains a canonical nuclear localization signal (NLS) (aa 56–63) within N-terminal subdomain 1 (Fig. 2) and is at least partially localized to the nucleus where it deiminates histones H3 and H4 [24, 35]. Despite lacking a canonical NLS sequence, PAD2 was also recently shown to localize to the nucleus and citrullinate histone H3 [39].

2.4. Calcium Dependency

The PADs are calcium-dependent enzymes [5]. There are five calcium binding sites in PAD4, with 2 being located in the C-terminal domain of the enzyme [24] and the remaining three being present in N-terminal subdomain 2. In the absence of calcium, the active site has a rather open concave structure with a highly acidic region with Cys645, the active site nucleophile, being pointed away from the active site [24, 40]. Upon binding calcium, the protein undergoes a series of conformational changes that ultimately results in the movement of key active site residues, including Cys645, into positions that are competent for catalysis [24, 38]. Although the pH optimum for PAD activity is 7.6, calcium activation of PAD4 is pH-independent between a pH of 6.0 and 8.5 [25].

2.5. PAD Substrate Targets

Not all arginine residues in a protein are equally likely to be citrullinated by PADs. A study investigating the citrullination of filaggrin and trichohyalin, known PAD substrates, was able to determine that approximately 95% and 60% of arginines were converted to citrulline within 3 hours of adding PAD to filaggrin (1:1000 ratio) or trichohyalin (1:30 ratio) substrates, respectively [21]. These results demonstrated that the secondary structure of a protein has an effect on the occurrence of citrullination. Filaggrin, which has a secondary structure consisting mainly of a β-turn, is citrullinated more frequently (~95%) than trichohyalin (60%), which is composed of a single α-helix [19, 21]. Likewise, Tarcsa *et al.* (1996) determined the amino acid sequences for filaggrin and trichohyalin and quantified the occurrence of each arginine residue being citrullinated found in each substrate [21]. For example, it was found that arginines located next to aspartic acid residues were citrullinated about 80–90% of the time, while arginines found next to glutamic acid residues were rarely citrullinated (0–5%) [19, 21]. Furthermore, arginine residues that are located next to an amino group or are flanked by proline are poorly citrullinated [19, 41].

Therefore, depending on the importance, location, structure, and abundance of the arginine residues in a protein, citrullination can have variable effects, such as changes in protein-

protein interactions or denaturation [13, 21]. PAD1 targets keratin and filaggrin [7]; PAD2 targets myelin basic protein (MBP) [30], vimentin [42], actin, and histones [39]; PAD 3 targets filaggrin, trichohyalin [7], apoptosis-inducing factor (AIF), and vimentin [43]; PAD4 targets multiple proteins involved in gene regulation (histones [24, 44], inhibitor of growth 4 (ING4) [45], p300 [46], p21 [47]) and apoptosis (nucleophosmin [48], nuclear lamin C [49]) ; and there are no known protein substrates of PAD6 (Table 1) [35]. Several of these protein targets of PAD citrullination will be described throughout the next sections that have a high arginine content (~10%) and/or have particular arginine-rich regions that are critical for their structure and function.

3. NORMAL PAD FUNCTION

3.1. Normal Cellular Processes Involving PADs

Under physiological conditions, PADs are usually inactive because calcium concentration is maintained at very low levels in the cell (10^{-8} to 10^{-6} M) [19, 50]. Normal functioning PADs become activated during certain events, such as apoptosis and terminal epidermal differentiation, where calcium levels are above the physiological concentration [19, 51]. Intriguingly, PADs function in gene regulation and other processes at physiological concentrations of calcium; therefore mechanisms to modulate the calcium-dependence of the enzymes must exist [19]. In this section, we will discuss four key cellular processes in which protein citrullination is known to play a role.

3.2. Apoptosis

Calcium acts as a signaling molecule to coordinate apoptosis; therefore, it is needed at high concentrations within apoptotic cells [52]. PADs are also activated by these higher concentrations of calcium in cells undergoing apoptosis. However, once activated, it is not fully understood how PADs determine their substrate specificity. One consideration is that PADs may target proteins that are arginine-rich or have arginine-rich regions within their amino acid sequence. In support of this idea, vimentin, an intermediate filament, is found to be citrullinated during apoptosis [51] and Inagaki *et al.* (1989) report that PADs citrullinate the non-α-helical head domain of the vimentin protein [6]. The vimentin protein is 466aa long with 9.2% arginine residues [53]. In this non-α-helical head domain (aa 2–95), there are 12 arginines that are citrullinated by the PADs [53]. When vimentin is citrullinated, the polymer disassembles and the monomers cannot rejoin properly to form the structural support and organelle anchor, triggering structural collapse and further completion of apoptosis [6, 19, 54]. For instance, PAD2 can induce apoptosis by citrullinating vimentin, specifically in macrophages. Overexpression of PAD2 leads to vimentin citrullination and apoptosis in Jurkat cells [42].

