Anthony P. Nicholas
Sanjoy K. Bhattacharya *Editors*

Protein Deimination in Human Health and Disease

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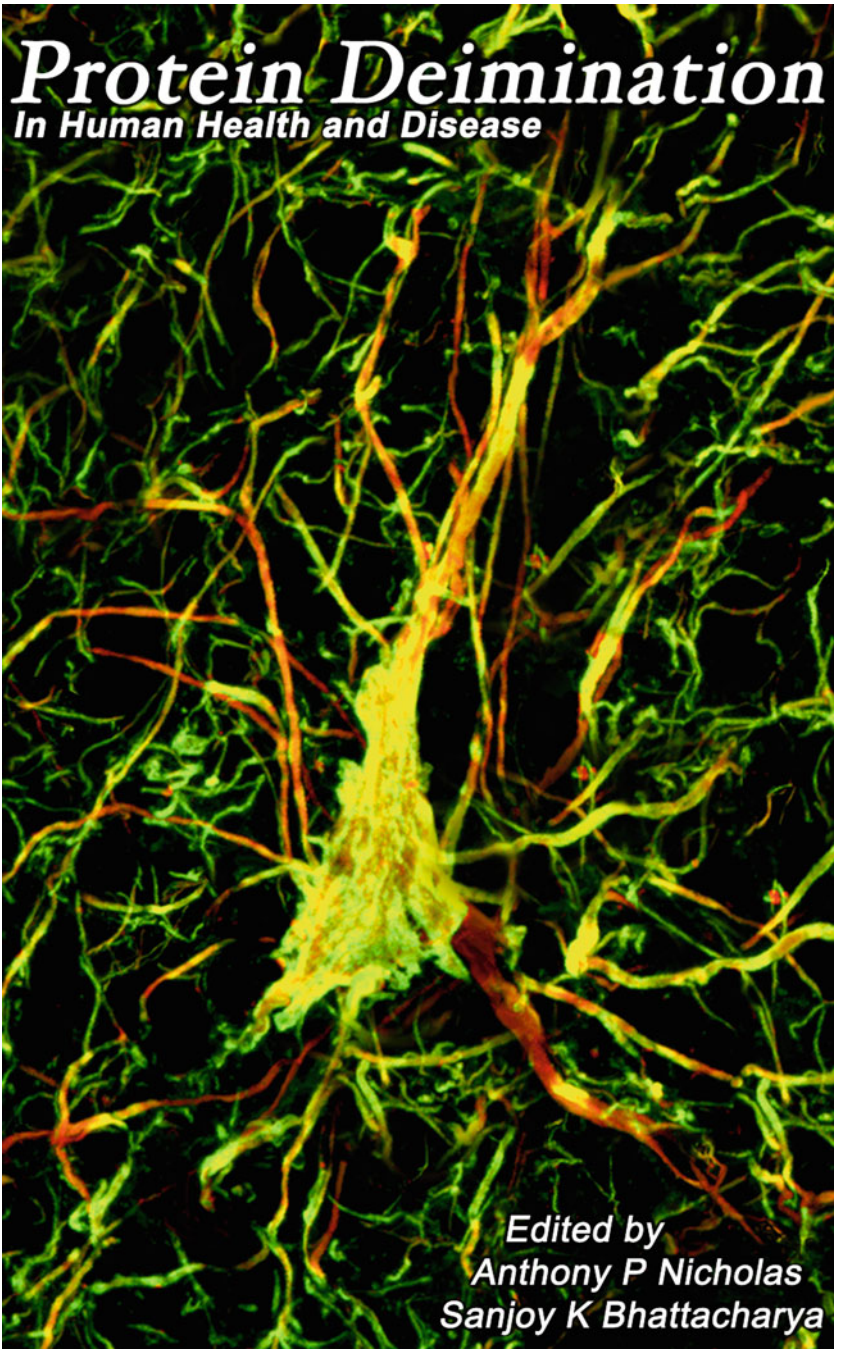
In Human Health and Disease



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Protein Deimination in Human Health and Disease

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Inside Cover: Confocal micrograph of immunofluorescent staining in a normal human cerebellum showing co-localization (*yellow*/*orange*) of glial fibrillary acidic protein (*green*) and deiminated proteins (*red*), using the F95 monoclonal antibody. Modified from Nicholas and Whitaker, Glia, Volume 37, pp. 328–336, Copyright 2002, Wiley-Liss, Inc.

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Professor Mario Moscarello, one of the founders of the deimination field, passed away on Thursday, August 8, 2013, at the age of 83 years, at Toronto Western Hospital, with his family by his side. Mario was a pioneer in myelin research, paving the way for a greater understanding of protein–lipid interactions and the role of post-translational modifications on these interactions throughout his scientific research career of 52 years. At the time of his passing, he was Professor Emeritus at the University of Toronto and Senior Emeritus Scientist at The Hospital for Sick Children. During his career, he supervised more than 80 students from around the world.

Mario received his M.D. degree in 1955 from the University of Toronto and subsequently entered graduate school, obtaining a Ph.D. in biochemistry. His early career involved active research in the biochemistry of amino acids in encephalomyelitis and the encephalomyocarditis virus. Perhaps this foundation, both in amino acid analyses and myelin changes, prepared him for his subsequent discovery of deimination of myelin basic protein.

In 1966, Mario began studying the isolation of acid-soluble proteins from myelin. By 1968, he started an intensive investigation of myelin proteins along with Dr. D. Denise Wood. This led to the discovery of the presence of peptide-bound citrulline in myelin proteins in 1971, coincident with G. E. Rogers' isolation of L-citrulline as a component of proteins from cells in hair fiber medullae and inner root sheaths of hair follicles. Mario showed that citrulline was present in acid hydrolysates of a protein fraction from normal human myelin and in the nonhydrolyzed protein as well, by direct colorimetric analysis. However, stemming from his deep familiarity with biochemistry, he further confirmed the presence of citrulline by protease digestion of myelin, chromatographic separation, and colorimetric confirmation, which was state of the art at that time. This is one of the very first landmark studies that placed L-citrulline within proteins on the map. Arguably and potentially unbeknownst to him this was also one of the early milestones for the field of deimination research.

Dr. Moscarello continued studying myelin, the interaction of myelin proteins with membrane lipids and, in 1976, showed that a nine-peptide sequence derived from myelin basic protein was encephalitogenic, but required more than a linear peptide to induce full encephalitogenic potential. In 1989, he demonstrated the lipid-aggregating properties of citrulline-containing myelin basic protein, another seminal discovery in deimination research. These studies were indicative of an important role for this posttranslational protein in basic biochemical alterations of neuronal membranes. Although Mario's lab had developed an antibody that distinguished citrullinated moieties from arginine, it was during a collaboration with the late John. N. Whitaker (then at University of Alabama at Birmingham) in 1992 that they distinguished the MBP C1 isomer from its less-cationic citrullinated isomers and the least-cationic C8 citrullinated isomer. In 1993, Mario began using the term "deimination" when he discovered the ability of the enzyme peptidylarginine deiminase from bovine brain to citrullinate (convert peptidyl-arginine to peptidyl-citrulline) human myelin basic protein. The discovery of this enzymatic activity was first made by Kubilus and Baden in 1983; however, the activity was never tested for modification of myelin basic protein until it was accomplished in the Moscarello laboratory. Another seminal discovery from Mario's group came in 1994, when they showed that myelin in multiple sclerosis was developmentally immature and highly citrullinated. This was the first report, which was published in the Journal of Clinical Investigation, describing the paradoxical increased deimination in the brains of infants and patients with multiple sclerosis, when compared with normal adults. Mario also showed similarities in posttranslational modification of myelin basic proteins between models of multiple sclerosis and Pelizaeus-Merzbacher disease, thus establishing a possibility of common denominators in different demyelinating disorders.

We would like to think that Professor Moscarello is survived not only by his family, but also by his work, and we believe that advancing the field of deimination research is the best way to keep his memory alive. With that thought, we dedicate this book to Professor Mario Moscarello, a great mentor and teacher who always instilled in his students the importance of leading a full life and to focus on the work at hand. Ironically, he always telegraphed this by referring to a lyric from the old spiritual entitled *Life's Railway to Heaven*, "Keep your hand upon the throttle, and your eye upon the rail." We will miss him dearly.

Preface

Deimination refers to the posttranslational conversion of protein-bound arginine into protein-bound citrulline. It is often interchangeably termed as "citrullination," which may also refer to the conversion of free arginine into citrulline. As a result, we have promoted the use of the word "deimination" to exclusively refer to the posttranslational modification (PTM) of protein-bound arginine for ameliorating some confusion for new investigators or researchers from other fields.

Despite being a relatively long-known PTM in mammals and other organisms, deimination has not been subject to rigorous research that some other PTMs have received, such as phosphorylation and glycosylation. Even sumoylation, a relatively newly discovered PTM, has about ten times more recorded published papers today. Currently, for a modification such as phosphorylation there are 10,000-fold more published papers, compared to deimination. In recent major PTM meetings, deimination either records no or only a token presence. For example, during the recent ASBMB-conducted PTM meeting in 2012, deimination was represented only by a single poster.

Two advances are expected to accelerate the pace of research on deimination: (a) the discovery of deiminated proteins with direct relationships to human disease and (b) the development of new reagents for assessment and quantification of deiminated proteins.

Usually the functions of a protein and its involvement in key biological processes spark interest in that protein, especially if a PTM is found to regulate the role of the protein in question. Unfortunately, early detection of deimination occurred in proteins that were primarily structural, during a time in which the study of structural cellular proteins was thought not to be particularly exciting. Although the first deiminated proteins were described in the late 1950s, almost 20 years went by before the enzymes responsible for this PTM, the peptidyl-arginine deiminases (PADs), were first discovered and later confirmed in almost all tissues of the human body.

Although PAD was found in the brain as early as the 1980s, showing to deiminate myelin structural proteins, the largest influx of researchers into the field up until that point did not occur until the late 1990s, after a direct association was found between the presence of deiminated proteins and the occurrence of rheumatoid arthritis. In fact, this disease is now primarily confirmed with a blood test that measures the amount of antibodies against deiminated proteins. As a result, the first chapters (Chaps. 1–6) of this book are dedicated to this topic, covering clinical aspects, the importance of anti-peptidyl-citrulline antibodies, and the roles of gum disease, smoking, and white blood cells themselves in the propagation and detection of this disorder.

Closely related to the joint, deimination is also involved in other related tissues, such as skin (Chap. 7) and hair (Chap. 8), playing important roles in the outer protection of the human body. The next eight chapters are dedicated to the nervous system, including the role of deimination in peripheral nerve development and responses to damage (Chap. 9). Also included are inflammatory diseases of the brain, such as multiple sclerosis (Chaps. 10 and 11), and neurodegenerative diseases, such as Creutzfeldt-Jakob disease (Chap. 12), Alzheimer's disease (Chap. 13), Parkinson's disease, amyotropic lateral sclerosis, and others (Chap. 14). Also included in the central nervous system is the spinal cord (Chap. 15) and eye (Chap. 16), in which deimination has been linked to several normal processes, as well as disease states.

Recently, increased PAD has been linked to cancer (Chap. 17). But probably the most interesting discovery within the last few years has been the role of deimination as a possible reverser of arginine methylation involved in epigenetic processes controlling the transcription of DNA (Chap. 18), since this mechanism may have ramifications for all of the prior normal processes and disease states linked to deimination. Thus, understanding the place of deimination vis a vis methylation on arginine residues of histone proteins that control the unwinding of the genetic code may be of immense biological significance.

On the other hand, confirmatory detection of deimination still remains a challenge. A rate-limiting step exists with the availability of reliable reagents and methods that enable verifiable detection of this PTM. Compounded with limitations in detection are problems with localization of peptidyl-citrulline moieties, which will need some additional development. For example, a current review on mass spectrometric methods used in this regard summarizes the current state of confirmatory detection (Chap. 19). Also, confounders such as the presence of peptidyl-homocitrulline, a PTM of lysine, must be acknowledged and accounted for, when studying deimination (Chap. 20). However, the most exciting and latest advancement in deimination research is the development and use of the first wave of PAD antagonists (Chap. 21), which is further highlighting how this PTM may be manipulated as new therapeutic interventions for a vast variety of human diseases in which increased deimination is believed to play a critical role. As evidenced in this book, teams of chemists, biologists, engineers, neuroscientists, and physicians have come together, with the promise of integrated collaboration that will hopefully prompt the development of new reagents and methods, as well as possible new treatments for devastating diseases that presently have few therapeutic options.

Ultimately, understanding how protein deimination is involved in human health and disease will hopefully be the focus of a new wave of investigators who will join us in uncovering the secrets of these altered proteins. As a first step, this book summarizes our current knowledge of this exciting and growing research field.

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Contents

Chapter 1
Physiological Pathways of PAD Activation and Citrullinated Epitope Generation

Amanda S. Rohrbach, Sanja Arandjelovic, and Kerri A. Mowen

Keywords Peptidylarginine deiminase • PAD • Calcium • Citrullination • Deimination • Disease • Rheumatoid arthritis

1.1 The Peptidylarginine Deiminase Family

The free amino acid form of citrulline was first isolated from watermelon (*Citrullus vulgaris*) over 70 years ago (Curis et al. 2005), while the peptidyl form of citrulline was first recognized within the hair follicle (Rogers 1962). Peptidylcitrulline is a noncoding amino acid that is generated through hydrolysis of peptidyl-arginine residues by Ca^{2+}-dependent peptidylarginine deiminase (PAD) enzymes, with ammonia released as a reaction by-product (Fig. 1.1). This process is referred to as deimination or citrullination. The conversion of arginine to citrulline results in only a small increase in molecular mass (less than 1 Da) but also converts the positively charged guanidino group on an arginine residue into the neutrally charged ureido group on the citrulline amino acid. The small mass difference between arginine and citrulline residues has made identifying sites of deimination challenging, especially on proteins isolated from cellular sources (Hao et al. 2009).

Although an approximate 1 Da change in mass may seem like a relatively minor difference, the conversion of charge from an arginine to a citrulline can have dramatic consequences on protein structure, proteolytic susceptibility, and protein–protein interactions (Vossenaar et al. 2003). For example, filaggrin is a highly basic

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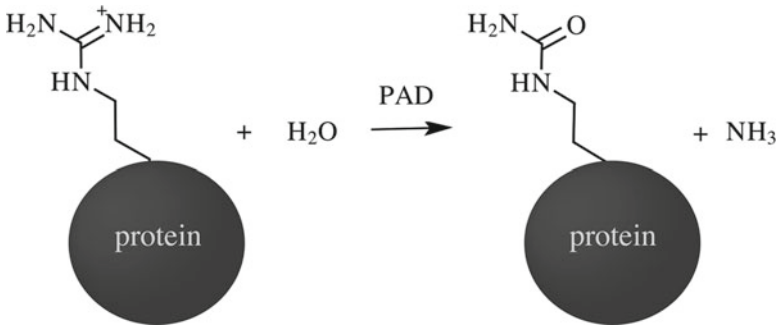

Fig. 1.1 The PAD enzymes catalyze hydrolytic deimination of protein substrates leading to the formation of peptidyl-citrulline and the release of ammonia

epidermal protein, essential for barrier function, and is first synthesized as a larger pro-filaggrin protein (Smith et al. 2006; Palmer et al. 2006). Deimination of filaggrin by skin-resident PAD proteins facilitates its proteolytic processing into free amino acids and other derivatives that make up "natural moisturizing factor," a mixture of natural hygroscopic agents that maintain epidermal hydration (Chavanas et al. 2006; Kamata et al. 2009). Additionally, citrullination could alter substrate receptivity to other posttranslational modifications, since arginine residues can be found within many enzyme consensus motifs (Papin et al. 2005). For instance, conventional protein kinase C enzymes can phosphorylate substrates containing serine or threonine with an arginine residue at the −3, −2, and +2 positions (Nishikawa et al. 1997). Thus, one could imagine that conversion of a consensus site arginine into a citrulline could impact a protein's posttranslational modification landscape. Not surprisingly, conversion of arginine residues to citrulline prevents methylation by members of the protein arginine methyltransferase (PRMT) family (Cuthbert et al. 2004; Wang et al. 2004). Finally, DNA-binding domains are often rich in positively charged arginine residues (Crane-Robinson et al. 2006), and therefore, citrullination could also regulate the association of transcription factors with their DNA response elements.

Since citrullination can lead to profound changes in protein function, it is not surprising that citrullination and the PAD enzymes have been implicated in numerous diseases. PAD enzymes and citrullination have been associated with rheumatoid arthritis (RA), multiple sclerosis, Alzheimer's disease, inflammatory bowel disease, psoriasis, and cancer (Gyorgy et al. 2006; Chumanevich et al. 2011). This chapter provides a general overview of the PAD enzymes and their known functions. In addition, we discuss possible physiological pathways that may contribute to the generation of citrullinated self-antigens, which could then prime the development of anti-citrulline peptide autoantibodies (ACPA) in RA.

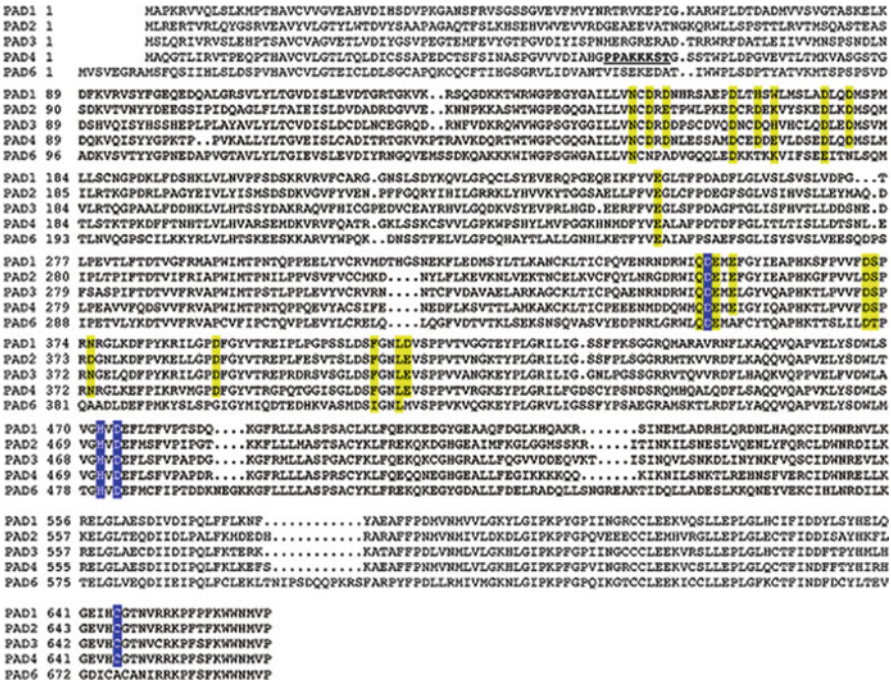

Fig. 1.2 Sequence alignment of the human PAD isozymes. Multiple sequence alignment of the human PAD amino acid sequences was created using ClustalW software (Thompson et al. 1994), and Strap (www.bioinformatics.org/strap) was used for the alignment layout. Calcium-binding residues and catalytic residues identified in the PAD4 crystal structure are highlighted in *yellow* and *blue*, respectively (Arita et al. 2004). The PAD4 nuclear localization sequence in *underlined* and in *bold* type

1.1.1 Introduction to the PAD Family

The enzymatic activity capable of generating peptidyl-citrulline was first identified in hair follicle extracts by Rogers and Taylor (Rogers and Taylor 1977). Since this discovery, five PAD enzymes (1,2,3,4/5,6), which are encoded by the *PADI* loci, have been identified in mammals, and they exhibit fairly high amino acid sequence homology (~41–55 %) (Fig. 1.2) (Chavanas et al. 2004). Mammalian *PADI* genes are co-localized within a single gene cluster, spanning a region of about 355 kb in length, on chromosome 1 in humans and on chromosome 4 in mice (Zhang et al. 2004). PAD5 was identified in mice and was later revealed to be the orthologue of human PAD4 (Vossenaar et al. 2003). Paralogues of the PAD enzymes appear in birds, amphibians, and bony fish. Phylogenetic analysis suggests that the ancestor of the *PADI* locus appeared in the last common ancestor shared by teleosteans and mammals (Balandraud et al. 2005). The genome of the bacterium *Porphyromonas*

gingivalis, a pathogen associated with periodontitis, also encodes a PAD enzyme, although the *P. gingivalis* PAD or PPAD appears to be evolutionarily unrelated to eukaryotic PAD enzymes (Shirai et al. 2001).

1.1.2 Regulation of PAD Activity

Members of the PAD family require high Ca^{2+} concentrations for their activity (Gyorgy et al. 2006). Not surprisingly then, treatment of cells with the Ca^{2+} ionophores, such as ionomycin, can induce the production of peptidyl-citrulline (Vossenaar et al. 2004; Nakashima et al. 2002). The divalent calcium cation requirement is specific, because other metal ions were unable to substitute for Ca^{2+} in an in vitro PAD activity assay where deimination of the arginine derivative *N*-α-benozyl-L-arginine ethyl ester (BAEE) was monitored by colorimetric change (Kearney et al. 2005). However, relatively little is known about physiological stimuli that induce this calcium-dependent PAD activity. Structural analysis of the PAD4 enzyme revealed five Ca^{2+}-binding sites that are fairly conserved amongst all PADs except PAD6 (Fig. 1.2) (Mechin et al. 2007; Arita et al. 2004). Binding of Ca^{2+} leads to a conformation change, moving the key catalytic C645 residue to the enzyme active site, where it is thought to exert a nucleophilic attack on the guanidium carbon atom of the target arginine (Arita et al. 2004). The thiolate anion C645 is essential for PAD4 activity because mutation of this residue is sufficient to abolish enzymatic activity (Knuckley et al. 2007). Notably, the haloacetamidine-bearing PAD inhibitors F- and Cl-amidine covalently bind to C645 and act as irreversible PAD inhibitors (Knuckley et al. 2007). Using in vitro studies, the optimal Ca^{2+} concentration for PAD2 and PAD4 is within the high micromolar to millimolar range (Kearney et al. 2005; Nakayama-Hamada et al. 2005). This level is far higher than the micromolar levels achieved following the opening of calcium release-activated channels (CRAC) after T cell receptor ligation, even higher than the sub-micromolar homeostatic intracellular Ca^{2+} levels (Feske 2007). Since PAD proteins require high micromolar levels of Ca^{2+}, one proposal is that cellular apoptosis exposes PAD proteins to high levels of extracellular Ca^{2+}, which promotes PAD activity. However, apoptosis alone is not sufficient to induce PAD activity, as the apoptosis-inducing agents camptothecin and staurosporine do not induce peptidyl-citrulline generation (Neeli et al. 2009). Perhaps, the PAD enzymes localize to a microenvironment within cells that has access to very high Ca^{2+} levels. Another possibility is that posttranslational modification of PAD enzymes or the association of the PAD enzymes with accessory proteins could lower the Ca^{2+} threshold requirement for enzymatic activity.

The expression pattern of mammalian PAD family members is fairly tissue restricted, suggesting that PAD expression is tightly controlled (see below and Fig. 1.3) (Vossenaar et al. 2003). However, a few studies have addressed the transcriptional regulation of the *PADI* genes. For example, PAD1 and PAD3 are both expressed in human skin and hair follicles (Mechin et al. 2007). PAD1 expression in normal human epidermal keratinocytes is regulated by Sp1, a transcription factor

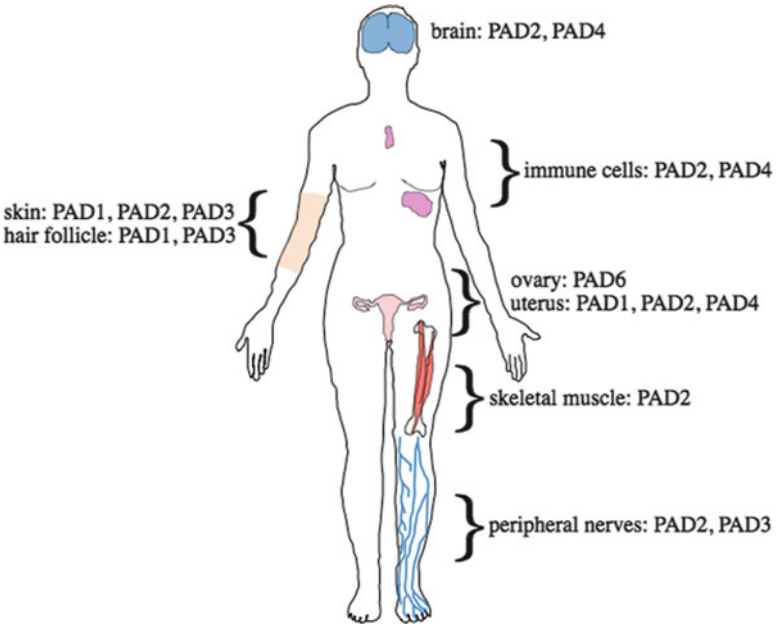

Fig. 1.3 Tissue distribution of the PAD isozymes. The expression patterns of the mammalian *PADI* genes in the brain, skin, hair follicle, immune cells (thymus and spleen, in *pink*), ovary, uterus, skeletal muscle, and peripheral nerves are depicted

known to regulate many genes, and by MZF1, a transcription factor with enriched expression in differentiated keratinocytes (Dong et al. 2008; Kamata et al. 2011). The transcription factors Sp1, Sp3, and NY-1 bind to the *PADI3* promoter and regulate its expression (Dong et al. 2006). PAD2 is expressed in many tissues and organs and is the most widely expressed mammalian PAD member (Vossenaar et al. 2003). In human keratinocytes, expression of the *PADI2* gene is controlled by a minimal GC-rich promoter, which is occupied by the ubiquitous transcription factors Sp1 and Sp3 (Dong et al. 2005). PAD4 expression is largely restricted to immune cells, especially granulocytes, but has also been found in some tumor lines (Jones et al. 2009). Treatment of the MCF-7 breast cancer cell line with estrogen leads to increased levels of the PAD4 protein (Cuthbert et al. 2004). Furthermore, estrogen-induced expression of PAD4 is regulated by cooperative binding of the AP-1, Sp-1, Sp-3, and NF-Y transcription factors to the PAD4 minimal promoter (Dong et al. 2007). The addition of granulocyte-inducing differentiation agents of dimethyl sulfoxide, retinoic acid, and vitamin D_3 leads to the appearance of peptidyl-citrulline after several days in culture, but little is known about the factors that induce PAD4 expression in immune cells (Nakashima et al. 1999, 2002). PAD6 transcripts are primarily found in oocytes and embryos (Horibata et al. 2012), and the oocyte-specific, homeobox-containing transcription factor Nobox is critical for expression of PAD6 (Choi et al. 2010). One caveat to studies that focus on the expression of

PADI transcripts is that, in some cell types, a disassociation has been occasionally observed between a particular PAD transcript and its protein levels (Vossenaar et al. 2004; Mechin et al. 2010). For example, PAD2 message was found in monocytes and macrophages, but the PAD2 mRNA appeared to be translated only in macrophages (Vossenaar et al. 2004). Conversely, the PAD4 protein was found in macrophages, but PAD4 mRNA was not expressed in detectable levels in these cells (Vossenaar et al. 2004).

Many PAD substrates have been described, including keratins, filaggrin, vimentin, myelin basic protein, fibrinogen, chemokines (CXCL8, CXCL10, CXCL11, CXCL12), p300, and histones (Jones et al. 2009). PAD1, PAD2, PAD3, and PAD4 can also auto-deiminate, which impairs their activity, at least when detected by in vitro assays (Mechin et al. 2010; Andrade et al. 2010). However, only a handful of studies have addressed PAD-mediated substrate recognition. Although PAD family members display a great degree of sequence similarity, direct comparison of PAD2, PAD3, and PAD4 enzymes using HL-60 lysates revealed that each enzyme has distinct substrate preferences (Darrah et al. 2012). Only PAD4 was able to citrullinate histone H3, while PAD2 was able to citrullinate β/γ actin (Darrah et al. 2012). Analysis of sites of PAD2 citrullination on β actin did not reveal a strict consensus sequence for citrullination, but arginines flanked by proline residues were not favored (Darrah et al. 2012). Indeed, the amino acids immediately flanking the arginine appear to be critical in determining whether an arginine can be citrullinated. Sequences containing proline residues adjacent to the target arginine are also unfavored for citrullination by PAD4 (Stensland et al. 2009). Interestingly, systematic examination of fillagrin- and histone H3-derived peptides to reveal favorable and unfavorable amino acid substitutions surrounding the PAD4 target arginine residue yielded a list of some overlapping, but also many non-overlapping, amino acids (Stensland et al. 2009). Co-crystallization of PAD4 with histone H3-1, H3-2, and H4 peptides demonstrated that PAD4 preferred sequences with a highly disordered conformation because the binding of PAD4 to these histone peptides induces a β-turn-like conformation (Arita et al. 2004). Overall, PAD4 has a broad sequence specificity, with a proposed consensus sequence of ϕXRXX, where ϕ denotes amino acids with a small side chain and X denotes any amino acid (Arita et al. 2004). Further studies to define the PAD consensus sites will be useful to identify new PAD substrates and to understand the physiological functions of the PAD enzymes.

1.1.3 Cross Talk Between PADs and Protein Arginine Methyltransferases

Arginine residues can also be subject to posttranslational modification by members of the PRMT family. PRMTs catalyze the addition of a methyl group from *S*-adenosylmethionine to guanidino nitrogen atoms on arginine residues (Bedford 2007). Three types of PRMTs have been subclassified based on the symmetry of

their reaction products. Type I PRMTs (1,3,4—also known as Carm1—6,8) catalyze asymmetric methylation of arginine residues, and type II PRMT5 catalyzes symmetric transfer of methyl groups to arginine residues (Krause et al. 2007). Both type I and type II PRMTs catalyze mono-methylation as a reaction intermediate. The type III PRMT7 catalyzes arginine mono-methylation as its end product (Zurita-Lopez et al. 2012). Modification of arginine residues by both PRMT and PAD family members suggests an intimate regulatory relationship between the two families. Interestingly, PRMT1 has been identified as a possible PAD4 substrate using protein arrays (Guo et al. 2011). Arginine methylation had long been thought to be a permanent posttranslational modification, since no demethylase had been identified. Two groups independently determined that, in addition to arginine, PAD4 could also target mono-methylated arginines on histone proteins from cellular sources. Arginine dimethylation appeared to be a more stable modification, as it is protective against PAD activity (Cuthbert et al. 2004). However, PAD4 acting as a demethylase has been called into question because chemically synthesized mono-methylated histone peptides containing potential target arginines are very poor substrates for PAD4 using in vitro assays (Kearney et al. 2005; Hidaka et al. 2005). Perhaps, the conversion of mono-methyl arginine to citrulline by PAD enzymes is context dependent, requiring associated proteins or additional posttranslational modifications to occur efficiently.

1.2 General Overview of the PAD Family Members

1.2.1 PAD1

PAD1 is cytoplasmic and expressed in all living layers of the epidermis (see Chap. 7), with graded expression increasing in intensity from the basal layer to the granular layer (Nachat et al. 2005a). In hair follicles (see Chap. 8), PAD1 is expressed in the cuticle and in the inner root sheath (Nachat et al. 2005b). Due to its localization, PAD1 is thought to be the primary enzyme responsible for filaggrin and keratin K1 citrullination in the skin epidermis (Senshu et al. 1996). The loss of charge due to citrullination leads to disassembly of the cytokeratin–filaggrin complex and facilitates break down of filaggrin to form "natural moisturizing factor" and maintain epidermal hydration (Chavanas et al. 2006; Kamata et al. 2009). PAD1 is linked to skin disease, because keratin K1 exhibits decreased citrullination levels in the epidermis of psoriasis patients (Ishida-Yamamoto et al. 2000).

1.2.2 PAD2

Of the five PAD enzymes, PAD2 is the most broadly expressed isoform (Vossenaar et al. 2003). PAD2 is also known as "skeletal muscle PAD," since it was first

isolated in large quantities from rabbit skeletal muscle (Takahara et al. 1986). PAD2 mRNA and protein have been detected within the skin epidermis, peripheral nerves, and several hematopoietic cell types (Vossenaar et al. 2004; Ying et al. 2009; Nagata and Senshu 1990; Keilhoff et al. 2008). Genes regulated by the NFκB pathway are involved in a wide range of physiological responses, including cell death, developmental processes, and inflammation (Perkins 2007). PAD2 has also been shown to target IKKγ, a kinase upstream of NFκB activation, and citrullination of IKKγ seems to suppress NFkB activation (Lee et al. 2010). Although PAD2 is expressed across many tissue types and has been shown to regulate NFκB-mediated signal transduction, it is important to note that PAD2 knockout mice are viable and fertile (Raijmakers et al. 2006), as verified by our own unpublished observations.

PAD2 is also expressed in tissues of the reproductive system, including the uterus, pituitary gland, and mammary epithelial cells (Cherrington et al. 2012; Senshu et al. 1989; Takahara et al. 1992). Although PAD2 has been described as a cytoplasmic protein, a fraction of PAD2 was also found to reside in the nucleus of mammary epithelial cells, where it is associated with chromatin and was discovered to citrullinate histone H3 at arginine position 26 (Cherrington et al. 2012; Zhang et al. 2012) (Nakashima et al. 2002). Interference with PAD2 expression using siRNA in the MCF-7 breast cancer cell line impairs estrogen receptor α (ERα)-driven gene expression, supporting the notion that PAD2 facilitates ERα-driven transcription (Cherrington et al. 2012; Zhang et al. 2012). Interestingly, high concentrations (millimolar amounts) of the cancer chemotherapeutic agent paclitaxel, which is used to treat many neoplasms including breast and ovarian cancers, can inhibit the enzymatic activity of PAD2 (Pritzker and Moscarello 1998). In fact, MCF-7 cells transfected with PAD2 siRNA exhibited a reduced proliferation rate in comparison to control cells (Cherrington et al. 2012). These studies suggest that PAD2 may be a target candidate for anticancer therapies. (See Chap. 17 for more information on this topic.)

Protein citrullination in the brain and spinal cord is a hallmark of multiple sclerosis (MS) (Gyorgy et al. 2006) and in the murine model of MS known as experimental autoimmune encephalomyelitis (EAE) (Kidd et al. 2008). In EAE and MS, MBP and glial fibrillary acidic protein (GFAP) are hypercitrullinated (Raijmakers et al. 2006; Nicholas et al. 2004; Moscarello et al. 2002). The current model for the role of citrullination in EAE and MS is that citrullination of the myelin protein components interferes with their association thought to directly contribute to myelin instability and degradation (Gyorgy et al. 2006). PAD activity is up-regulated in DM20 transgenic mice expressing extra copies of the myelin proteolipid protein DM20, and these mice develop spontaneous demyelination (Moscarello et al. 2002). Expression of PAD2 has also been documented in the CNS, including microglia (Asaga et al. 2002), astrocytes (Asaga et al. 2002; Sambandam et al. 2004), and oligodendrocytes (Akiyama et al. 1999). Indeed, PAD2 transgenic mice overexpressing PAD2 under the MBP promoter exhibit increased MBP citrullination and spontaneous demyelinating disease (Musse et al. 2008). While protein citrullination in the CNS of EAE animals is entirely dependent on the presence of PAD2, PAD2-deficient mice remain susceptible to EAE (Raijmakers et al. 2006). These

findings indicate that the role of PAD2 in EAE and MS is likely complex, and possibly, in the absence of PAD2, other PAD family members, like PAD4, can participate in disease pathogenesis. A more detailed discussion on the potential role of deimination in MS and EAE is presented in Chaps. 10 and 11.

1.2.3 PAD3

PAD3 expression is principally limited to the medullary and inner root sheath of the hair follicle with a localization overlapping that of its substrate trichohyalin (THH) (Nachat et al. 2005b). THH is a high-molecular-weight, α-helix-rich, structural protein of the hair follicle (see Chap. 8). After it is first synthesized, THH resides within soluble vacuoles, where it is stabilized by interactions between its α-helices (Gyorgy et al. 2006). Citrullination of THH is thought to promote its solubility and facilitate its cross-linking with cytokeratins and other THH molecules by transglutaminase, leading to directional hair growth (Gyorgy et al. 2006). PAD3 is also found in the granular and lower stratum corneum of the skin epidermis (see Chap. 7), where it may serve to collaborate with PAD1 to citrullinate filaggrin (Nachat et al. 2005a). Finally, the PAD3 transcript and protein are found in cells of the peripheral nervous system (Keilhoff et al. 2008) (see Chap. 9).

1.2.4 PAD4

PAD4 has been implicated in regulating inflammation (Nakayama-Hamada et al. 2005; Foulquier et al. 2007) and exhibits an expression pattern largely restricted to immune cell types, particularly macrophages and granulocytes (Foulquier et al. 2007; Asaga et al. 2001). Indeed, the addition of the granulocyte-inducing differentiation agents such as dimethylsulfoxide (DMSO) to the human promyelocytic HL-60 cell line leads to the acquisition of mature neutrophil properties and to the expression of PAD4 (Foulquier et al. 2007). Importantly, animals homozygous for the PAD deletion are viable, are fertile, and have no gross anatomical abnormalities (Hemmers et al. 2011). In addition, we have observed no perturbations in the development of any of the analyzed immune subsets in the absence of PAD4 (our unpublished observations).

Neutrophils are a critical component of the innate antimicrobial immune response (Nathan 2006; Borregaard 2010). The primary mission of the neutrophils is to seek and destroy pathogens. Upon recruitment to the site of infection, neutrophils can kill invading pathogens by phagocytosis, by release of preformed microbicidal granules, and by generation of reactive oxygen species (Flannagan et al. 2009; Nauseef 2007). Alternatively, neutrophils can kill extracellular pathogens by weaponizing their nuclear contents and releasing neutrophil extracellular traps (NETs) (Brinkmann et al. 2004).

NET structures are composed of decondensed chromatin decorated with antimicrobial mediators such as defensins, histones, neutrophil elastase, and myeloperoxidase (Urban et al. 2009; Wartha et al. 2007). In response to inflammatory stimuli, neutrophils can decondense their chromatin and actively expel their DNA, producing NETs that are decorated with granular and nuclear proteins, including citrullinated histones (Brinkmann and Zychlinsky 2007; Wang et al. 2009). Work from our lab and others have shown that PAD4 is essential for the production of NETs and NET-associated histone citrullination (Hemmers et al. 2011; Wang et al. 2009; Buono et al. 2005). In contrast, neutrophil phagocytic and chemotactic responses are unimpaired in PAD4-deficient mice (Hemmers et al. 2011; Li et al. 2010). PAD4-mediated histone citrullination is a hallmark of NET formation and is thought to play a mechanical role in NET configuration, where the conversion of positively charged arginine residues into the neutral citrulline amino acid by PAD4 promotes chromatin decondensation (Wang et al. 2009). PAD4-mediated NET formation is critical for controlling at least a subset of bacterial infections, because PAD4-deficient mice are more susceptible to infectious disease in a necrotizing fasciitis model (Buono et al. 2005). Through an unknown mechanism, the formation of NETs is coupled with the phagocytosis pathway because both myeloperoxidase and neutrophil elastase are essential for NET formation, though their exact function in NET formation is unclear. NET-mediated killing has been described for gram-positive and gram-negative bacteria as well as fungi. Targets include *Staphylococcus aureus*, Group A streptococci, *Salmonella enterica*, and *Candida albicans* (Brinkmann et al. 2004; Urban et al. 2009; Ermert et al. 2009). Proteomic studies have identified that PAD4 can be found in neutrophil-specific granules (Lominadze et al. 2005); however, it is still not known if PAD4 also contributes to neutrophil function and NET generation through other substrates.

Using in vitro studies, incubation of neutrophils with PMA, ionomycin, H_2O_2, lipopolysaccharide (LPS), and bacteria induces PAD4-mediated histone citrullination and NET formation (Hemmers et al. 2011; Wang et al. 2009; Buono et al. 2005; Neeli et al. 2008). Additionally, histone citrullination is sensitive to NADPH inhibitors, suggesting that PAD4 activation is downstream of ROS generation, but the precise signaling pathways by which ROS potentially activates PAD4 are unknown (Neeli et al. 2009; Denis et al. 2009). In contrast to published reports, we have been unable to demonstrate consistently that LPS stimulation induces PAD4 activity in murine bone marrow-derived neutrophils (BMDN) (Fig. 1.4), which is in line with several other studies reporting that LPS is incapable of inducing NET formation (Clark et al. 2007; Remijsen et al. 2011). We also detected citrullinated histone H4 in BMDN that were purified by gradient separation, but LPS stimulation did not increase histone H4 citrullination over the unstimulated, Ca^{2+}-incubated control sample (Fig. 1.4a). We speculated that the histone H4 citrullination we detected in BMDN, isolated using density gradient separation, was linked to the purification process. Thus, we purified BMDN using a gentler, magnetic bead separation method, where we could isolate largely untouched neutrophils via negative selection. Using these cells, PMA/ionomycin stimulation induced histone H4 citrullination, but LPS did not (Fig. 1.4b). To insure that our LPS preparation was properly

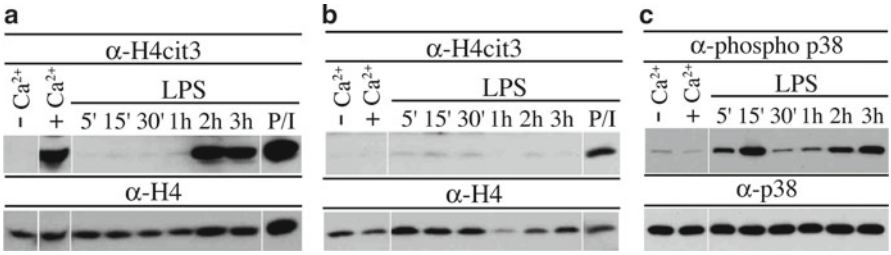

Fig. 1.4 LPS stimulation alone is not sufficient to induce PAD4-mediated histone H4 citrulline 3 generation in murine neutrophils. Murine bone marrow-derived neutrophils (BMDN) were isolated by density gradient sedimentation (**a**) or by negative selection using magnetic bead separation (**b**) and (**c**). BMDN were stimulated with LPS (1 μg/mL) in the presence of 2 mM of $CaCl_2$ in Lock's buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 0.1 % glucose) for the indicated times. As a control, cells were incubated in Lock's buffer in the absence ($-Ca^{2+}$) or in the presence ($+Ca^{2+}$) for 3 h. Cells were stimulated with PMA/ionomycin (P/I) as a control. (**c**) The activation of the TLR4 signaling pathway by LPS was determined by examining the lysates from (**b**) for phospho-p38

stimulating our neutrophils, we examined the activation of p38 by immunoblotting for phospho-p38 (Fig. 1.4c). These results do differ from previous studies, in terms of culture conditions and neutrophil populations used. In an earlier study with murine BMDN, the untreated negative control was prepared just after cell separation rather than incubating the untreated control samples in the presence of $CaCl_2$ for the entire culture period, as we have done (Li et al. 2010). Radic and colleagues performed their studies on LPS-induced histone citrullination using human neutrophils isolated from peripheral blood (Neeli et al. 2008, 2009). It is important to note that there are differences in the efficiency and kinetics of NET formation in human and murine neutrophils (Ermert et al. 2009). Thus, it is important to regard the sample preparation and stimulation conditions when comparing the results from different studies on LPS-stimulated neutrophils and histone citrullination. Perhaps, LPS binding to TLR4 provides a second signal that, by itself, is not sufficient to activate PAD4 in neutrophils. As a result, our findings suggest that another innate immunity sensor may be more relevant for activation of PAD4 in neutrophils.

PAD4 is unique in that it contains a classical nuclear localization sequence (NLS) and therefore can be found primarily within the nucleus, where it is a well-documented transcriptional regulator (Nakashima et al. 2002; Jones et al. 2009). In fact, PAD4 has been shown to associate with the transcriptional regulators HDAC1 and p300/CBP proteins (Denis et al. 2009; Lee et al. 2005). In general, PAD4 and PAD4-mediated histone deimination have been linked with transcriptional repression (Cuthbert et al. 2004; Wang et al. 2004). However, the interaction between PAD4 and p300 may also promote p300 activity and presumably enhance transcription (Jones et al. 2009). PAD4 is also recruited by p53 to repress the expression of select p53 target genes, and inhibition of PAD4 by chemical inhibitors or depletion via siRNA leads to cell cycle arrest and apoptosis (Yao et al. 2008; Li et al. 2008). These results and the expression of PAD4 in many tumor cells have led some to

speculate that PAD4 may be a target for cancer therapeutics (Chang and Han 2006; Slack et al. 2011). The potential regulation of cellular transformation is likely complex. Indeed, PAD4 also targets the tumor suppressor and p53-binding partner inhibitor of growth 4 (ING4). Citrullination of ING4 increases its susceptibility, thereby inhibiting p53-driven gene expression. Thus, more investigation will be necessary to understand the function of PAD4 in cellular growth and survival pathways.

1.2.5 PAD6

PAD6 was first identified as a highly abundant protein found in murine oocytes and embryos (Fig. 1.3). Hence, its original moniker was ePAD for "egg or embryonic PAD" (Wright et al. 2003). However, transcripts for the human PAD6 orthologue were also detected in ovary, testis, small intestine, spleen, lung, liver, skeletal muscle, fetal tissue, and peripheral blood leukocytes (Chavanas et al. 2004; Zhang et al. 2004). On the amino acid level, PAD6 shares ~42 % homology with the rest of the PAD family (Chavanas et al. 2004) and is missing several Ca^{2+}-binding residues that are conserved in the other PAD family members (Fig. 1.2) (Arita et al. 2004). In fact, evidence of PAD6 enzymatic activity in vitro has yet to be demonstrated (Snow et al. 2008).

Expression of PAD6 within the ovary is regulated by the oocyte-specific transcription factor, Nobox, which binds to regulatory elements within the PAD6 promoter and drives PAD6 expression during oogenesis (Choi et al. 2010). Although male PAD6−/− mice are fertile, PAD6 is a maternal effect gene and essential for female fertility. Zygotes derived from fertilized PAD6−/− oocytes arrest at the two-cell stage of embryonic development, prior to implantation (Esposito et al. 2007). In the oocyte and zygote, PAD6 localizes to cytoplasmic lattice structures and is essential for lattice formation (Esposito et al. 2007; Yurttas et al. 2008). PAD6-containing cytoplasmic lattices seem to be important for de novo protein synthesis, embryonic gene activation, and microtubule-mediated organelle reorganization within the zygote (Yurttas et al. 2008; Kan et al. 2011). An antibody against histone H4, with a citrullinated moiety at position 3, recognized a nonnuclear and unidentified deiminated protein within oocytes, and this reactivity was not present in PAD6−/− oocytes (Esposito et al. 2007). Since the protein expression profile between wild-type and PAD6−/− oocytes is similar (Yurttas et al. 2008), these findings suggest that PAD6 is active in vivo and either directly deiminates this substrate or PAD6 is required to activate another PAD present in oocytes. Interestingly, PAD6 is phosphorylated in the mature egg, and PAD6 phosphorylation is required for its interaction with tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHA), a molecule known to aid oocyte maturation in amphibians (Snow et al. 2008). Perhaps, the association with phosphorylation-dependent binding partners or a phosphorylation-induced conformational change facilitates enzymatic activity of PAD6 in vivo. In fact, the recombinant form of the arginine methyltransferase

PRMT5 is several hundredfold times less active than PRMT5 isolated from mammalian cells, likely due to the association of cellular factors that can promote PRMT5 enzymatic activity (Rho et al. 2001). Further enzymology and crystallography studies of PAD6 will be useful in determining whether PAD6 is an active deiminase and, if so, whether Ca^{2+} is required for its activity. Although it is unclear whether PAD6 is an enzymatically active PAD family member, collectively these findings demonstrate that PAD6 is essential for mammalian development.

1.3 PADs and RA

1.3.1 Anti-citrullinated Autoantibodies and RA

RA is a chronic, systemic inflammatory disease affecting approximately 2 % of the world population and is discussed in great detail in Chaps. 2–6. Briefly, this disorder is characterized by leukocyte invasion of the normally acellular synovial fluid and membrane, which thereby activates resident macrophages, mast cells, and synoviocytes, resulting in cell division and thickening of the joint lining (Firestein 2003). Plasma and synovial biopsy specimen from patients with RA contain high levels of citrullinated proteins (Chang et al. 2005; Makrygiannakis et al. 2006). In fact, anti-citrullinated peptide antibodies (ACPA) exhibit high specificity and sensitivity as diagnostic markers of the disease, suggesting that RA patients have defects in tolerance generated to citrullinated epitopes (Suzuki et al. 2007). Furthermore, the presence of intracellular and extracellular synovial citrullinated proteins correlates with inflammatory arthritis, as citrullinated proteins are found in RA patients but not in osteoarthritis patients (Foulquier et al. 2007; Kinloch et al. 2008; Lundberg et al. 2005). ACPA also develop in the murine CIA model of RA, and immunization with citrullinated collagen or administration of ACPA contributes to disease pathogenesis in mouse models (Lundberg et al. 2005; Kuhn et al. 2006).

It is now well known that PAD enzymes are activated during the inflammation process (Klareskog et al. 2008). Indeed, the presence of citrullinated proteins in the affected tissues of patients with RA, inflammatory bowel disease, and polymyositis, but not in healthy controls, supports the notion that deimination is linked with inflammation (Makrygiannakis et al. 2006). So far, PAD2 and PAD4 have been the only PADs detected in hematopoietic cells and in RA synovium (Nakayama-Hamada et al. 2005; Foulquier et al. 2007). Most PAD2- and PAD4-expressing cells within the RA synovium are positive for CD68, a marker of macrophages, neutrophils, and mast cells (Foulquier et al. 2007; Chang et al. 2005). Though variants of PAD4 are linked to RA in several Japanese and Korean cohorts, this association has not held true in most North American and European study groups (van der Helm-van Mil and Huizinga 2008). Despite the conflicting data regarding disease-contributing PAD4 haplotypes amongst different ethnic groups, the prevalence of ACPA in all ethnic groups supports the notion that aberrant PAD activity may contribute to RA pathogenesis (Klareskog et al. 2008).

It is possible not only that PAD enzymes could contribute to RA through self-antigen generation but also that the action of PAD enzymes could also contribute to the effector mechanisms of disease, such as immune cell recruitment and joint destruction. Recently, a subclass of ACPA that was especially reactive to citrullinated vimentin was also shown to stimulate osteoclastogenesis and bone resorptive activity, suggesting that ACPA might directly contribute to RA disease pathogenesis (Harre et al. 2012). The F_1 generation between the KRN TCR transgenic mouse specific for bovine RNase (Stensland et al. 2009; Bedford 2007; Krause et al. 2007; Zurita-Lopez et al. 2012; Guo et al. 2011; Hidaka et al. 2005; Nachat et al. 2005a, b; Senshu et al. 1996; Ishida-Yamamoto et al. 2000; Takahara et al. 1986; Ying et al. 2009; Nagata and Senshu 1990; Keilhoff et al. 2008; Perkins 2007) I-A^k and the nonobese diabetic (NOD) background spontaneously develop a progressive, inflammatory joint disease that is very similar to human RA (Kouskoff et al. 1996). The autoantigen in this model is glucose-6-phosphate isomerase (GPI), a ubiquitous cytoplasmic enzyme. Serum or purified anti-GPI autoantibodies are sufficient to transfer disease to healthy, wild-type mice, providing a model for the RA effector phase (Maccioni et al. 2002; Matsumoto et al. 1999). The region of murine chromosome 4 containing all PAD genes is linked to arthritis severity in the K/B×N model, with the highest associated SNPs being within the *PAD2* gene (Johnsen et al. 2011). Indeed, increased splenic expression of both PAD2 and PAD4 correlated with disease severity in the K/B×N model (Johnsen et al. 2011). Using PAD4-deficient mice generated in our laboratory, we found that K/B×N serum transfer arthritis is independent of PAD4 (Rohrbach et al. 2012). Perhaps the loss of PAD2 and PAD4 together may produce a more apparent phenotype in the K/B×N model. However, Willis et al. showed that the PAD inhibitor Cl-amidine provided therapeutic benefit in the collagen-induced arthritis model but had no benefit when arthritic disease was induced by the administration of anti-collagen antibodies (another model of the arthritis effector phase). Since the *Padi* locus is linked to disease severity in the K/B×N serum transfer model, it may be necessary to eliminate several PAD family members, either by targeting multiple locations within the PAD locus or by combining treatment with specific PAD inhibitors with targeted PAD alleles. Further studies will be necessary to fully dissect the role of the PAD enzymes in the effector phase of arthritis.

1.3.2 Citrullinated Epitope Generation

ACPAs can appear before the onset of disease and correlate with the most erosive form of RA (Raptopoulou et al. 2007). Several candidate anti-citrulline autoantigens have been identified, including citrullinated fillagrin, fibrin, vimentin, and α-enolase (Klareskog et al. 2008). There is a strong association between ACPA and the RA susceptibility major histocompatibility complex (MHC) II *HLA-DRB1* alleles (Klareskog et al. 2008). Indeed, conversion of arginine to citrulline increased peptide–MHC binding affinity in one of the *HLA-DRB1* genes (Fig. 1.5)

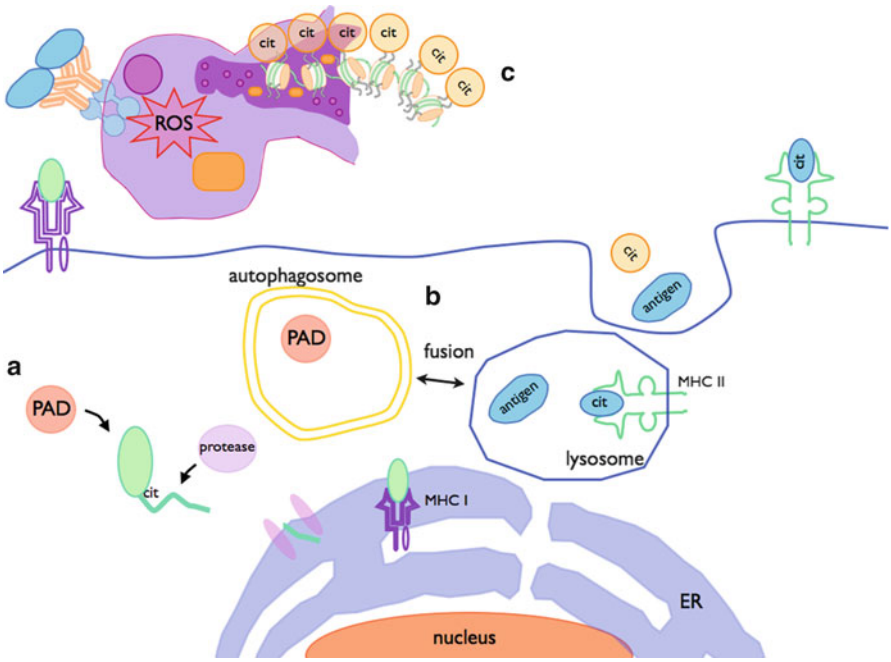

Fig. 1.5 Potential pathways that may contribute to the generation of citrullinated self-antigens. Citrullinated (cit) peptides could be shuttled to the MHC I or the MHC II antigen presentation compartments. (**a**) PAD-mediated protein citrullination could change the susceptibility to, or pattern of, cleavage by proteases. Cleaved peptides could then be shuttled into the ER for loading onto MHC I. (**b**) Extracellular proteins that are taken up by the lysosome could be citrullinated following fusion with the autophagosome, which is possible due to PAD residence in the autophagosome. Citrullinated proteins could then be cleaved and presented via the MHC II pathway. (**c**) NET formation, which can be triggered by immune complex recognition via Fcγ receptors, could allow for the release of active PAD enzymes and citrullinated self-proteins

(Klareskog et al. 2008). Though autoreactivity to protein citrullination is strongly associated with RA, the mechanism by which a healthy immune system tolerates citrullinated epitopes is unknown.

On average, each protein encoded by the human genome bears approximately 2.5 posttranslational modifications (Papin et al. 2005). To maintain tolerance, the immune system must consider the added diversity of potential self-antigens by post-translational modifications (Doyle and Mamula 2012). Since citrullination leads to a loss of a positive charge and potentially changes protease cleavage sites, inflammation-induced protein citrullination could result in the presentation of new self-epitopes (Fig. 1.5) (Vossenaar et al. 2003). In fact, deimination of filaggrin increases its susceptibility by the protease bleomycin hydrolase, an enzyme that also contributes to peptide generation for antigen presentation by MHC I (Kamata et al. 2009; Stoltze et al. 2000). Unanue and colleagues have elegantly shown that a CD4+ T cell response to citrullinated epitopes naturally develops following

immunization with the exogenous non-citrullinated antigen hen egg lysozyme (HEL) (Ireland et al. 2006). Autophagy is a process by which cytoplasmic proteins are engulfed by the membrane phagophore to be shuttled to the lysosome for degradation. One outcome of autophagy is the generation of peptides for MHC presentation (Kuballa et al. 2012). The presentation of citrullinated epitopes, at least from exogenous sources, is blocked by chemical inhibition of autophagy (Ireland and Unanue 2011). Investigation into intracellular and extracellular pathways that lead to the generation of citrullinated self-antigens and their presentation by MHC I and MHC II will be important for understanding the role of ACPA in RA.

Chronic joint inflammation in RA involves the influx of large number of inflammatory cells, including macrophages, mast cells, T cells, B cells, and neutrophils (Firestein 2003). NET formation by neutrophils, although critical for the full activation of the innate immune response (Buono et al. 2005), has also been implicated in inflammatory disease pathogenesis, including the autoimmune disorder lupus (Garcia-Romo et al. 2011), cystic fibrosis (Manzenreiter et al. 2012; Papayannopoulos et al. 2011; Marcos et al. 2010), sepsis (Clark et al. 2007), and thrombosis (Fuchs et al. 2007). Since PAD2, PAD4, and citrullinated proteins are found in the synovial fluid of RA patients (Kinloch et al. 2008), these enzymes can presumably act on extracellular proteins. For example, collagen and fibrinogen are both PAD substrates (Zhao et al. 2008; Yoshida et al. 2006), but the mechanism by which PAD molecules gain access to the extracellular space is unknown. Interestingly, it has been suggested that NETs offer a possible mechanism by which PAD4 may be liberated from the cell to generate citrullinated antigens and exacerbate inflammation (Jones et al. 2009; Dwivedi et al. 2012). Recently, Dwievedi et al. (2012) described hypercitrullination in neutrophils from arthritic patients as well as the specific reactivity of arthritic serum to activated neutrophils and citrullinated histones. The stimulus that induces PAD activity during autoimmune-mediated inflammation is currently unknown.

Because PAD4 activation requires Ca^{2+} and ROS generation, signaling through Fc receptors, which induce phagosome/granule fusion as well as NADPH oxidase assembly, may provide the necessary signals to induce PAD4 activity (Nimmerjahn and Ravetch 2008). This possibility would suggest that the relevant stimulus for PAD4 activation during an autoimmune response could be immune complex driven. Indeed, we have recently found that PAD4 activity and NETs are readily detected within the affected arthritic joint in a murine model of arthritis, which is induced by immune complexes (Rohrbach et al. 2012). In fact, the presence of deiminated histones in this model corresponded primarily to the infiltrating cells of the joint sublining, which is consistent with the expression pattern of PAD4 found in RA patients (Nakayama-Hamada et al. 2005; Rohrbach et al. 2012; Stahl et al. 2010). In a recent study, the interaction of neutrophil FcγRIIA with immune complexes induces NET formation (Chen et al. 2012). Furthermore, in autoimmune small-vessel vasculitis, anti-neutrophil cytoplasm antibodies (ANCA) triggered the formation of NETs, which promoted necrotic inflammation of blood vessels in this condition (Kessenbrock et al. 2009). Systemic lupus erythematous (SLE) is a systemic autoimmune disease characterized by the formation of pathogenic immune complexes.

When activated by autoantibodies, neutrophils isolated from SLE patients also produce NETs, exposing immune-stimulatory proteins and potential autoantigens, leading to the induction of type I interferons by plasmacytoid dendritic cells (Garcia-Romo et al. 2011; Hakkim et al. 2010; Villanueva et al. 2011). Collectively, these results support the notion that NET production can contribute to disease pathogenesis in inflammatory conditions and that immune complexes may drive NET formation during inflammation (Fig. 1.5). In a chronic setting, NETs might play a role in propagating an inflamed state, similar to what has been reported for NETs in chronic lung inflammation (Marcos et al. 2010). This is of special interest, since PAD4 dysregulation has been implicated in diseases like rheumatoid arthritis (Anzilotti et al. 2010; Suzuki et al. 2003), multiple sclerosis (Wood et al. 2008), and malignant tumors (Chang and Han 2006).

1.4 Conclusion

PAD enzymes contribute to RA through self-antigen generation, but PAD enzymes could also contribute to the effector mechanisms of this disease. In addition to RA, PAD enzymes and citrullination are associated with multiple sclerosis, Alzheimer's disease, psoriasis, and cancer (Gyorgy et al. 2006). Thus, the generation of isozyme-specific inhibitors and additional mouse models will be important to fully appreciate the physiological functions of PAD enzymes. Understanding the regulation of PAD activity may have broader implications in understanding the inflammation-linked disease states and may also shed light on the etiology of anti-citrulline autoantibodies.

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Chapter 2
From Citrullination to Specific Immunity and Disease in Rheumatoid Arthritis

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Keywords Citrullination • Anti citrullinated protein antibodies (ACPA) • Rheumatoid arthritis • Smoking • Shared epitope • Inflammation • Lungs • Periodontitis

2.1 Introduction

Citrullination (deimination) is a posttranslational modification occurring in a large variety of tissues and in a large variety of conditions and playing important roles in health and disease. It has originally been described as a physiological process in the skin and is thought to be critical in terminal differentiation of the epidermis, favoring interaction between structural proteins (Senshu et al. 1996, 1999) and facilitating proteolysis of filaggrin (Harding and Scott 1983; Pearton et al. 2002; Scott et al. 1982). Also, deimination is a physiological process during brain development, with elevated levels of citrullinated myelin basic protein, allowing high plasticity of the brain under 2 years of age (Moscarello et al. 1994) followed by a dramatic reduction in the levels of citrullination after 4 years of age to form very tight and compact myelin sheets (Beniac et al. 2000). Citrullination has been suggested to play a role in multiple sclerosis (Koch et al. 2013; Mohanan et al. 2012), cancers (Mohanan et al. 2012) and possible also in diseases of the reproductive organs (Horibata et al. 2012). It has also been mapped as a central event in the context of inflammation (Makrygiannakis et al. 2006; Vossenaar et al. 2004a) and linked to the pathogenesis of rheumatoid arthritis (RA) (Cantaert et al. 2006).

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This chapter focuses on the role of citrullination in breaking immune tolerance to first generate anti-citrullinated protein antibodies (ACPAs), which then lead to the onset of chronic joint inflammation. A more detailed discussion on known citrullinated autoantigens is included in Chap. 3.

2.1.1 Rheumatoid Arthritis Is a Chronic Inflammatory Disease with At Least Two Distinct Clinical Phenotypes

RA is a chronic inflammatory joint disease resulting from the complex interaction between genetic and environmental factors, with at least two distinct clinical phenotypes, according to the presence or the absence of ACPA, i.e., ACPA+RA and ACPA−RA (Klareskog et al. 2009). These two phenotypes strikingly differ both in terms of risk factors and disease mechanisms. Specific environment–gene interactions (such as smoking and presence of the HLA-DRB1 risk allele variants) (Huizinga et al. 2005; Klareskog et al. 2006; Pedersen et al. 2007; Ding et al. 2009; Lundstrom et al. 2009) and gene–gene interactions (such as interaction between HLA-DRB1 and PTPN22) (Kallberg et al. 2007) only confer risk for the development of ACPA+RA but not ACPA−RA. ACPA+RA has a more severe disease course with more comorbidities (such as cardiovascular disease) (Holmqvist et al. 2009, 2010; Lopez-Longo et al. 2009) and complications (such as bone destruction) (Ronnelid et al. 2005; van der Helm-van Mil et al. 2005; Syversen et al. 2010), as compared to ACPA−RA.

2.1.2 The Biochemistry of Tissue Citrullination

Citrullination is a posttranslational modification with conversion of arginine into citrulline by an enzymatic reaction catalyzed by peptidylarginine deiminases (PAD), extracellularly or in intracellular vesicles, and leading to a one Dalton mass reduction for each modified arginine residue. This reaction change the total charge of the proteins and their isoelectric points and influence their ionic and hydrogen bond forming capacity (Gyorgy et al. 2006; van Venrooij and Pruijn 2000; Tarcsa et al. 1996). There are five different PAD enzymes, with ubiquitous expression of the PAD-2 isotype and more tissue-restrictive expression of PAD-1 (skin and uterus), PAD-3 (hair follicles), PAD-4 (neutrophils and eosinophils), and PAD-6 (eggs, ovaries, embryos) (Vossenaar et al. 2003). Synovial membrane expresses only PAD-2 and PAD-4 enzymes (Nakayama-Hamada et al. 2005; Chang et al. 2005a). In vitro activation of PAD enzymes requires high levels of calcium difficult to achieve in vivo (Vossenaar et al. 2003; Takahara et al. 1986; Mechin et al. 2005), with the exception of extreme conditions such as cell death (Schwab et al. 2002). It is still possible, however, that in vivo, other regulation factors allowing activation of PAD enzymes exist, even at physiological levels of calcium.

2.1.3 Citrullination Is Linked to Environmental Risk Factors for ACPA+RA

Epidemiological studies have identified smoking as a risk factor for developing RA for more than 20 years ago (Heliovaara et al. 1993; Silman et al. 1996; Karlson et al. 1999; Stolt et al. 2003; Vessey et al. 1987). More recently this risk has been limited to only specific subsets of disease first defined as rheumatoid factor (RF)-positive RA (Stolt et al. 2003) and later ACPA+RA (Klareskog et al. 2006; Pedersen et al. 2006; Kallberg et al. 2011), suggesting a potential pathogenic link between smoking and the process of citrullination. Indeed histological studies in the lungs of healthy individuals demonstrated increased citrullination and expression of PAD enzymes in smokers as compared to nonsmokers (Klareskog et al. 2006; Bongartz et al. 2007; Makrygiannakis et al. 2008). These findings, discussed in more detail in Chap. 5, suggest that abnormal citullination outside the joints might be the initial event in starting the pathogenic autoimmune chain leading to arthritis. However smoking-induced lung citrullination by itself is not sufficient to explain all cases of ACPA+RA as far as there are also nonsmokers still developing this type of disease. Despite smoking being the major risk factor for RA, other environmental factors might also play a role. One example of another possible risk factor is bacterial infection leading to periodontitis (see Chap. 4 for more details). Periodontitis is a disease sharing several pathogenic traits with RA such as association with smoking, cytokine-driven chronic inflammation, and bone destruction (Routsias et al. 2011; Schenkein et al. 2007). The exact time and causality relationship between RA and periodontitis is still unclear, but the two diseases occur often in one and the same individual (Mercado et al. 2003; Rosenstein et al. 2004; de Pablo et al. 2009). One potential link between RA and periodontitis is induction of local gingival citrullination in periodontitis and local induction of ACPA, as recently suggested (Nesse et al. 2012). Also, cross-reactivity between antibodies to *Porphyromonas gingivalis* (the most common etiologic agent in periodontitis) and ACPAs suggests that the bacterial agent might contribute to generation of ACPAs through a molecular mimicry mechanism (Lundberg et al. 2008). No identified environmental factor alone (either smoking or bacteria) is sufficient for developing RA, since there are both smokers and individuals with periodontitis that never develop RA. Others host-specific traits in individuals already prone to develop arthritis (such as genetic traits) are essential for the progression of environment-triggered citrullination changes towards specific immunity and disease.

2.1.4 Citrullination of Antigens Might Lead to Breaking of Immune Tolerance in the Presence of Genetic Risk Factors for ACPA+RA

Smoking (and other factors) induces posttranslational modifications of proteins that theoretically could result in new epitope formation and break of immune tolerance.

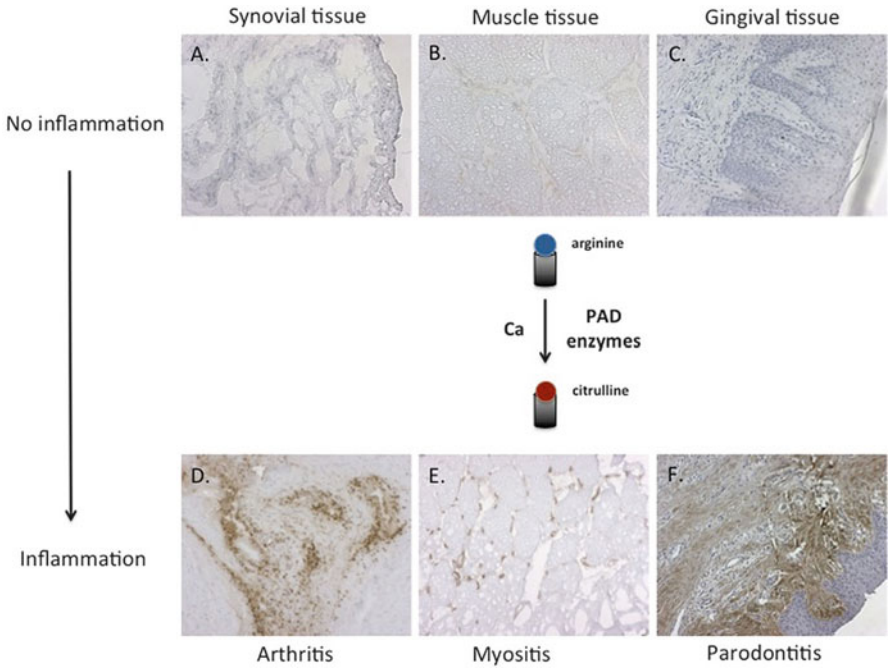

Fig. 2.1 Citrullinated proteins are expressed in a large variety of inflammatory tissues. Images represent brown immunohistochemical staining of citrullinated proteins in healthy synovial membranes (**a**), muscle (**b**), and gingival tissue (**c**), as compared to inflamed rheumatoid synovial membrane (**d**), polymyositis muscle (**e**), and periodontitis gingival tissue (**f**)

It is important to stress that anti-citrulline immunity, but not citrullination itself, is specific for RA, with citrullination being identified in a variety of inflamed tissues (Fig. 2.1) (Makrygiannakis et al. 2006). Better binding of the citrullinated forms as compared to native forms of a specific peptide might stabilize and improve antigen presentation in the context of specific HLA class II molecules and partially explain tolerance breaking with subsequent ACPA production (Hill et al. 2003; Snir et al. 2011). Recently it has been shown that three amino acid positions in HLA-DRβ1 and two additional amino acid positions in HLA-B and HLA-DP, all located within the peptide-binding grooves, confer risk for developing of ACPA+RA, further strengthening a potential functional impact on antigenic presentation of citrullinated peptides to T cells (Eyre et al. 2012).

We have preliminary reported on the identification of shared citrullinated peptides in the lungs and joints of patients with RA by mass spectrometry, and we are currently validating these peptides as targets of anti-citrulline immunity in RA patients (AI Catrina, personal communication). Other studies have focused on inflamed joint tissue (Tilleman et al. 2008; Chang et al. 2005b; Hermansson et al. 2010) and synovial fluid (Tabushi et al. 2008; Yoshida et al. 2006; Bang et al. 2007;

Kinloch et al. 2008; Raijmakers et al. 2012) and identifying fibrinogen, fibronectin, mutated vimentin, vimentin, alpha enolase, and collagen type II as potential ACPA epitope-bearing proteins. Despite little data on exact locations of citrulline residues in vivo, in vitro citrullination of target antigens and/or studies on ACPA recognition patterns have revealed several immunodominant citrullinated peptides: citrullinated enolase-1 peptide (Lundberg et al. 2008; Kinloch et al. 2005), citrullinated collagen type II (Uysal et al. 2009), citrullinated fibrinogen (Nakayama-Hamada et al. 2005; Sebbag et al. 2006), and citrullinated vimentin (Uysal et al. 2009; Vossenaar et al. 2004b; Asaga et al. 1998). Interestingly, it has been demonstrated that ACPA+RA displaying fine specificities for the immunodominant peptides of enolase and vimentin has a much stronger linkage to HLA-DRB1 than ACPA+RA lacking these reactivities, further strengthening the MHC class II-restrictive pattern of citrullinated antigen presentation (Lundberg et al. 2013; van der Woude et al. 2010a). It is still unclear whether regulation and control of the immune recognition of citrullinated epitopes take place during early thymic development or during peripheral immune responses.

2.1.5 From Citrullination and Autoimmunity to Autoimmune Joint Disease

Specific autoimmunity (ACPA) is present long before development of clinical joint inflammation (Nielen et al. 2004; Rantapaa-Dahlqvist et al. 2003) with very early isotype reservoir expansion and epitope spreading (Van der Woude et al. 2010b; Kokkonen et al. 2011; Verpoort et al. 2006; Ioan-Facsinay et al. 2008), resulting in the presence of a large variety of ACPA specificities already at disease onset. However, less is known on the origin of these antibodies and the mechanisms governing localization of the systemic antibody response to the joint. Recently, ACPA has been associated with the presence of extra-articular lung and gingival disease (Demoruelle and Deane 2011; Dissick et al. 2010) but not joint abnormalities in healthy individuals (van de Sande et al. 2011). We also have preliminary data (Fig. 2.2) showing subclinical tissue inflammation with local ACPA production in the lungs of RA patients already at disease onset (AI Catrina, personal communication). It is therefore possible that environmental factors (such as smoking, bacterial infections, and others) are inducing extra-articular citrullination with local generation of ACPA in hosts carrying certain HLA type II risk alleles that are then disseminated and circulated in the peripheral blood as still naïve bystanders. In this scenario, a second hit (still not identified) is needed to induce local changes in a joint, leading to synovial citrullination close to the clinical onset of joint symptoms. Trespassing over a certain threshold of citrullination and/or citrullination of specific immunodominant peptides shared with the original extra-articular emerging site will eventually lead to localization of the ACPAs to the joint and initiation of synovial chronic inflammation.

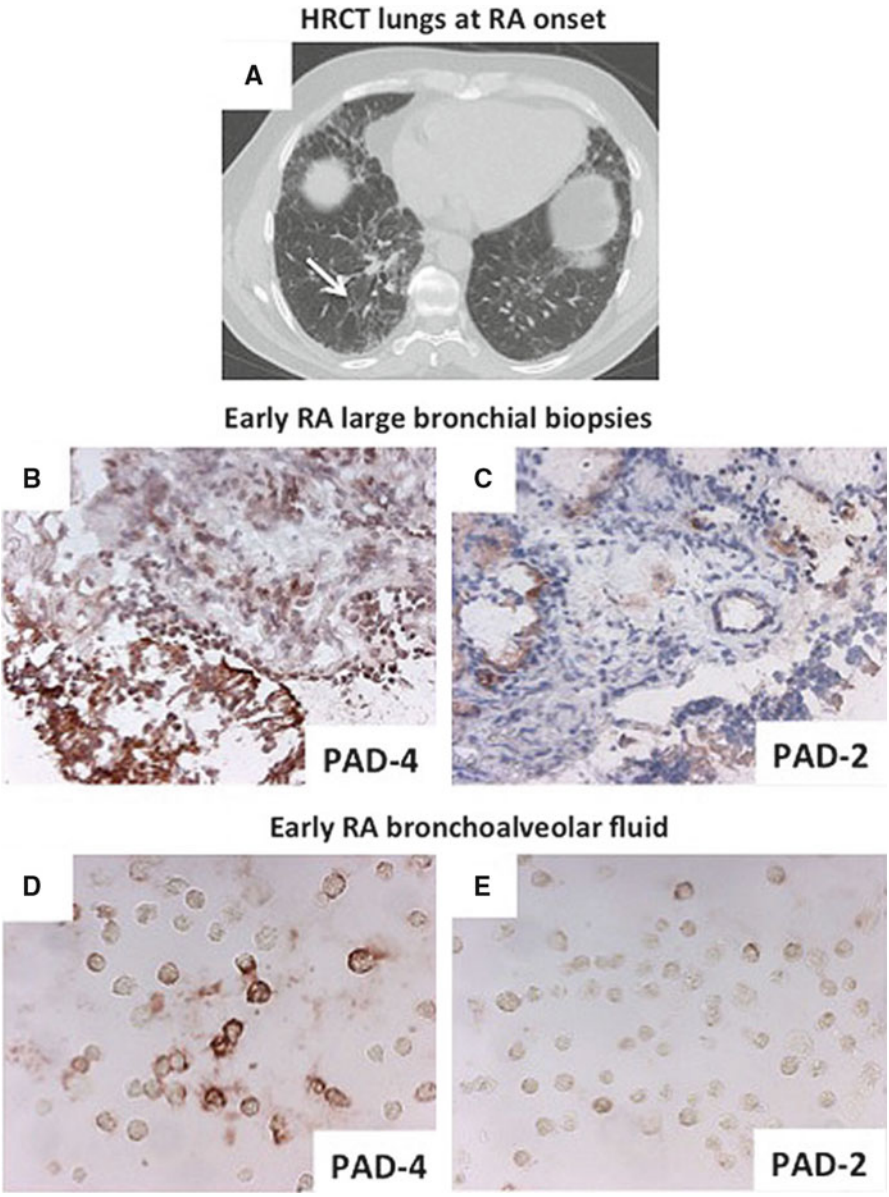

Fig. 2.2 Subclinical inflammatory changes in the lung are present in rheumatoid arthritis already at disease onset. Images showing fibrosis on high-resolution computer tomography (**a**, *arrow*) in a patient newly diagnosed with RA and red immunohistochemical staining of PAD-2 and PAD-4 enzymes in the large bronchial tissue (**b**, **c**) and bronchoalveolar fluid (**d**, **e**)

2.1.6 *Citrullination and Anti-citrulline Immunity in Perpetuation of Chronic Inflammation*

Citrullination as well as PAD2 and PAD4 enzymes are present in the inflamed synovium during all stages of the disease and correlate with local inflammation (Makrygiannakis et al. 2012; Foulquier et al. 2007), potentially contributing to synovial tissue damage. Few studies to address the mechanisms by which local citrullination could contribute to disease pathogenesis are currently available. In this respect, it has been recently shown that citrullinated fibronectin is less effective than native fibronectin in inhibiting the proteolytic activity of ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs able to degrade aggrecan and contribute to cartilage destruction in RA (Yan et al. 2013). Also, citrullinated fibronectin has been shown to impair adhesion and spreading of arthritic and non-arthritic synovial fibroblasts (Shelef et al. 2012).

Besides citrullination leading to changes in protein function, ACPAs themselves can also directly contribute to disease pathogenesis. Predominance of IgG1, but also IgM, among ACPA serotypes (Verpoort et al. 2006; Ioan-Facsinay et al. 2008) suggests continuous production of newly triggered B cells. Synovial fluid enrichment of ACPAs, as compared to peripheral blood in patients with established RA, demonstrates local continuous production of these antibodies at the site of inflammation (Snir et al. 2010). Utilizing a single B cell-based cloning technology, we were able to clone and express immunoglobulin genes from synovial fluid of ACPA+RA patients, demonstrating that a high proportion of B cells from these individuals are producing ACPAs (Amara et al. 2013).

Recent progress has demonstrated that ACPAs not only exist and are produced in the rheumatoid joint but also directly contribute to perpetuation of local inflammation. For example, it has been demonstrated that ACPA as well as immune complexes containing ACPA promote TNF production in monocyte-derived cell lines and macrophages derived from peripheral blood of healthy individuals (Clavel et al. 2008; Lu et al. 2010). It appears that this antibody effect is mediated via a toll-like receptor (TLR) 4- and MyD88-dependent mechanisms, while the effect of immune complexes is mediated through a TLR4- and Fcγ receptor (Fcγ R) IIa dependent mechanism (Sokolove et al. 2011). Recently, we demonstrated that ACPA could directly promote bone destruction in an FcγR-independent manner (Harre et al. 2012).

Despite these recent advances, the exact contribution of distinct ACPAs to inflammation and damage in and outside the joints in RA remains still elusive, and further investigation of the exact molecular pathways activated by ACPA and by ACPA immobilized in immune complexes with distinct citrullinated targets needs to be performed to address tissue-specific damage.

2.1.7 *Anti-citrulline Immunity as a Disease and Therapeutic Biomarker*

Detection of anti-cyclic citrullinated protein (CCP) antibodies, recognizing a large array of citrullinated peptides (Schellekens et al. 1998, 2000; van Venrooij et al. 2011), is currently used in clinical standard routine diagnostic testing and is the best known biomarker for RA with 95 % specificity (van Venrooij et al. 2011; Nishimura et al. 2007). Epidemiological studies have demonstrated that the presence of anti-citrulline immunity associates with worse prognosis (Ronnelid et al. 2005; Kastbom et al. 2004), more joint destruction (Forslind et al. 2004; Vencovsky et al. 2003; Meyer et al. 2003; Kroot et al. 2000), and comorbidities (Lopez-Longo et al. 2009). Recently, it has been suggested that immunity against specific citrullinated proteins, rather than broad anti-citrulline immunity, might play an important pathogenic role in defining specific clinical phenotypes of RA. For example, antibodies against citrullinated vimentin may be a better prognostic factor for radiographic progression, as compared to anti-CCP antibodies (Mathsson et al. 2008), while antibodies to citrullinated enolase seem to identify a specific pathogenic subset of RA with potential relevance for predicting clinical outcome (Mahdi et al. 2009). In fact, the presence of ACPA and high titers of ACPA might help identify patients benefiting most from rituximab therapy (Chatzidionysiou et al. 2011; Sellam et al. 2011; Narvaez et al. 2011; Lal et al. 2011). Similarly, one register study from France reported a better effect of abatacept in ACPA-positive RA independent of disease activity (Gottenberg et al. 2012).

2.1.8 *An Etiopathogenic Hypothesis on Citrullination and the Role of Anti-citrulline Immunity in the Development of ACPA+RA*

Data accumulated during recent years offer more insights into the longitudinal sequence of events leading from genes and environment to autoimmunity and chronic inflammation. Based on this hypothesis, external factors (such as smoking) are able to induce posttranslational extra-articular modifications of proteins (such as in the lungs). In the presence of specific HLA class II risk allele variants the new generated epitopes are more efficiently presented to the immune system and brake tolerance resulting in generation of ACPA long before disease onset. In the following years, isotype repertoire expansion and epitope spreading lead to the appearance of multiple ACPA specificities with the individual ACPA phenotype already present when the diagnosis of RA is finally made. A second hit, such as minor trauma or transient viral infection, might promote synovial citrullination with local emergence of the same citrullinated epitopes as originally present at the initiation site. In this way, ACPAs will localize in the joint and initiate antibody- and immune complex-mediated events, leading to chronic inflammation. Local synovial presence of

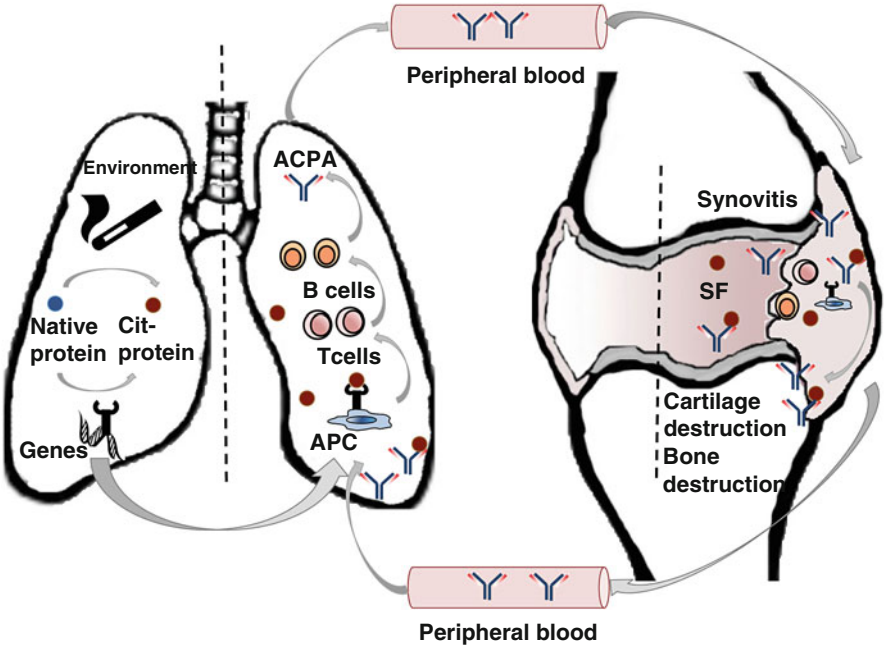

Fig. 2.3 The figure presents a hypothetical model on the pathogenic role of anti-citrullinated antibodies and the link between lungs and joints in rheumatoid arthritis. Abbreviations: *ACPA* anti-citrullianted protein antibodies, *APC* antigen-presenting cells, *cit-proteins* citrullinated proteins, *SF* synovial fluid

citrullinated antigens might facilitate local autoantigen-driven differentiation of B cells into ACPA-producing plasma cells, further contributing to the vicious circle of chronic inflammation and tissue destruction. A schematic presentation of the proposed pathogenic chain is illustrated in Fig. 2.3.

2.1.9 Clinical Consequences of the New Gathered Knowledge and Future Perspectives

The new knowledge gathered through both large epidemiologic and molecular biology studies of the anti-citrulline immunity in RA has contributed to major changes in the clinical understanding and management of the disease. We have today redefined the classification criteria for RA, changed the time of diagnosis and treatment to earlier stages of the disease, and highlighted the importance of active life changes. Also, we talk today about individuals at risk of developing RA, and recently EULAR has published recommendations for terminology and research in individuals at risk for RA (Gerlag et al. 2012). This new way of thinking might lead in the future to identification of healthy individuals at high risk for developing RA

in whom modulation of specific anti-citrulline immunity might be attempted through modification of lifestyle-specific, targeted T or B cell therapies, induction of immune tolerance, and/or direct modulation of the pathogenic effects mediated by ACPAs, with the final goal of preventing RA.

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Chapter 3
The Role of Synovial Citrullinated Proteins in the Pathophysiology of Rheumatoid Arthritis

Joyce J.B.C. van Beers and Ger J.M. Pruijn

Keywords ACPA • Arthritis • Citrullinated autoantigens • Citrullination • Joint inflammation • Rheumatoid arthritis

3.1 Introduction

A specific feature of rheumatoid arthritis (RA) is the production of anti-citrullinated protein antibodies (ACPA) by the majority of the patients (Schellekens et al. 1998). RA is an autoimmune disease which is characterized by chronic inflammation of the synovial joints. It can lead, when left untreated, to irreversible joint damage. In the synovial joints of RA patients, pannus tissue (containing many inflammatory cells) can be formed, which can invasively grow into the cartilage and bone and ultimately degrade these tissues.

Approximately, 75 % of RA patients have ACPA, compared to 1 % of healthy individuals (van Venrooij et al. 2011). The production of ACPA appeared to be an early feature of the disease process, because these autoantibodies often can already be detected prior to disease onset (Schellekens et al. 2000; Rantapaa-Dahlqvist et al. 2003; Kokkonen et al. 2011). Several immunoassays, which are commercially available, have been developed for the detection of ACPA in patient sera.

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The currently most frequently applied assay uses a synthetic cyclic citrullinated peptide (CCP2) and displays a high sensitivity for ACPA detection (Pruijn et al. 2010).

ACPA target citrullinated proteins, which are abundantly present in inflamed joints. Increasing evidence indicates that the immune response to citrullinated proteins and the formation of immune complexes plays an important role in the pathophysiology of RA. Nevertheless, protein citrullination is not a unique feature of joint inflammation in RA, but is commonly observed in inflamed tissues and this raises the question why particularly the joints are affected in RA. Possibly, the citrullinated proteins in these tissues contain specific structures (citrullinated epitopes) that are preferentially targeted by ACPA. This chapter will focus on our current knowledge of citrullinated autoantigens in the inflamed synovial joints.

3.2 From Citrullination to RA: What Is Going (wr)on(g)?

It is generally accepted that under certain conditions citrullinated proteins can become arthritogenic, possibly by inducing an immune response to such proteins. Autoantibodies to citrullinated proteins have been found to be associated with disease severity, but the exact role of the synovial citrullinome (i.e., all citrullinated proteins in the inflamed synovium) in the pathophysiology of RA is not yet known (Lundberg et al. 2005). In 2004, citrullinated antigens were proposed to play an important role in the pathogenesis of RA (Nijenhuis et al. 2004; Vossenaar and van Venrooij 2004; Zendman et al. 2006). It was hypothesized that the inflammatory cycle starts with a certain initial trigger (Fig. 3.1), which is likely to have an environmental origin, such as an infection (e.g., *Porphyromonas gingivalis* or viral infection) or exposure to toxic compounds (e.g., smoking) (Mikuls et al. 2004; Liao et al. 2009; Makrygiannakis et al. 2008). As a result of this event, peptidylarginine deiminase (PAD) enzymes can become activated, leading to citrullinated proteins, which in turn can elicit an immune response, resulting in ACPA production. As a result of another (secondary) trigger (e.g., infection, trauma), inflammatory cells will infiltrate the synovium, thereby introducing PADs, which are not detectably expressed in healthy joints. PADs will still be inactive due to the relatively low cytosolic calcium concentration within normal cells, but during cell death (many infiltrating cells will die during the inflammatory process) the intracellular calcium concentration rises to a level sufficient ($>10^{-5}$ M) for PAD activation (*step 1*) (Takahara et al. 1986). The activated PAD enzymes can citrullinate intracellular proteins (such as vimentin and histones) (*step 2*) (Menard et al. 2000; Vossenaar et al. 2004a). Under normal circumstances, dying cells would be removed by phagocytes, and as a consequence, the immune system would not "see" the citrullinated proteins. However, when the clearance system insufficiently removes the (remnants of the) dying cells (for example when one of the factors involved is defective or when too many cells are dying), the dead cells will become necrotic and release citrullinated proteins into the extracellular space. Also, the activated PAD enzymes will be released and will citrullinate extracellular synovial proteins, such as

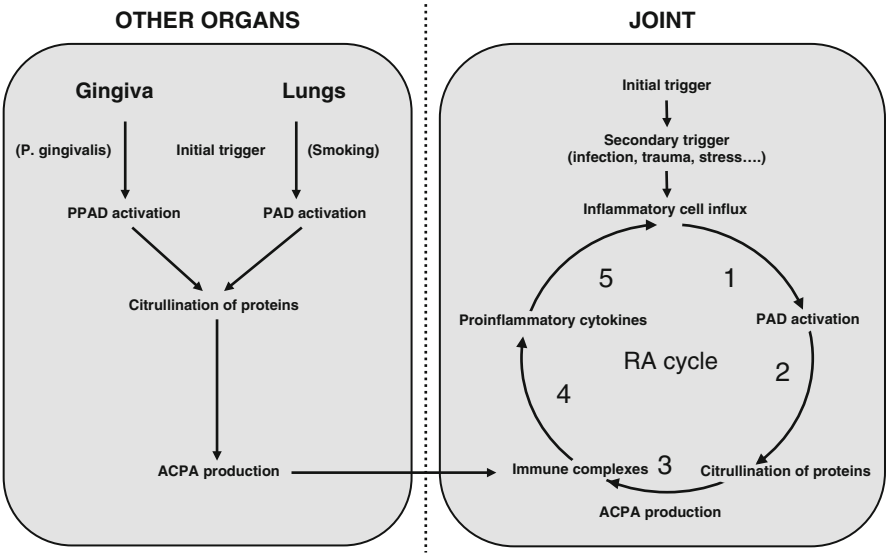

Fig. 3.1 The hypothesized role of citrullination in RA. An initial trigger (e.g., periodontal infection; see Chap. 4, smoking; see Chap. 5) can lead to activation of PAD enzymes, either in the joints or in other organs, leading to the citrullination of proteins. In susceptible individuals, this can lead to activation of the immune system and the production of autoantibodies (ACPA). When this occurs in other organs, ACPA may be transported to the synovium via the circulation. A secondary trigger (e.g., infection, trauma, stress) can lead to infiltration of immune cells, which contain PAD enzymes, in the synovium. After PAD activation (*step 1*), for example as a result of dying infiltrated cells, proteins will be citrullinated. In the case of defective clearance of cell remnants, activated PAD and citrullinated proteins will be released to the extracellular space, where PAD may citrullinate additional proteins (*step 2*). The presence of both ACPA and citrullinated proteins will lead to the formation of immune complexes (*step 3*), followed by the upregulation of proinflammatory cytokines (*step 4*). These cytokines will stimulate the inflammatory process and the infiltration of new immune cells (*step 5*), which might initiate a new cycle of citrullinated protein production and stimulation of the immune system

fibrin(ogen) (Masson-Bessiere et al. 2001). These events are not RA-specific, since they also occur during inflammatory processes in "healthy" individuals and other diseases, although it has been suggested that intracellular citrullinated proteins are RA-specific (De Rycke et al. 2005), whereas extracellular proteins are not (Vossenaar et al. 2004b). More importantly, citrullinated proteins will not always elicit an anti-citrullinated protein response and will not generally lead to the production of ACPA (Vossenaar et al. 2004b; Gyorgy et al. 2006). Only in some individuals (depending on their hormonal and genetic backgrounds) ACPA will be produced and chronic inflammation may develop. Smoking and possession of HLA-DR shared epitope (SE) alleles are established risk factors for the development of RA (Vessey et al. 1987; Gregersen et al. 1987). Smoking, for example, may trigger HLA-DR restricted immune reactions (Klareskog et al. 2006). Several HLA-DRB1 alleles have been found to be associated with RA. They share a consensus motif (Q/R K/R R A A)

which is located in the peptide binding pocket of these HLA class II molecules (de Vries et al. 2005). This binding pocket prefers negatively charged or neutral peptides (Gregersen et al. 1987; de Vries et al. 2005; Hill et al. 2003a). Individuals having these SE alleles will present citrullinated peptides more efficiently (to $CD4^+$ T lymphocytes) than the corresponding arginine-containing peptides (Hill et al. 2003b). In a susceptible environment, T cell activation (Hill et al. 2003b; Law et al. 2012; Snir et al. 2011) can subsequently activate B-cells to produce ACPA (*step 3*) (Bellatin et al. 2012). It is noteworthy to mention that Huizinga and coworkers found that the SE appears to be strongly associated with the production of ACPA in RA patients (Huizinga et al. 2005). The presence of ACPA and citrullinated proteins in inflamed joints will lead to the formation of immune complexes (antibodies bound to citrullinated antigens), which will result in complement activation and inflammatory cell activation (via Fc gamma receptor-mediated pathways) (*step 4*) (Reparon-Schuijt et al. 2001). This will ultimately lead to cytokine production, which constitutes the driving force for the perpetuation of a chronic inflammatory condition by, amongst others, recruiting novel inflammatory cells into the cascade (*step 5*) (Hueber et al. 2007).

3.3 Synovial Citrullinated Proteins and their Relationship with RA

The first studies to detect ACPA in patient sera were performed with citrullinated molecules that were not known to exist in the inflamed joints of patients, such as citrullinated filaggrin (Vittecoq et al. 2004), nucleophosmin/B23 (Hagiwara et al. 2002), eukaryotic translation initiation factor 4G1 (Okazaki et al. 2006), Epstein-Barr virus (EBV) nuclear antigen 1 (Pratesi et al. 2006; Pratesi et al. 2011) (Table 3.1), and citrullinated synthetic peptides from random peptide libraries (Schellekens et al. 1998). The identification of inflammation-associated citrullinated proteins severely lagged behind ACPA characterization.

Table 3.1 Citrullinated proteins in RA not detected in joint samples so far

Citrullinated protein	Recognized by ACPA	References
Aggrecan	n.d.[a]	Law et al. (2012), von Delwig et al. (2010)
Binding immunoglobulin protein (BiP)	Yes	Shoda et al. (2011)
Eukaryotic translation initiation factor 4G1 (eIF-4G1)	Yes	Okazaki et al. (2006)
Epstein-barr virus nuclear antigen 1 (EBNA-1)	Yes	Pratesi et al. (2006, 2011)
Far upstream element-binding protein (FUSE-BP)	Yes	Goeb et al. (2009)
Nucleophosmin (B23)	n.d.	Hagiwara et al. (2002)
Ribosomal protein S2 (RPS2)	n.d.	Guo et al. (2011)

[a]T-cell reactivity to a citrullinated aggrecan peptide has been observed

Although citrullinated proteins have been reported to occur in most, if not all inflamed tissues, formation of citrullinated protein-specific immune complexes would be largely restricted to RA, as a result of disease-specific production of ACPA. Because these complexes have been suggested to play an important role in the pathophysiology of RA, it is important to obtain a comprehensive overview of the citrullinated proteins present in the inflamed joints of RA patients and their recognition by ACPA. Although PAD enzymes display some substrate specificity, the released PADs may citrullinate any arginine-containing protein in the inflamed synovial joint. It is therefore not surprising that many citrullinated proteins have been found in synovial joint samples from RA patients (Okazaki et al. 2006; Chang et al. 2005; Suzuki et al. 2005; Burkhardt et al. 2005; Kinloch et al. 2005; Sebbag et al. 2006; Matsuo et al. 2006; Van Steendam 2010).

Recently, we have identified 53 distinct citrullinated proteins in synovial fluid (SF) samples of RA patients (van Beers et al. 2012; Raijmakers et al. 2012; van Beers et al. 2013). The citrullination of several of these proteins, fibrinogen, fibronectin, vimentin, albumin, and beta-actin, in the inflamed joints of RA patients confirms previous observations (Masson-Bessiere et al. 2001; Matsuo et al. 2006; Tilleman et al. 2008), although citrullinated fibrinogen alpha and beta-actin had not been detected in RA SF before, but only in synovial tissue. Citrullinated histones were previously detected in HL60-cells (Hagiwara et al. 2005). Six out of the 53 citrullinated proteins (alpha-fibrinogen, fibronectin, ApoE, MNDA, beta-actin, and CypA) were found in both RA SF samples analyzed in detail and three of these (ApoE, MNDA and beta-actin) were also frequently detected in SF from other RA patients. All identified citrullinated joint proteins are listed in Table 3.2. Until recently, little was known about the positions of the residues of these proteins that are citrullinated in RA synovia, because most of the previous studies focused on in vitro citrullinated proteins and/or cell-lysates. Our mass spectrometry data indicate that most of the synovial citrullinated proteins contain multiple residues that can be citrullinated.

For most of the citrullinated proteins in RA joints, it is still unknown whether they are targeted by ACPA and/or whether they play a role in the pathophysiology of RA. Here, we will focus on the best characterized citrullinated autoantigens: fibrinogen, vimentin, collagen type II, alpha-enolase, and fibronectin. In addition, three of the newly identified citrullinated proteins in RA joints, which are all targeted by ACPA, will also be discussed briefly.

3.3.1 Fibrin(ogen)

One of the best characterized citrullinated autoantigens is fibrinogen, a protein involved in blood clotting, in which thrombin catalyzes the conversion of fibrinogen into fibrin. Fibrinogen consists of two copies each of three polypeptide chains, α, β, and γ (Wolberg 2007). Fibrinogen has been demonstrated to be present in synovia, as well as in SF of RA patients (Snir and Widhe 2010; Takizawa et al. 2006). The

Table 3.2 Citrullinated synovial proteins

Citrullinated protein	Recognized by ACPA	References
40S ribosomal protein S16	n.d.	van Beers et al. (2013)
ADP-ribosylation factor 1	n.d.	van Beers et al. (2013)
Albumin	n.d.	Matsuo et al. (2006), van Beers et al. (2013)
Alpha-1-antitrypsin (serpin A1)	n.d.	van Beers et al. (2013)
Alpha-2-macroglobulin	n.d.	van Beers et al. (2013)
Alpha-enolase	Yes	Kinloch et al. (2005)
Antithrombin, chain A	n.d.	van Beers et al. (2013)
Apolipoprotein A-I	n.d.	van Beers et al. (2013)
Apolipoprotein A-IV	n.d.	van Beers et al. (2013)
Apolipoprotein B-100	n.d.	van Beers et al. (2013)
Apolipoprotein E	n.d.	van Beers et al. (2013)
Apolipoprotein L-I	n.d.	van Beers et al. (2013)
Asporin	n.d.	Matsuo et al. (2006)
(Mutant) beta-actin	Yes	Matsuo et al. (2006), van Beers et al. (2013), Darrah et al. (2012)
Cathepsin G, chain A	n.d.	van Beers et al. (2013)
Clusterin (apolipoprotein J)	n.d.	van Beers et al. (2013)
Collagen type II	Yes	Burkhardt et al. (2005)
Complement C3	n.d.	van Beers et al. (2013)
Complement component C4-A	n.d.	van Beers et al. (2013)
Complement factor B	n.d.	van Beers et al. (2013)
Complement factor H	n.d.	van Beers et al. (2013)
Complement factor H-related protein 1	n.d.	van Beers et al. (2013)
Coronin	n.d.	van Beers et al. (2013)
Cyclophilin A (peptidylprolyl isomerase A)	n.d.	van Beers et al. (2013)
ER60 precursor glucose-related protein 58 kDa	n.d.	Matsuo et al. (2006)
Fibrinogen, alpha chain	Yes	Sebbag et al. (2006), Raijmakers et al. (2012), van Beers et al. (2013)
Fibrinogen, beta chain	Yes	Sebbag et al. (2006), van Beers et al. (2013)
Fibronectin	Yes	van Beers et al. (2012)
Galectin-2	n.d.	van Beers et al. (2013)
Gamma-actin	n.d.	van Beers et al. (2013)
Gelsolin	n.d.	van Beers et al. (2013)
Glia maturation factor gamma	n.d.	van Beers et al. (2013)
Glyceraldehyde-3-phosphate dehydrogenase	n.d.	van Beers et al. (2013)
Hexokinase 3	n.d.	van Beers et al. (2013)
Histamine receptor	n.d.	Matsuo et al. (2006)
Histone cluster H1.3	n.d.	van Beers et al. (2013)

(continued)

Table 3.2 (continued)

Citrullinated protein	Recognized by ACPA	References
Histone H2A.J	n.d.	van Beers et al. (2013)
Histone H2B type 2-E	n.d.	van Beers et al. (2013)
Histone H3.3	n.d.	van Beers et al. (2013)
Ig mu chain C region	n.d.	van Beers et al. (2013)
Immunoglobulin lambda locus	n.d.	van Beers et al. (2013)
Inter-alpha-trypsin inhibitor heavy chain H2	n.d.	van Beers et al. (2013)
Inter-alpha-trypsin inhibitor heavy chain H4	n.d.	van Beers et al. (2013)
Keratin, type I cytoskeletal 9	n.d.	van Beers et al. (2013)
Keratin, type II cytoskeletal 1	n.d.	van Beers et al. (2013)
Kininogen 1 light chain	n.d.	van Beers et al. (2013)
Mitochondrial aldehyde dehydrogenase	n.d.	Matsuo et al. (2006)
Myeloid cell nuclear differentiation antigen	Yes	van Beers et al. (2013)
Myeloperoxidase	n.d.	van Beers et al. (2013)
Myosin 9	n.d.	van Beers et al. (2013)
Periostin	n.d.	van Beers et al. (2013)
Probable E3 ubiquitin-protein ligase MYCBP2	n.d.	van Beers et al. (2013)
Proteoglycan 4	n.d.	van Beers et al. (2013)
Profilin 1	n.d.	van Beers et al. (2013)
Protein disulfide-isomerase	n.d.	Matsuo et al. (2006)
Prothrombin (coagulation factor II)	n.d.	van Beers et al. (2013)
Pyruvate kinase	n.d.	van Beers et al. (2013)
Rho GDP dissociation inhibitor 2	n.d.	van Beers et al. (2013)
Serum amyloid A4	n.d.	van Beers et al. (2013)
Vimentin	Yes	van Beers et al. (2013), Malakoutikhah et al. (2011)
Vinculin	n.d.	van Beers et al. (2013)

first clue that citrullinated fibrinogen (citFib) might be an autoantigen in RA was found in 2001, when it was observed that the so-called RA-specific anti-fillaggrin antibodies (a subclass of ACPA) recognized citrullinated fibrin(ogen) (Masson-Bessiere et al. 2001). Several citrullinated residues, resulting from the conversion by either PAD2, PAD4 or both, in human fibrinogen have been identified (Nakayama-Hamada et al. 2005; Kubota et al. 2005) and a number of these were found to be important for the recognition of fibrinogen by autoantibodies (Sebbag et al. 2006; Snir and Widhe 2010; Zhao et al. 2008; Ho et al. 2010; van Beers et al. 2010) (Fig. 3.2). Recently, we identified several citrullinated residues in RA SF samples, a number of which were found for the first time (Raijmakers et al. 2012; van Beers et al. 2013) (Fig. 3.3). The presence of citFib is not specific for RA (Chapuy-Regaud

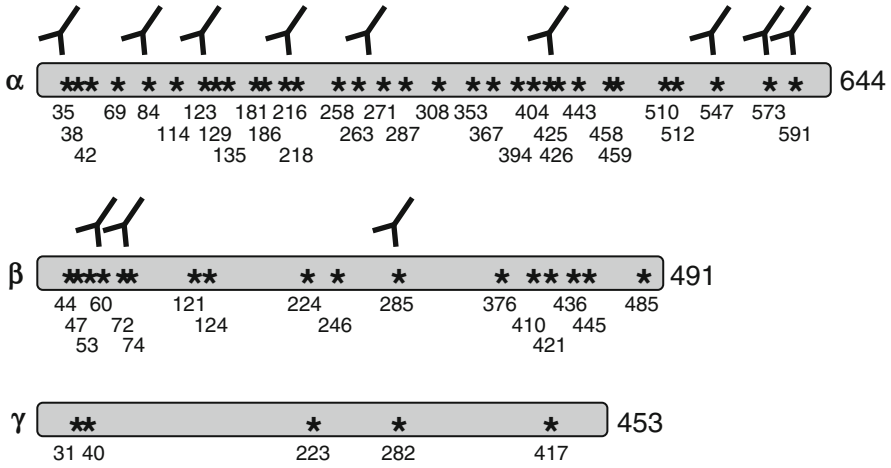

Fig. 3.2 Citrullination and auto-epitopes of human fibrinogen. Human fibrinogen was citrullinated in vitro by either the human PAD2, the human PAD4 or the rabbit muscle PAD2 enzyme, and citrullinated residues in the fibrinogen α, β, and γ chains (*asterisks*) were determined by mass spectrometry. The Y-shaped structures indicate ACPA recognition sites. Numbers refer to amino acid positions in each of the polypeptide chains (including the signal peptides and the peptides removed by thrombin cleavage)

et al. 2005), in contrast to the production of anti-citFib antibodies (Snir and Widhe 2009). An association of the presence of the latter antibodies with HLA-DRB1*04 SE alleles was found for RA patients (Snir and Widhe 2009; Auger et al. 2005). Furthermore, it was observed that circulating immune complexes containing citFib are present mainly in ACPA-positive RA patients and barely in control individuals (Van Steendam 2010; Zhao et al. 2008). These citFib containing immune complexes seem to contribute to the inflammatory response by stimulating macrophages (via Toll-like receptor 4 and Fcγ-receptor) to produce TNF, which plays an important role in maintaining inflammation in RA (Sokolove et al. 2011) (Fig. 3.4).

Besides the local production of anti-citFib antibodies in the synovial joint, anti-citFib antibodies may be produced in other parts of the human body. An interesting observation in this respect is the association of infections with *Porphyromonas gingivalis* with the development of rheumatoid arthritis *P. gingivalis* is the only prokaryote in which a deiminating enzyme (PPAD) has been detected and this enzyme has been demonstrated to citrullinate human fibrinogen (Wegner et al. 2010). (See Chap. 4 for more details.)

Fibrinogen, as a central player in the coagulation cascade, has been suggested to possess proinflammatory properties and in this way may contribute to the development of RA (Cirino et al. 1996). During coagulation, fibrinogen is converted to fibrin and fibrin deposits in RA joints are a pathological hallmark of the disease (Masson-Bessiere et al. 2001). It is known that in RA patients an imbalance between coagulation and fibrinolysis exists and that RA patients have difficulties in removing fibrin clots (Andersen and Gormsen 1970; Carmassi 1996). Citrullinated

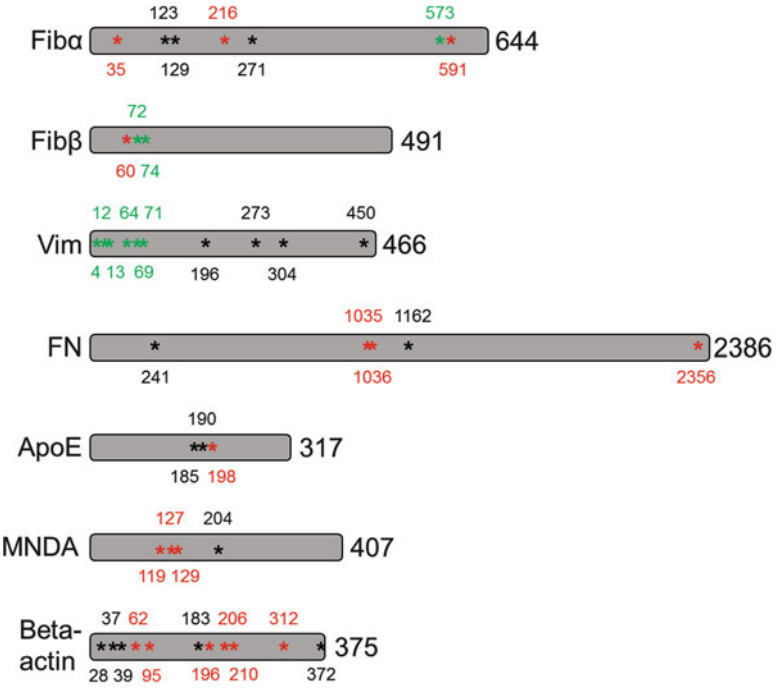

Fig. 3.3 Citrullinated residues identified in synovial proteins of RA patients. Citrullinated residues identified in fibrinogen α (Fibα), fibrinogen β (Fib β), vimentin (Vim), fibronectin (FN), apolipoprotein E (ApoE), myeloid nuclear differentiation antigen (MNDA), and beta-actin isolated from the synovial fluid of RA patients are indicated with *red* and *black asterisks*. Citrullinated residues located in regions known to be targeted by ACPA are indicated in *red*. Citrullinated residues in regions that have been demonstrated to be recognized by ACPA, but have not been identified in the RA synovium so far are marked in *green*. In view of its large size, FN is depicted on a different scale than that of the other proteins

fibrinogen might affect the balance between coagulation and fibrinolysis and may alter the antigenicity of fibrinogen as well (Nakayama-Hamada et al. 2008). In addition, fibrin clots or deposits can bind several proteins including actin filaments and fibronectin (Janmey et al. 1992; Corbett et al. 1997). The latter has binding sites for cell attachment (Corbett et al. 1997). It has been suggested that these fibrin complexes may stimulate pannus formation by acting as a scaffold and ultimately may lead to joint destruction (Fig. 3.4) (Sanchez-Pernaute and Largo 2003).

3.3.2 Vimentin

Vimentin is an intermediate filament protein involved in the organization of the cytoskeleton and can be secreted by activated macrophages (Mor-Vaknin et al. 2003). Citrullinated vimentin (citVim) was first identified as the Sa autoantigen, which was

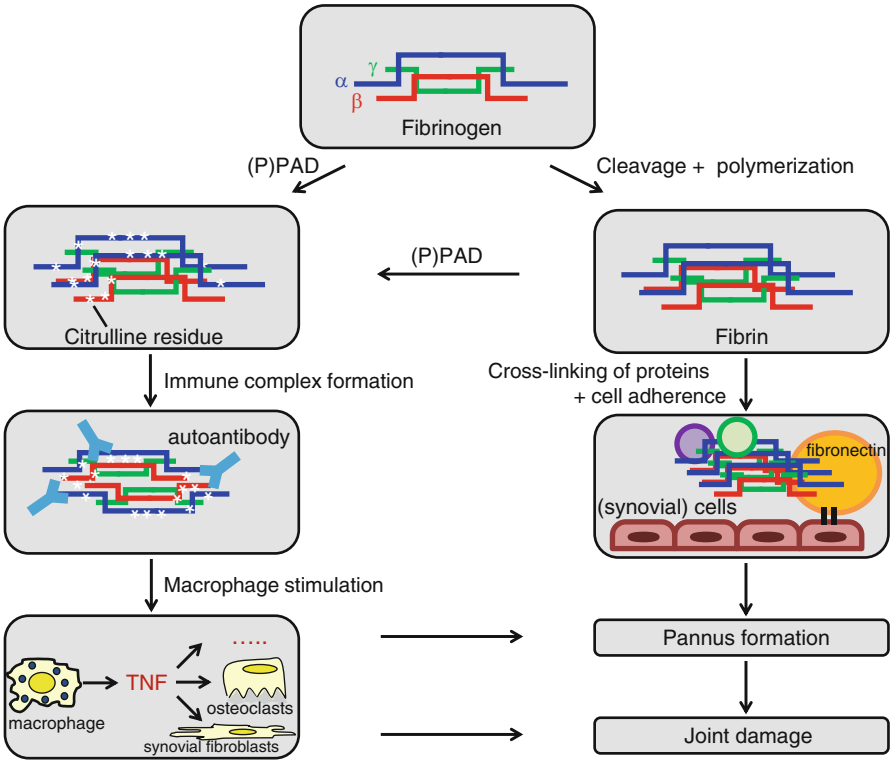

Fig. 3.4 The role of citrullinated fibrinogen in RA synovium. Fibrinogen is a hexamer containing two copies of the α, β, and γ polypeptides. Fibrinogen can become citrullinated (*white asterisks*) by PAD enzymes in the RA synovium. The presence of ACPA will lead to the formation of immune complexes, which stimulate macrophages to produce TNF. This can lead to the activation of osteoclasts and synovial fibroblasts and to pannus formation and irreversible joint damage. The cleavage of fibrinogen by thrombin initiates polymerization and cross-linking. The resulting fibrin matrix can bind several proteins (depicted by the *colored circles*), one of which is fibronectin. Fibronectin provides binding sites for cell attachment, leading to fibrin deposits, which have been suggested to contribute to pannus formation and joint damage

a very specific target for serum antibodies in RA patients and occurred in relatively high concentrations in placenta (Menard et al. 2000; Hueber et al. 1999; Hayem et al. 1999). In 2004, the Sa autoantigen was reported to be citVim (Vossenaar et al. 2004a). Since then, CitVim has been found both in the synovium and the SF of RA patients (Tilleman et al. 2008; Tabushi et al. 2008).

The B-cell response towards a specific citrullinated region (amino acids 59–74, VYATCitSSAVCitLCitSSVP) has been extensively studied, although vimentin citrullinated at these positions, or fragments thereof, have not been detected in RA synovium (Snir and Widhe 2010; van der Woude et al. 2010). RA-associated autoantibodies to a N-terminal citVim peptide, containing citrullinated residues at amino acid positions 4, 12, and 13, have been detected as well (van der Woude et al. 2010). Although

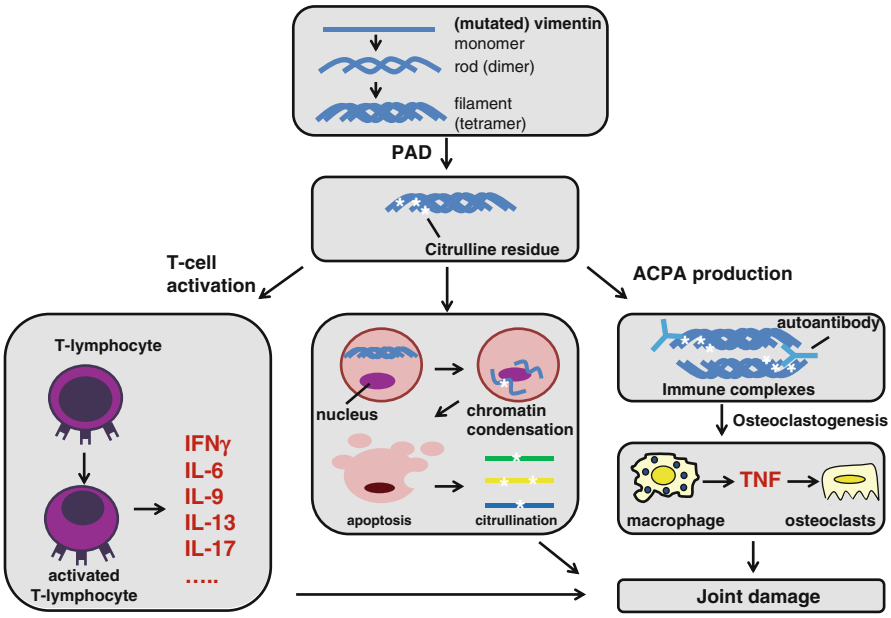

Fig. 3.5 The role of citrullinated vimentin in the RA synovium. Vimentin can form intermediate filaments via assembly of vimentin monomers. After citrullination (*white asterisks*) by PAD, vimentin can activate both T-cells and B-cells. It has been suggested that citrullination of vimentin can lead to filament disassembly and subsequently to apoptosis, which in turn, can lead to PAD activation, citrullination of autoantigens (depicted the *green*, *yellow*, and *blue bars*), and ultimately to joint damage. The mutation of a few codons in the vimentin gene may, after expression of the corresponding polypeptide, lead to a more antigenic protein. Anti-citrullinated vimentin antibodies can lead to osteoclast formation and finally joint damage

additional citrullinated residues have been identified in vimentin from RA SF samples, it is not yet known whether these reside in ACPA epitopes (Fig. 3.3) (van Beers et al. 2013). In 2010, the presence of autoantibodies against citVim was observed in sera as well as SF of patients with RA (Van Steendam 2010; Snir and Widhe 2010). These anti-citVim antibodies were found to be present in immune complexes (Van Steendam 2010), and an association between anti-citVim and the SE allele HLA-DRB1*04 was observed also for this protein (Snir and Widhe 2010). Furthermore, the role of T-lymphocytes is substantiated by the identification of citVim T-cell epitopes in HLA-DRB1*04 transgenic mice as well as in RA patients (Snir et al. 2011).

Citrullination may result in the disassembly of vimentin filaments, as observed in calcium ionophore-treated cultured mouse peritoneal macrophages, for example (Asaga et al. 1998). This can lead to shapeless citrullinated vimentin clusters around the nucleus of a cell, which may then trigger chromatin condensation and DNA fragmentation, leading to apoptosis (Asaga et al. 1998; Hojo-Nakashima et al. 2009; Durrant et al. 1999) (Fig. 3.5).

Bang and coworkers reported that mutated variants of vimentin can occur in RA patients, with amino acid substitutions at positions 16 and 59 and glycine residues

replaced by arginine residues. This protein, mutated citrullinated vimentin (MCV), appeared to be more highly reactive with RA autoantibodies than its non-mutated counterpart (Bang et al. 2007). Anti-MCV antibodies, like anti-CCP2 antibodies, are frequently present in sera as well as SF of RA patients (Snir and Widhe 2010).

It has also been demonstrated that the anti-MCV type of ACPA predicts bone-erosion in RA (Syversen et al. 2010). Most interestingly, antibodies against mutated citrullinated vimentin were recently reported to mediate osteoclast differentiation and bone resorption by inducing the production of TNF-α by osteoclast precursor cells of the monocyte/macrophage lineage (Fig. 3.5) (Harre et al. 2012).

3.3.3 Collagen Type II

Collagen type II (CII) is an important protein component of the articular cartilage and a characteristic feature of its structure is a triple helix. CII is frequently used as an inducer of arthritis in murine models (collagen-induced arthritis, CIA) and auto-antibodies against CII have been detected in RA patients (Burkhardt et al. 2005). Citrullination of CII appeared to increase its arthritogenicity and its recognition by antibodies present in sera and SF of RA patients (Lundberg et al. 2005; Burkhardt et al. 2005; Koivula et al. 2007; Yoshida et al. 2006). The presence of anti-citrullinated CII (citCII) has also been associated with HLA-DRB1*04 (Snir and Widhe 2009). Fragments of citCII have been found in SFs of RA patients (Yoshida et al. 2006) and a citrullinated synthetic triple helical peptide derived from CII (CitC1^{III}, containing citrullinated residues at positions corresponding to amino acids 361 and 368) was reported to be recognized by approximately 40 % of early RA patient sera. Citrullination at these positions of CII has not been identified in the RA synovium so far (Burkhardt et al. 2005; Snir and Widhe 2010). Also the activation of T-cells with a citrullinated peptide derived from collagen has been studied, but no differences were observed between the citrullinated and the corresponding non-citrullinated peptide, neither in healthy individuals, nor in RA patients (Law et al. 2012).

It is known that anti-CII antibodies can destabilize the synovial cartilage (Nandakumar et al. 2008). The citrullination of collagen and the generation of anti-citCII antibodies may augment this effect, although there is no experimental evidence to support this possibility yet. In addition, it has been suggested by Burkhardt and colleagues that autoimmunity to cartilage-specific modified self-proteins, such as citCII, might represent a critical intermediate step that helps to lower the threshold for the breaking of tolerance to other joint proteins (Burkhardt et al. 2005).

3.3.4 Alpha-Enolase

Alpha-enolase (or enolase 1) is an enzyme involved in glycolysis, hypoxia tolerance, growth control, fibrinolysis, extracellular matrix remodeling, and cell

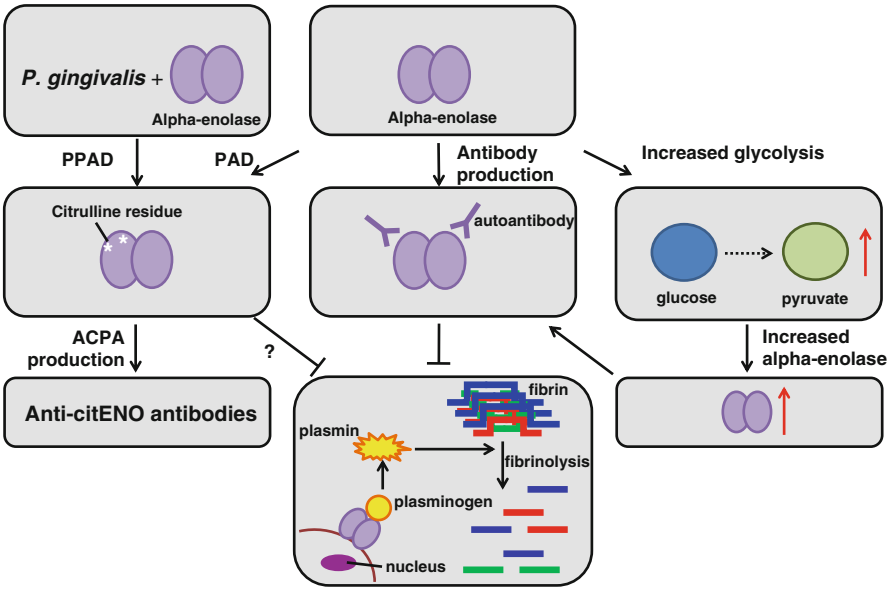

Fig. 3.6 The role of citrullinated alpha-enolase in the RA synovium. Both mammalian PAD and PAD expressed by *P. gingivalis* (PPAD) can citrullinate (*white asterisks*) alpha-enolase (*light purple dimer*). In RA, anti-alpha-enolase antibodies to the citrullinated, as well as the non-citrullinated protein, have been found. Plasminogen can bind to alpha-enolase at the cell surface. This will lead to the conversion to the proteolytically active plasmin, which is responsible for fibrinolysis and degradation of fibrin clots/deposits. Antibodies against alpha-enolase and possibly also the citrullination of alpha-enolase can inhibit the activation of plasmin and as a consequence the degradation of fibrin deposits. Alpha-enolase is involved in glycolysis and increased levels of glycolysis have been observed in RA patients. This may be accompanied by elevated alpha-enolase levels, which might contribute to anti-alpha-enolase antibody production and thus to the inhibition of fibrinolysis

migration (Miles et al. 1991; Pancholi 2001; Wygrecka et al. 2009). The homodimeric alpha-enolase is expressed by neutrophils, lymphocytes, and monocytes, which are all present in the inflamed synovium. Alpha-enolase can serve as a plasminogen-binding receptor, which can lead to the conversion to plasmin, an enzyme involved in the degradation of many blood plasma proteins, such as fibrin clots (Fig. 3.6). It has been shown that alpha-enolase expression at the cell surface is upregulated in RA patients (Bae et al. 2012). Like non-citrullinated CII, native alpha-enolase was reported to be an autoantigen in RA (Kinloch et al. 2005). Anti-alpha-enolase autoantibodies, however, are not very specific for this disease, because they are also present in several other autoimmune and inflammatory conditions (Saulot et al. 2002). In contrast, it was discovered in 2005 that antibodies to citrullinated alpha-enolase (citENO) display a much higher specificity for RA (Kinloch et al. 2005). In addition to citrullination by human PAD enzymes, PPAD was also found to citrullinate alpha-enolase peptides (Wegner et al. 2010).

Further analyses mapped the main citrullinated autoepitope in the N-terminal region of the protein (citENO peptide 1 or CEP-1) (Snir and Widhe 2009). The CEP-1 peptide contains citrullinated residues at alpha-enolase positions 9 and 15 (Kinloch et al. 2005). However, similar to the situation for the immunodominant citCII peptide, the corresponding citrullinated form of alpha-enolase has not been detected in the RA synovium so far. The CEP-1 peptide was identified by screening multiple citENO peptides with RA sera and antibodies to this peptide occur in approximately 50 % of the anti-CCP2-positive RA patients (Lundberg et al. 2008). Although the presence of citENO in the RA synovium has been reported, details about the citrullinated residues are still lacking (Kinloch et al. 2005, 2008).

Anti-CEP-1 antibodies were detected in sera as well as in SF of RA patients (Snir and Widhe 2010). Analyses by Snir and coworkers revealed an association between the presence of anti-CEP-1 antibodies and HLA-DRB1*04 SE alleles (Snir and Widhe 2009). In addition, associations between anti-CEP-1 antibodies and smoking in combination with SE alleles and between anti-CEP-1 antibodies and the PTPN22 gene, in combination with SE alleles, were observed in RA patients (Mahdi et al. 2009). Fisher and colleagues have addressed the question whether anti-CEP-1 antibodies can predict disease outcome, but they did not observe significant differences between anti-CEP-1-positive and anti-CEP-1-negative patients (Fisher et al. 2011).

Antibodies against non-citrullinated alpha-enolase inhibit the binding of plasminogen to alpha-enolase, leading to the inhibition of fibrin clot degradation (Moscato et al. 2000) (Fig. 3.6). It is not known whether anti-citENO antibodies have this capacity as well. Alternatively, citrullination of alpha-enolase itself may also affect the binding of plasminogen.

As already mentioned above, alpha-enolase is a glycolytic enzyme and increased glycolysis has been observed in RA synovial cells. This may trigger, via enzymes and products of the glycolysis pathway, autoimmunity (via alpha-enolase) (Saulot et al. 2002), angiogenesis (via pyruvic acid) (Lee et al. 2001), tissue destruction by metalloproteinases (via glucose-6-phosphate isomerase or GPI) (Yu et al. 2004), and apoptosis (possible link between glycolysis and apoptosis) (Otsuki et al. 2005), which are prominent features of the pathogenesis of RA. Citrullination of alpha-enolase might alter its function and may increase its antigenicity (Fig. 3.6) (Kinloch et al. 2005). Interestingly, citrullinated isoforms of additional glycolytic enzymes were recently found in RA SF: glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase. It remains to be investigated whether the citrullination of glycolytic enzymes contributes to the pathophysiology of RA and whether the latter enzymes are also targeted by ACPA.

3.3.5 Fibronectin

Fibronectin (FN) is a large glycoprotein, which is expressed by various cell types. The most basic function of FN is adhesion to other macromolecules (e.g., fibrin, DNA) and cells. In addition, FN is involved in wound healing, cytoskeletal

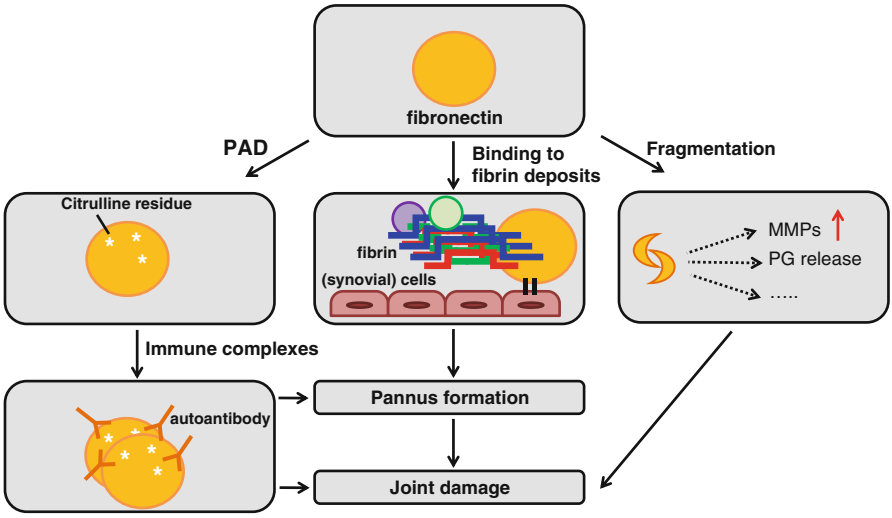

Fig. 3.7 The role of citrullinated fibronectin in the RA synovium. The citrullination (*white asterisks*) of fibronectin and the formation of immune complexes containing citrullinated fibronectin in ACPA-positive RA patients have been demonstrated previously. Fibronectin can (together with other proteins) bind to fibrin deposits and mediate the association of these complexes with the cell surface. This can contribute to pannus formation and joint damage. In addition, fragments of fibronectin have been detected in RA patients and these have been shown to increase proteinase (MMP) production and proteoglycan (PG) release from the cartilage matrix, which will also contribute to cartilage destruction and joint damage

organization, cell migration, phagocytosis, and embryogenesis (Hynes and Yamada 1982). In RA patients, FN has been detected in pannus tissue and in immune complexes present in sera (Herbert et al. 1987; Scott et al. 1981).

In 2005, citrullinated FN (citFN) was reported to be a potential autoantigen in RA, because it was found to be present in synovial tissue and SF of RA patients (Chang et al. 2005; Tabushi et al. 2008). A more detailed analysis of citFN from RA SF recently allowed the identification of five arginines that can be citrullinated in FN (Fig. 3.3) (van Beers et al. 2012). Autoantibodies present in RA sera frequently recognize epitopes containing citrullinated residues at positions 1,035, 1,036, and 2,356. Antibodies to the epitope containing the two flanking citrullines 1,035 and 1,036 were detected in 47 % of RA patients, were already present early in the disease and appeared to be associated with SE alleles. Interestingly, 2 % of RA patients showed reactivity with only the citFN peptide, but not with citrullinated peptides derived from vimentin, fibrinogen and alpha-enolase (van Beers et al. 2012). Therefore, anti-citFN peptides may have additional value for diagnostics and RA patient classification.

Fibronectin can bind to fibrin deposits, which are a hallmark of the inflamed joints in RA, and these FN-fibrin complexes can adhere to cells (Corbett et al. 1997). This may contribute to pannus formation by acting as a scaffold (Fig. 3.7). Fragments of FN can stimulate the production of multiple mediators of matrix destruction

(e.g., various cytokines and metalloproteinases), suggesting a role in articular cartilage destruction (Fig. 3.7) (Xie and Homandberg 1993; Homandberg et al. 1993). It is unclear whether and, if so, how citrullination of FN and the binding of antibodies to citrullinated FN affect these activities.

3.3.6 Apolipoprotein E

Apolipoproteins, which are involved in lipid binding and transport, have been previously detected in RA SF (Kinloch et al. 2008; Ananth et al. 1993). The concentration of certain apolipoproteins (ApoA1 and ApoB) was shown to be increased in RA SF, suggesting a role in the inflamed synovium (Ananth et al. 1993).

Citrullinated residues were detected ApoE at positions 185, 190 and 198 (Fig. 3.3). Autoantibodies against a citrullinated ApoE (citApoE) peptide (containing citrulline 198) were detected in RA sera, but not in sera from healthy individuals (van Beers et al. 2013). In contrast, using another citrullinated ApoE peptide, Chandra and coworkers did not observe differences in reactivity between RA patients and healthy individuals (Chandra et al. 2011). This suggests that the recognition of citrullinated molecules by ACPA is strongly influenced by the amino acid context of the protein in question.

3.3.7 Myeloid Nuclear Differentiation Antigen

Myeloid nuclear differentiation antigen (MNDA) is a member of the p200 or HIN-200 protein family, the expression of which can be induced by interferon (IFN). Other p200 family members have been implicated in other autoimmune diseases, such as systemic lupus erythematosus and systemic sclerosis (Mondini et al. 2007). A microarray analysis showed that MNDA mRNA was more highly expressed in white blood cells of arthritis patients than in those of healthy individuals (Gu et al. 2002). Furthermore, MNDA has been reported to bind to nucleophosmin/B23, which also has been identified as a citrullinated protein (Hagiwara et al. 2002).

Citrullinated MNDA (citMNDA) has recently been detected in RA SF samples (van Beers et al. 2013). Citrullinated residues were observed at positions 119, 127, 129 and 204 (Fig. 3.3). RA sera were shown to be predominantly reactive with a synthetic peptide containing citrullines at positions 127 and 129, and to lesser extent with a peptide containing the citrullinated residue at position 119 (van Beers et al. 2013).

3.3.8 Beta-Actin

Beta actin is one of six different human actin isoforms, which is a cytoskeletal protein of non-muscle cells, important for cell survival and involved in cell migration, movement, structure and integrity (Bunnell et al. 2011).

Matsuo and colleagues identified citrullinated mutant beta-actin in RA synovial tissue. Mutant beta-actin differs from wild-type beta-actin at position 139, where a valine residue is replaced by a methionine residue, and at position 295, where an alanine residue is replaced by aspartic acid residue (Matsuo et al. 2006). Recently, Darrah and coworkers have identified beta-actin as a substrate for PAD2 in ionomycin-activated neutrophils (Darrah et al. 2012), which results in citrullination at six residues (amino acids 176, 183, 196, 206, 312, and 372). The recently identified citrullinated beta-actin residues in our proteomic analysis of RA SF comprise five residues also identified by Darrah and coworkers (183, 196, 206, 312, and 372; Fig. 3.3) (van Beers et al. 2013). In addition, citrullinated beta-actin contains several epitopes recognized by RA autoantibodies and the most important epitopes appeared to be at positions 196, 206, and 210 (van Beers et al. 2013). The consequences of beta-actin citrullination for RA remain to be investigated.

3.4 The Diagnostic and Prognostic Value of Citrullinated Autoantigens

As described above, several citrullinated proteins have been reported to be targeted by the immune system in RA. The questions whether and, if so, how they contribute to the pathophysiology of RA and whether they are useful for improving the diagnosis and/or prognosis of RA are largely unanswered. In fact, it has been shown that the ACPA response in RA is very heterogeneous (Willemze et al. 2012). As a result, ACPA recognize multiple citrullinated proteins (van Beers et al. 2012; Snir and Widhe 2009; Willemze et al. 2012) and multiple epitopes (Snir and Widhe 2010) that differ among individual patients (van Beers et al. 2010, 2012; Willemze et al. 2012).

Several studies have addressed the use of these citrullinated antigens for diagnostic purposes, in particular to improve the sensitivity of ACPA detection. However, none of the resulting tests is superior to the CCP2 assay, when sensitivities are compared at stratified specificities (Coenen et al. 2007; Bizzaro et al. 2007). For example, Van der Cruyssen and colleagues investigated the diagnostic value of anti-citFib antibodies (AhFibA-ELISA) (Nielen et al. 2005) and compared the data with other ACPA tests, including a test based on peptides derived from filaggrin, including three different peptides, one of which corresponds to the peptide used in the first generation cyclic citrullinated peptide (CCP1) test and a commercially available CCP2 test. They observed that, for diagnostic purposes, the AhFibA test worked as well as the CCP2 test (Vander 2006).

Several researchers have investigated the diagnostic potential of anti-MCV antibodies and compared the results with anti-CCP2 data. ACPA detection by MCV led to similar sensitivities (Coenen et al. 2007; Bizzaro et al. 2007; Dejaco et al. 2006; Mathsson et al. 2008; Poulsom and Charles 2008), but comparable (Mathsson et al. 2008; Poulsom and Charles 2008) or lower specificities (Coenen et al. 2007; Bizzaro et al. 2007; Dejaco et al. 2006) than the anti-CCP2 test.

It is generally believed that anti-CCP2 antibodies cover reactivities with multiple citrullinated proteins (Ioan-Facsinay 2011). There is also evidence that the diversity of ACPA subspecificities (ACPA fine-specificity) is mainly generated prior to disease onset, probably at least in part mediated by epitope spreading. ACPA-positive undifferentiated arthritis patients already have a diverse set of antibodies reactive with distinct citrullinated antigens at the moment they enter the clinic (van der Woude et al. 2010; van de Stadt et al. 2011).

Overall, it is often difficult to compare the results of different studies as a result of clinical trial heterogeneity, choice of study populations and use of different cut-off values, even when the same peptides/tests are used. This implies that one has to be careful in drawing conclusions about the true diagnostic and prognostic value of ACPA tests.

A challenging new approach is to analyze ACPA profiles using multiple citrullinated antigens and epitopes simultaneously and changes therein during the course of the disease and upon treatment. In this respect, it is interesting to note that low and intermediate pretreatment levels of ACPA appear to be associated with a more favorable response to methotrexate treatment in recent onset, anti-CCP-positive arthritis patients, whereas high ACPA levels are associated with no or only a poor response (Visser et al. 2010). ACPA profiles can be monitored by using multiplex assays, for which several systems have been developed (Chandra et al. 2011; Lokate et al. 2007; Sokolove et al. 2012; Robinson et al. 2002) and it has recently been shown that biomarker profiles, including ACPA, predict the imminent onset of clinical arthritis (Sokolove et al. 2012).

3.5 Carbamylation Versus Citrullination of Proteins

When peptidyllysine is converted into peptidylhomocitrulline, this process, designated as carbamylation (see Chap. 20 for more detail), results in a structure that is chemically very similar to peptidylcitrulline (Fig. 3.8). Carbamylation of proteins can occur in urea-containing solutions via the spontaneous formation of isocyanic acid. Carbamylation can also occur in vivo, especially during inflammation, and is mediated by the formation of cyanate, which reacts with the lysine side chain to form the homocitrulline side chain. In addition, carbamylation can be driven by the enzyme myeloperoxidase, which is abundantly expressed in neutrophils and which uses thiocyanate to form homocitrulline residues, especially during inflammation. Interestingly, thiocyanate levels are elevated in smokers and smoking is an environmental risk factor for RA (Wang et al. 2007). It is feasible that carbamylation, in addition to citrullination, plays a role in RA, because it was observed that a subset of the ACPA-negative RA patients do show anti-carbamylated protein antibodies and these autoantibodies associate with more severe radiological damage in ACPA-negative RA patients (Shi et al. 2011).

RA autoantibodies are able to discriminate between carbamylated and citrullinated proteins, although their structures are very similar. Nevertheless, some sera

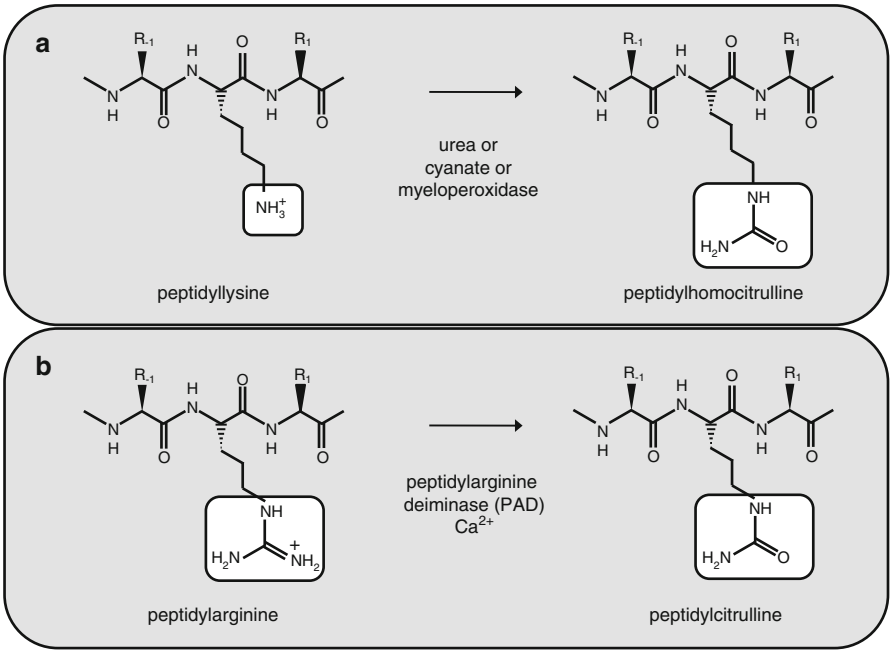

Fig. 3.8 Carbamylation mimicks citrullination. (**a**) Carbamylation (see Chap. 20 for more detail) is the posttranslational conversion of peptidyllysine into peptidylhomocitrulline and is driven by either urea (in vitro), cyanate (inflammation) or myeloperoxidase (inflammation). (**b**) Citrullination is the posttranslational conversion of peptidylarginine into peptidylcitrulline and is catalyzed by the Ca^{2+}-dependent peptidylarginine deiminase (PAD) enzymes. R_{-1} and R_1 refer to the side chains of the amino acids flanking the citrullinated residue

recognize both carbamylated and citrullinated proteins (Shi et al. 2013). Anti-carbamylated fibrinogen antibodies and anti-carbamylated vimentin antibodies have been identified in RA sera (Scinocca et al. 2011; Bang et al. 2011). Furthermore, it has been shown that immunization with carbamylated peptides derived from filaggrin, predisposes to the development of erosive arthritis. Mydel and colleagues identified carbamylated peptides by mass spectrometry in blood and SF samples of RA patients to quantify the amount of citrullinated and carbamylated residues expressed as absolute concentrations, but the identities of the carbamylated proteins were not disclosed (Mydel et al. 2010).

3.6 The Role of Citrullination in the Pathophysiology of RA Is Supported by Experiments in Mouse Models

Many animal models have been developed to study arthritis, the most frequently used mouse models being the streptococcal cell wall model, which represents an acute destructive arthritis, and the collagen-induced arthritis model, which

represents a more chronic type of arthritis (Billiau and Matthys 2011; Wooley 1991). The production of ACPA in these animal models has been debated, because conflicting results have been obtained (Vossenaar et al. 2004c; Kidd et al. 2008; Kuhn et al. 2006).

In view of the strong association of ACPA production in humans with shared epitopes, the role of citrullination and ACPA in RA has been studied in transgenic animals expressing human HLA-DRB1 alleles. Hill and coworkers observed that citFib can induce arthritis in HLA-DR4-IE transgenic mice, whereas native fibrinogen cannot (Hill et al. 2008). The immunization with citFib also led to the production of ACPA and the development of fibrinogen-reactive T-lymphocytes (Ho et al. 2010).

Besides citFib, citCII can induce arthritis in mice (Thiele et al. 2012) and the immunization with alpha-enolase citrullinated by PPAD induced autoimmunity and arthritis in the DR4-IE-transgenic mice (Kinloch et al. 2011). However, in case of citENO, also autoantibodies against native alpha-enolase were observed, suggesting that the response observed might be citrulline-independent.

An indication that ApoE might play a role in RA came from a recent study in which ApoE deficiency was shown to exacerbate collagen-induced arthritis (CIA) in mice (Postigo et al. 2011). It is tempting to speculate that the inflammation-associated citrullination of ApoE results in similar effects. A role for ApoE in the pathophysiology of arthritis is further supported by observations of Asquith and coworkers, who found that ApoE deficient mice are resistant to CIA development (Asquith et al. 2010). These contradictory observations, exacerbation of CIA in one study and the resistance to CIA in another, might be related to the use of different CIA models. For example, there were differences in both the mouse strains and the sources of collagen that was used in these studies.

3.7 Concluding Remarks

During the last decade, the involvement of citrullinated proteins and antibodies reactive with these proteins in a citrulline-dependent manner in the pathophysiology of RA has been well established. The initial trigger for the immune system to respond to citrullinated epitopes is still enigmatic, although increasing evidence suggests that environmental factors, such as smoking, are involved (see Chap. 5 for more detail). In combination with a susceptible genetic background, in which HLA alleles play a prominent role, adaptive immunity to citrullinated proteins will be generated. The production of ACPA and the citrullination of proteins in inflamed joints will lead to the formation of immune complexes, which subsequently stimulate the immune system and the inflammatory process. Although up to now, only citrullinated fibrinogen, fibronectin and vimentin have been found in immune complexes in RA. As a result, it is likely that most, if not all, citrullinated proteins in inflamed joints are targeted by ACPA and thus may contribute to immune complex

formation. When the resulting chronic inflammation is not repressed, this may lead to irreversible damage of cartilage and bone.

To understand the molecular aspects of this process in more detail, it is important to know which proteins are citrullinated in the inflamed joints of RA patients and which of these are targeted by the immune system. Recent data indicate that numerous proteins are citrullinated in the RA joints and that ACPA are probably reactive with the majority of these. As a consequence, the composition of immune complexes may be very diverse. The heterogeneity of ACPA raises the question whether or not a key citrullinated antigen exists that initially triggers the anti-citrullinated protein response, before, via epitope spreading, the heterogeneity is generated. ACPA occur in approximately 75 % of established RA patients and the "ACPA fine-specificity" differs among individual ACPA-positive patients. Intriguing questions are whether ACPA profiles correlate with clinical phenotypes and can predict the onset of clinical arthritis and whether ACPA profiling facilitates the subclassification of RA patients, which may aid the development of "patient-tailored" therapies in the future. The results that will be generated by multiplex systems, which now can be rationally developed based upon our knowledge of citrullinated autoantigens in the inflamed joint, might answer the latter question in the near future.

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Chapter 4
Protein Citrullination: The Link Between Rheumatoid Arthritis and Periodontitis?

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Keywords Rheumatoid arthritis • Periodontitis • *Porphyromonas gingivalis* • Protein citrullination

4.1 Introduction

Hippocrates was the first (400 BCE) to report on a case of successful treatment of "rheumatism" after the extraction of a tooth in a patient with joint pain (O'Reilly and Claffey 2000). Periodontitis is an infectious process with destruction of the supporting soft and hard tissue of the teeth (the periodontium) as a consequence. It is a multifactorial, bacterial driven, chronic inflammatory disorder that occurs in 10–15 % of the adult population, independent of ethnicity and geographic location (Jansson 2006). Clinical signs of periodontitis are bleeding gums, deepened

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periodontal pockets, suppuration, and, in an advanced stage, mobility of teeth and tooth loss due to extensive loss of alveolar bone.

Periodontitis is classified as chronic (slowly progressive) or aggressive (highly destructive). Further classification can be made on the extent (localized/generalized) and severity (mild/moderate/severe) of the disease (Armitage 2003). In addition, periodontitis is not a local disease, since the infected and necrotic epithelium surface area in severe periodontitis can amount up to 20 cm^2 and may lead to bacteremia that can result in a dental focal infection (Chiu 1999; Madianos et al. 2001; van Winkelhoff and Slots 1999). Also, severe periodontitis can lead to a continuous systemic inflammatory response; periodontitis patients have a lower hematocrit, lower number of erythrocytes, lower hemoglobin levels, and higher erythrocyte sedimentation rates compared to healthy controls (Hutter et al. 2001). Periodontitis also results in higher systemic levels of neutrophils, serum elastase, and inflammatory mediators such as C-reactive protein (CRP) and IL-6 (Loos et al. 2000; Wohlfeil et al. 2012). In addition, levels of soluble CD14, which mediates the response to lipopolysaccharide (LPS) in cells lacking membrane-bound CD14, are significantly higher in sera of patients with periodontitis than those of healthy subjects and decrease after treatment (Hayashi et al. 1999). Treatment of aggressive periodontitis leads to a significant decrease of circulating leucocytes, neutrophils, and platelets (Christan et al. 2002). Periodontal treatment in patients with diabetes mellitus type I or cardiovascular disease and periodontitis resulted in a decrease of markers of systemic inflammation as CRP, tumor necrosis factor alpha (TNFα), and interferon gamma (IFNγ) (Montebugnoli et al. 2005; Correa et al. 2010). Severe periodontitis has been associated with a number of chronic and inflammatory diseases such as diabetes mellitus (Mealey and Oates 2006), atherosclerosis, cardiovascular disease and stroke (Scannapieco et al. 2003), Crohn's disease and ulcerative colitis (Brito et al. 2008), and rheumatoid arthritis (Berthelot and Le 2010).

4.2 Rheumatoid Arthritis and Periodontitis

Rheumatoid arthritis (RA) is a chronic polyarthritis of autoimmune origin, with a prevalence of 0.5–1.0 % of adults in industrialized countries (Tobon et al. 2010). The pathogenesis is poorly understood, but the risk of developing RA is 50 % attributable to genetic factors (van der Woude et al. 2009). Smoking is the major known environmental risk factor for RA. Immune responses with several inflammatory cascades lead towards a final common pathway with persistent synovial inflammation and associated damage to articular cartilage and underlying bone as a consequence. Despite its predominant osteoarticular and periarticular manifestations, RA is a systemic disease and is associated with cutaneous and organ-specific extra-articular manifestations (Prete et al. 2011). Epidemiological studies show a significantly increased prevalence of periodontitis in RA patients (DePablo et al. 2008; Mercado et al. 2001), and also a higher prevalence of RA among patients with periodontitis has been noted (Mercado et al. 2000; Nesse et al. 2010). Moreover, disease severity

of RA seems to be related to the severity of periodontitis (Mercado et al. 2001; Marotte et al. 2006; de Smit et al. 2012a). Intervention studies point towards a beneficial effect of periodontal treatment on RA disease activity (Al-Katma et al. 2007; Ortiz et al. 2009), but more experimental clinical evidence is necessary to substantiate these observations (de Smit et al. 2012b).

4.2.1 Similarities Between RA and Periodontitis

There are remarkable similarities between RA and periodontitis. As mentioned in the previous paragraph, both diseases are multifactorial, chronic, destructive, inflammatory disorders characterized by deregulation of the host inflammatory response. Susceptibility to the diseases is influenced by shared genetic and lifestyle factors as the HLA-DRB1 shared epitope alleles and smoking (Kallberg et al. 2007; Bonfil et al. 1999). Both diseases are cumulative, i.e., severity, loss of function, and quality of life decrease with disease duration. Moreover, RA and periodontitis share pathological mechanisms in that both conditions are potentiated by an exaggerated inflammatory response featuring an increase in local and circulating pro-inflammatory mediators and resulting in soft and hard tissue destruction of the periodontium and synovium, respectively.

4.2.2 Autoimmunity to Citrullinated Proteins is Specific for RA

Autoimmunity to citrullinated proteins is very specific for RA and is considered of pathogenic significance (Vossenaar and van Venrooij 2004). Formation of anti-citrullinated protein antibodies (ACPAs; see Chap. 3) can precede clinical manifestation of RA (Nielen et al. 2004). The autoantibodies most frequently found in RA patients are the rheumatoid factor (RF), which are antibodies that bind to the constant domain of IgG molecules, and ACPAs. The majority of individuals with RA (50–80 %) have positive serum titers for IgM-RF and/or ACPAs. ACPAs have a higher specificity (98 %) and sensitivity (up to 80 %) for diagnosis of RA than IgM-RF (specificity 91 %, sensitivity 54 %) (Schellekens et al. 2000). ACPAs may be a better prognostic indicator than IgM-RF as well, since ACPA-positive RA is associated with increased joint damage and low remission rates (van der Helm-van Mil et al. 2005), suggesting a role for ACPAs in the pathophysiology of the disease. ACPAs exist in around 2 % of the normal population and are rare in other inflammatory conditions (Aggarwal et al. 2009). ACPAs were initially identified as antibodies against keratin (the so-called anti-perinuclear factor) in buccal epithelial cells (Nienhuis and Mandema 1964), and more recently, these autoantigens have been identified as citrullinated proteins (van Venrooij et al. 2004).

Citrulline is a nonstandard amino acid and is therefore not incorporated in proteins during translation. However, it can be generated by posttranslational modification (deimination) of protein-bound arginine by peptidylarginine deiminase (PAD) enzymes. This posttranslational modification has a significant impact on the structure and function of the target protein, usually resulting in unfolding due to the loss of positive charges from the arginine residues. Citrullination is an inflammation-associated phenomenon, occurring in a wide range of tissues. For example, this process is predominantly observed in proteins of the cytoskeleton and seems to represent a general regulatory mechanism, particularly occurring during apoptosis. So far, five isotypes of PAD have been described in humans (see Chap. 1 for more details). Briefly, all of these PAD enzymes rely strongly on the presence of calcium for their activity. Because of their calcium dependency, PAD enzymes are more likely to be active in the extracellular compartment. Whereas citrullination is associated with inflammation in general, the development of ACPAs is specific for RA. The specificity of ACPAs is therefore most likely the result of an abnormal (auto)immune response to these proteins in RA patients.

4.2.3 Role of Porphyromonas gingivalis

There are a number of postulated mechanisms by which infections can trigger autoimmune diseases, but most evidence obtained from animal models support the idea that cross-reactive immune responses cause autoimmunity due to "molecular mimicry" between microbiological and self-antigens (Getts and Miller 2010). Rosenstein et al. (2004) have hypothesized that *Porphyromonas gingivalis*, a major periodontal pathogen, plays an important role in the pathogenesis of RA. According to this theory, subgingival bacteria in periodontitis accumulate in a biofilm that comprises predominantly Gram-negative anaerobic rods. This group of Gram-negative, dark-pigmented anaerobic rods is strongly associated with destructive periodontal disease (Zambon 1996). The major pathogen in this group is *P. gingivalis* (van Steenbergen et al. 1989). The prevalence of this pathogen in severe periodontitis is up to 70 %, and *P. gingivalis* has rarely been isolated from subjects without periodontitis (van Winkelhoff et al. 2002), suggesting that this bacterium is not a normal inhabitant of a healthy periodontium (Griffen et al. 1998). To date, only prokaryotic PAD has been identified in *P. gingivalis* (McGraw et al. 1999). This bacterial PAD enzyme could act as a virulence factor based on its biochemical characteristics and properties. In contrast to human PAD, it can convert both peptidylarginine and free L-arginine and is not dependent on calcium (Shirai et al. 2001).

Known antibodies to citrullinated proteins, the abovementioned specific serological markers for RA, include anti-citrullinated keratin (previously the anti-perinuclear factor), anti-citrullinated vimentin (formerly known as the Sa-antigen), anti-citrullinated filaggrin, anti-citrullinated fibrin(ogen), and anti-citrullinated α-enolase antibodies (van de Stadt et al. 2011). Alpha-enolase is a multifunctional

protein, best known for its role in glucose metabolism and more recently as a plasminogen-binding protein on the surface of various mammalian and prokaryotic cells (Pancholi and Fischetti 1998; Wygrecka et al. 2009). In RA, the immunodominant epitope of human α-enolase is citrullinated-enolase-peptide-1 (CEP-1). This epitope (amino acids 5–21) demonstrates 82 % sequence similarity with CEP-1 of *P. gingivalis*, and amino acids 13–21 are 100 % identical. Antibodies that are affinity-purified for human CEP-1 cross-react with CEP-1 of *P. gingivalis* (Lundberg et al. 2008). Besides citrullination of its endogenous proteins, *P. gingivalis* is also able to citrullinate human fibrinogen and human α-enolase (Wegner et al. 2010). Thus, the immune system in patients with a periodontal infection with *P. gingivalis* is exposed to citrullinated antigens that might become systemic immunogens directly or via molecular mimicry and cross-reactivity. Periodontal infection with *P. gingivalis* could contribute to the total antigenic load of citrullinated proteins generated by host PAD during the inflammatory response and by bacterial PAD produced as a virulence factor of *P. gingivalis*. In a genetically susceptible host, this could result in a pathologic immune response, first resulting in the formation of ACPA, followed by joint inflammation (Hendler et al. 2010).

4.2.4 Porphyromonas gingivalis *in RA Patients*

Oral colonization by *P. gingivalis* in RA patients is barely considered. Scher et al. (2012) detected P. *gingivalis* more frequently in recently diagnosed, never treated RA patients in comparison with established RA patients or healthy controls. This can, however, be explained by the higher prevalence of severe periodontitis in RA patients. In a recent study, we found no difference in subgingival presence of *P. gingivalis* between established RA patients and non-RA controls matched for age, gender, smoking, and periodontal status (de Smit et al. 2012a).

Higher antibody titers against *P. gingivalis* in RA patients and a positive correlation with ACPA have been reported, suggesting that infection with periodontal pathogens may play a role in risk and progression of RA, but these studies did not consider the periodontal status or the microbiology of the RA patients (Hitchon et al. 2010; Mikuls et al. 2009; Ogrendik et al. 2005; Tolo and Jorkjend 1990). We could confirm elevated antibody titers against *P. gingivalis* in RA patients (de Smit et al. 2012a). In fact, we found higher anti-*P. gingivalis* titers in RA patients with severe periodontitis compared to matched non-RA controls with severe periodontitis and an equal distribution in subgingival *P. gingivalis*. No differences in anti-*P. gingivalis* titers between these groups were found in case of moderate periodontitis, but these RA patients still had higher titers compared to severe periodontitis patients without RA. Furthermore, in contrast to the findings of Dissick et al. (2010), we found no association between the presence of periodontitis and ACPA and/or IgM-RF levels.

4.3 Citrullinated Proteins in Rheumatoid Arthritis and Periodontitis

4.3.1 Citrullinated Proteins in Synovial Tissue

PAD2 and PAD4 are the only human PAD isotypes expressed in synovial tissue and synovial fluid of RA patients and patients with other arthritides (Foulquier et al. 2007; Vossenaar et al. 2004a). These PAD enzymes are mainly expressed by inflammatory cells; therefore, PAD2 and PAD4 are the most likely candidates to citrullinate synovial proteins in RA. Indeed, citrullinated proteins have been detected in RA synovial tissue (Baeten et al. 2001; Masson-Bessiere et al. 2001). The presence of citrullinated proteins in the inflamed synovium is not specific for RA, but rather, it may be an inflammation-associated phenomenon (Vossenaar et al. 2004b). Synovial fluid is identified as a site of citrullination of autoantigens as α-enolase (CEP-1), fibrin, and vimentin in inflammatory arthritis (Kinloch et al. 2008). The observations of higher ACPA levels in synovial tissue and synovial fluid compared to serum ACPA levels, and the presence of ACPA-producing plasma cells in synovial tissue, provide evidence for local ACPA production in the inflamed synovium (Masson-Bessiere et al. 2001).

4.3.2 Citrullinated Proteins in Periodontitis Tissue

As mentioned before, the discovery of ACPA in 1964, then called anti-perinuclear factor, was made on buccal late-stage epithelial cells (Nienhuis and Mandema 1964). In addition to the presence of citrullinated proteins in buccal epithelium, the periodontal epithelium may also contain citrullinated proteins. Recently, Nesse et al. (2012) assessed the presence and localization of citrullinated proteins in periodontal tissue of periodontitis patients without any other systemic disease. Clinically non-inflamed periodontal tissue was obtained from patients undergoing prophylactic removal of impacted third molars. The various epithelial cell samples were stained for the presence of citrullinated proteins using an anti-citrullinated protein rabbit polyclonal antibody (Ab5612; Millipore, MA, USA) and a mouse anti-citrullinated protein IgM monoclonal antibody (F95; kindly provided by A.P. Nicholas, University of Alabama, Birmingham, AL, USA). As positive controls, synovial tissue samples of RA patients, previously obtained during joint replacement surgery, and buccal epithelial cells from healthy donors known to be positive for anti-perinuclear factor staining were used.

In the stromal component of the periodontitis tissue samples, citrullinated proteins were detected in 53, 67, and 80 % of the samples using Ab5612, F95, or either antibody, respectively. Citrullinated proteins were detected in stroma of one-third of control tissue samples (Figs. 4.1 and 4.2). In epithelium, citrullinated proteins were detected in periodontitis and control tissue samples whenever using Ab5612. In

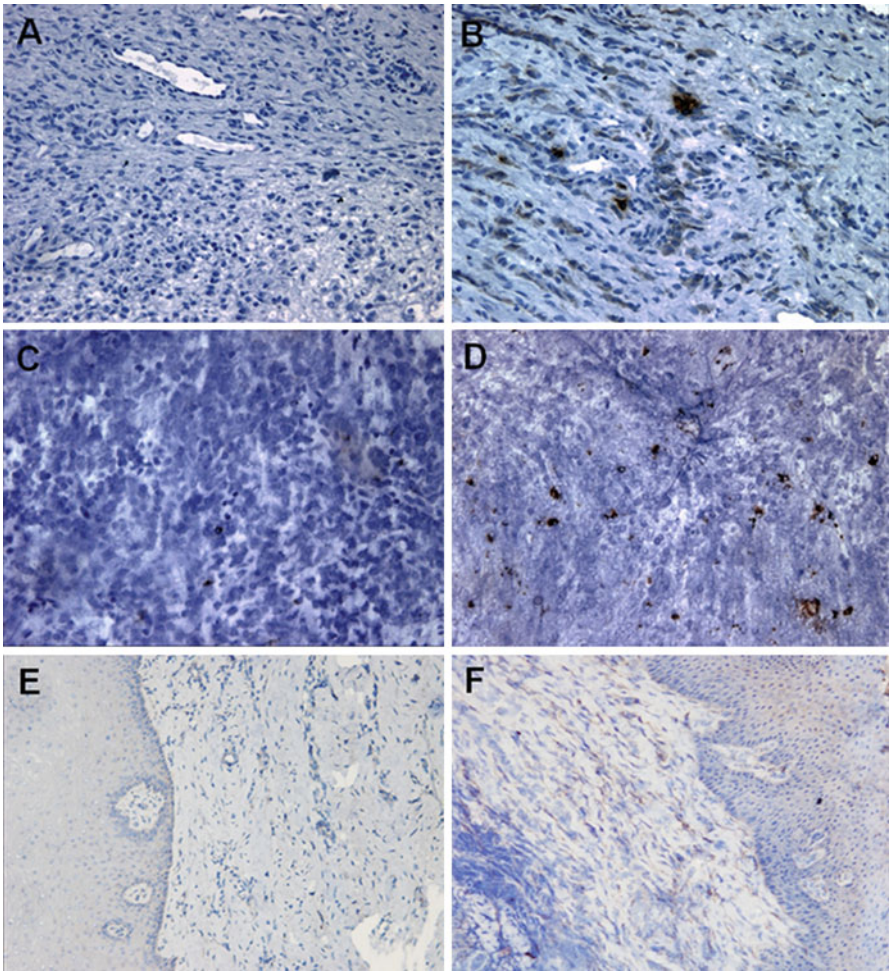

Fig. 4.1 Detection of deimination in human synovium (**a**, **b**) and periodontium (**c**–**f**) shows no false-positive immunostaining for citrullinated proteins using a rabbit IgG control, followed by secondary antibody in synovium of an RA patient (**a**) and periodontium of a periodontitis patient (**c**). Using rabbit anti-citrullinated protein polyclonal antibody Ab5612, *brown staining* indicates the presence of citrullinated proteins in synovium of an RA patient (**b**) and periodontium of periodontitis patient (**d**). Healthy periodontal tissue (**e**) immunostained negative for citrulline but positive (**f**) in the presence of inflammation (magnification 100×). Reproduced from Nesse et al. (2012)

contrast, when using F95, epithelium stained positive in periodontitis tissue samples only. Periodontitis may lead to the formation of additional citrullinated proteins (targeted by F95) in addition to the citrullinated proteins naturally present in non-inflamed periodontal epithelium (targeted by Ab5612 only). In addition, PAD4 was detected in neutrophils in periodontitis tissue using rabbit anti-PAD4 antibody (Ab38772) (Abcam, Cambridge, UK) (Fig. 4.3).

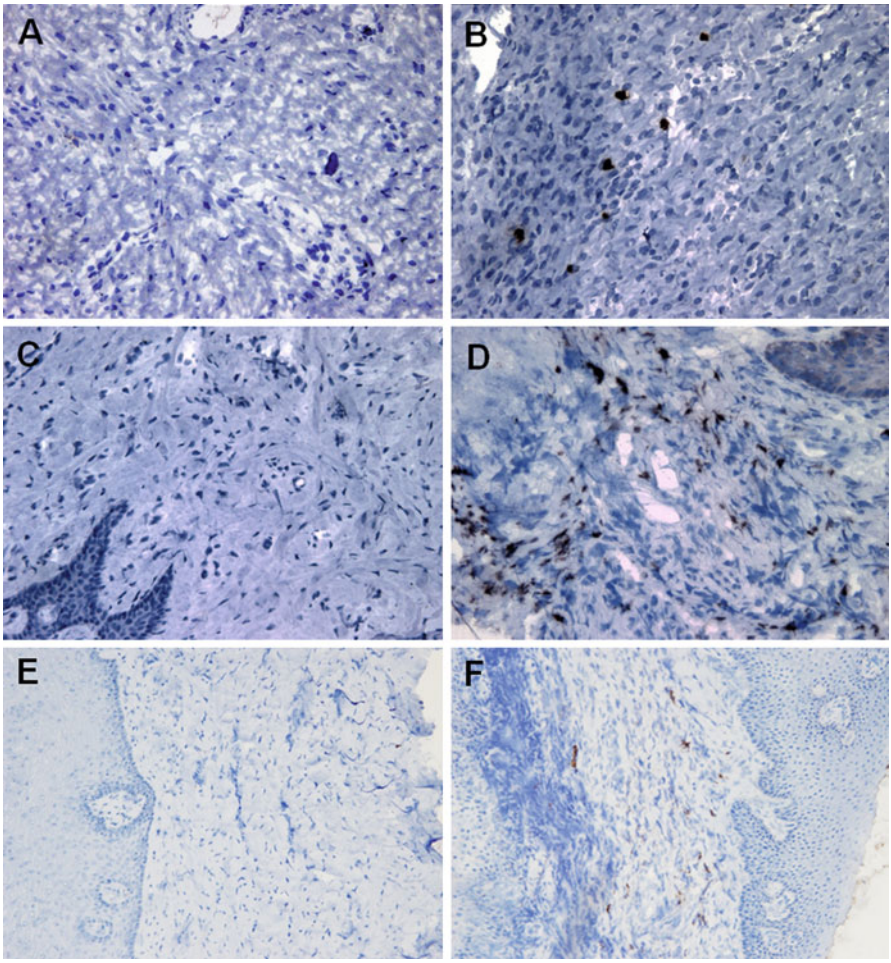

Fig. 4.2 Detection of deimination in human synovium (**a**, **b**) and periodontium (**c**–**f**) shows no false-positive immunostaining for citrullinated proteins using a mouse IgM isotype control, followed by secondary antibody in synovium of an RA patient (**a**) and periodontium of a periodontitis patient (**c**). Using the mouse anti-citrullinated protein monoclonal antibody F95, *brown staining* indicates the presence of citrullinated proteins in synovium of an RA patient (**b**) and periodontium of periodontitis patient (**d**). Healthy periodontal tissue (**e**) immunostained negative for citrulline but positive (**f**) in the presence of inflammation (magnification 100×). Reproduced from Nesse et al. (2012)

The buccal epithelial cells were positive for both Ab5612 and F95 (Fig. 4.4). Citrullinated proteins were detected in three-quarters of the synovial tissue samples of RA patients, irrespective of the antibody used. Western blotting with F95 resulted in a variety of similar bands of citrullinated proteins in periodontal tissue and in RA-affected synovial tissue with a prominent band around 100 kDa (Fig. 4.5).

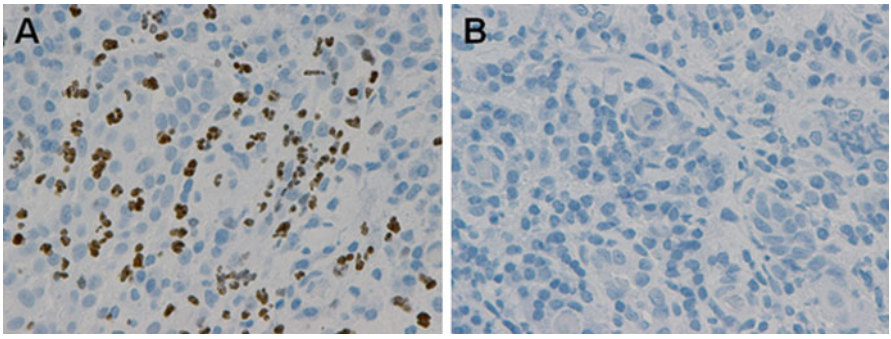

Fig. 4.3 *Brown staining* indicates the presence of PAD4 in periodontitis tissue using rabbit anti-PAD4 antibody (Ab38772) followed by secondary antibody staining. PAD4 was mainly detected in neutrophils, based on morphological characteristics of the nucleus (**a**). No false-positive immunostaining for PAD4 was shown after omitting the primary antibody (**b**)

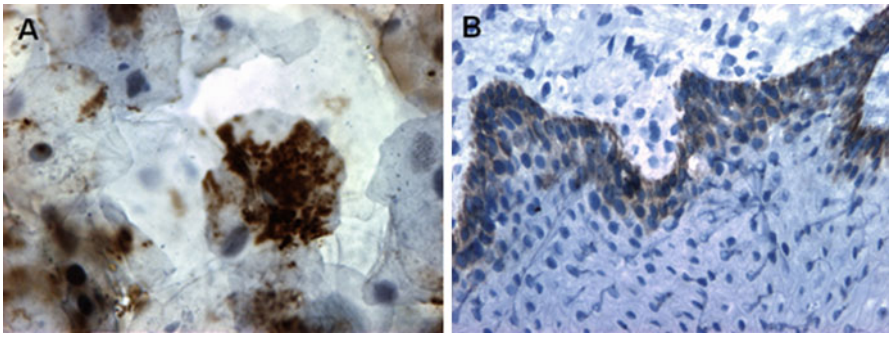

Fig. 4.4 Using the mouse anti-citrullinated protein monoclonal antibody (F95), buccal epithelial cells of healthy subjects, as originally described by Nienhuis and Mandema (1964), show speckled cytoplasmic, perinuclear, and nuclear brown immunostaining for citrullinated proteins (**a**; magnification 400×). Periodontal epithelium of a periodontitis patient (**b**) also shows brown F95 immunostaining, indicating the presence of citrullinated proteins (magnification 200×). Reproduced from Nesse et al. (2012)

Yucel-Lindberg et al. (2010) had similar observations, as citrullinated proteins were detected with a higher frequency in the subepithelial cells in periodontitis tissue (80 %) compared to healthy controls' biopsies (43 %), while no differences were observed in the epithelial layer. PAD2 was present in both epithelial and subepithelial layer with no differences in expression between periodontitis and healthy controls. The antibodies used were not described. These observations show that citrullination is a common process in periodontal epithelium, whereas citrullination in the stromal part of the periodontium is mostly inflammation dependent. Citrullinated proteins present in periodontal stromal tissue appear to be similar to those found in inflamed synovial tissue.

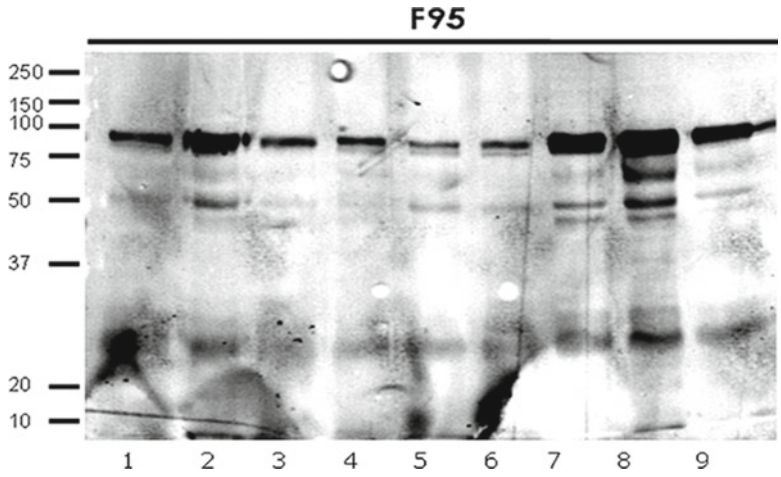

Fig. 4.5 Using Western blotting with the F95 antibody, citrullinated proteins were detected in periodontal tissue from an RA patient with periodontitis (*lane 1*), periodontal tissue from patients with periodontitis (*lanes 2–6*), and synovial tissue from RA patients (*lanes 7–9*). Reproduced from Nesse et al. (2012)

4.3.3 Autoimmunity in Periodontitis

Autoimmunity in particularly aggressive periodontitis has long been suggested. In periodontitis tissue, plasma cells are the most common cell type and represent about 50 % of all cells, while B cells comprise about 18 % (Berglundh and Donati 2005). Autoantibodies to extracellular matrix components such as type I collagen, fibronectin, and laminin have been detected in periodontitis tissue (Rajapakse and Dolby 2004; De-Gennaro et al. 2006). Higher levels of autoantibodies to type I collagen in periodontitis tissue than in serum point towards local antibody production (Rajapakse and Dolby 2004). Also, IgA-RF and IgM-RF production in periodontitis tissue is reported (Hirsch et al. 1989). Autoimmune reactions to native and post-translational modified self-antigens are exclusively found in aggressive periodontitis patients and not in chronic periodontitis patients. High antibody levels to citrullinated filaggrin-derived peptides were found in 8 % of aggressive periodontitis patients, and 16 % had borderline titers (Hendler et al. 2010). Preliminary results from our current studies show reactivity against candidate autoantigens in RA as citrullinated fibrinogen, citrullinated vimentin, and citrullinated alpha-enolase (CEP-1) in severe periodontitis patients. Further studies are necessary considering the role of autoantibodies in the regulation of host response in periodontitis patients. Current research focuses on the role of autoreactive B cells in periodontitis patients, discriminated by functional analysis.

4.4 Conclusion

Autoimmunity in periodontitis has long been suggested, whereas RA is considered a classical autoimmune disease. Up to now, it is unclear if the association between RA and periodontitis is patient related by an increased auto-inflammatory response triggered by the interaction of environment and genetics or if the periodontal pathogen *P. gingivalis* plays a unique role in its ability to citrullinate endogenous and host proteins.

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Chapter 5
From Genes and Environment to Anti-citrulline Immunity in Rheumatoid Arthritis: The Role of the Lungs

Dimitris Makrygiannakis

Keywords Lungs • Citrullination rheumatoid arthritis • Genes • Smoking • Shared epitope • Autoantibodies • Immunity • Bronchoalveolar lavage

5.1 Etiology of Rheumatoid Arthritis

The etiology of rheumatoid arthritis (RA) is still not known, but genetic susceptibility and environmental factors are important aspects for development of this complex disease. However, we should not forget that RA is a heterogenic condition, with variability both regarding phenotypic expression of disease as well as in the risk factor makeup (Padyukov et al. 2004; Klareskog et al. 2006; van der Helm-van Mil et al. 2007a).

In the pathogenesis of RA, genetic contributions play an important role and account for approximately 60 % of the variation in liability to disease (MacGregor et al. 2000). The most important genetic risk factor for RA is found within the HLA system. Research on the relationship between HLA and susceptibility to RA began in 1969, with the observation that lymphocytes from patients with RA were unaffected in mixed lymphocyte culture reactions against cells from other patients with RA (Astorga and Williams 1969). In 1976, it was proposed that the low responsiveness in RA was based on the sharing of genes within the HLA region (Stastny 1976), and 2 years later it was shown that HLA-DRw4 is more frequently present in rheumatoid factor-positive patients than controls (Stastny 1978). The presence of HLA-DR-expressing antigen-presenting cells in synovial tissue and their interactions with T cells were thus proposed as an important feature of RA pathogenesis (Klareskog et al. 1981). Later studies showed that several other HLA-DRB1 alleles

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were also associated with the disease. The products of these alleles appeared to share an amino acid sequence at position 70–74 in the third hypervariable region of the DRβ1 chain of the HLA-DRB1 molecule (QKRAA, QRRAA, or RRRAA). These residues are part of the ridge of the peptide-binding site; therefore, it has been postulated that the shared epitope motif itself is directly involved in the pathogenesis of RA by allowing the presentation of an arthritogenic peptide to T cells (Gregersen et al. 1987).

In addition, environmental factors are also relevant for the development of RA. For example, factors that possibly lead to RA occurrence via lung-associated pathogenetic mechanisms have been identified. It was already evident in the 1950s that in coal workers with pneumoconiosis there is a close association with RA (Caplan 1959). Later studies showed an association between silica exposure and the disease (Klockars et al. 1987; Calvert et al. 2003; Stolt et al. 2005, 2010). Asbestos exposure is another risk factor for RA (Olsson et al. 2004).

However, the strongest environmental factor associated with RA is smoking (Vessey et al. 1987; Silman et al. 1996; Uhlig et al. 1999; Criswell et al. 2002). Other studies have shown that the intensity and duration of smoking correlate with the risk of developing RA and that the increased risk remains even after some years from smoking cessation (Stolt et al. 2003; Karlson et al. 1999). Several studies have shown that smoking is a risk factor for RA in rheumatoid factor-positive patients (Uhlig et al. 1999; Stolt et al. 2003; Heliovaara et al. 1993; Costenbader et al. 2006), but the most striking finding regarding this was the demonstration of a gene–environment interaction between smoking and the shared epitopes for rheumatoid factor-positive, but not negative, RA (Padyukov et al. 2004). Smoking is also a risk factor for extra-articular manifestations of RA (Nyhall-Wahlin et al. 2006). A role of smoking in RA was even identified in animal models of the disease (Okamoto et al. 2011).

5.2 Autoantibodies in RA: Relevance to Citrullination

Among other factors, the autoimmune nature of RA is reflected by the presence of autoantibodies. The classic antibody associated with RA is known as the rheumatoid factor (RF), which is an antibody that is directed to the Fc portion of IgG (Dorner et al. 2004). This antibody has the disadvantage of being used in the diagnosis of RA, but it is not uniquely present in RA patients and can also be found in patients with other autoimmune and infectious diseases as well as in healthy (usually elderly) persons (Shmerling and Delbanco 1991).

On the other hand, anti-citrullinated protein antibodies (ACPAs) have received increasing attention in the last years as an alternative to RF. ACPAs can be found in approximately 60 % of RA patients (Schellekens et al. 2000) and have the obvious advantage in comparison with RF that they are highly specific for RA and are thus only rarely seen in other inflammatory conditions and the normal population (Schellekens et al. 1998, 2000).

Besides their diagnostic applications, clinical and experimental observations provide evidence that ACPAs may be related to important pathophysiologic processes in RA:

1. ACPAs can be found in blood samples of individuals who will develop RA years before the onset of disease, and increased frequencies as well as higher concentrations of antibodies were observed as the individuals approached the symptomatic onset of RA (Nielen et al. 2004; (Rantapaa-Dahlqvist et al. 2003). In fact, most individuals who will develop ACPA-positive RA have already developed their autoantibody status before disease onset (Nielen et al. 2004; Rantapaa-Dahlqvist et al. 2003).
2. The presence of ACPAs in RA is associated with greater disease activity (Kastbom et al. 2004; Vallbracht et al. 2004) and with more severe joint destruction (Vallbracht et al. 2004; Ronnelid et al. 2005) with ACPA positivity at the time of diagnosis being a predictor of more aggressive disease course (Kastbom et al. 2004). Moreover ACPAs are associated with extra-articular manifestations of RA (Turesson et al. 2007).
3. A sustained presence of IgM ACPAs in RA patients can be interpreted as a continuous activation of ACPA-reactive B cells within the course of disease (Verpoort et al. 2006). Moreover ACPAs seem to be produced topically in the inflamed joint compartment, the target organ of RA (Vossenaar et al. 2004a).
4. Another line of evidence for the pathogenic role of ACPAs in RA comes from experiments with animal models of RA. Monoclonal antibodies to citrullinated fibrinogen are not able to cause arthritis in naïve animals with no joint lesions but are able to enhance collagen-induced arthritis (Kuhn et al. 2006).

The term ACPAs includes a variety of different antibodies directed against different citrullinated peptides. In common clinical practice, measurement for ACPAs is performed with the help of the anti-cyclic citrullinated peptide (anti-CCP) assay which displays a high specificity for RA (Schellekens et al. 2000), even though it is detecting antibodies against an artificial citrullinated peptide. However, several recent reports have described autoantibodies against natural citrullinated proteins in RA sera (Burkhardt et al. 2005; Masson-Bessiere et al. 2001; Sebbag et al. 2006; Kinloch et al. 2005; Vossenaar et al. 2004b; Bang et al. 2007). A more detailed review of ACPAs and their antigens is given in Chap. 3.

5.3 Genes and ACPAs

The relationship between the strongest genetic determinant of RA, shared epitopes, and the most reliable serologic marker for the disease, ACPAs, has been a field of investigations during the last years. Shared epitope alleles are associated with ACPA-positive, but not ACPA-negative, RA (Klareskog et al. 2006; Huizinga et al. 2005). In other words, these studies refined the role of shared epitopes as risk factors for RA, showing that these motifs confer to a particular phenotype of RA.

Another study refined the role of shared epitopes even more by showing that these alleles do not confer the risk for RA directly but contribute to the development of ACPAs (van der Helm-van Mil et al. 2006). So it is ACPAs that explain the association between shared epitopes and RA. Strikingly, in the same study, ACPA-positive patients with shared epitopes had higher levels of ACPAs than ACPA-positive patients without them.

The observation that shared alleles confer a risk only for ACPA-positive RA suggests that the etiology of disease in these patients is different from those with ACPA-negative RA. Thus, in addition to all the differences regarding disease activity and bone erosions, they should be considered as different subsets of the disease. Interestingly, during recent years, a growing number of other genes and gene–gene interactions have also been associated with only ACPA-positive (Kokkonen et al. 2007; Plenge et al. 2007; Kallberg et al. 2007a) or ACPA-negative RA (Verpoort et al. 2005; Sigurdsson et al. 2007; Lorentzen et al. 2007). Similarly, environmental risk factors have also been found to differ between ACPA-positive and -negative RA (Klareskog et al. 2006; Pedersen et al. 2006, 2007).

5.4 A Gene–Environment Interaction and ACPAs

As previously mentioned, smoking is the strongest environmental factor linked to RA, while HLA-DRB1 shared epitope alleles are also a strong genetic predisposing factor. Our group identified a striking gene–environment interaction between smoking and presence of the HLA-DRB1 shared epitope alleles as a risk factor for only ACPA-positive RA (Klareskog et al. 2006). The results from our study were confirmed by more studies from the Netherlands (van der Helm-van Mil et al. 2007b), Denmark (Pedersen et al. 2007), and Malaysia (Too et al. 2012). In contrast, studies from North America obtained less clear results regarding the SE–smoking interaction, thus suggesting that there could be other confounding factors obscuring the associations observed in our study (Lee et al. 2007), such as variation in disease susceptibility due to differences in ethnic groups (Mikuls et al. 2010). The relative risk of developing ACPA-positive RA was much higher for smokers carrying two copies of the HLA-DRB1 shared epitope alleles than for nonsmokers with no shared epitope alleles, from a range of 20 (Klareskog et al. 2006) to 50 (Pedersen et al. 2007) times higher. On the other hand, no increased risk was observed in developing ACPA-negative RA. In a more recent study, dose-dependent correlations in this gene–environment interaction were found, showing that heavy smokers carrying two shared epitope alleles have 50 times higher relative risk for developing ACPA-positive RA than nonsmokers that were negative for shared epitopes (Kallberg et al. 2007b). Moreover, studies from our group have identified an interplay between two genes (HLA-DRB1 and PTPN22) and smoking (Kallberg et al. 2007a) in a similar pattern regarding the relative risk for developing ACPA-positive RA.

5.5 The Influence of Smoking on Citrullination in Human Lungs

The above findings describe a striking gene–environment interaction associated with an immunologically defined subgroup of RA. These findings are the basis from which molecular and immunological events should be studied in order to elucidate this interaction. In this context, our study showed that smoking is associated with increased presence of citrullinated proteins in bronchoalveolar lavage (BAL) cells of smokers in comparison to nonsmokers, but this was examined in a very limited number of individuals (Klareskog et al. 2006).

In a consequent study (Makrygiannakis et al. 2008), our aim was to extend our previous limited findings regarding the influence of smoking in protein citrullination in human lungs (Klareskog et al. 2006) in a new prospective trial, including larger number of individuals, but also to introduce a second lung compartment—bronchial mucosa—than just the alveolar compartment (BAL cells). We wanted also to study more events regarding citrullination, such as expression of the PAD2 and PAD4 enzymes, which are the main PAD enzymes associated with RA (Foulquier et al. 2007), but we also wished to examine expression of citrullinated proteins with an additional antibody (in comparison to the first study (Klareskog et al. 2006)) of particular interest for RA (Nicholas et al. 2003; De Rycke et al. 2005). We used a cohort of healthy smokers and a cohort of healthy nonsmokers where we obtained BAL cells and bronchial mucosal biopsies through bronchoscopy.

Regarding BAL cells (Fig. 5.1), using the same antibody (anti-modified citrulline—AMC) (Senshu et al. 1992) as in our previous study (Klareskog et al. 2006) we found a significant difference in the expression of citrullinated proteins: 56 % of smokers versus 7 % of nonsmokers showed expression of citrullinated proteins in BAL cells, while with the other antibody (F95) (De Rycke et al. 2005) there was again higher expression in smokers, but it did not reach statistical significance. The higher expression of citrullinated proteins in smokers was paralleled by higher expression of PAD2, while no difference regarding expression of PAD4 was observed. Around 90 % of BAL cells were macrophages.

Regarding bronchial mucosal biopsies (Fig. 5.2), we could unfortunately not use the AMC antibody, but with the F95 antibody we found that 100 % of smokers, but also 85 % of nonsmokers, were positive for citrullinated proteins, with similar amounts and distributions. On the other hand, a higher expression of PAD2 in BAL cells was observed in the biopsies as well. Similarly with BAL cells, no difference regarding expression of PAD4 was observed.

These experiments confirmed the results of our previous study (Klareskog et al. 2006), regarding expression of citrullinated proteins in the alveolar compartment (BAL cells) of smokers versus nonsmokers. Moreover we demonstrated for the first time that smoking is associated with higher expression of the PAD2 enzyme in two different lung compartments, alveolar (distal airways) and bronchial mucosal (proximal airways).