Likewise, histones and nucleophosmin are known targets of PAD4 and their citrullination can cause the nucleosome and nuclear lamina to collapse, also initiating apoptosis [18, 19]. The oligomerization of nucleophosmin is required for localization to the nucleolus and will prevent apoptosis by inhibiting p53 localization to the mitochondria (a key step in apoptosis) [55, 56]. Therefore, improper folding/binding caused by citrullination of nucleophosmin can induce apoptosis associated with p53 localization to the mitochondria. Also reported, PAD4

overexpression upregulates p53 and stimulates mitochondrial-associated apoptosis [57]. Additionally, Mizoguchi *et al.* (1998) reported the citrullination of a 70kDa nuclear protein localized on the periphery of the nucleus was linked to dissociation of the nuclear lamina during apoptosis [58]. Recently, PAD3 was found to be necessary for apoptosis-inducing factor (AIF)-mediated apoptosis [43]. Overall, PAD activation (due to amplified calcium levels during the early stages of apoptosis) can facilitate apoptotic cell death by citrullinating several nuclear and cytoskeletal proteins that can cause structural changes resulting in the disintegration of secondary and tertiary protein structures [6, 18, 19].

3.3. Structural Support

Additionally, PADs are involved in the organization of structural proteins in cells undergoing terminal epidermal differentiation [19, 59]. This differentiation is mediated by calcium, so there are usually higher concentrations of calcium, which in turn activates the PADs. Within the epidermis, PADs can citrullinate structural proteins, such as, keratin, filaggrin, and vimentin. Filaggrin, as an example, is 4061aa long with a relatively high arginine content (10.8%) [53]. Upon calcium-mediated terminal differentiation of epidermal cells, activated PADs can citrullinate these proteins, causing partial unfolding, which makes these proteins more susceptible to protease degradation [19, 60].

3.4. Gene Regulation

In cells not undergoing apoptosis, PADs can regulate gene expression [10, 35, 44–47, 61–63]. Most research on PAD involvement in gene regulation has been performed on PAD4. One of the best studied systems is the PAD4-mediated regulation of the p53 pathway. For instance, PAD4 citrullinates ING4 protein [45] and once modified, this protein does not bind to p53 and is more prone to protease degradation. Without the interaction of ING4, the transcriptional targets (e.g. p21) of p53 are suppressed (Fig. 3A) [45]. Another mechanism involves the citrullination of methylated arginine on histones. In this mechanism, PAD4 is recruited to gene promoters where it mediates the citrullination of histone H4 at R3 and histone H3 at R2, R8, and R17 and the modification is associated with gene repression [44]. As an example, PAD4, which interacts with the regulatory domain of p53, targets p21 at the p53-binding sites on the promoter region [47]. This interaction causes citrullination of histones on the p21 promoter region, ultimately repressing p21 transcription since p53 cannot properly bind to the p21 promoter region (Fig. 3B) [47]. This impaired binding is due to the changes in charge that can affect protein-protein interactions and hydrogen bond formation. Also, citrullination of the histones in the p53-binding sites on the promoter region may cause conformational changes that sterically hinder p53 binding. Li *et al.* (2010) correspondingly reported that PAD4 citrullinated histones on p53 target gene promoters, but they further found that PAD4 relied on the histone deacetylase, HDAC2, to repress the gene expression of p53 targets (Fig. 3B) [61]. It is still unknown how PAD4 is recruited to specific promoters or even how they are activated at physiological calcium levels [35, 62–63]. Wang & Wang (2013) propose a mechanism where PAD4 binds to the promoter region of p53 and represses target gene expression by histone citrullination during periods where the damage response pathways are not needed. Then, when these pathways are needed (i.e. in response to DNA damage), PAD4 dissociates from the promoter and the target genes are activated once again [35]. There is also evidence that PAD4 can act as a transcriptional

coactivator. In this process, PAD4 citrullinates the Arg2142 in the GRIP1 binding domain of the transcriptional coactivator p300. Once p300 is citrullinated, the interaction between it and GRIP1, another coactivator, is enhanced (Fig. 3C) [46]. Perhaps, in this case, the neutral charge of citrulline could be more complementary for the interaction between GRIP1 and p300. How the PADs get activated to modulate gene transcription is not known, but György *et al.* (2006) hypothesized that PADs may be active with strict substrate specificity at low levels of calcium and this could explain PAD involvement in gene regulation [19]. However, this is unlikely to be correct because different substrates have similar calcium dependencies [62].

PAD4 also plays a role in pluripotency as a transcriptional regulator of several key genes. PAD4 upregulates pluripotent markers (e.g. Klf2, Tcl1, Tcfap2c, Kit, Nanog) and downregulates differentiation markers (e.g. Prickle1, Epha1, Wnt8a) [8, 64]. In fact, citrullination of histones H1 and H3, by PAD4, regulates the induction of pluripotency and reprograming of stem cells during early embryonic development [8, 64]. Slade *et al.* (2014) also suggest that other PAD isozymes (PAD1–3) may impact pluripotency at other stages of differentiation since they showed individual expression in embryonic stem cells and induced pluripotent cells [8, 64].