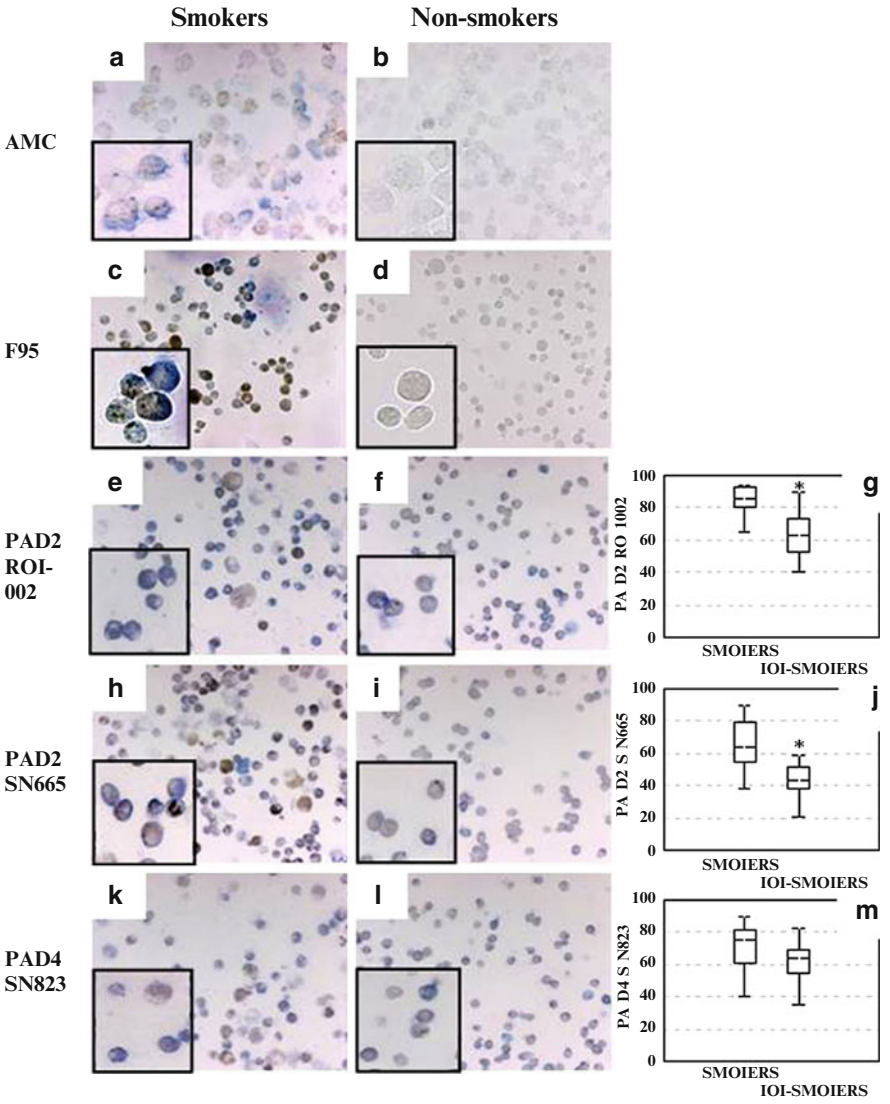

Fig. 5.1 Upregulation of citrullination and PAD2 expression in BAL cells of smokers. BAL cell samples of smokers and nonsmokers show alkaline phosphatase staining (*blue*) for citrullinated proteins (**a**–**d**) and PAD enzymes (**e**–**l**) without counterstaining (original magnification ×250). *Insets* show the same samples at higher magnification. Graphs (**g**, **j**, **m**) demonstrate quantitative analysis (% of positive cells) of BAL cell samples for PAD staining. Values represent the mean plus/minus SEM. $*p<0.05$ for expression in smokers versus nonsmokers. Reproduced from Makrygiannakis et al. (2008)

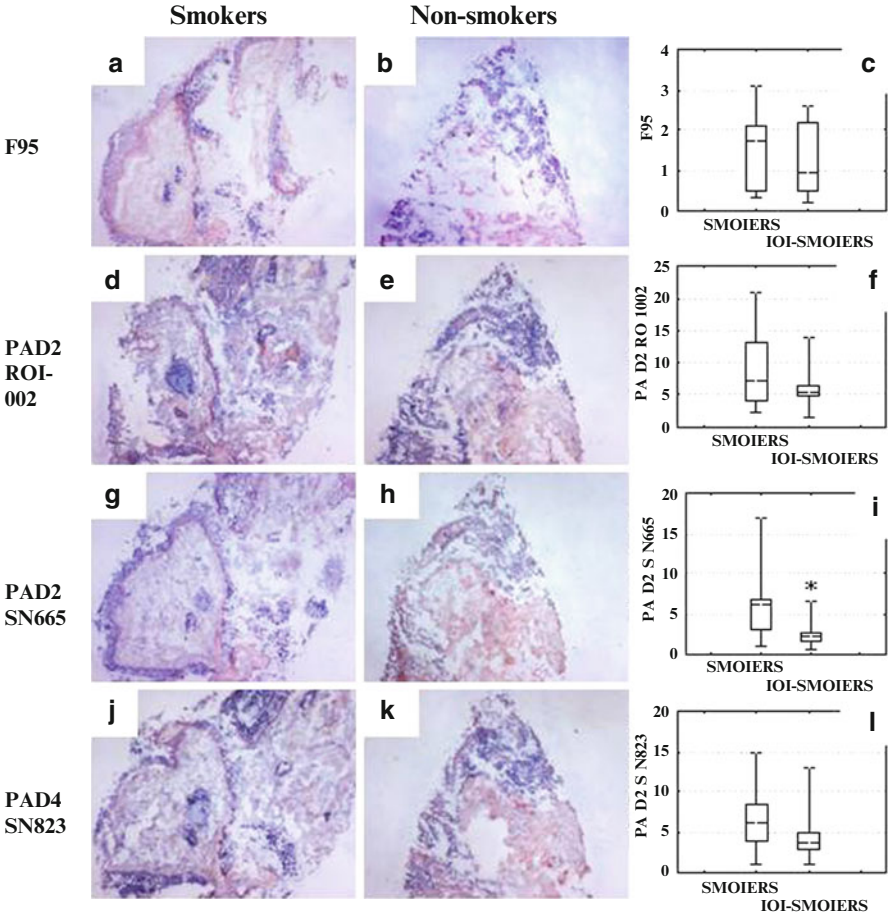

Fig. 5.2 Upregulation of PAD2 expression in bronchial mucosal biopsies of smokers. Frozen bronchial mucosal biopsy sections of smokers and nonsmokers show alkaline phosphatase staining (*blue*) for citrullinated proteins (**a**, **b**) with nuclear fast red counterstain and PAD enzymes (**d**–**k**) (original magnification ×60). Graphs show results of semiquantitative analysis of biopsy sections on a 5-grade scale for citrullinated protein (**c**) and PAD enzyme (**f**, **i**, **l**) staining. Values represent the median and whiskers the range, with $*p < 0.05$ for expression in smokers versus nonsmokers. Reproduced from Makrygiannakis et al. (2008)

In the alveolar compartment, higher expression of the PAD2 enzyme itself seems not to be enough as the citrullination reaction requires also intracellular calcium levels that can only exist in apoptotic cells in order that PAD enzymes get activated (Asaga et al. 1998). Smoking induces alveolar macrophage apoptosis (Aoshiba et al. 2001), a finding that provides a link between smoking, higher PAD2 expression, and induction of citrullination. An alternative/complementary explanation might be that smoking induces subclinical inflammation in healthy smokers (Linden et al. 1993) that might lead to increased citrullination (Makrygiannakis et al. 2006).

In bronchial mucosal biopsies, we observed, similar to the alveolar compartment, higher expression of PAD2. The higher expression of PAD2, but not PAD4, in both lung compartments could be related to the different maturation phases of alveolar and mucosal macrophages induced by smoking (Skold et al. 1996; Vossenaar et al. 2004c). However, we detected citrullinated proteins in biopsies of both smokers and nonsmokers, which could be explained either from the presence of citrullinated proteins in epithelial structures (Tsuji et al. 2003; Brouwer et al. 2006) or from the fact that lung mucosa represents a natural barrier for the human body and thus a constant ongoing degree of subclinical inflammation which could lead to citrullination (Makrygiannakis et al. 2006) might be present. This basal constitutional level of citrullination in both healthy smokers and nonsmokers might preclude identification of an additive effect of smoking through higher expression of PAD2 enzyme. With the help of the AMC antibody, a recent study (Bongartz et al. 2007) investigated the presence of citrullinated proteins in lung tissues from RA-associated lung disease, idiopathic interstitial lung disease, and control lung tissue from patients with lung cancer. They did not find any association for smoking and presence of citrullinated proteins, a result that agrees with our findings in lung tissue biopsies.

The alveolar (BAL cells) and bronchial mucosal compartments represent two different areas of the human respiratory system via distal and proximal airways, respectively. Smoking-induced citrullination in BAL cells takes place in an immunologically relevant compartment (Reynolds 2000). Thus, in a genetically susceptible smoker, citrullination of proteins in alveolar macrophages could result in enhanced presentation of citrullinated proteins to T cells, as a result of the higher affinity of citrullinated proteins with shared epitopes (Hill et al. 2003) but also of the effect of smoking in the antigen presentation capacities of BAL cells (Bratke et al. 2008). Activated T cells could migrate from the bronchoalveolar space to regional lymph nodes (Pabst and Binns 1995) and there interact with B cells for the production of ACPAs. The fact that IgA ACPA immunity is seen early during the development of the anti-citrulline immune response (Verpoort et al. 2006) indicates that immunity triggered from mucosal surfaces, such as the lung, may be of great relevance for RA ACPA immunity. Moreover, IgA ACPAs have been shown to precede RA development (Kokkonen et al. 2011). This finding is even more supported by the presence of ACPAs in patients with interstitial lung disease and no clinical evidence of connective tissue disease (Gizinski et al. 2006), since most of these individuals were smokers.

In conclusion, we identified a specific effect of smoking regarding expression of citrullinated proteins in the alveolar compartment which supports our previously proposed hypothesis for ACPA-positive RA pathogenesis that involves genes, environment, and immunity to post-translationally modified molecules (Klareskog et al. 2006). In a recent study, nanoparticles containing silicon dioxide, carbon black, or single-walled carbon nanotubes induced citrullination in cultured human cells and mouse lung tissues via PAD-dependent mechanisms, facts that provide molecular proof for the previous studies (Mohamed et al. 2012). Recent clinical studies are showing that among patients with RA, smokers are less likely to respond to treatment with methotrexate or TNF inhibitors (Saevarsdottir et al. 2011a; Saevarsdottir et al. 2011b; Soderlin et al. 2012), strengthening the possible connection between smoking and RA.

5.6 Hypothetic Model

Based on the aforementioned data, the following hypothetical model regarding pathogenesis of ACPA-positive RA could be proposed (Fig. 5.3), although it is probably not able to explain the pathogenesis of every case of ACPA-positive RA:

Stage 1: Smoking results in higher expression of the PAD2 enzyme in the alveolar lung compartment and in higher apoptosis of BAL cells, possibly resulting in higher calcium influx and activation of PAD2, with subsequent citrullination of proteins in BAL cells. In a genetically predisposed individual, this will result in enhanced antigen presentation (Hill et al. 2003) and activation of autoreactive T cells, which could migrate to regional lymph nodes and stimulate B cells for the production of ACPAs. Thus, in this hypothetic model, the production of ACPAs is initiated outside of the synovial compartment.

Stage 2: A second joint-specific event, such as infection, trauma, or excessive stress, could lead to clinical or subclinical inflammation in the joint, influx of inflammatory cells, and activation of PAD enzymes which results in citrullination of synovial proteins. Citrullination of RA-specific autoantigens attracts ACPAs from the circulation, so they enter into the joint, bind to the disease-specific citrullinated proteins (De Rycke et al. 2005; Baeten et al. 2001), and form immune complexes. In contrast, nonspecific citrullination would result in the resolution of synovial clinical or subclinical inflammation without further development into RA.

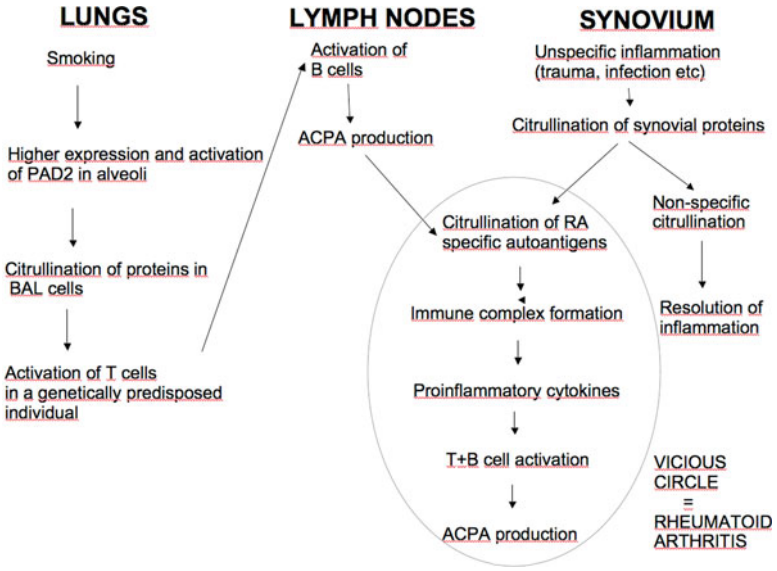

Fig. 5.3 Hypothetical model regarding the pathogenesis of ACPA-positive RA

Stage 3: ACPA-citrullinated protein immune complexes through binding to Fc receptors or complement could then result in the production of pro-inflammatory cytokines from inflammatory cells (Clavel et al. 2008) or further stimulation of antigen-presenting cells, which in turn could result in further stimulation of T and B cells and shift the main region of ACPA production from the periphery to the synovial compartment. A vicious cycle with continuous production of ACPAs, pro-inflammatory cytokines, inflammatory cell recruitment into the joint, further PAD activation, and further citrullination could result in the establishment and perpetuation of RA. In that context, the presence of various antibodies against citrullinated proteins exhibiting minimal cross-reactivity (Snir et al. 2009) in ACPA-positive RA may be the result of an epitope-spreading mechanism (Vanderlugt and Miller 2002).

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Chapter 6
Neutrophils and Their Contribution to Autoimmunity in Rheumatoid Arthritis

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Keywords Neutrophils • Granulocytes • Neutrophil extracellular traps (NETs) • Citrullination • Rheumatoid arthritis

6.1 Introduction

In recent years, one of the most exciting findings by researchers investigating the pathogenesis of rheumatoid arthritis (RA) has been the identification of citrullinated (deiminated) proteins as autoantigens (Klareskog et al. 2008; Schellekens et al. 1998; Sebbag et al. 1995). Anti-citrullinated protein antibodies (ACPA) can be found several years before the onset of arthritis (Rantapää-Dahlqvist et al. 2003). In patients with undifferentiated arthritis, the presence of ACPA is highly associated with progression towards RA. In fact, ACPA-positive RA patients generally develop a more severe disease, and the gene environmental interactions identified for RA such as smoking, HLA genotype and single-nucleotide polymorphism in the gene PTPN22 are all associated with autoimmunity to deiminated proteins (Kallberg et al. 2007). Taken together, these observations suggest that ACPA are likely to be involved in the pathogenesis of RA. However, since in many patients these auto-antibodies can be detected years before the onset of disease, it appears that ACPA alone are not sufficient to cause disease. We now have detailed information about many features of ACPA, including their affinity maturation, immunoglobulin isotypes, the citrullinated antigens they are specific for and the time course of their appearance (Amara et al. 2013; Lundberg et al. 2012; Rantapää-Dahlqvist

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et al. 2003; Suwannalai et al. 2012). Currently, there is also great interest in the triggers that lead to the transition from asymptomatic ACPA-positive individuals to the activation of disease symptoms and onset of RA. Changes observed at the onset of joint inflammation include an increase in the epitope spreading of ACPA and an increase in the range of isotypes (Ioan-Facsinay et al. 2008; van de Stadt et al. 2011; van der Woude et al. 2010).

6.2 The Role of Immune Complexes in RA

ACPA antibodies are unlikely to contribute to inflammation if they do not encounter the deiminated antigens for which they are specific. While direct activation of osteoclasts by anti-citrullinated vimentin antibodies was demonstrated in a recent study (Harre et al. 2012), most pro-inflammatory functions of such antibodies depend on formation of immune complexes. These will signal through Fc receptors on myeloid cells, such as macrophages and neutrophils, and together, with complement activation and cytokine production, fuel a rapid inflammatory response. In murine models, such as antibody-induced arthritis or collagen antibody-induced arthritis, the transfer of antibodies specific for glucose-6-phosphate isomerase (GPI) or through type II collagen, respectively, will lead to joint inflammation in susceptible strains (Maccioni et al. 2002; Nandakumar et al. 2003). One of these murine arthritis models, K/BxN, results from crossing KRN-TCR transgenic mice with NOD mice. The F1 K/BxN offspring develop spontaneous arthritis (Kouskoff et al. 1996). The antigen recognised by the KRN TCR, in the context of MHC-II I-Ag7, has been identified as GPI, a glycolytic enzyme that is widely expressed (Matsumoto et al. 2002). Sera from these mice contain GPI-specific antibodies, and transferring these into healthy as well as in lymphocyte-deficient mice induced joint inflammation. This model has been studied extensively to unravel the role of antibody-induced joint inflammation. Intriguingly, systemic presence of immune complexes here leads to joint inflammation.

Joint, kidney and vascular inflammation caused by immune complexes has been known historically as type III hypersensitivity. Why this is manifested particularly in the joint is not very well understood. Possibilities discussed include the effect of hydrostatic pressure or further mechanisms leading to a lack of clearance of immune complexes from the inflamed joint. In RA patients and in many elderly healthy controls, anti-immunoglobulin antibodies, known as rheumatoid factor (RF), can be detected in serum and, in the case of RA patients, synovial fluid. These are predominantly IgM anti-IgG antibodies, which are unlikely to directly cause disease, as they are found in many asymptomatic individuals. However, in patients with RA, precipitates of RF and their immunoglobulin antigens are found on the surface of the cartilage and are thought to lead to macrophage activation locally. Similarly, GPI and associated antibodies have also been shown to be associated with the cartilage surface in the K/BxN model. So among the manifold and complex interactions occurring in the inflamed joints of patients with RA, immune complex-mediated

immune activation should not be overlooked. The presence of citrullinated proteins has also been observed in precipitates on the surface of the synovial lining of RA patients, suggesting that these may also involve formation of immune complexes (De Rycke et al. 2005). When immune complexes were purified from the synovial fluid of RA patients and analysed for the antigens involved, citrullinated proteins were detected (Van Steendam et al. 2010).

Experiments using mice deficient in individual immunoglobulin receptors revealed that immune complexes binding to FcγRIII but not to FcγRII were necessary for arthritis development (Brunkhorst et al. 1992; Mihai and Nimmerjahn 2012). Furthermore it has been shown that the inflammatory response depends on the presence of neutrophils, macrophages and the alternative complement pathway, surprisingly less than the activation of the classical complement pathway. In vivo inhibition of the CXCR2 receptor led to significant reduction in RA, reflecting its important role in neutrophil recruitment. Further evidence regarding the role of neutrophils in this model comes from a recent report that has shown that expression of Syk, a kinase downstream of the Fcγ receptor, is necessary for development of arthritis (Elliott et al. 2011).

6.3 Expression of PAD isoforms in RA

While the presence of deiminated proteins in the rheumatoid synovium has been described (De Rycke et al. 2005; Makrygiannakis et al. 2006), we know relatively little about the cellular sources of citrulline-containing proteins in the inflamed joints of RA patients. An important step towards understanding the generation of these post-translationally modified proteins involves the enzymes responsible for the process of citrullination, peptidylarginine deiminases (PADs).

The five mammalian isoenzymes of PAD are highly homologous and functionally similar (see Chap. 1) but differ in their expression pattern throughout organ systems and cell types (Vossenaar et al. 2003). Myeloid cells such as neutrophils and granulocytes have been shown to express three of these PAD isoforms: PAD2, which is ubiquitously expressed, and PAD3, which so far mainly has been found in myeloid cells. All PAD isoforms need Ca^{2+} for their activation. The human PAD4 gene was originally named PAD5, but when the close similarity to murine PAD4 was observed, it was renamed to PAD4. Therefore, there is no human PAD5 isoform. However, at a later stage, another isoform was identified and named PAD6 to avoid confusion with previous work (Esposito et al. 2007; Vossenaar et al. 2003). Research into the regulatory mechanisms of PAD4 activity revealed that this enzyme autocitrullinates at ten sites clustered in three distinct regions (see Chap. 21 for more details), resulting in changes in protein structure and upregulation of enzyme activity (Slack et al. 2011). On the other hand, it has also been reported that citrullination of a cluster of arginines around the active site cleft can lead to auto- inactivation of the enzyme. In this study it was shown that, genetic variants of PAD4 differ in their degree of inactivation (Andrade et al. 2010).

Systematic investigation of the PAD isoforms present in the inflamed rheumatoid synovium has shown that predominantly PAD2 and PAD4 are expressed locally (Foulquier et al. 2007). Beyond its ability to citrullinated proteins, it is important to also note that PAD4 itself is an autoantigen (Takizawa et al. 2005). Among the cell populations found in the joint, PAD4 is particularly highly expressed in neutrophils. In this review, we focus on the contribution of neutrophils in the generation of deiminated proteins and their contribution to joint inflammation under that context.

6.4 The Role of Neutrophils in RA

The early stages of most inflammatory reactions are characterised by the influx of neutrophils into the tissue in which the response is triggered. In a study using a small sample of RA patients, it has been suggested that, in the earliest stages of synovial inflammation, neutrophils may predominate the infiltrate (Konttinen et al. 1985). In the case of chronically inflamed synovium of RA patients, however, we find only very low number of neutrophils in this location. In contrast to the low number of neutrophils in the synovial tissue, these are the most abundant immune cells in the synovial fluid (SF). In the joint space of RA patients, activated neutrophils encounter aggregates of immunoglobulins such as RF, both within the SF and deposited on the joint surface. These complexes of immunoglobulins engage Fcγ receptors on the surface of the neutrophil, triggering further activation (Pillinger 1995; Wright et al. 2010). Neutrophils isolated from RA SF show evidence of having initiated the production of reactive oxygen species (ROS) in vivo (Cedergren et al. 2007). Additionally, neutrophil granule proteins, such as myeloperoxidase and lactoferrin, have been found in the SF from RA patients (Edwards et al. 1988; Wong et al. 2008) and likely contribute to cartilage damage.

As we have recently shown, upon stimulation with TNFα, neutrophils in the synovial fluid shed the B cell-stimulating cytokine BLyS (BAFF) from their surface (Assi et al. 2007). This suggests that there is potentially a vicious cycle of activated neutrophils producing B cell-stimulating cytokines which contribute to more auto-antibody production and subsequently increased neutrophil stimulation by the resulting immune complexes. At the mRNA level, a range of cytokines was detected in neutrophils sorted directly from synovial fluid of RA patients, without in vitro stimulation or culture (Yeo et al. 2011). These data showed that many of the cytokines known to be produced by macrophages were also expressed in neutrophils, such as IFN-α1 and BAFF. Since macrophages are vastly outnumbered by neutrophils in RA SF, it is likely that these latter cells make a considerable contribution to the cytokines present in the SF.

In patients with early RA, synovial neutrophils show significantly lower levels of apoptosis compared to patients with other persistent forms of arthritis (Cascão et al. 2010). This may relate to high levels of anti-apoptotic cytokines such as GM-CSF and G-CSF (Raza et al. 2006). For example, it was shown that GM-CSF, at

concentrations similar to those found in SF, reduces the percentage of apoptosis induced by Fas ligation in inflammatory neutrophils (Renshaw et al. 2000).

Interestingly, some activated neutrophils in inflammatory sites were shown to express CD14, CD83 and MHC class II antigens (Iking-Konert et al. 2005), which are normally expressed by antigen-presenting cells (APCs) but not by resting neutrophils (Culshaw et al. 2008). MHC class II-positive neutrophils can present superantigens or antigens to T cells in an MHC class II-dependent or -restricted manner, respectively (Iking-Konert et al. 2005). Overall there is a large amount of evidence that neutrophils contribute to joint inflammation in RA by production of cytokines and free radicals and release of proteolytic enzymes. Beyond these mechanisms, more recently, NETosis, a novel process by which neutrophils can contribute to antibacterial immunity and autoimmunity, has been an intense focus of research (see Chap. 1). In the context of RA, this is of particular interest as it critically depends on deimination and involves the release of citrullinated proteins from neutrophils into the extracellular space.

6.5 The Role of NETosis in Inflammatory Disease

Neutrophils are able to employ several different mechanisms to eliminate microbes. These include phagocytosis and the release of antimicrobial molecules from granules (degranulation). In 2004, an additional antimicrobial process was described by a group in Berlin. Brinkmann and his colleagues reported that, upon activation by phorbol myristate acetate (PMA), lipopolysaccharide (LPS), interleukin 8 (IL-8) or Gram-positive and -negative bacteria, neutrophils release their chromatin into the extracellular space forming the so-called neutrophil extracellular traps (NETs) (Brinkmann et al. 2004). The main component of NETs is DNA associated with a number of proteins such as myeloperoxidase and neutrophil elastase. Using a proteomic approach, Urban et al. identified these proteins mainly as histones, components of various types of granules, cytoskeleton proteins and glycolytic enzymes (Urban et al. 2009).

Some morphological changes can easily be observed in vitro during NET formation. Activated neutrophils flatten, the characteristic lobular form of the nucleus vanishes and the chromatin decondenses (Brinkmann and Zychlinsky 2012). Subsequently, the nucleus and granules start losing their integrity during the first hour of activation until the nuclear membrane fragments into vesicles. This results finally in the mixing of decondensed chromatin with the cytoplasm followed by cell membrane rupture and release of NETs into the extracellular compartment.

Initially, the formation of NETs was described as a distinct form of active cell death and was therefore termed “NETosis” to distinguish it from apoptosis and necrosis. In contrast to apoptotic cells, no internucleosomal DNA fragmentation and exposure of phosphatidylserine or activation of caspases have been observed. Also, neutrophils undergoing NETosis do not appear to show any morphological characteristics of necrotic neutrophils (Fuchs et al. 2007).

Alternatively, additional models also suggested the release of NETs from viable neutrophils without cell lysis such as the release of mitochondrial DNA from intact neutrophils (Yousefi et al. 2009). Also, Yipp and co-workers recently observed neutrophils forming NETs in vivo while still maintaining features of live cells (Yipp et al. 2012).

6.5.1 Signalling Mechanisms Leading to NETosis

To date, very little is known about the cellular processes leading to NET formation. Since the discovery of NETs in 2004, there have been a number of studies published which found that the formation of extracellular traps does not seem to be restricted to neutrophils. Other cells such as eosinophils and mast cells were shown to release extracellular DNA attached to antimicrobial proteins (Yousefi et al. 2009; von Köckritz-Blickwede et al. 2008). In addition, the list of synthetic and physiological molecules as well as microorganisms able to induce NETs is growing. NETs were found to be induced by fungi, parasites and Gram-positive and Gram-negative bacteria. (For reference, see Brinkmann and Zychlinsky 2012 and Guimarães-Costa et al. 2012.)

The high diversity of stimuli found to induce NETosis so far makes it complicated to reveal common signalling pathways. Currently, the great majority of stimuli described to induce NET formation are dependent on ROS generation by NADPH oxidase. Thus, neutrophils from patients with chronic granulomatous disease (CGD) that lack this enzyme are not able to form NETs (Bianchi et al. 2009). When neutrophils of CGD patients are treated with hydrogen peroxide, however, the pathway downstream of NADPH oxidase can be rescued and the cells are able to produce NETs (Fuchs et al. 2007). In addition, myeloperoxidase (MPO) which is able to react with hydrogen peroxide and generate hypohalous acids was also shown to be essential for NET formation (Metzler et al. 2011). Accordingly, hypochlorous acid, one of the products generated by MPO, is sufficient for NET release (Palmer et al. 2012).

Moreover, MPO together with neutrophil elastase was recently shown to be involved in the chromatin decondensation observed during NETosis (Papayannopoulos et al. 2010). Both proteins seem to enter the nucleus by an unknown mechanism, where neutrophil elastase degrades histones. Subsequently, MPO synergises with neutrophil elastase in driving chromatin decondensation, independent of its enzymatic activity.

Concerning the signalling pathways upstream of NADPH oxidase, it has been proposed that the Raf–MEK–ERK pathway is implicated in NET formation (Hakkim et al. 2011), as is Rac2 (Lim et al. 2011). These observations may be explained by the finding that Rac2-deficient cells cannot form a functional NADPH oxidase complex to generate ROS.

6.5.2 NETs in Autoimmune Diseases

Whereas initially NET formation was regarded mainly as an additional defence mechanism against extracellular microorganisms, recent work suggests that these structures may also serve as putative sources of immunostimulatory proteins, with the potential of inducing autoimmunity and tissue damage.

Neutrophil components are well established as a source of autoantigens in a number of autoimmune conditions, primarily in small-vessel vasculitides (SVV) and systemic lupus erythematosus (SLE). For example, MPO and PR3 were discovered as the predominant autoantigens recognised by anti-neutrophil cytoplasmic antibodies (ANCA) in SVV (Lüdemann et al. 1990). Interestingly, ANCA were also shown to activate neutrophils in vitro (Chen and Kallenberg 2009) and are able to induce vasculitis in animal models (Pfister et al. 2004; Xiao et al. 2002).

In SLE, an autoimmune syndrome that is characterised by immune complex deposition, inflammation and organ damage (Tsokos 2011), auto-antibodies are primarily directed against nucleosomes (Bruns et al. 2000) but also against neutrophil components such as neutrophil elastase, MPO and LL-37 (Kaplan 2011). In addition, elevated plasma levels of defensins (Vordenbäumen et al. 2010) and HMGB1 (Ma et al. 2010) could be detected in SLE patients.

Thus, neutrophil-specific proteins appear to be targets for auto-antibodies in patients with systemic autoimmunity; however, the reason why these cells are targeted still remains unclear. One possible explanation could be related to neutrophil death at sites of inflammation. However, neutrophil death by apoptosis generally does not lead to release of intracellular constituents, unless the mechanisms involved in uptake and removal of these cells are compromised.

In this context, the discovery of NETs has exposed a new perspective on neutrophil research in the above-described autoimmune diseases. This is because a large proportion of NET components have been found to serve as autoantigens in systemic autoimmune diseases and also because of the potential of NETs in inducing tissue damage. For example, activated low-density granulocytes (LDGs) were isolated from SLE patients and characterised as playing pathogenic roles in endothelial damage and abnormal endothelial differentiation leading to accelerated atherosclerosis in SLE (Kaplan 2011; Villanueva et al. 2011). Also, there have been publications about an impaired serological DNase-I activity in 36 % of patients with SLE leading to a prevented degradation of NETs resulting in a more active disease state (Hakkim et al. 2010; Leffler et al. 2012).

Ex vivo, neutrophils releasing NETs have also been found in kidney biopsies from patients with ANCA-positive vasculitis (Kessenbrock et al. 2009). Also, increased NET formation has been reported in SLE (Garcia-Romo et al. 2011). Lande et al. described a mechanism by which antimicrobial peptides such as LL-37 were able to protect self-DNA in SLE patients from degradation by nucleases and, as such, stimulated self-DNA-induced triggering of TLR9 signalling in pDCs (Lande et al. 2011). In this study, these DNA–protein complexes induced an enhanced IFN-alpha synthesis by pDCs, in agreement with most current models of

lupus pathogenesis, in which an activation of the type I IFN pathway lowers the threshold for autoreactivity of both antigen-presenting and antibody-producing cells (Banchereau and Pascual 2006; Elkon and Stone 2011).

6.5.3 The role of NETS and PAD isoforms in RA

In the context of RA, it is of particular interest that the process of NETosis critically depends on citrullination and can therefore potentially lead to the generation of autoantigens driving the disease (Leon et al. 1981). The presence of large numbers of activated neutrophils in the synovial fluid suggests that the inflamed joint may be a site of NETosis. Indeed, the presence of extracellular DNA in the synovial fluid was recognized many years before the process of NETosis was defined. (Leon et al. 1981). There is a large number of remaining open question, though. These include which the key stimulants leading to NETosis in the joint are and how NETosis contributes to the profile of citrullinated autoantigens present in the joint. It needs to be considered that beyond the histone citrullination involved functionally in the process of NETosis, other proteins may be citrullinated. Furthermore it is possible that release of PAD isoforms from the neutrophil disintegrating after NETosis will release the entire spectrum of PAD isoforms. The contribution of these still remains undefined.

In contrast, the role of PAD 4 in histone citrullination has been researched in depth; Deimination of histones is catalysed by PAD4 and suggested to be an important step in the decondensation of DNA. This post-translational modification was first implicated in an epigenetic form of gene regulation in human HL-60 granulocytes (Wang et al. 2004). Later, Neeli et al. (2008) were able to show citrullinated histone H3 present within NET structures in LPS-stimulated neutrophils using confocal microscopy. In this publication, it was demonstrated that deimination of histone H2A, H3 and H4 in neutrophils that produce NETs, but not apoptosis, represents a response to inflammatory stimuli. This was also confirmed by Wang et al. who showed that histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation (Wang et al. 2009). In addition, Wang et al. proposed that the conversion of certain arginine residues to citrullines by PAD4 leads to a loss of positive charge of those residues and may thereby weaken the interaction between histones and DNA. This elimination of positive charge could be responsible for the unwinding of chromatin and, thus, may be partly responsible for the formation of NETs. PAD inhibitors such as Cl-amidine, in contrast, significantly reduced histone decondensation and NET formation in response to either ionomycin or *Shigella flexneri* (Wang et al. 2009). Moreover, neutrophils from PAD4$^{-/-}$ mice were shown to be incapable of forming NETs upon activation by different stimuli, and also no hypercitrullination of histone H3 was detectable (Li et al. 2010). In vivo, these mice had more severe bacterial infections than wild-type mice and also developed larger lesions when challenged with a *S. pyogenes* infection.

Deimination of histone H3 can be induced by a number of bacterial and inflammatory signalling molecules, such as LPS, TNF, *N*-formyl-methionine-leucine-phenylalanine (f-MLP), lipoteichoic acid (LTA) and hydrogen peroxide (Li et al. 2010; Neeli et al. 2008, 2009). Importantly, however, not all stimuli discovered to induce NETs so far were reported to also induce histone citrullination (Rohrbach et al. 2012). As a result, further research must be performed to reveal the exact mechanisms promoting histone deimination. The local activation of PAD4 in the synovium and to a larger extent in the synovial fluid during NETosis is potentially a source of autoantigens. These may be generated during the process of NETosis itself such as citrullinated histones or may be bystander proteins citrullinated because of PAD actviation.

In addition, the activation and additional functions of PAD4 also require further investigation. Recently, Neeli et al. reported cytoskeletal activity to be required for PAD4 activation in neutrophils (Neeli et al. 2009). In this publication, prior to LPS stimulation, pretreatment of neutrophils with nocodazole or cytochalasin D, both of which inhibit microtubule polymerisation, led to decreased histone citrullination and NET formation. How exactly the cytoskeleton contributes to histone deimination, however, is still unknown. Interestingly, in this context it was previously reported that PAD4 can be found not only in the cell nucleus of neutrophils (Nakashima 2002) but also in tertiary granules (Lominadze et al. 2005; Theilgaard-Mönch et al. 2005). Therefore, it is theoretically possible that cytoskeletal activity surrounding these structures could mediate the mixing of chromatin and granule contents and thus promote histone deimination.

Furthermore, because the generation of ROS is also required for NETosis, it is likely that ROS could also be involved in the initiation of PAD4 activation. For example, hydrogen peroxide was able to induce PAD4-dependent histone citrullination in neutrophils (Neeli et al. 2008). Also, when cells were pre-incubated with the NADPH oxidase inhibitor apocynin, LPS-induced deimination of histone H4 was decreased (Neeli et al. 2009).

Another recent study suggests that PAD4 may be able to regulate higher order chromatin structures in non-granulocytic cells. In this report, osteosarcoma U2OS cells which overexpressed PAD4 ejected extracellular web-like chromatin fibres, similar to those observed in NETs lacking treatment with calcium ionophores (Leshner et al. 2012). In cells transfected with mutant PAD4^{C645S}, however, no extracellular chromatin fibres could be detected. According to Leshner et al. gene regulation mechanisms in which citrullination may antagonise the binding of the protein HP1ß could underlie this phenomenon. HP1ß normally binds to histone H2K9me3 residues enabling the condensation of chromatin. As a result, histone hypercitrullination due to PAD4 overexpression may exclude HP1ß from binding to chromatin and thus lead to chromatin decondensation.

Neutrophils also express two other PAD isoforms, namely, PAD2 and PAD3 (Darrah et al. 2012). Interestingly, Darrah et al. also showed that the citrullination activity of each PAD isoform appears to be specific and directed preferentially against distinct substrates, independent of their cellular localisation. So, although PAD2 and PAD3 co-localise with actins in the cytoplasm, these enzymes were

found to generate distinct patterns of citrullinated proteins. Moreover, the finding that in cell lysates, actin and histone H3 are only deiminated by PAD2 and PAD4, respectively, strongly supports the existence of substrate specificity among PADs (Darrah et al. 2012). This means that although actin and histone H3 can be citrullinated by all PAD isotypes, each enzyme has a clear, intrinsic substrate preference. These results are also in agreement with data from Nakayama-Hamada et al. who demonstrated that human rPAD2 deiminates purified fibrinogen and filaggrin much more efficiently than human rPAD4, supporting the notion that PAD enzymes have either unique or preferential activity against macromolecular substrates (Nakayama-Hamada et al. 2005). The discovery that different PAD isoforms have distinct substrate specificities is very important in the context of RA.

Certainly, further research in the future has to confirm these observations and characterise the activation and potential function of PAD isotypes such as PAD2 and PAD3 in neutrophils and in inflammation in general.

6.6 Conclusion

The importance of autoimmunity to citrullinated proteins in RA, combined with the activation of PAD4 during NETosis, provides a new potential role for neutrophils in the pathogenesis of this and perhaps other inflammatory diseases. In RA, local generation of immune complexes containing ACPA and citrullinated autoantigens produced in neutrophils undergoing NETosis may be driving inflammation and chronicity in the affected joints. This area of research promises to be a future step forward in our understanding of this complex disease.

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Chapter 7
Deimination in Skin and Regulation of Peptidylarginine Deiminase Expression in Keratinocytes

Hidenari Takahara, Guy Serre, and Michel Simon

Keywords Peptidylarginine deiminase • Keratinocyte • Immunohistochemistry • Localization • Gene regulation • Differentiation • Skin

7.1 Introduction

The existence of citrulline in proteins was first described in the skin. In a paper published in Nature in 1958, George Rogers reported citrulline in a protein of hair follicles (Rogers and Simmonds 1958). Twenty-eight years later, Rothnagel and Rogers purified and characterised the corresponding protein and called it trichohyalin (Rothnagel and Rogers 1986). Since then, deiminated proteins have been detected in almost all cells, tissues and organs. The enzymes responsible for this posttranslational modification, the peptidylarginine deiminases (PADs), also known as protein-arginine deiminases, are becoming increasingly well known. Five types of PADs have been identified in humans and other mammals, the PAD1, 2, 3, 4 (also known as PAD5), and 6 (Vossenaar et al. 2003; Chavanas et al. 2004; Balandraud et al. 2005). They are encoded by five paralogous genes clustered on chromosome 1p35-36 and named *PADI*1, 2, 3, 4 and 6 (Vossenaar et al. 2003; Chavanas et al. 2004). The importance of PADs in many cellular processes is now recognised (Klose and Zhang 2007; Li et al. 2010; Struyf et al. 2009; Esposito et al. 2007) and PADs have

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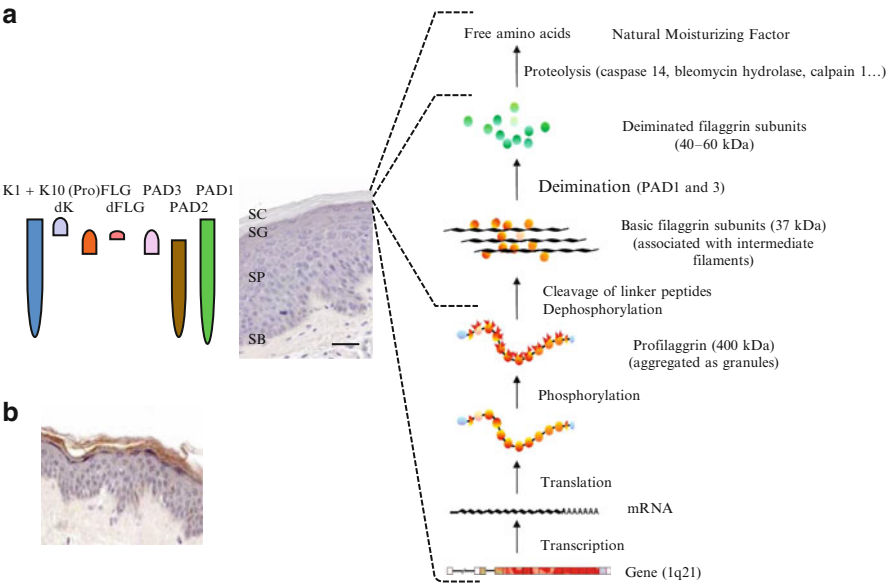

Fig. 7.1 Deiminated human epidermal proteins: involvement of PADs in the hydration of the *stratum corneum*. (**a**) The four epidermal keratinocyte layers are shown on a stained section of human skin (*center*): the *stratum corneum* (SC), *granulosum* (SG), *spinosum* (SP) and *basale* (SB). The immunodetection patterns of keratin (K) 1 and K10, profilaggrin and filaggrin ((pro) FLG), deiminated keratins (dK) and filaggrin (dFLG), and PADs in human epidermis are schematically indicated on the *left*. The complex metabolism of profilaggrin is schematically represented on the *right*: deimination of filaggrin, performed by PAD1 and PAD3, is essential for the degradation of the protein to free amino acids and the production of the natural moisturizing factor. (**b**) Immunochemical detection of deiminated proteins (in *brown*) in the *stratum corneum* of human epidermis. Scale bar = 40 μm

been involved in the pathogenesis of autoimmune diseases, e.g. rheumatoid arthritis (Klareskog et al. 2008; Sebbag et al. 2004), multiple sclerosis (Harauz and Musse 2007; Kim et al. 2003) and cancer (Slack et al. 2011a), that are described in more detail in other chapters of this book. Here, we report on the location of PADs expressed in skin; the mechanisms involved in the regulation of their expression and activity in keratinocytes, their skin targets and physiological roles; and, finally, their possible contribution to skin diseases.

The skin provides mechanical protection to the organism and is an important barrier for preventing the invasion of pathogens, the entry of exogenous substances including allergens and toxins, and the uncontrolled loss of body water and solutes. This so-called barrier function is performed by the epidermis (Madison 2003), a stratified squamous epithelium mainly composed of keratinocytes.

Terminal differentiation of keratinocytes is an oriented and complex program of gene expression from the proliferative basal layer of the epidermis to the upper horny layer, also named the *stratum corneum* (Fig. 7.1a). During their journey

through the spinous and granular layers, the cells sequentially turn specific genes on and off and undergo a series of structural and metabolic modifications. For instance, the expression of keratins KRT1 and KRT10 starts in the spinous layer, whereas filaggrin (FLG) is detected from the granular layer. Finally, the granular keratinocytes undergo a specialised form of programmed cell death called cornification. Cornification is characterised by (1) the elimination of all organelles and the nucleus; (2) the formation of a resistant and insoluble protein shell at the keratinocyte periphery, the cornified cell envelope; (3) the transformation of desmosomes, the intercellular junctional structures, into corneodesmosomes; and (4) the aggregation of the keratin intermediate filaments to form a macrofibrillar intracellular matrix. The resulting corneocytes are embedded in lipidic lamellae and form the thin, highly organised and resilient horny layer (Madison 2003; Candi et al. 2005). In order to maintain the thickness of the horny layer, the upper corneocytes detach from the skin surface during the strongly controlled process of desquamation and are replaced by newly differentiated cells. When human and rodent skin was probed with the anti-modified citrulline antibodies developed by Tatsuo Senshu (Yokohama, Japan), only the horny layer was stained (Senshu et al. 1996) (Fig. 7.1b).

7.2 PADs Expressed in Skin

7.2.1 In the Epidermis

The expression of only three *PADI* genes, i.e. *PADI*1, 2 and 3 (Guerrin et al. 2003; Nachat et al. 2005a), has been evidenced in human skin and epidermis by RT-PCR experiments. In cultured human primary keratinocytes, mRNAs encoding the same three PAD isotypes, but not PAD4 or PAD6, have also been detected (Méchin et al. 2010). In agreement with these findings, only PAD1, 2 and 3 have been immunodetected on skin sections with anti-peptide antibodies specific for each of the isoforms. PAD1 has been localised in the cytoplasm of keratinocytes throughout the whole human epidermis, with a higher expression in the granular cells and in the corneocyte intracellular matrix. In the granular cells, it is associated with keratohyalin granules and with keratin intermediate filaments. PAD2 has been detected in the cytoplasm of spinous keratinocytes and, with a more intense staining, at the periphery of granular keratinocytes. PAD3 is located in keratohyalin granules, in the cytoplasm of granular keratinocytes and in the matrix of the lower corneocytes (Figs. 7.1a and 7.2a). Immunoblotting experiments have confirmed these data. In particular, immunoblotting carried out on samples obtained from the superficial horny layer using adhesive tape stripping has evidenced that PAD1 is the only PAD isotype present in the upper corneocytes. The same pattern of PAD expression has been described in mouse

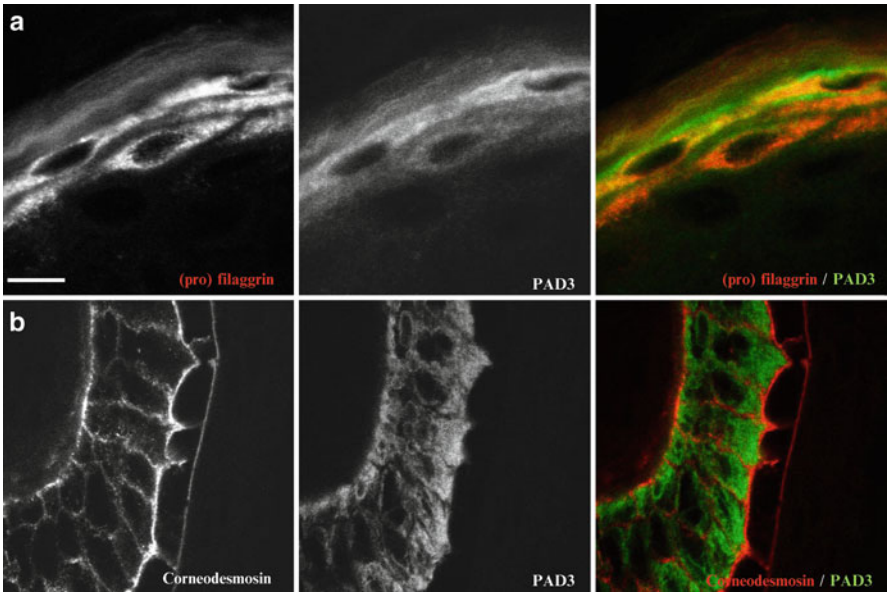

Fig. 7.2 Immunodetection of PAD3 in the epidermis and inner root sheath of hair follicles. Cryosections of human skin were analysed by confocal microscopy with anti-PAD3 rabbit antibodies, with a monoclonal antibody directed against profilaggrin and filaggrin ((pro) filaggrin) and with a monoclonal antibody specific for corneodesmosin. (**a**) In the epidermis, PAD3 is co-located with profilaggrin in the cytoplasm of the upper granular keratinocytes and with filaggrin in the lower corneocytes. (**b**) In the hair follicles, PAD3 is detected in the cytoplasm of keratinocytes in the Huxley layer of the inner root sheath. Bar: 10 μm

epidermis (Guerrin et al. 2003; Nachat et al. 2005a; Méchin et al. 2005; Coudane et al. 2011). In addition, the expression of PAD4 in rat epidermis has also been reported (Ishigami et al. 2001).

7.2.2 In Skin Appendages

PAD1 and PAD3 have been immunodetected in the concentric epithelial sheaths forming hair follicles at the anagen stage (Nachat et al. 2005b). PAD1 is expressed by differentiated keratinocytes, first those of the cuticle and Huxley's layer of the inner root sheath and second those of the companion layer between the inner and the outer root sheaths. PAD3 is expressed in cells of the inner root sheath (Fig. 7.2b) and the medulla. Both enzymes have been immunodetected in the keratinocyte cytoplasm.

PAD1 and PAD2 have also been observed in the secretory and myoepithelial cells of the sweat glands and in the arrector pili muscles (Nachat et al. 2005b). So far, no PADs have been detected in human sebaceous glands.

7.3 Regulation of PAD Expression in Keratinocytes

7.3.1 *Minimal Promoters of* PADI *Genes and Bound Transcription Factors*

Most of the genes expressed during the program of keratinocyte differentiation are regulated at the transcriptional level. This is probably also the case for the *PADI* genes. When differentiation of cultured human epidermal keratinocytes is induced by treatment with 10^{-7} M of 1-α,25-dihydroxy-vitamin D3 (VitD) for 24 h, higher amounts of mRNAs encoding PAD1 (~3-fold increase), PAD2 (~8-fold) and PAD3 (~10-fold) are detected (Méchin et al. 2010). Expression of *PADI*1, 2 and 3 mRNAs is also improved when the extracellular calcium concentration is increased (Méchin et al. 2010; Chavanas et al. 2008; Dong et al. 2005), another well-known way of inducing the differentiation of keratinocytes.

The minimal promoters of the *PADI*1, *PADI*2 and *PADI*3 genes have recently been delineated as short sequences of 195, 132 and 129 base pairs, respectively, upstream of the transcription initiation site (Dong et al. 2005, 2006, 2008). Electrophoretic mobility-shift assays, chromatin immunoprecipitation and small interfering RNA experiments have shown that binding of transcription factors of the ubiquitous stimulator protein (Sp) family, namely, Sp1 and Sp3, is crucial for the activity of these proximal promoters in keratinocytes (Dong et al. 2005, 2006, 2008). This is not surprising, since functional binding sites for Sp1 are frequently found in the promoter regions of genes expressed during the late steps of keratinocyte differentiation. These include the genes encoding keratin 1, transglutaminases 1 and 3, cornified cell envelope components and profilaggrin (Lee et al. 1996; Wong et al. 2005; Jang and Steinert 2002; Crish et al. 2006; Eckert et al. 2004; Markova et al. 2007).

The additional binding of MZF1 and NF-Y transcription factors is necessary to regulate the expression of *PADI*1 and *PADI*3, respectively (Dong et al. 2006, 2008). Also, the expression of both Sp1 and MZF1 is increased after calcium stimulation of keratinocyte differentiation (Dong et al. 2008; Wong et al. 2005). Interestingly, Sp1 and MZF1 are also involved in the regulation of the *BLMH* gene (Kamata et al. 2011). This gene encodes a neutral cysteine protease, bleomycin hydrolase, implicated downstream of PAD1 and PAD3 in the processing of FLG (see Sect. 5.2). This suggests a possible co-regulation of FLG processing-related enzymes. In addition, a proximal TATA-box is present in *PAD*1 and *PAD*3, but not *PAD*2, genes (Dong et al. 2005, 2006, 2008).

Since binding sites for Sp1, MZF1 and NF-Y have been identified in silico upstream of the transcription start site of the mouse orthologous genes (*Padi*1, *Padi*2 and *Padi*3) (Dong et al. 2005, 2006, 2008), the same transcription factors are probably involved in the regulation of both human and mouse PAD genes. As in humans, when wild-type, but not VitD receptor null, mouse keratinocytes are treated with an analogue of VitD (EB1089), *Padi*3 is up-regulated. This twofold activation occurs through the binding of activated VitD receptor–β-catenin complexes to two VitD response elements located ~3 kb upstream of the transcription initiation site (Pálmer et al. 2008).

7.3.2 Role of Non-coding Conserved Sequences

However, considered alone, Sp1 and NF-Y binding to its proximal promoter cannot explain the tight control of *PADI*3 expression in the granular keratinocytes, since these transcription factors are also involved in the regulation of *PADI*4 (Dong et al. 2007), the expression of which has not been detected in the keratinocytes. Therefore, other levels of *PADI*3 control have been suspected, in particular the role of non-coding evolutionarily conserved sequences.

The first to be identified was an 8-kb region located between *PADI*2 and *PADI*1, 42 kb upstream of *PADI2* and 37 kb upstream of *PADI1* (the two genes are in the opposite transcription orientation). This sequence groups together many potential transcription factor-binding sites and is in an open conformation state of the chromatin in differentiated keratinocytes. It is therefore likely to bind transcriptional activators (Chavanas et al. 2004, 2008). In addition, by the chromosome conformation capture technique, it has been shown to physically interact with the *PADI*3 promoter in the nuclei of differentiating keratinocytes through a chromatin loop spanning 86 kb (Chavanas et al. 2008). Several long-range enhancers and bound activators have been experimentally recognised in this region. Two segments of 346 and 245 bp, 1 kb distant from each other, cooperate in calcium-differentiated epidermal keratinocytes to enhance the activity of the *PADI*3 gene minimal promoter located 82 kb away (Adoue et al. 2008). They have no effect when tested independently but act in an orientation-independent and copy number-dependent manner. Their effect has not been observed in proliferative epidermal keratinocytes, in human fibroblasts or cervix adenocarcinoma HeLa cells or on the *PADI*2 minimal promoter. This strongly suggests that these two segments, called PAD intergenic enhancer segment 1 (PIE-S1) and PIE-S2, form a real *PADI*3 bipartite enhancer. PIE-S2 binds distinct transcription factors of the AP-1 family according to the differentiation state of keratinocytes, junD homodimer in proliferative cells and c-Jun homodimer in differentiated cells (Adoue et al. 2008). PIE-S1 contains an MIBP1/RFX1-binding site (Adoue et al. 2008), but binding of either of these two transcription factors has not yet been proved.

An additional non-coding conserved segment of 63 bp, called PIE, has also been shown to display strong enhancer activity on the *PADI*3 gene minimal promoter in calcium-differentiated keratinocytes (Chavanas et al. 2008). The enhancer activity of PIE does not depend on its orientation; it is low in proliferative keratinocytes and insignificant in HaCaT and Hela cells. It is low on the *PADI*2 minimal promoter and null on *PADI*1 and *PADI*4 promoter. To be active, PIE requires the binding of c-Jun and c-Fos, another transcription factor of the AP-1 family. In addition, PIE seems to interact functionally with the two CAAT boxes of the PADI promoter, probably through a direct interaction between c-Jun and NF-Y (Chavanas et al. 2008).

Long-range regulatory elements are important for the coordinated regulation of many clustered genes, at distances of up to 1 Mb from their cognate promoters and in several cell types (Li et al. 2002). Whether this is also a key mechanism in regulating genes essential for terminally differentiated keratinocytes is less certain.

However, a network of conserved non-coding sequences involved in the regulation of the numerous genes of the so-called epidermal differentiation complex in keratinocytes has been described recently (Martin et al. 2004; de Guzman Strong et al. 2010). The expression of the p63 gene is also controlled by a long-range keratinocyte-specific enhancer (Antonini et al. 2006).

When orthologous PADI genes from multiple mammalian species are aligned, a highly conserved 1 kb region is revealed in the *PADI*1 first intron, suggestive of a biological role (Ying et al. 2010). A 267 bp fragment of this region has been shown to enhance the activity of the *PADI*1 minimal promoter in an orientation-independent manner in both proliferative and calcium-differentiated human keratinocytes. Binding of p65 and p50 subunits of NF-kappaB transcription factor is necessary for this enhancer activity. A physical interaction between the *PADI*1 minimal promoter and these intronic conserved non-coding sequences, located 2.2 kb apart, has been evidenced, indicating chromatin looping (Ying et al. 2010). NF-kappaB involvement in keratinocyte differentiation and senescence is well known, as is its transcriptional effect via direct binding to response elements located in gene introns, including c-Fos (Bernard et al. 2004; Bell et al. 2003; Charital et al. 2009).

As a whole, these data show that the transcription of *PADI* gene during keratinocyte differentiation is under the control of multiple and complex regulatory mechanisms, including chromatin structure remodelling (Fig. 7.3).

7.3.3 Regulation at the Translational Level

As previously reported for other tissues or cells, including optic nerve cells (Bhattacharya et al. 2006) and monocytes (Vossenaar et al. 2004), PAD expression in keratinocytes seems to be also regulated at the translational level. For example, treatment of keratinocytes with VitD strongly increases the amount of PAD1, PAD2 and PAD3 mRNA but has no effect on the corresponding protein (Méchin et al. 2010).

7.4 Regulation of PAD Activity in Keratinocytes

The presence of one PAD in keratinocytes at a particular time does not necessarily mean that the deimination of proteins takes place at the same time. Although PADs are immunodetected in basal and suprabasal living epidermal keratinocytes, deiminated proteins are only immunostained in corneocytes. In addition, even though Hela and HaCaT cells express at least one PAD, we have not been able to detect deiminated proteins, even in cells cultured at a high extracellular calcium concentrations (Méchin et al. 2010). In the same way, no deiminated proteins have been detected in cells of sweat glands and arrector muscles, where PAD1 and PAD2 are expressed (Nachat et al. 2005b). We suspect that the local intracellular

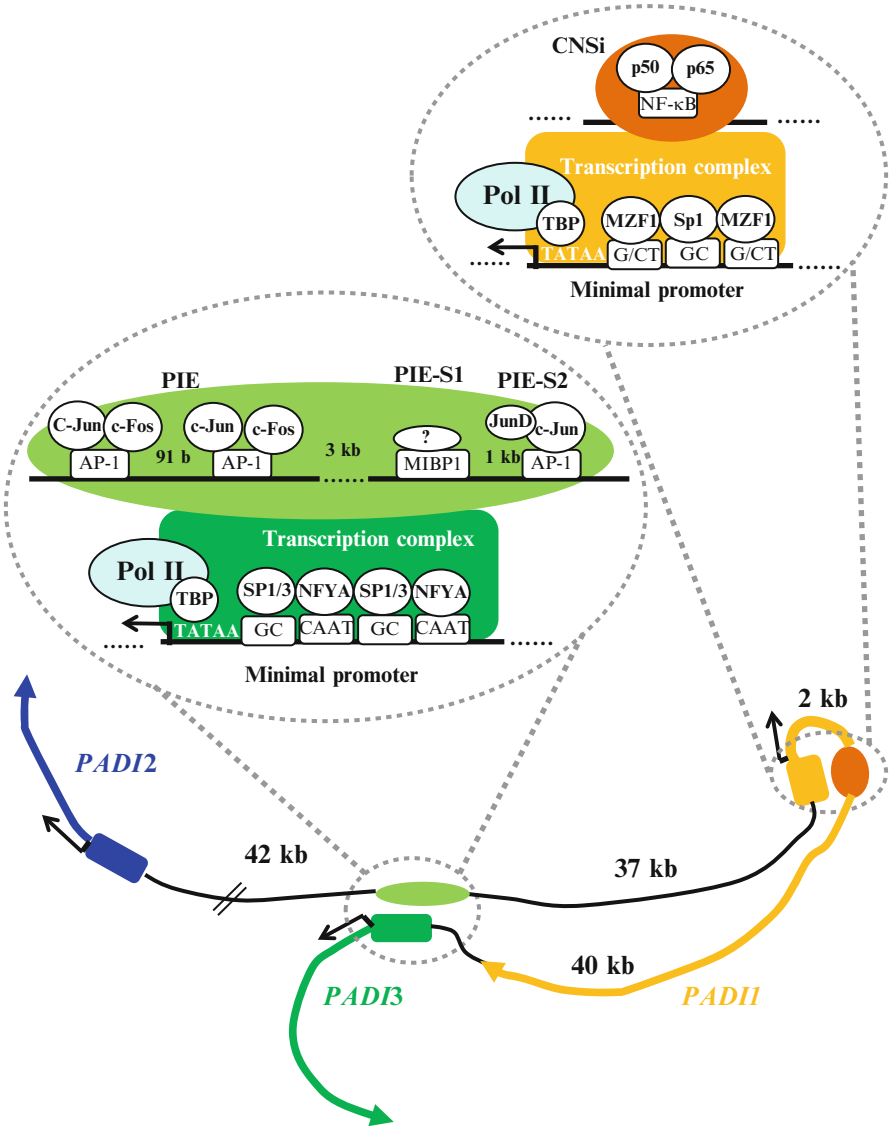

Fig. 7.3 Schematic model of the transcriptional regulation of *PADI*1 and *PADI*3 gene expression in human keratinocytes. Part of the *PADI* gene locus is located in shown: the 5′ region of *PADI*2, the intergenic region including two long-distance enhancers (namely, PIE and PIES1/S2), the entire *PADI*1 gene including the conserved non-coding sequence its first intron (CNSi) and the 5′ region of *PADI*3. Minimal promoters are indicated by *coloured rectangular boxes* and the orientation of transcription by *black arrows*. Bound transcription factors are shown, as are their binding sites. To allow activation of the *PADI*3 promoter by PIE and PIES1/S2 enhancers located 87 kb upstream, chromatin has to form a large loop to bring them into physical contact. Similarly, a chromatin loop allows the activation of *PADI1* transcription through an interaction between the transcription complex and p50/p65 NF-κB transcription factors bound 2 kb downstream on the CNSi. Transcription factors (MZF1, c-Jun, c-Fos, JunD, NFYA and Sp1/3), the TATA-box-binding protein (TBP) and RNA polymerase II complex (Pol II) are shown as *open circles* and the transcription factor-binding sites by *open boxes*. Note that the distances (in bases) are not drawn to scale

concentration of calcium plays a role in controlling PAD activity, this ion being required for their activity in vitro.

Auto-deimination of PADs may also be involved in the regulation of their activity. We have observed calcium-dependent auto-deimination of PAD1, 2 and 3 during in vitro incubations. This modification reduces, but does not suppress, their activity and changes PAD3 structure. In particular, the distances between the four major amino acids of the active site increase (Méchin et al. 2010). Considering the high sequence homologies between the three isotypes, this is probably true for PAD1 and PAD2 as well. Similarly, PAD4 is also auto-deiminated in vitro and in vivo in activated neutrophils. This posttranslational modification changes the structure of the enzyme and could either inactivate it or modulate its ability to interact with other histone-modifying enzymes (Slack et al. 2011b; Andrade et al. 2010).

7.5 Deiminated Proteins and Role of Deimination in Skin

7.5.1 In the Appendages

As already mentioned in the introduction (Sect. 1) to this chapter, the first protein shown to be deiminated was trichohyalin. Trichohyalin is a member of the S100 fused-type protein (SFTP) family (Henry et al. 2012). Like the other SFTPs, trichohyalin is a large protein (220 kDa in human) formed by three domains: an amino-terminal domain homologous to S100A proteins and containing two functional EF-hand calcium-binding sites (91 amino acids long), a highly charged central domain formed by a series of peptide repeats (1,581 amino acids long) and a short carboxy-terminal tail (50 amino acids long). Trichohyalin is preferentially expressed in the inner root sheath and medulla of hair follicles and in the granular layer of the epithelium of dorsal tongue papillae (Hamilton et al. 1991; O'Keefe et al. 1993). It initially accumulates as large cytoplasmic granules. Then it associates with keratin intermediate filaments. Later it becomes cross-linked to itself, to keratin head and tail domains and to several cornified cell envelope components through ε-(γ-glutamyl) lysine isodipeptide bonds catalysed by transglutaminases (O'Keefe et al. 1993). Trichohyalin serves as a strengthener of the envelopes and as an anchor between the envelope and the corneocyte cytoplasmic matrix. This forms a continuous hardened supramolecular structure conferring high mechanical strength on the inner root sheath (Steinert et al. 2003). Trichohyalin contains 435 arginine residues, many of them being citrullinated. Trichohyalin deimination modifies its α-helical structure, resulting in unfolding; makes it more soluble, inducing granule solubilisation; and makes it a better substrate for transglutamine 3 (Tarcsa et al. 1996, 1997). Because PAD3 and trichohyalin expression patterns are very similar, this isotype is certainly responsible for trichohyalin deimination. In addition, the tail of mouse inner root sheath-specific type-I keratin 27 (formerly K25irs3) and type-II keratin 71 (K6irs1) are also deiminated before the proteins are cross-linked by transglutaminase 3 (Steinert et al. 2003).

S100A3, a calcium- and zinc-binding protein, is another substrate of PAD3 in the hair follicles. S100A3 is located in the cuticle and the cortex of the hair shaft and is believed to be involved in hair shaft formation. In vitro deimination of S100A3 by PAD3 promotes the assembly of a homotetramer and increases its affinity for calcium ions (Kizawa et al. 2008). This is described in greater detail in Chap. 8.

As a whole, these data indicate that deimination in the hair follicles is important for the mechanical resistance of cells in the inner root sheath and hair shaft. No deiminated proteins have yet been identified in sweat gland or arrector muscle cells.

7.5.2 In the Epidermis

Several epidermal deiminated proteins have been characterized, and all of them are modified in the horny layer (Fig. 7.1a, b). The effect of deimination on their properties is starting to be unravelled and will be described below.

The major targets of PADs in the epidermis are two closely related SFTPs, i.e. FGL and FLG-2 (Henry et al. 2012). Their repetitive central domains show 45 % amino-acid sequence similarity and similar amino-acid compositions with particular high levels of serine, glycine, histidine, glutamine and arginine (70.4 and 74.2 %, respectively, of total amino acids). Both are specifically expressed in the granular layer of the epidermis in the form of large insoluble precursors (400 and 248 kDa) which accumulate in the cytoplasmic keratohyalin granules (Henry et al. 2012; Dale et al. 1990; Hsu et al. 2011). At the *stratum granulosum*/*stratum corneum* transition, they are proteolytically processed to smaller basic subunits that interact with and are believed to aggregate keratin intermediate filaments. In the lower *stratum corneum*, they are co-located in the corneocyte filamentous matrix (Henry et al. 2012; Dale et al. 1990; Hsu et al. 2011). In the upper *stratum corneum*, FLG and probably FLG-2 subunits are totally degraded by several proteases, including caspase-14, calpain-1 and bleomycin hydrolase (Hsu et al. 2011; Hoste et al. 2011; Kamata et al. 2009; Yamazaki et al. 1997). The resulting amino acids form part of the natural moisturizing factor, a mixture of osmotic molecules allowing water retention in the upper *stratum corneum* (Harding and Scott 1983; Rawlings and Matts 2005). Some of the amino acids are further modified. For example, trans-urocanic acid, involved in photoprotection since it absorbs part of ultraviolet radiation, is derived from histidine in a reaction catalysed by histidase (Barresi et al. 2011). In contrast, pyrolidone carboxylic acid, the most hygroscopic amino acid, derives from glutamine (Rawlings and Matts 2005). With a pKa of 3.9, it also contributes to the acidification of the superficial *stratum corneum*. This acidic pH is crucial for the antimicrobial activity of the layer, for its waterproof nature through the control of lipase activities and for the regulation of desquamation (Rawlings and Matts 2005; Harding et al. 2000).

FLG and FLG-2 deimination are thought to be necessary for their dissociation from the matrix. They also promote their proteolysis by calpain-1 and are a requisite for their proteolysis by bleomycin hydrolase (Hsu et al. 2011; Kamata et al. 2009). As a consequence, deimination participates in, and presumably controls, the

hydration of the upper epidermis and the epidermal barrier functions. On the basis of their enzymatic properties and their diffuse location within the fibrous matrix of the lower corneocytes (Méchin et al. 2005), PAD1 and PAD3 are probably the isotypes responsible for the deimination of FLG and FLG-2.

In the upper cornified layer, the head and tail of keratin K1 and K10 are deiminated (Senshu et al. 1996; Méchin et al. 2005; Ishida-Yamamoto et al. 2002). The enzyme involved is probably PAD1, since it is the only PAD isoform detected in this location (Méchin et al. 2005). The effect of deimination on the properties of these keratins is not known; however, it is concomitant with the observed ultrastructural modifications of the intracorneocyte fibrous matrix. Similarly to inner root sheath keratins, we could suspect that deimination precedes the cross-linking of these proteins to the cornified cell envelopes.

FLG has been known for a long time. It came back under the spotlight when nonsense mutations of its gene were shown to be responsible for ichthyosis vulgaris (OMIM 146700) and to be a high-risk factor for atopic eczema (OMIM #605803) (Smith et al. 2006; Palmer et al. 2006; Irvine and McLean 2006; Sandilands et al. 2007).

7.6 Deimination and Skin Diseases

Despite the accumulating data obtained on PADs by using skin as a model and the importance of deimination in skin physiology, few data are available concerning PAD implication in skin diseases. Lower amounts of citrullinated keratins have been detected in the epidermis of patients with epidermolytic hyperkeratosis (OMIM #113800; also known as bullous congenital ichthyosiform erythroderma) and psoriasis (Ishida-Yamamoto et al. 2000) (in addition to our unpublished data). However, in one study, paclitaxel, a well-known drug used in cancer therapy, but also an in vitro inhibitor of PAD, has been reported to improve severe psoriasis (Ehrlich et al. 2004). No further data has been published about this topic.

PAD could also be involved in skin tumorigenesis. Differential expression of the four genes encoding PAD1 (*PADI*1), laminin-γ2 (*LAMC*2), collagen type IV α1 (*COL4A*1) and collagen type I α1 (*COL1A*1) has been claimed as a predictive biomarker of squamous cell carcinomas of the oral cavity and oropharynx (Chen et al. 2008). In a genome-wide study concerning 930 Icelanders with cutaneous basal cell carcinoma, which is the most common cancer among Europeans, a single-nucleotide polymorphism in intron 13 of the PAD6 gene has been identified as a strong genetic risk factor (Stacey et al. 2008). This association has been replicated in an additional population from Eastern Europe. The estimated risk of the mutation carriers is 2.68 times that of noncarriers (Stacey et al. 2008). Finally, expression of PAD4, the isotype involved in gene expression regulation through deimination of histones, has been observed in skin carcinomas and extramammary Paget's disease (OMIM #167300) (Chang et al. 2009; Urano et al. 1990). Since PAD4 inhibition results in cell cycle arrest and apoptosis (Li et al. 2008) and since PAD4 represses the p53

target genes (Yao et al. 2008), this observation could be of relevance for skin tumours. A more detailed discussion on the role of deimination in cancer is discussed in Chap. 17.

7.7 Conclusion

PADs are increasingly considered as crucial molecular actors in cell physiology and human diseases. The data reported here highlight their importance in skin, particularly in the epidermis and hair follicles. However, more work needs to be done to definitively prove their contribution to skin diseases. Detailed analysis of mechanisms involved in controlling PAD expression and activity during keratinocyte differentiation indicates multiple levels of regulation. This indicates that deimination is a crucial post-translational modification of proteins that require tight control.

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Chapter 8
Importance of Citrullination on Hair Protein Molecular Assembly During Trichocytic Differentiation

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Keywords Calcium • Citrulline • EF-hand • PAD3 • S100A3 • S100 proteins • Tetramerization • Trichohyalin

Abbreviations

AMC	Anti-chemically modified citrulline
CE	Cornified envelope
Cit-	Citrullinated
KIF	Keratin intermediate filament
PAD	Peptidylarginine deiminase
SFTP	S100 fused-type protein

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TCHH	Trichohyalin
TCHHL1	Trichohyalin-like 1
TGM	Transglutaminase

8.1 Introduction

Both the hair fiber and the inner root sheath (IRS) are hardened keratinized epithelial tissues that form periodically within a mini-follicular organ. The hair follicle is a skin appendage able to regenerate the epithelial region in a cyclic manner (Myung and Ito 2012). This hair cycle is subdivided into three phases: anagen, the phase of active hair growth; catagen, the short transitional phase of regression; and telogen, the terminal resting phase. At the onset of anagen, germinative matrix cells arise from epithelial stem cells that reside in the bulge region of a follicle during telogen. Subsequently, these germinative cells differentiate into morphologically distinct trichocytes from which the hair and IRS compartments originate. In the hair compartment, the outermost cuticular cells form a thick cornified cell envelope (CE) structure termed the "A-layer," which provides protection from mechanical insult (Kalinin et al. 2002; Rogers and Koike 2009). The inner cortex comprises the largest mass and is primarily made up of hard α-keratin and keratin-associated proteins. At the center of the hair fiber, air within the medulla facilitates homeothermy. The hair compartment is surrounded by the IRS, which consists of the cuticle, Henle's layer, and Huxley's layer. The CE structure is obvious in the IRS cuticular cells but not in the cells of the Henle's and Huxley's layers (Hashimoto 1988). The diverse phenotypes of the trichocytes largely depend on the thickness of the CE and the mechanism of protein cross-linking (i.e., isopeptide bond or disulfide bond).

Protein deimination is a posttranslational modification catalyzed by peptidylarginine deiminases (PAD; EC 3.5.3.15) that irreversibly convert the positively charged guanidinium group of arginines in a natural substrate protein to neutral citrulline residues. Protein-bound citrullines were first found in mature IRS layers stripped from rat vibrissa follicles (Rogers 1958) and were subsequently detected in the alkali-resistant medulla of rabbit fur and medulla dissected from the quills of crested porcupines (Rogers 1962). A protein fraction derived from guinea pig hair follicles was first shown to exhibit Ca^{2+}-dependent PAD activity (Rogers and Taylor 1977).

Trichohyalin (TCHH) granules are cytoplasmic inclusion bodies that are considered the major hallmark of IRS differentiation (Vörner 1903). The major substrate protein produced by sheep TCHH granules is a keratin intermediate filament (KIF)-associated protein termed TCHH (Rothnagel and Rogers 1986). As both sheep and human TCHH carry an S100-like functional Ca^{2+}-binding domain (a pseudo EF-hand motif followed by a canonical EF-hand motif) at the N terminus that is fused to a larger repetitive peptide via a spacer sequence (Fietz et al. 1993; Lee et al. 1993), this multidomain protein is classified as a member of the S100 fused-type protein (SFTP) family (Kizawa et al. 2011). Meanwhile, several cDNAs that encode PAD isozymes responsible for Ca^{2+}-dependent protein deimination have been

cloned from various organs and species (Ying et al. 2009). In human hair follicles, type III PAD isozyme (PAD3) has been shown to be expressed in the three IRS layers and the medulla, coincident with TCHH distribution, as well as in the cuticular layer of the hair shaft (Kanno et al. 2000). Subsequently, S100A3, belonging to the Ca^{2+}-binding S100 protein family, was identified as the PAD substrate deiminated during cuticular differentiation (Kizawa et al. 2008).

In mammalian hair follicles, Ca^{2+}-dependent PADs catalyze the conversion of arginines into citrullines in S100A3, a cysteine-rich member of the S100 protein family, and TCHH, a member of the SFTP family. These irreversible posttranslational modifications were first described in the IRS and medulla and later in hair cuticles. In the IRS and medullary cells, arginine residues in the repetitive peptide domains of TCHH are converted to peptidyl-citrullines. Consequently, any α-helix-rich structures are unfolded due to decreased intramolecular ionic interactions caused by deimination. Therefore, citrullinated TCHH, which is susceptible to further introduction of isopeptide bonds, is cross-linked to KIF, the cornified envelope, or to itself in the IRS. Interconnected TCHH is predominantly deposited in amorphous vacuoles of the mature medulla. In cuticular cells, hair-dominant-type PAD specifically converts a symmetric pair of Arg51 on the S100A3 dimer to a citrulline pair. The citrullinated S100A3 dimer assembles as a homotetramer in the presence of Ca^{2+} and Zn^{2+}. This trichocytic pathway is likely to be associated with homeostatic Ca^{2+} and Zn^{2+} regulation during hair cuticular maturation. Differential formation of the CE and isopeptide bonds are a reflection of the peptidyl-citrulline content of each tissue within a hair follicle. This chapter documents literature dealing with Ca^{2+}-dependent modification of PADs that alter the solubility, susceptibility to cross-linking, and multimerization tendency of hair fibers and IRS-constituting proteins and outlines a protein deimination pathway leading to trichocytic differentiation. As a result, we summarize previous reports of pioneering research, updated with our current view of protein deimination in the hair follicle.

8.2 PAD Enzymes and Substrate Proteins in the Hair Follicle

PAD activity, which was first detected in guinea pig hair follicles (Rogers and Taylor 1977), has been detected in various tissues from multiple species. Five highly conserved paralogous genes encode PAD isozymes (gene symbols: *PADI*1-4 and 6, see Chap. 1 for more details) on human chromosome 1p35-36 (Ying et al. 2009). It was called PAD1, epidermis type; PAD2, muscle type; and PAD3, hair follicle type from their abundance in originally identified organs, before the cloning and characterization of all *PADI* genes was completed (Chavanas et al. 2004). Multiple PAD isozymes have been reported to be responsible for the deimination of common target proteins. For example, PAD1 and PAD3 both deiminate filaggrin in skin epidermis (Méchin et al. 2005). To elucidate the deimination process by PAD isozymes in vivo, a number of molecular probes have been generated for the comprehensive study of this enzyme family. The tissue distribution of each PAD isotype in human hair

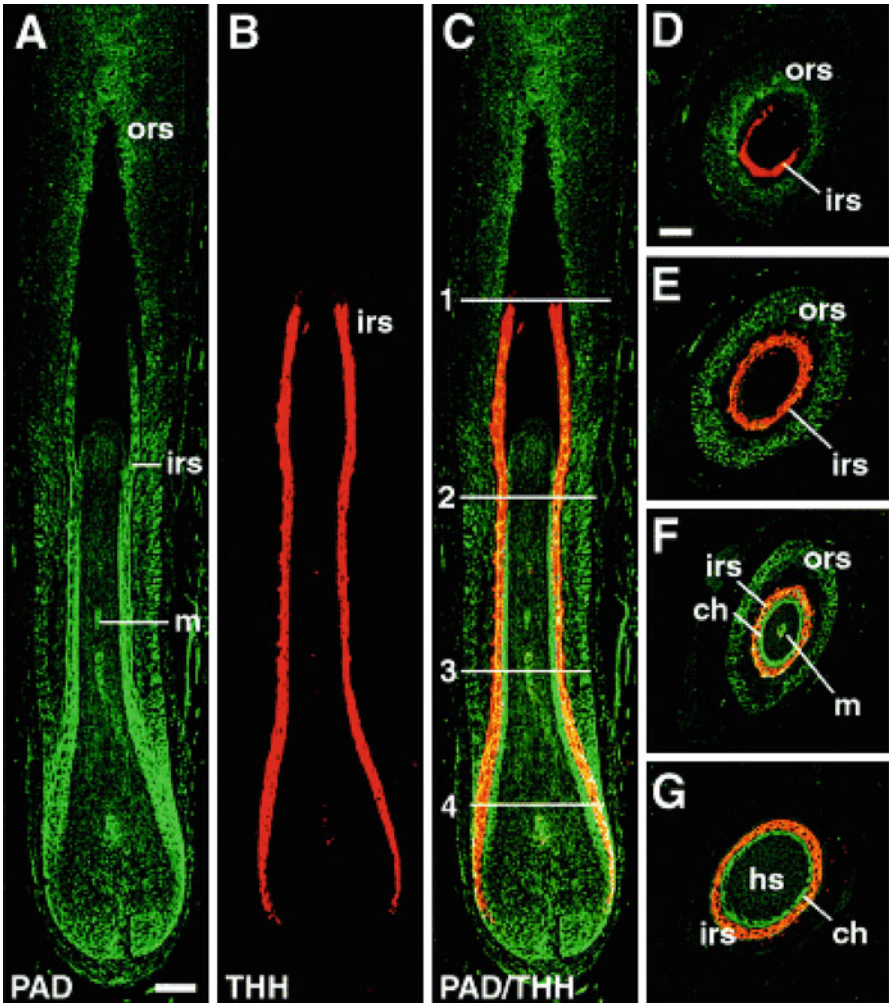

Fig. 8.1 Co-localization of PAD3 with THH in human hair follicles. Immunofluorescence staining of paraffin sections of human scalp hair follicle tissues with a rabbit anti-PAD3 antibody (**A**) or a mouse monoclonal anti-TCHH antibody (AE15) (**B**). (**C**–**G**) Double-immunofluorescence staining with anti-hPAD3 and anti-THH antibodies. The *numbers* in (**C**) correspond to the transverse sections shown in (**D**–**G**) as follows: 1—**D**, 2—**E**, 3—**F**, and 4—**G**. PAD3 was co-expressed with TCHH in the IRS between the supramatrix and the keratogenous zone and in the medulla. TCHH was also localized to the ORS and cuticle of the hair. *ch* cuticle, *m* medulla, *irs* inner root sheath, *ors* outer root sheath, and *hs* hair shaft. Reproduced from Kanno et al. (2000). © Nature Publishing Group

follicles in anagen phase (Figs. 8.1 and 8.2) has been analyzed by immunohistochemistry using isozyme-specific antibodies (Kanno et al. 2000; Nachat et al. 2005, b; Kizawa et al. 2008). PAD1, PAD2, and PAD3 were found to be expressed in the hair cuticular cells as well as in all three types of IRS cells. PAD3 is dominantly expressed in the medulla, while PAD1 and PAD2, but not PAD3, are present in the hair cortical cells. No staining of PAD4 or PAD6 was observed in hair follicles.

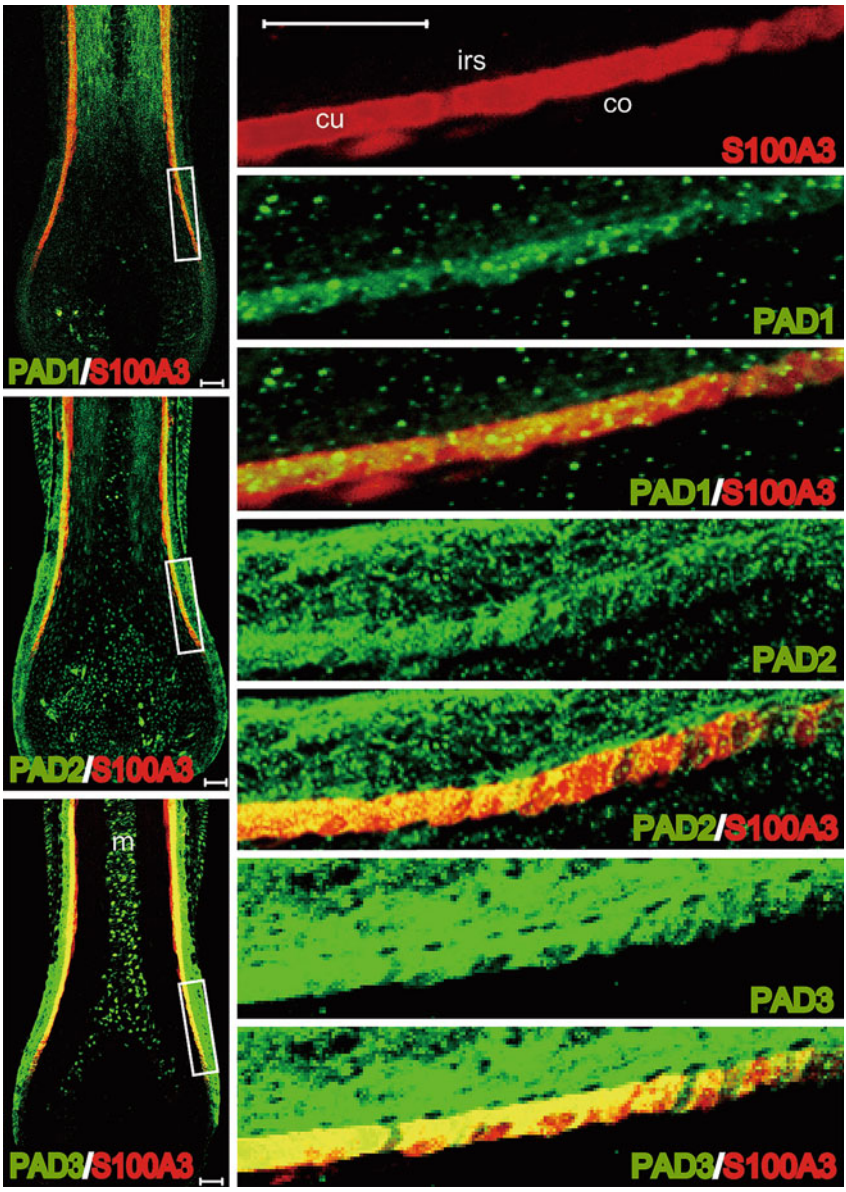

Fig. 8.2 Co-localization of PAD isozymes with S100A3 in human beard follicles. Dual-immunofluorescence labeling of paraffin sections of plucked human beard follicle tissues with rabbit antibodies to PAD1-3 and biotinylated anti-S100A3 antibody. Sections were subjected to confocal microscopy. The *boxed* areas in three left panels are shown at a higher magnification in the panels on the right. Signals of each of the PADs (*green*) are visible as the granular patches in the lower follicle. In all cases, the cuticle is labeled with S100A3 (*red*), which has a cytoplasmic distribution. PAD isozyme staining differs in extent in the inner root sheath as well as in the cuticle. Cuticular staining is punctate with PAD1, partially cytoplasmic with PAD2, and fully cytoplasmic with PAD3. This pattern almost completely overlaps that of S1000A3 staining. *cu* cuticle, *co* cortex, *m* medulla, *irs* inner root sheath. Scale bar: 50 μm. Reproduced from Kizawa et al. (2008). © The American Society for Biochemistry and Molecular Biology

Two representative natural substrates of PAD enzymes in hair follicles are members of distinct but closely related gene families (Kizawa et al. 2011). S100A3 is a member of the S100 protein family, which includes more than 20 genes in humans, whereas TCHH is a member of the SFTP family, which includes proteins such as filaggrin and trichohyalin-like 1 (TCHHL1). Seventeen functional S100 genes, including S100A3, and all seven SFTP genes, including TCHH, are clustered together with CE precursors, e.g., involucrin and loricrin (Candi et al. 2005), in the epidermal differentiation complex on human chromosome 1q21 (Marenholz et al. 1996). S100A6, S100A8/S100A9, and TCHHL1 have been reported to be expressed in the medulla and in IRS cells coincident with TCHH expression (Wood et al. 1991; Schmidt et al. 2001; Kizawa et al. 2005a; Wu et al. 2011), but their post-translational modifications in hair follicle have not yet been characterized. It was previously reported that several citrulline-containing peptides in mouse IRS cell digests (refer to Sect. 8.5.1) were derived from type I and II IRS keratins other than TCHH (Steinert et al. 2003). A sequence search (blastp) of the listed partial amino acid sequences in the current database (http://blast.ncbi.nlm.nih.gov/) revealed that these peptides correspond to the translation products of the mouse type I keratin genes, K25, K27, and K28, and the type II keratin gene, K71 (Langbein et al. 2006).

8.3 Localization of Protein Deimination in the Hair Follicle

Coincident with the asynchronous cornification of the three morphologically distinct cell types in the IRS, a chemically modified citrulline (AMC) antibody (Senshu et al. 1999) labeled proximal cells in the Henle's layer, then the IRS cuticle cells, and distal cells in the Huxley's layer (Nachat et al. 2005b). Mouse monoclonal AE15, AE16, and AE17 (refer to Sect. 5.1) antibodies differentially recognize TCHH at different stages of maturation (O'Guin et al. 1992). Dual-immunofluorescent microscopy with AE15, which stains TCHH proteins throughout the TCHH granules, showed that TCHH co-localized with PAD1 and PAD3 in three cellular layers of the IRS (Fig. 8.1) (Kanno et al. 2000). In medullary cells, PAD3, which is the dominantly expressed PAD isozyme, is most likely responsible for TCHH deimination. Immuno-electron microscopy showed that AE16 specifically recognizes TCHH on the surface of the granule (O'Guin et al. 1992). Released TCHH co-localized with PAD3 in the Huxley's layer of the IRS (Nachat et al. 2005b). In addition to TCHH, the major IRS keratin pairs expressed in all three IRS layers (Langbein et al. 2006) were also shown to be PAD substrates (Steinert et al. 2003). Antibodies were prepared by immunizing guinea pigs with synthetic peptides corresponding to the carboxyl terminus of mouse K27 (anti-K27 antibody) and carboxyl terminus K27, within which arginine had been replaced by citrulline (anti-Cit-K27 antibody). Immunoreactivity of the anti-Cit-K27 antibody was observed in cells at a later differentiation stage than those stained with anti-K27 antibody in all IRS layers of the mouse hair follicle. This time point appears to correspond to the onset of protein deimination in each IRS tissue. It is also possible

that TCHHL1, another SFTP family member, may be a PAD substrate, based on its similar distribution pattern. Furthermore, the restricted distribution of TCHH1 compared to TCHH in the central and distal zones of the IRS suggests a close association with the terminal differentiation of IRS cells (Wu et al. 2011).

In addition to medulla and IRS layers, the hair cuticular cells have shown to be labeled with both antibodies to AMC (Kizawa et al. 2011) and to PAD3 isozyme (Kanno et al 2000). S100A3 was expressed profoundly in the cuticular cells and, to a lesser extent, in the cortical cells (Kizawa et al 1998). S100A3 is considered to be a major deiminated protein present in hair cuticular cells. The distribution pattern of granules with PAD1 immunoreactivity in the hair cuticular layer was clearly different from the nuclear and cytoplasmic distribution of S100A3. The granular patchy signals of the PAD2 and PAD3 isozymes appeared prior to the appearance of the S100A3 signal but subsequently co-localized with the cytoplasmic distribution of S100A3 (Fig. 8.2). Cytoplasmic distribution of PAD3 at an earlier cuticular differentiation stage than PAD2 almost completely co-localized with S100A3. Thus, PAD3 appears to be primarily responsible for the deimination of S100A3 within the cuticular cells, although it is clear that PAD2 also participates in this process (Kizawa et al. 2008). Although keratins from the IRS and epidermis have been reported to be major substrates of PADs (Steinert et al. 2003; Nachat et al. 2005a), no information is available regarding the deimination of major hair keratins derived from cortical cells. The hardened rigid structure of these proteins is thought to be conferred by highly cross-linked disulfide bonds rather than isopeptide bond formation, which requests preliminary introduction of deimination (refer to Sect. 5.1). This is reflected in the lower distribution of both PAD enzymes and deiminated proteins in the hair cortex than in other keratinized tissues.

8.4 Selective Arg/Cit Conversion in Hair Substrate Proteins

8.4.1 Substrate Specificity of PAD Isozymes

Synthetic arginine derivatives, in which the α-amino group has been substituted with other groups, were shown to be converted to the corresponding citrulline derivatives to varying extents (Méchin et al. 2005). A consensus sequence recognized preferentially by PAD enzymes has not yet been identified, however. For structural hair proteins to fold and unfold properly, PAD enzymes must selectively convert target arginine residues in each substrate protein to peptidyl-citrullines. The procedure for isolating TCHH from wool follicle (Fietz et al. 1993) or porcine tongue (Hamilton et al. 1992) is based on its low solubility in water. Therefore, characterization of the biochemical properties of hair substrates in vitro has been limited. Human TCHH is a high-molecular-weight protein with a mass of 220 kDa (O'Guin et al. 1992). Domain 8 corresponds to residues 1250–1849 in the protein (THH-8), to which KIF is exclusively cross-linked as well as to domain 6 (Steinert et al. 2003). In addition, it has been expressed in *E. coli* and subjected to in vitro modification

(Tarcsa et al. 1996). A number of studies focused on target arginine residues in either synthetic or natural substrates have been performed using native enzymes derived from various species. The deimination rate of THH-8 by rabbit PAD2 isolated from skeletal muscle reached a plateau at 20–25 % under an enzyme/substrate ratio of 1:50. This rate increased to 65 % when the enzyme/substrate ratio was increased to 10:1. Our knowledge of the substrate specificities of each PAD isozyme has been further advanced by the use of active highly purified recombinant PAD enzymes. A hair follicle dominant-type human PAD3 recombinant enzyme was produced and subjected to in vitro modification of THH-8 (Kanno et al. 2000). At an enzyme/substrate ratio of 1:50, 10–20 % of the arginines in THH-8 are converted to citrullines after 24 h. These results suggest that PAD3 might be responsible for conversion of the restricted arginine residues in THH-8. Further deimination appears to be catalyzed by rabbit PAD2 derived from skeletal muscle, even though critical comparison of the results obtained with enzymes of different purity is inappropriate.

In contrast to TCHH, S100A3 is a small (11 kDa) soluble protein that contains only four arginines (Engelkamp et al. 1993; Kizawa et al 1996; Kizawa et al. 2002). Previously, rabbit muscle PAD2 was reported to specifically convert Arg63 in the active site of the Kunitz-type soybean trypsin inhibitor (Takahara et al. 1985). As PAD enzymes are absent in soybean, this specific demination was not a case that naturally occurs in vivo. We were able to conclusively demonstrate that Arg-51 in S100A3 is specifically converted to Cit51 in hair cuticles (Kizawa et al. 2008). S100A3 was isolated from cuticles delaminated from hair fibers by water agitation (Swift and Bews 1974) and immature cuticles in plucked beard follicles. Analyses of S100A3 by 2D-PAGE showed that several spots shifted in the acidic direction. Western blot analyses using an AMC antibody revealed that peptidyl-arginines were introduced into S100A3 by PAD enzymes. Different proportions of isoelectric S100A3 variants were identified in the extracts of plucked immature beard follicles and in mature hair cuticles (net conversion rates: 19 % vs. 59 %). Thus, naturally occurring S100A3 appeared to have experienced progressive deimination during cuticular maturation. In vitro modification of recombinant S100A3 by recombinant PAD isozymes resulted in different conversion rates (Fig. 8.3). Peptide mapping of the endoproteinase Lys-C digest of modified S100A3 revealed that PAD1 and PAD2 convert Arg3, Arg22, and Arg51 completely, and Arg-77 partially, to citrullines. By contrast, PAD3 specifically converts Arg51. Naturally occurring S100A3 appears to have a higher proportion of Cit at position 51 than at other sites. Taken together, PAD3 specifically converts Arg51 to Cit51 in S100A3 in vivo. To address the substrate specificity of this protein-modifying enzyme family, each substrate and responsible isotype(s) must be experimentally determined.

8.4.2 Structural Insights into PAD Target Recognition

Equilibrium dialysis experiments first showed that one rabbit PAD2 molecule is capable of reversibly binding at least three Ca^{2+} ions (Takahara et al. 1986). Later, the crystal structures of Ca^{2+}-bound PAD4 and the substrate complex conclusively

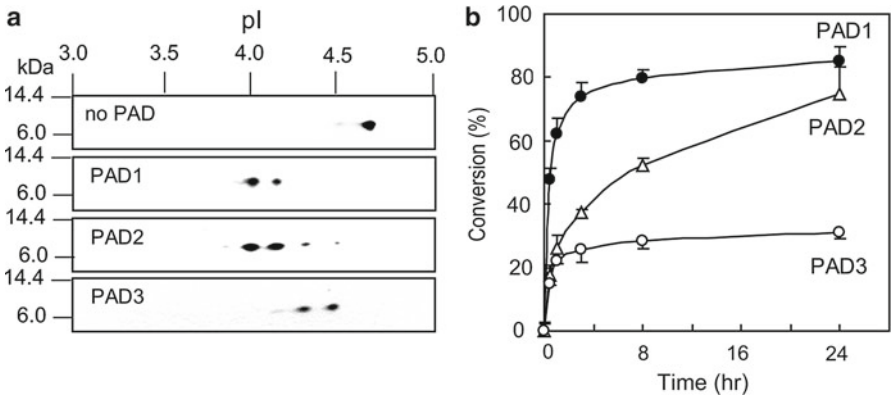

Fig. 8.3 In vitro citrullination of human recombinant S100A3 by PAD isozymes. (**a**) Selected areas from 2D-PAGE of S100A3. Recombinant S100A3 produced in *E. coli* (1 μg; p*I* 4.7) was incubated with recombinant PAD1, PAD2, or PAD3 (25 mU each) at 37 °C for 24 h with 10 mM $CaCl_2$. Resultant isoelectric variants indicate that PAD1 and PAD2 are capable of converting three or four arginines in S100A3 (p*I* 4.1 and 4.0), whereas PAD3 converted only one or two arginines (p*I* 4.5 and 4.3). (**b**) Time course of conversion. Citrulline contents in S100A3 modified by PAD1, PAD2, and PAD3 plateaued at approximately 80, 70, and 30 %, respectively, within 24 h. Shown are the averaged percentages and S.D. values of three independent experiments. Reproduced from Kizawa et al. (2008). © The American Society for Biochemistry and Molecular Biology

demonstrated that both have five Ca^{2+}-binding sites per PAD molecule (Arita et al. 2004). Although the Ca^{2+}-binding sites of other isozymes have yet to be determined, most of the PAD4 residues that were shown to participate in Ca^{2+}-binding were conservative. All of the Ca^{2+}-binding sites of PAD4 are coordinated in a topology that is quite different from that of the EF-hand-type structural domain (i.e., a helix–loop–helix topology). Two Ca^{2+}-binding sites are located at the bottom of the catalytic site. An active cleft capable of recognizing a peptidyl-arginine residue is formed upon the binding of Ca^{2+} to these sites. Binding of Ca^{2+} ions to the three N-terminal Ca^{2+}-binding sites of PAD4 induces an N terminus conformational change; however, this has no effect on its catalytic activity, and these Ca^{2+} ions appear to function in stabilizing the structure of PAD enzymes. (See Chap. 21 for more details.)

Several residues of PAD4 that interact with the backbone atoms of the substrate protein at the $(N-2)$, $(N-1)$, and $(N+1)$ positions proximate to the target arginine (Arita et al. 2006) are not conserved in other isotypes. The N-terminal arginine in all known substrates is not modified by any of the PAD isotypes. It is generally accepted that the activities of PAD enzymes are markedly affected by both the substrate structure and sequence in the vicinity of the target arginine. The conversion rates in THH-8 were markedly lower than those of filaggrin. Rabbit PAD2 converts 20–25 % of the arginines in THH-8 to citrulline, whereas it converts >95 % in filaggrin (Tarcsa et al. 1996); and recombinant PAD3 converts 10–15 % in THH-8, whereas it converts ~60 % in the filaggrin repeat unit (Kanno et al. 2000). PAD enzymes can rapidly modify filaggrin, which possesses only a *b*-turn secondary structure, but the modification of α-helix-rich TCHH is significantly slower. The

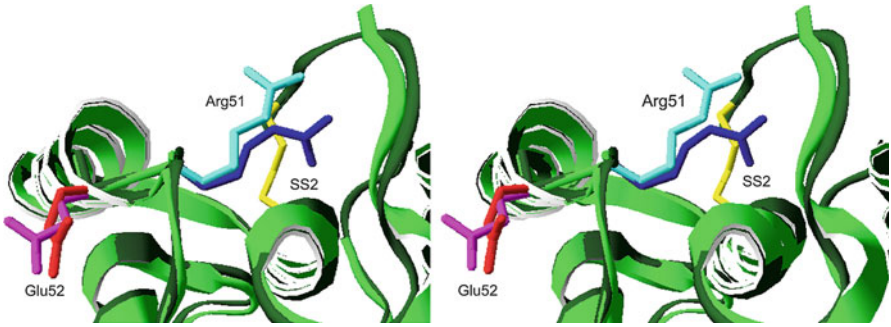

Fig. 8.4 Stereo view of a peptidylarginine residue in S100A3 targeted by PAD3. The properly folded backbone structure of S100A3 (PDB code, 3NSI, in *dark green* colors) was superimposed on the unfolded form (1KSO, *bright green* colors) by fitting helices III and IV. Inversely orientated side chains of Arg51 (*dark* and *light blue*) in the tail of the hinge region and Glu52 (*red* and *pink*) in the head of helix III are indicated on both 3NSI and 1KSO. Note that a disulfide bridge between Cys81 and Cys99 (SS2, *yellow*), which connects the C-terminal domain to helix IV, positions the guanidinium group of Arg51 to an inward orientation in 3NSI

lower conversion rate of THH-8 can also be attributed, in part, to the finding that more than 30 % of the arginines in THH-8 are followed by glutamic acid moieties and to the frequency of consecutive arginine sequences in THH-8. While most peptidyl-arginines in THH-8 were converted to peptidyl-citrullines in vitro, the conversion rate of peptidyl-arginine followed by a glutamate (i.e., X-Arg-Glu) on an α-helix is very low (<5 %) (Tarcsa et al. 1996). Furthermore, in the special case of two or three consecutive arginines, the first were modified to citrullines, but the conversion rates of the second and/or third arginines lagged behind. Several TCHH peptides isolated from the IRS carry X-Cit-Glu sequences (Steinert et al. 2003) that are probably located on the random coil structure or become located there upon deimination of other sites. Furthermore, Arg51, followed by Glu52, in S100A3 was preferentially converted by PAD3 (Kizawa et al. 2008). The crystal structures of S100A3 (PDB code: 1KSO, 3NSO, and 3NSI) show that Arg51 is located on the end of the loop structure fused to helix III (residues 52–64) (Fritz et al. 2002; Unno et al. 2011). Previously determined crystal structures of PAD4 (PDB code: 1WD8, 1WD9, and 1WDA) show that Asp350, His471, Asp473, and Cys645 participate in the target recognition (Arita et al. 2004). The catalytic site is conserved in other PAD isozymes, which are responsible for deimination of S100A3 and TCHH in the hair follicle. Considering that Asp350 and Asp473 in PAD4 form hydrogen bonds and a salt bridge with the NeNh1 and Nh_2 atoms of the guanidyl group of the target arginine, an electric repulsion (or attraction) may exist between the Asp residue in the active site and the carboxyl group (or guanidinium group) located near to the target guanidyl group of the substrate protein. On the other hand, -Arg51-Glu52- in S100A3 may be accessible to the active site of PADs, because these side chains adopt a reverse orientation (Fig. 8.4).

The affinity of PAD3 for Arg-51 in S100A3 was shown to be largely affected by the formation of two disulfide bridges (Fig. 8.5) (Unno et al. 2011). Of the ten

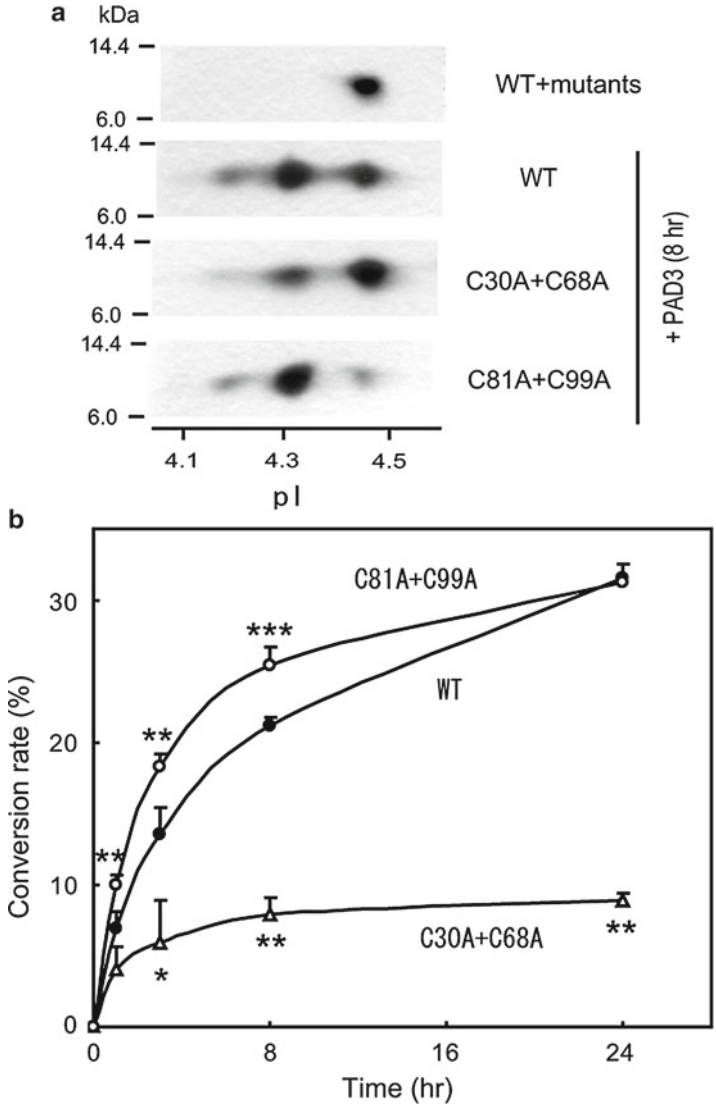

Fig. 8.5 Effect of disulfide folding on the specific citrullination of Arg51 in S100A3 by PAD3 in vitro. (**a**) Selected area of 2D-PAGE. Silver-stained gels showing a co-migrated spot of untreated *Sf21*-derived recombinant protein mixture (total, 1 μg) and WT, C30A + C68A, and C81A + C99A (1 μg each) (Kizawa et al. 2013b) treated with recombinant PAD3 enzyme (25 mU) in the presence of 2 mM $CaCl_2$ at 37 °C for 8 h. The p*I* shift from 4.5 to 4.3 corresponds to the selective conversion of Arg51 into citrulline by PAD3. (**b**) Time course of conversion. The conversion ratio was calculated by the relative intensities of the immnospots on 2D-PAGE. Statistical differences from WT-S100A3 are indicated by $*p<0.05$, $**p<0.01$, and $***p<0.001$. The averaged percentages of maximum activity are presented with standard deviations. Reproduced from Unno et al. (2011). © Elsevier B.V

cysteine residues, Cys-30 and Cys-68 form SS1, which connects two Ca^{2+}-binding loops. SS1 formation in S100A3 is essential for Ca^{2+} binding and Arg51 conversion by PAD3. This suggests that a Ca^{2+}-dependent conformational change (e.g., reorientation of the helix III) is induced during catalysis. By contrast, SS2 formation between Cys81 on helix IV and Cys99 in the C-terminal coil shields Arg51 in S100A3. This structural environment appears to be different from that of Arg63 in the active site of the soybean trypsin inhibitor, located on the external loop. Considering that mutagenic disruption of SS2 (C81A + C99A) increases the Ca^{2+} affinity and reactivity of Arg51, S100A3 may not form SS2 before PAD binding. The intracellular redox state appears to be critically regulated for preserving an optimal catalytic environment. Unfortunately, the little structural information presently available on the three PAD isoforms (PAD1, PAD2, and PAD3) in the hair follicle neither account for their different substrate specificities nor allow elucidation of the consensus recognition site of the target proteins. Further study is necessary to reveal the structural accommodation between S100A3 and recently crystallized PAD3 (Unno et al. 2012).

8.5 Consequences of Protein Deimination in the Hair Follicle

8.5.1 Cit-TCHH Susceptibility to Transglutaminase

Transition from an electron donor group of peptidyl-arginine to an acceptor group of peptidyl-citrulline confers significant structural and mechanical effects on the target proteins by altering their inter- and intramolecular ionic or hydrophobic interactions (Vossenaar et al. 2003). Natural substrates of PAD enzymes are directed to either aggregate into or disassociate from protein complexes based on changes in their net charges. For example, citrullinated subunits of filaggrin (Kamata et al. 2009) and filaggrin-2 (Hsu et al. 2011) have been reported to become susceptible to hydrolysis into free amino acids in skin epidermis. In the central nervous system, citrullinated myelin basic protein becomes susceptible to digestion by cathepsin D (Pritzker et al. 2000). In hair follicles, modification by PAD2 significantly increases the solubility of THH-8 (from 60 to >200 mg/ml) and TCHH from intact pig tongue (from ~3 to 25 mg/ml). The high α-helix content (~86 %) maintains the rigid structure of THH-8, but more than half of the α-helical structure is lost following 25–40 % Arg/Cit conversion (Tarcsa et al. 1996). The accelerated solubility of Cit-TCHH can disperse an insoluble particulate suspension (>1 μm) into a soluble form in vitro, thus also promoting TCHH droplet dissolution in vivo.

TCHH has been proven to be a natural substrate of both PADs and transglutaminase (TGM; EC 2.3.2.13) in the IRS and medullary cells (Tarcsa et al. 1997). N^{ε}-(g-glutamyl)lysine cross-links in citrulline-containing proteins were first derived from the medulla (Harding and Rogers 1971). Sequential modification of THH-8 by PAD and TGM was conducted in vitro (Tarcsa et al. 1997). Following PAD

modification, TGMs introduce abundant N^{e}-(g-glutamyl)lysine isopeptide bonds in TCHH. Incorporation of [^{14}C]putrescine into THH-8 was most efficiently accomplished by TGM3 and to a far lesser extent by TGM1 and TGM2, thus confirming that TCHH is preferentially cross-linked by TGM3 in vitro. After digestion with Asp-N, the resultant peptides were sequenced and the radioactivity in each cycle was measured. PAD modification of THH-8 significantly increases the rate of cross-linking by TGM3. Peptide sequences revealed that, while a limited number of glutamines were labeled before PAD modification, nearly all glutamines in Cit-THH-8 are equally targeted by the cross-linking reaction. In addition, immunostaining with a Cit-K27 antibody was diminished following TGM3 expression in the IRS of mouse hair follicle (Steinert et al. 2003). These results suggest that PAD and TGM reactions occur sequentially in a restricted region of each IRS tissue. Of the seven known active TGMs in human (Lorand and Graham 2003), TGM1, TGM2, TGM3, and TGM5 have been shown to be expressed in hair follicles. Although TGM3 is detected in the IRS and medulla of mouse pelage follicles (Tarcsa et al. 1997), its expression pattern in human hair follicles is unclear (Thibaut et al. 2005). Although the TGM isozymes responsible for introducing isopeptide bonds into TCHH and IRS keratins have not been conclusively identified in human hair follicles, TGM1 and TGM5 appear to cooperate during IRS differentiation.

Considering the absence of KIF in the medulla, its vacuolated structure was envisaged to be primarily comprising denatured interconnected TCHH aggregates (Tarcsa et al. 1997). To identify the cross-linked partners of TCHH in the IRS, trunk skin dermis from newborn albino mice was harvested and suspended in urea (Steinert et al. 2003). The IRS-rich material was passed through a nylon gauze filter (pore size: ~0.2 mm) to remove hair fibers and then subjected to stepwise digestion using trypsin, Asp-N protease, and proteinase K. The resultant peptides were subjected to amino acid analyses to quantify isodipeptide cross-linking. Branched peptides with two or more arms were subjected to sequence analyses. The resultant phenylthiohydantoin derivatives were identified based on the results of database searches. TCHH was shown to be cross-linked with another TCHH, IRS keratins, and CE proteins (e.g., involucrin, repetin, small proline-rich proteins, and late cornified envelope proteins). It was also cross-linked with desmoplakin, envoplakin, and epiplakin, which link KIFs to desmosomal cadherins (Steinert et al. 2003). Glutamine and lysine in both the head and tail regions of Cit-IRS keratins (K25, K27, K28, and K71) were variably cross-linked with those in Cit-TCHH domains 6 and 8. Immuno-electron microscopy with the TCHH monoclonal antibody AE17 showed that TCHH is distributed with a regular 400 nm periodicity along KIFs in the IRS (O'Guin et al 1992). Recently, the C-terminal 13 amino acid sequence in the tail domain 9, which is conserved among species, was shown to be essential for keratin adherent ability of mouse TCHH in keratinocyte cell lines, though neither deimination nor isopeptide cross-links within the functionally critical sites has yet been determined. Based on these observations, Takase and Hirai (2012) proposed a principal role of the C-terminal tail domain of TCHH in adhesion to keratins. Following aggregation of N-terminal parts (i.e., N-terminal S100-like domain and following repetitive peptide domains) in TCHH, KIFs bundle and form meshes.

Mature TCHH likely plays a role in lateral aggregation, precise alignment, and stabilization of IRS KIF bundles. TCHH–TCHH and TCHH–CE protein links were distributed among domains 2–5 but were rare in domains 6 and 8. Intra-TCHH cross-links frequently appeared in the least ordered domain 5. Although these extensive deiminations have been envisaged to occur after TCHH–KIF cross-link formation, it has not been determined which PAD isozymes are responsible for cross-link formation between the TCHH–KIF, TCHH–TCHH, and TCHH–CE proteins. TCHH peptides were derived from the entire sequence, except for the N-terminal S100-like domain carrying two EF-hand motifs, and there was no evidence of cross-linking between TCHH and the S100 proteins. The fate of the N-terminal S100-like Ca^{2+}-binding domain in TCHH is still unknown but may be proteolytically cleaved from the repetitive peptide domains, as has been previously reported to occur in filaggrin (Presland et al. 1997).

8.5.2 Zn^{2+} with Ca^{2+} Causes Assembly of a Cit-S100A3 Tetramer

Three PAD isozymes are known to be expressed in distinct epithelial components in human hair follicles (Nachat et al. 2005b; Kizawa et al. 2008). Little is known about the structural consequences of PAD activity on each target substrate or the functional impacts of selective Arg/Cit conversion. Analysis of posttranslational modification of S100A3 carrying only four arginines examined the functional significance of Arg/Cit conversion by multiple PAD isozymes in vivo. The initial specific conversion of Arg-51 in S100A3 by PAD3 results in the assembly of a homotetramer. Subsequently, PAD2 exhibits broad catalytic activity for S100A3, resulting in extensive conversion of other sites (Kizawa et al. 2008). Because PAD3 is predicted to form a dimer (Unno et al. 2012), its catalytic reaction proceeds in a heterotetrameric substrate–enzyme complex (left side of Fig. 8.6). At present, it is not known whether an active site cleft of PAD3 recognizes Arg51 in S100A3 or two active sites on a dimeric enzyme simultaneously recognize the target Arg51 pair within the intermediate complex. A symmetric Cit-51 pair on the S100A3 dimer surface allows interaction of the apo-dimers, which is prevented by the electric repulsion between the positive charges of Arg-51 in non-modified dimers. The structural and functional roles of the other deiminations remain undefined.

It has been proposed that the Ca^{2+} ions bound to an S100A3 tetramer (Kizawa et al. 2008, 2011) or an S100-like Ca^{2+}-binding domain of SFTP (Vossenaar et al. 2003) are transferred to Ca^{2+}-dependent protein-modifying enzymes. Although intracellular Ca^{2+} oscillation (<1 mM) activates Ca^{2+}-dependent protein-modifying enzymes, it appears insufficient for assembling a Cit-S100A3 tetramer. However, a single Zn^{2+} ion accelerated Ca^{2+}-dependent S100A3 tetramerization (Kizawa et al. 2013a).

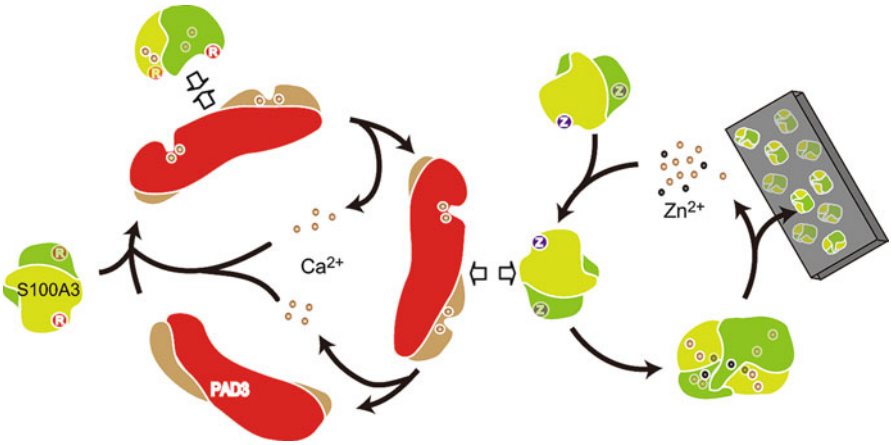

Fig. 8.6 S100A3/PAD3 pathway associated with Ca^{2+}/Zn^{2+} homeostasis in hair cuticular cells. Dimeric PAD3 specifically converts a symmetric Arg51 pair (R in *red circles*) on the S100A3 dimer in the presence of Ca^{2+} ions. In addition to stationary bound Ca^{2+} ions in PAD3 (not shown), two Ca^{2+} ions bound to each subunit form the active site cleft (i.e., eight Ca^{2+} ions reversibly complex with an S100A3/PAD3 heterotetramer). Dimeric S100A3 with Cit51 (Z in *purple circles*) disassociates from dimeric PAD3 and then associates with another dimer formed in a previous cycle in the presence of Ca^{2+} and Zn^{2+}. Each Cit-S100A3 subunit is capable of binding two Ca^{2+} and one Zn^{2+} ions in a homotetramer. Either intra- or intermolecular disulfide bridge formation of S100A3 in response to the environmental redox state (Unno et al. 2011) or an excessive amount of Zn^{2+}, which disrupts the α-helices (Kizawa et al. 2013a), ultimately liberates Ca^{2+} and Zn^{2+}. Resultant cation pools are essential for the activation of Ca^{2+}-dependent protein-modifying enzymes, including PAD3. As the amount of S100A3 deposited into the subcuticular cellular component (*gray rectangle* on the *upper right*) increases, local levels of intracellular Ca^{2+}/Zn^{2+} increase

S100A3 has redox state-dependent affinity for Zn^{2+}: $K_d = 11$ μM under aerobic conditions (Föhr et al. 1995), 1.5 μM under anaerobic conditions (Fritz et al. 1998), and 4 nM in the presence of glutathione (Fritz et al. 2002). A $(Cys)_3His$-type Zn^{2+} complex is coordinated by the preformed Zn^{2+} pocket composed of Cys83, Cys86, His87 (Fritz et al. 2002), and a Cys93 recruited from the C terminus (Unno et al. 2011). The binding states of the Ca^{2+} or Zn^{2+} ions to each S100A3 subunit within a homotetramer have been shown to be propagated by repositioning of helix III and rearrangement of the C-terminal tail domain (Kizawa et al. 2013a). Cit-S100A3 dimers disassociated from dimeric PAD3 most likely assemble as a homotetramer after relocation to the epithelial cellular compartments with both Ca^{2+} and Zn^{2+} ions (right part of Fig. 8.6). At present, it is difficult to envisage the entire Ca^{2+}/Zn^{2+} system associated with citrullination-mediated S100A3 tetramerization. Further studies will be necessary to investigate the cellular pathways underlying epithelial Ca^{2+}/Zn^{2+}-homeostatic regulation of cuticular cell differentiation in hair follicles.

8.6 Summary and Outlook

Mature S100A3 and TCHH are ultimately deposited in the mature endocuticle (Takizawa et al. 1999) or medulla (Tarcsa et al. 1997) of keratinized hair or shedding IRS cells (O'Guin et al. 1992). Both PADs and substrates are thought to play the following important roles during the terminal differentiation of each tissue.

1. *S100A3 in hair cuticles*: Although the final fate and ultimate structure of S100A3 remain unknown, bound Ca^{2+} and Zn^{2+} ions disassociated from S100A3 are recycled for activation of the protein-modifying enzymes and assembly of an S100A3 homotetramer. S100A3 is predicted to play a role in sequestering Ca^{2+} and may also be a source of Zn^{2+}, which is required for the formation of a thick epithelial barrier.
2. *TCHH in the medulla*: TCHH becomes dispersed and is predicted to form self-interconnected disordered aggregates in the medulla. The resultant vacuolated amorphous deposits fulfill a critical function in thermal regulation.
3. *TCHH in IRS*: Cit-TCHH has been shown to be cross-linked with itself or with KIF and CE components. The resultant isopeptide bonds confer a rigid structure to IRS cells and are thought to determine the shape of the internal hair shaft.

Hair protein deimination confers significant functional and structural changes during hair fiber and sheath maturation processes. A number of human genes encoding hair-specific PAD substrate proteins are reported to be associated with human hair morphology (straight, wavy, or curly) as well as with disorders of hair loss (Shimomura 2012). Heterozygous mutations in IRS keratins (K71 and K74) are reported to cause autosomal dominant woolly hair (Wasif et al. 2011; Fujimoto et al. 2012), and TCHH gene variants are reported to be associated with hair morphology (Medland et al. 2009). Although some deiminated proteins have emerged as key targets of the immune response in rheumatoid arthritis (Suzuki et al. 2007), no direct evidence indicating an association of protein deimination with human hair morphology and disease has yet been reported. The underlying mechanisms by which antigenic deiminated proteins arise might be relevant to the pathogenesis of various immune diseases. Although TCHH was reported to be a major autoantigen in the sera of the patients with alopecia areata (Leung et al. 2010), it has not been determined whether deimination of TCHH is related to human diseases. The resistance of hair cuticles against mechanical insults has shown to be conferred, at least in part, by disulfide cross-links of S100A3 (Inoue et al. 2000; Kizawa et al. 2005b). As a result, it is also conceivable that the extents of deimination in S100A3, TCHH, and other substrate proteins may affect hair texture. High-throughput analyses of hair protein variants at post-synthetic stages, e.g., enrichment of citrulline-containing peptides (Tutturen et al. 2010), will be required for critical diagnoses of project participants and patients. Altogether, a better understanding of hair protein deimination would lead to development of new therapeutic strategies for the treatment of human hair diseases, perfection of personal hair texture, and recuperation of healthy hair.

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Chapter 9
Deimination in the Peripheral Nervous System: A Wallflower Existence

Gerburg Keilhoff and Anthony P. Nicholas

Keywords Citrullination • Deimination • Dorsal root ganglia • Histone • Myelin • Nitric oxide synthase • PAD • Peripheral nervous system • Satellite cell • Schwann cell

9.1 Introduction

Searching in Medline for "deimination/citrullination" and "peripheral nervous system" (PNS) results in a total of nine citations found (Ishiyama et al. 2001; Tommasi et al. 2006; Harauz and Musse 2007; Musse and Harauz 2007; Wood et al. 2008; Musse et al. 2008; Bhattacharya et al. 2008; Ahmed et al. 2010; Homchaudhuri et al. 2010), suggesting that deimination in the PNS is unimportant. This, however, is neither reasonable nor accurate.

Deimination or citrullination is a posttranslational modification of proteins in which selected arginine residues are converted into citrullines. This reaction commonly occurs and is performed by a family of enzymes called peptidylarginine deiminases (PADs). It must be distinguished from the formation of free citrulline as part of the urea cycle or from the nitric oxide synthase (NOS)-mediated conversion of L-arginine as a substrate to nitric oxide and its "by-product" of free citrulline. Known substrates to be deiminated by the PAD enzymes under physiological conditions are, among others, cytoskeletal proteins like cytokeratin and fillagrin, histones, and myelin basic protein (MBP). Other proteins, like GFAP, vimentin, or fibrin, were found to be citrullinated during cell death and tissue inflammation (for review

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see Gyorgy et al. 2006; Thompson and Fast 2006; Mechin et al. 2011). Histones, MBP, and GFAP, however, are common proteins of the PNS. Moreover, the expression of the PAD isoenzymes 2 and 3 in Schwann cells, the myelinating cells of the PNS, was demonstrated (Keilhoff et al. 2008). Thus, a lack of the substrate as well as of the enzyme for deimination/citrullination in the PNS can now be excluded. The following is a review of what we presently understand about the PNS as well as the role of deimination in this area of the body in normal healthy conditions and during injury and disease.

9.2 Peripheral Nervous System

The PNS refers to parts of the nervous system outside the brain and spinal cord. Generally, the definition of the PNS is inconsistent. Consensus suggests that this is part of the "somatic" nervous system, which controls body movements via skeletal muscles and includes the spinal nerves with their roots and branches and peripheral nerves with their neuromuscular junctions as well as their respective ganglia. The cranial nerves are also units of the somatic PNS, however, with the exception of second cranial (optic) nerve. Since it is directly attached to the retina, which is considered an embryonic extension of the brain, the optic nerve instead belongs to the central nervous system (CNS). Consistent with this idea, the optic nerve fibers are covered with myelin produced by oligodendrocytes of the CNS, whereas PNS fibers are ensheathed by myelin produced by Schwann cells. As a result, reports on deimination in the eye by Bhattacharya and co-workers can be reviewed elsewhere (Bhattacharya et al. 2006, 2008; Bhattacharya 2009) (see Chap. 16).

Although deimination/citrullination in spinal cord neurons has been reported (Lange et al. 2011; Raijmakers et al. 2006; Asaga and Senshu 1993; Nicholas et al. 2004, 2005), motor neurons of the spinal cord anterior horn as part of the motor unit are sometimes discussed with the PNS; they also belong to the CNS, making them similar to the optic nerve in this regard.

The autonomic (vegetative) nervous system, consisting of the sympathetic ("fight or flight") and parasympathetic ("rest and digest") components, as well as the enteric nervous system ("second or abdominal brain"), all together innervating smooth muscles, cardiac muscles, and glandular epithelia, are sometimes referred to as a "second" or a "special" component of the PNS. We, however, judge them to be independent to this nomenclature, which is, on the other hand, a hindrance to be included in this minireview.

As a result, the cellular elements of the somatic PNS that will be addressed include:

1. The ganglion cells: Sensory neurons, bringing afferent information from the periphery into the CNS.
2. Two types of glial cells: (a) Schwann cells, myelinating the PNS axons and covering neuromuscular junctions (terminal Schwann cells), and (b) satellite cells, surrounding the neurons in ganglia, thus establishing a specialized microenvironment.

3. Different sensory receptor cells.
4. Fibrocytes, producing the connective tissue sheaths of the nerve fibers (epineurium, perineurium, endoneurium).

9.3 Distribution of PADs in the PNS

Details concerning PAD expression in the aforementioned cellular PNS structures are more than incomplete. Although evidence for deiminated proteins in the PNS was first seen in the trigeminal nerve and ganglion of the rat in 2003 (Nicholas et al. 2003), the most extensive study of deimination in the PNS to date was published by us in 2008, providing detailed expression patterns of deiminated proteins and PAD enzymes in rat and human peripheral nerves and Schwann cells (Keilhoff et al. 2008).

Therefore, cultivated rat and human Schwann cells and slices of fetal, juvenile and normal, and degenerating and regenerating adult sciatic nerves were subjected to RT-PCR, Western blot, and immunohistochemical analysis of the PAD subtypes. Moreover, citrullinated proteins in these specimens were explored. Albeit our focus was directed on Schwann cells, observations relevant to fibrocytes were also made, as a quasi-like by-product. As a result, cultures of pure fibrocytes were studied in parallel to exclude false-positive Schwann cell results by contamination of these cells.

The main PCR findings (Fig. 9.1a) were as follows:

1. PAD1 transcripts were lacking in Schwann cells and fibrocytes.
2. High amounts of the PAD2 transcript were found in juvenile rat and fetal human sciatic nerve. Adult rat and human sciatic nerves expressed reduced levels of PAD2 mRNA. Expression of PAD2 transcripts in rat sciatic nerves was induced in specimens undergoing either degeneration or regeneration. Schwann cell cultures clearly expressed the PAD2 transcript. Cultivated fibroblasts, however, were (almost) free of the PAD2 transcript.
3. PAD3 mRNA was moderately detectable in rat juvenile and human fetal sciatic nerve samples, in both with decreasing tendency in dependence on age and with only a marginal activation potential. Cultured Schwann cells expressed the PAD3 transcript only at moderate levels. Again, cultivated fibroblasts were (almost) free of the PAD3 transcript.
4. The PAD4 message was (hardly) detectable only in rat material, concrete in the juvenile, and slightly more in degenerating and regenerating adult sciatic nerves. Both types of cultures were free of PAD4 mRNA.

The translation of PAD transcripts into their respective proteins was demonstrated by Western blot analysis (Fig. 9.1b) and immunocytochemistry (Fig. 9.2A, B; Table 9.1).

1. After 10 days in vitro, rat and human spindle-shaped Schwann cells, being only S100-immunopositive, did not express any PAD proteins, not even after activation with KCl or LPS/IFNγ. A subpopulation of the S100-labeled Schwann cells, co-expressing MBP, however, revealed PAD2 immunostaining almost completely, and about half of them were also PAD3 positive. A further subpopulation of rat

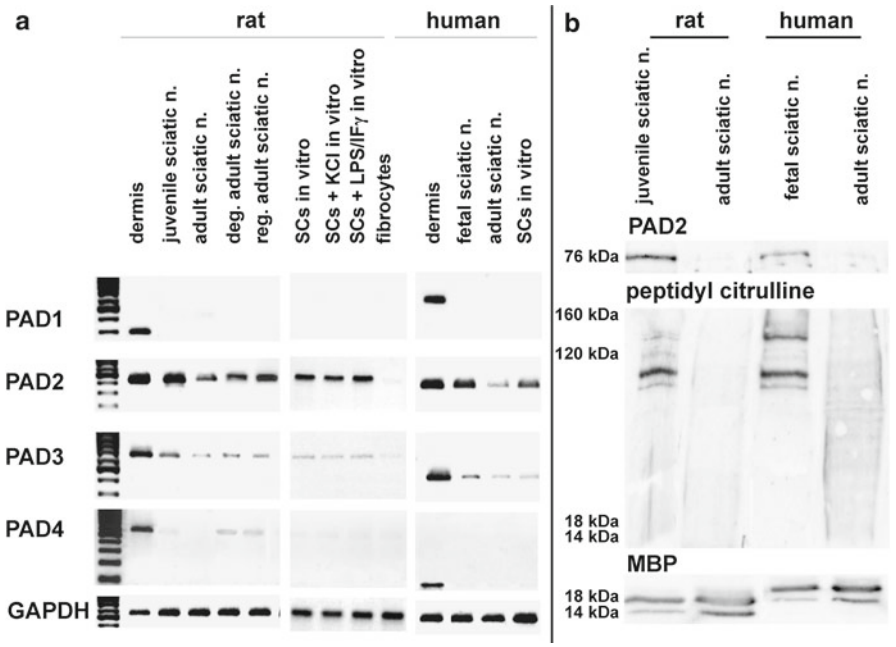

Fig. 9.1 (**a**) *RT-PCR*. Skin samples were used as positive controls for mRNA PAD1 expression, which was restricted to the dermis. PAD2 transcript was found in all specimens, excluding cultured fibrocytes, with the highest amounts in the PNS found in juvenile (postnatal day 3) rat and fetal (24 week of gestation) human sciatic nerves. PAD2 mRNA expression in untreated adult specimens was low but could be induced by regeneration and degeneration processes. PAD3 transcripts were seen in all studied specimens, although with different intensities. Traces of PAD4 transcript were demonstrable only in rat material, specifically in the juvenile and degenerated/regenerated adult sciatic nerve. GAPDH served as a positive control for mRNA input. (**b**) *Western blot analysis*. PAD2 immunosignal and multiple F95-immunoreactive bands, representing multiple citrullinated moieties, were demonstrated in both rat juvenile and human fetal sciatic nerves but not in their respective adult specimens. Modified from Keilhoff et al., Developmental Neurobiology, Volume 68, pp. 101–114, Copyright 2008, Wiley-Liss, Inc.

and human Schwann cells, immunopositive for $p75^{NTR}$ and CD104 and often also immunoreactive for NG2 and nestin, were also immunopositive for PAD2 (Fig. 9.2A(b,g)) and PAD3 (Fig. 9.2A(c,h)). PAD1 (Fig. 9.2A(a,f)) or PAD4 (Fig. 9.2A(d,i)) immunostaining was not detectable at all.

2. Fibroblasts, regardless of whether found in poor fibroblast cultures or as the remaining cells in Schwann cell cultures, were always free of any PAD immunosignal.
3. Adult rat sciatic nerves never demonstrated an immunolabelling with any of the PAD antibodies (Fig. 9.2B(b)). However, $p75^{NTR}$-positive structures in human fetal and rat juvenile sciatic nerves were immunostained for PAD2 (Fig. 9.2B(a)) and PAD3 (Fig. 9.2B(e)). In degenerating (Fig. 9.2B(g)) and regenerating (Fig. 9.2B(i)) adult rat nerves, PAD2 immunosignal was up-regulated in Schwann cells. The PAD1 and PAD4 signal was not detectable in any of the PNS cellular specimens.

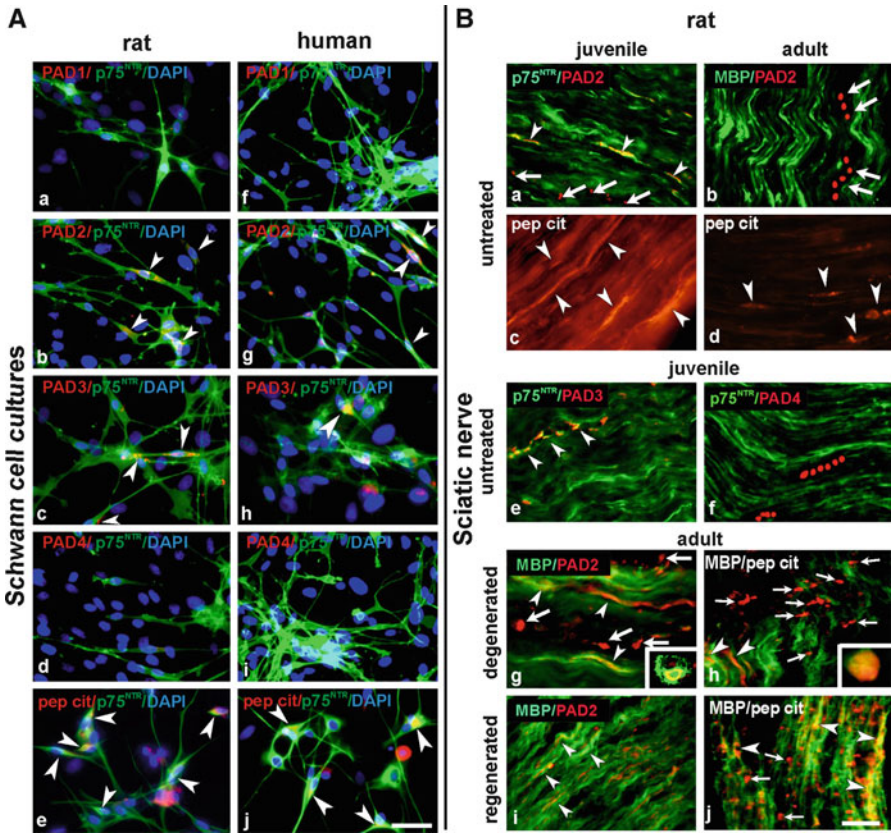

Fig. 9.2 (**A**) Rat and human Schwann cells, identified by $p75^{NTR}$ immunostaining (*green*) and co-stained for various PAD isozymes (*red*), were free of PAD1 (a,f) and PAD4 (d,i) immunofluorescence, whereas PAD2 (b,g) and PAD3 (c,h) immunosignals as well as peptidyl-citrulline staining (red, e,j) were found regularly (*arrowheads*). Cultures were used after 10 days in vitro. Nuclear counterstain is shown in *blue* (DAPI). Bar = 50 µm. (**B**) In samples of juvenile rat sciatic nerve (a), $p75^{NTR}$-positive structures were co-immunostained (*yellow*) for PAD2 (*arrowheads*). PAD2 immunostaining was also seen in white blood cells (*arrows*). In normal adult rat sciatic nerve (b), PAD2 immunosignal was restricted to white blood cells (*arrows*), compared to MBP (*green*). Respectively, a clear peptidyl-citrulline immunosignal with F95 (*red*) was found in juvenile specimens (c, *arrowheads*), whereas citrullinated proteins were rare in untreated adult specimens (d, *arrowheads*). Juvenile rat sciatic nerves also contained $p75^{NTR}$-positive structures co-expressing PAD3 (e, *arrowheads*), whereas PAD4 staining was found only in white blood cells (f). In degenerating (g, 5 days after reconstruction) and regenerating (i, 3 weeks after reconstruction) adult rat sciatic nerves, PAD2 immunosignal (*red*) marks MBP-positive (*green*) structures (*arrowheads*) and macrophages (*arrows*). Respectively, in both degenerating (h) and regenerating (j) adult nerve fascicles, peptidyl citrulline (*red*) immunosignal co-stained (*yellow*) MBP-immunopositive (*green*) structures (*arrowheads*) and macrophages (*arrows*). Insert g: PAD2/ED1 double-labeled macrophages; Insert (e): peptidyl citrulline/ED1 double-labeled macrophages. Bar = 350 µm. Modified from Keilhoff et al., Developmental Neurobiology, Volume 68, pp. 101–114, Copyright 2008, Wiley-Liss, Inc.

Table 9.1 Strength of immunoreaction of different antigens in respective PNS specimens

Protein		PAD1	PAD2	PAD3	PAD4	nNOS	eNOS	iNOS	Pep-cit
Rat	Dermis	+++	+++	+++	+++	nd	nd	nd	++
	Juvenile nerve	–	++	+/++	+	nd	nd	nd	+++
	Adult nerve	–	+	–	–	nd	nd	nd	+
	Adult nerve deg	–	++(+)	–	++	nd	nd	nd	+(+)
	Adult nerve reg	–	++(+)	–	++	nd	nd	nd	+++
	Cerebellum cultures	–	+++	+	–	++	–	+	+++
	SZ cultures	–	++	+	–	+	–	(+)	+
	SZ cultures*	–	++	+	–	++	–	++	+
	DRG neurons	–	+	++	(+)	+	–	–	+
	DRG neurons**	–	+(+)	++	(+)	++	–	–	+
	DRG satellite cells	–	+	+	(+)	–	–	+	–
	DRG satellite cells**	–	+	+	(+)	–	–	+	–
Human	Dermis	+++	+++	+++	++	nd	nd	nd	++
	Fetal nerve	–	++	+	+	nd	nd	nd	+++
	Adult nerve	–	+	–	–	nd	nd	nd	+
	SZ cultures	–	++	+	–	+	–	(+)	+

nd, not determined; –, no immunoreactivity; (+), immunoreactivity only sporadically expressed in the respective cell type; +, immunoreactivity expressed in the respective cell type; ++, immunoreactivity increasingly expressed in the respective cell type; +++, immunoreactivity strongly expressed in the respective cell type; SZ cultures*, LPS or K^+ activated; **, DRG 4 days after sciatic nerve transection; nerve deg, degenerating nerve; nerve reg, regenerating nerve; DRG, dorsal root ganglia. Modified from Keilhoff et al., Developmental Neurobiology, Volume 68, pp. 101–114, Copyright 2008, Wiley-Liss, Inc.

4. Western blot analysis revealed a PAD2-positive band in human fetal and rat juvenile sciatic nerve but not in adults (Fig. 9.1B), suggesting that deimination is more active during PNS development.

Concerning differential PAD expression in sensory neurons and satellite cells of the dorsal root ganglia (DRG), there are no literature findings, although one report showed no obvious immunohistochemical staining for citrullinated proteins within rat DRG cells (Nicholas et al. 2003). Thus, we have to draw on our own previously unpublished preliminary data on adult rat DRG (Fig. 9.3, Table 9.1). Using the previously mentioned antibodies, we were able to demonstrate the following:

1. PAD2 was clearly expressed in some of the NeuN-positive sensory neurons. The staining was localized in the cytoplasm as well as in the nucleus. PAD3 was

Fig. 9.3 (continued) (**b**, *hollow arrows*) as well. In satellite cells, the subcellular distribution was unclear (*hollow arrowheads* in **f**). PAD3 (**c**, **g**) immunostaining was found almost exclusively in the nucleus of neurons, while only very few nuclei of satellite cells could be identified as being PAD3 positive (**g**, *arrowhead*). PAD4 (**d**, **h**) was seen only rarely in the cytoplasm of neurons (**h**, *hollow arrow*) and occasionally co-localized with GFAP (**h**, *arrows*). (**i**) Peptidyl-citrulline immunolabelling (*red*) was occasionally found in the cytoplasm of neurons (*arrows*), probably in the cytoplasm of satellite cells (*hollow arrowheads*) and in the nuclei of some neurons (*asterisks*)

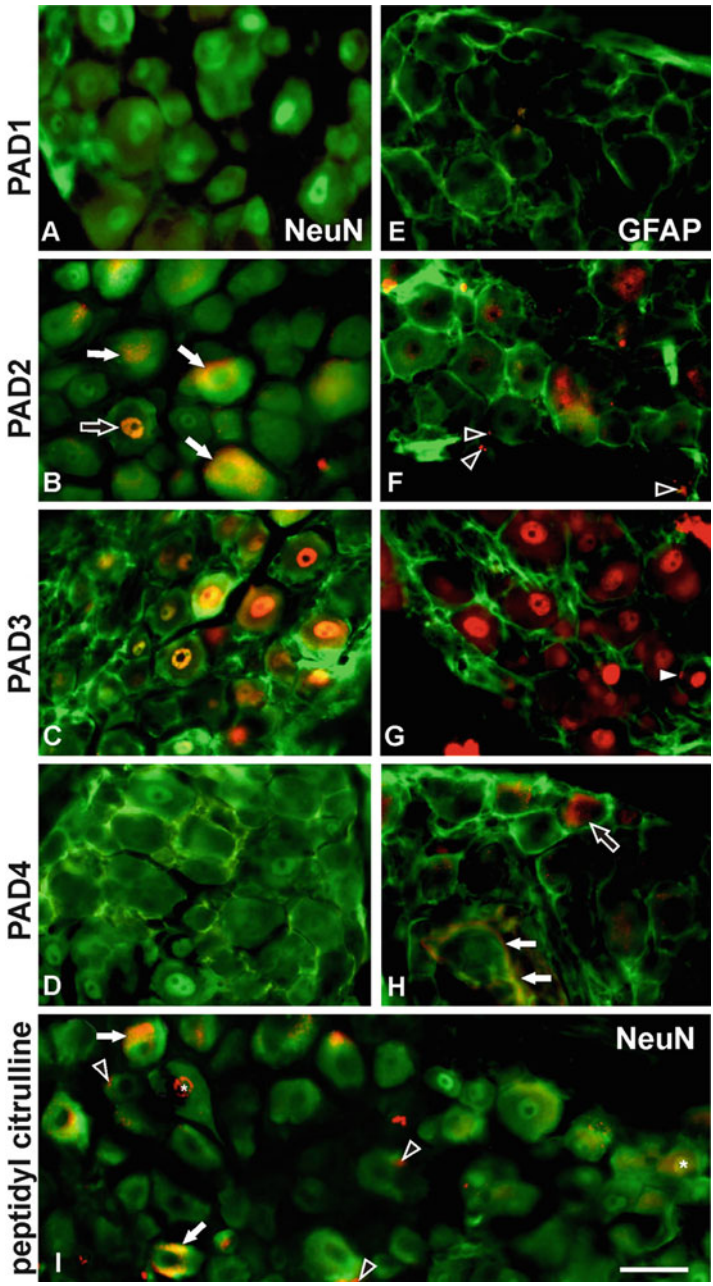

Fig. 9.3 Immunohistochemical evaluation of dorsal root ganglia (DRG) cells (*green*) included neurons identified by NeuN immunolabelling (**a**–**d**, **i**) and satellite cells by GFAP immunostaining (**e**–**h**), compared to immunostaining of various PAD isozymes (**a**–**h**, *red*) and peptidyl-citrulline (**i**, *red*). In these two cell types, PAD1 (**a**, **e**) co-staining was not evident. PAD2 (**b**, **f**) co-staining was evident in both cell types. In neurons, it was detected in the cytoplasm (**b**, *arrows*) and the nucleus

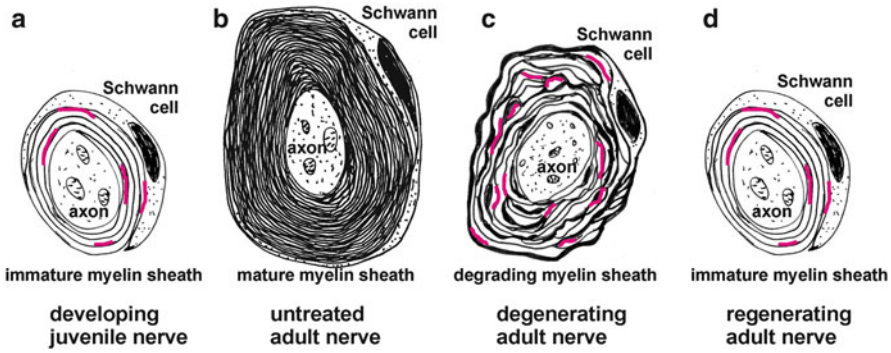

Fig. 9.4 Differential distribution of deiminated proteins in peripheral nerves. In developing juvenile nerves (**a**), the immature myelin sheath is packed regularly, but loosely, and contains citrullinated proteins (indicated in *pink*), including MBP. (**b**) Healthy adult myelin is packed regularly and tightly and does not contain citrullinated proteins. (**c**) After an insult, myelin sheaths lose their compactness, showing irregular profiles, myelin ovoids, and debris. Thus, phagocytosis of debris is achievable. This process could be induced, among others, by PAD activation and subsequent citrullination of respective proteins, including MBP. (**d**) In contrast, a regenerating nerve is comparable to a developing one. Again the myelin sheath is regularly but loosely packed. With progressing regeneration and remyelination, citrullination decreases and myelin compacts normally (or near normally) once again

clearly expressed in the nuclei of nearly all sensory neurons. PAD4 was seen only very rarely and then in the neuronal cytoplasm. PAD1 was not expressed in DRG neurons.

2. In the satellite cells, PAD1 staining was undetectable. PAD2 was seen in GFAP-positive ensheathing cells but only rarely, whereby the subcellular allocation is still uncertain. PAD3 was seen, also rarely, but clearly in the nuclei of ensheathing cells. PAD4 was inconstantly detectable by co-staining with GFAP.
3. Sciatic nerve transection seemed to result in a moderate PAD2 induction in neurons. The other PAD isoforms were unchanged.

9.4 Distribution of Peptidyl-Citrulline in the PNS

To detect peptidyl-citrulline moieties, the F95 antibody (Nicholas and Whitaker 2002) was used (Figs. 9.1b and 9.2A,B; Table 9.1).

1. In juvenile rat and human fetal sciatic nerve samples, the F95 antibody detected multiple deiminated proteins with a most prominent band at 120 kDa. This band appeared to have a similar molecular weight as alpha-dystroglycan. An additional band at about 105 kDa coincided with immunoreactive neuronal adhesion molecule L1, whereas the 160 kDa band appeared to have a similar molecular weight as neurofilament 160. However, peptidyl-citrulline signals at the levels of the main MBP bands (14 and 18 kDa) were not detectable (Fig. 9.1b).

2. Whereas immunostaining of uninjured adult sciatic nerves of either species did not show much F95-labelling (Fig. 9.2B(d)), juvenile (Fig. 9.2B(c)), degenerating (Fig. 9.2B(h)), and, even more, regenerating (Fig. 9.2B(j)) adult rat sciatic nerves were characterized by an induction of deimination. Here, the peptidyl-citrulline signal may depict newly formed myelin, whereas, in the degenerating specimens, myelin debris would most likely be the main source of this immunosignal.
3. In rat and human Schwann cell cultures, peptidyl-citrulline staining was demonstrable in about 30 % of $p75^{NTR}$-expressing Schwann cells (Fig. 9.2A(e,j)). The peptidyl-citrulline staining remained unchanged when the cultures were stimulated by LPS/IFNγ or KCl.
4. Cultured fibrocytes did not have any detectable peptidyl-citrulline immunostaining.
5. Unlike a limited previous report in rat DRG (Nicholas et al. 2003), peptidyl-citrulline staining was demonstrated in about 35 % of NeuN-expressing sensory neurons. There were no significant differences between pre- and post-injured DRG (Fig. 9.3I, Table 9.1).

9.5 Citrulline Immunohistochemistry and the Pitfall of NOS

To exclude false-positive free citrulline immunostaining potentially produced by NOS, the expression patterns of the NOS isoforms were detected in parallel with the previously described experiments (Table 9.1).

1. Schwann cells immunopositive for nNOS could be found regularly under control conditions as well as after LPS/IFNγ or KCl stimulation in vitro. Under control conditions, Schwann cells immunopositive for iNOS were seen only rarely, if at all, whereas LPS/IFNγ or KCl was able to stimulate their iNOS expression significantly. Schwann cells immunopositive for eNOS were missing in untreated as well as in stimulated cultures.
2. These results were confirmed by RT-PCR analysis. Untreated cultivated Schwann cells expressed the mRNA transcript of nNOS only. The expression of nNOS and iNOS, but not eNOS, mRNA could be stimulated by LPS/IFNγ and KCl.
3. Cultured fibrocytes were free of any NOS immunoreactivity, and their RT-PCR did not yield any clear bands.
4. In DRG, all three NOS isoforms were found by RT-PCR as well as by immunohistochemistry. In uninjured rats, nNOS expression was restricted to a subpopulation of small- and medium-sized neurons. iNOS was, if at all, slightly expressed by satellite cells and macrophages/microglia, and eNOS was found exclusively in lining of the vessels. Sciatic nerve transection induced the expression of nNOS in neurons markedly; eNOS appeared to be unaffected, whereas iNOS was marginally up-regulated in macrophages/microglia.
5. In untreated sciatic nerves, NOS immunostaining was not a prominent feature. Sciatic nerve transection induced the expression of iNOS in Schwann cells and

resident and invading macrophages significantly. When regenerating, an enhanced nNOS immunosignal was found in these nerves, probably generated by Schwann cells and outsprouting nerve fibers.

If detected by the F95 antibody, citrulline immunosignal would be generated also by NOS, and its activation should be able to enhance the immunostaining. That was not the case, further validating the specificity of the F95 antibody against protein-bound citrulline (see Chap. 14), which depends only on PAD activity and was not inducible by LPS/IFNγ or KCl.

9.6 Possible Importance of Protein Deimination in the PNS

Human and rat Schwann cells express PAD2 and PAD3 mRNA and protein, with the highest amounts seen in immature cell stages. Moreover, as a result of degeneration and regeneration, an induction of PAD2 mRNA and protein takes place, and, subsequently, citrullination was demonstrated in the PNS, at least in developmental stages and during repair.

These findings coincide well with the concept that protein citrullination plays a physiological role in plasticity of the nervous system. In the CNS, it is well known that the degree of MBP deimination (citrullination) is high in early development (Moscarello et al. 1994). Even if MBP is primarily a component of CNS myelin and since we were unable to demonstrate MBP deimination in juvenile/fetal or adult sciatic nerves, a developmental aspect of citrullination in the PNS should not be excluded. Thus, the citrullinated protein in both rat juvenile and human fetal sciatic nerve specimens at 120 kDa may be alpha-dystroglycan. Expressed in Schwann cells, this protein is crucial for myelination and nodal architecture. Mice with a dystroglycan-defective Schwann cells are characterized by abnormal sorting of axons, disrupted laminin binding, dysmyelination or demyelination, axonal loss, and aberrant nerve conduction (Saito et al. 2003). Furthermore, the neuronal adhesion molecule L1, being a candidate for the deiminated protein at about 105 kDa, plays a role in developmental neurite outgrowth and pruning (Skaper et al. 2001). Future of these suspected deiminated PNS proteins would need to be verified in future experiments utilizing extensive proteomic analysis.

Regardless, the present results also demonstrate a clearly evident involvement of PAD enzymes in PNS degeneration and regeneration processes. Immediately after a peripheral nerve injury, Wallerian degeneration ensues. This process involves degradation and removal of axons and myelin from the distal segment of the injured nerve and is initiated by activated/reprogrammed Schwann cells. A decompaction of myelin lamellae is required to enable its attack by Schwann cells, and later on by microglia and macrophages, and to facilitate access of proteases to myelin proteins (Stoll and Muller 1999). Impaired Wallerian degeneration, on the other hand, leads to a hampered entry and growth of new axons into Bands of Büngner with the consequence of impaired peripheral nerve regeneration. From the CNS it is known that

citrullinated myelin is disorganized and less compacted. All three protein candidates mentioned above for citrullination in the PNS are involved in developmental neuritogenesis, including myelination. Moreover, the structural changes of citrullinated myelin resulted in a changed modifiability by other agents, itself being essential for correct myelination (Kim et al. 1997; Pritzker et al. 2000a, b; Wang et al. 2004). Thereby, not only methylation of the MBP itself is essential. Epigenetic control of histones has been shown to be crucial for Schwann cell differentiation (Heinen et al. 2012). Suppression of histone methyltransferase reduced the length of Schwann cell processes. These morphological changes were accompanied by downregulation of myelin genes and a hampered myelination capacity in vitro. Histones, on the other hand, are among the preferred substrates of PAD enzymes, including PAD2 (Zhang et al. 2012), which we have found in the cells of the PNS. Thus, citrullination appears to be involved in PNS development, degeneration, and regeneration. The role of deimination in epigenetic mechanisms is discussed further in Chap. 18.

9.7 Why Deimination in the PNS Leads a Wallflower Existence?

Nevertheless, deimination in the PNS is not presently a "hot topic" in the scientific literature, but why? One of the most decisive reasons is certainly the fact that multiple sclerosis (MS), a disease of the CNS having a prevalence between 2 and 150 per 100,000 (Rosati 2001), is clearly related to MBP deimination and is thus being intensively explored. In the existing mass of these MS studies, PNS myelin was occasionally examined as a negative control or not at all.

Secondly, the suggested involvement of autoimmunity to peripheral myelin proteins, especially MBP, in the pathogenesis of Guillain–Barre' syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) could not be clearly proven so far (Csurhes et al. 2005).

Both facts are related to the important qualitative difference in composition of CNS and PNS myelin. Although MBP, the positively charged deiminase substrate, is an extrinsic membrane protein of both CNS and PNS myelin (Lees et al. 1984; Lemke 1988), their quantities are significantly different. In the CNS, about 30–40 % of the total myelin is composed of MBP, where it is essential to retain the cytoplasmic leaflets in the myelin sheet closely together, thus forming the major dense line. In the PNS, only 5–15 % of myelin is composed of MBP, and it seems that it does not play a major role in myelin compaction in this location. Supporting this idea is that a lack of MBP did not result in a defective major dense line of PNS myelin in the MBP knockout shiverer mouse (Kirschner and Ganser 1980). Instead, there is evidence that the type 1 transmembrane P0 glycoprotein, accounting for more than 50 % of the PNS myelin (Quarles 2002), contributes much more than MBP to the formation of the major dense line in PNS myelin. In fact, P0 seems to be important

for the initial/early phase of major dense line formation and MBP for their stabilization and maintenance. Correspondingly, the expression of MBP occurs significantly later than that of P0 in the PNS (Martini et al. 1995). Finally, in an emergency such as the failure of MBP, P0 alone is able to realize myelin compaction (Martini et al. 1995). Collectively, these findings suggest that P0 glycoprotein should be able to compensate for even a massive loss of MBP functionality, as resulted from the deimination of all of its arginines.

Next, protein deiminases, recently discussed as "new players in the developmentally regulated loss of neuronal regenerative ability" (Lange et al. 2011), seemed not to be of high interest for the PNS scientific community. Perhaps this is due to the attitude that, unlike the spinal cord, the regenerative capacity of the mammalian PNS is preserved over the life-span. The question whether the developmentally regulated loss of neuronal regenerative ability in the CNS is more a cause for or yet rather an effect of PAD activity remains, however, unanswered. The same applies to the PNS. Is the preserved regenerative capacity (of course only partly, if at all) a result of lower PAD expression, or does the regenerative apparatus depress PAD expression? Interestingly, all known PAD sequences identified in piscine, amphibian, and avian species are closely related to PAD2, being the isotype with the broadest tissue distribution, including the PNS, in mammalian species (Vossenaar et al. 2003; Rebl et al. 2010). As the piscine and amphibian CNS have, however, a respectable regenerative capacity, a causative nexus between PAD expression and regenerative capacity must be discussed with caution.

9.8 What the Future Should/Could Bring in Regard to PNS Deimination?

Based on the fact that the posttranslational modification by deimination/citrullination is involved in basic cellular processes like gene regulation, cell development, and differentiation as well as in pathophysiological processes of severe diseases of different human organ systems (see Mechin et al. (2011) for review), it seems incredible that the PNS is excluded from this discussion. This is even more unbelievable since PAD2 and PAD3 were clearly expressed in Schwann cells and DRG neurons. From this location, they could be involved in developmental and regenerative/degenerative processes, as mentioned in Chap. 15 (Lange et al. 2011). The outcome of peripheral nerve regeneration is often far from satisfying. Schwann cells are main and global players in this process (Ide 1996; Allodi et al. 2012; Bosse 2012). They start the obligatory Wallerian degeneration with degradation and phagocytosis of the myelin sheath. They generate Bünger Bands, the pathfinding structures for the outsprouting nerve. They produce a regeneration-supporting microenvironment producing growth factors and cytokines and assist the reconstruction of the motor endplate (Sugiura and Lin 2011). Thus, there arise enough opportunities for regulatory interventions by PAD enzymes, still to be elucidated.

Strongly linked to the processes of degeneration/regeneration are neuropathies, which may also be distinct major processes for PAD therapeutic intervention. There are many factors that can cause the more than 100 types of peripheral neuropathies, each having its own characteristic set of symptoms, patterns of development, and prognoses. Some neuropathies are caused by inflammation resulting from immune system activities. They include the acute inflammatory demyelinating neuropathy (GBS) as well as the CIDP. For the inflamed CNS, it was shown that PAD2 and PAD4 enzymes were markedly up-regulated and that once inflammation is established, citrullination of target autoantigens can allow an expanded repertoire of T cells to contribute to pathology (Carrillo-Vico et al. 2010). Certainly these same processes may also be relevant in the PNS.

From neuropathies, there is a direct link to the large problem of neuropathic pain, bringing the PAD-expressing DRG neurons and satellite cells up for discussion once again, if currently even hypothetically. Thus, there is evidence that augmented Gap junction-mediated coupling of satellite cells contributes to the lowering of pain threshold in mice (Warwick and Hanani 2013). The epigenetic regulation of Connexins, the Gap junction proteins, however, has been considered (Oyamada et al. 2013), and rapid alterations in satellite cell GFAP (PAD substrate) expression seem to be in close temporal association with neuronal hyperexcitability and neuropathic pain (Zhang et al. 2009). Additionally, a functional correlation between deimination and deacetylation of histones (PAD substrate) was demonstrated (Denis et al. 2009). Inhibition of histone deacetylation, on the other hand, causes analgesia (Chiechio et al. 2009).

Last, but possibly not least, there is increasing evidence that PAD4 and possibly PAD2 have potential roles in tumor progression and tumor-associated inflammation (see Chap. 17 for more details). When treated with PAD inhibitors, cell lines and mice tumor growth were significantly suppressed and citrullination of histones again seemed to play a key role. Thus, PAD enzymes regulate transcriptional activity and modulate the inflammatory microenvironment via cytokine citrullination (Mohanan et al. 2012). In fact, activation of the growth factors EGFR, FGFR (Blair et al. 2011), or BDNF (Kramer et al. 2010) may promote proliferation and invasive behavior of Schwannomas.

Taken together, we propose growing interest in deimination in the PNS, offering widespread evidence for deimination in developmental, degenerative, regenerative, and inflammatory processes, which may be a basis for future clinical therapies as well.

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Chapter 10
Deimination in Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

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Keywords Multiple sclerosis • Experimental autoimmune encephalomyelitis • Myelin basic protein • Glial fibrillary acidic protein

10.1 Introduction

Multiple sclerosis (MS) is a chronic immune-mediated neurodegenerative disease of the central nervous system (CNS) affecting approximately 0.1 % of Caucasians of north and central European ancestry (Sadovnick et al. 1996). Most patients present initially with an inflammatory relapsing-remitting disease course (RRMS), which accounts for approximately 90 % of all cases, followed by a progressive disease course, secondary progressive MS (SPMS), where there is a gradual increase in disability (Lublin and Reingold 1996). Approximately 65 % of all patients with RRMS go on to develop SPMS. This progression from RRMS to SPMS takes an average of 19 years from the initial onset of the disease (Rovaris et al. 2006). Clinically, symptoms in people with MS include visual disturbance, sensory and motor impairment, balance disturbance, sphincter dysfunction, cognitive impairment, pain, and fatigue, which vary depending where in the CNS these lesions develop (Compston and Coles 2008). For more details, see Chap. 11.

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The pathological hallmark of MS is focal areas of demyelination in the CNS white matter, initially, mediated predominantly by T cells and to lesser extent B cells, in which myelin, oligodendrocytes, and axons are destroyed (Compston and Coles 2008; Zuvich et al. 2009; Trapp and Nave 2008). In active lesions, a disturbance of the blood–brain barrier, and release of pro-inflammatory cytokines and chemokines promotes the inflammatory response. Chemokines are a family of secreted proteins that activate and attract leukocytes to sites of inflammation and have been demonstrated to play a role in the recruitment of leukocytes across the blood–brain barrier in MS (Cannella and Raine 1995; Huang et al. 2000; Rot and von Andrian 2004; Charo and Ransohoff 2006). Limited remyelination can occur, which helps to restore conduction in some of the affected axons, resulting in a degree of functional recovery (Trapp and Nave 2008). In this phase, a proportion of preexisting lesions show gradual expansion at their margins, characterized by the infiltration of inflammatory cells, mainly consisting of T cells and activated microglia, referred to as chronic active plaques or lesions (Prineas et al. 2001; Lassmann et al. 2007). The normal-appearing white matter (NAWM) outside of these lesions shows evidence of diffuse inflammation with microglial activation but no obvious pathology.

10.2 Antibodies and Multiple Sclerosis

The presence of oligoclonal immunoglobulin in cerebrospinal fluid (CSF) of patients with MS is one of the important diagnostic indicators of this condition (Berger and Reindl 2007). Oligoclonal bands (OCBs) of immunoglobulins are observed in up to 90 % of patients following isoelectric focusing of CSF; however, these bands are also observed in other neurological conditions and in multiple myeloma patients (Compston and Coles 2002; Bourahoui et al. 2004; Mayringer et al. 2005; Fryden et al 1978; Kostulas et al. 1987; Pranzatelli et al. 2011; Zent et al. 1998; Hovenga et al. 2000; de Larrea et al. 2009). The finding of OCBs in CSF remains the most reliable immunological test used to support the clinical and MRI features in establishing the diagnosis of MS (Weber and Hemmer 2010).

However, the target antigens of these immunoglobulins have not yet been fully characterized. Of the candidate autoantigens, myelin basic protein (MBP) is the most extensively studied. MBP is one of the principal components of the myelin sheath and is the second most abundant protein in the CNS (Boggs 2006). Another candidate autoantigen is myelin oligodendrocyte glycoprotein (MOG), which is present on the outer layer of the myelin sheath and is thus more accessible to antibody-mediated attack (Brunner et al. 1989). MS patient antibodies to MOG and MBP have been identified through incubating three MS postmortem brain tissue sections with gold-conjugated MOG and MBP peptides, where reactivity was observed in actively demyelinating lesions and no reactivity was observed in the NAWM of these patients (Genain et al. 1999). Both MBP and MOG can act as autoantigens to trigger a rodent model of MS, experimental autoimmune

encephalomyelitis (EAE) (Gold et al. 2006). Increased antibody titers against MBP and/or MOG have also been reported in serum and CSF of patients with MS (Reindl et al. 1999; Schmidt et al. 2001; Antel and Bar-Or 2006). However, most antibodies detected in the CSF of MS patients are also found in other neurological and systemic conditions and to a lesser extent in healthy controls. Therefore, it is controversial as to whether antibodies to myelin proteins are greater in MS patients than in normal controls.

In the CNS of MS patients, changes in CNS protein structures via deimination may play an important role in disease pathogenesis, perhaps through the generation of novel epitopes to which the immune system is intolerant. Deimination or citrullination is a process whereby the guanidino group of arginine is hydrolyzed to a ureido group and ammonia, producing a deiminated amino acid, citrulline. This results in the loss of a positive charge and an altered secondary and tertiary structure of the protein (Harauz and Musse 2007; Musse et al. 2006). This posttranslational modification is carried out by a family of five citrullinating enzymes known as the peptidylarginine deiminases (PADs) (Rogers et al. 1977; Vossenaar et al. 2003; Chavanas et al. 2004). PAD2 as well as PAD4 are present in synovium and brain tissue and are both upregulated in the CNS in EAE (Musse et al. 2006; Harauz and Musse 2007). A number of PAD substrates which may be of importance in MS have been identified including MBP, glial fibrillary acidic protein (GFAP), nuclear proteins, and most recently members of the CXC chemokine family (Vossenaar et al. 2003; Hagiwara et al. 2002; Nicholas et al. 2004; Proost et al. 2008; Loos et al. 2008; Struyf et al. 2009; Mortier et al. 2010).

10.3 Peptidylarginine Deiminases and Citrullination of MBP in MS

MBP, in addition to other proteins in the CNS, undergoes numerous posttranslational modifications, the most extensive of which is citrullination (Finch et al. 1971; Wood and Moscarello 1989; Moscarello et al. 2007). Excess citrullination has been reported in the CNS in postmortem MS brain tissue (Mastronardi et al. 2006; Nicholas and Whitaker 2002; Raijmakers et al. 2005). MBP isolated from the CNS of MS patients was found to be more highly citrullinated than MBP isolated from CNS tissue of adults without neurological disease and was similar in the extent of citrullination as that observed in children aged under 4 years, indicative of a role of deimination in the development of mature myelin (Moscarello et al. 1994; Wood et al. 1996). By using MBP isolated from postmortem NAWM of patients with MS and controls and fractionation of the MBP by column chromatography, Moscarello et al. found that 18 % of MBP was citrullinated in control tissue compared to 45 % of MBP in patients with MS (Moscarello et al. 1994). Further studies by the same group found that in Marburg's disease, an acute, fulminate, and rare form of MS, as much as 90 % of MBP is deiminated, showing that in this more severe form of the disease, almost all of the MBP is citrullinated (Wood et al. 1996). Specifically, this

study found that citrullinated MBP, in both control and patients with chronic MS, contained 6 citrulline residues and 13 arginine residues, whereas citrullinated MBP in patients with Marburg disease contained 18 citrulline residues and only 1 arginine residue.

PADs 2 and 4 are the most common PADs found in the brain. In support of the increased citrullination of MBP described above, levels of both PAD2 and PAD4 in myelin isolated from NAWM of seven patients with MS were found to be increased significantly compared to white matter from six control subjects using immunoslot blots with antibodies specific to PAD2 and PAD4 (Wood et al. 2008). The same method was also applied using the F95 antibody specific for citrullinated proteins, which also showed increased citrulline in the myelin isolated from NAWM of these patients compared to control tissue (Nicholas et al. 2004). By using mass spectrometric analysis of intact human MBP and its citrullinated forms, it has recently been shown that PAD2 can citrullinate 18 out of 19 arginine residues whereas PAD4 can only citrullinate 15 out of the 19 arginine residues present in MBP (Wood et al. 2008). Using proton MR spectroscopy, Oguz et al. found that citrulline peaks occurred more frequently in 27 patients with early-onset MS, compared to 23 control cases (Oguz et al. 2009). This further suggests a role of increased citrullination of myelin proteins in the pathophysiology of MS and possibly other demyelinating diseases.

PAD2 in MS NAWM has been shown to be threefold higher than in control subjects, accompanied by a twofold increase in deimination compared to control subjects (Mastronardi et al. 2007). NAWM from 12 MS patients and white matter from 3 controls were assessed by immunoslot blots and measurement of PAD2 levels using anti-PAD2 antibody. Further western blot analysis confirmed that this increase was consistent with increased citrullination of MBP.

10.3.1 Proteolytic Enzyme Cleavage of Citrullinated MBP

Deimination of MBP increases its susceptibility to degradation by proteinases, which are reported to be elevated in the CNS of MS patients, in particular around active plaques and in the CSF (Cuzner and Davison 1973; Einstein et al. 1972; Richards and Cuzner 1978). Macrophages and reactive astrocytes have been previously shown to produce the proteinase cathepsin D (Allen and McKeown 1979; Prineas and Wright 1978). Studies carried out by Cao et al. showed that MBP-C6, the citrullinated isomer of MBP which contains six citrulline residues, is digested 4 times faster by cathepsin D than the unmodified form of MBP and 35 times faster in MBP-C18, which contains 18 citrulline residues and is found in the Marburg type of MS, releasing numerous peptides through cleavage at the Phe–Phe linkages in the MBP protein (Cao et al. 1999). The peptides generated by cathepsin D cleavage, which contain the immunodominant epitopes of MBP, have been detected in the CSF of patients with MS (Whitaker 1977; Whitaker and Granum 1980). By incubating various MBP species, containing different amounts of citrulline per mole of

MBP, with cathepsin D, Pritzker and colleagues were able to show that the MBP species containing the greatest amount of citrulline per mole of MBP were digested at a much faster rate, as determined by mass spectrometric analysis of MBP peptides following digestion (Pritzker et al. 2000). In addition, using a three-dimensional atomic structure model of human MBP, Pritzker et al. were able to show that this molecule became significantly more extended and open in structure, the more citrullinated it was (Pritzker et al. 2000). Following this study, the authors concluded that this more open structure would allow cathepsin D better access to Phe–Phe linkages in MBP, which would account for the increased digestion of citrullinated MBP.

Using Cys scanning, spin labeling, EPR spectroscopy, and site-specific proteolysis, Musse et al. were also able to show that, in the membrane-bound state, the primary immunodominant epitope, V83-Y92, of recombinant murine MBP-C6 is more highly surface exposed than unmodified recombinant murine MBP (Musse et al. 2006). In addition, in the presence of cathepsin D, recombinant murine MBP-C6 showed enhanced proteolysis. These results suggest that citrullination of MBP not only impedes membrane adhesion and assembly activity of this protein but also exposes an immunodominant epitope in the membrane-bound protein to proteases. From this study, the authors concluded that greater surface exposure and greater cleavage of the citrullinated protein by enzymes would lead to increased release of the immunodominant epitope, which could then prime the innate immune-derived cells of the CNS and sensitize peripheral T cells (Musse et al. 2006; Musse and Harauz 2007).

D'Souza et al., using western blot analysis at 0, 1, 4, and 24 h, showed that stromleysin-1-digested MBP-C6 isolated from MS NAWM at a faster rate compared with unmodified MBP isolated from MS NAWM. In addition, the unmodified MBP from MS patients was shown to be digested more rapidly in the presence of stromelysin-1 than unmodified MBP from control white matter. Using a monoclonal anti-MBP antibody, western blots showed the release of one peptide, resulting from the digestion, reacting very strongly with the antibody, which recognizes residues 84–89, the major immunodominant MBP epitope. Furthermore, this same peptide containing the immunodominant epitope was released following incubation of myelin from both normal white matter and MS NAWM with stromelysin-1, with this peptide being released at a greater extent from MS myelin compared with normal myelin. From this study, the authors proposed that this increased susceptibility of MBP-C6 to stromelysin-1 cleavage in MS is due to a change in conformation, and hence its localization in the lipid bilayer, as citrullinated MBP, has been shown to be more highly surface exposed than the unmodified form, therefore increasing its susceptibility to proteolytic cleavage and contributing to myelin breakdown in MS (Musse et al. 2006; Musse and Harauz 2007; D'Souza and Moscarello 2006). In addition, increased MBP-C6 is reported to destabilize the myelin sheath, which may allow enhanced digestion of MBP through greater accessibility of the enzyme stromelysin and other proteinases. In support of this, an earlier study found that the Marburg form of MBP (MBP-C18) caused fragmentation of lipid vesicles, also indicating that deimination destabilizes myelin (Boggs et al. 1999).

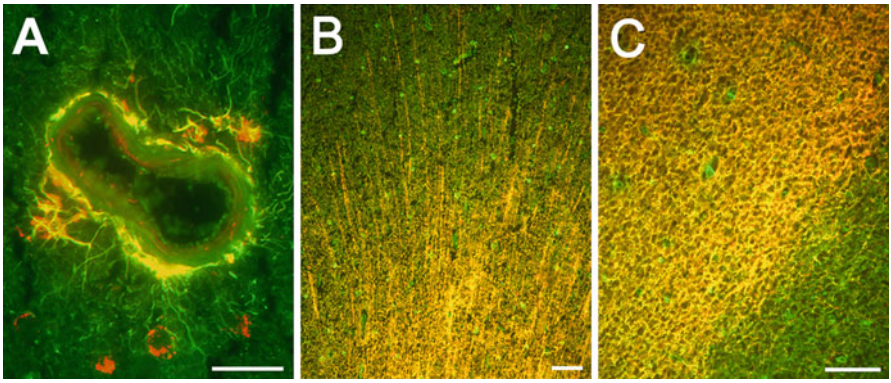

Fig. 10.1 Using dual-color immunofluorescence examination of chronic SPMS brains, co-localization (*yellow*) of GFAP (*green*) and deiminated proteins identified by the F95 antibody (*red*) was often seen (**a**) in astrocytes encircling a central blood vessel within the cores of "burned-out" plaques, suggesting an important role for deiminated proteins in distal astrocyte filaments and footpads. In NAWM from these patients (**b**), radiations of GFAP/F95-positive immunoreactive processes extended from the *white* matter (*bottom*) into the *gray* matter (*top*). However, the most intense GFAP/F95 co-localization was observed (**c**) in SPMS white matter (*upper left*) at the interface adjacent to old plaques (*lower right*), suggesting that deiminated GFAP in this location may have served to help wall off inflammatory lesions in an earlier, more active phase of this disease. Scale bars = 50 μm. Modified from Nicholas et al., Journal of Comparative Neurology, Volume 473, pp. 128–136, Copyright 2004, Wiley-Liss, Inc.

10.3.2 Citrullination of GFAP in MS

More recently, we have shown that GFAP, in addition to MBP, is citrullinated in MS. GFAP was deiminated in higher amounts, in both the NAWM and lesions of patients with SPMS, compared with the white matter of control brain tissue (Nicholas et al. 2004). Using antibodies against citrullinated proteins and GFAP, together with the aid of confocal microscopy, the NAWM of patients with SPMS was highly deiminated, compared to white matter taken from control patients (Nicholas et al. 2004), and this citrullination also co-localized with GFAP in astrocytes surrounding central blood vessels within old "burned-out" plaques (Fig. 10.1).

In an expanded study with increased sample numbers and including active MS lesions, we have also recently shown extensive deimination of GFAP in the lesions of patients with progressive MS (Fig. 10.2). Using dual staining with antibodies against citrullinated proteins and GFAP, we found that lesions taken from nine patients with MS were also highly citrullinated, compared to white matter taken from six control individuals with no history of neurological disease (manuscript submitted). Through western blotting and using the same antibodies against citrullinated proteins and GFAP together, we were able to confirm that the majority of this deimination occurred in GFAP (Fig. 10.3).

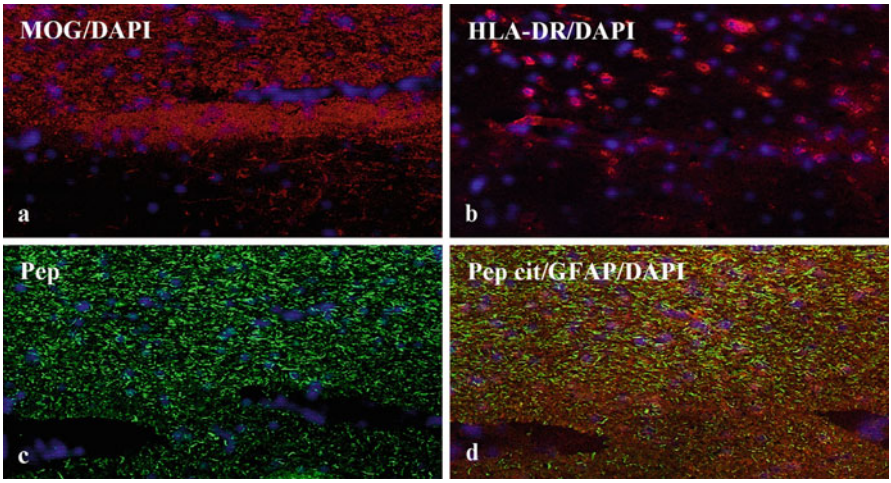

Fig. 10.2 Citrullinated protein expression (pep cit; *green*), myelin (MOG; *red* in **a**), microglia (HLA-DR; *red* in **b**), and GFAP (*red* in **d**) in a chronic active MS lesion (MS58 P1D3) was visualized using single- and dual-staining immunofluorescence microscopy of serial tissue sections combined with DAPI (*blue*) demonstrating cell nuclei. (**a**) Complete myelin loss was observed (*bottom*), along with (**b**) activated microglia and (**c**) absence of immunoreactive staining for citrullination. (**d**) Low levels of citrullination (*green*) were observed in the absence of extensive astrocyte processes (GFAP; *red*). Scale bar = 50 μm

10.3.3 PAD4 Expression in MS

PAD4 is the only isoenzyme to carry a functional nuclear localization signal which enables it to translocate from the cytoplasm to the nucleus (Nakashima et al. 2002). PAD4 has also been specifically found to citrullinate nuclear proteins; histones H2A, H3, and H4; and nucleophomin/B23 (Hagiwara et al. 2002) (see Chap. 18 for more details). When histone proteins are deiminated, gene transcription is generally suppressed, suggesting that deimination causes a tightening of chromatin structure, leading to repression of gene transcription (Shimoyama et al. 2010). In a study by Cuthbert et al. PAD4 was found to inhibit transcription of estrogen-responsive genes in MCF-7 cells. Levels of PAD4 in MCF-7 cells were shown to rise in response to estrogen, followed by deimination of the pS2 promoter by PAD4, which coincides with downregulation of this gene (Cuthbert et al. 2004). Wang et al. showed that incubation of purified substrates H3 and H4 with PAD4 in vitro generated citrullinated H3 and H4, which coincided with a dramatic reduction in H3 Arg^{17} and H4 Arg^{3} methylation (Wang et al. 2004). This effect was also mimicked when HL-60 granulocytes were incubated with PAD4 in vivo, in the presence of calcium ionophore.

PAD4 was found to be elevated in NAWM from 17 patients with MS, compared to 9 control subjects (Mastronardi et al. 2006). This was shown by fractionation of these samples into membrane-containing, non-microsomal, and nuclear fractions,

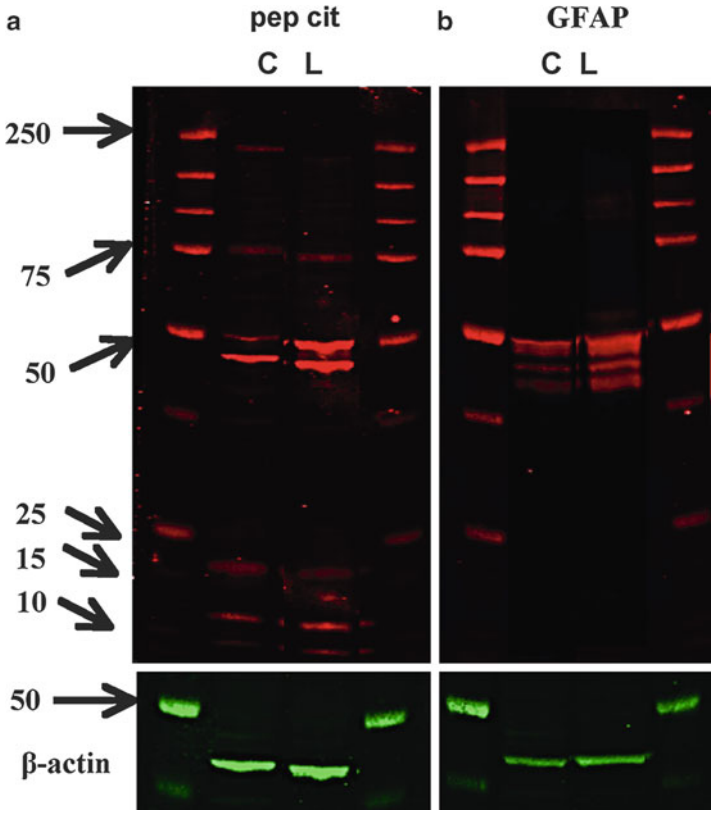

Fig. 10.3 Western blot analysis of proteins extracted from control white matter (C) and MS lesional (L) brain tissue is shown for peptidyl-citrulline (*red*) and GFAP (*green*). (**a**) Multiple bands of citrullinated proteins using the F95 antibody were observed, (**b**) with two of those bands identified as citrullinated GFAP isoforms. The first and last lanes represent standard molecular weight markers, with β-actin antibody used as a loading control (*green*)

followed by quantitation of the amount of PAD 1–4 antibody binding. The nuclear fraction contained a 3.5-fold increase in the level of PAD4 in patients with MS compared to controls. Through western blot analysis using anti-PAD4 antibody, this was attributable to increased PAD4 in the MS NAWM. Using an antibody against citrullinated proteins, this increase in PAD4 was found to be accompanied by an increase in citrullinated proteins in brain tissue taken from patients with MS, whereby strong nuclear labeling in NAWM from MS patients was seen compared to controls (Mastronardi et al. 2006). This increase in PAD4 was also accompanied by an increase in nuclear histone H3 citrullination, as shown by immunostaining of MS and control tissue with an antibody against citrullinated protein, which revealed strong nuclear staining of cells in the MS white matter. These findings were confirmed by western blot analysis, which showed a great abundance of citrullinated H3 in MS NAWM, with only traces in white matter from controls. This citrullination of histones greatly affects the chromatin structure and function, as deimination of

arginine residues of histones decreases their positive charge, which compromises its ability to interact with DNA and possibly resulting in apoptosis of affected cells (Moscarello et al. 2007; Wang et al. 2004).

10.4 Chemokines and Citrullination

Proost et al. (2008) were the first to identify deimination of the chemokine CXC ligand 8 (CXCL8) expressed by human peripheral blood mononuclear cells (PBMCs). Furthermore, chemokines have since been reported to be citrullinated including CXCL10, CXCL11, and CXCL12, and most recently, Mortier et al. identified citrullinated CXCL5 (Loos et al. 2008; Struyf et al. 2009; Mortier et al. 2010). All of these chemokines have been previously reported to play a role in the migration of T cells and monocytes into the CNS (Proost et al. 2008; Loos et al. 2008; Struyf et al. 2009; Mortier et al. 2010) as well as CXCL12 having a proposed role in engraftment of neural stem cells (Carbajal et al. 2010). Thus, deimination of chemokines may also play a role in the pathogenesis of MS and other inflammatory neurological diseases.

In general, the effect of chemokine citrullination may lead to a decrease in their proteolytic cleavage, resulting in a reduction in their binding affinity and intracellular signaling through the corresponding chemokine receptor. The heparin-binding capacity of both citrullinated CXCL10 and CXCL11 is significantly reduced compared to their native form, and since sequestration of chemokines and formation of a chemokine gradient are essential for inflammatory cell recruitment, reduced binding of the deiminated forms to the glycosaminoglycan in the extracellular matrix would also contribute to decreased inflammatory cell recruitment (Loos et al. 2008). In functional in vivo and in vitro assays, citrullination of chemokines led to decreased inflammatory cell migration, when compared to their non-citrullinated isoforms, suggesting that PAD enzymes play an anti-inflammatory role through citrullination of chemokines (Proost et al. 2008; Loos et al. 2008; Struyf et al. 2009; Mortier et al. 2010). Since in MS, CXCL10 protein, induced by interferon-γ, is expressed in actively demyelinating MS lesions, predominantly by macrophages within plaques and by reactive astrocytes in the surrounding parenchyma, these conditions promote recruitment of activated $CD4^+$ T cells expressing CXCR3 across the blood–brain barrier (Simpson et al. 2000a, b). As a result, citrullination of locally expressed chemokines may be an important mechanism to further reduce T cell and monocyte migration into the CNS.

10.5 Citrullination and Enhanced HLA-DR Binding

The conversion of arginine to citrulline has been shown to enable a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0101, *0401, *0404, and *1001 MHC class II molecules (Hill et al. 2003; James et al.

2010). Furthermore, this deimination of peptides is necessary in order to elicit $CD4^+$ T cell responses, suggesting that "altered-self" epitopes are only presented to T cells when certain arginine residues have been converted to citrullines (Hill et al. 2003; James et al. 2010). Since in multiple sclerosis there is an association between HLA type, including HLA-DRB1, this may indicate that citrullination of MBP and other CNS proteins may result in peptides which have higher affinity for HLA-DRB1, which may then trigger autoreactive $CD4^+$ T cells in an analogous manner as reported in rheumatoid arthritis (Naito et al. 1972; Jersild et al. 1973; Barcellos et al. 2006; Disanto et al. 2011). This is an area for future research in MS.

10.6 Regulation of Peptidylarginine Deiminases and Citrullination

It is not known whether excess citrullination is a primary or a secondary event to the inflammatory process in MS or whether the regulation of PAD isoforms may be part of the genetic susceptibility to MS. Single-nucleotide polymorphisms in the PAD4 gene, also associated with the autoimmune disease rheumatoid arthritis, increase mRNA stability, suggesting that this could result in greater PAD4 protein expression and hence increased citrullination of proteins (Suzuki et al. 2003). Increased PAD2 protein expression in human astrocytes in vitro has also been reported in response to increased intracellular calcium levels when cells were subjected to elevated pressure or in response to hypoxia (Bhattacharya et al. 2006a, b; Sambandam et al. 2004).

A number of pathological processes, including excitotoxicity, occur in the CNS of patients with MS, which would lead to raised intracellular calcium ions in neurons and glia (Shideman et al. 2006; Smith 2007). Large numbers of activated macrophages are present in inflammatory demyelinating sites within MS lesions. Since these cells contain PAD enzymes and there is increased cell death due to raised intracellular calcium ions, this would lead to activation of PAD enzymes when released from dying cells (Bhattacharya et al. 2006a). Thus myelin proteins may be citrullinated both intracellularly, during myelin degradation following phagocytosis, as well as extracellularly, following release of PAD enzymes from dying cells. In addition, significant hypomethylation of the PAD2 promoter has also been found to occur in MS NAWM compared to controls, which may lead to increased PAD2 expression and subsequent increase in citrullination, as hypomethylation leads to increased gene transcription (Mastronardi et al. 2007).

10.7 Diagnostic Detection of Anti-citrullinated Protein Antibodies

Autoantibodies directed against citrullinated proteins, commonly known as anti-citrullinated protein antibodies (ACPA), are detected early on in the onset of rheumatoid arthritis, and individuals with a positive ACPA are prone to an increased risk

of future development of disease and a more severe course (Avouac et al. 2006; Kroot et al. 2000; Meyer et al. 2003; Vencovský et al. 2003). Testing for ACPA in individuals first presenting with rheumatoid arthritis-associated symptoms is now routinely carried out, and the detection of ACPA at an early time point has allowed the use of disease-modifying antirheumatic drugs at an early stage of disease, which has led to decreased morbidity. (A more detailed discussion regarding ACPAs in rheumatoid arthritis can be found in Chap. 3.)

In MS, early studies have found antibodies against deiminated MBP in the sera of patients (De Seze et al. 2001; Bodil Roth et al. 2008). With the increasing evidence of citrullination of a number of CNS proteins, in particular MBP and GFAP in MS CNS tissue, further studies assessing ACPA in MS patients may allow an early diagnosis and inform long-term prognosis, which would have therapeutic implications allowing early initiation of disease-modifying therapies with the aim of reducing disease progression and improving the quality of life of affected individuals. Continuing research into ACPA in MS may contribute to further elucidating the role of citrullination in disease pathogenesis and help in developing new therapeutic targets, such as PAD inhibitors (see Chaps. 11 and 21 for greater detail).

10.8 Citrullination in Experimental Autoimmune Encephalomyelitis

For many decades, EAE has been widely used as an animal model of MS. There are several variations of EAE induction such as immunization of animals with spinal cord homogenates, individual myelin proteins, or by adoptive transfer of myelin-specific T cells (Mix et al. 2010). The oligodendrocyte-derived myelin proteins, MBP and MOG, as well as smaller encephalitogenic peptides of MBP, MOG, and proteolipid protein (PLP) can act as antigens to trigger EAE (Gold et al. 2006; Mix et al. 2010). Transgenic animal models of spontaneous demyelination have also been developed in an attempt to imitate MS, without the bias of a specific peptide for induction (Mastronardi et al. 1993; Ellmerich et al. 2005; Pöllinger et al. 2009; Mix et al. 2010). Although EAE models do not directly mimic the complex pathogenesis of MS, their similarities have allowed the investigation of CNS inflammation and tests for potential MS therapies. There is now an emerging understanding of the role of protein deimination in the CNS derived from studies on EAE models of MS.

High levels of citrullination, demonstrated previously in postmortem MS tissue (Moscarello et al. 1994), generated interest in the ability of citrullinated MBP to elicit an immune response in EAE. Cao et al. (1998) demonstrated that active EAE induction in Lewis rats with a citrullinated peptide of guinea pig MBP (MBP-C8) generated distinct subsets of MBP-C8-specific T cells. When isolated and used in adoptive transfer EAE experiments, these cells led to severe EAE and furthermore were able to re-induce EAE in animals that had previously undergone induction of EAE with the non-citrullinated peptide. The group hypothesized that this was due to regulatory T cells being generated specifically to the non-citrullinated peptide,

thereby allowing the MBP-C8 T cells to evade this control, leading to inflammation in the CNS.

Carrillo-Vico et al. (2010) performed similar experiments using MOG (35–55 pMOG) peptides which were citrullinated at T cell receptor (TCR) contacts at residues 41 and/or 46 and compared these to the wild-type (WT) peptide. Only one of the three modified peptides (pMOG41cit) was able to induce an encephalitogenic response, and disease severity was lower than that induced using WT MOG peptide. After immunization with pMOG41cit, a distinct T cell population was developed which responded only to pMOG41cit and a smaller population that also cross-reacted with WT pMOG. Experiments whereby mice were pre-tolerated against pMOG41cit resulted in maintained susceptibility to development of EAE induced by WT pMOG. Conversely, mice pre-tolerated using WT pMOG did not develop EAE when immunized with pMOG41cit. The group concluded that citrullinated MOG peptides are not necessary to initiate EAE, although they may increase the severity of ongoing disease. This supports the hypothesis of Cao et al. (1998) that wild-type and deiminated peptides generate distinct T cell responses. It may be that citrullinated peptides do not generate a regulatory T cell (T_{reg}) population and/or induce T cell tolerance. This is another area of research that requires further investigation.

The antibody response to citrullinated proteins in EAE has also been investigated by Kidd et al. whereby EAE induced with the native peptide PLP 139–151 in SJL mice led to epitope spreading to both native and citrullinated MBP peptides. Autoantibodies generated in vivo against citrullinated epitopes of MBP were 20 times more reactive than against the native epitopes (Kidd et al. 2008). This also supports the hypothesis that deimination occurs early in EAE development, generating neoantigens as targets for antibody production and epitope spreading to several myelin components, thus contributing to disease progression.

10.8.1 MBP and GFAP Deimination in EAE

We have previously demonstrated citrullinated MBP and GFAP in the normal rat brain using immunohistochemical localization (Nicholas et al. 2003) and increased citrullinated protein expression in the CNS of mice with MOG-induced EAE (Nicholas et al. 2005). The amount of citrullination increased with worsening disease symptoms and was shown to co-localize with both MBP and GFAP (Nicholas et al. 2005), with astrocytes surrounding extracellular plaques that were both immunoreactive for citrullinated proteins (Fig. 10.4). Raijmakers et al. (2005) also demonstrated hypercitrullination of proteins in PLP-induced EAE mice, and immunohistochemistry showed that the areas of the spinal cord with most citrullinated proteins correlated with areas of demyelination. The amount of citrullinated protein was highest in the relapse phase of EAE, compared with acute-phase EAE, which might be due to remyelination attempts, as increased citrullination is associated with myelination during development of the CNS. By using western blotting

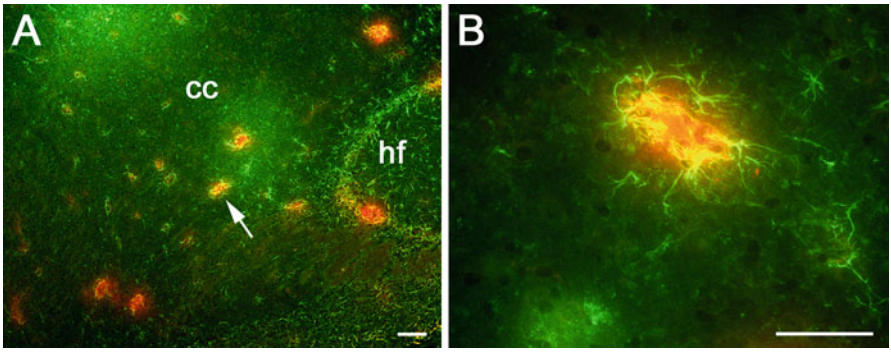

Fig. 10.4 In merged images of EAE brains, immunoreactive patches of citrullinated proteins (*red*) were surrounded by astrocytes immunoreactive for GFAP (*green*). In low magnification (**a**), within the corpus callosum (cc) and near the hippocampal formation (hf), these lesions were numerous during active disease. When one of these patches (**a**, *arrow*) is magnified (**b**), co-localization (*yellow*) of deiminated protein (*red*) and GFAP (*green*) demonstrate astrocyte processes within the plaque, perhaps representing phagocytosis of deiminated MBP by these glial cells. Scale bars = 50 μm. Modified from Nicholas et al., Journal of Comparative Neurology, Volume 486, pp. 254–266, Copyright 2005, Wiley-Liss, Inc.

and specific antibodies, the citrullinated proteins were identified as MBP and GFAP (Rajimakers et al. 2005). The increase in citrillunated GFAP was due to not only an increase in GFAP expression but also an increase in the number of citrulline residues per GFAP molecule. Grant et al. (2007) also identified increased citrullination of GFAP from spinal cord of MBP-induced EAE and showed citrullination specifically at the Arg27 residue by iTRAQ mass spectrometry.