3.5. Immune Response

PADs 2 and 4 are involved in inflammatory immune responses. PAD4 is mainly expressed in macrophages, neutrophils, and eosinophils whereas PAD2 is highly expressed in macrophages [16]. In macrophages, PAD2 becomes activated due to increased levels of calcium [65]. Also, PAD4 is involved in the citrullination of histones in neutrophils. After activated PAD4 has translocated to the nucleus of neutrophils, hypercitrullination of histones can trigger the production of neutrophil extracellular traps (NETs) that trap bacteria and other pathogens. The release of NETs can provoke an autoimmune response to NET-associated nuclear antigens and granule proteins, leading to a pro-inflammatory form of programed cell death termed ‘NET-osis’ [40, 66, 67].

4. PAD INHIBITORS

4.1. Development of F-Amidine, the First Bioactive PAD Inhibitor

Thompson and colleagues reported the first bioactive PAD inhibitor in 2006 when they described the synthesis and characterization of F-amidine. The structure of F-amidine is based on benzoylarginine amide (BAA), one of the best small molecule substrates identified for the PADs [25, 68]. Since the PADs preferentially hydrolyze a positively charged guanidinium, Thompson hypothesized that replacement of one of the amino groups with a methylene fluoride would generate a compound that could covalently modify the enzyme (Fig. 4A) [68]. The resulting flouroacetamidine containing compound maintains the positive charge and most of the hydrogen bonding capabilities of the substrate guanidinium, but due to the electron withdrawing nature of the fluorine can undergo reaction with a cysteine residue present in the active site of the enzyme [68]. This compound, F-amidine, inhibits PAD4 with a k_{inact}/K_I value of 3000 $M^{-1}s^{-1}$ and was the most potent pan-PAD inhibitor at the time. Further experiments demonstrated that F-amidine irreversibly inhibits PAD4 by

modifying Cys645, the active site cysteine [69]. F-amidine is bioavailable and has been used to confirm that PAD activity can inhibit gene expression [68].

4.2. Cl-Amidine

Building on the success of F-amidine, Thompson and colleagues next synthesized a series of compounds in which they optimized the identity of the warhead by replacing the fluorine with either a chlorine or hydrogen. They also optimized the side chain length by synthesizing compounds in which the side chain is varied between two and four methylene units [69]. From these compounds, Cl-amidine was found to be the most potent compound with a k_{inact}/K_I value of 13000 $M^{-1}s^{-1}$ for PAD4 [69]. Subsequent work showed that it inhibits all four active PAD isozymes with similar potencies [11]. Cl-amidine is structurally identical to F-amidine except that it has chlorine substituted for the fluorine in the haloacetamidine warhead (Fig. 4B) [11, 69]. By contrast, compounds that lacked an electron withdrawing group or had shorter or longer side chains were rather poor PAD inhibitors [69].

Cl-amidine, like F-amidine, preferentially inhibits the activated, calcium-bound form of the PADs [69] and based on detailed mechanistic studies [13] both compounds irreversibly inhibit the enzyme via nucleophilic attack on the amidine carbon, which results in the formation of a tetrahedral intermediate that is stabilized by proton donation from His471, the general base in the normal PAD-catalyzed reaction. The sulfur then attacks the halide bearing carbon to form a three member sulfonium ring. Proton abstraction and collapse of the tetrahedral intermediate cleaves the sulfonium ring to form the stable thioether adduct [40].

Since its development, Cl-amidine has been the most widely used PAD inhibitor and studies indicate that it is bioavailable and can inhibit PAD4 activity found in the nucleus [69]. Remarkably, Cl-amidine was cytotoxic to cancer cell lines (MCF-7, HL-60, and HT29) and showed minimal cytotoxic effects on noncancerous cell lines (NIH 3T3 and HL-60 granulocytes) [70]. Furthermore, Cl-amidine triggered the differentiation of the leukemic HL-60 cell line into HL-60 granulocytes [70]. As will be discussed later on in this review, Cl-amidine has been tested on numerous disease models and shows promise for the treatment and/or prevention of many diseases.

4.3. Other PAD4 Inhibitors

In order to detect other PAD4 inhibitors, a competitive screening assay was developed using Rhodamine-conjugated F-amidine. This assay overcomes the challenges of traditional PAD activity assays because it does not require strong acids, high temperatures, and toxic reagents that are normally necessary to detect the citrulline product of the reaction [71]. Using this assay, multiple drugs that are typically used to treat RA were evaluated for their ability to inhibit PAD4. Streptomycin was found to be a competitive inhibitor whereas minocycline and chlorotetracycline were found to be mixed inhibitors that did not bind to the active site [71]. These drugs can potentially provide scaffolds for the next generation of PAD inhibitors [71, 72].

4.4. PAD Specific Inhibitors

In disease, it has been shown that only certain PADs may be dysregulated (e.g. PAD4 in RA); therefore, the development of PAD specific inhibitors has been explored. Structure activity relationships (SAR) were generated for F-amidine and Cl-amidine to identify modifications that would increase interisozyme selectivity. One outcome of these efforts was the identification of o-F-amidine and o-Cl-amidine, which contain an orthocarboxylate on the benzoyl ring of the parent structures [11]. Both compounds are significantly more potent than the parent compounds and also exhibited PAD specificity. For example, o-F-amidine selectively inhibits PAD1, whereas o-Cl-amidine preferentially inhibits PADs 1 and 4 [11]. Additional studies identified D-o-F-amidine and D-Cl-amidine as a pair of PAD1 specific compounds that possess increased *in vivo* stability [12]. As of 2010, Cl4-amidine and F4-amidine, which were tested previously on PAD4, are the strongest PAD3 specific inhibitors [13]. TDFA, a tripeptide composed of threonine, aspartate and F-amidine is the most potent PAD4 specific inhibitor to be reported [73]. Additionally, in 2005, Kearney *et al*. showed that different metals inhibit PAD activity. In their studies, they found that manganese, samarium, and zinc did not allow the PADs to structurally change into the catalytically active form in the presence of calcium [25]. These findings are another step in the development of new and more specific PAD inhibitors.

5. PAD DYSREGULATION IN DISEASE

5.1. Diseases Associated with PAD Dysregulation

PADs have a role in maintaining many vital cellular processes; therefore, it is necessary for a balance in PAD activity or these processes can become uncontrollable with potentially deleterious side effects. Due to this delicate balance, it is not hard to believe that there are numerous diseases associated with PAD dysregulation and abnormal levels of citrullination. In this section, we will discuss our current understanding of the role that PADs play in these diseases even though much (i.e. specific cause for dysregulation) is still to be discerned. We will also discuss specific PAD isozymes that are associated with certain types of diseases, such as PAD1 is linked to psoriasis [15] and PADs 2 and 4 are heavily involved with neurodegenerative and inflammatory [16–17] diseases and cancers [34].

5.2. Causes of PAD Dysregulation

Not much is known about what causes PADs to become dysregulated, but there are several proposed scenarios that may lead to abnormal levels of citrullinated proteins. For example, (i) PAD activity may become uncontrolled at extreme levels of calcium and may lose target specificity [19]. The lack of target specificity could cause loss of activity and even total denaturation in arginine-rich proteins. As well, (ii) increased translation of PADs could also explain for increased levels of citrullination; however, it is unknown what might cause the increased translation [19, 74]. (iii) There is abnormal TNFα signaling in Rheumatoid Arthritis (RA) and Ulcerative Colitis (UC), TNFα can induce PAD4 translocation [75], and PADs can citrullinate TNFα [76]. Taken altogether, perhaps RA and UC result, in part, from PAD dysregulation [17]. Finally, (iv) PAD4 has been seen to autocitrullinate and this may be a form of self-regulation. Although autocitrullination is not shown to interfere with specificity or activity levels of the enzyme, it does affect the enzyme’s interaction with other

proteins involved in the PTM of histones [62]. Once again, as discussed in Section 2.5, the amino acid sequence and structure surrounding arginine residues can also influence susceptibility to citrullination [19, 21].

5.3. PADs in the CNS

5.3.1. Normal Function—PAD2 is the predominant isozyme found in the nervous system [14, 30, 35]. While PAD4 does not normally target CNS substrates, it has been discovered in the cytoplasmic granules of neuronal cells [77]. PAD2 and 4 are normally in the inactive state in the adult CNS [14], but are active during development [78].

5.3.2. Alzheimer's disease—PAD 2 and 4 become activated in the CNS during neurodegenerative processes [14]. Alzheimer's disease is linked to an increase in abnormal accumulation of misfolded proteins in the hippocampal region of the brain. Interestingly, an increased amount of PAD2 and citrullinated proteins are found in the hippocampus of patients suffering from Alzheimer's [14]. The structural proteins vimentin and glial fibrillary acidic protein (GFAP) are some of the proteins found to be citrullinated in the hippocampus [19, 38, 79, 80]. Likewise, PAD4 and citrullinated proteins were colocalized in regions of neurodegeneration and inflammation in Alzheimer's patients [77].

5.3.3. Prion Disease—Similar to Alzheimer's disease, prion diseases involve misfolded proteins alongside deterioration of many structures in the brain [79]. Calcium levels are dysregulated in these diseases and are likely the reason for high amounts of active PADs and citrullinated proteins in the CNS [79].