10.8.2 PAD Expression in EAE

For citrullination to occur within the CNS, PAD enzymes must also be expressed. Both PAD2 and PAD4 have been detected in the normal mouse CNS and in EAE (Raijmakers et al. 2005; Mastronardi et al. 2006; Carrillo-Vico et al. 2010), although whether or not these are upregulated in EAE is not straightforward as some findings are conflicting (Raijmakers et al. 2005; Carrillo-Vico et al. 2010). Since these authors used different antigens to trigger EAE, it could be that different models elicit a different response, or it may be due to the different techniques used for detection.

Raijmakers et al. 2005 used immunoblotting of proteins and RT-PCR of extracted mRNA from spinal cord sections of mice with PLP-induced EAE to demonstrate that the increase in citrullination present in EAE was not due to an increase in the levels of PAD2 or PAD4. Based on this, the authors concluded that the hypercitrullination observed in EAE must be mediated by activation of PAD2 and PAD4

already present in the CNS. However, Carillo-Vico et al. (2010) showed by real-time RT-PCR that mRNA for both PAD2 and PAD4 were upregulated following EAE induction by pMOG, although protein levels were not assessed.

Musse et al. (2008) investigated the role of PAD2 in demyelinating disease by developing transgenic mice overexpressing PAD2 under the control of an MBP promoter. These mice showed a reduction of myelin compared to non-transgenic mice, with a more severe clinical disease course than spontaneously demyelinating transgenic (ND4) mice. In addition, clinical symptoms of demyelination in PAD2 overexpressing mice presented earlier at 6 weeks compared to 3 months in the ND4 mouse model. Levels of PAD2 were compared to control mice, with PAD2 transgenic mice showing significantly increased PAD2 activity at 2 months of age, along with a ~2.5-fold increase in citrullinated MBP at 3 months of age. Staining with luxol fast blue, the optic nerve white matter of these mice showed areas of myelin breakdown, along with a reduction in myelin. All of these changes were more pronounced in homozygous mice possessing 30 copies of the cDNA encoding the PAD2 gene, compared to the heterozygotes possessing only 15 copies of this gene. PAD2 mRNA levels showed similar changes with a fourfold increase in PAD2 mRNA in transgenic, compared to non-transgenic mice, which increased to 5.3-fold in homozygous PAD2 transgenic mice. This overexpression of PAD2 also led to an increase in the expression of PAD4 (but not PAD1 or PAD3) compared with control mice, with an accompanied relative increase in histone H3 citrullination.

PAD4 has previously been found to be upregulated in brain extracts in EAE models induced with either MBP or PLP compared to control groups as well as a rise in nuclear PAD4 in the preclinical stage of demyelination in the spontaneously demyelinating ND4 transgenic mouse model (Mastronardi et al. 2006). There was also an association between high TNFα levels, increased nuclear PAD4, and histone H3 citrullinated proteins. Furthermore, incubation of an oligodendrocyte cell line with TNFα caused PAD4 translocation into the nucleus (Mastronardi et al. 2006).

Transgenic mice have also been used to demonstrate the deletion effects of the PAD2 gene on active MOG-induced EAE (Raijmakers et al. 2006). These authors showed that the time to onset of EAE, incidence, and severity were not impaired in the knockout mice and that neither citrullinated GFAP nor MBP in these mice was detected by western blotting or immunohistochemistry. It was concluded that PAD2 expression and citrullination are not required in the disease progression of EAE; however, if PAD activity and citrullination is an early event in demyelination (as indicated in the PAD2-overexpressing mouse) leading to conformational changes and exposure of new epitopes, then induction of EAE with encephalitogenic peptides of myelin proteins generating a T cell response may bypass this event. It is therefore not unexpected that PAD2 knockout has no effect on EAE development in that setting.

Further support of citrullination as an early event in demyelination was also seen when paclitaxel, a noncompetitive inhibitor of PAD (Pritzker and Moscarello 1999), was able to delay the onset and severity of EAE in active and passive transfer models (Cao et al. 2000). This may be due to its effects on microtubule stabilization, preventing T cell infiltration, but may also be caused by its downregulation of

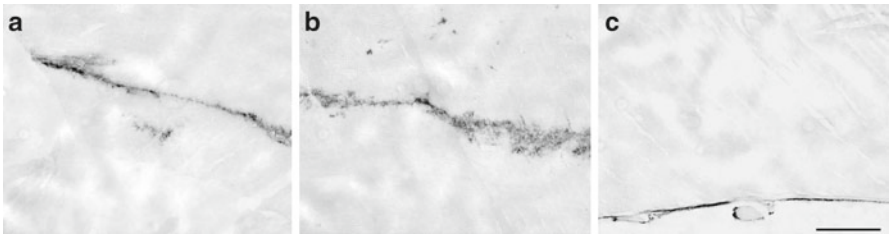

Fig. 10.5 In spinal cords with MOG-induced EAE, large lesions containing deiminated protein identified by F95 immunostaining are no different between control mice (**a**) and PAD2 knockout mice (**b**; courtesy of Dr. Brian Werneburg, Boehringer Ingelheim Pharm., Inc.), supporting the contention that EAE clinical deficits are no different without the presence of PAD2 and that deimination still takes place in these animals. In contrast, control mice without EAE (**c**) do not have these lesions but do express some surface immunostaining for deiminated proteins, as expected. Scale bars = 100 μm

citrullination. In theory, this process could perhaps maintain compact myelin structure, preventing proteolysis and neoepitope exposure. Paclitaxel also inhibits MBP synthesis, including stromelysin (Hui et al. 1998), an enzyme able to digest MBP with a higher affinity for citrullinated MBP compared with native protein (D'Souza and Moscarello 2006), meaning that the data presented in the study by Cao et al. (2000) is difficult to interpret. Since the PAD2 knockout mouse study by Rajimakers et al. (2006) showed no effect on EAE induction or severity, this suggests that paclitaxel may not have exerted its effects through PAD2 inhibition.

However, other more specific PAD inhibitors such as Cl-amidine (Willis et al. 2011) have been recently examined in four murine demyelinating models of MS (see Chap. 11 for more details). These results clearly demonstrate attenuation of disease by Cl-amidine (Moscarello et al. 2013) and suggest that deimination may have still been present in PAD2 knockout mice with EAE, putting into question previously published results (Raijmakers et al. 2006). In fact, using the F95 antibody to detect peptidyl-citrulline (Nicholas and Whitaker 2002), spinal cord lesions in control versus PAD2 knockout mice with EAE were identical (Fig. 10.5), suggesting that another PAD isozyme besides PAD2 may be responsible for increased deimination in these animals with EAE.

10.9 Summary of EAE and MS Findings

Our recent studies have identified two major citrullinated proteins in MS, MBP and GFAP, which have the potential to influence the disease process (Fig. 10.6). The conversion of arginine to citrulline in MBP alters the charge of the protein, as for each arginine converted to citrulline there is a loss of one positive charge, leading to partial unfolding of the protein and weakening interaction with phospholipids; therefore myelin sheaths are not as tightly packed as in normal myelin (Beniac et al.

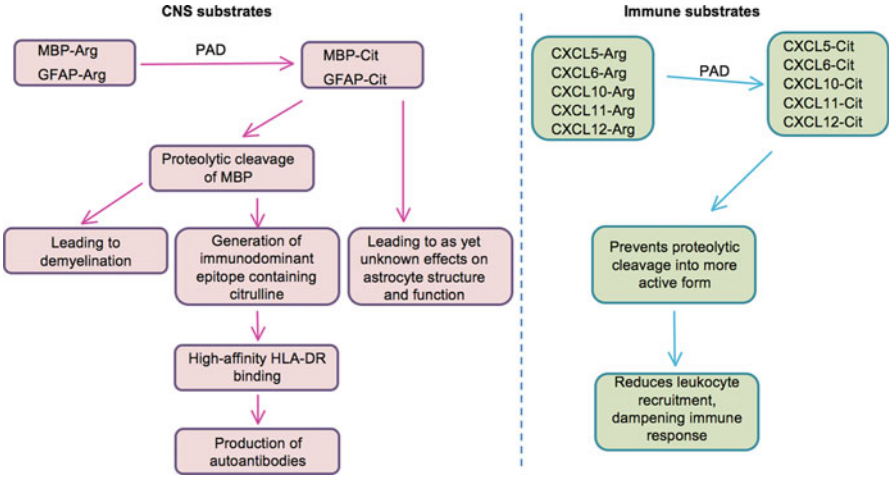

Fig. 10.6 Summary of the effects of citrullination on the immune response in MS. Citrullination of MBP increases its susceptibility to degradation by proteinases releasing new epitopes, which may lead to autoantibody production. The effects of citrullination of GFAP are as yet unknown but may affect astrocyte structure and function. Citrullination of chemokines prevents their proteolytic cleavage into more active forms and may therefore affect leukocyte recruitment into the CNS. Key: Arg, arginine; Cit, citrulline; HLA, human leukocyte antigen; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein

2000). Deimination of MBP increases its susceptibility to degradation by proteinases, such as cathepsin D, which has been shown to release numerous peptides at the Phe–Phe linkages in the MBP protein, in particular, generating immunodominant epitopes containing citrulline (Cao et al. 2000). Conversion of arginine to citrulline has been shown to enable a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*401 MHC class II molecule and subsequent activation of $CD4^+$ T cells (Hill et al. 2003). Overall, this could lead to the production of autoantibodies targeting the myelin sheath. Although less is known regarding the conversion of arginine to citrulline in GFAP, it is expected to have implications on the structure and function of GFAP-positive astrocytes. Secondary to this, recent evidence has identified the CXC chemokines as targets for citrullination, which has been shown to prevent proteolytic cleavage of chemokines into their more active form, resulting in reduced leukocyte recruitment, and therefore may also play a role in disease pathogenesis (Proost et al. 2008; Loos et al. 2008, 2009; Struyf et al. 2009; Mortier et al. 2010).

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Chapter 11
Protein Hypercitrullination in CNS Demyelinating Disease Reversed by PAD Inhibition

Mario A. Moscarello

Keywords Demyelination • Multiple sclerosis • Neurodeneration • Autoimmunity • Myelins • Posttranslational modifications • Citrulline • Deimination • PAD inhibitor

11.1 Introduction

Protein deimination (the conversion of peptide-bound arginine residues to citrulline) is a posttranslational modification which has important consequences for both protein structure and function. In this reaction shown in Fig. 11.1, the quanidino group of arginine is deiminated to citrulline. Each arginine deiminated is accompanied by the loss of positive charge, since citrulline is neutral. Thus, the deimination of several arginines in a protein results in large changes in total charge, causing major changes in protein structure and function. This will be abundantly demonstrated in this article, highlighting myelin basic protein (MBP) and its effects on myelin structure and stability. These devastating effects on myelin suggest a basic mechanism preceding demyelination in multiple sclerosis (MS), the most common neurological disease of humans, affecting about two million people worldwide.

The family of enzymes that carry out this reaction are called peptidylarginine deiminases (PADs), of which, five are known all located on a single site lp36.1 on chromosome 1 in humans (see Chaps. 1 and 21 for more details). Of the five isozymes, PAD2 and PAD4 are present in brain. PAD4 is unique because it carries a nuclear localization signal which allows it to be translocated to the nucleus, where

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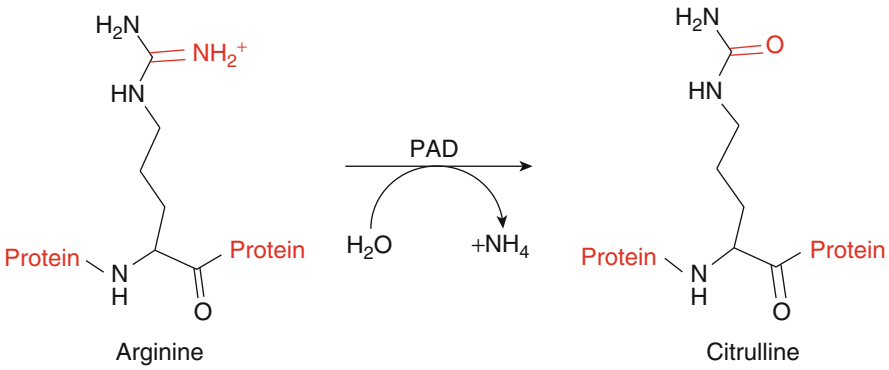


Fig. 11.1 Protein deimination by PAD enzymes

it deiminates histones (Vossenaar et al. 2003, Cuthbert et al. 2004, Hagiwara et al. 2002, Nakashima et al. 2002).

We have demonstrated that PAD activity was consistently elevated in normal-appearing white matter (NAWM) extracts from brain of MS patients. The nuclear targeting of PAD4 was associated with increased TNFα. Exposure of oligodendrocytes in culture to TNFα resulted in the appearance of PAD4 in the nucleus and citrullination of histone H3 (Mastronardi et al. 2006). The consequences of this deimination were the loosening of interactions between histone and DNA, contributing to apoptosis of oligodendrocytes and demethylation of the PAD2 promoter with important consequences for demyelination. Oligodendrocyte apoptosis is an important mechanism in the pathogenesis of MS and may be an initiating factor in some cases (Ludwin 2006).

Both PAD2 and PAD4 are localized to myelin in the central nervous system (CNS). By immunogold electron microscopy, we showed that, in mouse optic nerve, PAD2 was found in clusters in myelin sheath, the axon, oligodendrocytes and significantly at the peri-axonal space (the contact area between myelin and the oligodendrocyte processes). Deimination of MBP at this site loosens the contacts, giving rise to a "dying back" and retraction of the processes of the oligodendrocytes (Ludwin 2006). In in vitro studies with recombinant PAD2 and PAD4, we reported that MBP with 19 arginyl residues is an excellent substrate for both enzymes (Wood et al. 2008). In contrast to PAD2, PAD4 was found in single gold particles in both the cytoplasm and nucleus, the latter finding consistent with the nuclear localization of PAD4.

11.2 Citrulline

The free amino acid citrulline was isolated for the first time in 1914 from the watermelon, *citrullus vulgaris* (Koga and Ohtake 1914). Until recently, it was considered a curiosity because it is a noncoded amino acid, not involved in protein synthesis.

In recent years, its importance has been underlined by its occurrence in proteins, the detailed studies of PAD enzymes are responsible for this (Fig. 11.1) and its association with the pathogenesis of many diseases. In order to understand the role of citrulline in pathophysiology of disease, a brief summary of its physical and chemical properties will be provided here (see Curis et al. Amino Acids 2005 for more details). Citrulline (CAS 372-75-8) is a colorless solid with a melting point of 222 °C. It has an asymmetric carbon atom and is therefore present as two enantiomeric forms. Its natural form is the L configuration. It has a molar mass of 175 $gmol^{-1}$. Its chemical properties result from both its amino acid function and the ureido group of the aliphatic chain. At alkaline PH, both carboxylic acid and amine groups can act as Lewis donors, which mean they can complex metal ions. Since there is no codon in the genetic code for citrulline, its presence in proteins results from the deimination of arginine. This important reaction has been shown to be involved in regulatory reactions such as allosteric regulation of enzymes (Eronina et al. 1996). Citrulline is unique from other amino acids because of the presence of an ureido group and can be considered a substituted urea. In contrast, free citrulline has an important role in the urea cycle and in the formation of nitric oxide. Here we are concerned only with protein-bound citrulline, which is completely independent of the free citrulline pathway.

11.3 Protein-Bound Citrulline

In an early observation (Finch et al. 1971), we showed that citrulline was present in a myelin protein fraction. Subsequent isolation and purification of MBP localized the citrulline to this protein. However, isolation of MBP, although migrating as a single band on SDS-PAGE at 18.5 kDa, several protein bands were observed at alkaline pH in urea-alkaline gels. These represented the micro heterogeneity of MBP, resulting from a large number of posttranslational modifications, including phosphorylation, deamination, C-terminal arginine loss, arginine methylation (both mono- and dimethyl-arginine), and deimination (for a review see Wood and Moscarello 1997). Of the various components resolved on urea-alkaline gels, one component, C-8 (termed C-8 for convenience) was recovered from the void volume of a CM52 cation exchange column at pH 10.5. Since this material was found in the void volume, it was the least cationic of the components. Further purification on HPLC yielded a single protein, which we used for sequencing. At this point, we then adopted more descriptive names for the various components. Instead of components 1, 2, 3, etc. representing the individual fractions from the CM-52 column, we adopted the term “charge isomers” for the various MBP components. These are not isomers in the mass spectrometric sense, since the various components differ in mass depending on the modifications. Deamination increases the mass by 1 Da, methylation increases the mass by 14 Da and deimination increases the mass by 1 Da for each citrulline formed from arginine.

Complete sequence analysis of MBP using the Edman degradation technique showed that six consistently (and sometimes seven) of the arginyl residues were converted to citrulline in the C-8 component from normal MBP (Wood and Moscarello 1989). The arginine residues deiminated included Arg9, Arg25, Arg33, Arg122, Arg130, Arg159, Arg170, with three clustered at the N-terminus and four at the C-terminus. Thus, the deimination of seven arginyl residues resulted in a loss of seven positive charges on MBP.

In addition to loss of positive charge, the presence of citrulline has other effects. As a substituted urea, it has many of the properties of urea. Urea has rigid electron-rich planer structure and is a good chelator, so it can sequester divalent cations, which are important in myelin stability. Urea forms a mixed clathrate-like structure around hydrocarbons, resulting in increased solubility.

Furthermore, deimination of MBP has been shown to produce marked effects on protein conformation (folded structure) (Libich et al. 2003a, b; Lamensa and Moscarello 1993; Bates et al. 2002; Beniac et al. 1999; Pritzker et al. 2000), generating a more open structure. Some of the consequences of this more open structure have been reported. Thus, proteolytic digestion by Cathepsin D, a brain protease implicated in lesion formation in MS (Cuzner and Davison 1973), is increased. In fact, degradation correlated well with the extent of MBP deimination. MBP Cit_6 was degraded 4 times more rapidly than MBP Cit_0, whereas MBP Cit_{18} (seen in Marburg disease) was degraded 35 times faster. When bovine MBP was deiminated in vitro with PAD2, MBP Cit_{15} was digested 3.5 times faster than MBP Cit_0. This increased susceptibility to protease digestion generates immunogenic peptides (neo-epitopes), stimulating the immune response.

An unexplained degradative response was reported by D'Souza et al. (2005). They found that the isolated and purified component 1 (C-1) from MS tissue degraded spontaneously in vitro without proteases. Since the MBP had been extracted from brain in the usual way with 0.2 N H_2SO_4, contaminating proteases were not responsible. The fact that the MBP (C-1) isolated from MS tissue degraded more rapidly than that from normal brain suggested that deimination may be involved. The significance of these observations may be related to the postulated "molecular mimicry" hypothesis: that the initial sensitized T-cells appeared in the periphery as a result of a viral infection, which contained a protein with some sequence homology to a myelin antigen (possible MBP), and then sensitized T-cells, which recognized and cross reacted with the myelin antigen. Alternatively, we suggested (D'Souza et al. 2005) that the increased susceptibility of deiminated MBP to "autocatalysis" may be the mechanism, rather than molecular mimicry. The significance of these interactions is still not understood at this time.

11.4 Protein–Protein Interactions

The effects of deimination on protein–lipid interactions are well documented (Boggs et al. 1982). In a number of studies involving X-ray diffraction (Brady et al. 1981a, b), electron spin resonance (Boggs et al. 1981), circular dichroism

(Epand et al. 1974a, b), and NMR (Deber et al. 1986), it was demonstrated that citrullinated MBP prevented compaction of the myelin bilayer, resulting in membrane destabilization and subsequent degradation. MBP has a number of interactions with other proteins, both structural and enzymatic, which are also affected by deimination. The significance of these interactions is also not fully understood at this time.

11.4.1 MBP Self-Assembly

Many attempts to crystallize MBP have been made, but none have succeeded. Using transmission electron microscopy and one-dimensional assemblies of monolayers containing a nickel chelating lipid and phosphoinositides, paracrystalline arrays of 4.8 nm spacing were obtained with recombinant mouse MBP. After deimination in vitro, an average of nine citrullines (MBP Cit_9) resulted in the best ordered domains in planer, crystalline assemblies (Ishiyama et al. 2001). The significance of these observations is not known.

11.4.2 MBP and PLP Interactions

MBP accounts for 30–35 % of total CNS myelin proteins, whereas proteolipid protein (PLP) accounts for about 50 %. Using a purified PLP, termed lipophilin, incorporated into phosphatidycholinc vesicles, MBP was unable to aggregate these vesicles. However, citrullinated MBP was highly effective in this function, suggesting a specific interaction between lipophilin and deiminated MBP (Wood and Moscarello 1989).

11.4.3 MBP and Phospholipase C Interactions

Phosphatidylinositol-specific phospholipase C (PI-PLC) isolated from bovine brain was stimulated two- to threefold by addition of MBP (Tompkins and Moscarello 1991). When the positive charge on MBP was reduced by phosphorylation or deimination, the stimulatory effect was also reduced, suggesting that these interactions may play a regulatory role.

11.4.4 MBP Calmodulin Interactions

Component 1 (C-1), in its most unmodified form, bound calmodulin in a Ca^{2+}-dependent manner at a 1:1 ratio, as determined by inherent tryptophan fluorescence and light scattering. When citrullinated MBP was used, the changes in fluorescence

were better modeled by a two-site equation, suggesting that deimination resulted in a conformational change, which exposed a second binding site (Polverini et al. 2004) for additional Ca^{2+} ions to be sequestered by this interaction. Since PAD enzymes are Ca^{2+} dependent, these binding sites for Ca^{2+} may also have an important role in regulating PAD activity.

11.4.5 MBP and Actin

MBP has been shown to polymerize actin and bind it to lipid bilayers by electrostatic interactions (Boggs et al. 2005), whereas the deiminated form of MBP showed less ability to bind to lipid bilayers.

11.4.6 MBP and Tubulin

The unmodified C-1 assembled tubulin in a dose-dependent manner, creating bundles (Hill et al. 2005). As the level of modification increased, the ability to assemble tubulin was enhanced, in contrast to the observations with actin mentioned above.

The interaction of MBP with these various proteins, many of which are cytoskeletal proteins, such as actin and tubulin, supports a role for MBP and MBP–protein interactions in signal transduction in both oligodendrocytes and myelin. By redistribution of the lipids in myelin, signals can be transmitted through the myelin stack (Dyer 1993). It has been demonstrated that these interactions are dependent on MBP, since they are absent in the oligodendrocytes of "shiverer mice," which lack CNS MBP (Boggs et al. 2008). Lipid redistribution may adversely affect membrane reorganization, leading to degeneration. A role for MBP in signal transduction is compromised by deimination, affecting the ability of MBP to polymerize and bundle actin and bind it to the membrane surface. For a full discussion of MBP-cytoskeletal interactions, see Boggs (2008).

A number of interactions with other proteins and MBP have been documented. The possible significance of these various interactions cannot be decided at this time. However, it appears consistent that a change in the charge of MBP by deimination affects these interactions, adding to the regulatory role of this modification. The above-mentioned protein–protein interactions represent indirect effects on signal transduction mechanisms. More immediate and direct effects have been demonstrated in experiments involving phosphorylation, GTP binding and ADP-ribosylation reminiscent of G protein reactions, which may have important roles in oligodendrocyte–myelin interactions.

The activities of several protein kinases have also been associated with myelin. These include cyclic AMP-dependent protein kinase (Johnson et al. 1971; Miyamoto 1975; Miyamoto et al. 1978); Ca^{2+}-phospholipid-dependent protein kinase (Turner et al. 1982; Murray and Steck 1986), and calmodulin-dependent kinase

(Cheung 1980; Sulakhe et al. 1982). Since MBP contains a number of seryl and threonyl residues, a large number of sites can be phosphorylated by the various kinases, giving rise to a variety of charged isomers. Two of the sites have been shown to be notable. Ser7 was refractory to acid phosphatase, because it forms ionic interactions with Arg 5 and Arg 9, thus stabilizing a β-structure (Ramwani and Moscarello 1990). Thr 98, which forms part of the SH3 domain, is phosphorylated by MAP kinase. We have demonstrated that this site is phosphorylated in vivo. Using an antibody that reacts specifically with phosphorylated Thr 98, we localized this phosphorylated site to the major dense line of myelin by immunogold electron microscopy (Yon et al. 1996).

The triproline region, TPRT98 PPP, is an SH3 binding site, shown to bind Fyn-NRTK, a src tyrosine kinase that is of considerable interest, because it is involved directly in MBP gene expression (Sperber et al. 2001). No deiminated arginyl residues were found in this area of the MBP sequence. In fact, the presence of methylated arginine at R107 precludes deimination. One report claims to have shown that methylated arginine was deiminated (demethyliminated), producing citrulline (Wang et al. 2004) by PAD4, but has not been confirmed. In an extensive recent analysis using a large number of synthetic substrates in vitro, no deimination of methylated arginine was observed (Raijmakers et al. 2007). Thus, the presence of methylated arginine 107 in close proximity to the SH3 binding domain prevents deimination, thereby protecting this site.

This binding mechanism has been studied by several techniques, including "docking" (for review see Polverini et al. 2008). Positively charged amino acids in the ligand interact with acidic residues in the binding pocket. Thus R104 (mouse MBP), the methylated R, binds to ASP118 in n-Src loop. The other arginine (R94) makes a salt bridge with Asp99. These interactions (and those involving methylated Arg104) explain the inability of PAD enzymes to deiminate Arg residues in the binding site.

The presence of the SH3 binding domain strongly supports a role for MBP in signal transduction. Signalling by G proteins is characterized by GTP binding and ADP ribosylation. Both of these interactions have been reported in MBP from human CNS. In 1988 Chan et al. reported that human MBP from normal brain, bound one mole of GTP/mole of MBP. The binding site was shown to be the glutamine/residue at position number 3. This was analogous to the binding of GTP to the α-subunit of heterotrimeric G proteins. ADP-ribosylation of G proteins occurs by the transfer of the ADP-ribose moiety from NAD+ to an acceptor amino acid by an ADP-ribosyl transferase, an activity found in cholera toxin. A number of G proteins have been ADP-ribosylated, including the α-subunit of transducin (Van Dop et al. 1984) and ADP-ribosylation factors in bovine brain (Tsai et al. 1988). We have extended these studies using a specific brain protein MBP, component 1 (C-1) of human MBP (the most unmodified of the components), the most positively charged, and therefore it is eluted last from the CM52 column. In the presence of cholera toxin, 1 mol of C-1 accepted 2 mol of ADP-ribose. The critical sites were identified as Arg9 and Arg54 (Boulias and Moscarello 1994). Two other components, C-2 (one positive charge less than C-1) and C-3 (two positive charges less than C-1),

also accepted 2 mol of ADP-ribose/mole protein. However component 8 (the most common citrullinated isoform of MBP) could not be ADP-ribosylated. Lack of ADP-ribosylation of Arg9 can be readily explained by deimination at this site; however, Arg54 is not known to be deiminated. Conformational factors such as shown for Arg177 of G actin may account for this (Vandekerckhove et al. 1987). We next showed that these ADP-ribosylation studies have relevance in vivo, by demonstrating ADP-ribosyltransferase activity in myelin. Freshly prepared myelin from human white matter was used as the source of enzyme. Several myelin proteins were ADP-ribosylated, including some in the 40–50 kDa range, which are probably α-submits of G proteins.

We briefly mentioned phosphoinositide/phospholipase C (above), which forms part of the signal transduction system in myelin. When the α-submit of the heterotrimeric G protein exchanges GDP for GTP, it dissociates from the complex and interacts with PI-PLC. The activated complex then hydrolyzes phosphoinositol-4,5-bis phosphate, PIP2 to yield inositol-1,4,5-trisphosphate (IP_3) and 1,2 diacyl-*sn*-glyceral (DAG) (Berridge 1984). The second messenger DAG, stimulates protein kinase C. In 1991, we purified PI-PLC from bovine brain and human myelin. The preferred substrate was PIP2. The activity of the enzyme was stimulated more than twofold by component-1 (C-1) of MBP, but was not stimulated by the deiminated component C-8. Here again, citrullination of MBP has myriad of effects on biological mechanisms. Since the difference between C-1 and C-8 is primarily due to deimination of 6–7 arginyl residues, we concluded that arginyl residues are important for the activity of these enzymes. Chemical modification of 13 of 19 arginyl residues with 1, 2-cyclo hexanedione decreased the ability of C-1 to stimulate PI-PLC. A synthetic MBP peptide (residues 24–33) containing three arginyl residues (25, 31, 33) stimulated the enzyme as well as C-1. In contrast, deimination of Arg25 and Arg31 decreased the stimulation and deimination of seven arginine residues on C-1 and completely abolished stimulation (Tompkins and Moscarello 1993). The important role of arginyl residues in these reactions suggests that deimination is an important mechanism of metabolic control. The ratio of Arg/Cit may have a regulatory function.

In summary, MBP, originally called "the encephalitogenic protein," has been studied extensively as a candidate autoantigen in MS. The immunological studies are numerous and will not be discussed here. Here we review other roles of MBP and the effect of deimination of MBP on these interactions. We focused on deimination because this posttranslational modification is considerably increased in MS patients and appears to correlate with disease severity. Understanding this role in affecting the integrity of the demyelinating unit in MS, consisting of the myelin sheath and the oligodendrocyte, is essential. The present state of knowledge has been summarized above. Thus, MBP has an important role to play in the integrity of the myelin sheath via protein–lipid interactions between positively charged residues (such as arginines) and negatively charge phosphoryl groups of lipids. Deimination of these arginyl residues to citrulline, results in the formation of a neutral amino acid that compromises the ability of MBP to carry out its function of compacting the myelin bilayer (review, Boggs et al. 1982). This less compact myelin is more susceptible to degradation.

An important role for MBP in signal transduction has been described, with both indirect and direct effects. The indirect effects include binding to calmodulin in a Ca^{2+}-dependent fashion, polymerization of actin that binds it to lipid bilayers and assembly of tubulin bundles. Direct effects on signal transduction have also been reported, which are similar to those of G proteins. Thus, MBP binds GTP at one site (Gln 3), is ADP-ribosylated at two sites (Arg 9 and 54), and interacts with PI-PLC, which generates IP_3 and DAG. These roles in signal transduction (both indirect and direct) are compromised by deimination of arginyl residues, emphasizing that this modification has severe consequences for the proper functioning of MBP. Controlling the extent of deimination by controlling the activities of the PAD enzymes responsible, represents a sound therapeutic strategy.

11.5 Significance of Posttranslational Modifications

After synthesis, proteins are modified in many different ways, mostly by the addition of various groups. Some modifications, such as glycosylation, disulphide bond formation, and lipidation, are stable and become part of the mature protein. Other modifications are transient, allowing proteins to take part in regulation of function. They may be modified at more than one site. For example, MBP can be modified at several Ser and Thr residues by phosphorylation. Glycosylation and deimination also occur on several sites; so a large number of combinations are possible, imparting considerable flexibility on the protein to interact with a variety of ligands and to participate in many metabolic reactions. These posttranslational modifications are found on at least 24 sites. Some sites contain several modifications. For example, Thr98 may be phosphorylated or glycosylated, Arg9 may be citrullinated or ADP-ribosylated and Arg107 may be monomethylated, symmetrically dimethylated or asymmetrically dimethylated. The role of methylated arginine is not known, although it appears to be involved in signal transduction by interaction with protein modules such as SH2, SH3, and ubiquitin-binding domains. Tudor2 domains bind to symmetric dimethyl-arginine, rather than monomethyl- or asymmetric dimethyl-arginine (Sprangers et al. 2003). Arg107, which contains these modifications, is positioned near the SH3 domain (the triproline region, TPRTPPPSQGKGR). A role for arginine methylation has never been demonstrated. Since methylated arginine cannot be deiminated (Raijmakers et al. 2007), we suggest that methylation preserves the guanidine group, allowing it to interact with the adjacent SH3 domain.

Fyn tyrosine kinase, which contains an SH3 domain and is a member of the src family of non-receptor protein tyrosine kinases, was reported to have a role in myelination (Sperber et al. 2001). Fyn^{-1} mice were shown to have decreased myelin formation and decreased MBP formation. Full length Fyn with a single mutation Lys 296 → Arg is devoid of kinase activity and was unable to support myelination, similar to that seen in the absence of Fyn. A role for Fyn in MBP gene expression was postulated, involving its kinase activity.

The association of MBP to the membrane surface was shown to bind Fyn, showing that the SH3 domain of MBP is accessible, even when MBP is bound to the vesicle, suggesting that MBP could bind SH3 domain proteins to the oligodendrocyte membrane (Polverini et al. 2008). Therefore, posttranslational modifications have an important role in both maintaining membrane integrity and regulating metabolic reactions.

11.6 Rethinking Multiple Sclerosis: The Role of Citrulline

As the most common demyelinating disease of human adults, MS is characterized by a patchy degradation of CNS myelin, which heals by scar formation, called "plaques." The cause is unknown, although genetic, immunological, and environmental factors are suggested to play a role (Noseworthy 1999). It is a heterogenerous disease characterized into four distinct patterns based on careful analyses of 282 biopsy samples (Lucchinetti et al. 2000). Pattern 1 showed inflammatory demyelination with macrophages; pattern 2 showed demyelination with infiltration with T-cells; pattern 3 was characterized by loss of oligodendrocytes by apoptosis; while pattern 4 also showed oligodendrocytes loss, but was rare.

The most common theory is the autoimmune hypothesis, which postulates that MS arises by sensitization of T-cells in the periphery, which travel through a disrupted blood brain barrier to attack and destroy CNS myelin. It is based largely on the animal model of MS, experimental autoimmune encephalomyelitis (EAE), initially induced by injection of MBP into a susceptible host (Alvord 1970). Since then, several other CNS proteins have been shown to induce EAE, including PLP (Hashim et al. 1980), myelin-associated glycoprotein (MAG) (Sospedra and Martin 2005), myelin oligodendrocyte glycoprotein (MOG) (Lebar et al. 1986), transaldolase, and S100 (Sospedra and Martin 2005). The initial sensitization of T-cells in MS has been suggested to involve "molecular mimicry," in which a viral antigen with some sequence homology to a myelin protein sensitizes T-cells; however, this mechanism has never been shown to be operative in MS.

Genetics studies have been inconclusive. Much of these reports seeking a relationship between disease causality and genetic variants have involved single-nucleotide polymorphisms (SNPs) in the hope of identifying susceptibility loci. So far 20–30 SNPs have been reported and the search is continuing for more (Bahreni et al. 2010). In a recent report by the "International Multiple Sclerosis Consortium" a further 29 susceptibility loci have been reported. Since immunologically relevant genes were over represented, mapping close to the identified loci, support for an immunological theory was suggested (International Multiple Sclerosis Genetics Consortium and the Welcome Trust 2011).

Current therapies, which are all immune-based, reduce the frequency of relapses, but do not delay disease progression (Steinman and Zamvil 2006). Disease heterogeneity provides evidence for non-autoimmune biochemical abnormalities, whose role in the complex pathogenesis of MS remains unclear. An alternative to

immune-based therapies is the regulation of neurobiological pathways, which can be regulated by small molecules.

In early observations, we reported that MBP contained the noncoded amino acid citrulline and that, even in normal brain, citrullinated MBP accounted for 20 % of the total MBP, while in chronic MS it accounted for 45 % (Moscarello et al. 1994) and in fulminating MS it accounted for 90 % of the MBP (Wood et al. 1996).

With the demonstration of increased amounts of citrullinated proteins in several major diseases, the generation, metabolism, and regulation of citrullinated proteins have become a major focus of study (Vossenaar et al. 2003). In rheumatoid arthritis, citrullinated protein antibodies are found in 80 % of patients. Altered amounts of citrullinated proteins are also found in Alzheimer's disease (Ishigami et al. 2005), psoriasis (Ehrlich et al. 2004), open angle glaucoma (Bhattacharya et al. 2006), scrapie (Jang et al. 2008), and Creutzfeld–Jacob disease (Jang et al. 2010). In prion disease, deimination of PrP leads to rapid acquisition of Prp^{sc}, the disease-associated form (Young et al. 2009). In spinal cord injury, an important role for PAD enzymes has been suggested by demonstrating that PAD inhibition with a general PAD inhibitor Cl-amidine, decreased PAD activity, reduced apoptosis of neural stem cells, and improved regeneration (Lange et al. 2011).

Since deimination is also elevated in MS, inhibition of PAD enzyme activity should attenuate disease. To this end we have recently shown that the active-site PAD inhibitor, 2-chloroacetamidine (2CA) attenuated disease, decreased the amount of citrullinated protein, and decreased PAD activity in the brains of four animal models of MS, two neurodegenerative and two autoimmune (Moscarello et al. 2013). Since no one model reproduces all facets of MS, the EAE models reflect pattern 2 pathology, and the neurodegenerative models reflect pattern 3 pathology. Patterns 2 and 3 account for 80 % of the MS cases. Therefore, our models are highly relevant. In addition to MBP, histones are also citrullinated, after TNF-induced translocation of PAD4 to the nucleus, affecting chromatin structure and resulting in apoptosis of oligodendrocytes (Mastronardi et al. 2006). Therefore, increased protein citrullination represents a basic mechanism in demyelination involving several neurobiological systems (Fig. 11.2). Since the active sites of PAD enzymes are very similar, drugs directed at the active site would inhibit all PADs. Success of this approach will give strong support to the neurodegenerative theory of MS, in which the autoimmune component arises after the initial degradation of myelin, thus exacerbating the disease.

There are two consequences from these immunological processes: (1) MBP peptides are released and the immune response is generated with T-cell activation, (2) peptides in which arginine has been deiminated represent neo-epitopes, which then generate their own immune responses, (3) T-cell activation induces inflammatory demyelination. Therefore, therapy for MS requires a three-pronged attack, by decreasing citrullinated proteins, apoptosis, and T-cell pools.

Accompanying the increased citrullination of MBP, an increase in both PAD enzyme activity and amount of PAD protein has been demonstrated in NAWM from brains of MS patients (Moscarello et al. 2013). An increase in PAD protein suggests an increase in PAD transcription, which in turn is controlled by the PAD promoter.

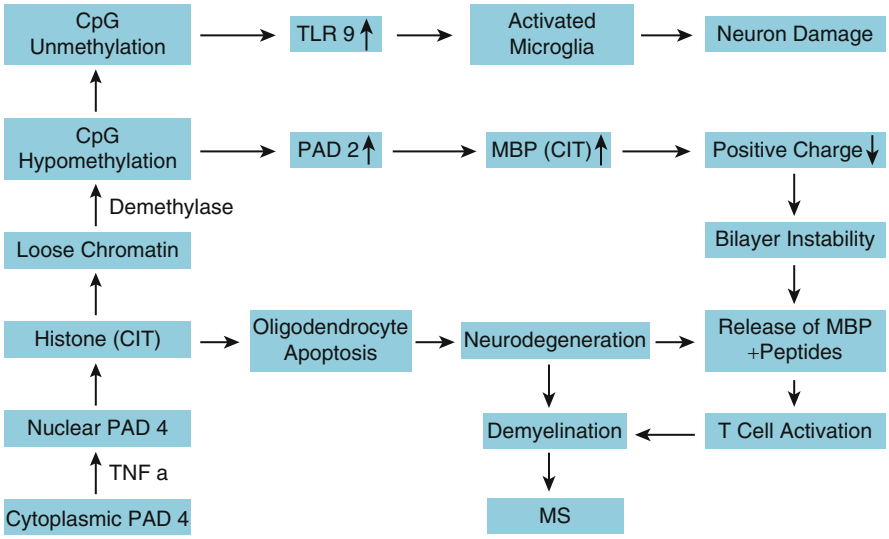


Fig. 11.2 Pathogenesis of MS

Isolation and sequencing of the PAD2 promoter showed that it was hypomethylated. In fact, cytosine methylation of CpG islands in MS tissue was 1/3 of that in normal brain. The CpG content was 74 %, much higher in normal brain than in MS. Hypomethylation of the PAD-2 promoter increased transcription of PAD2 accounting for the increased PAD2 protein in MS NAWM. Measurement of the DNA demethylase activity in NAWM showed that it was elevated twofold in MS tissue accounting for the decreased methylation of the promoter of PAD2 (Mastronardi et al. 2007a, b). The reaction sequence suggested by these data is increased DNA demethylase activity → decreased methylation → increased PAD2 transcription → increased PAD2 enzyme → increased citrullinated MBP.

A second PAD enzyme, PAD4 is also localized in brain (Wood et al. 2008). Again, PAD4 is different from other PAD enzymes because it contains a nuclear localization signal which allows it to be transported from the cytoplasm to the nucleus, where it deiminates histone (Mastronardi et al. 2006) in the presence of TNFα. As mentioned above, DNA demethylase demethylates methylcytosine in CpG islands of the promoter. As a consequence, interactions between histone and DNA are less effective. Translocation of PAD4 into the nucleus then deiminates histone, which disrupts its chromatin interactions, resulting in subsequent degradation of DNA and apoptosis of the oligodendrocyte. Apoptosis of oligodendrocytes results in neurodegeneration reflecting pattern 3 pathology. Deiminated histone induces NET formation which activates T-cells (Tillak et al. 2012).

A further effect of unmethylated CpG DNA (the result of increased DNA demethylase activity) increases the Toll-like receptor 9 (TLR9), which is increased in EAE and causes microglial activation that damages neurons. Activated microglia also produce TNFα, causing translocation of PAD4 to the nucleus (see above).

Microglia have been implicated to have a major role in cortical demyelination (Peterson et al. 2001). The mechanism of demyelination in chronically demyelinated axons involves an increase in axoplasmic Ca^{2+}. Increased Ca^{2+} in the axons stimulates PAD activities (which are Ca^{2+}-dependent enzymes). The search for citrullinated proteins in these axons would represent an important contribution to the pathogenesis of demyelination. Since cortical lesions do not contain inflammatory cells, studies of these lesions may provide important support for a neurodegenerative mechanism.

11.6.1 Rationale for Novel Therapies

MS is a heterogeneous disease of the human CNS, affecting about two million people worldwide. The cause is unknown and our understanding of the disease process (pathogenesis) is incomplete. The pathological picture shows evidence of both neurodegenerative and immunological/inflammatory processes. The neurodegenerative component involves the formation of plaques (areas of myelin degradation) and axonal loss responsible for much of the long-term disability (Trapp and Nave 2008). The immunological component involves the presence of perivascular infiltration with hematogenous cells (T-cells), a leaky blood brain barrier and an association with the HLA chromosomal region, reflecting the possibility of susceptibility genes (Okensberg et al. 2008). The mechanism by which sensitization of T-cells in the periphery takes place has not been elucidated and the failure of immune-based therapies to affect the disease process has shifted emphasis to the evolving concept that MS is primarily a neurodegenerative disease with secondary inflammatory demyelination. In addition, the presence of cortical lesions in MS without the presence of hematogenous leukocytes and the intact blood–brain barrier (Peterson et al. 2001) supports a neurodegenerative mechanism. We believe that the mechanism involves the interplay of a number of metabolic processes. One of these with widespread effects is the protein citrullination, which is represented schematically in Fig. 11.2. The value of this scheme is the identification of PAD enzymes as therapeutic targets.

11.6.2 A Novel Therapeutic Target for MS

Current therapies (see Lopez-Diego and Weiner 2008 for review), all of which are immumodulatory, reduce the frequency of relapses, but have not reached the goal of stopping or preventing the disease. As a result, the underlying pathology continues (Steinman and Zamvil 2006; Trapp and Nave 2008). Commonly used drugs include glatiramer acetate (Copaxone, Co-Polymer I), which was synthesized as a random polymer of glutamic acid, tyrosine, alanine, and lysine-based on the molar ratios of these amino acids in MBP. Its widespread binding to a diverse group of HLA molecules results in varied effects on the immune system. Mitoxanthrone, an anthracene

dione, is an anti-neoplastic agent approved for use in more aggressive disease to destroy leukocytes. Interferon β requires frequent injections, may generate neutralizing antibodies, and has many side effects including flu-like symptoms. Natalizumab (Tysabri) is a monoclonal antibody to α4β1 integrin, and thus blocks T-cells from crossing the blood–brain barrier. It was withdrawn after three cases of leukoencephalapy resulted in death. The leukoencephalapy was caused by the release of the ubiquitous and usually innocuous JC virus by an unknown mechanism. Caution is required in its use. It has been reintroduced but at lower dose.

Two oral therapies have been reported recently. Fingolimod is a sphingosine-1-phosphate receptor modulator that prevents the egress of lymphocytes from lymph nodes. It used in MS and resulted in an improved relapse rate. Adverse effects included bradycardia, atrioventricular conduction block, macular edema, and altered liver enzymes. Cladribine (2-chlorodeoyadenosine triphosphate) inhibits DNA synthesis and repair. It preferentially attacks lymphocytes, because these cells show a high ratio of deoxycytidine kinase to 5′-nucleotidase. It produces rapid and sustained reductions of $CD4^+$ and $CD8^+$ cells. Again, relapse rates were improved in MS patients. Toxic effects included lymphocytopenia and herpes zoster. None of the above-mentioned treatments affect the ongoing disease process, which continues relentlessly.

In our studies, we have identified a novel target, the PAD enzymes. Our animal studies are presented below (Moscarello et al. 2013). We used 2-chloroactamidine (2CA), an irreversible PAD active site inhibitor in four animal models of MS, two neurodegenerative and two autoimmune, representing patterns 3 and 2, respectively, of MS. In all models, disease was attenuated and remyelination was observed. Although high concentrations of 2CA were required, it was not toxic. Because 2CA binds covalently to the enzyme, its major contribution has been to define PAD enzymes as therapeutic targets. The thrust of future research is to prepare other PAD inhibitors, which are reversible and can be used at lower concentration. In fact, we have identified four other PAD inhibitors that are active in the micromolar range and are now being tested.

11.7 PAD Expression and Activity in White Matter from MS Patients

We compared protein citrullination, PAD protein expression and activity in extracts of normal brain and of NAWM from MS patient brains using the slot blot method. PAD protein expression was elevated in NAWM from MS brain (Fig. 11.3a) with a corresponding increase of PAD activity and protein citrullination (Fig. 11.3b, c). Following addition of 2CA to NAWM extracts of MS brain, PAD activity declined to normal levels, demonstrating that 2CA was effective in human brain extracts (Fig. 11.3c).

Using recombinant PAD2 and PAD4 enzymes, we then measured the residual activity with BAEE in the presence of increasing concentration of 2CA (Fig. 11.4a).

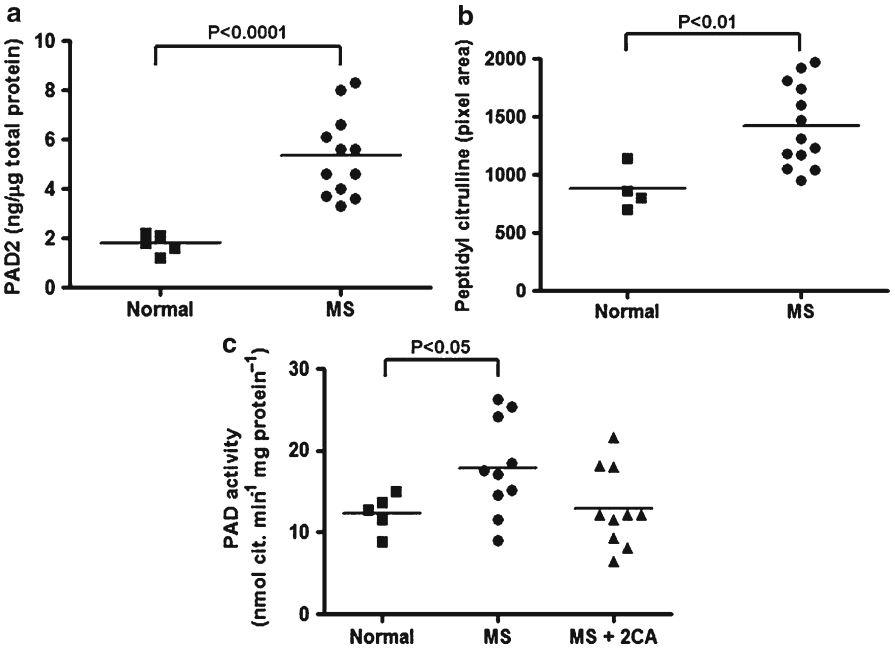
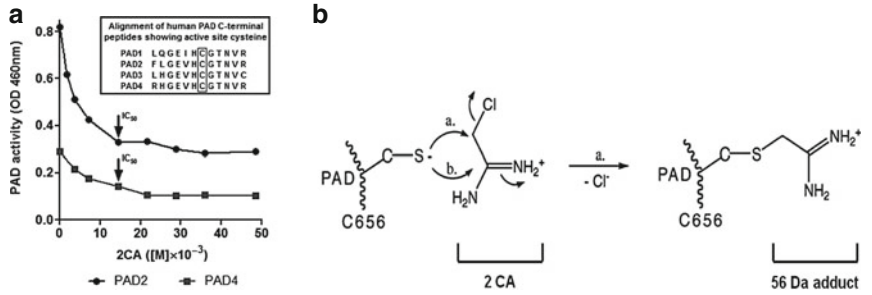


Fig. 11.3 PAD activity in NAWM of MS patients. (**a**) Quantification of PAD2 protein in white matter from normal and MS brain by immunoslot blot ($n=5$, $p<0.0001$). (**b**) Citrullinated protein in white matter from normal and MS brains by immunoslot blot as pixel density ($n=4$, $p<0.01$). (**c**) PAD enzyme activity in normal and MS tissue, with or without preincubation with 2CA ($n=5$, $p<0.05$). Each dot is one patient analyzed three times. The means (*bar*) for all the MS patients were compared with the normal. Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

A rapid decrease of activity was observed with both enzymes, more marked with PAD2, yielding an IC50 14.4 mM.

Human PAD enzymes are well conserved in the C-terminus, containing the active-site region (Fig. 11.4a inset). To identify the 2CA binding site, recombinant human PAD2 was reacted with 2CA. Trypsin digests of 2CA-reacted and unreacted enzyme were compared by LC-ESI-QToF-MS/MS analysis. We identified a tryptic peptide in 2CA-reacted PAD2 with a mass of 1386.68 Da, while the corresponding unreacted peptide had a mass of only 1330.45 Da. The difference of 56 Da corresponds to the addition of one molecule of acetamidine (Fig. 11.4c). As shown in Fig. 11.4b, we propose the mechanism of the reaction is acetamidine binding to Cys 656 in a covalent bond between the cysteine sulfur and the carbon of the acetamidine in a covalent interaction, as originally described by Stone et al. (2005). Fragmentation analysis (Fig. 11.4c) mapped the relevant peptide (^{650}FLGEVH**C**GTNVR) to the C-terminal region containing the active-site cysteine (^{656}C). A similar analysis identified the same active-site cysteine in recombinant PAD4 (unpublished). Fragmentation analysis determined that the y-ions 688-326-17 (NH3) and in PAD2 and PAD4, respectively, contained the cysteine-adduct (Fig. 11.4c).



c

Not Modified				2CA Modified			
Sequence	**y**	**y^{++}**	**y***	**Sequence**	**y**	**y^{++}**	**y***
F				**F**			
L	1184.5841	592.7957	1167.5575	**L**	1240.6215	620.8144	1223.5950
G	1071.5000	536.2536	1054.4735	**G**	1127.5374	564.2724	1110.5109
E	1014.4785	507.7429	997.4520	**E**	1070.5160	535.7616	1053.4894
V	885.4359	443.2216	868.4094	**V**	941.4734	471.2403	924.4468
H	786.3675	393.6874	769.3410	**H**	842.4050	421.7061	825.3784
***C**	649.3086	325.1579	632.2821	***C**	705.3461	353.1767	688.3195
G	546.2994	273.6534	529.2729	**G**	546.2994	273.6534	529.2729
T	489.2780	245.1426	472.2514	**T**	489.2780	245.1426	472.2514
N	388.2303	194.6188	371.2037	**N**	388.2303	194.6188	371.2037
V	274.1874	137.5973	257.1608	**V**	274.1874	137.5973	257.1608
R	175.1190	88.0631	158.0924	**R**	175.1190	88.0631	158.0924

Fig. 11.4 (**a**) PAD2&4 inhibition curves in the presence of increasing 2CA concentrations. Insert: PAD1-4 enzymes contain a common C-terminal active-site Cys residue (656Cys) bound by 2CA, confirmed by ESI mass spectrometry of tryptic digests of PAD2-acetamidine adducts. (**b**) A schematic of the nucleophilic reaction between 2CA and the 656Cys residue in the active site of PAD2, adapted from (Stone et al. 2005). (**c**) Summary of peptide fragment atomic masses for 2CA modified and native PAD2. Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

To determine if 2CA crosses into the brain, we generated monoclonal antibodies to our therapeutic target using p^{650}FLGEVH**C***GTNVR, carrying the *acetamidine adduct on the ^{656}cysteine residue. Antibody mAb4E12 recognized 2CA modified, but not unmodified PAD2 in Western blots. We injected 2CA i.p. twice per week into transgenic mice over-expressing PAD2 in white matter (Musse et al. 2008). Brain sections were stained with mAb4E12 and developed with gold-labeled anti-mouse IgG for immuno-electronmicroscopy (Fig. 11.5). Drug-PAD2 adducts were scattered in myelin sheaths and nuclei of oligodendrocytes, indicating that the drug reached its target molecules in the brain. Clusters of gold particles were found in the periaxonal region (white arrows), while gold label was absent in similarly stained sections from untreated PAD2 transgenics.

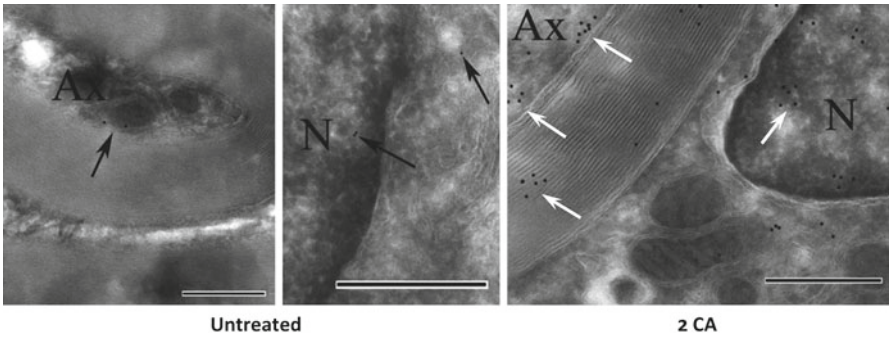


Fig. 11.5 mAb4E12 (anti-2CA adduct)-Immunogold-labeled optic nerve cryosections from control and 2CA-treated PAD2 transgenic mice. Minimal labeling in untreated mice: *arrows* in left two panels. Numerous gold particles in nuclei (N) and cytoplasm of oligodendrocytes, myelin, and axonoplasm (Ax) of 2CA-treated mice (bar: 500 nm). Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

11.8 Disease Attenuation by 2CA in Demyelinating Mouse Models

We employed four independent mouse models of demyelination, two non-autoimmune transgenic (Musse et al. 2008; Mastronardi et al. 1993) and two induced autoimmune models of demyelinating disease (Iglesias et al. 2001; Tuohy et al. 1995), thus comparing 2CA responses across different genetic backgrounds, disease causes, and progression programs.

11.8.1 Survival of Transgenic ND4 and PAD2 Over-Expresser Mice with 2CA

The first of the neurodegenerative models tested was the ND4 transgenic mice (DM20 overexpressors), which are normal from birth until ~10–12 week of age, when they develop a non-autoimmune, primary progressive, ultimately fatal CNS demyelinating condition (Mastronardi et al. 1993; Johnson et al. 1995). Disease progression is associated with increased myelin PAD expression and hypercitrullination of myelin (Moscarello et al. 2002) and histone H3 proteins (Mastronardi et al. 2006). ND4 mice received i.p. PBS, 2CA (5 mg/kg) or 2CA + 10 mg/kg B12 every other day, beginning well before disease onset (2 months, Fig. 11.6a), or early after disease onset (3.5 months, Fig. 11.6b). In another group with early treatment onset, 2CA injections were stopped after 6 weeks, while B12 injections continued (Fig. 11.6c).

Early and prolonged 2CA injections starting at 2 months of age essentially prevented disease, with or without B12 (Fig. 11.6a). Most untreated mice were

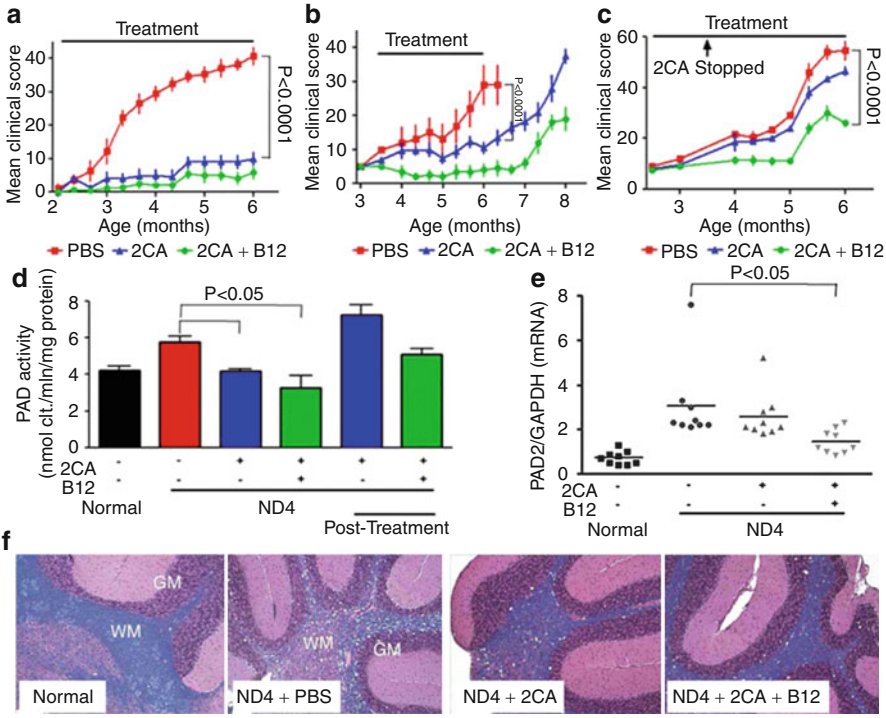


Fig. 11.6 2CA attenuates demyelinating disease in ND4 mice. (**a**) ND4 mice treated with PBS, 2CA (5 mg kg^{-1}), or 2CA+B12 (5 mg kg^{-1} and 10 mg kg^{-1}) starting at 2 months before disease onset ($n=5$, $p<0.0001$). (**b**) ND4 mice treated at disease onset ($n=4$, $p<0.0001$). (**c**) Stopping 2CA, but continuing B12 at 3.5 mol in ND4 mice ($n=5$, $p<0.0001$) demonstrating that B12 alone does not attenuate disease. (**d**) PAD activity in brains from animals is shown in Fig. 11.6b ($n=5$, $p<0.05$). The first four bars were from animals at 6 months of age, whereas the post-treatment animals were 8 months of age. (**e**) PAD2 RT-PCR in white matter extracts of normal, PBS, 2CA, and 2CA+B12 treated ND4 mice ($n=9$, $p<0.05$). (**f**) LFB and hematoxylin stain of cerebella from normal, PBS, 2CA, and 2CA+B12 treated ND4 mice (40×). WM, white matter; GM, grey matter. Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

sacrificed with severe disease by 6 months, while none died in the treatment groups. Treatment of early disease at 3.5 months prevented progression, but mild disability continued, unless animals received 2CA plus B12. However, fully progressive clinical disease reemerged promptly after therapy cessation at 6 months (Fig. 11.6b). The temporal link of disease protection by 2CA and relapse after therapy cessation suggests PAD-mediated citrullination and disease progression are a basic mechanism in the transgene-driven pathogenesis. Abbreviated 2CA treatment (2–3.5 months) was not very effective, but continued B12 injections generated milder disease and delayed progression (Fig. 11.6c). We previously reported that B12 by itself has little effect in the ND4 mouse (Mastronardi et al. 2004), which we confirm again here. Overall, 2CA induced dramatic disease attenuation, which did, however, require continued injections due to obvious persistence of pathogenic transgene expression.

To determine how improved clinical scores were related to PAD activity, 6-month-old mice from the treatment groups in Fig. 11.4b were examined (Fig. 11.6d). 2CA normalized white matter PAD activity in brain homogenates, with considerable overshoot (and rapid disease progression) in such animals after therapy cessation. B12 further reduced PAD hyperactivity, preventing the *post*-therapy overshoot (Fig. 11.6d). PAD2 gene expression (Fig. 11.4e) paralleled PAD activity, suggesting that disease-induced elevations are regulated at the transcriptional level, where PAD promoter hypomethylation may explain PAD overexpression, as well as the positive impact of B12 adjunct therapy (Mastronardi et al. 2007a).

Luxol-fast-blue staining of myelin (Fig. 11.6f) shows impressive myelin deficits and pronounced vacuolization in untreated ND4 mice, which were dramatically improved in treated mice. When treatment was removed, myelinolysis reemerged in both treatment groups, albeit milder in those that received B12. Morphological changes in myelin structure were analyzed by transmission electron microscopy of optic nerve cross-sections from 6-month-old mice (described in Fig. 11.7a–f). In non-transgenic (normal) CD1 littermates (Fig. 11.7a), axons (Ax) are well myelinated, with myelin of uniform thickness. ND4 mice show wide areas of myelin loss, degradation, and nude axons (Fig. 11.7b). 2CA treatment (Fig. 11.7c) clearly improved this morphology, with few axons seriously affected, but many still show thinning of the myelin thickness. Addition of B12 resulted in a morphological picture virtually indistinguishable from normal (Fig. 11.7d). When treatment stopped at 6 months with analysis at 8 months, showed reappearance of disease (Fig. 11.7e–f) due to the continuing presence of the transgene.

To quantify the above myelin changes, we calculated G-ratios (axon diameter/fiber diameter) from ~500 non-contiguous semi-thin sections per treatment group (Moscarello et al. 2013). Compared to healthy littermates (G-ratio 0.74 ± 0.13) ND4 mice showed a reduction in optic nerve myelin thickness: G-ratio 0.96 ± 0.3 ($p < 0.0013$). In 2CA-treated ND4, myelin thickness was slightly improved and showed less variation (G-ratio: 0.9 ± 0.15) ($p < 0.015$), while 2CA+B12 treatment nearly normalized myelin thickness to a mean G-ratio of 0.81 ± 0.18 ($p < 0.015$). These treatment data are typical for remyelination, where the original myelin thickness is never re-achieved.

Transgenic mice over-expressing PAD2 under control of the MBP promoter (Musse et al. 2008) also develop a progressive demyelinating phenotype (Fig. 11.8). Treatment with 2CA or 2CA plus B12 had little effect on early disease, but subsequent treatments prevented the second, more aggressive disease phase equally. PAD activity was high in transgenic white matter, and 2CA, with or without B12, normalized tissue enzyme activity levels (Fig. 11.8). B12 alone had little effect in this model.

11.8.2 Attenuation of Acute MOG-EAE with 2CA

EAE was induced in C57BL/6 mice with 100 μg of MOG35-55 peptide, emulsified in Freund's complete adjuvant and 300 ng of pertussis toxin. At the earliest sign of

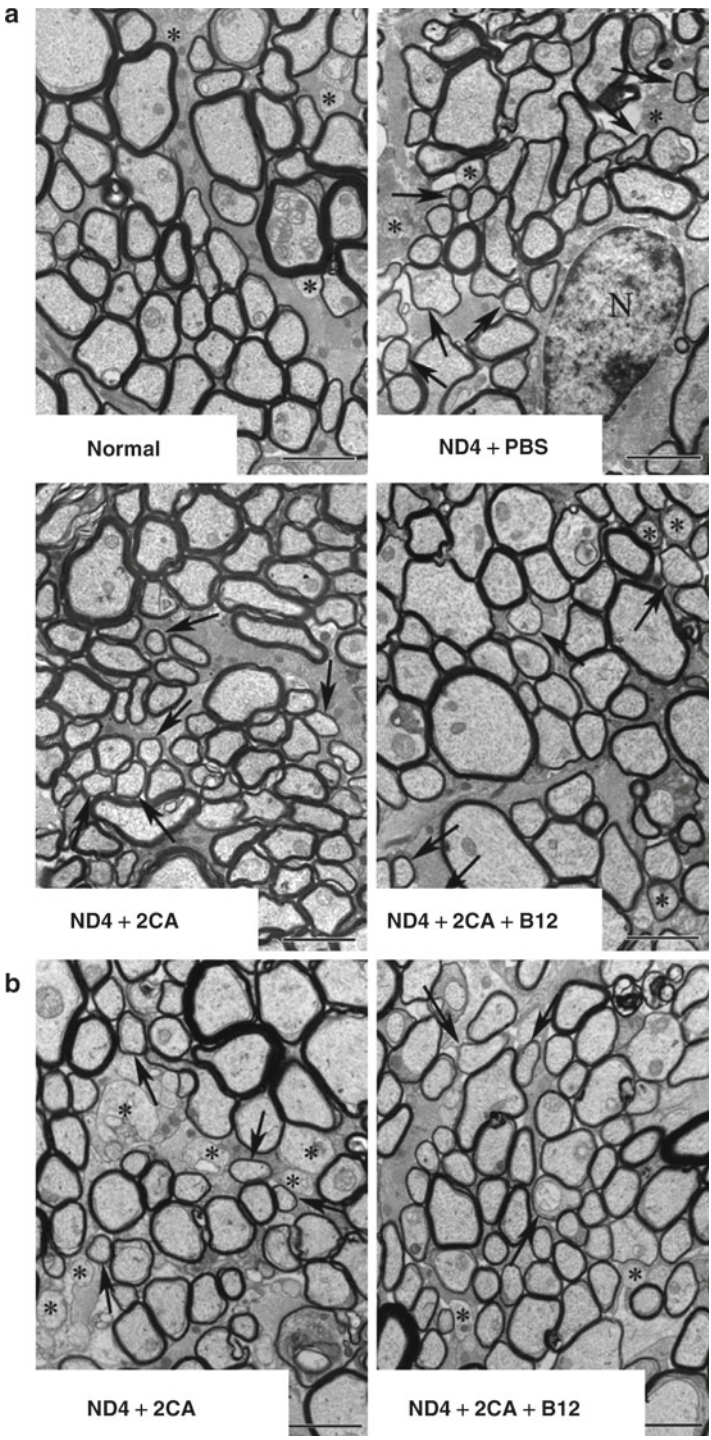


Fig. 11.7 (**a**) Transmission EM of optic nerve sections from normal, PBS, 2CA, and 2CA+B12 treated ND4 mice at 6 mol (bar: 2 μm). (**b**) Transmission EM of optic nerve sections from

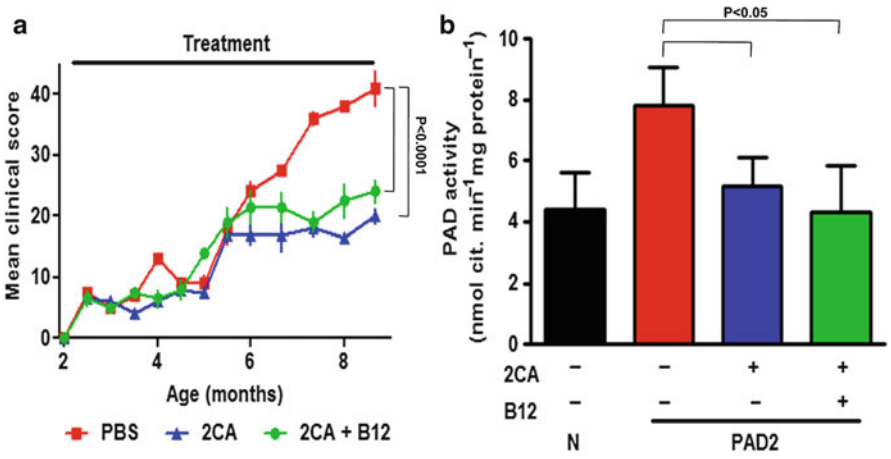


Fig. 11.8 2CA attenuates PAD2 Overexpressor. (**a**) Demyelinating disease in PAD2 transgenic mice treated with PBS, 2CA, or 2CA+B12 starting at 6 months of age ($n=5$, $p<0.0001$). (**b**) PAD activity in brain extracts of PAD2 transgenic mice treated with PBS, 2CA, or 2CA+B12, and non-transgenic littermates ($n=4$, $p<0.05$). PAD activity is reduced to normal levels by treatment. Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

disease, usually 9 days post-immunization, groups of mice received 2CA or 2CA plus B12 as before (Fig. 11.9a). Untreated mice developed rapidly progressive disease and were sacrificed when moribund (~day 19). 2CA and 2CA plus B12 treatment did not affect the disease course observed in untreated controls until ~ day 14, when progression halted and recovery began, leaving ~50 % survival by day 30 (Fig. 11.9b). When 2CA treatment was started before immunization, disease lethality was zero.

Despite the severity of disease, there was relatively little histopathology in the brain. However, vacuolar demyelination and lymphoid infiltration were prominent in the spinal cord (Fig. 11.9c left panel arrows). In contrast, surviving 2CA and 2CA plus B12 recipients showed much improved, virtually normal spinal cord histology (Fig. 11.9c, center and right panels). More sensitive immunofluorescence still detected scattered $CD3^+$ T-cells in treated animals, but the heavy T-cell clusters in control animals were absent (Fig. 11.10a), suggesting that one possible effect of 2CA may be suppression of T-cell expansion. White matter of untreated EAE mice had elevated PAD activities, which were normalized after 2CA (Fig. 11.10b). Spinal cord PAD activities of untreated mice reached threefold elevations, but reductions to normal levels were once again observed following treatment (Fig. 11.10c).

Fig. 11.7 (continued) 2CA and 2CA+B12 treated ND4 mice, 2 mol after treatment cessation (bar: 2 μm). *Asterisks* show nude axons, *arrows* show thin myelin sheaths. Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

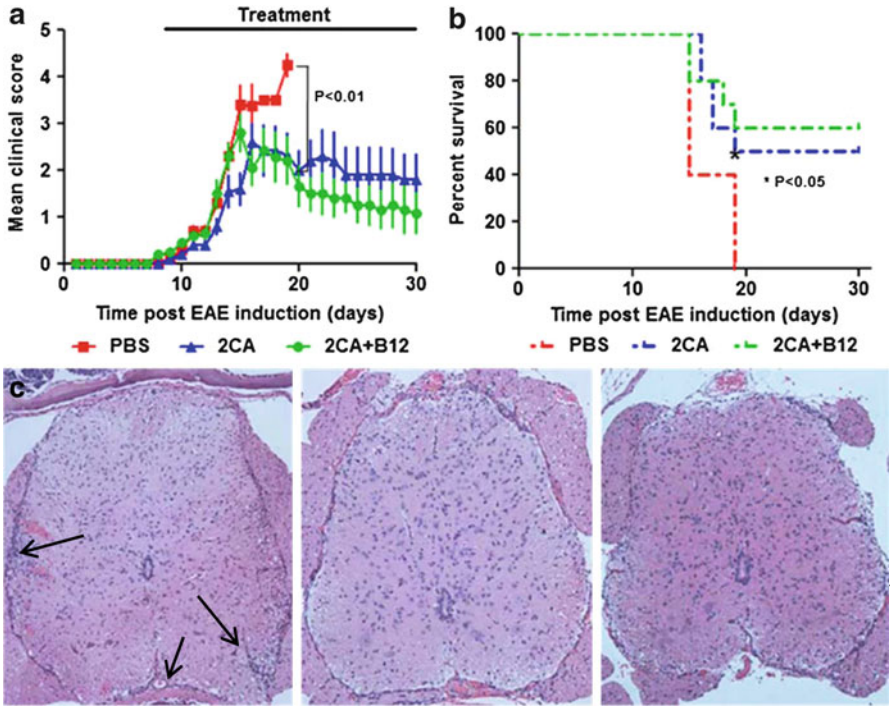


Fig. 11.9 2CA attenuates acute EAE. (**a**) Clinical progression ($n=5$, $p<0.0001$) and (**b**) survival ($n=5$, $p<0.01$) during acute MOG-EAE in C57BL/6 mice treated with PBS, 2CA, or 2CA+B12. (**c**) H&E stain of spinal cord treated with PBS, 2CA, or 2CA+B12 (40×) showing decreased cellular infiltration after treatment (*arrows*). Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

of scattered CD3+ T-cells in treated survivors (Fig. 11.10a) may be either anergic or nonspecific bystanders with little pathogenicity, since there were no relapses after therapy cessation in CD3+ T-cell population. Our conclusion of immune expansion as a major 2CA target may also explain the lack of B12 effects in acute MOG-EAE, where time is short for remyelination.

11.8.3 Attenuation of Chronic Relapsing EAE (crEAE) by 2CA

Since acute MOG-EAE is a rapid and aggressive disease, we also decided to include a crEAE model that better mimics the relapsing-remitting course common in humans with MS. Disease was induced in SJL mice with a proteolipid protein peptide (pPLP139-155, 200μg/mouse) (Tuohy et al. 1995; Mastronardi et al. 2004). Treatments with 2CA and 2CA plus B12 were started at disease onset (d10-11) with

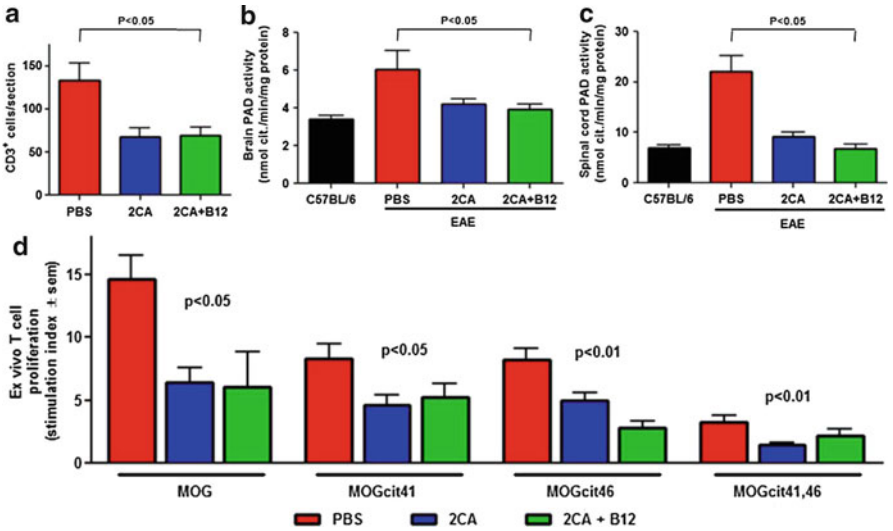


Fig. 11.10 (**a**) Spinal cord CD3+ lymphocytes in MOG-EAE mice treated with PBS, 2CA, or 2CA+B12 ($n=12$, $p<0.05$). (**b**) PAD activity in brain ($n=4$, $p=$n.s.). (**c**) Spinal cord extracts ($n=4$, $p<0.05$) showing threefold increase in EAE reduced to normal levels by 2CA. (**d**) Ex vivo recall proliferative splenocytes responses of C57BL/6 mice with peak disease stimulated with MOG35–55 and MOG35–55 citrullinated at residue 41 (MOGcit41), residue 46 (MOGcit46), and residues 41 plus 46 (MOGcit41,46) ($n=8$, $p<0.05$ and $p<0.01$). Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

different treatment schedules indicated in the top bars of Fig. 11.11. Attenuation of disease was observed in all treated mice (Fig. 11.11a). In contrast to our previous findings, where B12 showed synergistic effectiveness in interferon-β-treated crEAE mice (Mastronardi et al. 2004), there was little added benefit when B12 was combined with 2CA.

After pilot experiments suggested steep excursions at disease onset, we followed PAD activity over the disease course in 20 mice (Fig. 11.11b). PAD activities indeed showed a sharp rise at disease onset, peaking at the height of the initial disease phase, and declining to still above-background levels thereafter. Protein citrullination in white matter was consequently elevated, a process prevented by 2CA (Fig. 11.11c). Besides strain differences, the different EAE induction modes critically reflect choice of the inducing myelin peptide, thus involving a cognate recognition event in $CD4^+$ and/or $CD8^+$ T-cells with potentially encephalitogenic T-cell receptors (Zhu et al. 2006). The extent of disease was closely reflected by the extent of cellular infiltration in white matter sections (Fig. 11.12d) confirmed by measuring $CD3^+$ T-cell invasion around blood vessels (Fig. 11.12a). As with the MOG-EAE model, 2CA also prevented and/or reduced T-cell tissue infiltrates in the chronic relapsing model of EAE (Figs. 11.11d and 11.12a).

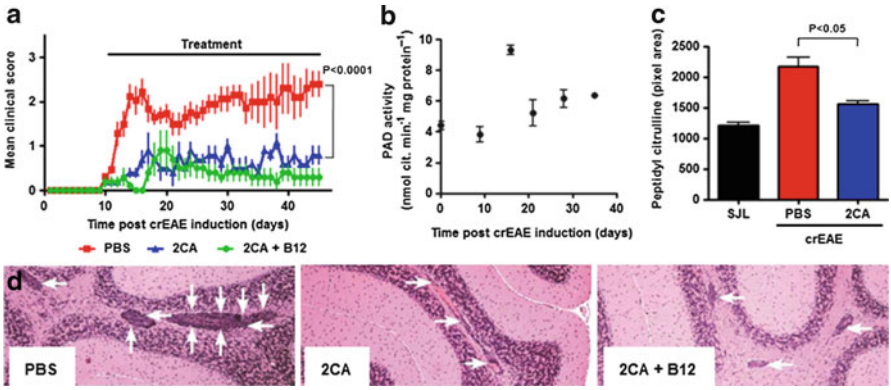


Fig. 11.11 2CA attenuates chronic relapsing EAE (crEAE). (**a**) Clinical crEAE progression in SJL mice treated with PBS, 2CA, or 2CA+B12 ($n=5$, $p<0.0001$). (**b**) PAD activity in SJL brain extracts during crEAE ($n=4$). (**c**) Protein citrullination in brains of SJL animals ($n=5$, $p<0.0001$) after termination of the experiment at 42 days. (**d**) H&E stain of SJL brain (40×) showing infiltration of cells (*white arrows*). Note the decrease after treatment. Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

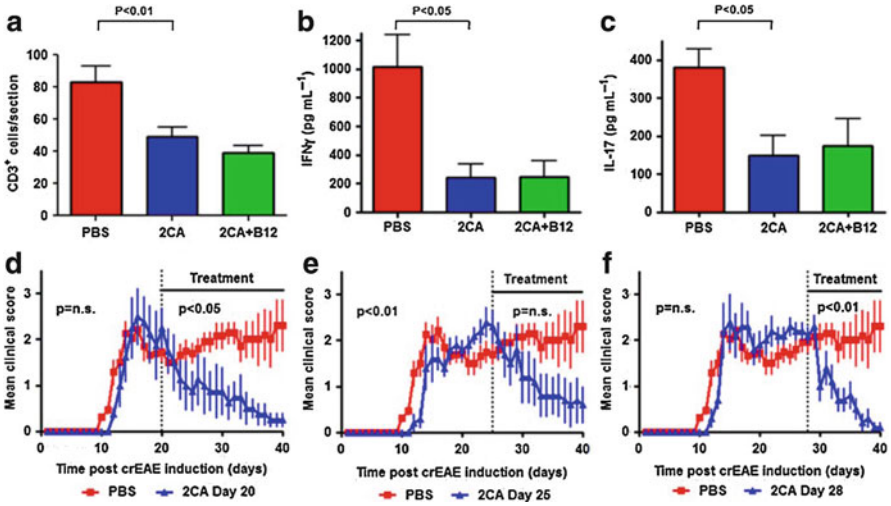


Fig. 11.12 (**a**) CD3+ lymphocytes in SJL spinal cord ($n=12$, $p<0.01$). (**b**) IFN-γ ($n=3$, $p<0.05$) and (**c**) IL-17 ($n=3$, $p<0.05$) secretion following in vitro stimulation of SJL splenocytes with PLP139-155. (**d**, **e**, **f**), Progression of crEAE following treatment with PBS or 2CA, beginning on day 20, 25, or 28. ($n=5$, $p<0.0001$). Modified from Moscarello et al., Disease Models and Mechanisms, Volume 6, pp. 467–478, Copyright 2013, The Company of Biologists, Ltd.

We also compared systemically distributed pPLP139-155-specific T-cells of treated and untreated mice, via their release of ex vivo, peptide-induced effector cytokines IFNγ and IL-17 (Fig. 11.12b, c). The dramatic depression of cytokine responses to near background values was interpreted to suggest that 2CA interfered with recruitment and/or expansion of the "inciting" T-cell repertoire.

The chronic phase of crEAE allowed us to examine 2CA effects in greater detail (Fig. 11.12d–f). For example, PAD inhibition was effective in abrogating disability at all stages of disease progression (Fig. 11.12d, f). The prompt decline of clinical signs following 2CA application in mice with crEAE implies a very rapid local and systemic (Fig. 11.12b, c) loss of effector cells, following inhibition of continued protein deimination. Tissue half-life times of citrullinated proteins are unknown, as is the persistence of the relevant, processed peptides in local antigen presenting cells. The rapidity of 2CA's beneficial effects therefore makes direct actions of the drug on T-cells and/or APC likely, since both cell lineages express PAD (Liu et al. 2006).

11.9 Concluding Remarks

MS is the most common demyelinating disease of humans with persistent unmet therapeutic needs. Pathologically, it is a heterogeneous disease, which shows the features of several processes, notably, neurodegeneration, apoptosis, and T-cell activation. Successful therapy requires normalization of all three features by a single therapeutic agent, which can only be possible if a common biological pathway can be identified as the target.

Since MBP is a putative autoantigen in MS and the major myelin protein responsible for maintaining the compaction of the lipid bilayers and the integrity of the myelin sheath, we have studied extensively the mechanism by which this occurs and the interactions required. We have discovered that the positive charge on MBP residing in 19 arginyl and 12 lysyl residues is affected by several posttranslational modifications, all of which decrease the positive charge. Since stability of myelin compaction requires strong interactions between the basic residues on MBP and the negatively charged phosphates of the phospholipids, loss of positive charge results in decreased myelin compaction, shedding of MBP and proteolytic digestion to activate the immune response. We studied citrullination of MBP, an irreversible modification on myelin and found loss of compaction that correlated with the extent of deimination. This mechanism may explain demyelination.

A second feature of MS is apoptosis of oligodendrocytes. Deimination of histones is a similar mechanism as the one above, in which a highly basic protein (histone) interacts with the negatively charged phosphate groups of DNA to form a compact chromatin structure. Deimination of histones, which is well known, compromises these tight interactions, exposing the DNA to nuclease digestion. In this case, the responsible enzyme is PAD4, which can be translocated into the nucleus because of its nuclear localization sequence.

A third feature in MS is T-cell infiltration, also found in acute and chronic EAE. Whereas treatment of features 1 and 2 of MS shows decreased hypercitrullination

by PAD inhibition, the mechanism of action of PAD inhibition in decreasing T-cell pools is not completely understood at this time. The recent involvement of PAD enzymes in the production of neutrophil extracullular traps (NET) by deiminating histones (Li et al. 2010), and the subsequent demonstration that NETS can directly prime T-cells, reducing their activation threshold (Tillak et al. 2012), provides strong support for the important role of PAD enzymes in this process as well.

Since the above-mentioned features found in patterns 2 and 3 of MS account for 80 % of the MS cases studied by Lucchinetti et al. (2000), PAD enzymes represent a promising target for therapeutic intervention. The active sites of all PAD enzymes are almost identical, thus a single drug should attenuate all processes. This three-pronged attack on myelin destabilization, apoptosis and T-cell expansion, suggests that PAD enzyme antagonism represents a promising approach to therapy.

Although the above-mentioned studies of MBP citrullination have been well documented, other effects, such as those on signal transduction and where signal transduction fits into the complex pathogenesis of MS, are unknown. The identification of MBP as a small "G" protein has been shown by its ability to bind GTP and to be ADP-ribosylated. Other signal transduction characteristics include the SH3 binding domain and as a substrate for protein kinases, notably MAP kinase, all of which are negatively affected by deimination of MBP. Future investigations will determine where signal transduction and its regulation by citrullination fit into the pathogenesis of MS. The commonality of the "hypercitrullination syndrome" is maintained.

What do these studies add to the ongoing debate about the origins of MS? Is it immunologically based, utilizing T-cells sensitized in the periphery and that invade the brain and destroy myelin (the outside-in hypothesis), or is it primarily a neurodegenerative process, using an "inside-out" hypothesis with a secondary immune response (Stys et al. 2012). The data collected over the years favors a primary neurodegenerative etiology in our opinion.

Although the mechanism of signal transduction in axons and myelin is not understood, deimination clearly supports an "inside-out" etiology for MS. Significantly, PAD enzymes have been localized to the periaxonal space, where the oligodendrocyte contacts myelin. Disruption of the signal transduction mechanism would lead to an altered distribution of lipids and microtubules and to neurodegeneration, where myelin destabilization by citrullinated MBP, coupled with the release of myelin antigens and stimulation of the immune response, the full picture of MS is manifest. The commonality in all these neurobiological processes is protein hypercitrullination, mediated by PAD enzymes. Therefore, PAD enzymes represent a promising therapeutic target by small molecule PAD inhibitors.

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Chapter 12
Deimination in Prion Diseases

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Keywords Peptidylarginine deiminase 2 • Creutzfeldt-Jakob disease • Scrapie • Prion • Enolase • Neurodegeneration • Subcellular fraction • Calpain • Reactive astrocytosis

12.1 Introduction

The accumulation of misfolded proteins, altered redox homeostasis, and the post-translational modification of proteins have been implicated in various pathological states of progressive neurodegenerative disorders such as Alzheimer's disease (AD, see Chaps. 13 and 14), Parkinson's disease (see Chap. 14), and prion diseases. Also known as transmissible spongiform encephalopathies, prion diseases are a group of chronic, fatal neurodegenerative disorders that affect the central nervous system

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(CNS) of humans and animals (Prusiner 1998). Animal prion diseases include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in mule deer and elk, transmissible mink encephalopathy, and feline spongiform encephalopathy in domestic and exotic cats. Human prion diseases include kuru, classical Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia, and variant CJD (vCJD). To study these diseases, numerous animal models have been established by experimental transmission studies in rodents such as mice, hamsters, and bank voles (Tateishi 1996; McKintosh et al. 2003; Nonno et al. 2006).

Prion diseases are characterized by various neurological symptoms and common histopathological features, including spongiform degeneration of the CNS, reactive gliosis, neuronal loss, and the accumulation of disease-associated misfolded prion proteins (termed PrP^{Sc}) in the CNS (Prusiner 1998). Prion diseases involve the conversion of the cellular prion protein (PrP^{C}) into an abnormal proteinase K-resistant and infectious isoform (PrP^{Sc}), which is the principal molecular basis of prion diseases (McKintosh et al. 2003; Prusiner 1991). A growing number of studies suggest that posttranslational modifications of PrP^{C} and other structural proteins are responsible for the conversion of PrP^{C} into PrP^{Sc} (Otvos and Cudic 2002; Dear et al. 2007). The accumulation of PrP^{Sc} in the CNS may be responsible for neuronal loss and/or astrocytosis (Cronier et al. 2004), and these changes are consistently observed during the neurogenesis and neuropathology of prion diseases.

To date, a sum of evidence suggests that perturbations of intracellular Ca^{2+} homeostasis and Ca^{2+}-related proteins play an important role in synaptic dysfunction and eventual neuronal loss in neurodegenerative diseases. Prion infection and the absence of PrP^{C} disrupts Ca^{2+} homeostasis through the alteration of receptor-mediated intracellular Ca^{2+} responses (Wong et al. 1996; Takenouchi et al. 2007) and the regulation of local Ca^{2+} movements (Lazzari et al. 2011), which suggests a role for Ca^{2+} in neuronal cell death characteristic of prion diseases. In addition, increased peptidylarginine deiminase 2 (PAD2), the abnormal accumulation of various deiminated proteins, and the impairment of intracellular Ca^{2+} homeostasis have been detected in various neurodegenerative conditions (Asaga and Ishigami 2001; Ishigami et al. 2005). PADs irreversibly catalyze the posttranslational deimination (citrullination) of peptidylarginine to peptidylcitrulline in a Ca^{2+}concentration-dependent manner, and these enzymes are directly affected by Ca^{2+} homeostasis (Inagaki et al. 1989; Vossenaar et al. 2003). This chapter reviews our recent findings that suggest a possible role for PAD-mediated deimination in the pathological changes associated with prion diseases.

12.2 The Puzzle of PrP Conversion: Potential Role of Deimination

The accumulation of conformationally altered and aggregated PrP underlies a key pathological event that leads to several devastating transmissible prion diseases in humans and animals (Colby and Prusiner 2011). The most common form of human

prion disease, sporadic CJD (sCJD), may be caused by somatic mutations of human PrP gene (PRNP) or the posttranslational modification of PrP, followed by the spontaneous conversion of PrP^{C} to PrP^{Sc} (Prusiner 1989; Borchelt et al. 1990; Collinge 1997; Beck et al. 2004). PrP^{C} is highly enriched in CNS neurons, but this protein is also detected in glial cells, such as astrocytes and oligodendrocytes (Moser et al. 1995), and various peripheral tissues (Manson et al. 1992). PrP^{C} is a membrane glycosylphosphatidyl inositol-anchored glycoprotein that is expressed on the cell surface (Stahl et al. 1987; Caughey et al. 1989). The secondary structure of PrP^{C} consists of a long flexible N-terminal tail, a globular domain with three α-helices and several pairs of small antiparallel β-sheet stands, and a short carboxy-terminal tail (Riek et al. 1997). Spectroscopic analysis has revealed that PrP^{C} contains approximately 42 % α-helices and 3 % β-sheets, whereas PrP^{Sc} contains 30 % α-helices and 43 % β-sheets (Pan et al. 1993). This conformational transition of PrP may be a crucial step for the generation of PrP^{Sc} in vivo.

However, it is still in debate, whether the accumulation of PrP^{Sc} in vivo directly causes prion diseases. The "protein-only" or "prion hypothesis" suggests that an abnormal conformational conversion of PrP, PrP^{Sc}, acts as an infectious agent that initiates self-propagation by recruiting other PrP^{C} molecules, and it is this form that underlies many of the prion diseases (Prusiner 1991; Jackson et al. 1999; Corsaro et al. 2012). This hypothesis has been highly controversial for decades, but recent studies have generated infectious PrP^{Sc} using in vitro conversion techniques in both cell-free and cell culture systems (Castilla et al. 2005; Deleault et al. 2007; Barria et al. 2009; Wang et al. 2010; Legname et al. 2004; Makarava et al. 2010).

Among the posttranslational modifications, PAD-mediated deimination contributes to the structural and functional diversity of proteins by altering the net loss of positive charge, conformational change, the isoelectric point (*pI*) value, and electrophoretic mobility (Tarcsa et al. 1996; Jang et al. 2012). Interestingly, the posttranslational deimination of arginine residues in recombinant ovine PrP (ovPrP) was shown to yield a higher content of secondary β-sheets and amyloid congophilic structures, with an increased resistance to trypsin digestion, and the loss of PK sensitivity in the presence of copper, compared with non-deiminated PrP (Young et al. 2009). This study further suggested that deimination of R151 and R154 in the helix 1 region of ovPrP leads to the destabilization of PrP native folding and that the trypsin-resistant conformation of deiminated ovPrP acts as a template for the conversion of unmodified PrP. Earlier studies demonstrated that arginine residues, such as R25 and R37 in the N-terminus of PrP^{C}, are posttranslationally modified, which suggests that such changes may occur during PrP conversion (Prusiner 1991; Turk et al. 1988; Hope et al. 1988; Safar et al. 1990). Moreover, previous reports have suggested that deimination on the N-terminal region of PrP modulates its physiological functions, such as the copper-binding affinity of the octarepeats, cell surface trafficking and its interaction with cellular proteins (Nunziante et al. 2003; Zomosa-Signoret et al. 2008). Although PAD-mediated deimination does not commonly share the specific consensus motif, further characterization is required to fully understand the substrate specificity of PAD enzymes and involvement of deiminated PrP prior to, and after, the conformational change of PrP^{C} to PrP^{Sc} in vivo.

12.3 PAD-Mediated Deimination in the Pathogenesis of Prion Diseases

The accumulation of deiminated proteins and/or abnormal PAD activation has been reported in various neurodegenerative conditions, including AD, multiple sclerosis (MS, see Chaps. 11 and 12), hypoxia, and kainic acid-evoked neurodegeneration (Asaga and Ishigami 2001; Ishigami et al. 2005; Vossenaar et al. 2003; Sambandam et al. 2004). We recently analyzed PAD2 expression and the profiles of deiminated proteins in a widely used animal model, ME7 scrapie-infected mice, to investigate the role of PAD-mediated deimination in prion diseases (Jang et al. 2008). This study demonstrated that various molecular weights of deiminated proteins, specifically those between 10 kDa and 150 kDa, were markedly increased in whole brains and various dissected brain regions, including the cerebral cortex, hippocampus, striatum, and brainstem, of scrapie-infected mice. In correlation with these data, the expression levels of PAD2 protein and mRNA in these mice were also up-regulated in most brain regions and similar results were observed in the brains of 87 V scrapie-infected mice and 263 K-infected hamsters (unpublished data), suggesting that an increase in PAD-mediated deimination after scrapie infection is a general phenomenon. These data demonstrated that PAD2 enzyme activity reached maximum levels at the end stage of the disease, which suggests a correlation between abnormal PAD2 activation and disease development.

To further characterize PAD2 and deiminated proteins in the pathogenesis of human prion diseases, we also examined the profiles of deiminated proteins in the frontal cortex of patients with sCJD, using Western blot analysis and immunohistochemical staining (Jang et al. 2010). The accumulation of various deiminated proteins paralleled increased PAD2 expression and enzyme activity that were detected in the brains of sCJD patients, which is consistent with the data from an experimental mouse model of prion disease (Jang et al. 2008). In addition, no significant differences in specific deiminated proteins or the accumulation levels of deimination were observed between prion disease and other neurodegenerative diseases such as AD.