5.3.4. Multiple Sclerosis—In the CNS, the MBP is essential for maintaining the myelin sheath, which is involved in neuronal signal transduction throughout the CNS [19, 79]. Signal transduction requires the myelin sheath to act as an insulator with the MBP holding a positive charge [18]. In Multiple Sclerosis (MS), the myelin sheath is degraded, leading to decreased signaling. PAD isozymes 2 and 4 are involved in the pathology of MS [19, 30, 35, 38, 75]. It is thought that PAD4 is brought to the CNS by infiltrating macrophages during diseased states and may citrullinate proteins that are not naturally targeted in the CNS [81]. The excessive citrullination of the MBP is thought to be a major contributor to the disease [19, 30, 38, 82]. It has been established that up to 90% of MBPs are citrullinated in severe forms of the disease [38, 83]. Citrullination causes MBP to partially unfold and make them more susceptible to degradation by proteases, like cathepsin D [19, 83, 84]. Also the change from positively charged arginine to neutral citrulline is not optimal for the electrical specifications required for proper signal transduction [19, 84]. The high frequency of arginine residues (10.3%) in the amino acid sequence of MBP may also explain why it is targeted by PADs [85]. While the PAD2 knockout still develops experimental autoimmune encephalomyelitis (EAE), a preclinical animal model of MS, overexpression of PAD2 gives MS-like symptoms, suggesting that multiple PAD isozymes are involved in the etiology of this disease [86].

5.4. PADs in the Epidermis

5.4.1. Normal Function—PAD1 is highly expressed in the epidermis where it citrullinates keratin K1and filaggrin [7, 19, 35, 38]. These proteins aid in the maintenance of homeostasis and structural flexibility in the dermis and epidermis [7, 18, 38]. In normal skin, keratin K1 is citrullinated at low levels and degraded to prevent cornification.

5.4.2. Psoriasis—Psoriasis is a type of dermatitis characterized by excessive proliferation and atypical epidermal differentiation which contribute to the flaky, dry patches of skin [19, 38]. The possible cause of the disease can be explained due to the fact that there is no citrullinated keratin K1 found in skin samples from patients with psoriasis [15, 18–19, 38]. The lack of citrullinated proteins causes excessive cornification and an inflammatory response [15, 18–19, 35, 38]. Psoriasis is the only disease linked to PAD1 dysregulation and it is unknown what triggers its decreased enzymatic activity [15, 18, 35, 38].

5.5. PADs in the Synovial Joints

5.5.1. Normal Function—PAD2 and PAD4 mRNA and protein are expressed in peripheral blood from both RA patients and healthy control patients [16]. In healthy patients as compared to the RA patients, the PAD isozymes were inactive since the monocytes and macrophages, where the PADs were located, were not involved in an inflammatory response [16]. Also, there were fewer macrophages and monocytes found in the joints in healthy patients. These PAD enzymes are typically dormant in the leukocytes and synovial joints until they are activated during an inflammatory immune response.

5.5.2. Rheumatoid Arthritis—RA is a chronic inflammatory disease of the joints that is thought to be caused or intensified by PAD dysregulation. In patients with RA, an increase in PAD2 and 4 translation is observed alongside increased enzyme presence and activation [19, 74]. Vossenaar et al. (2004) developed a model for PAD infiltration into the synovial joints of RA patients where PAD2 and PAD4 are expressed in monocytes and macrophages that are recruited to the joint [16]. Their proposed model shows that the activated macrophages and monocytes, containing the inactive PADs, are eventually degraded in the joints and undergo apoptosis where the PAD isozymes become activated as calcium levels rise. PAD activation then causes cellular protein (e.g. vimentin) citrullination and the PADs can seep out of the cell to target extracellular proteins [16]. For example, PAD2 and 4 can citrullinate fibrin in the affected joints, producing an immune response. Also, PAD4 has been shown to citrullinate antithrombin, a thrombin inhibitor. Thrombin is involved with the onset of RA; therefore, the citrullination of antithrombin may further lead to RA [87].

In many inflammatory diseases, such as RA, inflammation is a result of an atypical immune response to bacteria and other stresses (e.g. citrullination) [19, 88]. NETs are found to be dysregulated in RA, causing an increase in antibodies against citrullinated proteins [89]. Correspondingly, there are high levels of antibodies against citrullinated proteins found in the blood of RA patients, possibly explaining the atypical immune and inflammatory responses correlated with RA [18, 89]. Importantly, the presence of these antibodies is the most specific diagnostic for the disease and they are present in the sera of RA patients 4–5 years, on average, before clinical symptoms appear [90]. This suggests that dys-regulated

PAD activity is potentially disease initiating. Despite the specific causes of RA still being unknown, there have been several haplotypes of PAD4 that are correlated with an increased risk of developing the disease [91].

5.6. PADs and their Role in NET Formation

As mentioned in Section 3.5, PAD4 is involved in a unique form of cell death, 'NETosis'. Released NETs actively trap bacteria and other pathogens that prompt this type of immune response. This immune response is a common defense mechanism against foreign microbes. The effects of NET dysregulation play a role in a number of other autoimmune diseases, including psoriasis [92], systemic lupus erythematosus (SLE) [93], and RA as previously discussed. Interestingly, pro-inflammatory IL-17 is released during NET formation in psoriasis, SLE, and RA, indicating that this aberrant immune response triggers an inflammatory response [89, 92, 94].

5.7. PADs in the Colon

5.7.1. Normal Function—There is no data on the normal function of PADs in the human colon. Perhaps the presence and dormancy of PADs in normal colonic tissue resembles that in the healthy, non-arthritic patients, as described in Vossenaar *et al.* (2004), where the inactive PADs are present in macrophages and monocytes [16].

5.7.2. Ulcerative Colitis—UC is another inflammatory disease where PADs 2 and 4 are involved in the pathogenesis of the disease. UC is characterized by chronic, relapsing periods of inflammation in the colon. The exact cause of the disease is unknown but it has been proposed that an atypical immune response is triggered by an inflammatory response to several cytokines produced by activated macrophages [88]. In UC, similar to RA, high levels of anti-citrullinated protein antibodies are found in the blood [17]. Thus, UC could be caused by an autoimmune response to improperly folded proteins caused by excessive citrullination. The specific PAD4 haplotypes that have been linked to genetic susceptibility of RA have similarly been linked to UC [91].

5.8. PADs in Cancer

PAD dysregulation is also associated with cancer [34]. For example, PAD2 levels are elevated in luminal breast cancer cell lines [95]. Likewise, since PAD4 regulates gene expression as a corepressor, its dysregulation is thought to be linked to various cancers, such as breast, renal, and colorectal cancer [34, 47]. Chang *et al.* (2009) found that PAD4 protein and mRNA expression levels were significantly increased in more than 12 types of cancers, including breast, lung, liver, esophageal, colorectal, renal, bladder, and ovarian malignant tumors collected from patients [34]. There were elevated levels of PAD4 found in these cancerous tissues, similar to the increased PAD4 levels in the tissues of patients suffering from inflammatory diseases (i.e. RA, UC) [34, 96]. Interestingly, in benign tumors and non-cancerous inflamed tissue samples, PAD4 was not expressed, except tissue samples from the gastrointestinal tract [34]. These higher PAD levels in tumor versus non-tumor tissues suggest that abnormal PAD activity/citrullination can cause a dysregulation of gene expression. When tumor suppressor genes are the targets of PAD4 dysregulation, tumorigenesis can occur. In contrast to results indicating that PAD4 overexpression

upregulates p53-mediated apoptosis, PAD4 has been shown to promote tumorigenesis by suppressing p53 and disrupting the cell cycle and apoptosis [57]. Similarly, PAD4 citrullination of the intermediate filament, cytokeratin, is determined to interrupt apoptosis [57]. Overall, these findings support the premise that PADs promote tumorigenesis.

Alternatively, Stadler *et al.* (2013) reported that PAD4 negatively regulates tumor invasiveness in breast cancer models *in vitro* and *in vivo* [97]. In this study, PAD4 citrullinated glycogen synthase kinase-3β (GSK3β) in MCF-7 breast cancer cells and in a tumor xenograft animal model. Upon PAD4 downregulation, there was an increase in epithelial-to-mesenchymal transition (EMT) and tumor invasiveness [97]. These results indicate a complex role of PAD4 in tumorigenesis with contradicting roles between various cancer types.

5.9. PAD Inhibitors Used in Diseases

In addition to using PAD inhibitors to study PAD biology, many labs that work on models of diseases caused by PAD dysregulation are testing the potency and safety of these inhibitors to treat and prevent their disease of interest. For example, 2-chloroacetamidine was found to reverse the effects of PAD activity in numerous mouse models of MS [98]. Likewise, current inhibitors that show efficacy against PAD2 and especially PAD4 have been tested on models of RA. Excitingly, Cl-amidine, a pan-PAD inhibitor, was found to reduce clinical symptoms and disease severity in an *in vivo* mouse model of RA [99].

The progress made in using PAD inhibitors for the treatment of RA should be translatable to other hyperactive immune and inflammatory diseases associated with PAD dysregulation. As evidence of translation to other diseases, Cl-amidine was found to prevent and treat UC when given in a mouse model of UC [17]. Additionally, Cui *et al.* (2013) reported that Cl-amidine induced the upregulation of several tumor suppressor microRNAs, in HCT116 colon cancer cell line, that are reportedly downregulated in cancers (e.g. miR-16, let-7) [100–103]. Cl-amidine also shows proven efficacy in a multitude of other disease models including: cancer [70, 100, 104], SLE [105], a spinal cord injury model [106], atherosclerosis [107], and hypoxia [108]. Altogether, these findings demonstrate the potential for PAD inhibitors as disease treatments and preventatives.

CONCLUSION

As discussed in this review, the Protein Arginine Deiminases catalyze the citrullination of protein embedded arginine residues, a PTM that has many functional implications on target proteins. Citrullination by PADs affects vital cellular responses involving cell death (via apoptosis and NET-osis), terminal epidermal cell differentiation, and gene regulation. PADs have complex roles in these processes and the particular regulatory mechanisms that control PAD activation/inactivation need to be explored. To better understand the mechanisms and physiological functions of PADs, a series of potent pan-PAD and isozyme-specific inhibitors have been used. Likewise, these PAD inhibitors have been tested on *in vitro* and *in vivo* disease models that are associated with aberrant PAD regulation. Many of these studies have seen success in treatment and/or prevention of diseases including: rheumatoid arthritis, multiple sclerosis, ulcerative colitis, and cancer. Future research on typical PAD

activity and atypical involvement in disease will certainly continue to expand as more encouraging results spotlighting effective PAD regulation are published.