Current knowledge of the molecular and pathological features of PAD enzymes is limited (see Chap. 21), but elevated PAD2 in the hippocampus during AD progression suggests that PAD2 activation is correlated with the onset of neurodegenerative change in this disease (Ishigami et al. 2005). However, it is still not known whether PAD2 activation is a cause or effect of prion disease progression. The activation of PADs, which catalyze the conversion of arginyl residues to citrullyl residues, is induced by elevated levels of intracellular Ca^{2+} or the leaking of these enzymes into the extracellular space due to cell membrane disruption (Takahara et al. 1986; Nijenhuis et al. 2004). The half-maximal activity (*Ka* value) of PAD enzymes is observed at 40–60 μM Ca^{2+}, and this Ca^{2+} concentration is reduced to 15–20 μM by the supplementation of phosphatidylserine with diacylglycerol (1,2-diolein), which supports the interaction of phospholipids with PAD in a membranous environment (Takahara et al. 1986). These findings suggest that PAD-mediated deimination occurs at extremely high Ca^{2+} concentration ranges, as compared to the physiological range

of 10^{-8} to 10^{-6} M, but factors other than Ca^{2+} may enable PAD function at physiological concentrations of Ca^{2+} within the cell (György et al. 2006). A recent study has provided important new insight that the interaction of the N-terminus of PrP^C (23–89) with membrane regions containing high concentrations of anionic phosphatidylserine in an acidic pH produces a morphological change from an exclusively random coil to a significant increase in β-sheet structures (Boland et al. 2010). Thus, PAD-mediated deimination of PrP may occur in disrupted cell membranes where PAD is exposed to phosphatidylserine. The increase in intracellular Ca^{2+} levels, which acts at activity and transcriptional levels, is the primary regulator of PADs. However, steroid hormones such as estrogen and progesterone, epidermal growth factor, insulin, vitamin D, retinoic acid, F- and Cl-amidine, paclitaxel, and a reducing environment also modulate PAD activity (Takahara et al. 1992; Cherrington et al. 2010; Méchin et al. 2007; Luo et al. 2006; Chumanevich et al. 2011; Pritzker and Moscarello 1998; Fujisaki and Sugawara 1981; Kubilus and Baden 1983). Therefore, the regulation of PAD2 activity is highly complex with multiple interdependent control points. The question of whether PAD regulation of PrP deimination controls PrP conversion requires further investigation.

12.4 Subcellular Localization of PAD2 and Deiminated Proteins in Prion Diseases

Five isotypes (PAD1-4 and 6) of PAD with distinct substrate and tissue specificities in humans and rodents (see Chaps. 1 and 21) have been identified (Vossenaar et al. 2003). PAD2 protein is expressed throughout the nervous system and has been identified in neurons (Jang et al. 2011; Shimada et al. 2010) and glial cells, such as astrocytes (Jang et al. 2011; Vincent et al. 1992), microglia (Vincent et al. 1992; Asaga et al. 2002), and oligodendrocytes (Vossenaar et al. 2003; Akiyama et al. 1999). PAD2 expression is also detected in cultured Schwann cells (Keilhoff et al. 2008). Reactive astrogliosis and neuronal cell death are remarkable pathological features of prion diseases. Therefore, our findings are interesting, since PAD2 expression was predominantly localized within glial fibrillary acidic protein (GFAP)-positive reactive astrocytes and a few B4-isolectin-positive microglial cells in the brains of scrapie-infected mice and sCJD patients (Jang et al. 2008). Other studies have reported the abnormal activation and/or up-regulation of PAD2 primarily in reactive astrocytes in the hippocampal region of AD patients (Ishigami et al. 2005) and in human astrocytes exposed to prolonged hypoxia (Sambandam et al. 2004), which is consistent with our results. PAD2 overexpression in transgenic mice leads to activation of astrocytes and microglia via the accumulation of deiminated proteins (Musse et al. 2008). This, in turn, is accompanied by demyelination, suggesting that a relationship exists between increased levels of PAD2 expression in glial cells and the pathogenesis of MS and MS-like diseases. These results suggest that PAD2 expression in glial cells may be controlled by disease-related factors or abnormal environmental conditions.

We performed subcellular fractionation and immunogold electron microscopic examination in the brains of normal and scrapie-infected mice to elucidate the changes and correlations in the expression and localization patterns between PAD2 and deiminated proteins. The majority of PAD2 was localized within the cytosol of scrapie-infected and control mice, and low levels were observed in the membrane fractions containing mitochondria and microsomes (Jang et al. 2011). PAD2 enzyme activity was also increased significantly in each subcellular fraction, including mitochondria, microsomes and cytosol, compared to controls. This observation may be explained by a previous report showing that the activity and Ca^{2+} sensitivity of PAD was increased at the cell membrane (Takahara et al. 1986). Various sizes of deiminated proteins were also abundantly present in the microsomal and mitochondrial fractions of scrapie-infected brains, despite a relatively low level of PAD2 expression. In contrast, only one band predominated in the cytosolic fraction (at approximately 50 kDa) of scrapie-infected brains (Jang et al. 2011). These findings suggest that the expression level and localization of PAD2 are not directly correlated with hyperdeimination or the accumulation of deiminated proteins.

PADs are widely distributed in mammalian tissues. PAD2 exhibits a cytoplasmic distribution, but PAD4, which deiminates histones, primarily resides in the cell nucleus (Vossenaar et al. 2003; Mastronardi et al. 2006; Nakashima et al. 2002). We observed a surprising increase in PAD2 expression, which was detected at similar levels in cytosolic fractions, and accumulated deiminated proteins in the nuclear fractions of scrapie-infected brains (Jang et al. 2011). However, nuclear PAD4 was detected at a very low level and no changes in expression levels were observed in the nuclear fractions of control versus scrapie-infected brains. Our extended study demonstrated that PAD4 and deiminated histone H4 were not detected in whole-brain homogenates or the nuclear fractions from non-CJD and sCJD brains (Jang et al. 2011), which suggests that PAD2 is the predominant PAD isoform in prion disease-affected brains.

PAD2 and PAD4 are expressed in the brains of MS patients, and an increase in nuclear PAD4 and protein deimination have been observed in myelin (Musse et al. 2008; Mastronardi et al. 2006; Wood et al. 2008). PAD4 contains a nuclear localization signal (NLS) at the N-terminal domain (56-PPAKKKST-63) of its amino-acid sequence (Arita et al. 2004), which presumably translocates PAD4 to the nucleus. The over-expression of PAD4 alone generally does not induce its translocation, as additional factors, such as tumor necrosis factor-α (TNF-α) have been shown to be required (Musse et al. 2008). We assessed the sufficiency of PAD2 over-expression alone for nuclear localization because we observed increased expression levels of PAD2 in humans and a mouse model of prion diseases. We also found high levels of PAD2 in the nuclear fractions of H4 human neuroglioma cells that stably expressed human PAD2 protein (Jang et al. 2011). These results suggest that PAD2 over-expression is sufficient to target this protein into the nucleus, but further studies are required to confirm this result. Unlike PAD4, PAD2 does not possess a NLS sequence (Arita et al. 2004); therefore, PAD2 translocation to the nucleus may be mediated by interactions with other molecules that contain a NLS or additional unknown factors. The role of PAD4 cannot be excluded, but our study demonstrated

that PAD2 was expressed in the cytosol and nucleus, whereas nuclear PAD4 was barely detectable in prion disease. Therefore, it will be of interest to determine whether the increase in deiminated nuclear proteins is caused by nuclear PAD2 or PAD4. In addition, the factors required for nuclear PAD2 localization need to be identified and characterized in future studies.

Finally, we performed electron immunogold labeling on hippocampal sections from the brains of control and scrapie-infected mice to identify the subcellular compartment-specific localization of PAD2 and deiminated proteins. The hippocampus, which is the most damaged region in ME7 scrapie-infected brains, exhibited significantly different PAD2 expression in control and scrapie-infected mice (Jang et al. 2008). Electron microscopic analysis further revealed that the increase in PAD2 and deiminated proteins in scrapie-infected brains was widely distributed among astrocytes and neurons and that the immunogold particles were located in most cellular compartments, including the mitochondria, endoplasmic reticulum (ER), astrocytic glial filaments, nuclei, and Golgi apparatus, as well as freely in the cytoplasm. This result is consistent with our previous studies where PAD2 was detected in reactive astrocytes in the brains from humans with AD and prion diseases and in cortical neuronal cells and Purkinje cells in the cerebellum during disease development and aging (Ishigami et al. 2005; Jang et al. 2008; Jang et al. 2010; Shimada et al. 2010). We also demonstrated that a high density of immunogold particles in astrocytes and neurons of scrapie-infected mice could be found in nuclear structures, including the nuclear membrane and perinuclear heterochromatin. These results suggest that the localization of PAD2 and its substrates in neuronal and glial cells are controlled under appropriate conditions and that PAD2 activation is regulated by, not only intracellular Ca^{2+} levels, but also other factors, which lead to increased levels of cell type-specific deiminated proteins.

12.5 Implication of Identified Deiminated Proteins in the Pathogenesis of Prion Diseases

The occurrence of deiminated proteins is associated with disease development and progression, and three distinct deiminated proteins have been described previously: GFAP in AD (Ishigami et al. 2005), MBP in MS (Wood et al. 1996), and α-enolase in rheumatoid arthritis (RA) (Kinloch et al. 2005). Using two-dimensional gel electrophoresis (2-DE) and MALDI-TOF mass spectrometry analyses, we identified various deiminated candidates in the brains of scrapie-infected mice and patients with sCJD. These included mitochondrial proteins, such as malate dehydrogenase 2 and voltage-dependent anion channel 1, neuron-specific proteins, such as neuron-specific enolase (NSE), astrocyte-specific proteins such as GFAP, cytoskeletal-associated proteins, such as GFAP, MBP, and vimentin, and energy metabolism-associated proteins such as α-enolase, NSE, and aldolase C (Jang et al. 2008, 2010). However, we could not identify all of the deiminated spots because of their low concentration and/or inability to match in a subsequent database search. These candidate proteins

Table 12.1 Deiminated candidates in brains of patients with sporadic Creutzfeldt–Jakob disease and scrapie-infected mice

Identification	CJD	Scrapie	Functions and localization
Vimentin	+	–	Intermediate filament, ubiquitously
Glial fibrillary acidic protein	+	+	Intermediate filament, ubiquitously
Tubulin, beta 2	–	+	Microtubule protein, ubiquitously
Myelin basic protein	+	+	CNS myelination, CNS and hematopoietic cells
Cofilin 1, non-muscle	–	+	Actin-binding protein, cytosol and nucleus
Enolase 1, alpha	+	–	Energy metabolism, cytosol
Enolase 2, gamma neuronal	–	+	Energy metabolism, cytosol
Aldolase 1, A isoform	+	+	Energy metabolism, cytosol
Aldolase 3, C isoform	–	+	Energy metabolism, cytosol
Malate dehydrogenase 2	–	+	Energy metabolism, mitochondria
Phosphoglycerate kinase 1	+	–	Energy metabolism, cytosol
Voltage-dependent anion channel 1	–	+	Channel, mitochondria
Peroxiredoxin 1	–	+	Antioxidant, cytosol
Cyclophilin A	+	+	Protein folding, cytosol
Carbonyl reductase 1	+	–	Arachidonic acid metabolism, cytosol and microsome

Modified from Jang et al. Am J Pathol (2008), Acta Neuropathol (2010)

are related to cellular energy production-related pathways and cellular structure (Table 12.1), and the results indicated that GFAP, MBP, aldolase, enolase, and cyclophilin A were detected in the brains of scrapie-infected mice and sCJD patients. This section focuses on the possible role(s) of these deiminated proteins in the pathogenesis of prion diseases.

12.5.1 Deiminated Cytoskeletal-Associated Proteins

Increased deiminated GFAP and MBP have been identified in degenerative disorders. We also observed extensive reactive astrocytosis with increases in GFAP expression and deiminated GFAP in prion diseases (Jang et al. 2008, unpublished data). In reactive astrocytes, GFAP acts as a major intermediate filament protein in addition to vimentin and nestin (Schiffer et al. 1986; Lin et al. 1995). PAD2 was observed in the glial filaments of scrapie-infected astrocytes (Jang et al. 2008) and GFAP was shown to be necessary for the morphological retention and distribution of reactive astrocytes during prion disease (Gomi et al. 2010). Therefore, the deimination of GFAP may play a role in the pathogenesis of prion diseases. Deiminated MBP is an arginine-rich protein with 19 arginine residues in its protein sequence and is rapidly degraded by cathepsin D (Pritzker et al. 2000), an enzyme that is elevated in scrapie (Diedrich et al. 1991). These results may explain the decreased expression levels of MBP, although its deiminated form was abundant in scrapie-infected mice (Jang et al. 2008).

12.5.2 Deiminated Energy Metabolism-Associated Proteins

A physiological connection has been demonstrated between PrP^C and aldolase C (Strom et al. 2006), and aldolase A and C have been identified as PAD substrates in prion disease (Jang et al. 2008). Aldolases are ubiquitous enzymes that exist as three isoforms (A, B and C), with different distributions being observed in vertebrate tissues. Aldolase A is a candidate auto-antigen in RA (Ukaji et al. 1999) and AD (Mor et al. 2005), and its deficiency is associated with hemolytic anemia (Kishi et al. 1987). Aldolase C is a new deiminated protein candidate that is primarily expressed in astrocytes and Purkinje cells (Jang et al. 2008; Popovici et al. 1990; Walther et al. 1998; Dandoy-Dron et al. 2000). We and others have demonstrated that the expression of aldolase C protein, which has been termed scrapie-responsive protein 2 (scrg2) or zebrin II (Ahn et al. 1994), is increased in the brains of scrapie- and BSE-infected mice at the terminal stage of the disease (Jang et al. 2008; Kishi et al. 1987; Dandoy-Dron et al. 1998). Although it remains to be elucidated, the increased expression of aldolase C and its deimination may be related to Purkinje cell degeneration in PrP-null mice ($Prnp^{0/0}$) or reactive astrogliosis in prion diseases.

Enolase is a highly conserved glycolytic/gluconeogenic enzyme and multifunctional protein that was formerly classified as the soluble brain-specific protein 14-3-2, which is identical to NSE (Kaiser et al. 1989), and exhibits a variety of cellular functions. It can act as a neurotrophic factor (Takei et al. 1991; Hattori et al. 1995), an endothelial hypoxic stress protein (Aaronson et al. 1995), a c-Myc binding protein and transcription factor (Ray and Miller 1991; Subramanian and Miller 2000), and a strong plasminogen (PGn) binding protein (Nakajima et al. 1994; Pancholi and Fischetti 1998). Three major isoforms of enolase are differentially, but widely, distributed in mammalian cells, including ubiquitous α-enolase (ENO1), muscle-specific β-enolase (ENO3), and neuron-specific γ-enolase (ENO2, NSE). ENO1 and NSE are auto-antigens and/or diagnostic markers of RA (Kinloch et al. 2005; Mahdi et al. 2009), MS (Almeras et al. 2004; Forooghian et al. 2007), Hashimoto's encephalopathy (Yoneda et al. 2007) and other autoimmune diseases (Pancholi 2001), neuronal injuries in ischemic stroke (Anand and Stead 2005) and status epilepticus (DeGiorqio et al. 1996), and early stage CJD (Jimi et al. 1992; Evers et al. 1998; Kropp et al. 1999; Kohira et al. 2000).

We recently investigated novel potential physiological roles of enolases using newly developed mouse monoclonal antibodies specific for deiminated enolase peptides (Jang et al. 2012) and observed increased levels of deiminated ENO1 and NSE in the frontal cortex of sCJD and AD patients. We also demonstrated that the PAD-mediated deimination of enolase modulates its three different physiological functions, including: (1) the inhibition of glycolytic ENO1/NSE activities, which may disrupt the biochemical balance in energy metabolism; (2) the induction of enolase degradation by Ca^{2+}-dependent calpain-1 activation, which is associated with impaired intracellular Ca^{2+} homeostasis in various neurodegenerative diseases (Vosler et al. 2008); and (3) the elevation of enolase-plasminogen binding affinity, which may increase the activation of plasminogen (Redlitz et al. 1995) and regulate neurotrophic activity in neurons (Butterfield and Lange 2009).

12.5.3 Deiminated Cyclophilin Proteins

We further demonstrated that cyclophilin A (CypA) is a deiminated protein candidate in prion-affected brains. Cyclophilins participate in a wide range of pathological conditions and cellular dysfunctions, such as cancer, angiogenesis, atherosclerosis, hepatitis B and C viral infections, ER stress, and neurodegenerative diseases (Lee and Kim 2010). Cyclophilins, which include seven major proteins in humans, are widely distributed in most cellular compartments, including the cytoplasm (CypA, CypNK, and Cyp40), nucleus (CypA, CypE, CypG, CypH, CypJ, Cyp60, and RanBP2), ER (CypB and CypC), mitochondria (CypD), and various membranes (Galat 2004; Wang and Heitman 2005). CypA is a ubiquitously expressed peptidylprolyl *cis–trans* isomerase (PPIase) that was originally discovered as a major target for the immunosuppressive drug cyclosporine A (Handschumacher et al. 1984). CypA possesses diverse biological functions and affects signal transduction, cell cycle regulation and protein folding, assembly and trafficking (Göthel and Marahiel 1999). Treatment with cyclosporin A, a fungal peptide immunosuppressant that is used as an inhibitor of cyclophilins, induces the accumulation of protease-resistant PrP aggregates, which suggests a physiological role of cyclophilins in normal PrP metabolism (Cohen and Taraboulos 2003). Furthermore, CypA from scrapie-infected brains stimulates cytokines released from microglia and astroglia through its interaction with CD147, an extracellular receptor (Tribouillard-Tanvier et al. 2012). Although CypA truncation contributes to glial activation during the process of prion infection, it is also possible that the deimination of CypA may be an important factor in the regulation of glial activation and accumulation of protease-resistant PrP aggregates in the pathogenesis of prion diseases and the development of spontaneous and inherited prion disorders.

Further investigation is required to characterize these candidate proteins and their potential physiological and pathogenic functions in prion diseases because there is currently not enough evidence to support the involvement of other deiminated candidates in prion diseases.

12.6 Conclusions

The delineation of the molecular and cellular mechanisms of PAD-mediated deimination, which is one of the enzymatic posttranslational modifications associated with prion diseases, and its importance in various physiological and pathological processes, is the subject of intensive study in current research. The physiological consequences of PAD2 activation and the roles of the identified candidates in different cellular brain compartments in normal and neurodegenerative conditions remain unknown, but PAD2 may play a pivotal role in the onset and progression of prion diseases via the abnormal disruption of Ca^{2+} homeostasis and/or an increase in disease-related deiminated proteins (Fig. 12.1). In addition, the characterization of deiminated proteins and the development of specific antibody probes may be useful as diagnostic and prognostic biomarkers for various human neurodegenerative diseases.

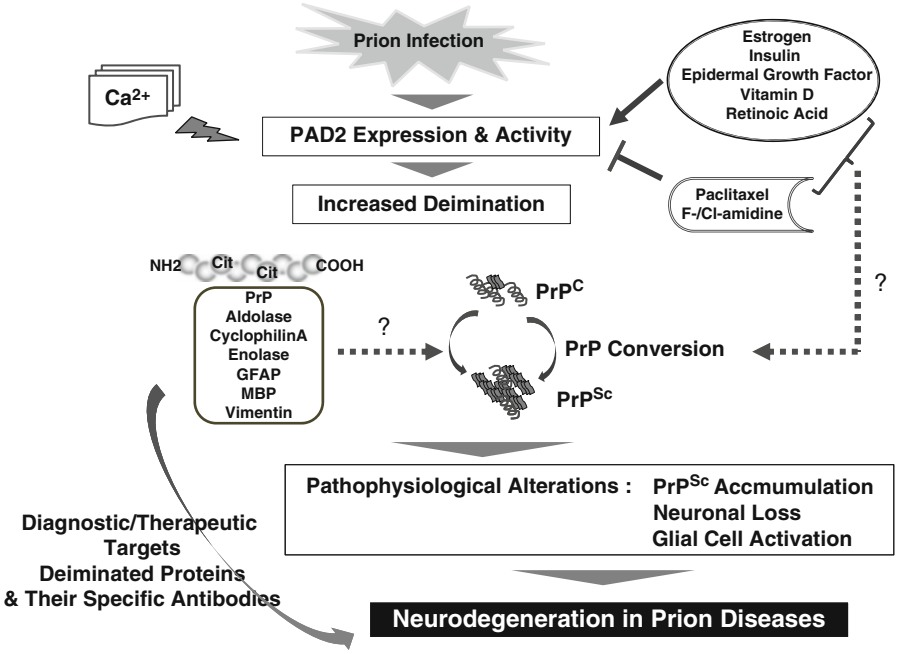

Fig. 12.1 Overview of protein deimination in the pathogenesis of prion diseases. Prion infection disrupts Ca^{2+} homeostasis that directly affects PAD2 expression and its activity. PADs can be regulated by different molecules including some hormones (such as estrogen and insulin), epidermal growth factor, vitamin D, retinoic acid, paclitaxel, and F-/Cl-amidine. Activation of PAD2 is involved in the posttranslational deimination of various cellular proteins, as well as conversion of α-helix-rich PrP^{C} to β-sheet-rich insoluble PrP^{Sc}, which leads to pathophysiological alterations including PrP^{Sc} accumulation, neuronal loss, and glial cell activation, followed by neurodegeneration in prion diseases. Deiminated proteins and their specific antibodies may be useful for the development of diagnostic/therapeutic target(s) in further examining these disorders

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Chapter 13
Deimination in Alzheimer's Disease

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Keywords Alzheimer's disease • Aging • Astrocyte • Cerebellum • Citrullinated (deiminated) proteins • Glial fibrillary acidic protein • Neurodegenerative disorder • Peptidylarginine deiminase • Purkinje cells • Vimentin

Abbreviations

anti-CCP	Anti-cyclic citrullinated peptide
anti-MVC	Anti-mutated citrullinated vimentin,
AD	Alzheimer's disease
BAEE	Benzoyl-L-arginine ethyl ester
Bz-L-Arg	Benzoyl-L-arginine
Calbindin	Calbindin-D-28K
CNS	Central nervous system
DAB	3,3′-Diaminobenzidine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
NFT	Neurofibrillary tangles

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MAP2	Microtubule-associated protein 2
MBP	Myelin basic protein
Nef3	Neurofilament 3
PAD	Peptidylarginine deiminase
RA	Rheumatoid arthritis
RT-PCR	Reverse transcriptase-polymerase chain reaction
SP	Senile plaque

Peptidylarginine deiminases (PADs) are a group of posttranslational modification enzymes that citrullinate (deiminate) protein arginine residues in a calcium ion-dependent manner. Enzymatic citrullination abolishes positive charges of native protein molecules, inevitably causing significant alterations in their structure and functions. Deiminated protein provides the important physiological advantage of forming a cornified layer of skin that covers the human body (see Chap. 7 for more details). Despite this beneficial function, deimination also has a negative side, because the accumulation of these proteins in the brain is a possible cause of Alzheimer's disease (AD). In this chapter, we introduce PADs and their protein citrullination function, which is now considered critical for advancing research on aging and neurodegenerative disorders, especially AD.

13.1 Introduction

Numerous posttranslational modification enzymes participate in age-associated diseases. However, little attention has been paid to one of these groups, the peptidylarginine deiminases (PADs, EC 3.5.3.15) (Kubilus et al. 1980; Rogers and Simmonds 1958; Kubilus and Baden 1983; Ishigami et al. 1996). These PAD enzymes function to citrullinate (deiminate) protein arginine residues in a calcium ion-dependent manner, yielding citrulline residues. Enzymatic deimination abolishes positive charges of native protein molecules, inevitably causing significant alterations in their structure and functions (Imparl et al. 1995; Lamensa and Moscarello 1993; Tarcsa et al. 1996). Citrullinated protein performs the important physiological act of cornification, which thickens the protective layer of skin that covers the human body (Senshu et al. 1995, 1999). However, the detrimental aspect of deiminated protein is its accumulation in the brain, constituting a possible cause of Alzheimer's disease (AD) (Ishigami et al. 2005), prion diseases (Jang et al. 2008, 2010, 2012), Parkinson's disease (Nicholas 2011), and multiple sclerosis (Moscarello et al. 2007). Research on PADs and citrullinated proteins is devoted to precisely and usefully untying the threads of this pathway, in the expectation of contributing to humanity the capacity for healthful longevity. This chapter describes the current state of studies on PADs' expression and protein deimination whose understanding is critical for advancing research on the neurodegenerative disorders, especially AD. For additional data linking deimination to other neurodegenerative diseases besides AD, please see Chaps. 12 and 14.

13.2 Peptidylarginine Deiminases (PADs)

The group of enzymes collectively called PADs convert protein arginine residues to citrulline residues in the presence of calcium ions (Kubilus et al. 1980; Rogers and Simmonds 1958; Kubilus and Baden 1983; Ishigami et al. 1996). Early reports described three types of PADs termed "PAD I" or "epidermal type," "PAD II" or "muscle type," and "PAD III" or "hair follicle type" (see Chaps. 1 and 21), each of which differs in relative activities towards synthetic substrates such as benzoyl-L-arginine ethyl ester (BAEE) or benzoyl-L-arginine (Bz-L-Arg), antigenic properties, and distribution in mammalian tissues (Watanabe et al. 1988; Terakawa et al. 1991). Subsequently, cDNA cloning analyses revealed the existence of five isoforms of PADs (PAD1, PAD2, PAD3, PAD4, and PAD6) in rodents (Ishigami et al. 1998). These isoforms displayed nearly identical amino acid sequences, which are conserved with approximately 59–71 % homology (Ishigami et al. 1998; Tsuchida et al. 1993; Watanabe and Senshu 1989; Nishijyo et al. 1997; Rus'd et al. 1999), but appeared to have different tissue-specific expression, as evidenced by assessment with the reverse transcriptase-polymerase chain reaction (RT-PCR) or Northern blot analysis (Ishigami et al. 2001). Rat PAD1 mRNA was detected only in the epidermis and stomach, while that of rat PAD3 appeared mainly in the epidermis, ovary and hair follicles, whereas rat PAD2 and PAD4 were more widely expressed, for example, in the epidermis, lung, spleen, stomach, kidney, ovary, and uterus (Fig. 13.1). Only in the epidermis were four PAD mRNAs identified, indicating that these enzymes play functionally important roles during terminal differentiation of epidermal keratinocytes.

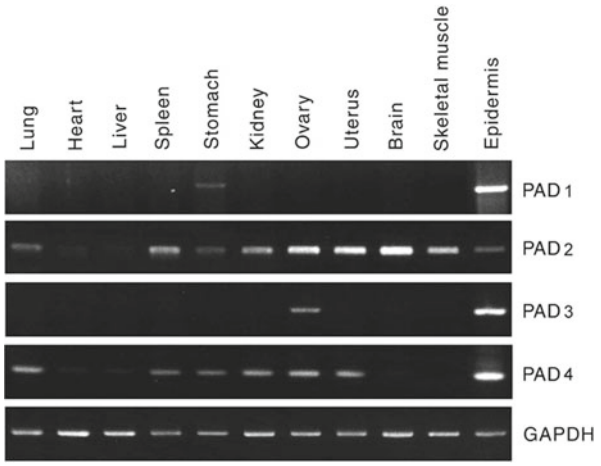

Fig. 13.1 Expression of PAD1, PAD2, PAD3, and PAD4 transcripts in various rat tissues analyzed by RT-PCR. Expected sizes were 631 bp for rat PAD1, 428 bp for rat PAD2, 648 bp for PAD3, 205 bp for PAD4, and 788 bp for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reproduced from Ishigami et al. (2001) with permission from Biomedical Research Press

Concerning human tissues, all five types of PAD noted above have been cloned to date, i.e., PAD1 (Guerrin et al. 2003), PAD2 (Ishigami et al. 2002), PAD3 (Kanno et al. 2000), PAD4 (Nakashima et al. 1999), and PAD6 (Chavanas et al. 2004). However, the tissue-specificity of these human PADs is poorly delineated. PAD3 was found in both the inner and outer root sheaths of the hair follicles (see Chap. 8), where citrullination of trichohyalin occurs in the process of keratinization (Kanno et al. 2000). PAD4 was present in human myeloid leukemia HL-60 cells induced to differentiate into granulocytes by retinoic acid and later found in peripheral blood granulocytes (Nakashima et al. 1999; Asaga et al. 2001). Lately, identification of the PAD4 gene and citrullinated proteins in tissues from patients with rheumatoid arthritis (RA) strongly suggested that these are major factors in the pathogenesis of RA (Suzuki et al. 2003; Liu et al. 2011). In fact, PAD4 was named as an autoantigen in some RA patients, based on recognition of the conformation-dependent epitopes of PAD4 (Takizawa et al. 2005; Zhao et al. 2008; Halvorsen et al. 2008; Serdaroglu et al. 2008; Wagner et al. 2009). Moreover, anti-deiminated protein/peptide antibodies, such as anti-cyclic citrullinated peptide (anti-CCP) antibodies (Serdaroglu et al. 2008) and anti-mutated citrullinated vimentin (anti-MVC) antibodies (Wagner et al. 2009), displayed a strong sensitivity and specificity that contributed to the diagnosis of RA (see Chaps. 2, 3 and 6).

13.3 Detection of All Citrullinated Proteins

To promote a study of PAD and citrullinated proteins, it was essential to establish a method that would detect all deiminated proteins. However, since PAD replaces the imino group, which is double-bonded to the guanido carbon atom of arginine residues with an oxygen atom, radioisotopic techniques are not applicable for our purposes here, despite their usefulness with kinases, phosphatases, acetylases, and other enzymes. Therefore, we have developed a sensitive method for locating citrulline residues in proteins by generating an antibody that binds specifically to chemically modified citrulline residues. For that purpose, we modified citrulline residues in enzymatically deiminated histones by incubation with diacetyl monoxime and anti-pyrine in a diluted H_2SO_4 and H_3PO_4 mixture and used that substance as an immunogen in rabbits (Senshu et al. 1992). The resulting rabbit polyclonal antibody was further affinity purified by using a modified citrulline column. Chemical modification of deiminated residues was based on one of the known color reactions of citrulline (Zarabian et al. 1987; Boyde and Rahmatullah 1980). However, the detailed chemical structure of the product has not been elucidated. Therefore, all citrullinated proteins were detected by western blot analysis (Fig. 13.2) and immunohistochemical methods using this antibody. However, it was essential to modify citrulline residues by incubation with diacetyl monoxime and anti-pyrine in a strong acid mixture on the membrane after transferring the electrophoresed proteins and specimen onto a slide glass. As little as 3–10 fmol of citrulline residues dotted onto the membrane were detectable, regardless of the backbone protein molecules

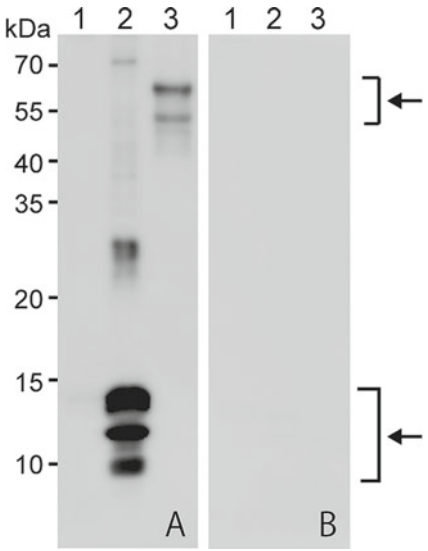

Fig. 13.2 Detection of citrullinated proteins by western blot analysis using anti-modified citrulline antibody. For the detection of citrullinated proteins, it is essential to modify citrulline residues by incubation with diacetyl monoxime and antipyrine in a diluted H_2SO_4 and H_3PO_4 mixture on the membrane after transferring the electrophoresed proteins. Signals were detected in (*A*) the chemically modified membrane, but not detected in (*B*) a membrane without chemical modification. *Lane 1*, Histones from calf thymus; *Lane 2*, Citrullinated histones; *Lane 3*, Human skin cornified cell lysate. *Arrows* indicate modified citrullinated histones (*Lane 2*, 10–15 kDa) and citrullinated keratins (*Lane 3*, 55–65 kDa)

(Senshu et al. 1992). The establishment of this reliable methodology previously led to the identification of multiple deiminated proteins (Senshu et al. 1995; Ishigami et al. 2005; Jang et al. 2008, 2010, 2011) and enabled the following experiments.

13.4 PADs Expression in the Central Nervous System (CNS)

Numerous proteases and posttranslational modification enzymes participate in neurodegeneration, such as that in patients with AD and Parkinson's disease (Keller et al. 2000; Maccioni et al. 2001). Among the five isoforms of PADs, PAD2 and PAD4 are known to occupy the CNS, although PAD2 is the main member of this enzyme group expressed in the CNS (Kubilus and Baden 1983; Watanabe et al. 1988; Terakawa et al. 1991). Both of these isoforms are present in the myelin sheath, and hypercitrullination of myelin basic protein (MBP) resulted in a loss of myelin sheath integrity in patients with multiple sclerosis (Moscarello et al. 1994; Wood et al. 2008; Musse et al. 2008). Moreover, PAD4, the nuclear isoform of this family of enzymes, is involved in histone deimination in brain tissues of multiple sclerosis

victims (Mastronardi et al. 2006). Immunocytochemical studies have localized PAD2 in glial cells, especially astrocytes (Asaga and Ishigami 2000; Vincent et al. 1992; Asaga and Ishigami 2001), microglial cells (Vincent et al. 1992; Asaga et al. 2002), and oligodendrocytes (Akiyama et al. 1999). Additionally, PAD2 expression was later detected in cultured Schwann cells (Keilhoff et al. 2008). Since deiminated proteins were rarely located in the enzyme-positive glial cells examined by using the anti-modified citrulline antibody method described above (Senshu et al. 1992), we assumed that PAD2 is normally inactive (Senshu et al. 1992; Boyde and Rahmatullah 1980; Jang et al. 2011). However, glial fibrillary acidic protein (GFAP) was highly susceptible to the attack of PAD2 in excised rat brains deliberately left at room temperature (Asaga and Senshu 1993). Moreover, under hypoxic conditions (Asaga and Ishigami 2000) and during kainic acid-evoked neurodegeneration (Asaga and Ishigami 2001; Asaga et al. 2002), PAD2 became activated in regions undergoing neurodegeneration and functioned to deiminate various cerebral proteins, suggesting that protein citrullination occurs in neurodegenerative processes. These findings provided a clue that PAD2 normally remains inactive, but becomes active and deiminates cellular proteins when the intracellular calcium balance is upset during neurodegenerative changes.

13.5 Abnormal Accumulation of Citrullinated Proteins in Brains of AD Patients

The pathological presentation of AD involves the selective death of pyramidal neurons and an accumulation of two main abnormal protein aggregates, senile plaques (SPs) and neurofibrillary tangles (NFTs) (Katzman 1986; Smith 1998). Although NFTs and SPs are found in many areas of the cerebrum, they are concentrated mainly in the hippocampus and cerebral cortex. The former site actually appears to be more important, since pathological indices are first localized in that region (Maccioni et al. 2001). Our report indicates that levels of PAD2 are more than threefold higher in the hippocampus than cortex of rat brains (Asaga and Ishigami 2000).

To elucidate the involvement of protein citrullination in the progress of AD, we examined whether citrullinated proteins are produced in the brains of patients with AD (Ishigami et al. 2005). By western blot analysis using anti-modified citrulline antibody, we detected deiminated proteins of varied molecular weights in hippocampal tissues from patients with AD, but not normal humans (Fig. 13.3). Two of the citrullinated proteins were identified as vimentin and GFAP using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry (Ishigami et al. 2005). Although deimination of vimentin and GFAP seems to be much more specific than that of other intracellular proteins, the physiologically importance of these in the brains of AD patients is still unclear. However, vimentin and GFAP were highly susceptible to the attack of PAD2 in vitro; for example, deimination of vimentin induced disassembly of intermediate filaments (Inagaki et al. 1989).

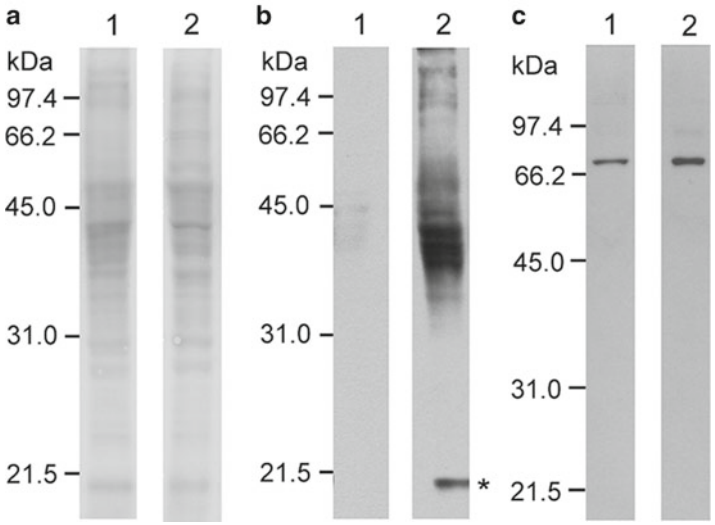

Fig. 13.3 Western blot analysis of citrullinated proteins and PAD2 in hippocampi from the brains of AD patients and normal controls. (**a**) Typical protein profiles detected by amido black staining. (**b**) Citrullinated protein profiles. (**c**) Immunoreactive PAD2 profiles. *Lane 1* age-matched control; *Lane 2* brain section from AD patient. *Asterisk* indicates the citrullinated myelin basic protein (MBP). Reproduced from Ishigami et al. (2005) with permission from John Wiley & Sons

We also identified citrullinated MBP, which is an authentic marker of oligodendrocytes, in the AD-afflicted hippocampus (Ishigami et al. 2005). Moreover, we found PAD2 localized in a stage-specific, immature oligodendrocyte from a rat's cerebral hemisphere in vitro (Akiyama et al. 1999). Elsewhere, PAD2 cDNA was highly expressed in myelin sheath assembly sites, as others reported after using a combination of subcellular fractionation and suppression subtractive hybridization (Gould et al. 2000). Moreover, PAD enzyme and citrullinated MBP proved to be relatively enriched in immature myelin and MBP citrullination played an important part in myelin development and in the human demyelinating disease, multiple sclerosis (Moscarello et al. 1994; Moscarello et al. 2002). Many investigators have suggested that myelin breakdown may be a contributing factor to the pathologic effects of AD (Bartzokis 2004; Tian et al. 2004); possibly then, MBP deimination participates in myelin breakdown.

Interestingly, PAD2 was detected in hippocampal extracts from AD-positive and normal brains, but the amount of PAD2 in AD tissue was markedly greater. Histochemical analysis revealed citrullinated proteins throughout those hippocampal samples, especially in the dentate gyrus and stratum radiatum of CA1 and CA2 areas (Fig. 13.4). However, normal hippocampus did not contain measurable citrullinated proteins. Nevertheless, PAD2 immunoreactivity was ubiquitous throughout both AD-affected and normal hippocampal areas. Still, PAD2-enrichment coincided well with deiminated protein-positivity. Double immunofluorescence staining revealed that citrullinated protein- and PAD2-positive cells also coincided with

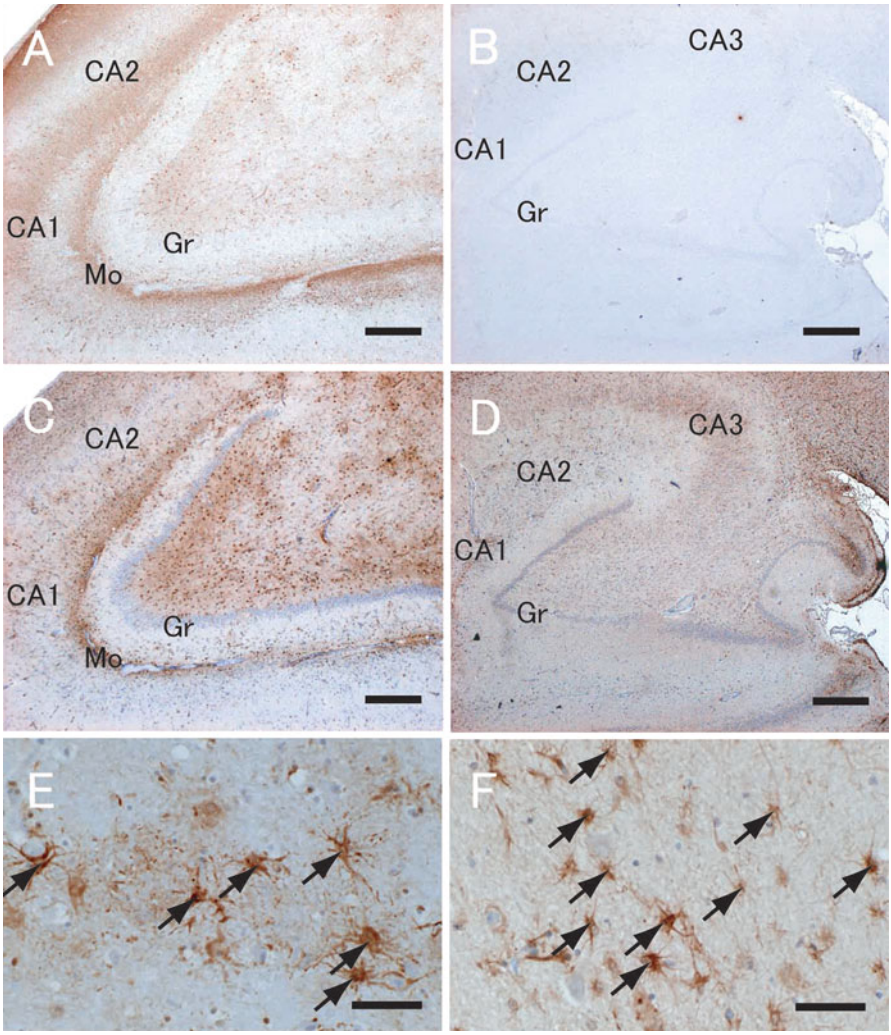

Fig. 13.4 Immunohistochemical staining of citrullinated proteins and PAD2. Hippocampal sections from brains of AD patients (**a**, **c**) and controls (**b**, **d**) were stained for citrullinated proteins (**a**, **b**) and PAD2 (**c**, **d**). (**e**) Higher magnification of (**a**). *Arrows* indicate the citrullinated protein-positive cells. (**f**) Higher magnification of (**c**). *Arrows* indicate the PAD2-positive cells. *Gr* granule cell layer, *Mo* molecular cell layer. Scale bars: **a**–**d** = 500 μm; **e** and **f** = 50 μm. Reproduced from Ishigami et al. (2005) with permission from John Wiley & Sons

GFAP-positive cells, but not all GFAP-positive cells were immunoreactive for PAD2 (Fig. 13.5). Like GFAP, PAD2 was distributed mainly in astrocytes. These collective results, the abnormal accumulation of deiminated proteins and abnormal activation of PAD2 in the hippocampi of patients with AD, strongly suggest that PAD has an important role in the onset and progression of AD and that citrullinated proteins may become a useful marker for human neurodegenerative diseases.

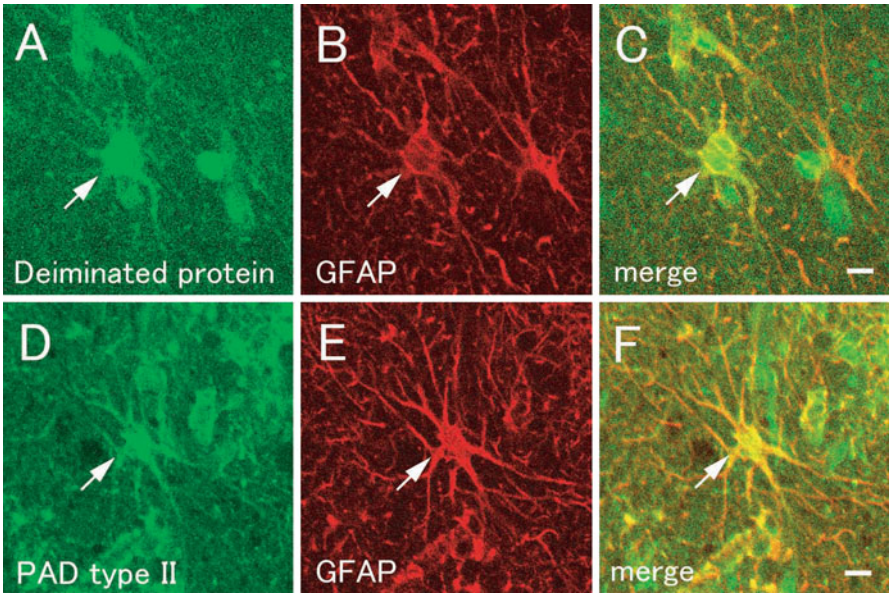

Fig. 13.5 Identification of citrullinated protein-positive and PAD2-positive cells by double immunofluorescence staining. Sections of AD hippocampus were double immunostained with monoclonal anti-GFAP antibody and with polyclonal anti-modified citrulline IgG or polyclonal anti-human PAD2 antibody. The primary antibodies were visualized with anti-rabbit Alexa Fluor 488™ (*green*) and anti-mouse Alexa Fluor 594™ (*red*). (**a**, **d**) Alexa 488 (*green*) for citrullinated protein (**a**) or PAD2 (**d**). (**b**, **e**) Alexa 568 (*red*) for GFAP. (**c**, **f**) Merged views for (**a/b**) and (**d/e**). *Arrows* indicate coincident positions (*yellow*). Scale bar = 5 μm. Reproduced from Ishigami et al. (2005) with permission from John Wiley & Sons

The mechanism(s) by which deiminated proteins occur in the hippocampus during AD remains unclear. Possibly PAD2 becomes activated, abundant and functional only in the process of AD, since PAD2 content increased notably in hippocampi of AD patients compared to normal subjects. Although PAD2 was also present in hippocampal extracts from normal subjects, that enzyme remained in a steady state during which no enzyme activation occurred. For enzyme activation, the intracellular calcium concentration must become elevated. No other factors can regulate PAD activity in vivo or in vitro, to the best of our knowledge. A loss of neuronal calcium homeostasis leading to increases in the intracellular calcium concentration has been proposed to play a major role in hypoxic and ischemic brain injury (Hossmann 1999; Choi 1988). In fact, experimental results with simulated ischemia in a primary culture of astrocytes indicated that an influx of extracellular calcium contributes to astroglial injury during ischemia (Haun et al. 1992). Our former report showed that PAD2 activated and citrullinated various cerebral proteins under hypoxic conditions (Asaga and Ishigami 2000) and during kainic acid-evoked neurodegeneration (Asaga and Ishigami 2001; Asaga et al. 2002). Abnormal PAD activation resulted in random protein deimination, which could then trigger the onset of neurodegenerative disease. Therefore, the development of an inhibitory drug specific for PAD could conceivably prevent the onset and progression of neurodegeneration.

13.6 Age-Related Changes of PAD2 in the Mouse Brain

Understandably, abnormal protein deimination by PAD2 has been closely associated with the pathogenesis of neurodegenerative disorders such as AD. Protein citrullination in these patients is thought to develop during the initiation and/or progression of disease. However, the contribution of changes in PAD2 levels, and consequent deimination, during developmental and aging processes remained unclear. Therefore, we measured PAD2 expression and localization in the brain during those processes (Shimada et al. 2010).

PAD2 mRNA was expressed in the brains of mice after 15 days of embryonic development, and GFAP mRNA expression first became evident just 1 day later (Fig. 13.6). Our previous reports indicated that PAD2 appeared mainly in glial cells, especially astrocytes (Asaga and Ishigami 2000, 2001), microglial cells (Asaga et al. 2002), and oligodendrocytes (Akiyama et al. 1999). However, since we detected PAD2 earlier than GFAP, PAD2 must be expressed in cells other than glial cells, possibly astrocytes. Microtubule-associated protein (MAP2) and neurofilament 3 (Nef3) were also expressed at an early embryonic stage in amounts that increased slightly until birth and remained almost constant until the 7th postnatal day (Fig. 13.6). Thus, PAD2 expression did not correlate with GFAP, MAP2, or Nef3 expression, indicating that PAD2 must appear at specific, but still unknown, stages and conditions of glial and neuronal cell differentiation. In addition, no citrullinated proteins were detected during this developmental process.

In the cerebral cortex, cerebellum, and hippocampus, PAD2 mRNA expression increased significantly as the mice aged from 3- to 30-months-old (Fig. 13.7). That is, PAD2 mRNA levels at the 30-month-old mark were 1.5-fold to 1.6-fold higher than in 3-month-olds. Although GFAP mRNA expression also increased significantly during aging, the increase of GFAP did not correlate closely with that of PAD2, because GFAP in 30-month-old mice was 2.7-fold to 4.7-fold higher than that in 3-month-olds, far exceeding the increase of PAD2. Moreover, Nef3 mRNA expression did not change during aging. Since the change of PAD2 expression levels during aging did not correlate with those of GFAP or Nef3, PAD2 must be expressed only at certain times and under appropriate conditions by neuronal cells and glial cells including astrocytes (Asaga and Ishigami 2000, 2001), activated microglial cells (Asaga et al. 2002), and stage-specific immature oligodendrocytes (Akiyama et al. 1999). Again, no citrullinated proteins were detected during this aging process.

13.7 Localization of PAD2 in the Cerebral Cortex, Cerebellum, and Hippocampus

Immunohistochemical staining of PAD2, GFAP, and MAP2 provided new insight into the characteristics of PAD2-positive cells in the cerebral cortex, hippocampus, and cerebellum of 3-month-old mice (Fig. 13.8). In the cerebral cortex and

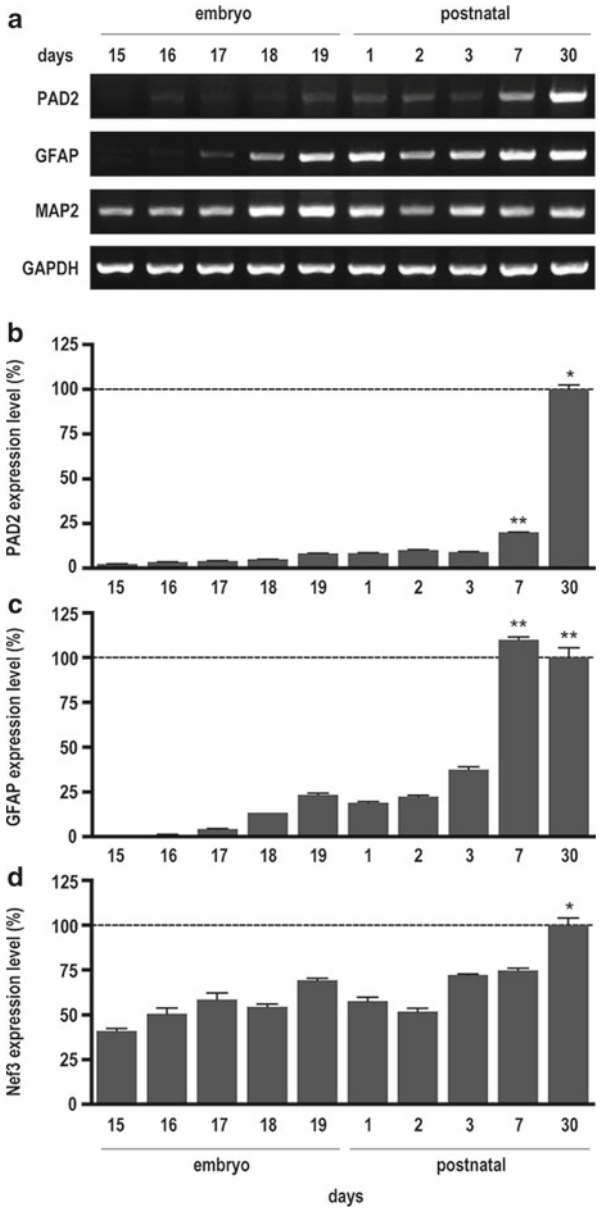

Fig. 13.6 Developmental changes of PAD2, GFAP, MAP2, and Nef3 mRNA expression levels in the whole brains of mice. (**a**) RT-PCR of PAD2, GFAP, MAP2, and GAPDH. (**b–d**) Quantitative RT-PCR analysis of PAD2 (**b**), GFAP (**c**), Nef3 (**d**). Data from quantitative RT-PCR are shown as the percentage of each value with the 30th postnatal day taken as 100 % and representing a mean ± SEM of five animals. *$p < 0.05$ as compared to 15–19 days of embryonic life and 1–3 or 7 days after birth. **$p < 0.05$ as compared to 15–19 days of embryonic life and 1–3 days after birth. Reproduced from Shimada et al. (2010) with permission from John Wiley & Sons

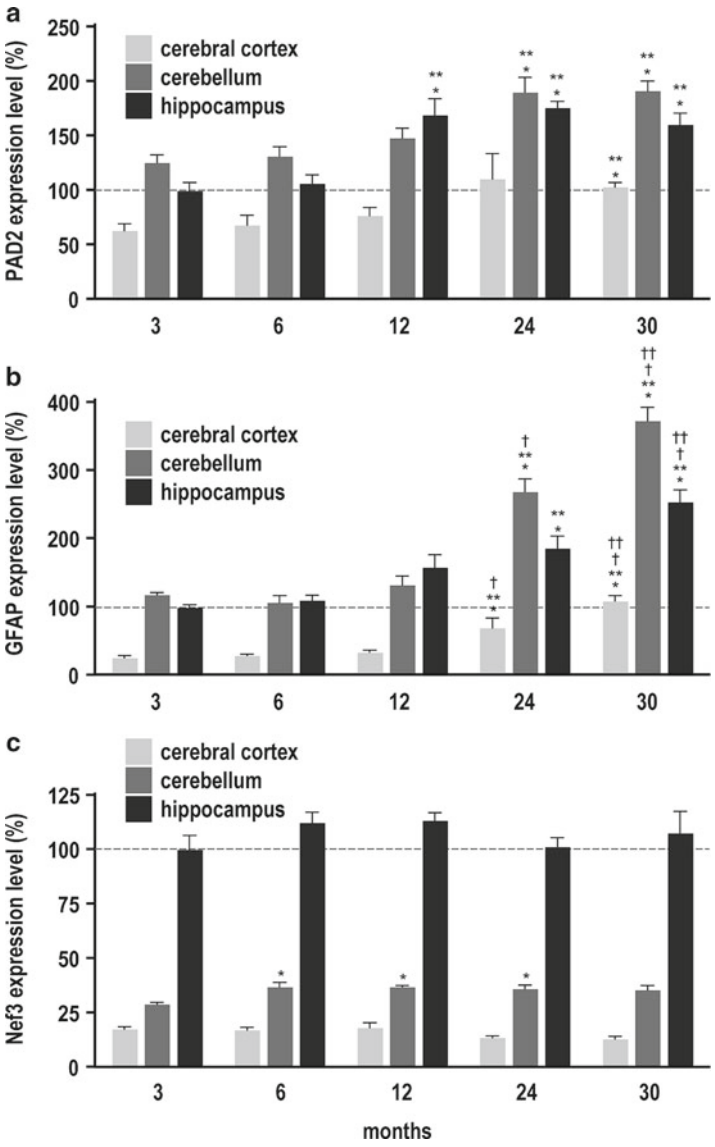

Fig. 13.7 Age-dependent changes of PAD2, GFAP, and Nef3 mRNA expression in the brain. Total RNA from the cerebral cortex, cerebellum, and hippocampi of 3, 6, 12, 24, and 30-month-old mice was prepared. Quantitative RT-PCR analysis of PAD2 (**a**), GFAP (**b**), and Nef3 (**c**) was carried out. As the endogenous control, GAPDH was quantified simultaneously to normalize each raw data set. Data are expressed in percentages as values in the hippocampi of 3-month-old mice as 100 % and represent a mean ± SEM of five animals. $^{*}p < 0.05$ as compared to 3-month-old mice. $^{**}p < 0.05$ as compared to 6-month-old mice. $^{\dagger}p < 0.05$ as compared to 12-month-old mice. $^{\dagger\dagger}p < 0.05$ as compared to 24-month-old mice. Reproduced from Shimada et al. (2010) with permission from John Wiley & Sons

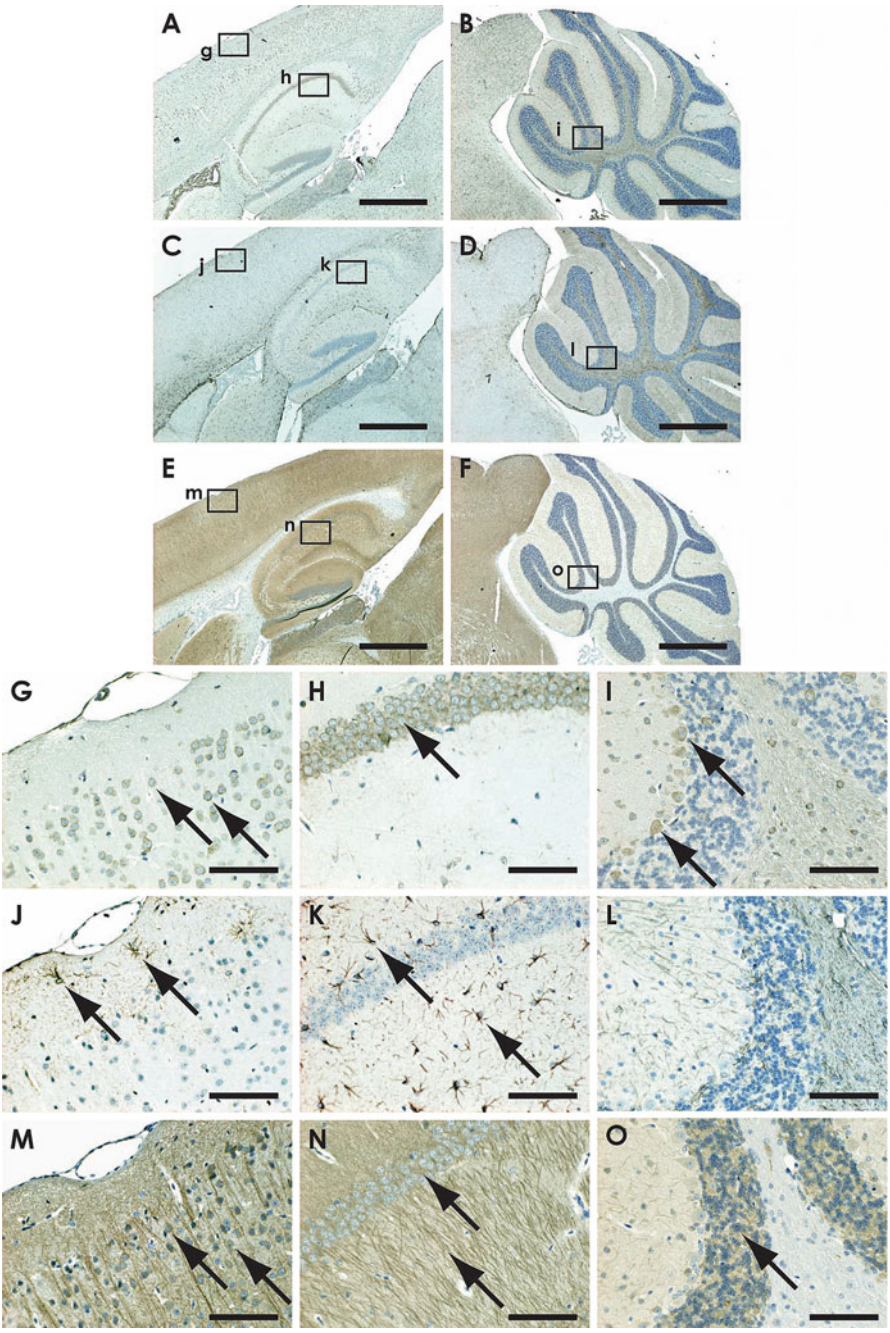

Fig. 13.8 Immunohistochemical staining of PAD2, GFAP, and MAP2 in the cerebral cortex (**a**, **c**, **e**, **g**, **j**, **m**), hippocampus (**a**, **c**, **e**, **h**, **k**, **n**), and cerebellum (**b**, **d**, **f**, **i**, **l**, **o**) of 3-month-old mice. Each brain section was stained with PAD2 (**a**, **b**), GFAP (**c**, **d**), and MAP2 (**e**, **f**) antibody. The *square area* of g–i in **a** and **b**, *j–l* in **c** and **d**, and *m–o* in **e** and **f** were magnified for presentation in **g–i**, **j–l**, and **m–o**, respectively. *Arrows* indicate typical stained objects. Scale bars = 1 mm in **a–f** and 100 μm in **g–o**. Reproduced from Shimada et al. (2010) with permission from John Wiley & Sons

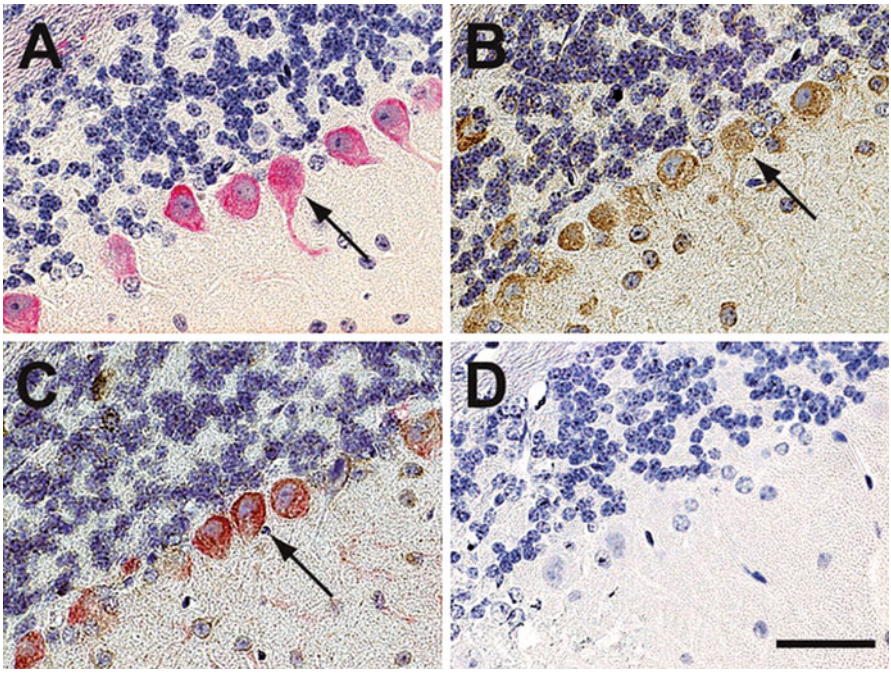

Fig. 13.9 Identification of PAD2-positive cells in the cerebellum by double immunostaining. Sections of cerebellum from 3-month-old mice were double immunostained with PAD2 and calbindin. (**a**) Alkaline phosphate substrate as a chromogenic substrate (*light magenta*) for calbindin; (**b**) 3,3′-diaminobenzidine (DAB) as a chromogenic substrate (*brown*) for PAD2; (**c**) Double immunostaining of PAD2 and calbindin; (**d**) Mouse and rabbit IgG were used for control staining. *Arrows* indicate Purkinje cells. Scale bar = 100 μm. Reproduced from Shimada et al. (2010) with permission from John Wiley & Sons

hippocampus, PAD2-positive signals were detected in neuronal cell bodies that co-stained with MAP2, but not in dendrites. Not only was MAP2 staining positive in both neuronal cell bodies and dendrites, but the cerebral cortex, hippocampus, and cerebellum were also MAP2-positive. However, GFAP-positive cells, which are considered to be reactive astrocytes, were PAD2-negative in the cerebral cortex, hippocampus, and cerebellum.

In the cerebellum, PAD2-positive staining was present on morphologically characteristic Purkinje-like cells along dense granule cell layers that were not positive for either MAP2 or GFAP. To confirm that these were actually Purkinje cells, we performed double immunostaining with PAD2 and calbindin-D-28K (calbindin), a known marker of Purkinje cells and limited to localization in those cells (Servais et al. 2005; Whitney et al. 2008). Calbindin staining was evident as a light magenta coloration on the alkaline phosphate substrate (Fig. 13.9). These Purkinje cells appeared as huge round-shaped cell bodies located between the bottom of the molecular layer and surface of the granule cell layer of cerebellar cortex. PAD2 was

stained brown by the 3,3′-diaminobenzidine (DAB) used as a chromogenic substrate (Fig. 13.9). Double immunostaining enabled detection of both calbindin- and PAD2-positive cells in the same Purkinje cells from the cerebellum, thus assuring the existence of PAD2 in clearly identified Purkinje cells of the cerebellum. Additionally, the characteristic localization of PAD2, GFAP, and MAP2 in the cerebral cortex, hippocampus, and cerebellum did not change during aging from 3 months to 30 months. Thus, PAD2 localized in neuronal cells of the cerebral cortex and Purkinje cells of the cerebellum (Shimada et al. 2010).

13.8 PAD2 in Purkinje Cells of the Cerebellum

PAD2 has been documented in Purkinje cells of the cerebellum (Shimada et al. 2010). The cerebellum primarily functions to process sensory information in the control of coordinated movement. Purkinje cells of the cerebellum are its sole output neurons and are important as the integrators and fine-tuners of diverse input signals (Cheron et al. 2008). Accumulated evidence indicates that the dynamic transfer of Ca^{2+} plays a key role in the function of Purkinje cells (Matsushita et al. 2002; Erickson et al. 2007). Intracellular Ca^{2+} concentrations become elevated via voltage-dependent calcium channels of plasma membranes or inositol-1,4,5-triphosphate-dependent Ca^{2+} release from intracellular Ca^{2+} storage sites, such as the endoplasmic reticulum (Cheron et al. 2008). Elevations of intracellular Ca^{2+} activate an intracellular signal cascade leading to such functional events such as neurotransmitter release (Cheron et al. 2008). The relevance of this background is that PAD enzyme requires about a 100-fold higher than normal intracellular Ca^{2+} level for its activation (Inagaki et al. 1989). Since Purkinje cells store large amounts of Ca^{2+} corresponding to physiological stimuli (Matsushita et al. 2002), conceivably those intracellular Ca^{2+} concentrations become elevated transiently in specific, limited areas, such as near the endoplasmic reticulum and plasma membrane. When such conditions prevail, PAD2 enzymes would become activated and citrullinate various proteins, leading to cell death (Asaga et al. 1998). In fact, quantities of PAD2 and citrullinated proteins have been shown to increase in the brain in vivo during abnormal conditions such as scrapie-infection of mice and AD in humans (Ishigami et al. 2005; Jang et al. 2008).

13.9 Aspects of PAD2 Expression and Protein Citrullination in Neurodegenerative Disorders

The mechanism by which citrullinated proteins occupy the hippocampus during AD remains unclear. Possibly PAD2 becomes activated, abundant, and functional only in the process of AD, since the amount of PAD2 increased notably in hippocampi of the persons with AD compared to normal subjects. Although PAD2 was also present in hippocampal extracts from normal subjects, that enzyme remained in a steady

state during which no apparent activation occurred. For enzyme activation, the intracellular calcium concentration must become elevated. To the best of our knowledge, no other factors can regulate PAD activity in vivo or in vitro. A loss of neuronal calcium homeostasis leading to increases in the intracellular calcium concentration has been proposed to play a major role in hypoxic and ischemic brain injury (Hossmann 1999; Choi 1988). An influx of extra cellular calcium could contribute to astroglial injury during ischemia, as suggested on the basis of experimental results with simulated ischemia in a primary culture of astrocytes (Haun et al. 1992). Our reports showed that PAD2 activated and citrullinated various cerebral proteins under hypoxic conditions (Asaga and Ishigami 2000) and during kainic acid-evoked neurodegeneration (Asaga and Ishigami 2001; Asaga et al. 2002). Clearly, from the weight of evidence now available, abnormal PAD activation that results in random protein deimination has the potential to trigger a neurodegenerative disease such as AD.

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Chapter 14
Ongoing Studies of Deimination in Neurodegenerative Diseases Using the F95 Antibody

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Keywords Aging • Alexander disease • Alzheimer's disease • Amyotrophic lateral sclerosis • Creutzfeldt–Jakob disease • Diffuse Lewy body disease • Glioma • Parkinson's disease

14.1 Introduction

During the late 1990s and in collaboration with the laboratory of Dr. John N. Whitaker, then chairman of the Department of Neurology at the University of Alabama at Birmingham (UAB), a series of antibodies against deiminated proteins were developed using hybridoma technology. Although the ultimate goal of using

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these antibodies as clinical biomarkers of disease severity in multiple sclerosis (MS) was not fulfilled at that time, in part due to Dr. Whittaker's tragic bicycle accident and death in 2001, these antibodies were subsequently tested with immunohistochemistry in a number of disease states of the central nervous system versus normal control tissue. One of these antibodies, known as F95, was made against a synthetic peptide antigen (Peninsula Laboratories) consisting of ten peptidyl-citrullines attached to two glycines and a cystine, known as the deca-citrullinated protein (DCP). As a mouse monoclonal antibody of IgM/kappa class, F95 has been used in hundreds of experiments to date, for its ability to recognize multiple natural and synthetic proteins containing peptidyl-citrulline moieties in western blots, immunoprecipitation, and immunohistochemistry.

Presently, hybridoma cells that produce the F95 antibody are housed at the Epitope Recognition Immunoreagent Core, which is part of the Division of Genetic and Translational Medicine at UAB, and are maintained in culture using established protocols for the propagation of hybridoma cell lines (Harlow and Lane 1988). In brief, the cells are passaged in standard tissue culture media (such as RPMI 1640 or DMEM) supplemented with 10 % fetal calf serum, glutamine, and commonly used antibiotics such as Pen/Strep or gentamycin. The cells are split, as necessary, to maintain a density from 1×10^{-5} to 1×10^{-6} cells per ml. Purification of the IgM, kappa F95 antibody from culture media, or bioreactor supernatant can be achieved by dialysis against distilled water or precipitation with ammonium sulfate (Andrew et al. 1997). Additional methods for IgM purification include size exclusion chromatography (Andrew et al. 1997) and affinity chromatography using mannan-binding protein (Nevens et al. 1992). Alternatively, high concentrations of relatively pure F95 antibody can be obtained by humanely propagating the cell line in immunocompromised mice (i.e., nude or RAG2 knockout mice). These animals are euthanized as soon as the presence of tumor is evident but prior to the onset of physical distress. At that time, serum and ascites fluids are collected for antibody purification. The amount of F95 in biological fluids is then measured by quantitative ELISA performed on wells coated with synthetic cyclic citrullinated peptide (CCP). We routinely test each aliquot of F95 using the CCP2 ELISA kit available from Euro Diagnostica, Malmö, Sweden.

14.2 Characterization of the F95 Antibody

In our initial study of the F95 antibody (Nicholas and Whitaker 2002), ELISA characterization was done to determine binding affinities to various natural and synthetic antigens (Fig. 14.1). For example, stronger F95 immunoreactivity was seen using human and guinea pig myelin basic protein (MBP) isoforms containing numerous peptidyl-citrulline moieties (such as the C8 isoform) than MBP isoforms that contained fewer peptidyl-citrullines (such as the C1 isoform). Commercially generated (Peninsula Laboratories) peptide fragments of C8 containing peptidyl-citrullines, as well as DCP, also bound heavily to F95, while carrier molecules such as keyhole limpet hemocyanin, maleimide-activated bovine serum albumin (BSA), or ovalbumin alone were unreactive.

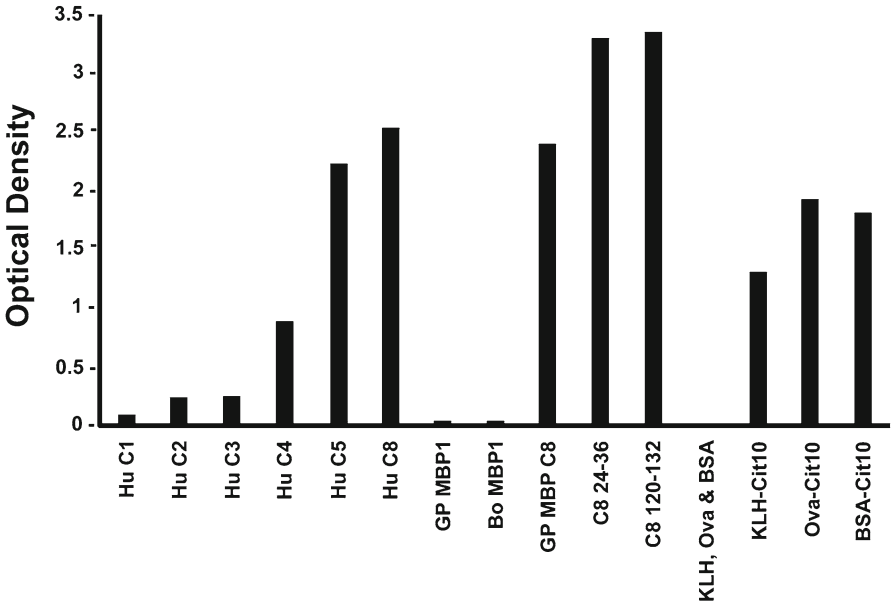

Fig. 14.1 ELISA results characterizing the binding of the monoclonal F95 antibody demonstrated its recognition of citrullinated epitopes in various proteins. Reaction of F95 hybridoma supernatant diluted 1:50 was performed with each antigen plated at a concentration of 0.4 μg/well and expressed as the average optical density of duplicates at 405 nm after 10 min (*Hu* human, *GP* guinea pig, *MBP* myelin basic protein, *Bo* bovine, *KLH* keyhole limpet hemocyanin, *Ova* ovalbumin, *BSA* bovine serum albumin, *Cit10* deca-citrullinated protein). Modified from Nicholas et al., Glia, Volume 37, pp. 328–336, Copyright 2002, Wiley-Liss, Inc.

Fig. 14.2 Dot immunoblots showed that the F95 antibody reacted strongly with a synthetic peptide containing ten citrullines (Cit10) but much less so with a synthetic peptide containing two citrullines (Cit2). In contrast, F95 did not react with BSA or free citrulline (cit)

Dot blots also showed intense F95 immunoreactivity for DCP and mouse brain extracts, but not to free citrulline (De Rycke et al. 2005). Using similar techniques, previously unpublished dot blot experiments showed that F95 reacted with DCP (Cit10), and less so with a synthetic peptide containing two peptidyl-citrullines (Cit2), but not at all with BSA or free citrulline (Fig. 14.2).

In addition, we also recently tested the F95 antibody against deiminated glial fibrillary acidic protein (GFAP) in immunoblots. Recombinant human GFAP was prepared as described previously (Perng et al. 2006) and dissolved in 8 M urea/5 mM EDTA. An aliquot was deiminated by diluting tenfold into 25 mM Tris–HCl/1 mM DTT/20 mM $CaCl_2$ (pH 7.6) and adding a 1:50 ratio of human recombinant peptidyl-arginine deiminase (PAD) type 1 enzyme (Cayman Chemical). Following incubation for 60 min

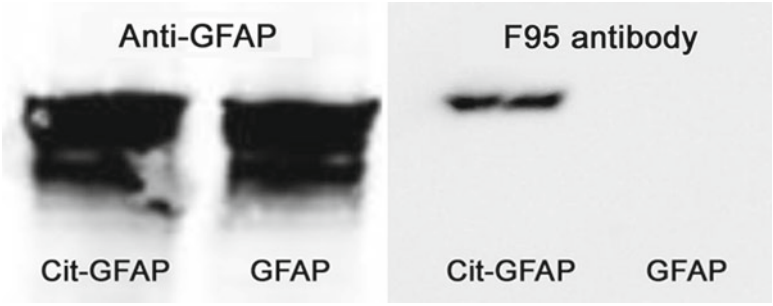

Fig. 14.3 Detection of citrullinated GFAP by the F95 antibody in immunoblots using recombinant human GFAP (75 ng) samples that had been deiminated (Cit-GFAP) or untreated (GFAP) showed that an antiserum against total GFAP recognized both samples (*left*), while the F95 antibody only reacted with Cit-GFAP (*right*)

at 25 °C, the reaction was quenched by diluting with an equal volume of 250 mM EGTA/5 % SDS/0.1 mg/mL BSA. For the immunoblot, samples were run on a 4–20 % SDS gel (Pierce), transferred to Amersham Hybond ECL nitrocellulose (GE Healthcare), and blocked in phosphate-buffered saline (PBS) plus 3 % BSA for 1 h followed by further blocking in a mixture of PBS and 5 % nonfat dry milk for 1 h. The F95 antibody was then added to the membranes at a 1:2,000 dilution in 0.1 % Tween-20/5 % nonfat dry milk in PBS (PBST) and incubated overnight at 4 °C. The membranes were then washed three times in PBST, incubated for 1 h at room temperature with an anti-mouse IgM peroxidase-conjugated antibody (Jackson Immuno Research) diluted 1:4,000 in PBST, washed three times in PBST buffer, and given a final brief rinse in PBS. Citrullinated GFAP immunosignals were developed by incubation for 2 min in SuperSignal ECL (Thermo Scientific) and visualized using a Kodak Image Station 4000 MM. To detect total GFAP, membranes were placed in PBST and incubated for 1 h at room temperature with a polyclonal anti-GFAP antibody (Dako) diluted 1:5,000. Treatment with secondary antibody was as described above, using an anti-rabbit IgG 800 nm IR dye secondary antibody (LI-COR Biosciences) diluted 1:15,000, and signals were detected using an Odyssey IR Imager.

These results showed that F95 reacted specifically to citrullinated GFAP but not to non-deiminated GFAP (Fig. 14.3). Further evidence for the specificity of F95 for peptidyl-citrulline was that preabsorption of F95 with DCP blocked immunostaining of brain (Nicholas and Whitaker 2002) and rheumatoid arthritis synovial tissues (De Rycke et al. 2005).

14.3 Examination of Citrullinated Proteins in Normal Human and Rodent CNS Using F95 Immunostaining

Since it was well known that approximately 20 % of all MBP in normal human brains consisted of the C8 citrullinated isomer (Moscarello et al. 1994), it was not surprising that F95 lightly stained white matter in the brains of normal humans

(Nicholas and Whitaker 2002; Nicholas et al. 2004), rats (Nicholas et al. 2003) and mice (Nicholas et al. 2005). Using an antibody raised against PAD from rat skeletal muscle (PAD2), Vincent et al. was the first to map the location of this PAD isozyme in the rat CNS (Vincent et al. 1992), showing cellular staining in what appeared to be astrocytes, some neurons, and possibly microglia as well. This contrasted with a later study showing PAD2 immunostaining primarily in immature oligodendrocytes (Akiyama et al. 1999).

Using the F95 antibody, the presence of deiminated proteins in a subset of astrocytes was first established in the brains of normal humans (Nicholas and Whitaker 2002) and rats (Nicholas et al. 2003). Similar to MBP (Harauz and Musse 2007), this evidence suggested that GFAP may also exist in various citrullinated isoforms, even in non-diseased brains. Over the past decade, numerous studies using F95 and other antibodies have confirmed that astrocyte intermediate filament proteins such as GFAP are common targets for deimination and that citrullination of GFAP in particular is increased in numerous neuropathological conditions (Nicholas et al. 2004, 2005; Grant et al. 2007; Ishigami et al. 2005; Jang et al. 2010).

14.4 Examination of Citrullinated Proteins in Neurodegenerative Diseases Using the F95 Antibody

All patients that provided tissue to the various brain banks (see Acknowledgements) associated with these experiments did so with written premortem informed consent in accordance with the Helsinki declaration's ethical code of the World Medical Association. In addition, the local Institutional Animal Care and Use Committees at UAB and Ruhr-University Bochum also approved the methods used in these studies to ensure compliance with specific pathogen-free animal welfare organizational guidelines as well as national, state, and local government regulations.

Although the F95 antibody has previously been used to identify deiminated proteins in multiple species, including human, mouse, rat, and chick, and in various tissues including brain, spinal cord, peripheral nerve, joint, lung, and gums, this chapter primarily demonstrates recently published and unpublished findings of deiminated proteins in various neurodegenerative diseases. As a result, our past work using F95 to study MS and its animal model, experimental autoimmune encephalomyelitis (EAE), has been reviewed elsewhere (in Chap. 10).

14.4.1 Brain Deimination During Aging: The Inevitable Neurodegenerative Disease

One of our unpublished observations was that F95 immunostaining in the brains of humans and rodents varied between individuals. After altering multiple parameters in our staining protocols, we finally deduced that F95 staining was routinely more robust in older individuals. In fact, our best staining in rats was when retired male

breeders over 12 months old were used (Nicholas et al. 2003). Although one report suggested that deimination actually decreased in the eye during the aging process (Bhattacharya 2009), another study showed that PAD2 protein and mRNA steadily increased in mouse brains up to 30 months of age (Shimada et al. 2010). This study also showed that PAD expression differed in different brain areas, with the highest levels in the cerebellum, and that PAD2 immunoreactivity was present in both astrocytes and neurons (Shimada et al. 2010). These mirrored our previous findings of variable F95 staining in different regions of normal rat brains, even justifying our then controversial observation of deiminated proteins in Purkinje cells and other neurons (Nicholas et al. 2003).

To determine if brain protein deimination increased during aging, we compared the expression of citrullinated brain proteins from young versus older humans and mice using the F95 antibody. Thus, six normal-appearing male C57BL/6J mice (Charles River) were used. Three were 8 weeks of age, while the other three were retired breeders, approximately 10–12 months old. These animals were housed in a controlled environment with food and water available ad libitum. At the appropriate age, the mice were then anesthetized and transcardially perfused with ice-cold saline and their brains were removed, flash frozen with dry-ice vapor, and stored at −70 °C until used. In contrast, frozen human brain specimens that were free of disease were obtained from the National Neurological Research Specimen Bank in Los Angeles, California. These blocks were from five control individuals of various ages ranging from 29 to 71.

Tissue processing for western blot analysis of this material was identical to methods described previously (Nicholas et al. 2003). In order to isolate deiminated proteins from these specimens, samples were immunoprecipitated with F95 using methods that were modified from those that we also described previously (Nicholas et al. 2005). Briefly, a 20 % slurry stock of protein-G sepharose (PGS) beads (Sigma) was prepared, using 100 mM sodium borate buffer (pH 8.0) containing 0.02 % sodium azide. The beads were then washed three times with ice-cold sodium borate buffer, followed by centrifugation for 1 min at 5,000 rpm, after which the supernatant was removed. Then, a mixture of 200 µl of F95 ascites fluid with 600 µl of sodium borate buffer was added to the beads, and the PGS bead–antibody suspension was incubated overnight at 4 °C with gentle rotation. The next day, the PGS–F95 antibody-coupled beads were washed again as described above and then incubated in 600 µl of a freshly prepared solution containing 20 mM dimethyl pimelimidate-dihydrochloride (Sigma) in 200 mM sodium borate buffer (pH 9.0) for 30 min at room temperature with gentle rotation, followed by brief microfugation and gentle removal of the supernatant. In order to block nonspecific sites, 600 µl of 200 mM ethanolamine (pH 8.0) was then added to the PGS–antibody-coupled beads and incubated for 2 h at room temperature with gentle rotation, followed by brief microfugation, gentle removal of the supernatant, and two additional washing steps with sodium borate buffer as described above. After all remaining buffer was removed, 200 µg of solubilized brain proteins that were previously diluted 20-fold with an ice-cold "RIPA" buffering solution consisting of 1 mM of the chelating agent ethylenediaminetetraacetic acid with 75 mM NaCl, 25 mM Na_2SO_4, 0.5 %

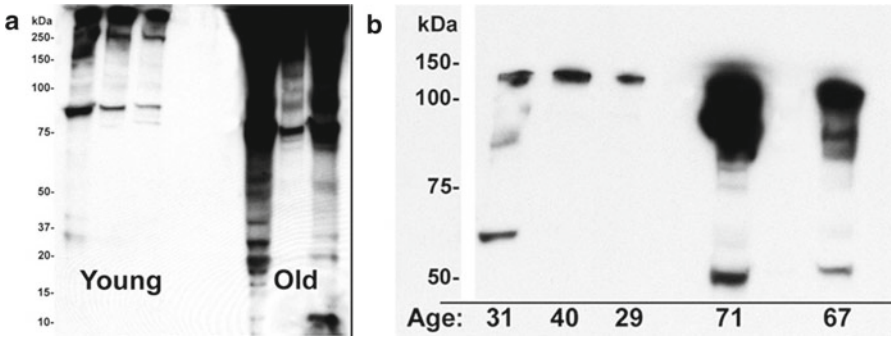

Fig. 14.4 F95 western blot analysis of brain extracts from three young (8 weeks of age) versus three old (10–12 months of age) mice (**a**) and of young versus old normal human frontal cortical extracts (**b**) that were previously immunoprecipitated with F95 prior to immunoblotting with F95 clearly showed more deiminated proteins in aged brain samples. Note that cognitive testing of these subjects was not performed, although the human specimens came from brains reportedly without a histological diagnosis of Alzheimer's disease. Locations of standard molecular weights in kDa are depicted to the *far left* of each blot

sodium deoxycholate, and 0.5 % Triton X-100 (pH 7.2) were added to the PGS beads coupled with F95 antibody and incubated overnight at 4 °C with gentle rotation. The next day, the beads were washed twice with 800 µl of RIPA buffer, mixed with a solution containing 100 mM dithiothreitol to reduce disulfide bonds, combined with 4 % SDS and 15 % glycerol in 1.25 M Tris–HCl, and boiled for 5 min with agitation every minute. Following vigorous vortexing for 2 min and centrifugation for 5 min, the remaining supernatant contained the F95-immunoprecipitated (deiminated) proteins.

Tris–HEPES 4–20 % gradient gels (Precise Protein gels) were then run under reducing conditions using 10–15 µg of SDS soluble total or F95-immunoprecipitated brain proteins from AD and control brains in separate lanes along with pre-stained Precision Plus Protein Markers (BioRad). After gel electrophoresis, protein extracts were transferred to nitrocellulose membranes (BioRad) for 2 h at room temperature and then incubated with a blocking buffer consisting of 5 % powdered nonfat skim milk and 0.05 % Tween in 50 mM TBS (pH 7.4) for 1 h at room temperature on a shaker platform. Each piece of nitrocellulose was then separately incubated overnight in a 1:1,000 dilution of F95 in blocking buffer, at 4 °C on a shaker platform. The next day, the membranes were incubated in a 1:1,000 dilution of donkey anti-mouse IgM secondary antiserum (Jackson Immuno Research). Protein bands were characterized and identified after being reacted for 30–60 s on Bio Max MR-1 film (Kodak) using a chemiluminescent HRP substrate (Millipore Immobilon Western).

Using these techniques, we provide evidence for the first time that the amounts of deiminated proteins in general are higher in aged mice and human brains (Fig. 14.4). This suggests that increased citrullination may be part of the natural process of brain senescence and would require that any future experiments studying deimination in neurological diseases should include controls that are age matched.

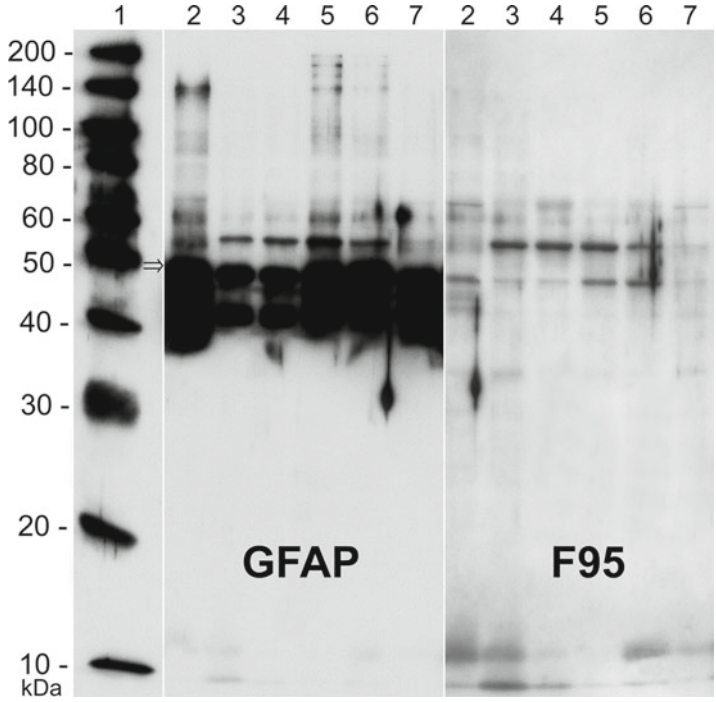

Fig. 14.5 Western blot analysis for total GFAP and citrullinated proteins (F95) from 1-year-old (*lanes 2–4*) and 2-year-old (*lanes 5–7*) Alexander diseased patients (*lanes 3–6*) and age-matched controls (*lanes 2* and *7*). Western blots were run using 10 μg of protein per lane of acidic brain extract from the cerebrum (*lanes 2*, *3*, and *5*) and cerebellum (*lanes 4*, *6*, and *7*) using techniques described previously (Nicholas et al. 2004). *Lane 1* represents standard molecular weight markers. The *arrow* depicts the expected molecular weight of human GFAP (approximately 50 kDa)

14.4.2 Preliminary Examination of Brain Protein Deimination in Human Alexander Disease and an Alexander Disease Animal Model

Since GFAP appears to be a key deiminated protein in several neurodegenerative diseases (Nicholas et al. 2004, 2005; Grant et al. 2007; Ishigami et al. 2005; Jang et al. 2010), we also examined brain specimens from individuals with Alexander disease, a neurodegenerative disorder linked to mutations in GFAP (Brenner et al. 2001). Cerebral and cerebellar samples from two infant patients with Alexander disease and age-matched controls were obtained from the University of Maryland Brain and Tissue Bank for Developmental Disorders and processed as previously described to isolate acidic brain proteins (Nicholas et al. 2004). Western blot analysis of these samples for both GFAP and F95 is shown in Fig. 14.5. Using 10 μg protein per lane, F95 staining indicated the presence of considerably more deiminated proteins in brain samples from Alexander disease patients (lanes 3–6) than

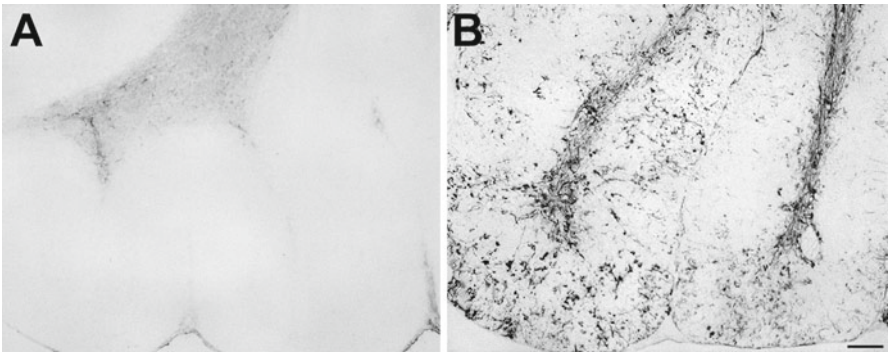

Fig. 14.6 F95 immunoreactive staining in sections of cerebellum from a wild-type control (**a**) and an Alexander disease mouse model that over-expresses human GFAP (**b**) demonstrates evidence for increased citrullinated proteins in the transgenic animals. Scale bar = 100 μm

age-matched controls (lanes 2 and 7). Some of these F95-positive bands from diseased patients appeared to be of similar molecular weights (especially at approximately 55 kDa) to those immunoreactive for GFAP, suggesting that they are a GFAP isoform that is deiminated in the diseased state. No obvious differences were seen in the number of F95-positive bands from the Alexander diseased patients, but the intensity of the 50 kDa bands from the 2-year-old patient (lanes 5 and 6) was increased compared to those from the 1-year-old patient (lanes 3 and 4). These results suggest that deimination may be increased in Alexander disease and that some of these proteins may be citrullinated isoforms of GFAP.

In addition, we also immunohistochemically examined a mouse model of Alexander disease in which GFAP is over-expressed (Messing et al. 1998), using techniques previously described (Nicholas and Whitaker 2002; Nicholas et al. 2003, 2004, 2005). Compared to control mice, GFAP-over-expressing mice showed vastly increased F95 staining (Fig. 14.6), particularly in astrocytes that also expressed immunoreactivity for GFAP (Fig. 14.7). Although these collective results suggest that deiminated GFAP may be increased in Alexander disease, it remains to be determined if GFAP is indeed the protein involved and, if it is, whether the increased level is due to greater deimination of GFAP or due to simply more GFAP being present.

14.4.3 Preliminary Examination of Brain Protein Deimination in Alzheimer's Disease and the APPswe/HuPS1-A246E Mouse Animal Model

Increased deimination of GFAP and vimentin have also been shown in the hippocampus of patients with Alzheimer's disease (AD) (Ishigami et al. 2005). In addition, increased PAD2 immunoreactivity was also observed in astrocytes of the hippocampus (Ishigami et al. 2005) and entorhinal cortex (Acharya et al. 2012) of AD patients compared to age-matched controls.

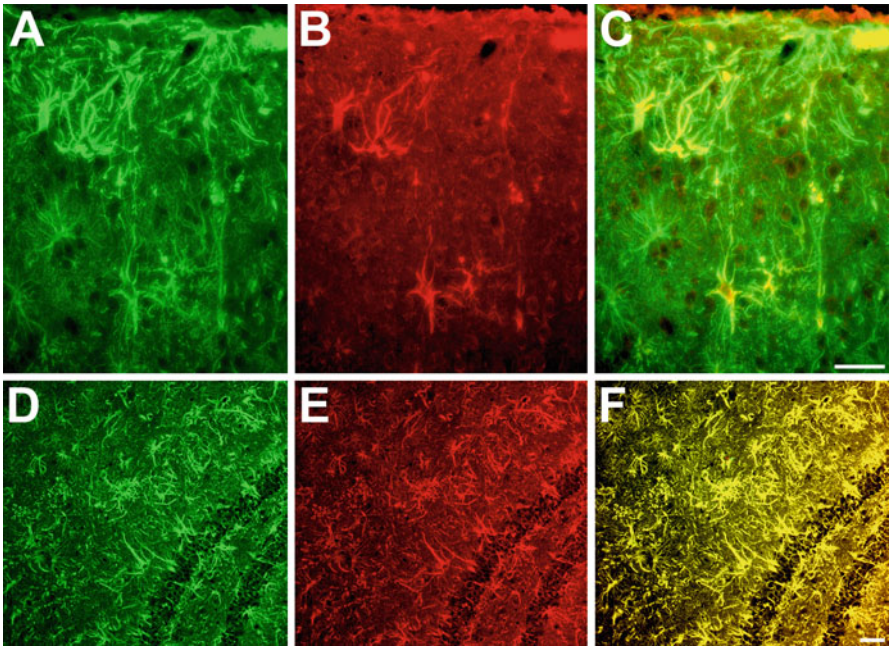

Fig. 14.7 Co-localization (*yellow*) of GFAP (*green*) and F95 (*red*) immunofluorescent staining in the telencephalon (**a**–**c**) near the cortical surface (*top*) and in the hippocampus (**d**–**f**) of GFAP-overexpressing transgenic mice demonstrate that most reactive astrocytes were also immunopositive for deiminated proteins. Scale bars = 50 μm

Using the F95 antibody, we also observed similar findings of large reactive astrocytes containing deiminated proteins in the frontal cortex of AD patients (Fig. 14.8a, b) obtained from the Brain Donation Program Bank associated with the Alzheimer's Disease Research Center at UAB. Some of these glial cells were especially concentrated at the grey matter–white matter interface (Fig. 14.8b), mirroring similar published findings of ours seen in the brains of patients with secondary progressive MS (Nicholas et al. 2004). However, in addition, F95 staining was also seen in extracellular plaques (Fig. 14.8c, d), suggesting that the beta amyloid peptide might be deiminated in AD (Nicholas 2013).

In order to examine if deiminated beta amyloid is increased in AD brains, protein samples of frontal cortex from four AD patients and two age-matched controls were prepared as previously described (Nicholas et al. 2003, 2004, 2005). These samples were then examined with western blot analysis using a mouse monoclonal antibody against human beta amyloid 1–42 (Signet) after immunoprecipitation with the F95 antibody to isolate deiminated proteins (Nicholas et al. 2005). Evidence for deiminated beta amyloid was seen in all samples; however, in general, these bands appeared to be more plentiful and of lower molecular weights from AD brains compared to those of age-matched controls (Fig. 14.9).