Acknowledgments

This work was supported by National Institutes of Health Grant 1R01CA151304.

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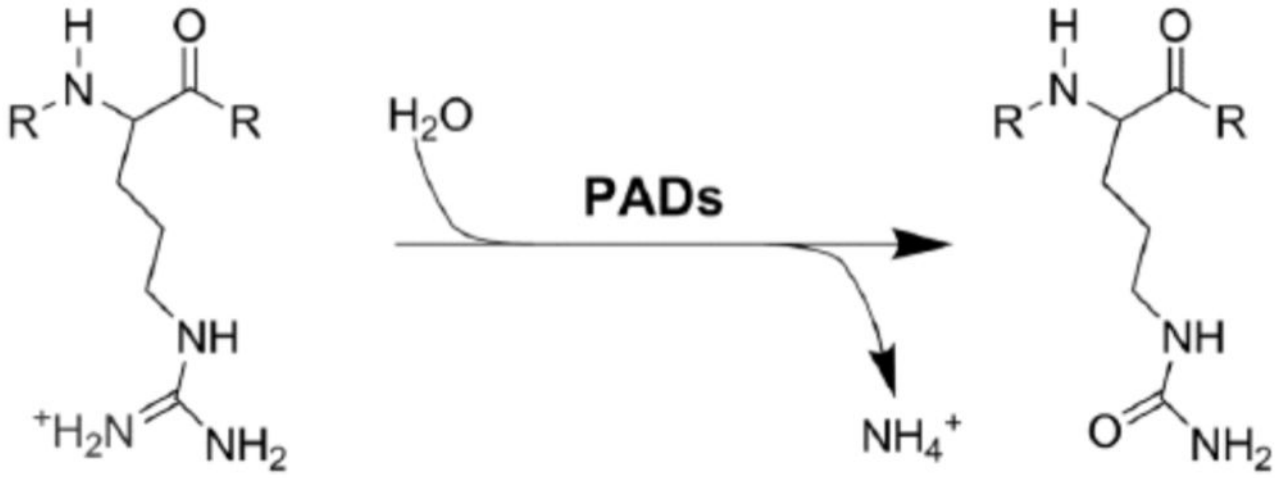

Fig. 1. The process of citrullination

The primary amine group of peptidyl-arginine is hydrolyzed upon interaction with the cysteine of the deiminating enzyme and is replaced to form a keto group. The end products of this reaction are peptidyl-citrulline and free ammonium.

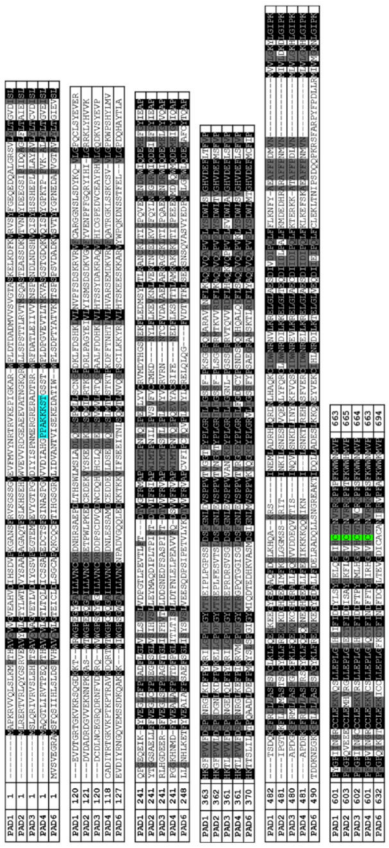

Fig. 2. Human PAD isozyme sequence alignments

Amino acid sequence alignment of the PAD isozymes with approximately aa 1–300 and 301–663 containing the N-terminus and C-terminus domains, respectively. Highlighted in black are amino acids that are conserved in all 5 isozymes, while gray highlighted amino acids are conserved in 4 of the 5 isozymes. In PADs 1–4, the important cysteine residue (Cys645) in the active site is highlighted in green and, in PAD4, the nuclear localization signal (NLS) sequence is highlighted in blue.