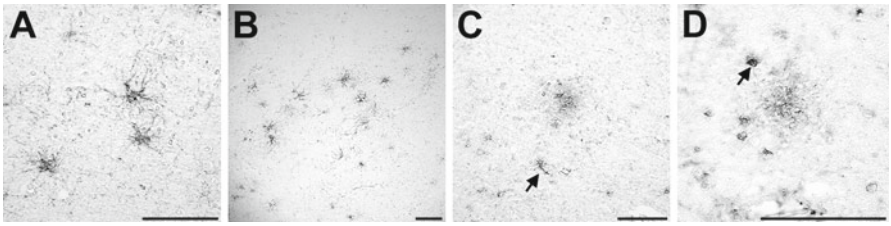

Fig. 14.8 In Alzheimer's disease frontal cortex, evidence for deiminated proteins is seen in large reactive astrocytes (**a** and **b**), some of which are found in a band at the interface between the grey and white matter (**b**). Also immunostained for deiminated proteins are extracellular amyloid plaques (**c** and **d**), which are anatomically near reactive astrocytes (*arrow* in **c**) and suspected neuronal nuclei (*arrow* in **d**) that also are immunoreactive for deiminated proteins. Scale bars = 50 μm

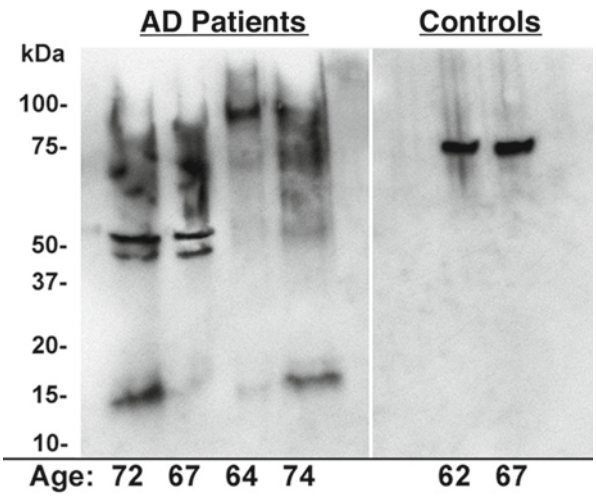

Fig. 14.9 Using immunoprecipitation with F95, deiminated proteins were first isolated from frontal cortical extracts of four AD and two elderly control patients without AD and then probed with a monoclonal antibody against beta amyloid. Although expression of deiminated beta amyloid varied, all specimens expressed evidence for the citrullinated form of this peptide. However, in general, the bands from AD brains appeared to be more abundant and of lower molecular weights as compared to similar specimens from age-matched control patients without AD. The locations of standard molecular weights in kDa are depicted to the *far left* of both blots

Using dual-color immunofluoresence of AD brains (Fig. 14.10), co-localization of F95 and beta amyloid immunoreactivities were seen in amyloid plaques that were less dense (Fig. 14.10a), and these were usually surrounded by astrocytes containing deiminated proteins (Fig. 14.10a, b). This close anatomical association between astrocytes and extracellular plaques supports the notion that these glial cells may function in part to phagocytize this material (Akiyama et al. 1996; Wegiel et al. 2000; Wyss-Coray et al. 2003) in an attempt to clear it from the brain (Wyss-Coray et al. 2003; Frautschy et al. 1992). Supporting this idea is a very recent study showing

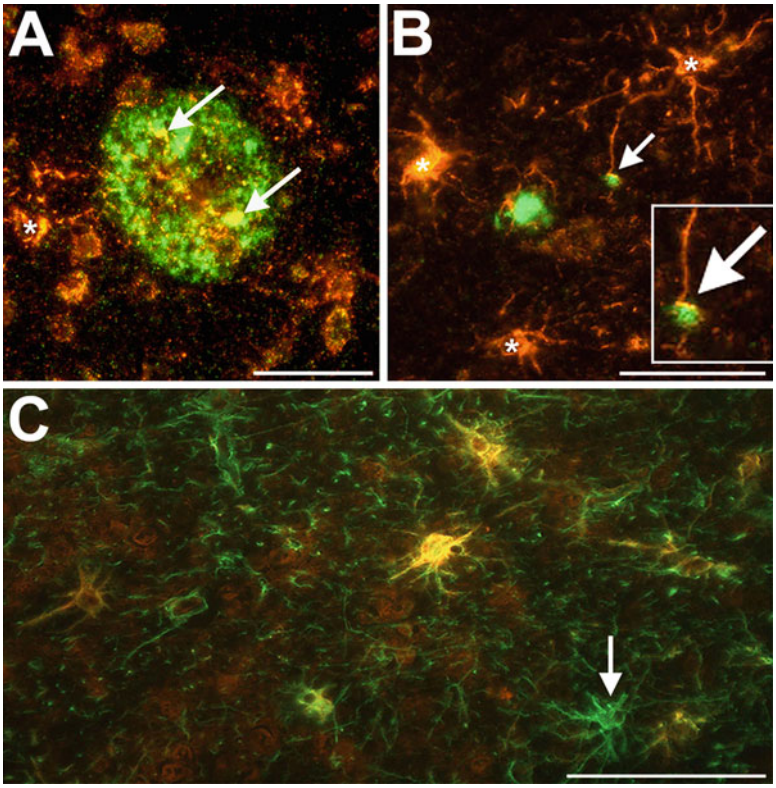

Fig. 14.10 Co-localization (*yellow*) of deiminated proteins (*red*) with other antigens (*green*) in AD brains included beta amyloid (**a** and **b**) and GFAP (**c**). Extracellular plaques (**a** and **b**) are usually surrounded by astrocytes (*asterisks*) and contain beta amyloid (*green*), some of which is deiminated (*yellow*) and occasionally in contact with astrocyte footpads (*arrows* in **b** with higher magnification in insert). This finding suggests that protein deimination in the terminal processes of these glial cells may be required to successfully phagocytize beta amyloid. In addition (**c**), GFAP-immunopositive (*green*) astrocytes in AD brains often contain deiminated protein (*red*) by demonstrating co-localization (*yellow*) with F95, but citrullinated proteins are not present in all astrocytes (*arrow*) in these areas. Scale bars = 50 μm

co-localization of F95 with GFAP at the ventricular surface and surrounding small intraparenchymal blood vessels in AD frontal cortex (Nicholas 2013). Co-localization was also seen in this location between F95 and beta amyloid in astrocytes (Nicholas 2013). In addition, F95 and beta amyloid immunoreactivities were also observed in larger extraparenchymal blood vessels in AD brains, but they were in different layers of these structures and not co-localized, suggesting that the amyloid angiopathy seen in some cases of AD is primarily of the non-deiminated form of beta amyloid (Nicholas 2013).

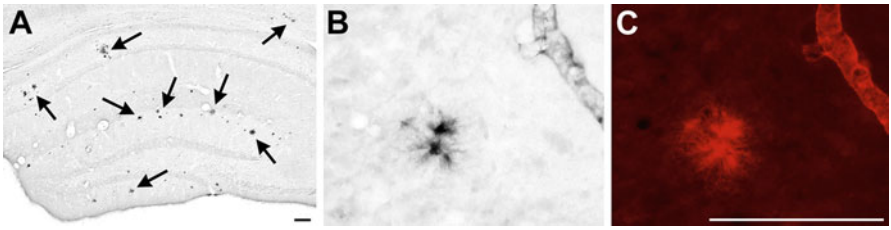

Fig. 14.11 F95 staining (**a** and **b**) in the hippocampus of a cognitively impaired 12-month-old APPswe/HuPS1-A246E transgenic mouse showed numerous intracerebral lesions (*arrows*) reminiscent of extracellular plaques. Higher magnification of this material (**b** and **c**) suggests that F95-immunoreactive deiminated proteins are present in blood vessels (*upper right*) and plaques (**b**) that also contain amyloid, identified by Congo red counterstaining (**c**). Scale bars = 100 μm

Another potential function of these reactive astrocytes containing deiminated intermediate filaments in AD brains may also be to change the shapes of these glial cells in order to physically constrain certain types of inflammatory lesions (Xu et al. 1999), such as extracellular plaques seen in AD (Ishigami et al. 2005; Nicholas 2013) as well as in MS (Nicholas et al. 2004) and EAE (Nicholas et al. 2005). Again, our finding of co-localization of GFAP- and F95-immunoreactivities in reactive astrocytes (Fig. 14.10c) supports previous experiments suggesting that increased deimination of this intermediate filament is commonly seen in AD brains (Ishigami et al. 2005).

However, to further examine the possibility that extracellular plaque proteins such as beta amyloid may be deiminated in AD, we also examined F95 immunostaining in a well-established double-transgenic mouse model of AD containing mutations of amyloid precursor protein and presenilin 1 (Borchelt et al. 1996). After 12 months, when these APPswe/HuPS1-A246E mice (obtained from the MMRRC) were shown to demonstrate numerous intracerebral plaques accompanied by cognitive deficits (Liu et al. 2002), these animals were anesthetized and transcardially perfused with ice-cold saline followed by 4 % paraformaldehyde, and their brains were removed, as previously described (van Groen et al. 2006). After postfixation for 4 h followed by cryoprotection in 30 % sucrose, coronal free floating sections of these brains were cut (30 μm) and incubated overnight at 4 °C with a 1:1,000 dilution of mouse ascites fluid containing the F95 monoclonal antibody in PBS with 0.3 % Triton X-100 (Keilhoff et al. 2008). The following day, these sections were processed for immunoperoxidase labeling and subsequently counterstained with 0.2 g/100 ml Congo red (Sigma) to localize amyloid.

Similar to what we observed in human AD brains (Fig. 14.8c, d), multiple extracellular plaques were immunolabeled with F95 in these transgenic mice (Fig. 14.11a, b), as well as some small blood vessels that were occasionally stained (Fig. 14.11b). Since these structures were also co-labeled with Congo red (Fig. 14.11c), these results suggest that deiminated proteins may be present in amyloid-containing areas. For additional discussion of deiminated proteins in AD, see Chap. 13.

14.4.4 Preliminary Examination of Brain Protein Deimination with F95 in Creutzfeldt–Jakob Disease

In 2010, Jang and collaborators were the first to demonstrate increased PAD activity in the frontal cortex of four patients with Creutzfeldt–Jakob disease (CJD) versus controls (Jang et al. 2010). Although deimination in prion diseases is discussed more thoroughly in Chap. 12, this landmark study showed increased immunoreactivity for PAD2 in reactive astrocytes that also contained increased levels of citrullinated proteins. In addition, using two-dimensional gel electrophoresis and MALDI-TOF mass analysis, various citrullinated proteins were demonstrated in these CJD brain samples, including vimentin, GFAP, enolase, phosphoglycerate kinase, and MBP.

In collaboration with the National Prion Disease Pathology Surveillance Center at Case Western Reserve University, autopsy tissue from a 63-year-old female patient with confirmed CJD was fixed in formalin for at least 10 days and then immersed in 98 % formic acid for 1 h at room temperature before being again placed in formalin for 48 h prior to standard processing for paraffin embedding. Sections (8 μm) of cerebellum from this patient were deparaffinized, rehydrated, and washed. Antigen retrieval was then performed using 10 mM citrate buffer in a microwave pressure cooker set for 15 min at 20 % power. After 1 h, the sections were cooled for 20 min before being rinsed in water, followed by two 15-min washes in Tris-buffered saline with 0.05 % Tween 20 (TBST), prior to F95 immunolabeling.

The staining procedure began with the blocking of endogenous peroxidase by the immersion of the sections in 8 % hydrogen peroxide in water for 10 min, followed by two 10-min washes, before incubating in a blocking solution of 10 % normal goat serum in TBST for 10 min. The slides were then incubated with the F95 anti-peptidyl-citrulline IgM mouse monoclonal antibody for 1 h at room temperature followed by two 10-min washes in TBST, before incubating with Dako Envision + System horseradish peroxidase (HRP)-labeled polymer anti-mouse antisera for 30 min. Diaminobenzidine was used to visualize the immunoreactivity.

Although cellular localization of increased deiminated proteins was previously seen in astrocytes and neurons in the frontal cortex of CJD patients (Jang et al. 2010), the present experiment demonstrates unique patchy F95 staining of what appears to be myelinated axons in the deep cerebellar white matter (Fig. 14.12). This finding supports previous evidence for increased deimination of MBP in CJD brains using protein analysis techniques (Jang et al. 2010). Certainly, examination of more areas of CJD brains with the F95 antibody may be warranted.

14.4.5 Preliminary Examination of Brain Protein Deimination with F95 in Primary Human Astrocytic Tumors

Since astrocytes seemed to be a common target in numerous neurodegenerative diseases for increased deimination (Nicholas et al. 2004; Ishigami et al. 2005; Jang et al. 2010; Acharya et al. 2012; Sambandam et al. 2004), we also examined

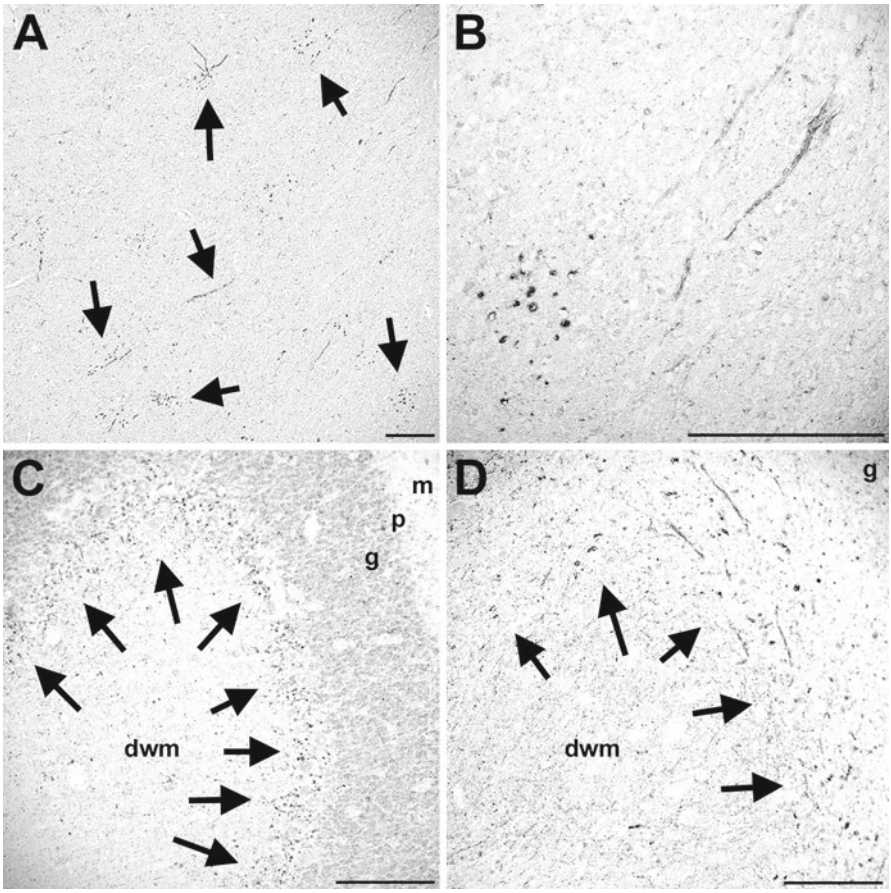

Fig. 14.12 Examination of F95 immunostaining with F95 in the cerebellum of one CJD patient showed spuriously stained structures (**a**) in the white matter near the dentate nucleus (not shown). Higher magnification (**b**) showed F95-immunostained profiles reminiscent of myelinated axons seen longitudinally (*upper right*) and in cross section (*lower left*). In addition (**c** and **d**), the highest concentration of F95-imunoreactive structures (*arrows*) was seen in the deep white matter (dwm) immediately adjacent to the granular cell layer (g). Scale bars = 100 μm. p = Purkinje cell layer; m = molecular cell layer

World Health Organization (WHO) grade I and IV astrocyte tumors with the F95 antibody. Using frozen pathological samples, courtesy of Dr. Yancey Gillespie and the Pathology Department at UAB, grade I and IV astrocytic tumors were prepared for both immunohistochemistry and western blot analysis as previously described (Nicholas and Whitaker 2002; Nicholas et al. 2004; Ishigami et al. 2005; Jang et al. 2010; Acharya et al. 2012; Sambandam et al. 2004).

In samples of grade I pilocytic astrocytomas (Fig. 14.13a, b), F95 staining was sparse but was sometimes found in what appeared to be inclusion bodies. Co-localization of F95 with GFAP immunofluorescence in these structures

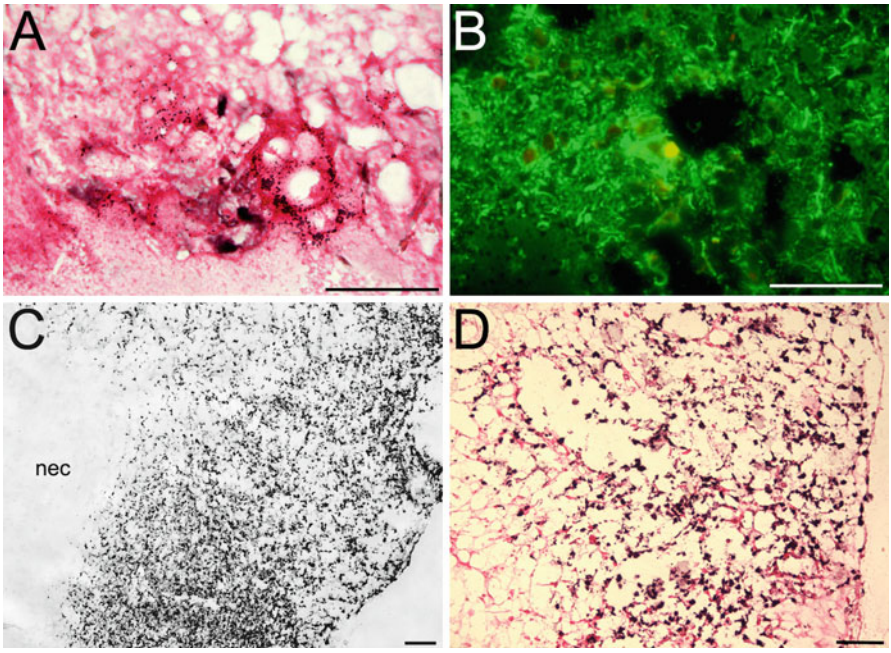

Fig. 14.13 F95 immunostaining of WHO grade I (**a** and **b**) and IV (**c** and **d**) astrocytic tumors. In pilocytic astrocytoma samples (**a**) counterstained with eosin (*pink*), deiminated proteins appeared as sparse, black inclusion bodies. With dual-color immunofluorescence histochemistry, (**b**) deiminated proteins (*red*) often co-localized (*yellow*) with immunofluorescent staining for glial fibrillary acidic protein (*green*), suggesting that some of these inclusions may be Rosenthal fibers. In contrast, F95 staining (*black*) was much more extensive in glioblastoma samples (**c** and **d**), especially when adjacent to necrotic (nec) areas (**c**). With eosin (*pink*) counterstaining (**d**), F95 staining was cytoplasmic, nuclear, or both. Scale bars = 100 μm

(Fig. 14.13b) suggested that these may be Rosenthal fibers. In contrast, samples of grade IV glioblastoma multiforme (Fig. 14.13c, d) demonstrated F95 immunostaining that was more plentiful and intense, especially around areas of necrosis (Fig. 14.13c). Although particular cell types were not identified, both cytoplasmic and nuclear labeling were seen (Fig. 14.13d). This data supports a previous finding of increased PAD4 staining in undescribed "astrocytomas" (Chang and Han 2006) and the idea that increased deimination may play an important role in many types of cancer (see Chap. 17 for more details). Western blot analysis of five different glioblastoma specimens (Fig. 14.14) showed that deiminated proteins were more abundant in these samples compared to controls, but the F95-positive bands were of higher molecular weights as compared to most GFAP isoforms. Future studies will focus on identifying these deiminated proteins.

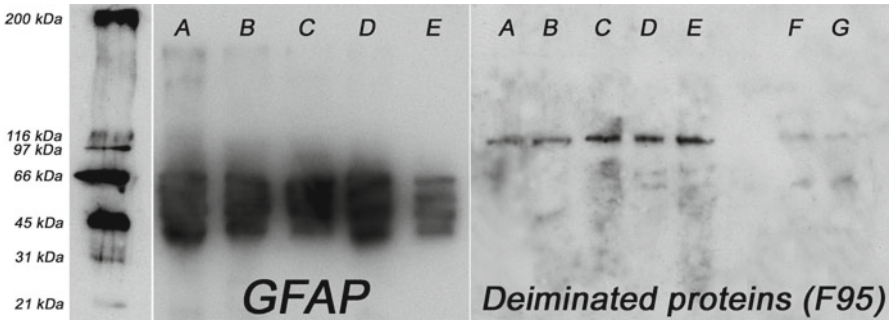

Fig. 14.14 Western blot analysis of protein specimens from five different patients with grade IV glioblastomas (**a**–**e**) showed multiple bands for glial fibrillary acidic protein (GFAP). Deiminated proteins were also found in these specimens, but the most intense bands were of a higher molecular weight (markers at *far left*) compared to most GFAP isoforms. In addition, the relative amounts of deiminated proteins from patients with glioblastoma were greater than those from two normal control brain samples (**f** and **g**). *Lane 1* represents standard molecular weight markers

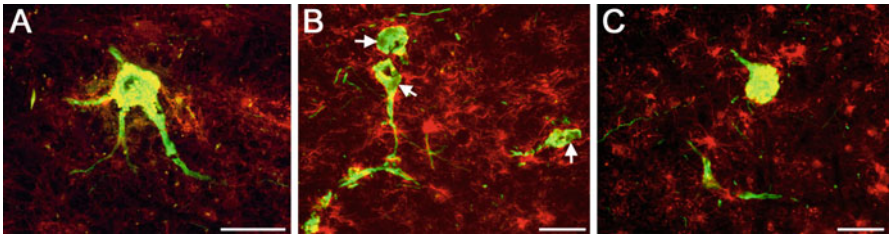

Fig. 14.15 Co-localization (*yellow*) of F95 (*red*) and tyrosine hydroxylase (*green*) was seen in dopamine cells of the substantia nigra of patients with PD (**a** and **b**), but occasionally was also seen in age-matched controls (**c**). *Arrows* indicate patches of neuromelanin pigment. Scale bars = 50 μm

14.4.6 Preliminary Examination of Brain Protein Deimination in Diffuse Lewy Body Disease Versus Parkinson Disease and an Animal Model of Alpha-Synuclein Over-Expression

Recently, our laboratory published the finding that surviving dopamine neurons in the substantia nigra of patients with Parkinson's disease (PD) contain extensive cytoplasmic immunoreactivity for deiminated proteins, which were not confined to inclusions known as Lewy bodies (Nicholas 2011). Deiminated protein immunoreactivity was also seen in numerous large reactive astrocytes in this area of the brain, both in PD patients (Fig. 14.15a, b), as well as age-matched controls (Fig. 14.15c), and these glial cells seemed to be in intimate contact with dopamine neurons

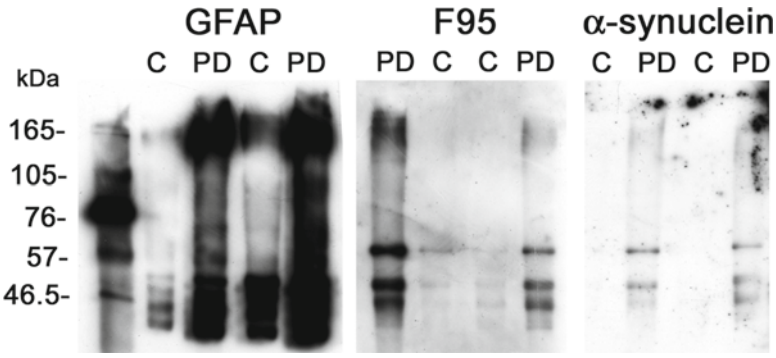

Fig. 14.16 Western blot analysis of brain protein samples showed evidence of higher levels of GFAP, deiminated proteins (F95), and α-synuclein from patients with PD as compared to age-matched controls (C). Note that some bands immunoreactive for F95 are of similar molecular weights to those immunoreactive for α-synuclein and GFAP. *Lane 1* represents standard molecular weight markers

(Nicholas 2011). We reported previously that these dopamine neurons, only in PD patients, contained deiminated proteins (Nicholas 2011). However, after examining more substantia nigra specimens, occasionally evidence for deimination was also seen in these neurons from elderly control patients (Fig. 14.15c). This suggests that perhaps these cells are vulnerable to deimination in general, especially in elderly individuals. Another possibility is that some of these control patients had preclinical PD, prior to brain donation. Future studies on similar specimens with and without PD are therefore warranted.

Using techniques already established (Nicholas et al. 2004, 2005; Nicholas 2011), our next experiments focused on trying to obtain clues on the identity of the deiminated neuronal protein in PD brains. Since α-synuclein is known to be the major toxic protein that aggregates in PD substantia nigra dopamine neurons (Eriksen et al. 2003), we used antibodies against this protein and GFAP and compared western blots from brain samples of PD patients and controls from the Harvard Brain Bank to similar immunoblots using F95 (Fig. 14.16). Although these samples were not immunoprecipitated with F95 to isolate deiminated proteins, these results showed evidence for increased GFAP, F95, and α-synuclein in PD brains, compared to controls. Even though monomers of α-synuclein contain no arginines, similar molecular weight bands for F95 and α-synuclein were observed, suggesting that an unknown deiminated protein bound to α-synuclein aggregates may be present in PD brains.

In contrast to PD, frontal cortex specimens from three additional patients with the pathological diagnosis of diffuse Lewy body disease (DLBD) who donated their brains to the Harvard Brain Bank were also examined with F95 immunohistochemistry. Although these frozen specimens were not of ideal histological quality, two unexpected findings were seen (Fig. 14.17). In the grey matter, F95-positive extracellular plaques, similar to those seen in AD (Figs. 14.8c, d, and 14.10a) and APPswe/HuPS1-A246E mice (Fig. 14.11), were observed (Fig. 14.17a). In the white

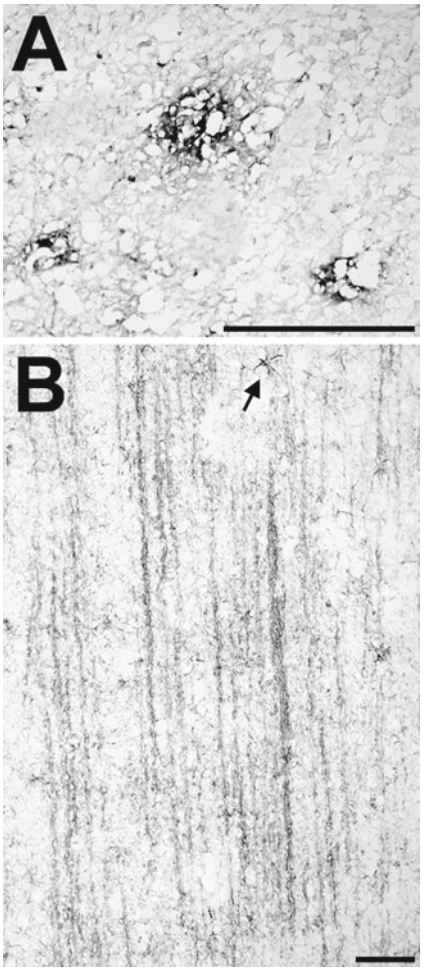

Fig. 14.17 F95 immunostaining showed plaque-like labeling in grey matter (**a**) and radial fiber-like labeling in the white matter (**b**) in the frontal cortex of two patients with diffuse Lewy body disease. Reactive astrocytes (*arrow*) positive for F95 were also occasionally seen in these specimens. Scale bars = 100 μm

matter, streaks of longitudinally running F95-immunolabeled structures were also seen (Fig. 14.17b). Interestingly, these were very similar to GFAP-immunoreactive fibers observed in the normal-appearing white matter of secondary progressive MS patients (Nicholas et al. 2004). Although a few reactive F95-positive astrocytes were also seen in these specimens (Fig. 14.17b), no immunostaining for deiminated proteins was observed in cortical neurons from these DLBD patients. As in other neurodegenerative diseases examined with the F95 antibody described in this chapter, more experiments with additional patients and additional brain areas should be investigated in future studies.

To further explore deimination as it may relate to possible α-synuclein aggregation, we also examined a transgenic mouse model of PD which over-expressed doubly mutated (A30P, A53T) human α-synuclein, driven by the chicken β-actin

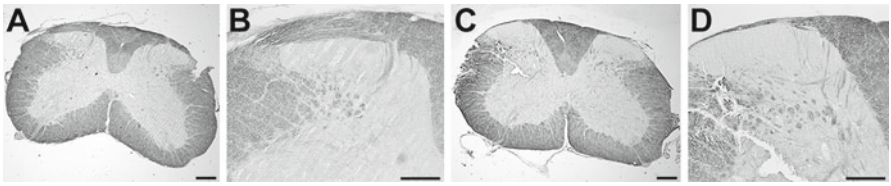

Fig. 14.18 No definitive differences were seen in F95 immunostaining of cervical spinal cords from controls (**a** and **b**) versus mice that over-express α-synuclein (**c** and **d**). (**b**) and (**d**) = higher magnification of the left dorsal horns of (**a**) and (**c**), respectively. Scale bars = 100 μm

promoter and possessing an additional deletion of exon3 in the parkin gene (Mendritzki et al. 2010). Cervical spinal cords from these animals and control littermates were cut into 18 μm thick sections, which were then deparaffinized. Antigen retrieval proceeded with 0.01 M citrate buffer (pH 6.0) steaming for 5–10 min, followed by incubation in 3 % normal serum and then with a 1:500 dilution of ascites containing the F95 antibody. Sections were immunostained with biotinylated secondary antiserum (1:300, Axxora), ABC reagent (1:100 Axxora), and silver–gold intensification. Although these mice were shown to demonstrate motor deficits, as well as glial pathology in their spinal cords (Mendritzki et al. 2010), F95 immunohistochemistry showed no difference in expression of deiminated proteins in spinal cords from these mice, as compared to controls (Fig. 14.18). Even though this negative finding is disappointing, certainly, other animal models of PD have yet to be similarly explored.

14.4.7 Preliminary Examination of Spinal Cord Protein Deimination in Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease causing spasticity and weakness due to degeneration of upper and lower motor neurons, respectively, often leading to death within 3–5 years after diagnosis due to a lack of effective treatment options (Morren and Galvez-Jimenez 2012). The field of ALS research has seen a number of remarkable advances during recent years, in particular, the discovery of pathogenic mutations in TAR DNA-binding protein 43 (TDP-43), fused-in sarcoma (FUS), ubiquilin-2, and most recently C9ORF72 (abbreviation for the open reading frame 72 on chromosome 9), substantiating a relationship between classic ALS and frontotemporal degeneration (Ludolph et al. 2012). In addition, the pathophysiology of ALS has been recently linked to the RNA-binding proteins FUS and TDP-43 (Van Langenhove et al. 2012), as well as HuR (Lu et al. 2009).

To determine if there was differential expression of these molecules versus deiminated proteins, lumbar spinal cords were obtained at autopsy from three patients

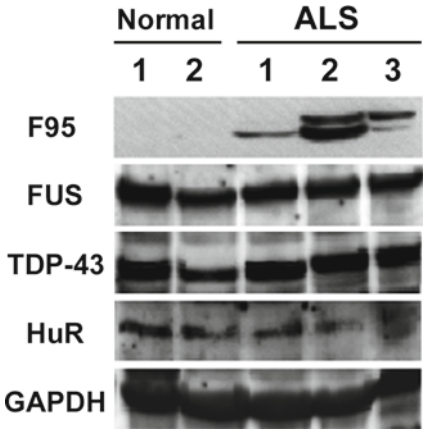

Fig. 14.19 Western blot analysis of spinal cord samples from normal controls and patients with ALS using the F95 antibody showed evidence of increased deiminated proteins (at approximately 50 kDa), only in ALS specimens. In contrast, no significant differences were seen between all patient spinal cord samples for FUS, TDP-43, HuR, or GAPDH. These results were representative of three independent experiments

with idiopathic ALS via the Department of Pathology at UAB. Similar age-matched control specimens were obtained from UAB and the University of Maryland brain bank. Protein extracts of this material were prepared using the M-Per kit (Pierce) and quantified with a bicinochoninic acid protein assay kit (Pierce). Gel electrophoresis was then performed on 50 μg protein samples from each patient per lane, and these were then transferred onto polyvinylidene fluoride membranes (Millipore) for western blotting. Besides F95, the blots were probed side by side with antibodies to the following targets: HuR (Santa Cruz), FUS (Proteintech), TDP-43 (Cell Signaling), and the housekeeping gene product GAPDH (Cell Signaling).

Interestingly, there were no differences in any of the probed samples, except for F95, in which deiminated proteins were significantly increased in all three ALS spinal cord protein samples compared to controls (Fig. 14.19). Again, the identity and cellular localization of these deiminated proteins in ALS patients still remain to be elucidated.

14.5 Conclusion

Using the F95 antibody as a probe, this chapter provides tantalizing evidence that deimination of brain proteins is increased in aging and a number of neoplastic and neurodegenerative diseases. With this data, future studies will focus on trying to identify these citrullinated proteins and their possible roles in the pathophysiology of these various neurological diseases.

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Chapter 15
The Role of Deimination as a Response to Trauma and Hypoxic Injury in the Developing CNS

Patrizia Ferretti, Sigrun Lange, Kin Pong U, and Gennadij Raivich

Keywords Brain • Chick • Deimination • Citrullination • Development • Human • Hypoxia–ischemia • Injury • Mouse • Neural stem cell • Neonate • PAD inhibitor • Peptidylarginine deiminase (PAD) • Trauma • Spinal cord • Stroke

In higher vertebrates, including birds and mammals, damage after birth to the central nervous system (CNS), be it brain or spinal cord, results in permanent disability. In contrast, regeneration in developing embryos can occur until relatively late developmental stages. A key question in regenerative biology and medicine concerns the mechanisms underlying the transition from a regeneration-permissive to a non-permissive state, with the view that such understanding can help devise strategies for reducing damage and aiding repair following injury to the CNS. Another important question concerns whether the same pathways are activated in response to different types of neural insult and could be targeted for the development of much-needed novel therapeutic approaches. Peptidylarginine deiminases (PADs) are emerging as new early players in the response to neural damage and may also play important roles during development. Here we discuss findings concerning the

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putative role of PADs in response to neural damage and evidence that PAD inhibition can reduce the secondary injury response and tissue loss in different models of injury to the developing and perinatal/neonatal CNS.

15.1 Introduction

Neural damage is often a condition of the young, which not only is due to congenital defects but also can be frequently caused by traumatic injuries to the CNS (brain and spinal cord) and perinatal hypoxia–ischemia. In adult birds and mammals, neural injuries do not spontaneously repair themselves. Hence they have serious implications for the quality of life in affected individuals and for their carers and thus have considerable socioeconomic implications. In contrast, significant ability to regenerate the spinal cord is found in lower vertebrates and during development in birds and mammals (amniotes) (Ferretti et al. 2003; Tanaka and Ferretti 2009). Chick and marsupials, which develop much of their CNS during the postnatal period while in the pouch, have provided good models to study loss of spinal cord regeneration in amniotes. Because of the accessibility of their embryos, the times when regenerative capability in these species is lost have been very well established, and this allows researchers to address the question of why regenerative capability is lost with development and investigate the molecular basis underlying such loss (Ferretti and Whalley 2008).

In this review we briefly discuss some of our recent published and unpublished work concerning the putative role of PAD enzymes in changes of regenerative ability and in their response to damage of the developing CNS, with a focus on traumatic injury and hypoxia–ischemia.

15.2 Importance of PAD Enzymes

PADs are a family of highly conserved enzymes that act on target proteins to convert arginine residues to citrulline, a process known as deimination or citrullination. Deimination is calcium dependent and leads to the loss of a positive charge. This can change the protein conformation and consequently its structure, function, and interaction with other proteins. Deimination has been reported to be altered in some neurodegenerative and skin diseases and in rheumatoid arthritis (Chavanas et al. 2004; Gyorgy et al. 2006). In theory, PADs can citrullinate any arginine on a protein, although preference for deimination of certain primary and secondary protein structures has been reported (Gyorgy et al. 2006; Wood et al. 2008). For example, β-turns are efficiently citrullinated, whereas an arginine sandwiched between two prolines is not. Among PAD substrates are structural proteins, such as intermediate filaments and intermediate filament-associated proteins, nuclear proteins, such

as histones (Wang et al. 2004), and extracellular proteins, such as fibrin and fibronectin (Gyorgy et al. 2006).

In mammals, there are five PAD genes (PAD1–4 and PAD6) that cluster on the same chromosome, with PAD2 slightly further away (Balandraud et al. 2005). Only three PAD genes, apparently homologous to mammalian PAD1, 2, and 3, have been identified in chick (Balandraud et al. 2005; Chavanas et al. 2004; Vossenaar et al. 2003). In mammals, there appears to be some overlapping function among PADs (Wood et al. 2008). With the exception of PAD6 (also known as ePAD), which is expressed in oocytes and early embryos, all PADs are expressed to some extent in the nervous system (Esposito et al. 2007; Keilhoff et al. 2008; Vossenaar et al. 2003). PAD2 is considered to be the main PAD in the brain, but PAD3 is also present at significant levels and was detected in some cultured neurons and also in neural stem cells, both from chick and human CNS (Keilhoff et al. 2008; Lange et al. 2011; U et al. submitted). PAD4, like PAD2, has been detected in myelin and has also been shown to translocate to the nucleus where it can citrullinate histone H3 (Mastronardi and Moscarello 2005; Mastronardi et al. 2006; Wood et al. 2008). PAD2 and PAD3 lack a classical nuclear localization signal, but they can also be found in the nucleus of neural cells, although there is no information on how their translocation is regulated (Lange et al. 2011; U et al. submitted; Zhang et al. 2012). For further information on the PAD family of enzymes, see Chaps. 1 and 21.

15.3 Deimination in Normal and Injured Developing CNS

Spinal cord injury has an annual incidence of 15–40 cases per million worldwide and often occurs in young people: over 50 % of spinal injuries occur in patients from 16 to 30 years old. We use the chick spinal cord as a model of spinal injury because it is very accessible, and in the later, non-regenerating stages of development, it responds to traumatic injury in a similar fashion to the human spinal cord by forming a large fluid-filled cavity. In contrast, the injury site in the mouse spinal cord is filled in with cells and connective tissue (Inman et al. 2002; Zhang et al. 1996).

Chick embryos can effectively repair relatively mature spinal cords, where formation of descending neuronal tracts is complete, and recover function. Damage after a pivotal point in development, at around embryonic day 13 (E13), however, results in irreversible structural and functional damage, as in adult birds and mammals (Fig. 15.1a–c) (Hasan et al. 1991, 1993; Shimizu et al. 1990; Sholomenko and Delaney 1998; Whalley et al. 2006, 2009). The ability to regenerate the spinal cord, as well as the loss of this ability with development and evolution, is undoubtedly due to a combination of several factors. One of the significant changes we have observed to occur with spinal cord development in response to injury is the extent of secondary tissue damage at the injury site. Occurrence of apoptosis and subsequent cavitation, which is observed also in adult rats and humans in response to injury, is extensive during non-regenerating stages but either absent or limited during regenerating periods (Fig. 15.1) (Beattie et al. 2000; Berry and Logan 2000; Emery et al. 1998;

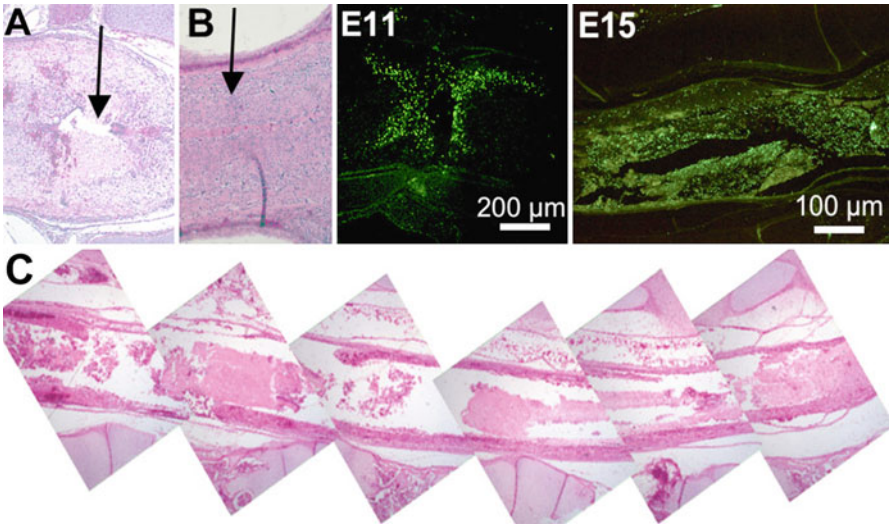

Fig. 15.1 Changes in the chick spinal cord in response to injury during development. (**a**, **b**) E11 spinal cord 24 h and 4 days after injury, respectively. The arrows indicate the injury site. (**c**) Even more massive tissue disruption is apparent in E15 spinal cord 24 h after injury. TUNEL staining (*upper right panels*) 24 h after injury at E11 and E15 also showed much more extensive apoptosis after injury at E15. Modified from Ferretti and Whalley (2008) and Whalley et al. (2006)

Velardo et al. 2000; Whalley et al. 2006). Reduction of tissue loss is a crucial first step for supporting functional recovery following CNS damage and provides a suitable environment for axonal regrowth (Whalley et al. 2006). Inhibition of factors involved in cell death pathway(s) upstream of death effectors would potentially provide a powerful tool to reduce cavitation and maintain a more permissive environment for repair. A chick gene annotated as PAD3 appears to be such a factor.

PAD3 is expressed in the chick spinal cord (Fig. 15.2a). It was initially identified in gene profiling experiments aimed at discovering molecules differentially regulated in the chick spinal cord in response to injury at permissive (E11) and non-permissive (E15) stages for regeneration. A modest PAD3 transcript increase occurred 24 h after spinal cord injury at E11. In contrast, a sixfold up-regulation was observed following injury at E15. Consistent with this finding, increased deimination was found to occur as early as 2 h after injury at E15 but not at E11 (Lange et al. 2011).

Subsets of both neurons and glia were found to be PAD3 positive, and an increase in deimination was observed in these different cell types in response to injury. Strikingly, the increase in PAD activity detected by an antibody to deiminated proteins, the F95 antibody (Nicholas et al. 2003), paralleled the increase in apoptosis and its distribution around the injury site (Lange et al. 2011). Crucially, a pan-PAD inhibitor, Cl-amidine (Luo et al. 2006), applied either before or after injury, significantly reduced not only deimination but also apoptosis and cavitation of the injured spinal cord (Fig. 15.3). Furthermore, no changes in response to injury were observed in the PAD2 transcript or other PADs detected in the chick spinal cord. Altogether, these

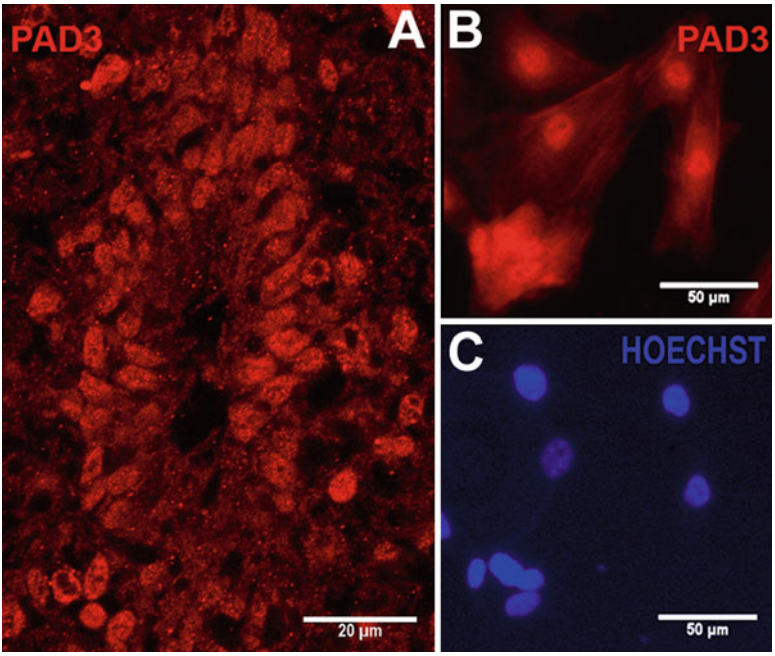

Fig. 15.2 PAD3 protein expression in chick and human detected by immunochemistry. (**a**) Many cells surrounding the central canal in E12 chick spinal cord are PAD3 positive, with strong nuclear expression. (**b**) PAD3 is detected both in the nucleus and cytoplasm of human neural stem cells derived from embryonic brain. (**c**) Corresponding nuclear staining of (**b**)

findings placed PAD3 as the putative PAD isozyme involved in the apoptotic response. This has also been recently found to be the case for human PAD3 in an in vitro model (Fig. 15.2b, c), suggesting that although chick PAD3 shares a similar degree of homology with human PAD3 and PAD4, its function, at least in the context discussed here, is more similar to human PAD3. Indeed, human PAD3 over-expression in vitro was found to increase cell death, both in control cells and in cells treated with thapsigargin, which was used to mimic the increase in cytoplasmic calcium occurring in vivo following traumatic injuries (U et al. submitted) .

As mentioned above, deimination increases in oligodendrocytes following injury at non-regenerating stages and isomers of myelin basic protein (MBP) are well-known PAD targets. This is important, since changes in myelin components also appear to contribute to loss of regenerative ability (McBride et al. 2003). The stages permissive for chick spinal cord regeneration precede myelination, and myelin components have been shown to inhibit axonal regeneration in several systems. Nevertheless, it should be noted that the myelinated spinal cord of certain lower vertebrates maintains regenerative capability (Clarke and Ferretti 1998; Tanaka and Ferretti 2009). MBP was amongst the proteins immunoprecipitated with the F95 antibody against peptidyl-citrulline (see Chap. 14) from E15 injured spinal cords

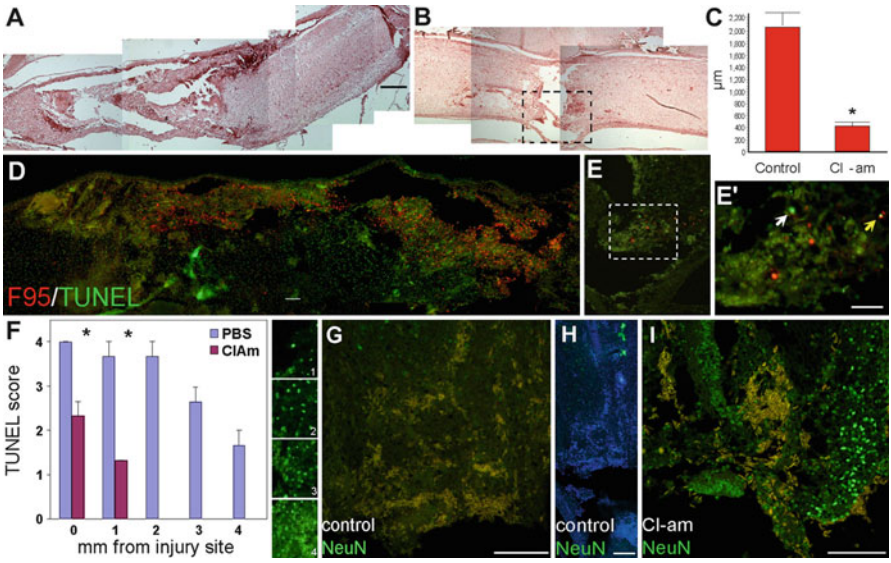

Fig. 15.3 Cl-amidine treatment reduces cell death and tissue loss and increases neuronal survival in injured E15 chick spinal cord. (**a**) Control spinal cord injury (hematoxylin and eosin staining); note large cavity. (**b**) Injured spinal cord treated with 80 mg/kg Cl-amidine (hematoxylin and eosin staining); note the reduction in cavity size. (**c**) Quantitative analysis of cavity size ($n=6$; m ± sd); note significant reduction (ANOVA, $p \leq 0.001$) following Cl-amidine treatment. (**d**) F95 (*red*) and TUNEL (*green*) co-labelling of injured control spinal cord; note extensive deimination and apoptosis. (**e**) F95 (*red*) and TUNEL (*green*) co-labelling of injured Cl-amidine-treated spinal cord. (**e′**) Higher magnification of the *dotted box* in (**e**); note the reduction in both deimination and apoptosis in the treated spinal cord; the *white arrow* points to an apoptotic cell and the *yellow arrow* to a cell double labelled for TUNEL and F95. (**f**) Density of TUNEL-positive cells in control and Cl-amidine-treated animals scored at different distances from the injury ($n=4$; m ± sd). The *boxes* indicate the scoring criteria used to evaluate the extent of apoptosis from very low/negative (1) to high (4). Note significant reduction ($^*p \leq 0.04$; Student's *t*-test) following Cl-amidine treatment close to the injury side and absence of TUNEL-positive cells from 2 mm from the injury site in Cl-amidine-treated spinal cords. (**g**, **h**) Control spinal cord injury stained for NeuN (*green*) shows no NeuN reactivity detected close to the injury site, though some positive neurons (**h**) can be detected distal from the injury site. (**i**) Cl-amidine-treated injured spinal cord shows that NeuN reactivity is maintained close to the injury site, unlike in phosphate buffer saline controls. Scale bars = 260 µm (**a**, **b**, **d**); 130 µm (**e**); 65 µm (**e′**); 100 µm (**g**, **i**); 250 µm (**h**). From Lange et al. (2011)

but not from injured cords treated with Cl-amidine. It is tempting to speculate that deimination of MBP is one of the mechanisms contributing to increased cell death and loss of regenerative capability, possibly through an increased inflammatory response. This hypothesis is consistent with a recent study showing that oligodendrocytes and microglial cells exposed to deiminated MBP peptides undergo apoptosis (Shanshiashvili et al. 2012).

Additional PAD targets we identified by mass spectrometry following immunoprecipitation of proteins from injured spinal cords, with either anti-PAD3 or F95 antibodies, include histones 2 and 3 and a number of cytoskeletal proteins, such as

actin and tubulin, that have been proposed to regulate apoptosis (Franklin-Tong and Gourlay 2008; Lange et al. 2011; Rovini et al. 2011). While histone deimination suggests a role for epigenetic regulation in response to injury, changes in deimination of cytoskeletal components may provide a link between survival/apoptosis and cell motility and possibly the ability of injured neurons to regrow axons.

15.4 PAD Inhibition Reduces Neuronal Damage in Neonatal Hypoxic Ischemia

Neonatal hypoxic ischemia occurs at a frequency of 1–8 cases per 1,000 births in the western world and to a higher extent in less developed countries, resulting in various degrees of neural impairment in 80 % of surviving infants (Perlman 2006). As with adult stroke, there is a lack of effective therapeutic approaches. Furthermore, current understanding is limited regarding the molecular mechanisms underlying perinatal/neonatal brain damage caused by hypoxic-ischemic events and its ensuing neurological deficits. Roles have been proposed for white matter phagocytes during development, for the TNF gene cluster, and for pH changes in hypoxia–ischemia (Hristova et al. 2010; Kendall et al. 2011a, b). Epigenetic mechanisms may also play an important role (see Chap. 18), but only one study on DNA methylation has so far looked into their possible function in this model (Kumral et al. 2012).

Based on our findings in spinal cord injury (Lange et al. 2011), we hypothesize that deimination plays a crucial role in other models of neuronal insult. We have therefore started to investigate changes in deimination in the Rice–Vannucci model of unilateral occlusion of the left common carotid artery and 60-min hypoxia in 8 % oxygen (Rice et al. 1981). Brains of 7-day-old (P7) mice that had undergone the procedure were examined 48 h later, when microglial activation is high and tissue damage is already apparent and can be quantified. All hypoxic-ischemic brains showed an increase in deimination. While the more intense F95 staining was found in the hippocampus, some reactivity was also observed in cortex, striatum, thalamus, and piriform cortex. Intraperitoneal injection of a single dose of Cl-amidine (60 mg/kg, the highest tolerated dose with no mortality) immediately after hypoxia significantly reduced infarct size in the striatum (Fig. 15.4a). Reduction in the extent of damage was also observed in some Cl-amidine-treated animals (Fig. 15.5a, b). Neuronal loss, as estimated from cresyl violet-stained sections, was also reduced in the treated groups and so was apoptosis, as indicated by the decrease in the number of TUNEL-positive cells (Figs. 15.4b, c and 15.5a–d). Finally, quantification of activated microglia cells detected by αMβ2 antibody showed a significant reduction in microglia activation in Cl-amidine-treated brains (Figs. 15.4d and 15.5e, f). The fact that Cl-amidine-induced reduction in tissue damage and microglial activation in hypoxic-ischemic brains was not statistically significant in all brain areas studied may be due either to differences in the evolution of secondary injury response in

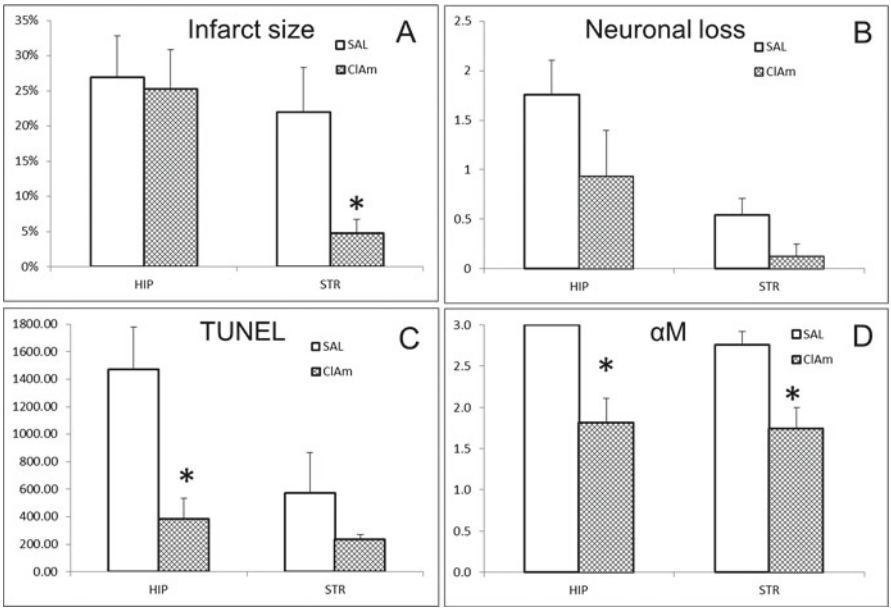

Fig. 15.4 PAD inhibition reduces tissue damage following severe neonatal hypoxia–ischemia (60 min). Hypoxia–ischemia in P7 mice ($n=5$) and tissue analysis (10 sections/brain) of hippocampus (HIP) and striatum (STR) 48 h after the insult were performed as previously described (Hristova et al. 2010; Kendall et al. 2011a, b). (**a**) Infarct size is significantly reduced in the striatum of brains treated with 60 mg/ml Cl-amidine (ClAm; $*p=0.04$) as compared to saline-injected controls (SAL). Infarct size was measured in ten coronal sections from each forebrain (200 μm apart) stained with cresyl violet using the Optimas 6.2 image analysis software (Meyer Instruments Inc.) to calculate the percentage of surviving brain tissue as left/right volume to estimate the reduction in infarct size in treated versus control brains. (**b**) Reduction in neuronal loss is observed in hippocampus and striatum upon PAD inhibition as compared to controls but did not reach statistical significance ($*p=0.18$ and 0.09, respectively). Neuronal damage in control- and Cl-amidine-treated tissue was assessed by cresyl violet staining. Neuronal loss was estimated on a scale from 0 to 4 (0 = no damage; 1 = minimal evidence of damage without evidence of infarct; 2 = small infarct, <50 % of the affected region; 3 = large infarct, >50 % of the affected region; 4 = total neuronal loss). (**c**) The number of TUNEL-positive cells was significantly reduced in the hippocampus ($p=0.02$) upon PAD inhibition, as compared to controls. Reduced cell death was also observed in the striatum, albeit not statistically significant. Cell death involving DNA fragmentation was quantified by counting TUNEL-positive nuclei in each brain region. (**d**) Significant reduction in microglial activation (αM) was detected in both hippocampus ($p=0.01$) and striatum ($p=0.006$) following Cl-amidine treatment, compared to controls. Activated microglial cells were detected using the αMβ2 antibody (Serotec). Score for microglial activation was on the scale from 0 to 3 (0 = no activation, 1 = focal activation, 2 = mild diffuse activation with occasional phagocytic macrophages, 3 = widespread activation with predominant phagocytic macrophages)

different brain regions or to the small sample size ($n=5$/group) in this initial study, or both.

Altogether, this first pilot study on deimination in neonatal hypoxic ischemia shows that protein deimination increases in the areas most affected by

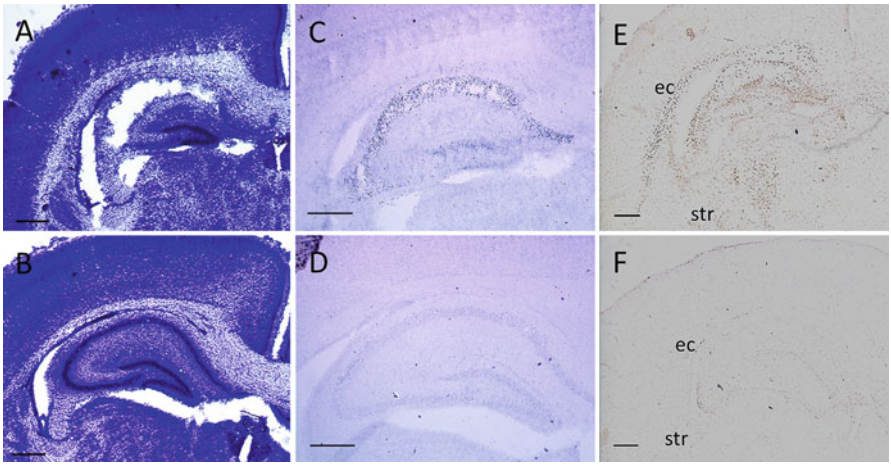

Fig. 15.5 PAD inhibition reduces tissue damage in neonatal hypoxic ischemia. Damage in saline- (**a**, **c**, **e**) and Cl-amidine-treated (**b**, **d**, **f**) brains was assessed 48 h after hypoxia–ischemia. (**a**, **b**) Cresyl violet staining shows neuronal loss in hippocampus in saline controls (**a**) versus Cl-amidine-treated animals (**b**). (**c**, **d**) Dying cells detected by TUNEL in hippocampus of saline control (**c**) and Cl-amidine-treated brains (**d**). Cell death is dramatically reduced upon PAD inhibition (**d**). (**e**, **f**) Microglial activation detected by αMβ2 immunoreactivity in brains of saline control (**e**) and Cl-amidine-treated (**f**) animals. The strong microglial activation seen in hippocampus, external capsule (ec), and striatum (str) in saline controls is greatly reduced by Cl-amidine. Scale bar: 1 mm

hypoxic-ischemic insult and that some reduction in cell death and tissue loss is achieved by inhibiting PAD activity. This parallels the findings in the chick spinal cord and hNSCs, supporting the notion that PAD inhibition early after CNS damage could provide a novel therapeutic avenue (Lange et al. 2011). In addition, it shows a role for PAD(s) in the inflammatory response, as indicated by the regulation of microglia activation, which appears to play such an important role in the progression of this pathology.

Our findings suggest that deimination contributes significantly to tissue damage after perinatal hypoxia–ischemia, although the question of whether PAD3 is the main mediator of cell death in this model remains to be elucidated (Fig. 15.6). In-depth studies are under way to determine the pattern of expression of the different PAD isozymes, the time course of protein deimination in different brain regions following hypoxia–ischemia, and the identity of PAD targets in the injured CNS.

15.5 Deimination in Neural Development: Friend or Foe?

Protein deimination is emerging as a very important posttranslational modification in several contexts in adult mammals (Gyorgy et al. 2006), but detailed information on expression patterns and roles of PADs during development, including in the

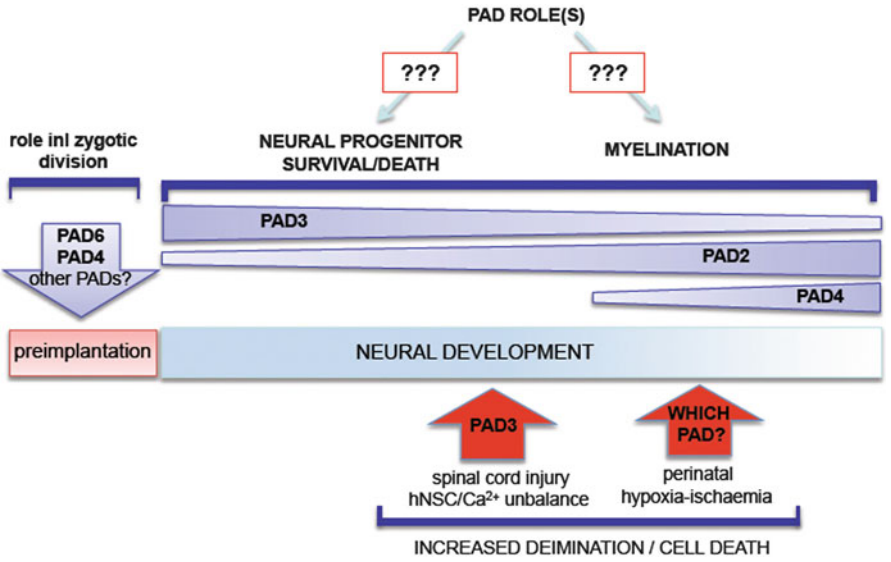

Fig. 15.6 Cartoon summarizing current knowledge on the role of deimination, as a response to trauma and hypoxic injury in the developing CNS and in normal development. Although the exact roles of PAD enzymes in myelination and neural progenitor survival and death are not fully understood, PAD2, 3, and 4 appear to be involved in these processes. In addition, PAD4 and 6 (and perhaps others) also appear to have a role in zygotic division in preimplantation embryos In response to CNS injury, deimination in general is increased, with PAD3 apparently involved in the responses to neural damage in chick spinal cord trauma and increased in cell death induced by cytoplasmic Ca^{2+} in human neural stem cells. Although increased deimination is also observed following hypoxic-ischemic damage in the neonatal mouse, the PADs involved with this process have not yet been characterized

CNS, is still lacking (Fig. 15.6). (For a more detailed overview of PAD enzymes in general, see Chaps. 1 and 21.)

PAD6, which is expressed in oocytes and very early embryos, appears to be crucial for protein translation in the two-cell zygote, by regulating localization of ribosomal components; and female mice lacking this isozyme are sterile (Esposito et al. 2007; Yurttas et al. 2008). PAD4, that has been recently shown to be also expressed in preimplantation embryos, does not appear to compensate for this function (Brahmajosyula and Miyake 2011). A role for PAD(s) in early development has also been supported by evidence that their inhibition using Cl-amidine affects progression of development (Brahmajosyula and Miyake 2012). Furthermore, histone deimination, which is also inhibited by Cl-amidine, has been recently reported in mammalian oocytes and preimplantation embryos. However, the absence neither the absence of PAD6 nor of PAD4 alone is sufficient to block histone deimination at these early stages of development (Kan et al. 2012). It is currently not known whether these two isozymes can compensate for each other or if PAD-dependent regulation of chromatin structure and transcriptional activity is

mediated through other PADs. Nonetheless, there is now substantial evidence for a role of these isozymes in oocytes and preimplantation embryos. A better understanding of the involved pathways might help to elucidate some instances of human infertility and possibly provide novel therapeutic avenues.

PAD2 levels of expression in the mouse brain increase with development and this seems to be the case also in the developing human CNS (Shimada et al. 2009); U and Ferretti, personal communication). Single knockout of PAD2 does not produce obvious developmental defects nor impairs evolution of experimental autoimmune encephalomyelitis (Raijmakers et al. 2006). This could be either due to lack of activity in normally developing embryos or to compensatory effects of other PAD isozymes requiring further studies to address these issues. In contrast, PAD2 over-expression results in CNS hypomyelination (Musse et al. 2008).

In the developing chick spinal cord, we have reported expression of both PAD2 and PAD3 and observed the presence of deiminated proteins, though to a lesser extent than after injury (Lange et al. 2011; Lange and Ferretti, in preparation). Studies on deimination in the adult nervous system have mainly focused on myelin, but it is currently not known which proteins are physiologically targeted by PADs during spinal cord development. The fact that deimination in chick spinal cords can be detected several days before the onset of myelination is strongly indicative of additional roles for this posttranslational modification. Hence other PAD targets are likely to be important early in development. For example, several histones were identified by mass spectrometry following immunoprecipitation of injured spinal cord protein extracts with the anti-PAD3 antibody, consistent with nuclear localization of PAD3 in both chick and human neural precursors (Fig. 15.2) (Lange et al. 2011; U et al. submitted). Whether this reflects a role for PAD3 and/or PAD2 in the regulation of histone deimination and transcription in the developing CNS, a function played by PAD4 in other cell types (Wang et al. 2004), has yet to be determined. Altogether, developmental regulation of PADs in the CNS and presence of deiminated proteins support the hypothesis that PADs play some role(s) in neural development, as proposed for skin epidermal morphogenesis (Tsuji et al. 2003).

15.6 Conclusions

Our understanding of the roles of PADs in the developing CNS is still in its infancy, but given the momentum this field is gaining, much progress could be made in a relatively short time through effective collaborations, and reagent and transgenic model sharing. In contrast, there is mounting evidence that PAD3 activation is associated with increased cell death, secondary injury response, and tissue loss following CNS damage, whether it is caused by a crush injury, as in the chick spinal cord model, or by hypoxia–ischemia, as in the neonatal murine model (Fig. 15.6). Inhibition of this pathway using a pan-PAD inhibitor appears to reduce neural damage in both models. Hence, novel drugs targeting the appropriate PAD isozyme or its

relevant targets will be excellent candidates for the prevention of neural impairment in neonates and children following traumatic injury or oxygen deprivation.

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Chapter 16
Deimination in Ocular Tissues: Present and Future

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Keywords Retina • Aging • Ocular deimination • Glaucoma • Age-related macular degeneration

16.1 Introduction

Deimination refers to the posttranslational conversion of protein-bound arginines into protein-bound citrullines, catalyzed by peptidyl arginine deiminase (PAD) enzymes (Vossenaar et al. 2003). Lack of a carrier tRNA for citrulline has established deimination as a posttranslational modification. Deimination and citrullination are interchangeably used for conversion of arginine into citrulline. To distinguish free arginine citrullination from protein-bound citrullination, we prefer referring to the latter as deimination (Bhattacharya 2009). Throughout this chapter we use deimination to refer to the conversion of protein-bound arginine modification into citrulline.

The function of deimination in neuronal tissues is just emerging (see Chaps. 9–15 for more details). Among these neuronal tissues, eyes have a special place for three reasons: (1) about 38 % of all nerve inputs to the brain originate from the eyes, (2) eyes are highly accessible tissue, and (3) eyes are immuno-privileged tissues. Eyes are also the most complex of neural tissue in terms of cell diversity and complexity of organization. In addition, eyes provide distinguishing differences in the organization of cells, compared to other central nervous system (CNS) (brain and

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spinal cord) tissues, as well as from that of peripheral nervous system tissue. Due to similarities as well as differences in cellular organization compared to other neuronal tissues, eyes are poised to provide contrast and additional insight into biological functions that deimination imparts. Malfunction of different ocular parts is known to contribute to over 400 documented diseases and disorders. It is also evident that some known diseases or disorders are a continuum rather than a discrete single disease. Thus, understanding the role of deimination and its aberration in different regions of the eyes in disease or disorders will provide additional insight into deimination, regarding its regulation and function in other cells and tissues. We present an account of the current state of understanding of existence of deimination and its roles in eyes. Unique and complex organization of eyes and ocular tissues will help unravel roles that deimination plays in other neuronal tissues in the future.

16.2 PAD Enzymes in the Eyes of Different Species

Organisms with eyes present a spectrum, from compound eyes (invertebrates) to complex eyes (birds and mammals) (Bhattacharya 2009). The organisms bearing eyes show differences with respect to the presence of PAD activity. In many invertebrates that have compound eyes, we could not detect the presence of PADs (data not shown) using immunoblots. The fruit fly Drosophila genome has been sequenced (Ensembl database; www.ensembl.org). A search in the Drosophila genome database showed a lack of a PAD transcript. In amphibians and fish, specifically in frogs (*Xenopus*) and in zebra fish, the existing PAD transcript resembles that of PAD2 of higher mammals (Ensembl database). Three and four transcripts have been found in zebra finches and chickens, respectively (for which genome has been sequenced: Ensembl database). In zebra finches (*Taeniopygia guttata*), all three transcripts remain uncharacterized. However, they resemble PAD2 and PAD3 (accession numbers H1A259 and H1A200, respectively, in Uniprot database; www.uniprot.org). In chickens, the transcripts resemble PAD1, PAD2, and PAD3 (Ensembl and Uniprot database). A novel additional transcript found in chickens also resembles PAD3. Turkey (*Meleagris gallopavo*), whose genome has been sequenced, has four uncharacterized transcripts resembling PAD2 and PAD4 (Ensembl database), and their translated protein products resemble PAD1 and PAD2 (Uniprot database). In higher mammals, such as mice and humans, there are 13 and 19 transcripts, respectively, encompassing gene and protein products for PAD1–4 and PAD6, respectively.

16.3 Regions, Types, and Origins of Cells in the Eye and Deimination

The outermost layer of the eye is the cornea, which meets with the extension of the iris and ciliary epithelium at the limbus. Roughly around this junction, fibrous strands of the zonule (zonule of Zinn, ciliary zonule) hold the lens. The lens divides

Table 16.1 Eye cell types and their origin in mammals (mouse in particular)

Origin	Region of the eye	Cell type
Periocular mesenchyme	Cornea, trabecular meshwork, ciliary body, iris	Corneal endothelium and stroma, trabecular meshwork, Schlemm's canal, sclera, ciliary body muscles, iris stroma, extraocular muscles, and ocular blood vessels
Ectoderm	Retina, optic cup, and stalk	Retinal pigment epithelium (RPE), optic cup, optic stalk
Mesodermal	Endothelial lining of ocular blood vessels	Endothelial cells of blood vessels
Endodermal	Optic nerve, vessels	Subset of neurons

Based on Gage et al. (2005) Investigative Ophthalmology and Visual Science 46:4200–4208

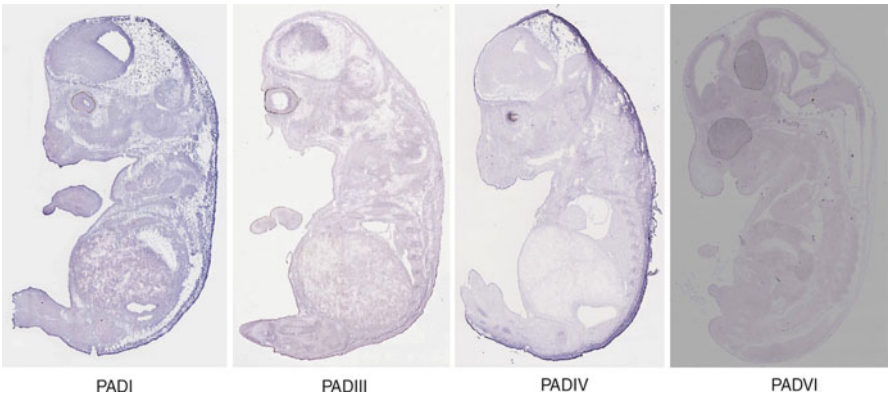

Fig. 16.1 Different PAD transcripts in the mouse (mammalian) eye during development. In situ hybridization on E14.5 C57BL6/J embryo for different PAD transcripts as indicated was performed (Visel et al. 2004; Diez-Roux et al. 2011) using appropriate probes (adopted from Genepaint.org)

the eye into anterior and posterior chambers. The anterior chamber is filled with aqueous humor, and the posterior chamber is filled with more viscous vitreous humor. The cornea and lens are the transparent tissues, which confer refractive and magnifying power to the eye. Each of these layers harbors a few different types of cells. The cornea bears epithelial, stromal, and endothelial cells. The lens possesses a lens capsule, epithelium, fiber cells, and nucleus. The retina is one of the most complex layers and possesses the choroid, Bruch's membrane, the pigment epithelium, outer and inner photoreceptor segments, outer and inner nuclear layers, outer and inner plexiform layers, and ganglion cell layers. The back part of the eye is encased by sclera. This casing, in the anterior direction, is completed by the cornea. All of these layers as well as the iris and ciliary body comprise several cell types. The origin of these cells from periocular mesenchyme, ecto, meso, and endodermal cell layers (Hu et al. 2003; Gage et al. 2005) has been presented in Table 16.1.

On embryonic day 14.5, PAD transcripts (Visel et al. 2004; Diez-Roux et al. 2011) have been found in the eyes of C57BL6/J mice (Fig. 16.1). However, it is

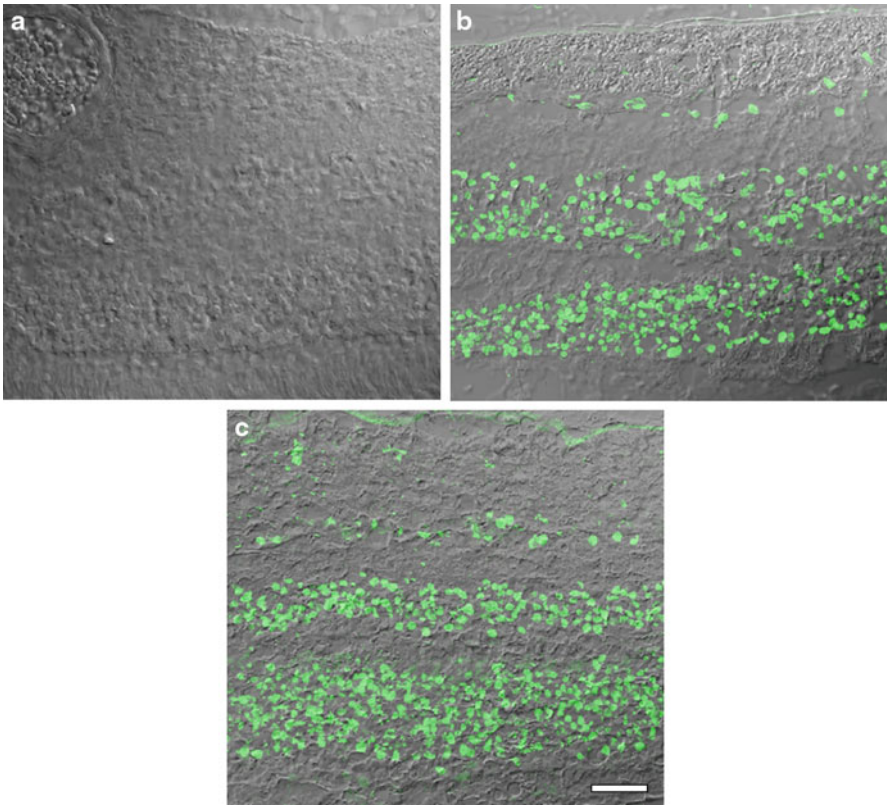

Fig. 16.2 Deimination in the human retina. Representative confocal images of retinal deimination on paraffin-embedded sections were probed with modified anti-citrulline antibody (*green*) after 2,3 butanedione and antipyrene treatment, as recommended by the manufacturer (Millipore Inc., Bellerica, MA). (**a**) No immunostain was seen in a differential contrast image (DIC), control image of retinal tissue from a 77-year-old Caucasian male donor, using secondary antibody but no primary antibody. (**b**) Image of retina from the same patient as in (**a**) showed positive immunoreactivity using the anti-citrulline antibody. (**c**) Image of retina from a 75-year-old Caucasian female probed with anti-citrulline antibody also showed positive immunoreactivity. A bigger, same size scale bar as in figure 16.3 = 40 μm

difficult to ascertain in the primordial eye which cells will eventually express the message and protein. Although expression of different PAD transcripts has been found in mouse eyes (Fig. 16.1), deimination has also been recorded in different regions of human eyes. However, different human donors have shown tremendous variation in retinal tissue, which has been subjected to more studies than other regions of the eye (Fig. 16.2). With control animals such as F344BN rats, several retinal layers show elevated deimination (Bhattacharya et al. 2008) in young animals compared to older ones (Fig. 16.3).

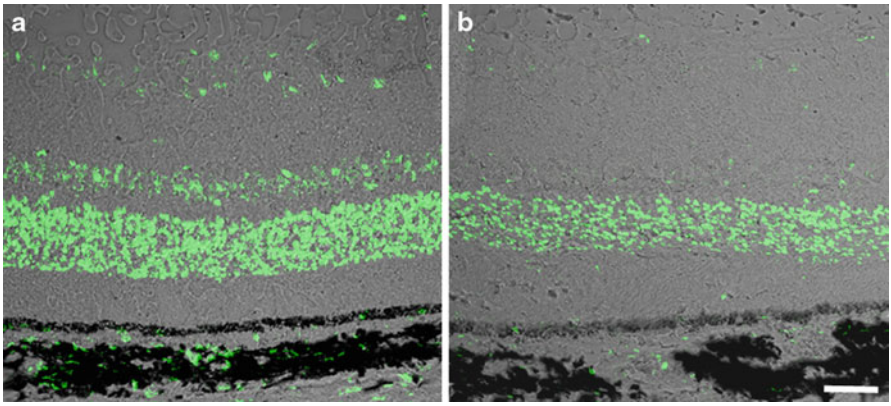

Fig. 16.3 Representative confocal images of deimination in the F344BN rat retina. F344Bn rat retina was probed with an anti-citrulline antibody (*green*) and merged with differential contrast image (DIC). Representative images of a retina from a 3-month-old animal (**a**) versus a 24-month-old animal (**b**) provide evidence for decreased retinal deimination during aging. Bar = 40 μm

16.4 Deimination in Normal Aging Eyes and in Ocular Diseases

There is no known enzyme that reverses the protein-bound arginines into protein-bound citrulline, rendering deimination as a long-term modification. It was found that the level of deimination is increased in neuronal tissue in neurodegenerative diseases (Moscarello et al. 1994, 2002; Bhattacharya et al. 2006). Eyes are regarded as extensions of the CNS, and, hence, it was expected that elevated deimination would be associated with neurodegeneration. It was also found that infants have a higher level of deimination in the brain than normal adults (Moscarello et al. 1994) suggesting a decrease in deimination in normal aging. Control F344BN rats, widely used in aging studies, indeed showed that young animals show elevated deimination (Bhattacharya et al. 2008) in many cell layers in the retina compared to aged adults (Fig. 16.3). This contrasts against findings in human and mice brains that the level of deimination actually undergoes an increase with aging (see Chap. 14). It remains to be investigated whether a perceived age-associated decrease in deimination in the eyes is due to reduced expression or increased turnover or a combination of both during aging. Consequences of altered deimination in different aged tissues also need to be investigated. Expression of certain proteins such as SMP30 has been shown to undergo a decrease with aging and has been attributed to oxidative stress (Maruyama et al. 2005). It is possible that, at least partly, the reduced expression of select proteins in normal aging in the eye results in decreased deimination levels.

Increased deimination has been found in ocular tissues (as well as in other CNS tissues such as the brain and spinal cord) in systemic neurodegenerative diseases

that have ocular manifestations, including demyelinating diseases such as multiple sclerosis (MS) (Moscarello et al. 1994, 2002; Ding et al. 2012; Enriquez-Algeciras et al. 2013). (See Chaps. 10 and 11 for more details.) Increased deimination in ocular tissues, particularly in specific layers of the retina and optic nerve, has also been observed in glaucoma (Bhattacharya et al. 2006). Consistent with these results, elevated PAD2, the main deiminase enzymatic activity found in neuronal systems, was also found to be elevated in glaucomatous optic nerve tissue by mass spectrometric proteomic analyses (Bhattacharya et al. 2006). As yet, deimination levels have not been investigated in all ocular tissues or in most ocular diseases and disorders. Recently, deimination was investigated in age-related macular degeneration (AMD) using cadaveric eyes (Bonilha et al. 2013). Control cadaveric eyes in this study were found to possess variability, and, like the eyes in glaucoma or MS, the AMD eyes displayed elevated deimination levels in retinal and RPE lysates; however, there was no significant change in the levels of PAD2 in these samples (Bonilha et al. 2013).

Modulated levels of deimination have been shown in CNS tissue in a number of other neurodegenerative diseases such as Parkinson's (Nicholas 2011), Alzheimer's (Acharya et al. 2012; Kaiser et al. 2010; Ishigami and Maruyama 2010), and prion diseases (Jang et al. 2012) (see Chaps. 12–14). However, the modulation of deimination (and concomitant assessment of visual impairment) in ocular tissues remains to be investigated in these systematic diseases, as in other eye diseases.

16.5 Deimination and Traumatic Injuries

Prior to elaborate molecular and kinetic characterization of PAD enzymes (see Chap. 21), arginine deiminase was implicated in wound healing in rats (Albina et al. 1988). Elevated deimination has been postulated to be a hallmark of organ injury as well as a marker of differentiation of some tissues such as the epidermis (Maruyama et al. 2005). (For a more detailed review of protein citrullination in skin, see Chap. 7.) In fact, elevated deimination and deiminases were found in the clot and regenerating tissue during cutaneous wound healing in mice (Coudane et al. 2011). Elevated deimination and up-regulation of PAD3 were also found in an avian model of spinal cord injury (Lange et al. 2011) (see Chap. 15). In addition, elevated deimination has been detected in kinate-induced neuronal injuries in the brain (Asaga and Ishigami 2001; Asaga et al. 2002). In kainite-induced injuries as well as in traumatic injuries to CNS tissue, the elevated deimination appeared to be due to activation of astrocytes or other astroglial cells (Asaga et al. 2002; Sambandam et al. 2004; Algeciras et al. 2008). Recent investigation shows persistence of swollen neurons in the optic nerve tissue for a long residence time. Could the elevated deimination that has been earlier observed as a hallmark of organ injury elsewhere (Maruyama et al. 2005) be present in these swollen neurons? Future systematic investigation will yield answers to such questions and also provide insight into the molecular mechanisms associated with such elevated deimination in injured cells, tissues, and organs.

16.6 Cell Type-Specific Control of Deimination

Elevated deimination has been found in CNS tissue (brain, spinal cord) in MS and in the optic nerve (glaucoma) (Moscarello et al. 1994; Bhattacharya et al. 2006). Based on these observations, elevated deimination can be attributed to neurodegenerative diseases. However, closer examinations of cadaveric CNS tissues in infants and normal adults also revealed elevated deimination during development, which is decreased in adulthood (Moscarello et al. 1994). Elevated deimination during development was further supported by a study in an animal model (Bhattacharya et al. 2008), that is, F344BN rats (Fig. 16.3). It was conjectured that such contrasting observations, showing elevated deimination associated with development and neurodegenerative disease, can perhaps be explained on the basis of cell type-specific over-expression of deiminases in development and diseases (Bhattacharya 2009; Bhattacharya et al. 2008). It was postulated that over-expression of deiminase in development occurs in neurons, while in the diseased state this occurs in astroglial cells (Bhattacharya 2009). In the brain, the neurons are always surrounded by a large number of astroglial cells. Glaucoma and demyelinating diseases led to investigation of deimination in the eye. The retina offers a glimpse into neurons (retinal ganglion cells) in an environment that is not rich in astroglial cells. This led to the discovery that, in the retinal ganglion cells (RGCs), decreased deimination occurs during neurodegenerative diseases, with simultaneous increased deiminase expression in astroglial cells, resulting in an accumulation of deiminated proteins in CNS tissues. We postulated that this decreased deimination occurs in all or most generic neurons, just like that observed in RGCs (Ding et al. 2012; Enriquez-Algeciras et al. 2013). In the brain, non-cell-specific inhibition of PADs has been shown to reverse protein hypercitrullination and multiple sclerosis (Moscarello et al. 2013) (see Chap. 11). This is based on the observation of hyper-deimination that is likely contributed by astroglial cells. It is important to note that visual impairment in these MS models has not been characterized, nor has assessment of deimination been made in the ocular tissues of the optic nerve tract (Moscarello et al. 2013). In a genetic model of demyelination, functional electrophysiological and noninvasive structural studies have shown progressive visual impairment, even prior to the onset of clinical symptoms (Enriquez-Algeciras et al. 2011). Based on these findings, RGC-specific delivery of viral vectors, leading to increased over-expression of PAD2, resulted in increased deimination and promotion of dendritic outgrowth. These two features are thought to underlie the observed improvement in visual function on RGC-specific PAD2 over-expression in demyelinating disease (Enriquez-Algeciras et al. 2013). An RNA export binding factor (REF) has been specifically identified and shown to undergo decreased neuronal protein deimination in demyelinating disease (Ding et al. 2012). RGC-specific PAD2 over-expression results in restoration of normal deimination levels for REF, contributing to increased dendritic protein synthesis and increased transport of select mRNA onto the mitochondrial surface (Ding et al. 2012; Enriquez-Algeciras et al. 2013). Overall, reinstatement of normal deimination of REF underlies the dendritic or the proximal growth of neurons and restoration of visual function in a demyelinating disease model (Enriquez-Algeciras et al. 2013).

16.7 Conclusions

Deimination is a long-term irreversible posttranslational modification. It has been shown to occur only on a handful of proteins so far that include nuclear, cytosolic, and extracellular matrix proteins (Bhattacharya 2009). However, the function of this process in normal cellular physiology remains poorly understood. Elevated deimination has been shown in a handful of neurodegenerative diseases in the eyes. It remains to be explored in other ocular diseases, disorders, or traumatic injuries. Elsewhere, deimination has been shown to be a hallmark of organ injury (Maruyama et al. 2005) which remains to be shown systematically in the ocular tissues. In general, all regions of the eye need to be probed for the presence of deimination and deiminases in normal conditions as well as in diseases and disorders. Careful investigation is necessary in the eye due to the diversity of cell types and their specific organization, compared to other CNS tissues. For example, in MS, simple inhibition of PAD activities has been shown to reduce hyper-deimination and provide some functional improvement in four mouse models of the disease (Moscarello et al. 2013). In contrast, improved visual function has been shown using RGC-specific over-expression of PAD2 (Enriquez-Algeciras et al. 2013), illustrating the complexity of the situation wherein hypo- and hyper-deimination often occur simultaneously (Enriquez-Algeciras et al. 2013). In many systemic diseases, ocular manifestations occur first. Furthermore, ocular manifestations are also often linked to disease progression. Thus, from a therapeutic development point of view, investigation of deimination in eyes is poised to provide insight into the complexity and best outcome for therapies tailored for cell specificity.

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Chapter 17
PAD Enzymes in Female Reproductive Tissues and Cancer Pathogenesis

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Keywords Peptidyl-arginine deiminase 2 • PAD2 • PAD4 • Histone citrullination • Breast cancer • Carcinoma • Reproductive tumors • Hormonal regulation of PADs • Female reproductive tissues

17.1 Introduction

Increasing clinical evidence now suggests that peptidyl-arginine deiminase (PAD) enzymes, especially PAD4 and possibly PAD2, have important roles in tumor progression. Further linking PADs and cancer are recent findings showing that treatment of cancer cell lines and mouse models of disease with PAD inhibitors significantly suppresses tumor growth and, interestingly, also inflammation. Current functional target proteins for PAD-catalyzed citrullination in cancer include transcription factors, co-regulators, and histones, the latter of which alter gene expression patterns in multiple cancerous cell lines. As the novel relationship between PADs, inflammation, and cancer unfolds, next-generation isozyme-specific PAD inhibitors may have therapeutic potential to regulate both the inflammatory tumor microenvironment and tumor cell growth. In this chapter we first discuss expression patterns of PADs in reproductive tissues, focusing on their endocrine regulation, as this appears to have major implication for expression, catalytic activity, and tumorigenesis. Next, the chapter details our current understanding of the molecular pathophysiological roles of PADs in cancer. Finally, we discuss the evolving role of PADs in inflammation, in the context of tumorigenesis, with discussion of the potential of new isozyme-specific PAD inhibitors to serve as adjuvant therapy for malignancies.

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17.2 PAD Expression and Function in Reproductive Tissues

Modern transcriptomic data strongly supports the expression of PADs in female reproductive tissues, confirming some of the earliest PAD studies. Massively Parallel Signature Sequencing, which can determine absolute gene expression profiles, was utilized to evaluate mRNA expression patterns across a broad range of mouse tissues (Barrett et al. 2009). Of the more than 50 tissues analyzed, expression levels of PADs 1, 2, 4, and 6 were highest in female reproductive tissues such as the uterus, cervix, and vagina (GEO Profiles GDS868). Our current understanding of PAD expression patterns in female reproductive tissues is illustrated in Fig. 17.1.

It is now well established that early or long-term exposure to estrogens, as with postmenopausal hormone replacement therapy (HRT), increases the risk of ovarian, endometrial/uterine, and breast cancers (Bernstein 2006). Further linking PADs, estrogen, and female reproductive cancers is the presence of estrogen response elements (EREs) in the PAD1 (two EREs), PAD3 (one canonical ERE), and PAD4 (one canonical ERE) gene promoters (Bourdeau et al. 2004). Interestingly, PAD2, which does not appear to contain an ERE, is estrogen regulated in uterine and mammary tissue (Horibata et al. 2012). PAD2's estrogen responsiveness may potentially be explained by estrogen receptor α (ERα) interactions with the transcription factor Sp1, which binds the PAD2 promoter at multiple sites (Dong et al. 2005). Given the correlation between newer transcriptome data, literature showing estrogen

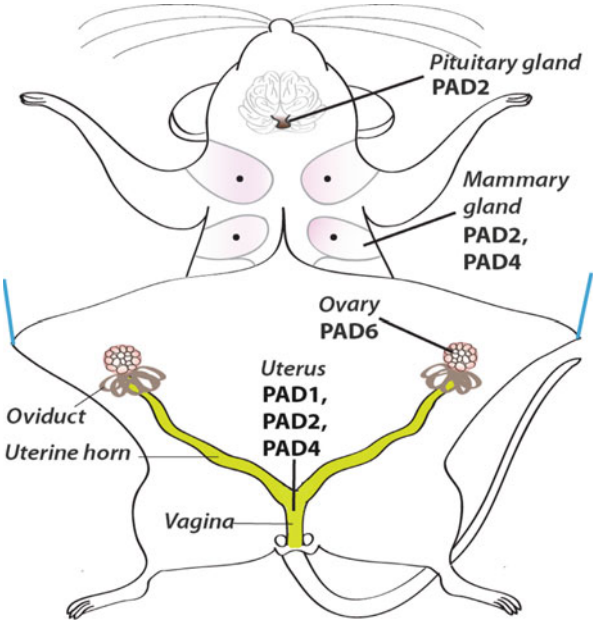

Fig. 17.1 PAD isozyme expression patterns in female reproductive tissues. Reproduced with permission (Horibata et al. 2012)

regulation of PADs, and the mitogenic properties of estrogens in female reproductive cancers, we feel this warrants a further discussion of the potential of PAD enzymes and citrullinated proteins as mediators of reproductive tumorigenesis.

17.2.1 Pituitary Gland

Initial studies in the PAD field focused on a single enzyme termed "PAD" or "skeletal muscle PAD," before the discovery of the different isoforms of this family. The isoform detected in the pituitary appears to be PAD2, based on the cDNA sequence (Watanabe et al. 1988; Watanabe and Senshu 1989). Whole pituitary lysates display a strong sexual dimorphism, with a high level of PAD enzymatic activity in the female rat pituitary versus that of males. PAD enzymatic activity was low in 3-week-old female pituitaries, but following sexual maturation at 4 months of age, activity increased substantially, suggesting hormone-dependent regulation of PAD expression (Senshu et al. 1989).

During the rodent estrous cycle, serum estrogen (17β-estradiol) concentrations gradually increase during metestrus, peak at proestrus, and then rapidly decline during estrus (Butcher et al. 1974). From a reproductive perspective, this spike in estrogen is critical to trigger a surge in luteinizing hormone (LH) release from gonadotropes in the anterior pituitary gland, a critical step for ovulation. Estrous cycle-staged rats showed twofold higher PAD activity levels in pituitary lysates during proestrus and estrus versus the metestrus and diestrus stages. The rise in 17β-estradiol serum levels, just prior to increased PAD activity, indicates that fluctuations in this hormone during the estrous cycle play an important role in PAD expression in the rodent pituitary (Senshu et al. 1989). Supporting this role, ovariectomy of rats results in a substantial decrease in pituitary PAD activity, which can be restored by repeated injections of exogenous 17β-estradiol (Senshu et al. 1989; Watanabe et al. 1990). Interestingly, PAD mRNA levels did not directly correlate with enzymatic activity in the pituitary, which the authors speculate may be due to translational regulation or stability of PAD transcripts (Watanabe et al. 1990). Regulation of PAD2 at the mRNA level has also been documented in other systems (Vossenaar et al. 2004). Studies have confirmed that only 17β-estradiol, and not other steroid hormones, causes a dose-dependent increase in PAD biosynthesis and activity. In the somato-lactotrope rat pituitary-derived MtT/S cell line, PAD levels and activity were four- to fivefold higher than controls, following 17β-estradiol treatment, while testosterone, progesterone, and corticosterone treatment had no effect (Nagata and Senshu 1990). Insulin also increases PAD biosynthesis and activity in MtT/S cells in a dose-dependent manner (Nagata et al. 1992). The increase in insulin-induced PAD expression levels occurs prior to prolactin biosynthesis, suggesting that elevated PAD levels may be important in terms of reproductive physiology to regulate prolactin expression by lactotropes in the anterior pituitary gland. Furthermore, immunohistochemical and immunofluoresecent studies on rat anterior pituitary glands localized PAD expression to prolactin-secreting lactotropes

(Akiyama et al. 1989). A significant increase in PAD activity occurs from day 7 of pregnancy through day 14 in the rat pituitary, although estrogen levels remain low during this time in pregnant rats. This observation suggests that other factors may be regulating PAD expression in the pituitary during pregnancy.

Prolactinomas, derived from prolactin-secreting lactotrope cells in the anterior pituitary, are the most common pituitary tumors and generally occur in women 20–40 years of age who present with hyperprolactinemia (Prabhakar and Davis 2008). Normally during pregnancy, estrogen strongly induces proliferation of lactotrope cells and initiates prolactin biosynthesis, critical steps for lactation. Studies have shown that prolonged estrogen exposure can induce prolactinomas in rats (Elias and Weiner 1984; Sarkar 2006). Given estrogen's role in regulating PAD2 expression in lactotrope cells, it is interesting to speculate that PAD2-catalyzed citrullination of proteins may play a functional role in the initiation and neoplastic growth of prolactinomas.