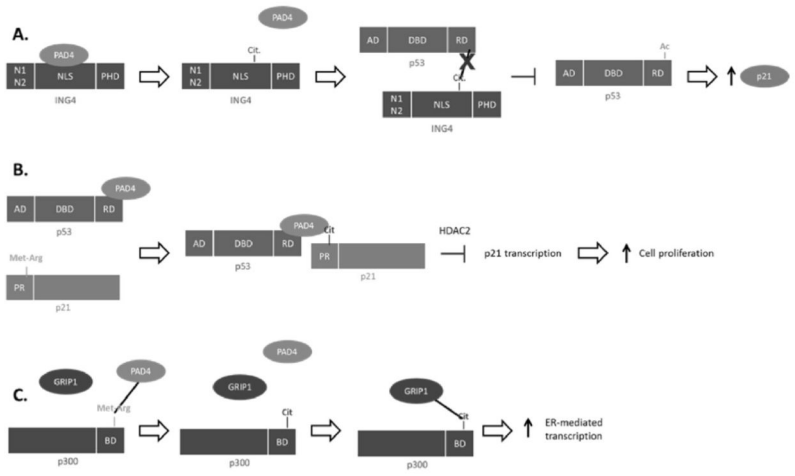

Fig. 3. Mechanisms of gene regulation involving PADs

A) Activated PAD4 citrullinates ING4 at the nuclear localization signal (NLS) region. Therefore, the regulatory domain (RD) of p53 cannot bind to the NLS of ING4, leading to inhibition of p53 acetylation and inhibition of subsequent upregulation of p21. **B)** PAD4 interacts with the regulatory domain of p53. The PAD4 attached to p53 is recruited to the promoter region (PR) of p21 where PAD4 citrullinates methylated arginine on histones at the promoter region. This citrullination, along with HDAC2, inhibits p21 transcription, resulting in an increase in cell proliferation and inhibition of cell cycle arrest. **C)** PAD4 citrullinates methylated arginine on the GRIP1 binding domain (BD) of p300. This leads to increased binding to GRIP1 and results in increased ER-mediated transcription.

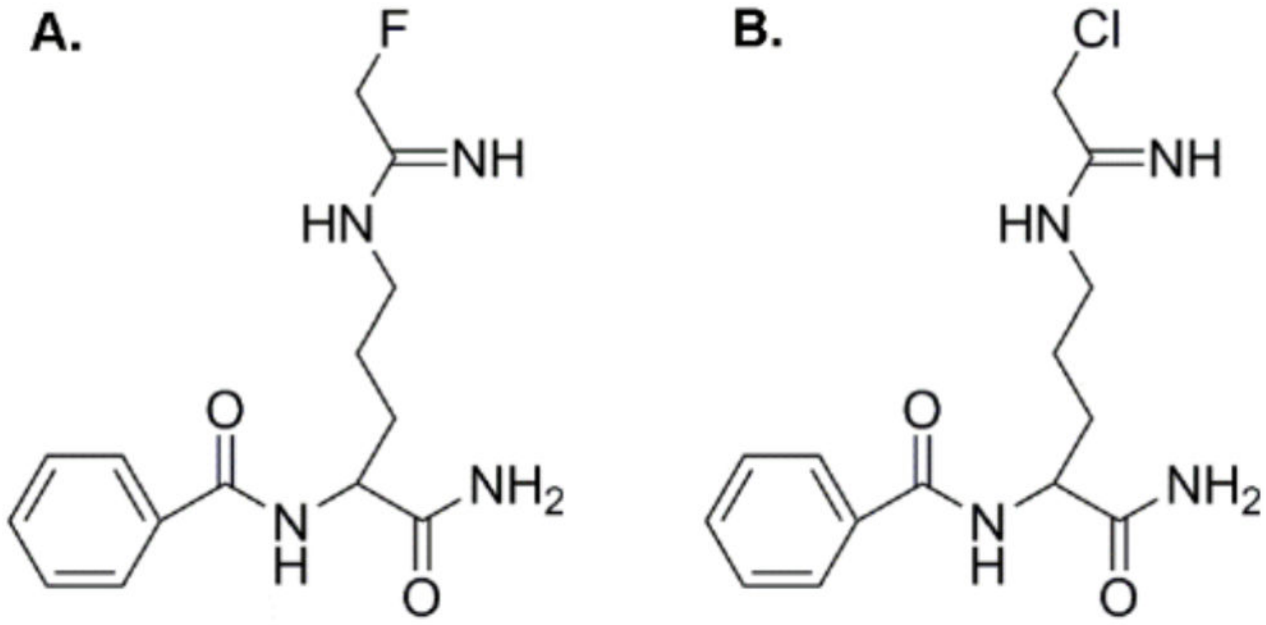

Fig. 4. Structures of PAD inhibitors F-amidine and Cl-amidine

A) F-amidine and **B)** Cl-amidine share similar structures to arginine. Also these compounds are positive H bond donors that can sterically fit in the active site of PADs.

Table 1

PAD target substrates. Known substrates that are targeted by the individual isozymes of the PAD family of isozymes.

Isozyme	Substrates
PAD1	Keratin, filaggrin [7]
PAD2	Myelin basic protein [30], vimentin [42], actin, histones [39]
PAD3	Filaggrin, trichohyalin [7], apoptosis-inducing factor, vimentin [43]
PAD4	Histones [24, 44], ING4 [45], p300 [46], p21 [47], nucleophosmin [48], nuclear lamin C [49]
PAD6	None known