17.2.2 Uterus

Although the uterus was one of the first tissues investigated in PAD biology, surprisingly little is currently known about the functional role of PADs in this tissue. PADs 1, 2, and 4 are expressed in uterine tissue and localize to luminal and glandular epithelia. PAD1 appears to peak during proestrus, while PAD2 and 4 peak during the estrus phase of the estrous cycle (Horibata et al. 2012; Terakawa et al. 1991). For all PAD isoforms, ovariectomy results in the loss of expression, but it is restored with exogenous estrogen treatment (Terakawa et al. 1991; Takahara et al. 1989, 1992; Rus'd et al. 1999; Horibata et al. 2012). The differences in peak expression between estrous cycle stages of the different PAD family members may be explained by cross-reactivity of early PAD antibodies.

Older literature and newer data clearly indicate strong estrogen regulation of PAD enzymes in rodent uterine tissue. For example, Takahara et al. showed that enzymatic activity in uterine tissue was three- to fourfold higher during estrus than during diestrus, although this likely represents the combined activity of PADs 1, 2, and 4 (Takahara et al. 1992). In a more recent study, ovariectomized mice were treated with exogenous estrogen and uterine samples harvested at 0.5, 2, 6, 12, and 24 h post treatment. Microarray analysis of uterine samples shows that estrogen treatment increases uterine PAD1 and PAD2 expression by >3-fold within 2 h of treatment, while 12 h is required for this level to increase in PAD4 (Hewitt et al. 2003). Expression of PAD mRNA levels was also compared in the uteri of ovariectomized wild-type, αERKO (ERα knockout mice) and KI/KO (ER-binds mutant) mice after estrogen treatment (O'Brien et al. 2006). Both αERKO and KI/KO mice uteri show decreased levels of PAD1, 2, and 4 expression, compared to wild-type uteri at 2 and 24 h, indicating that ERα is involved in the expression of these PAD family members. Thus, multiple PAD family members are expressed in uterine tissue in an estrogen-dependent manner, but the normal physiological function of PADs in this tissue is still unclear.

During the estrous cycle, the growing follicle produces increasing amounts of estrogen, which peaks just prior to ovulation. Known as the proliferative phase, this steady rise in estrogen is critical for the growth and regeneration of the endometrium, to prepare an optimal environment for blastocyst implantation. Given that PADs are highly expressed during this phase, they presumably play a functional role in endometrial proliferation during the normal estrous cycle. Estrogen responsiveness of the family members also implies that PADs may be involved in estrogen-regulated pathophysiological conditions, such as endometriosis or endometrial cancers. Endometriosis is characterized by hormone-driven proliferation of endometrial cells outside the uterine cavity, which can lead to pelvic pain and infertility (Bulletti et al. 2010).

Endometrial cancers are the most common cancer of the female genital tract and are classified into type I endometrioid, which are estrogen regulated, and type II non-endometrioid neoplasms (Ulrich 2011; Yeramian et al. 2013). Prolonged exposure to estrogen is believed to contribute to the development of most type I cancers originating from luminal and glandular epithelial cells of the endometrium. Thus, in uterine tissue, estrogen-regulated PAD expression and resulting citrullinated proteins may contribute to the development of endometrial cancer. To date, only PAD4 expression has been examined in endometrial cancers and was detected in carcinomas and adenocarcinomas (Wang et al. 2010). Based on these current findings, it would be highly valuable to examine expression of PAD1 and PAD2 in endometrial cancers as well and explore the functional role of PADs in uterine tissue.

17.2.3 Ovary

PAD6 is highly expressed in germ cells within the ovary and was first identified due to its absence of expression in somatic tissues versus high expression levels in oocytes and preimplantation embryos (Wright et al. 2003). PAD6 null mice have developmental arrest, occurring at the two-cell stage of embryonic development (Esposito et al. 2007). Normally, PAD6 localizes to a poorly characterized structure, termed cytoskeletal lattices, in oocytes and early embryos and associates with α-tubulin at the lattices (Yurttas et al. 2008). Deletion of PAD6 causes altered microtubule formation, a dramatic suppression of stable microtubules, and defective microtubule-mediated organelle repositioning during oocyte maturation (Kan et al. 2011).

PAD4 also appears to be expressed in oocytes and ovarian cancers. Analysis of pig and murine oocytes found that PAD4 is primarily localized to the cytoplasmic compartment; however, upon progression to the late germinal vesicle stage, PAD4 translocates to the nucleus and localizes to the metaphase I and II spindles (Brahmajosyula and Miyake 2011). Citrullinated proteins, believed to be histone H3, were detected in chromatin of germinal vesicle and metaphase I and II oocytes, indicating a possible role for PAD4 in embryonic development. PAD4 expression also appears to be upregulated in ovarian tumors. For example, PAD4 is highly expressed in serious cystadenocarcinoma, clear cell cancer, mucinous cystadenocarcinoma, dysgerminoma, squamous cell tumor, signet-ring cell carcinoma,

endodermal sinus tumors, germ cell tumors, and immature teratomas. In contrast, PAD4 expression was absent or low in granulosa cell tumors, malignant thecoma, ovarian cystadenoma, and normal ovarian tissue (Wang et al. 2010). Furthermore, these authors showed that PAD4 expression was estrogen regulated in SKOV-3 cells, a human ovarian adenocarcinoma-derived line.

Although PAD4 does not appear to be expressed in normal ovarian tissues outside of oocytes, it may function in an estrogen-dependent fashion in the ovarian tumorigenesis. As with other female reproductive cancers, certain types of ovarian neoplasms are estrogen regulated and prolonged hormone exposure has at least a moderate effect on the risk of ovarian cancer (Beral et al. 2007; Hein et al. 2013). Prolonged estrogen exposure, as with precocious puberty or HRT, may result in dysreglation of PAD enzyme expression, altering citrullination of proteins, which, in turn, leads to increased risk of ovarian cancers.

17.2.4 Mammary Gland

Recent investigations suggest an important role for PAD enzymes in both the normal function of the mammary gland and breast cancer tumorigenesis. In this section, we focus on reviewing the tissue expression patterns and data supporting hormonal regulation of PAD expression in the normal mammary gland, while discussion of these enzymes in breast cancer at the molecular level will be the focus of a later section. To date, PAD2 and 4 appear to be the main isoforms expressed in this tissue.

PAD2 is expressed in mouse, dog, cat, and human mammary glands (Horibata et al. 2012; Cherrington et al. 2010, 2012a). In the canine mammary gland, PAD2 expression initiates during estrus, but the mRNA and protein levels peak during diestrus. During this stage, PAD2 expression is confined to luminal epithelial cells and a portion of staining is detected in small, punctate foci in the nuclei correlating with elevated levels of citrullinated proteins (Cherrington et al. 2010). PAD2 is also expressed in epithelial cell populations within the mouse mammary fat pad in an estrous cycle-dependent manner. In contrast to the dog mammary gland, PAD2 expression is detected during all stages of the estrous cycle, with the highest expression observed at estrus (Horibata et al. 2012). In mice, ovariectomy eliminates PAD2 expression in mammary epithelial cells, but expression can be rescued with exogenous estrogen treatment. Divergent expression patterns between dogs and mice may be due to unique estrous cycle stage lengths and relative hormone levels, especially given that the canine luteal phase can last up to 100 days (Concannon 1986). During this phase, the dog can undergo pseudopregnancy in which prolactin levels rise and the mammary gland proliferates and actively lactates, even in the absence of pregnancy (Concannon 1986). Interestingly, estrogen levels are relatively low during pseudopregnancy, suggesting that other growth factors or hormones may be regulating PAD2 expression. For example, in canine mammary tissue, highest PAD2 expression correlates with wheat germ agglutinin

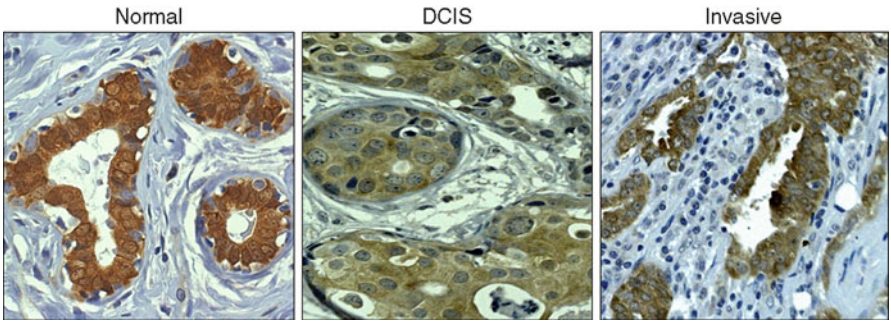

Fig. 17.2 Immunohistochemical staining of PAD2 in normal human mammary gland, a DCIS lesion, and invasive carcinoma

staining, a marker for active lactation (Cherrington et al. 2010). Thus, expression of PAD2 is not only estrogen responsive but during lactation, and possibly pregnancy, also regulated by different factors. PAD2 is also expressed in the epithelia of terminal ductal-lobular units of human breast tissue (Fig. 17.2) and localizes to luminal epithelial cells, similar to expression in other species (Cherrington et al. 2012a, b). Lastly, a portion of PAD2 localizes to the human breast epithelial cell nuclei, suggesting a potential role in gene regulation, which will be discussed in a subsequent section.

Although multiple studies have investigated the molecular role of PAD4 in gene regulation in MCF-7 cells, there has been little emphasis on normal reproductive function or mammary tissue expression patterns for this enzyme. We recently examined PAD4 expression patterns in mouse, dog, and human mammary tissue, in an effort to address this issue. In the mouse mammary, fat pad PAD4 is expressed at low, but detectable, levels in luminal epithelial cells during the estrus phase of the estrous cycle. In ovariectomized mice, exogenous estrogen replacement strongly increases PAD4 expression levels, primarily in the nuclei of mammary epithelial cells, compared to the placebo-treated control (Horibata et al. 2012). PAD4 is also expressed in the canine mammary gland and localizes in luminal epithelial cells. In contrast to the mouse, PAD4 expression in dogs is highest during the anestrus phase of the estrous cycle, when estrogen levels are relatively low, suggesting that other growth factors or hormones may also regulate PAD4 expression (Cherrington et al. 2010). Similar to PAD2 expression in the mammary gland, the difference in PAD4 expression patterns between mice and dogs may be due to different estrous cycle stage lengths and relative hormone level differences between the two species. In terms of normal human breast tissue, recent work by Stadler et al. shows that PAD4 is expressed in alveolar end units and appears to localize to luminal epithelial cells (2013). Overall, PAD4 is expressed in mammary epithelial cells from multiple species and appears hormonally regulated; however, the role of this enzyme in normal physiological function in the mammary gland is unclear.

17.3 PAD Expression and Pathophysiology in Cancer

Studies have examined PAD4 expression patterns in a wide range of invasive carcinomas and, to a lesser degree, PAD2 in mammary tumors (Wang et al. 2010; Cherrington et al. 2012a; Chang and Han 2006; Chang et al. 2009). Unfortunately, a clear picture of PAD2 and PAD4 expression has not emerged. Although there are indications that the loss of PAD2 staining in the nucleus is associated with progression of invasive carcinomas, other studies suggest that PAD4 expression is elevated across numerous tumor types. In general, there is a lack of information regarding expression of various PAD isoforms in a wide range of tumor types.

17.3.1 PAD2 Expression in Tumors

To the best of our knowledge, no work has been done to characterize PAD2 expression patterns in tumors, with the exception of mammary tumors. Comparative analysis of normal human, canine, and feline mammary tissue found strong nuclear and cytoplasmic PAD2 staining in luminal epithelial cells. As mammary tumors progress and show morphological characteristics of carcinomas in humans and dogs, PAD2 expression in the nucleus diminishes but is retained in the cytoplasm (Cherrington et al. 2012a). Currently, there are no characterized cytoplasmic target proteins for PAD-catalyzed citrullination in mammary tumors or information regarding its function in tumorigenesis. Interestingly, PAD2 expression in feline mammary tumors was purely cytoplasmic, with no signal detected in the nucleus (Cherrington et al. 2012a). Given the highly aggressive nature of feline mammary tumors, the loss of nuclear PAD2 may lead to uncontrolled proliferation, increasing carcinomatous behavior and tumor progression. In terms of human breast cancer, PAD2 expression is detected in normal breast tissue, DCIS lesions, and invasive carcinomas (Fig. 17.2). However, no significant correlation was detected between staining intensity and age, survival time, and estrogen or progesterone status of the tumors (Cherrington et al. 2012a). Interestingly, PAD2 expression in invasive carcinomas partially colocalizes with cytokeratin, suggesting that this intermediate filament may be a cytoplasmic target for PAD2-catalyzed deimination (Fig. 17.3). We believe that further investigation of PAD2 expression patterns in a wide variety of tumors would be a valuable addition to our understanding of PADs in cancer tumorigenesis.

17.3.2 PAD4 Expression in Tumors

PAD4 expression, and frequently activity, was elevated in neoplastic cells from breast carcinomas, lung adenocarcinomas, hepatocellular carcinomas, esophageal carcinomas with squamous differentiation, colorectal adenocarcinomas, renal carcinomas, ovarian adenocarcinomas, uterine carcinomas, uterine adenocarcinomas, and bladder carcinomas (Chang and Han 2006). In contrast, PAD4 expression was

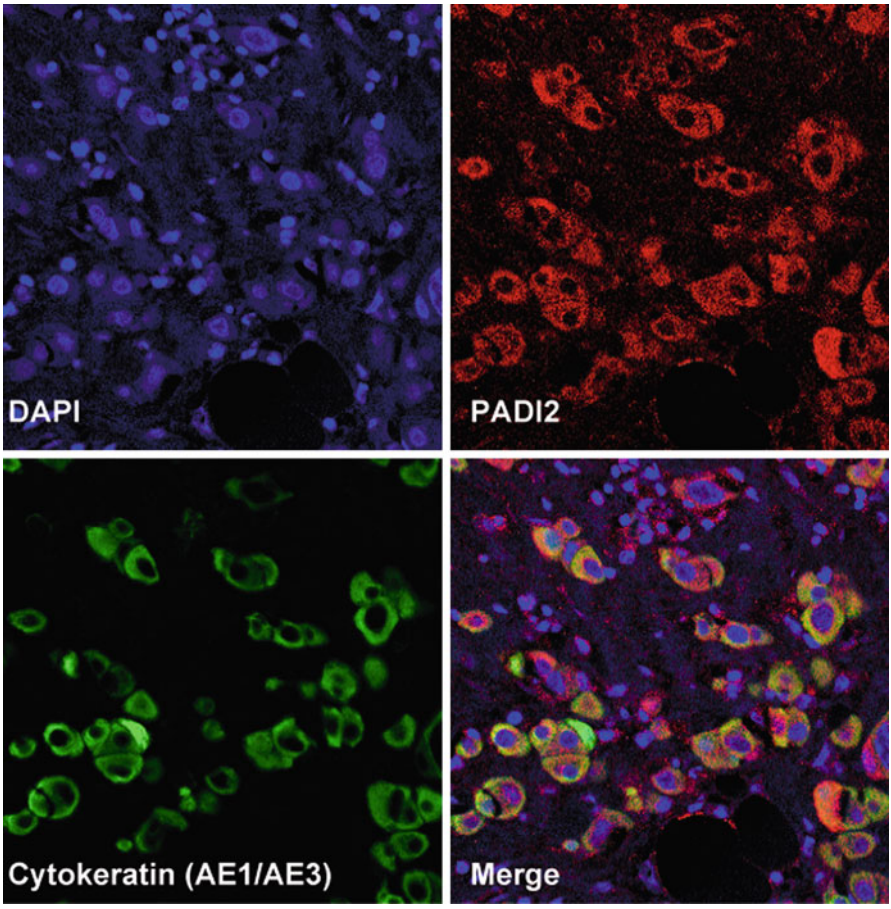

Fig. 17.3 PAD2 immunofluorescent staining (*red*) partially co-localizes with cytokeratin (*green*) in luminal mammary epithelial cells of invasive carcinomas

absent or minimal in benign gastric and uterine leiomyomas, hyperplastic conditions of endometrium, cervical polyps, teratomas, hydatidiform moles, hemangiomas, lymphatic proliferative conditions, schwannomas, and neurofibromas (Chang and Han 2006; Chang and Fang 2010). Western blotting and immunohistochemical studies confirmed the presence of citrullinated proteins in tumors. The authors also detected colocalization of PAD4 with cytokeratin and hypothesized that citrullination of cytokeratin makes it more resistant to caspase cleavage, leading to disruption of apoptosis in tumor cells (Chang and Han 2006). Interestingly, high PAD4 expression in carcinomas was correlated with patients who also had elevated serum citrullinated anti-thrombin levels and PAD4 activity, suggesting that this enzyme may serve as a prognostic clinical biomarker (Chang et al. 2009). Further investigation is needed to characterize PAD4 expression patterns in various tumors and to determine if expression correlates with tumor grade, progression, and clinical prognosis.

17.4 The Molecular Mechanisms of PADs in Cancer Cells

A growing body of evidence now indicates that PAD4 citrullinates and, thereby, alters the function of many important cellular molecules, such as co-regulators, transcription factors, and histones in cancer cells. In contrast, less is known about the molecular role of PAD2 in cancer, although recent work indicates that PAD2 can play a role in epigenetic gene regulation in cancer cells (Cherrington et al. 2012b). PADs are also present in the cytoplasmic compartment of cancer cells and may function to modulate intracellular signaling pathways and cytoskeletal reorganization via citrullination.

17.4.1 Molecular Actions of PAD2 in Cancer Cells

Previous work investigating PAD2 at the molecular level has focused on its role in neurodegenerative diseases such as multiple sclerosis (Chaps. 10 and 11), glaucoma (Chap. 16), and rheumatoid arthritis (Chaps. 2–6) (Pritzker et al. 2000; Cafaro et al. 2010; Chang et al. 2005). Our work was the first to characterize PAD2 expression, regulation, and function in breast cancer cell lines (Cherrington et al. 2012b; Zhang et al. 2012; McElwee et al. 2012). Examination of PAD2 expression in mammary tissue from multiple species detected the presence of punctate staining in the luminal epithelial cell nuclei. To rule out potential cross-reactivity with nuclear PAD4, the PAD2 antibody was tested for isoform specificity and did not show cross-reactivity with PAD4 or any other PAD isoform (Cherrington et al. 2010). In canine mammary gland epithelial cells, robust PAD2 expression during diestrus strongly correlated with citrullination of histone H3 at arginine residues 2, 8, and 17 but not histone H3 at arginine 26 or histone H4 at arginine 4 (Cherrington et al. 2010). This data was the first to support a molecular role for PAD2 in histone tail citrullination.

Given the novel potential role for PAD2 in gene regulation, studies next examined PAD2 expression and function in MCF7 breast cancer cells. In MCF7 cells, a portion of PAD2 is associated with chromatin and shows partial colocalization with acetylated histone H3 at lysine 9, which is a marker for active gene expression, suggesting that PAD2-catalyzed histone deimmination is associated with gene activation, rather than repression (Cherrington et al. 2012b). Unlike PAD4, which contains a nuclear localization signal, the mechanism by which PAD2 translocates to the nucleus is still unclear. Truncation analysis studies indicate that a region between amino acids 278 and 140 either targets the PAD2 protein to the nucleus or is necessary for a protein–protein interaction with the binds partner responsible for its nuclear translocation (Cherrington et al. 2012b).

Using stable shRNA, non-targeting control, and PAD2-depleted MCF-7 cells, microarray analysis detected a set of significantly up- and down-regulated genes. In PAD2-depleted versus control cells, up-regulated genes included PTN, GJA1, JAM2, and SLCO1A2, while down-regulated genes included MAGEA12, Nostrin, and TFAP2A (Cherrington et al. 2012b). Both PAD2 and citrullinated histone H3,

at arginine residues 2, 8, and 17, were shown to associate with the PTN and MAGEA12 gene promoters in vivo, by chromatin immunoprecipitation. Interestingly, both PTN and MAGEA12 are important molecules in cancer biology. PTN is a heparin-binds growth factor important for tumor angiogenesis and is also described as a proto-oncogene (Perez-Pinera et al. 2007; Papadimitriou et al. 2009). MAGE proteins, including MAGEA12, are expressed in multiple types of cancers and are thought to promote survival and proliferation (Simpson et al. 2005; Miranda 2010). Although the phenotype of a cancer cell with decreased PAD2 is unclear, we speculate that PAD2 is necessary for normal epigenetic gene regulation, and its loss results in alterations in gene expression, contributing to carcinoma-like cellular behavior.

17.4.1.1 PAD2- and Estrogen-Mediated Gene Expression

Estrogen and its receptor play important roles in the initiation and progression of breast cancer and serve as a target for many therapies. PAD2 expression is estrogen regulated in mammary epithelial cell populations in vivo, but, until recently, the functional significance of this regulation was unclear (Horibata et al. 2012; Calvo et al. 2012). Work by Zhang et al. indicates that PAD2, rather than PAD4, appears to regulate expression of estrogen-responsive genes by citrullination of histone H3 at arginine residue 26 in MCF7 cells (Zhang et al. 2012). Interestingly, PAD2 has a sixfold higher catalytic activity for histone H3 at arginine residue 26 than does PAD4. In MCF-7 cells, deimination of histone H3 at arginine residue 26 colocalizes with ERα, resulting in the activation of over 200 gene promoters that show higher levels of the H3 citrulline 26 modification following estrogen stimulation. Estrogen treatment leads to enhanced interaction between PAD2 and the ER, suggesting that ERα recruits PAD2 to gene promoters, where it deiminates histone H3 at the arginine 26 residue, facilitating an open chromatin status at target genes (Zhang et al. 2012). In terms of ER+ breast cancers, PAD2-catalyzed histone citrullination of H3 at citrulline 26 may alter gene expression profiles in cancer cells to promote uncontrolled proliferation, leading to breast cancer progression.

17.4.1.2 PAD2 Regulation by Epidermal Growth Factor

Similar to estrogen, epidermal growth factor (EGF) and over-expression of its receptor, HER2/ERBB2, have well-characterized mitogenic effects on breast tumor proliferation and is a significant therapeutic target by Herceptin/Trastuzumab. Multiple lines of evidence suggest a relationship between PAD2 and EGF. For example, treatment of canine mammary epithelial CMT25 cells with EGF resulted in increased expression of PAD2 and an increase in citrullination of histone H3 at arginine residues 2, 8, and 17 (Cherrington et al. 2010). In addition, PAD2 was identified as one of 29 genes over-expressed in HER2/ERBB2-positive primary breast tumors by gene expression profiling (Bertucci et al. 2004). Gene expression profiles of mice with MMTV-neu tumors, which over-express the ErbB-2/HER2

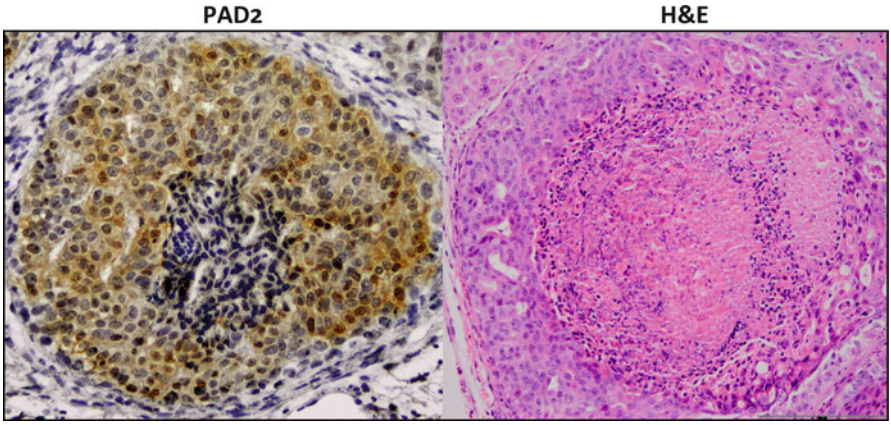

Fig. 17.4 PAD2 immunostaining is localized to luminal mammary epithelial cell populations surrounding the necrotic center of MCF10DCIS xenograft tumors

protein, have approximately a twofold increase in PAD2 mRNA expression in hyperplastic mammary epithelia. In primary neu tumors, PAD2 expression is up-regulated fourfold, when compared to normal mammary epithelia (Montanez-Wiscovich et al. 2009). Recent RNAseq data also shows that PAD2 mRNA expression is significantly correlated with HER2/ERBB2 status in numerous luminal breast cancer cell lines (McElwee et al. 2012). In the MCF10AT model of breast cancer progression, PAD2 expression peaks in the MCF10DCIS cell line, which recapitulates a preinvasive ductal carcinoma in situ (DCIS) lesion. Inhibition of PAD2 in these cells, using the pan-PAD inhibitor Cl-amidine, strongly decreases expression of cell cycle genes implicated in tumor progression, such as p21, GADD45α, and Ki67 (McElwee et al. 2012). Mouse xenograft studies using MCF10DCIS cells showed strong PAD2 staining in luminal epithelial cells surrounding the necrotic center of the tumor (Fig. 17.4). Interestingly, daily treatment of the mice with the Cl-amidine resulted in a significant reduction in tumor volume and prevented the loss of the basement membrane (McElwee et al. 2012). Thus, PAD2 seems to be a downstream target of HER2/ERBB2 signaling in breast cancer cells, but the results of this activation remain unclear. Although the mechanistic function of PAD2 in breast tumor progression may be gene regulatory in nature, the identities of which transcriptional proteins are deiminated and what genes are affected remain to be determined.

17.4.2 Molecular Actions of PAD4 in Cancer Cells

In contrast to PAD2, more is currently known about the molecular function of PAD4, which alters gene expression in different cancer model cell lines (Wang et al. 2004; Li et al. 2008). Co-activator-associated arginine methyltransferase 1 (CARM1)

and protein arginine *N*-methyltransferase 1 (PRMT1) have well-documented roles in methylating histone H3 at arginine 17 and H4 at arginine 4, respectively, which primarily result in reduced levels of gene expression (Wysocka et al. 2006). For many years, it was not known if this methyl modification was permanent or reversible. In a paradigm shifting study for the field of PAD biology, Wang et al. showed that human PAD4 can convert methyl-arginine residues on histone H3 and H4 tails to the nonstandard residue citrulline to regulate expression of the estrogen-responsive pS2 gene promoter in MCF7 cells (Wang et al. 2004). Edman degradation- and histone tail citrullination-specific antibodies now indicate that PAD4 can deiminate multiple arginine residues on histone H2A, H3, and H4 (see Chap. 18 for more details).

We believe that PAD4 is recruited to specific gene promoters by relevant transcription factors, followed by histone deimination, resulting in modification of chromatin status. Multiple studies now show that PAD4 citrullination of histone tail arginine residues leads to chromatin decondensation, resulting in an open chromatin conformation and increasing access to the gene promoter by the required transcriptional machinery (Wang et al. 2009; Tanikawa et al. 2012). There is also a growing body of evidence that PAD4 can citrullinate transcription factors such as Elk-1 and co-activators like p300 to modify gene expression in cancer cell lines (Zhang et al. 2011; Lee et al. 2005). Although a complete picture of the role of PAD4 in gene regulation of cancer progression is far from clear, a major mechanism appears to be regulating the activity of the critical tumor-suppressor protein p53.

17.4.2.1 PAD4- and Estrogen-Mediated Gene Regulation

Understanding the functional mechanism and target genes regulated by PAD4 following estrogen stimulation should provide valuable knowledge regarding the role of PAD4 in female reproductive cancers. The human PAD4 promoter was characterized in MCF7 cells and found to be estrogen responsive. Mechanistically, the authors concluded that ERα binds to two upstream EREs and ERα mediates enhancement of AP-1, Sp1, and NF-Y transcription factor levels, resulting in estrogen regulation of the PAD4 promoter (Dong et al. 2007). Functionally, expression of PAD4 was found to inhibit activity of an ERE-luciferase promoter construct with estrogen treatment in a dose-dependent manner (Wang et al. 2004). This initial observation lead the authors to conclude that PAD4 was primarily repressive on gene expression; however, newer studies indicate that PAD4 can also mediate gene activation (Zhang et al. 2011). In addition, PAD4 also functions in ER-mediated gene transcription by citullinating the co-activator, p300 (Lee et al. 2005). Arginine residue 2142 in the C-terminal region of p300 is methylated by CARM1, which prevents p300 from interacting with the glucocorticoid receptor-interacting protein (GRIP1), a member of the p160 family of co-activators. PAD4 can also remove the methyl mark from p300, altering the conformation and activity of the co-activator complex to regulate ER-mediated gene transcription (Lee et al. 2005). Given the relationship between PAD4 and estrogen in breast cancer cell lines, we believe that there is a strong likelihood that PAD4 plays a prominent role in tumorigenesis in many female reproductive cancers.

17.4.2.2 PAD4- and Epidermal Growth Factor-Mediated Gene Expression

In addition to estrogen, the EGF intracellular signaling pathways appear to use PAD4 as a cofactor to regulate target gene expression in breast cancer cells. A non-biased, genome-wide approach showed that PAD4 is enriched at transcriptional start sites following EGF stimulation of MCF7 cells (Zhang et al. 2011). Mechanistically, the treatment of MCF-7 cells with EGF leads to PAD4-mediated citrullination of the transcription factor Elk-1, a member of the ETS oncogene family, which binds to a DNA motif in the serum-response element on the c-fos gene promoter. Deimination of Elk-1 results in increased levels of phosphorylation of the transcription factor by the signaling kinase ERK1/2. In the proposed model, citrullination and phosphorylation of Elk-1 induce conformational changes that allow for increased histone acetylation, resulting in the subsequent activation of a range of targets, including the immediate early gene c-fos (Zhang et al. 2011). This work was the first to indicate that PAD4 was primarily associated with actively transcribed genes, revising the concept of histone tail citrullination as primarily a repressive modification. Interestingly, several of the DNA-binds elements associated with EGF/PAD4 activity correspond to recognition sites for transcription factors with known roles in mammary gland function, such as STAT1, 3, and 5. Although it is unknown if PAD4 is involved, Elk-1 can interact with BRCA1a and BRCA1b, which may have important ramifications in terms of breast cancer tumorigenesis (Chai et al. 2001).

17.4.2.3 PAD4- and p53-Mediated Gene Expression in Cancer Cells

Multiple studies now indicate a relationship between PAD4- and p53-dependent gene expression in cancer cells. For example, PAD4 and HDAC2 interact with p53 on the p21 gene promoter to regulate gene expression (Li et al. 2010). Given the critical role of p53 in regulating expression of p21, which tightly controls cell cycle progression through G_1, PAD4 association with p53 appears to have a very significant role in cancer biology. Doxorubicin-induced DNA damage in U2OS cells causes PAD4 and HDAC2 to disassociate from the p53 target gene promoters p21, GADD45, and PUMA (Li et al. 2010). Normally, p21, GADD45, and PUMA play critical roles in responding to DNA damage by regulating cell cycle progression, stress response, and apoptosis. Simultaneous with PAD4 and HDAC2 disassociation, there is an increase in histone lysine acetylation and arginine methylation at the target gene promoters. Thus, aberrant PAD4 expression in cancerous cells may lead to repression of a specific subset of genes critical to initiate normal cell cycle arrest and apoptotic pathways, without which, unchecked cellular proliferation ensues.

A study by Tanikawa et al. showed that the level of citrullination of histone H4 at arginine 3 is inversely correlated with p53 protein expression and with tumor size in non-small-cell lung cancer (Tanikawa et al. 2012). The p53–PAD4 pathway mediates citrullination of histone H4 at arginine 3 and appears to lead to localized chromatin decondensation around sites of DNA damage to augment p53-mediated

apoptosis. Furthermore, nuclear lamin C, which provides a critical structural and transcriptional role within the nucleus, was deiminated in response to DNA damage and nuclear fragmentation. Although PAD4-null mice did not display any distinct neoplasias, they appeared resistant to apoptotic stimuli in the thymus and also showed a consistent reduction in cleaved caspase-3 expression (Tanikawa et al. 2012). With DNA damage, citrullination of histone H4 at arginine 3 may function as part of an "apoptotic code" that may be used therapeutically to detect a range of damaged or cancerous cells. Recent immunofluorescence studies also support a role for the deimination of histone H4 at arginine 3 in the nucleus of epithelial cells undergoing morphological changes associated with various stages of apoptosis in comedo-DCIS xenograft sections (Mohanan et al. 2012).

Lastly, PAD4-catalyzed citrullination of the tumor-suppressor protein, inhibitor of growth protein 4 (ING4), impacts p53-mediated gene expression (Guo and Fast 2011). Normally, ING4 binds to p53 and enhances p53-mediated transcription to arrest uncontrolled cellular proliferation. PAD4 interacts with and citrullinates ING4, disrupting its ability to associate with p53. Functionally, citrullination inhibits the ability of ING4 to acetylate p53 at lysine 382, causing down-regulation of p53-mediated transcriptional activity, as detected by a decrease in p21 gene expression (Guo and Fast 2011). Overall, PAD4 is highly correlated with p53-mediated gene expression at the level of the histone tail, and co-activator citrullination supports an important role of PAD4 in tumorigenesis and as a potential therapeutic target.

17.5 The Role of PADs in Chemokine Signaling and Inflammation in Cancer Progression

Inflammation promotes cancer by generating a microenvironment favorable for neoplastic progression. Epidemiological studies have shown that high levels of circulating acute-phase inflammation-associated proteins, at 3 years post treatment, are associated with elevated risk for breast cancer recurrence and mortality (Pierce et al. 2009). A direct link between circulating inflammatory markers and breast cancer metastasis has also been documented (Ahmed et al. 2006; Bachelot et al. 2003; Cole 2009; Salgado et al. 2003). Within the tumor microenvironment, pro-inflammatory cytokines can promote tumor growth and facilitate metastasis by altering the tumor cell phenotype and by regulating nearby endothelial cells, tumor-associated macrophages, and fibroblasts. Infiltration of immune cells into the tumor can also accelerate tumor progression (Coussens and Werb 2002). Cyclooxygenase (COX) is an important mediator of inflammation that has been implicated in lung, breast, and colorectal carcinogenesis. Carcinogenic stimuli, such as tobacco smoke, UV irradiation, and pro-inflammatory cytokines, can induce constitutive COX2 expression (Cebola and Peinado 2012). Epidemiological studies have found that NSAIDs, such as aspirin and ibuprofen, which inhibit COX2, can reduce the risk of cancer (Arber and Levin 2008). Exciting new data indicates that PAD-mediated protein citrullination may regulate the inflammatory microenvironment and cytokine expression.

17.6 The Role of PADs in Inflammation

Many autoimmune diseases associated with PADs, such as rheumatoid arthritis and ulcerative colitis, result in part from protein citrullination within inflamed tissues (Anzilotti et al. 2010; Chang et al. 2005). The PAD inhibitor, Cl-amidine, appears to suppress these inflammatory symptoms in mouse models of colitis (Chumanevich et al. 2011). In cultured RAW 264.7 macrophage cells, PAD2-mediated protein citrullination regulates the NF-κB and COX pathways (Lee et al. 2010). Another strong link between PADs and inflammation is PAD4-catalyzed neutrophil extracellular trap (NET) formation in inflamed tissues, as part of the innate immune response (see Chaps. 1 and 6). Following activation of neutrophils, PAD4-mediated histone hypercitrullination promotes chromatin decondensation and subsequent NET formation (Wang et al. 2009; Chumanevich et al. 2011). In HL60 granulocytes, PAD4 can rapidly convert multi-lobular heterochromatic nuclei into a more round euchromatic nuclear architecture, suggesting a direct role for PADs in heterochromatic-euchromatic interchange (Wang et al. 2009).

While PAD activity is primarily associated with autoimmune-mediated inflammatory events, recent studies suggest that PAD-mediated citrullination is also elevated in a variety of inflammatory states like COPD and myositis, which are less autoimmune in nature (Ruiz-Esquide et al. 2012; Kilsgard et al. 2012; Makrygiannakis et al. 2006). Perhaps the strongest demonstration that PAD-mediated deimination can facilitate non-autoimmune inflammatory events is that PAD activity is strongly up-regulated in inflamed tissue following a sterile skin punch biopsy procedure in mice (Coudane et al. 2011). Overall, PAD-mediated citrullination of proteins appears to play a critical role in the inflammatory processes in a range of pathologies.

17.7 The Role of PADs in Chemokine Signaling

Chemokines recruit leukocytes to the site of inflammation. PAD2 and PAD4 likely regulate chemokine signaling, since they are the only isoforms expressed in peripheral blood mononuclear cells such as NK cells, T cells, B cells, and monocytes (Vossenaar et al. 2004; Asaga et al. 2001; Nakashima et al. 2002). The chemokine, CXCL10, binds to the CXC chemokine receptor 3 (CXCR3) on natural killer (NK) cells to facilitate their recruitment to inflamed target tissues (Luster 2002). Interestingly, CXCL10 levels are highly elevated in the serum of early rheumatoid arthritis patients and in the cerebrospinal fluid of multiple sclerosis patients, two diseases highly correlated with increased PAD activity (Hueber et al. 2007; Sorensen et al. 2002). Supporting a role for PADs in chemokine signaling, the recruitment of lymphocytes was reduced following citrullination of arginine residue 5 in CXCL10 by PAD2, suggesting possible impairment of immunoregulation by chemokine deimination (Loos et al. 2008).

The chemokine receptor system can be dramatically modified in neoplasms, especially at the invasive edges of growing tumors, to act as pro-angiogenic and pro-desmoplastic factors. Chemokines including CXCL1, 2, and 8 can exert effects on tumor cell growth. The ability of chemokines to activate or repress cellular signaling pathways depends upon posttranslational modifications such as phosphorylation and glycosylation. In addition, proteolytic processing of the N-terminus of chemokines also modifies their activity, depending on the type of chemokine and protease involved. For example, CCL2, CXCL7, and CXCL10A are C-terminally processed (Wolf et al. 2008). Importantly, PADs have recently been shown to citrullinate CXCL5, CXCL8, CCL17, and CCL26, thereby altering the inflammatory microenvironment (Proost et al. 2008). Previous studies on deiminated CXCL8 (cit-CXCL8) focused predominantly on its leukocyte function and found that the arginine at position 5 was citrullinated in 14 % of natural leukocyte-derived CXCL8 (Proost et al. 2008). Investigators speculated that the amount of cit-CXCL8 produced by nonimmune cells, including cancer cells, could be substantially higher, because these cells express the CXCL8 isoform, which contains the additional N-terminal arginine residue that is a strong target for PAD2 (Yoshimura et al. 1987; Foulquier et al. 2007; Van Damme et al. 1988). Given the compelling links between inflammation and cancer progression, these observations support the hypothesis that PAD-mediated citrullination of chemokines plays an important role in regulating the inflammatory milieu of the cancer microenvironment.

17.8 Therapeutic Potential of PAD Inhibitors in Cancer

The treatment of cancer cell lines with PAD inhibitors decreases neoplastic cell viability while not affecting the growth of normal cells (Slack et al. 2011). Cl-amidine and F-amidine display low micromolar cytotoxicity in multiple tumor cell lines, such as osteosarcoma U2OS and a variety of breast cancer cell lines (Li et al. 2008; Slack et al. 2011; Luo et al. 2006a, b; Knuckley et al. 2008). These compounds also induce the differentiation of HL-60 cells, a leukemic cell line, making them more susceptible to drug treatments (Slack et al. 2011). Cl-amidine can act synergistically with the anticancer drug doxorubicin, enhancing the efficiency of neoplastic cell death. In breast cancer cell lines, such as MCF7 cells, Cl-amidine also regulates the expression of the tumor-suppressor protein OKL38 in a p53-dependent manner, by decreasing histone deimination at the OKL38 promoter (Yao et al. 2008).

Recently, YW3-56, a Cl-amidine derivative with increased cell permeability, was shown to significantly suppress cancer cell growth and also reduce tumor size in mice xenograft models (Wang et al. 2012). This compound affects the expression of cell proliferation and cell death genes, in addition to regulating macro-autophagy in cancer cells. The authors concluded that the drug likely inhibited the mTORC1 pathway. Given that PAD inhibitors are well-tolerated in disease models, a new generation of PAD inhibitors may serve as novel cancer therapeutics (Chumanevich et al. 2011). Since Cl-amidine, F-amidine, and YW3-56 display limited selectivity,

it is unclear whether the inhibition of one or more PADs is required for the in vivo effects of these compounds. Although a more detailed review of PAD inhibitors is discussed in Chap. 21, the development of new, more selective compounds, such as the PAD4-selective inhibitor TDFA and the PAD1-selective inhibitor o-F-amidine, will undoubtedly prove to be useful in addressing this question.

17.9 Conclusions

Given the recent findings about the developing role of PAD2 and PAD4 in cancer biology, it will be interesting to determine whether they function in a synergistic manner to promote tumor progression. The observation that both PAD2 and PAD4 expression appear to be regulated by estrogen suggests that these two PADs may work together in some capacity to mediate estrogen-responsive tumor progression. Furthermore, PAD2 expression in mammary epithelial cells is regulated by EGF, and PAD4 regulates EGF-induced ELK1 target gene activation (Cherrington et al. 2010; Zhang et al. 2011). Again, these findings suggest that PAD2 and PAD4 may cooperate to mediate HER2/EGFR signaling and have prominent roles in EGF-mediated tumor growth. Currently, our understanding of the role of PADs in cancer is in the early stages. A large-scale screening of different types of tumors for the expression of all PAD isoforms would greatly advance our knowledge. Finally, significant work remains to be done to understand the functional role of PADs and protein citrullination at the cellular level in different types of cancer cells. Molecular studies such as the ones investigating the relationship between PAD4 and p53 strongly suggest that PAD proteins play an important role in tumorigenesis. A growing body of evidence now indicates an important role of PADs in cancer, and it will be interesting to see if new-generation PAD inhibitors can serve as anticancer therapeutics.

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Chapter 18
The Role of Protein Deimination in Epigenetics

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Keywords Epigenetics • Chromatin • Posttranslational modification • Deimination

18.1 Introduction

The first description of "epigenetics" was made by C. H. Waddington in 1942 to include a whole complex of developmental processes between genotype and phenotype (Waddington 1942). Since then, a plethora of epigenetic mechanisms for gene regulation have been discovered, with many conserved from yeasts to higher animals. As a result, the definition has evolved to include mechanisms of gene regulation that are inheritable mitotically and/or meiotically but not dictated by the DNA sequence itself (Feil and Fraga 2011). At present, Waddington's insight still holds true, in that epigenetic regulation of gene expression involves a number of processes that are methodically organized to allow spatial and temporal regulation of gene expression.

Accumulating evidence suggests that epigenetic factors exemplified by histone posttranslational modification (PTM) cooperate with DNA sequence to control a diverse range of biological processes, including cell differentiation and reprogramming, organism development, and tissue homeostasis. Protein deimination of

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nucleosomal histones is a relatively less-characterized form of histone PTM. However, it appears to play a unique and incompletely understood role in epigenetics. At a molecular level, histone deimination directly antagonizes arginine methylation on histone tails, thereby interfering with the transcriptional consequences of such an effect. In addition, deimination can also affect the activity of important histone-modifying enzymes, such as the acetyl transferase p300, consequently amplifying the transcriptional outputs. In physiological or pathological conditions, histone deimination mediates chromatin decondensation and elicits a cell-specific innate immune response in neutrophils.

In this chapter, we first review histone PTMs and DNA methylation and consider the potential consequences that deimination has on histones and chromatin, either directly or indirectly, through regulating the activities of histone-modifying enzymes. In addition, we elaborate on the potential roles that histone deimination might play in cell function. Finally, we end this chapter with a discussion regarding the potential epigenetic mechanisms through which protein deimination may be involved to affect health and disease.

18.2 Epigenetics: Modification of Histone Tails and Genomic DNA

We know that genomic DNA of eukaryotic cells is packaged into fundamental units called "nucleosomes," characterized by an average of 146 bp of DNA surrounding a protein octamer composed of histones (two copies of H2A-B2B dimer and two copies of H3-H4 dimer) in each, with the N-terminal tails of the core histones extending towards the outside space (Luger et al. 1997). Polymers of nucleosomes are further packed by linker histones (H1) and non-histone nuclear proteins (i.e., HMG family proteins) to form chromatin fiber that can be further compacted to higher order structures (Luger et al. 2012). Based on this complex nuclear architecture, each cell type is able to favor or inhibit transcription by regulating the accessibility of DNA binding sequences to RNA polymerases, transcription factors, and other regulatory proteins. The epigenetic mechanisms utilized for this purpose include covalent modification of DNA nucleotides, PTM of histones, and replacement of canonical histone subunits with histone variants. Multiple enzymes or effector proteins are specifically responsible for "writing," "reading," or "erasing" these marks and ultimately lead to changes in chromatin structure and gene expression (Bernstein et al. 2007; Strahl and Allis 2000).

18.2.1 Posttranslational Modification of Histone Tails

The crystal structure of the nucleosome core particle was first resolved in 1997 and two major characteristics were immediately revealed. The first one was that the N-terminal tails of histone H3 and H4 (but not H2A and H2B) were extruded from

the core particle (Luger et al. 1997), which explains why most of the functionally important histone PTMs take place on the N-terminals of H3 and H4. However, PTMs on H2A and H2B, as well as inside the globular domains of core histones, also exist and may play a role in regulating the chromatin structure (Tan et al. 2011). The second major characteristic was that interaction of DNA with the core histones was mediated not only through hydrogen bonds but also through electrostatic interactions between the negative charges of the DNA phosphate-backbone and the positively charged N-terminal tails of histones H3 and H4. Consequently, it became clear that modulation of these interactions between DNA and histones could affect the packaging of DNA and therefore gene expression.

There are at least two major mechanisms through which histone PTMs are able to affect chromatin structure and transcription. Besides their ability to alter DNA–histone interaction or inter-nucleosomal interactions, modified histones can also serve as docking sites for proteins capable of recognizing modified histones and, in turn, mediate changes in local chromosomal architecture or DNA bases, such as methylation (Kouzarides 2007). Based on this assumption, it was proposed that distinct PTMs of amino acids on histone tails may individually, or in a combinatorial fashion, determine the transcriptional response of a cell to extracellular signals or intracellular changes, such as DNA damage (Rando 2012; Strahl and Allis 2000). To date, more than 100 modified amino acids on histones have been collectively described and they can be subject to at least 12 types of PTMs (Table 18.1). We will briefly review modifications of lysine residues here and then focus on arginine methylation and deimination.

18.2.1.1 Modification of Lysine Residues and Their Functional Roles

Lysine acetylation is one of the most widely distributed PTMs on core histones tails. Acetylation of 18 lysine residues on specific locations of the four core histones have been confirmed in vivo using modification-specific antibodies and genome-wide ChIP-sequencing approaches (Wang et al. 2008) (Fig. 18.1). Lysine acetylation on histones is a reversible and highly dynamic process. Addition or removal of acetyl groups is carried out by two families of proteins of antagonistic enzymatic activities. The first group are histone acetyltransferases (HATs), which include p300/CBP, GCN5, PCAF, and others. They are responsible for adding acetyl groups using acetyl-CoA as co-factor. The second group includes histone deacetylases (HDACs) which are responsible for removing acetyl groups. Histone acetylation adds negative charges, therefore weakening DNA–histone interactions, resulting in more relaxed chromatin structure, and making DNA more accessible to transcription factors and other protein complexes. Conversely, deacetylation restores the positive charge of histones and favors nucleosome compaction, thereby limiting the access of gene-regulatory proteins to DNA. Besides modulating DNA–histone interactions, histone acetylation allows recruitment of proteins carrying the bromodomains that can "read" the acetylation marks specifically and regulate gene expression. A number of genetic and biochemical studies have revealed that the amount of histone acetylation is overwhelmingly correlative to gene activation, while deacetylation is

Table 18.1 Principal posttranslational modifications of amino acids on histone tails

Type of PTM	Subtypes of modification	Residues modified	Regulatory enzymes	Reference
E ADP-ribosylization	Mono- and poly-ADP-ribosylization	H1E2, H1E14, H1E116, H2BE2	Poly(ADP-ribose) polymerases and Poly(ADP-ribose) glycohydrolases	Messner and Hottiger (2011)
K acetylation	NA	H2AK5, H2AK9, H2BK5, H2BK12, H2BK20, H2BK120, H3K4, H3K9, H3K14, H3K18, H3K23, H3K27, H3K36, H4K5, H4K8, H4K12, H4K16 and H4K91	HATs and HDACs	Wang et al. (2008)
K biotinylation	NA	H2AK9, H2AK13, H2AK125, H2AK127, H2AK129, H3K4, H3K9, H3K18, H4K8 and H4K12	Biotinidase and holocarboxylase synthetase	Hassan and Zempleni (2006)
K crotonylation	NA	Multiple	Unknown	Tan et al. (2011)
K methylation	Mono-, di-, and tri-methylation	H3K4, H3K9, H3K27, H3K36, H3K79, H4K20	HMTs and KDMs	Greer and Shi (2012)
K sumoylation	NA	H2BK6, H2BK7, H2BK16, H2BK17, H4K5, H4K8, H4K12, H4K16, H4K20	Sumoases	Bannister and Kouzarides (2011)
K ubiquitination	NA	H2AK119 and H2BK123	Ubiquitinases	Bannister and Kouzarides (2011)
P isomerization	NA	H3P38	Proline isomerases	Bannister and Kouzarides (2011)
R methylation	Mono-, asymmetric di- and symmetri di-methylation	H3R2, H3R8, H3R17, H3R26, H4R3	PRMTs	Di Lorenzo and Bedford (2011)
R deimination	NA	H3R2, H3R8, H3R17, H3R26, H4R3	PADs	Thompson and Fast (2006)
S phosphorylation	NA	H2AS1, H2AS121, H2AS129, H2AS139, H2BS10, H2BS14, H2BS32, H2BS33, H2BS36, H3S10, H3S28, H4S1	Protein kinases and phosphatases	Banerjee and Chakravarti (2011)
T phosphorylation	NA	H2AT119, H3T3, H3T6, H3T11, H3T45	Protein kinases and phosphatases	Banerjee and Chakravarti (2011)

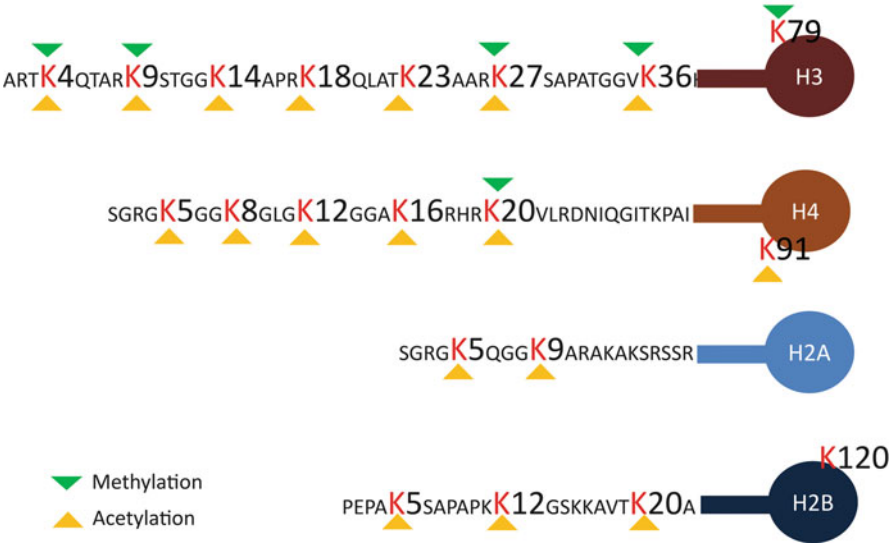

Fig. 18.1 Histone-lysine modifications. The diagram depicts key lysine residues that can be either acetylated or methylated on the tail of nucleosomal histones

correlative to gene repression (Eberharter and Becker 2002). Specifically, 12 of the 18 acetyl-lysine residues on histones have been found to coexist in large groups of genomic regions defining known gene promoters. Conversely, regions with minimal transcriptional activities are characterized by depletion of the histone acetylation marks (Wang et al. 2008).

Lysine residues on histones can also be methylated (Fig. 18.1). However, unlike acetylation, different forms of methylation can exist at the same residue of lysine. Specifically, three forms of methylation can occur at each site of a lysine residue, namely mono- (me), di-(me2), and tri-(me3) methylation. Each modification is regulated by two groups of enzymes or enzyme complexes with antagonizing activities, namely the histone methyltransferases (HMTs) and lysine demethylases (KDMs). HMTs catalyze the transfer of one or two methyl groups from S-adenosylmethionine (SAM) to the ε-amino group of lysine residues and are characterized by the presence of a SET domain, with the exception of the H3K79 HMT Dot1L. KDMs mediate the removal of methyl groups from the methylated lysines. They include amine oxidases, which utilize flavin adenine dinucleotide (FAD) as a cofactor (i.e., LSD1), and the jumonji-C domain-containing proteins, which use Fe^{2+} and α-ketoglutarate as cofactors.

The molecular mechanisms by which histone-lysine methylation is responsible for gene regulation do not involve changes in the charge of histones and therefore does not interfere with DNA–histone interaction. In this case, lysine methylation serves as binding sites for the recruitment of effector proteins (Greer and Shi 2012). Many protein domains can specifically bind to certain types of methylated lysine

side chains on histones, including (1) the chromodomains, which can bind to methylated H3K4, H3K9, H3K27, or H3K36; (2) PHD domains, which can bind to methylated H3K4 or H3K9; (3) WD40 repeats, which can bind to methylated H3K9 and H4K20; and (4) Tudor domains, which can recognize methylated H3K4 and H4K20, among others (Yun et al. 2011).

In agreement with the high specificity of each form of lysine methylation, interpretation of the "histone-lysine methylation code" is often context-dependent. For example, while H3K4 methylation is generally associated with active gene expression, H3K9 and H3K27 methylation is often linked to gene silencing. In addition, even among the different forms of methylation at the same site, different marks may encode different information. For instance, peaks of H3K4me3 represent active or primed proximal regulatory regions of genes, while peaks of H3K4me define distal regulatory regions that work as enhancers (Barski et al. 2007). Additionally, although H3K4me3 and H3K4me2 are generally considered as active marks, they can be linked to gene repression. For example, when the PHD domain-containing protein ING2 binds to H3K4me3 or H3K4me2, the HDAC-containing complex is recruited to initiate histone deacetylation and gene repression (Shi et al. 2006). In another case, the active mark H3K4me3 and the repressive mark H3K27me3 can coexist at promoters of many developmentally regulated genes in embryonic stem cells, as well as certain adult stem cells, marking a group of genes that are primed in stem cells that would be turned on or off permanently as the stem cells differentiate, depending on which lineage they enter (Bernstein et al. 2006; Cui et al. 2009).

18.2.1.2 Modification of Arginine Residues and Their Functional Roles

Similar to lysine residues, arginines are also positively charged amino acids and can be subject to PTMs inside a cell. Specialized enzymatic activities catalyze mono-(one) or di-(two) methylation of arginine residues on histones. However, an additional level of complexity exists for arginine methylation in that either symmetric or asymmetric di-methylation can occur on the same residue, depending on whether the two methyl groups are positioned on the same (symmetric) or different (asymmetric) guanidino groups of the arginine residue. Enzymes responsible for histone arginine methylation are the protein arginine methyl transferases (PRMTs). While all PRMTs can mediate mono-methylation, only the type I PRMTs (PRMT 1, 2, 4, and 6) catalyze the formation of asymmetric dimethyl arginines (me2a) on histone residues, while the type II PRMTs (PRMT5 and possibly PRMT7) generate symmetric dimethyl arginines (me2s) (Di Lorenzo and Bedford, 2011) (Fig. 18.2).

Histone arginine methylation has some unique characteristics. For example, most of the arginine residues on the tails of H3 and H4 are in close proximity to functionally important lysine residues that can be modified by either acetylation or methylation (Fig. 18.2). Therefore, this arrangement is particularly favorable for functional cross-talk between adjacent arginine and lysine residues. In addition, symmetric or asymmetric di-methylation on the same arginine is functionally antagonistic. For instance, R2 on H3 can either be asymmetrically or symmetrically di-methylated, but

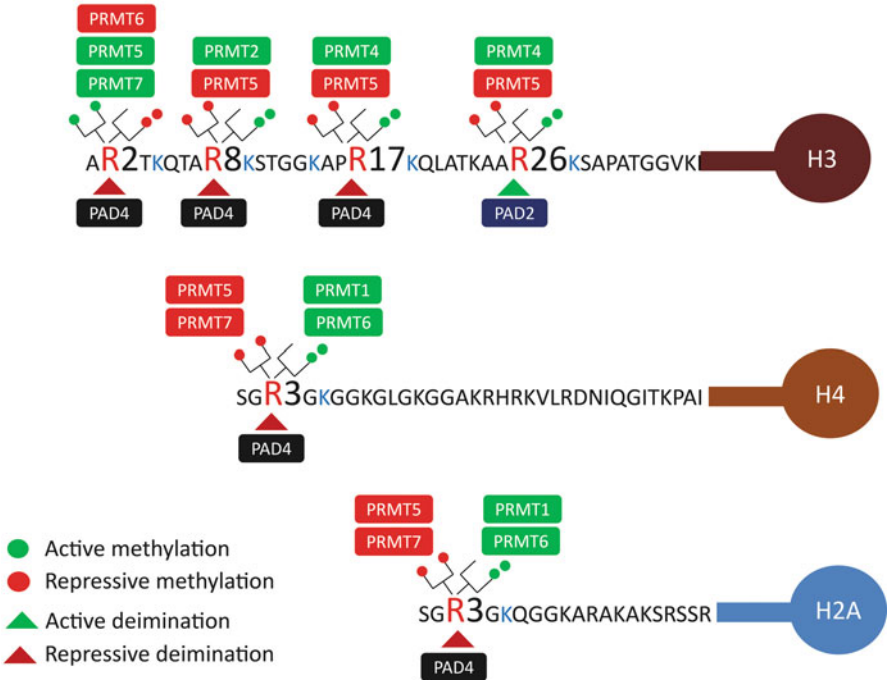

Fig. 18.2 Histone arginine modifications. The diagram shows key arginine residues that can be either methylated or deiminated on the tail of nucleosomal histones. The enzymatic activities responsible for these modifications are also indicated

these changes mediate distinct functional outcomes. More specifically, H3R2me2a, produced by PRMT6, antagonizes the generation of the active mark H3K4me3 by preventing the binding of the HMT complex containing WDR5, ASH2, and SET1 to the tail of H3 (Guccione et al. 2007; Hyllus et al. 2007; Kirmizis et al. 2007). This is in agreement with reports that H3R2me2a and PRMT6 negatively regulate gene expression in different cellular contexts. For instance, the expression of p53 is down-regulated in senescent cells (Neault et al. 2012). As a result, cyclin-dependent kinase (CDK) inhibitors are negatively regulated in a human cancer cell line (Kleinschmidt et al. 2012) and pluripotency-related genes are inhibited in differentiating embryonic stem cells (Lee et al. 2012). In contrast, H3R2me2s, produced by PMRT5/7, facilitates the binding of WDR5 to the tail of H3 and thus promotes the generation of H3K4me3 (Migliori et al. 2012).

Genome-wide studies also suggest a different distribution of these two marks. H3R2me2a is distributed throughout the gene body and 3′ end of most genes, as well as in heterochromatic regions, but it is not detected at core gene promoters, characterized by the presence of H3K4me3 marks (Kirmizis et al. 2007). In contrast, 1/3 of the H3R2me2s marks are detected around the transcriptional start sites (TSS) of active genes and the majority of them are localized to the nucleosomes

directly upstream of the transcription initiation sites (Migliori et al. 2012). Therefore, H3R2me2a and H3R2me2s carry exclusive functions at gene promoters by influencing the occurrence of tri-methylation at its neighboring lysine residue. In addition, mono-methylation of H3R2 (H3R2me) has also been observed in living cells, throughout the coding regions of genes and correlated to active transcription (Kirmizis et al. 2009).

Adding still more complexity to the H3R2 code, the versatile protein UHRF1 (Unoki et al. 2009) has been found to bind to the unmodified form of H3R2 with its PHD domain and this interaction is disrupted by methylation (Rajakumara et al. 2011). Another two important arginine residues that can be methylated on histone H3 are H3R17 and H3R26. The PRMT4-mediated methylation of these two residues is often clustered at promoter regions of the genes regulated by nuclear hormone receptors, such as the estrogen-responsive promoter pS2, which is often used as a molecular model to study histone arginine modifications. Due to its being recruited by active nuclear hormone receptors, PRMT4 is also named coactivator-associated protein arginine methyltransferase 1 (CARM1) and facilitates the transcriptional initiation mediated by activated nuclear receptors (Bauer et al. 2002; Ma et al. 2001; Wu and Xu 2012).

On histone H4, antagonistic effects of asymmetric and symmetric di-methylation also exist. Like H3R2, H4R3 can be modified by either asymmetric or symmetric di-methylation, mediated by PRMT1 or PRMT5/7, respectively. However, unlike H3R2, asymmetric di-methylation of H4R3 is linked to gene activation, while symmetric di-methylation is linked to gene repression. The active H4R3me2a mark can be recognized by the Tudor domain-containing protein TDRD3, which in turn acts as a genome-wide transcriptional coactivator (Yang et al. 2010). The repressive mark H4R3me2s, in contrast, can be recognized by the DNA methyltransferase DNMT3a, which mediates gene silencing by inducing DNA methylation (Zhao et al. 2009). Despite abundant evidence showing that arginine methylation is a dynamic PTM of histone tails, no arginine demethylase has been identified to date; however, a report that the Jumonji-C domain-containing protein JMJD6 has arginine demethylase activity awaits further confirmation (Chang et al. 2007).

An alternative mechanism in which cells opt to counteract the effects of arginine methylation is arginine deimination. Deimination is a process in which the imine residue of the guanidino group of an arginine is oxidized into a ketone group, so that methylation of that arginine can no longer occur. As a consequence, the arginine is converted into a citrulline, a nonstandard amino acid. For this reason, deimination is synonymous with citrullination in the research literature. It has been shown that deimination is most likely to occur on unmodified arginine side chains of a histone, although mono-methylated arginines can also be converted into citrulline. Therefore, unlike demethylation, deimination is not really a process that turns a methylated arginine back to its originally unmodified form. Rather, it is an irreversible process that prevents the occurrence of arginine methylation (Cuthbert et al. 2004; Wang et al. 2004). Given the important regulatory role of arginine methylation on transcription, the occurrence of deimination on histone tails is likely to bear stable consequences on the transcriptional program of gene expression.

18.2.2 Methylation of Cytosines on Genomic DNA

Another very important source of epigenetic regulation of gene expression that can be affected by changes in arginine methylation is DNA methylation, which defines the addition of methyl groups on cytosine residues of genomic DNA. It has been shown in various contexts that DNA methylation is indispensable for establishing and maintaining cell identity and tissue physiology; therefore, it has been implicated in human health and disease (Heyn and Esteller 2012). Cellular enzymes performing this type of modifications are the DNA methyltransferases (DMMTs), which include the de novo methyltransferases (DNMT3a and DNMT3b) and DNMT1, responsible for maintaining the existing DNA methylation pattern by using one methylated strand as a template to replicate the methylation pattern on the other strand during DNA synthesis or duplication. The special DNMT3L lacks a catalytic domain, but is able to form complexes with DNMT3a/b and facilitate the enzymatic functions of the latter (Cheng and Blumenthal 2008). For a long time, study of gene regulation has focused on the DNA methylation events occurring at conserved genomic regions characterized by "CG" sequences called "CpG islands," found with high frequency within certain DNA regions. Hypo-methylation of DNA around TSSs has been correlated with active transcription, while hyper-methylation with gene silencing (Eckhardt et al. 2006; Illingworth et al. 2008).

Mechanistically, DNA methylation is often associated with repressive chromatin and favors the recruitment of proteins such as MeCP2, which, in turn, allows the recruitment of other proteins required for heterochromatin formation (Agarwal et al. 2007). Recently, many newly developed genome-wide sequencing techniques have expanded our knowledge. It is now clear that DNA methylation is not restricted to gene promoters or to CpG islands. One study even suggested that DNA methylation in the gene bodies contributed to active transcription (Lister et al. 2009). Another study confirmed these findings and reported that DNA methylation in intergenic regions and gene body facilitated transcription by antagonizing the silencing action of Polycomb group proteins (Wu et al. 2010). As a result, it is becoming unlikely that DNA methylation is a stable modification as once thought. However, the existence of well-characterized DNA "demethylation" machinery remains to be found. The recent findings on the role of the ten-eleven-translocation (TET) proteins on DNA methylation provided a first clue towards the identification of enzymes counteracting DNA methylation. It is now well-defined that the TET proteins can mediate the conversion of methyl cytosines (5mC) into 5-hydroxymethylcytosines (5hmC), 5-formylcytosines (5fC), and 5-carboxylcytosines (5caC). After that, some default mechanisms involved in DNA repair can remove the bulky groups out of the cytosines, thereby restoring the unmodified DNA. In addition, some evidence suggests that the main product of the TET protein reaction, 5hmC, can exist as stable marks inside the cell nucleus and become a novel type of epigenetic landscape, independent of the 5mC marks (Williams et al. 2012; Wu and Zhang 2011).

18.3 Protein Deimination in Epigenetics

Histone deimination was first discovered in human granulocyte-differentiated HL-60 cells that were catalyzed by PAD4, a member of a calcium-dependent enzyme family called peptidyl arginine deiminases (PADs) (Hagiwara et al. 2005). The PAD family includes five members: PAD1, 2, 3, 4, and 6 (see Chaps. 1 and 21 for more details). Although the distribution and substrates of PADs are tissue-specific, change of protein structure is a consequence of all forms of protein deimination. Specifically, PAD1 is mainly expressed in the uterus (see Chap. 17) and epidermis (see Chap. 7), where it modulates the flexibility of keratin cytoskeleton by inducing deimination on keratins and keratin-related proteins during the maturation of keratinocytes. In contrast, PAD2 is expressed in a wide range of tissues, including skeletal muscle, secretory glands, and the nervous system and is involved in deiminating many tissue-specific or cell type-specific structural proteins, including the oligodendrocyte-specific myelin basic proteins (MBP), thereby affecting the stability of the myelin sheath (see Chap. 11). Expression of PAD3 is primarily located in hair follicles (see Chap. 8), where it deiminates trichohyalin, an important structural protein in the inner root sheath cells of hair follicles. PAD4 is expressed in white blood cells (see Chap. 6), and its levels are elevated in many malignant tumors (Vossenaar et al. 2003). PAD4 is unique for its possession of a conserved nuclear localization signal (NLS), giving it the ability to translocate into the nucleus and deiminate histones. However, recent evidence suggests that PAD2 can also deiminate histones (Zhang et al. 2012). The latest member, PAD6, (PAD5 does not exist, since it is mis-nomenclature for the human PAD4) is restricted to germ cells (see Chap. 17) and has been shown to regulate microtubule organization in oocytes and the proper development of early embryos (Kan et al. 2011; Yurttas et al. 2008).

Consistent with the role of PTMs on histones in modulating chromatin structure and gene expression, histone deimination is likely to affect these processes by antagonizing histone arginine methylation. The antagonistic roles of arginine methylation and deimination on the regulation of gene expression were originally described by two groups, both working in the MCF7 human mammary epithelial cell line (Cuthbert et al. 2004; Wang et al. 2004). In these cells, estrogen treatment results in the induction of gene expression associated with arginine methylation mediated by CARM1. Deimination of four arginine residues (R2, R8, R17, and R26) on the N-terminal tail of histone H3, mediated by the enzyme PAD4, was identified as repressive and antagonistic of the hormone-dependent effects (Cuthbert et al. 2004; Wang et al. 2004). Therefore, it was proposed that PAD4 activity prevented the occurrence of the estrogen-mediated changes of histone arginine methylation. Despite the results of these two seminal studies, the precise mechanism involving PAD4 as a repressive factor of gene expression might vary, depending on the cell type or environmental context. A role of PAD4 in retaining quiescent gene expression was described not only for the repression of estrogen-dependent genes in the absence of ligand but also for p53 target genes, such as p21, GADD45, and PUMA, in the absence of DNA damaging stimuli (Denis et al. 2009; Li et al. 2008, 2010b).

Also in this case, gene expression was directly correlated with arginine methylation at active promoters, while citrullination, mediated by PAD4, was associated with repression (Li et al. 2008).

Besides the role of deimination as antagonistic for arginine methylation, it is becoming increasingly clear that additional mechanisms might be involved. In the case of PAD4, for instance, the enzyme has been shown to form complexes with the histone deacetylase HDAC2, therefore promoting histone deimination and deacetylation (Li et al. 2010b). Indeed, binding of PAD4 to chromatin was shown to require histone deacetylation (Li et al. 2010b) and the cooperation of PAD4 and HDAC in establishing or maintaining a repressive chromatin state was further supported by the evidence that treatment of cells with HDAC and PAD inhibitors exerted a synergistic effect on gene expression (Denis et al. 2009; Li et al. 2010b). Besides the interference with activating arginine methylation and binding to HDAC2, a third mechanism has been proposed, involving the ability of PAD4 to form complexes with ING4, a protein with the ability to bind the NLS of p53 and shuttle the molecule into the nucleus, where p53 can exert its transcriptional activity (Guo and Fast 2011). Interestingly, recent data have shown that PAD4 expression can be activated by p53 and likely participates in feedback loops that are lost or deregulated in cancer cells (Tanikawa et al. 2009). Based on the discussed role of PAD4 as repressor of the p53 gene-regulatory network, it should be noted that PAD4 is highly expressed in malignant tumor tissues and cancer cell lines (Chang and Han 2006). Since normal cell lines appear to be relatively insensitive to PAD4 inhibition, this molecule represents an interesting pharmacological target for reactivation of p53-dependent gene expression in cancers characterized by a deficiency in this pathway (Slack et al. 2011).

Besides the role of PAD family members in repressing gene expression by deimination of arginine residues on histone tails, PAD molecules also have the ability to modulate transcription indirectly by deimination of critical arginine residues on histone-modifying enzymes. One example of this is the deimination of the HATs p300/CBP during nuclear receptor (NR)-mediated gene activation (Lee et al. 2005). NRs are a large family of transcription factors undergoing conformational changes upon ligand binding and form large coactivator complexes that are required for transcriptional initiation (Chen et al. 2000). The arginine methyltransferase CARM1 (PRMT4) and p300/CBP are important components of these coactivator complexes (York and O'Malley 2010). CARM1-mediated methylation of multiple arginine residues on p300/CBP has been shown to be essential for protein–protein interactions and the formation of NR coactivator complexes (An et al. 2004; Chen et al. 2000; Lee et al. 2002). PAD4-mediated deimination of arginine residues on p300/CBP competes with CARM1 activating changes, thereby affecting NR-dependent gene expression. Despite the vast literature supporting a role of PAD4 in repression of gene expression, a recent study in MCF7 cells has also reported a role for PAD4 in activating E2F1, in response to treatment with EGF (Zhang et al. 2011).

Thus a complex picture is emerging, with PAD4 as a downstream effector of multiple pathways. In response to steroids, PAD4 acts as a repressive enzyme, but in response to other growth factors such as EGF, it acts as an activating enzyme.

The activating effect of PAD4 is achieved through citrullination of the transcription factor ELK1, followed by its phosphorylation and recruitment of HATs (Zhang et al. 2011). It is important to mention that until very recently, the only deiminase that had been implicated in regulation of gene expression was PAD4. This notion was based on the reported exclusive cytoplasmic localization of PAD2, even though both molecules were capable to convert arginine residues into citrulline on synthetic substrates (Raijmakers et al. 2007). The nuclear detection of PAD2 and concomitant increased histone citrullination in canine mammary cells stimulated with EGF (but not with estrogen or progesterone) suggested that this molecule could also play a role in epigenetic regulation of gene expression (Cherrington et al. 2010). Similar findings were reported in human MCF7 cells, where PAD2 was found on the promoter of actively transcribed genes in over-expressing cells (Cherrington et al. 2010) and in cells stimulated with estrogens (Zhang et al. 2012). Thus, distinct PAD family members could play distinct roles in MCF7 cells, with PAD4 and PAD2 mediating the deimination of distinct arginine residues with differential transcriptional outcomes in response to distinct stimuli.

Using biochemical methods and genome-wide approaches, a novel role for PAD2 and the histone mark (H3Cit26) was revealed in estrogen-responsive cells, either from the mammary epithelium or from murine uterine epithelium (Zhang et al. 2012). The estrogen-dependent citrullination of R26 residues on histone H3 was abrogated by the depletion of estrogen receptor (ER) or by silencing PAD2 (but not PAD4). The H3Cit26 mark was mainly localized to genes containing conserved ER binding sites and resulted in chromatin decondensation. Using recombinant histones and PAD proteins, PAD2 was found much better at deiminating H3R26 than PAD4 (Zhang et al. 2012). However, how H3Cit26 contributes to opening local chromatin structure remains to be addressed. A possible scenario is the cross-talk between H3Cit26 and H3K27Ac, since acetylation of K27 is a prerequisite for citrullination of H3R26 (Zhang et al. 2012). In this case K27Ac and R26Cit would synergize to prevent the recruitment of silencing complexes and therefore promote transcriptional activation. See Chap. 17 for further discussion.

18.4 Protein Deimination in Health and Disease: Hints on Epigenetics

Based on these considerations, we may conclude that there is no simple, universal role for arginine deimination in modulating transcription. The consequences may be cell-context specific and signal dependent. One possible way of modulating gene expression is direct histone deimination. Since the conversion of arginine into citrulline prevents the occurrence of both asymmetric and symmetric di-methylation of histone arginines, the final functional readout of histone deimination would not only depend on which arginine residues are being deiminated, as in a case where histone deimination is a form of independent code, but may also and probably more importantly depend on which form of arginine di-methylation (asymmetric vs. symmetric) is being prevented. For this reason, the field awaits comprehensive and systematic

studies on PADs/histone deimination and PRMTs/arginine methylation in different tissues and in distinct pathological conditions, in order to conclusively define the roles of histone deimination in regulating chromatin dynamics and gene expression.

18.4.1 Deimination and Chromatin Decondensation in Response to "Danger" Signals

A very interesting role of histone deimination in modulating innate immunity has been proposed. In response to bacterial infection, activated neutrophils secret a "NET" (neutrophil extracellular trap) composed of granules containing anti-bacteria peptides, cytokines, and decondensed chromatin into the extracellular environment (Brinkmann et al. 2004). Neutrophils are considered as part of the innate immune system against invading pathogens (see Chap. 6). During this process, histones undergo massive deimination and it is believed that this modification is crucial for the rapid decondensation of chromatin in response to bacterial infection. Mice with genetic deletion of PAD4 were shown to be more susceptible to bacterial infection, compared to wild-type controls, and were characterized by failure to decondense chromatin and secrete NETs, despite ongoing neutrophil activation (Li et al. 2010a; Wang et al. 2009). The ability of PAD4-mediated histone deimination to induce chromatin decondensation was not limited to NET formation in response to bacterial infection, but has also been observed in response to other "danger" signals, such as DNA damage (Tanikawa et al. 2012). This suggests that histone deimination can be used by a cell to "sense a danger signal" in response to extracellular stimuli and immediately respond by massive histone deimination and chromatin decondensation. Although the consequence of these changes in gene expression has not yet been studied in detail, it is intriguing that cells have developed very diverse strategies to decondense chromatin in response to physiological or pathological stimuli. Perhaps local recruitment of complexes containing PAD2 or PAD4 is carefully associated and dependent on other histone marks, such as acetylation, which is associated with transcriptional responses to physiological stimuli. In contrast, rapid and massive deimination may represent a fast response to emergency situations the cell might face in response to danger signals from the environment.

18.4.2 Multiple Sclerosis

Protein deimination has been implicated in autoimmunity, including rheumatoid arthritis (Masson-Bessiere et al. 2001; Suzuki et al. 2003; Yamada et al. 2005) and multiple sclerosis (Mastronardi et al. 2006), where the conversion of arginine to citrulline residues in auto-antigens has been proposed to be causally related to the disease. Multiple sclerosis (see Chaps. 10 and 11) is characterized by an immune attack on components of myelin, the fatty membrane that ensheaths axons in the central nervous system (de Seze et al. 2001; Moscarello et al. 2007; Nicholas et al.

2005). It is known that MBP, an important structural component of the myelin sheath, carries many arginine residues that can be subject to high degrees of deimination. The conversion of arginines into citrulline results in loss of positive charges on MBP (Musse et al. 2008) and mis-incorporation into the myelin membrane (Beniac et al. 2000), where it affects its stability (Cao et al. 1999) and antigenicity (Deraos et al. 2008; Shanshiashvili et al. 2012; Zhou et al. 1993, 1995). In MS patients, the detection of deiminated MBP has been linked to higher levels of the PAD2 enzyme in myelin-forming cells (Mastronardi et al. 2007; Moscarello et al. 1994). Besides the well-documented role of PAD2 in modifying MBP, deimination has also been reported in the nuclei of the myelin-forming cells, where PAD4 accumulates in response to TNF-α stimulation (Mastronardi et al. 2006). The functional consequences of histone deimination in these cells, whether it is a localized event modulating transcription, or a massive event resulting from the detection of a "danger signal" by the cell, remains to be determined and will likely be the goal of future studies.

18.5 Conclusion and Perspectives

It is becoming increasingly evident that epigenetic modifications are the response of a given cell to changes in the environment. Unlike genetic changes, which imply mutations, amplifications or deletions and are stable, epigenetic changes are highly dynamic and somewhat stable, although they can be altered by extrinsic stimuli. Therefore, it is not surprising that epigenetic changes modulate susceptibility, onset and progression of human diseases. Understanding mechanisms governing epigenetic regulation under physiological and pathological conditions has been proven to be able to facilitate disease diagnosis, treatment, and prevention (Maunakea et al. 2010). Protein deimination is a PTM of proteins, including histones, transcription factors, and chromatin modifying enzymes, that can directly or indirectly modulate gene expression. Distinct PADs may be expressed in different cell types and differentially respond to extracellular signals. However, the localized or diffuse effect of deimination on chromatin, the temporal modulation of the response, and the amino acid specificity on distinct proteins will need further characterization. As a result, a clear definition of the role of histone deimination in physiological and pathological conditions remains to be further evaluated.

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Chapter 19
Identifying Citrullination Sites by Mass Spectrometry

Jennifer E. Grant and Hong Li

Keywords Citrullination • Arginine deimination • Mass spectrometry • Posttranslational modification

19.1 Introduction

There are a wide variety of posttranslational modifications (PTMs) that may occur to a protein's amino acid side chain, including phosphorylation, methylation, and acetylation, to name only a few. Judicious use of mass spectrometry as part of a carefully designed proteomic workflow can identify many of these covalent modifications, even including arginine citrullination, which represents one of the smallest PTMs by quantifying mass. During citrullination, which is the deimination of arginine, replacement of one of the guanidinium NH groups of arginine with an oxygen atom results in a mass addition of 0.9848 Da (Fig. 19.1a). Yet even this very small addition of mass results in potentially large consequences to a protein, including changes in its isoelectric point (pI), hydrophobicity, and three-dimensional structure (Gyorgy et al. 2006; Kinlock et al. 2008).

It has been widely documented that citrullination events may create autoantigens suspected in immune disorders such as rheumatoid arthritis (Kinlock et al. 2008; Suzuki et al. 2007; van Venrooij and Pruijn 2000) and multiple sclerosis (Moscarello et al. 2007; Musse et al. 2006; Anzilotti et al. 2010). Despite the importance of this PTM in disease processes, the identification of citrullination sites within peptides has lagged behind that of other common PTMs for several reasons.

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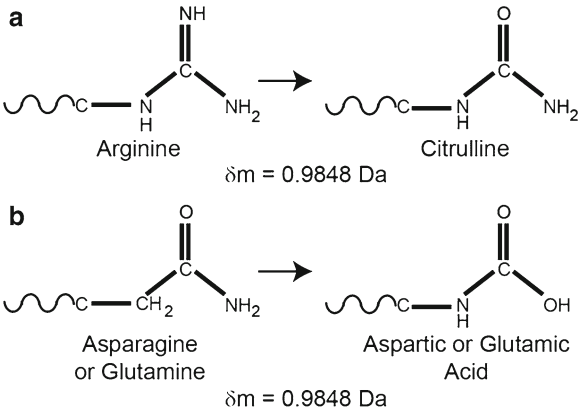

Fig. 19.1 Arginine deimination. Deimination of arginine to citrulline (**a**) results in the exchange of a nitrogen for an oxygen, and a mass addition of 0.9848 Da. Deamidation of asparagine or glutamine (**b**) also results in a mass addition of 0.9848 Da

One complicating factor is that citrullination confers a mass shift that is minute in size compared to the size of an average peptide (1,000 Da, or 0.001 %) generated through trypsin digestion of full-length proteins. This is noteworthy, since trypsin digestion is routinely performed in conjunction with analysis by liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS). Moreover, differentiation between citrullination and deamidation of asparagine or glutamine (Fig. 19.1b) in MS/MS spectra of a peptide may be ambiguous in the absence of reasonably complete MS/MS fragmentation information for the peptide in question.

The purpose of this review is to provide an overview of successful strategies used to identify and quantify protein citrullination using mass spectrometry workflows and to provide an overview of the strengths and weaknesses of the instruments commonly employed in these studies.

19.2 Experimental Considerations

Those wishing to pursue identification of citrullination sites will have to choose between two major strategies: those that detect arginine deimination sites directly, based on high resolution mass (accurate mass) measurement of the citrullinated residue within peptide fragments, and those that utilize chemical derivatization to increase the mass changes of deiminated residues from arginines for unambiguous detection by MS/MS. The purpose of this review is to provide the reader with an overview of successful strategies for the identification of specific arginine citrullination sites and offer guidance on method choice.

19.2.1 Bottom-Up Versus Bottom-Down

Both approaches rely on “bottom-up” strategies that require proteolytic digestion of full-length polypeptides into more manageable component peptides that, in turn, may then be sequenced by LC/MS/MS. This is in contrast to “top-down” methods

that rely on unique fragmentation processes within highly specialized mass spectrometers to break the intact protein down into more manageable component peptides. “Bottom-up” strategies rely on protease selection (Siuzdak 2006), which may include chymotrypsin, endoproteinase Glu-C, endoproteinases Lys-N and endoproteinase Lys-C, and trypsin, taking advantage of the unique sequence cleavage specificity of each enzyme. It is worthwhile to note that the use of trypsin presents both a challenge and an opportunity, because this protease cleaves peptide bonds after arginine or lysine. Deimination affects the digestion pattern by removing the positive charges on arginines. This results in a missed cleavage when citrullinated arginine is present, which may lead to individual trypsin peptides that are significantly longer than anticipated.

Peptide digests can be prepared from complex mixtures or can be performed on isolated polypeptide complexes. Since citrullination is thought to contribute to the progression of diseases, such as multiple sclerosis (see Chaps. 10 and 11) or rheumatoid arthritis (see Chaps. 1–6), biological samples are often derived from complex nervous system tissue or synovial tissue, respectively. Preparations from cultured cells have also been employed to analyze histone citrullination (Hagiwara et al. 2005) and a targeted method for studying peptide antigens that may be displayed by immune cells has been reported (Seward et al. 2011). In any case, fractionation of peptide species by employing LC strategies, either on-line or off-line, to MS, is typically performed to reduce analyte complexity and increase signal-to-noise ratios for more sensitive detection of deiminated peptides.

19.2.2 Choosing the Appropriate MS Device

The experimenter must choose the MS instrument most suitable for a given experiment. In general, MS data are recorded as m/z values, reflecting the ratio of mass to charge for a given molecule. The predominant charge status observed for an ionic species is greatly influenced by the machine employed. Typically, one collects both MS spectra (one dimension of MS detection by peptide molecular weight only) and MS/MS spectra. The goal of the MS experiment is to identify potential peptides by their molecular mass, which may already provide hints as to possible addition of extra mass values due to PTMs. MS/MS analysis of citrullinated peptides may be performed to confirm the presence of deimination and identify the specific amino acid residue that was modified. Presently, many different MS platforms have been employed in monitoring peptide citrullination. Three popular instrument types applied in this analysis include Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS instruments (Marshall et al. 1998; Comisarow and Marshall 1974; Guan and Marshall 1995), Matrix-Assisted Laser Desorption Ionization MS machines, coupled with Time of Flight Detection in two dimensions (MALDI TOF/TOF) (Cornish and Cotter 1993; Cotter et al. 2007), and Orbitrap MS devices (Hu et al. 2005; Makarov 2000; Hardman and Makarov 2003).

Mass accuracy, mass resolution, and data acquisition time are three factors that drive instrument selection. Expressed in parts-per-million (ppm), mass accuracy refers to the deviation of a measured mass from its calculated value and is an important consideration when trying to detect citrulline directly within peptides. The more accurate the mass measurements, the smaller mass measurement errors will be, creating ideal conditions for unambiguous identification of deiminated peptides. Given the relatively minute mass addition of 0.9848 Da, corresponding to a single change of one argnine to citrulline, accurate mass measurements must be very precise in this case. The mass resolution of an instrument describes the mass difference between two analytes that is required to fully differentiate the two ion signals similar in mass/charge. Finally, whether or not it is practical to perform a complex proteomics experiment with a given device is often limited by whether the experiment can be performed within a reasonable timeframe, typically dictated by the length of the LC separation required to provide adequate resolution.

Often, one finds a tradeoff between data acquisition time and mass accuracy. As a result, faster MS/MS data acquisition ensures higher detection sensitivity for sequencing low abundant PTM-containing peptide ions, albeit often with less mass accuracy and resolution. All three parameters are important qualitative considerations when working with both MS spectra and MS/MS spectra, where the latter allows peptide sequencing and PTM localization.

19.2.2.1 MS Using FT-ICR

Out of all the instrument types that can be used to study protein citrullination, FT-ICR instruments are capable of offering the highest mass accuracy (~ 0.1 ppm) and mass resolution, which is advantageous when studying citrullination using accurate mass methods. These devices utilize Penning traps, relying upon the principle that these ions rotate at a frequency proportional to m/z when subjected to a magnetic field. Wanczek (1989) FT-ICR instruments are very sensitive and provide high resolving power. They may also allow multistage MS (MS^n) analysis, which relies on the ability of FT-ICR instruments to retain ions, so that multiple rounds of fragmentation analysis can be performed. These features are important for distinguishing between peptide fragment ions that share great similarity in mass. With FT-ICR machines, peptides are often sequenced using collision-induced dissociation (CID), which generates y- and b-ion series (Biemann 1990), although other methods for generating peptide-sequencing ions are also effective. A drawback is that acquisition times can be significant, due to instrumental procedures, and these instruments require a magnet and are expensive to maintain. Examples of the use of FT-ICR instruments to study citrullination events include the work of Hermansson et al. (2010) as well as that of Goëb et al. (2009), among others.

19.2.2.2 MS Using MALDI TOF/TOF (Matrix-Assisted Laser Desorption Ionization with Time of Flight Detection in Two Dimensions)

The MALDI TOF/TOF MS device, taking advantage of two stages of time of flight analysis, can generate tandem mass spectra by utilizing CID dissociation to fragment molecules, generating mostly the y- and b-ion series. Compared to FT-ICR devices, MALDI TOF/TOF instruments can perform both ion selection and MS/MS analysis fairly rapidly, but do not routinely provide the same level of mass accuracy, although carefully calibrated instruments can provide a mass accuracy of 5–10 ppm. An example of the use of MALDI TOF/TOF methods to identify sites of citrullination include the work of Grant et al. (2007) and Tutturen et al. (2010).

19.2.2.3 MS Using the Orbitrap Device

A relatively recent acquisition to the research armamentarium is the Orbitrap MS instrument, which allows exquisitely precise and rapid mass measurements. In one study, mass accuracy for the Orbitrap was better than 5 ppm using very large datasets and, with internal mass calibration, mass accuracy exceeded 1 ppm (Makarov et al. 2006a, b). In some cases, dynamic range of relative analyte concentrations, at which 5 ppm mass accuracy could be attained, was one order of magnitude higher than that of the TOF instruments. In addition, the Orbitrap allows fragmentation of peptides by electron transfer dissociation (ETD) (Syka et al. 2004), when combined with an electrospray ionization (ESI) source. Generating fragmentation patterns complimentary to those observed by CID methods, this ionization technique is known to leave PTMs largely intact, which is a great advantage for labile PTMs such as phosphorylation. Generally, data acquisition with this instrument requires more time than TOF instrumentation, yet less time than FT-ICR methods. An example of the use of an Orbitrap to study citrullination can be found in the work of Creese et al. (2011) and Van Beers et al. (2010).

Although three MS types were emphasized here in order to showcase some of the factors affecting instrument selection, citrullination studies can be performed using other hybrid methods, including quadrupole TOF (De Ceuleneer et al. 2011) and quadrupole linear ion trap devices (Hao et al. 2009). It is important to bear in mind that technological improvements occur frequently, greatly impacting the sensitivity, accuracy, and data collection times of these instruments. As new devices and approaches emerge, the ability to detect citrullination sites may improve at a rapid pace.

19.3 Direct Strategies for Detection of Sequence-Specific Citrullination Sites

The concept of measured accurate mass relies on the fact that each element in the periodic table has a unique and precise calculated exact mass and that the molecular formula of a given peptide can be determined uniquely when the mass of a peptide

or peptide fragment is dependably measured. As MS technology has improved, the standards for exact mass measurements are periodically raised. Current standards indicate that an accurate mass measurement should be accurate to 5 ppm, although 10 ppm has been commonly accepted in the past. Accurate mass measurements rely on careful calibration of a sensitive instrument and the ppm accuracy needed to make an accurate mass determination depends on the operating characteristics of the MS device employed, as well as the molecular mass of the analyte (Sack et al. 1984; Webb et al. 2004). Popular instrumental choices for making highly accurate mass determinations include FT-ICR and Orbitrap instruments.

19.3.1 Accurate Mass Strategies for the Identification of Citrullination Sites

Accurate mass measurements, however, do not guarantee that one and only one molecular structure can be confirmed. Since many peptides share very similar masses, the use of accurate mass measurements alone to identify a peptide presents difficulties (Kind and Fiehn 2006). This concept holds true for small molecule metabolites as well (Stanley et al. 2011; Kumari et al. 2011). Therefore, confirmation of peptide sequences through the analysis of MS/MS spectra is strongly encouraged.

At present, the majority of citrullination studies using accurate mass have been performed in vitro on either full-length polypeptides or synthetic peptides that have been deiminated using a specified isoform of peptidyl arginine deiminase (PAD). For example, (Van Beers et al. 2010) performed a comprehensive study identifying 42 citrullination sites within the fibrinogen hexamer. This work is notable because of the thorough use of in vitro deimination and digestion strategies to generate an inclusive fibrinogen citrullination map. These authors deiminated human fibrinogen with one of three PAD isozymes, then isolated fibrinogen α, β, and γ chains using SDS-PAGE analysis. Followed by in-gel digestion with a variety of proteases, 86 % sequence coverage was observed for each chain. In this case, LC MS/MS was performed using either a Linear Ion Trap Quadrupole (LTQ) Orbitrap XL device, equipped with ETD, or LTQ FT-ICR.

The authors noted that out of a possible 81 arginines present within the fibrinogen α, β, and γ chains, human PAD4 was able to citrullinate only 28 of them (35 %). In contrast, both the human and rabbit muscle isoforms of PAD2 were able to deiminate 39 of these residues (48 %), suggesting that PAD4 is more sensitive to the sequence-dependent context surrounding a given arginine. Interestingly, human and rabbit muscle PAD2 shared only 26 specific citrullination sites. Clarification of the amino acid context that promotes deimination of a peptide sequence by PAD4 and PAD2 has yet to be achieved, although the importance of the four residues immediately surrounding an arginine has been reported (Stensland et al. 2009).

19.3.1.1 The Pros and Cons of Accurate Mass Strategies

In general, accurate mass approaches rely on highly precise and accurate tandem mass spectra to identify deiminated peptides, a process that is demanding in terms of instrument performance and calibration. Even with an accurate mass measurement, it is important to confirm peptide identity by generating tandem mass spectra (Kind and Fiehn 2006). Fairly complete fragment ion series are required to confidently identify the peptide and the citrullination site. Depending on analyte complexity, this approach may require screening of thousands of peptide species in order to identify deiminated ones. Manual confirmation of citrullination events within the mass spectrum is often required.

An advantage to this approach is that the experimenter does not need to introduce potentially complex chemical derivatization steps, which might demonstrate bias towards particular citrullination sites over others. Additional examples of the use of accurate mass strategies to identify peptide deimination events include works by Seward et al. (2011) and Hagiwara et al. (2005).

19.3.2 Quantification of Deimination in Diseases Using Accurate Mass Strategies

Quantification of the extent of peptide citrullination in an experimental model of an autoimmune disease is often desirable, in order to shed light on the precise molecular nature of disease progression. However, there are only a limited number of articles addressing quantification of differential protein deimination in health and disease at present. Hardman and Makarov (2003) used accurate mass measurements (within 10 ppm) of citrullinated peptides recovered from in vitro deiminated fibrinogen to develop benchmarks that allowed detection of citrullinated fibrinogen peptides by the distinct retention times and mass measurements during LC/MS, a strategy formalized as the "Accurate Mass and Time" strategy (Smith et al. 2002; Zimmer et al. 2006). In this study, LC/MS was performed on a linear ion trap/ion cyclotron resonance MS device, which conferred high mass accuracy. As a result, MS/MS data were acquired on the two most abundant species in the total ion chromatogram.

This study provided 44 % sequence coverage for all fibrinogen polypeptides, where fibrinogen was harvested from the synovial fluids of RA patients. In order to be considered as a potential biomarker for arthritis-related deimination, peptides had to meet stringent criteria that guarded against false positives. First, prospective peptides containing Gln or Asn were disqualified, in order to exclude possible deamidation events. Additionally, the MASCOT database search software must indicate high confidence in the peptide and the MS/MS assignment must identify the citrullinated peptide with a threshold value higher than 25.

The authors discovered two deiminated peptides that satisfied the criteria: the fibrinogen α-chain peptide Fibα[559–575] and the β-chain peptide Fibβ[52–77]. Considered potential biomarkers, the levels of these two peptides in diseased and control samples were then quantified. Using High Performance Liquid Chromatography (HPLC), the authors established retention times using fibrinogen peptides that had been citrullinated in vitro using PAD from rabbit muscle, as well as the retention times for the unaltered forms of the peptides. Then, the retention time data was used to identify each pair of deiminated and non-deiminated peptides in the in vivo samples harvested from RA patients. Mass spectrometric analysis confirmed that the peptides were associated with the appropriate chromatographic retention times.

Based on comparison of the area-under-the-curve (AUC) measurements for the appropriate pairs of chromatographic peaks, the percent citrullination of both peptides were calculated in the in vivo RA samples for three RA patients. The Fibα[559–575] peptide was found in both its deiminated and unmodified forms, and the degree of in vivo citrullination was calculated to be between 1.4 and 2.5 %. In contrast, the second fibrinogen β-chain peptide, Fibβ[52–77] was found at levels ranging from less than 0.1 to 1.2 %. These data further underscore how relatively rare deimination events may be in vivo.

The previous paper utilized reproducible retention time-specific HPLC peak-area data to generate quantitative conclusions. In fact, it is very difficult to maintain identical LC retention times for the species present in complex peptide mixtures (Kind and Fiehn 2006). Alternative methods that are independent of reproducible peptide LC retention times are valuable when quantifying citrullinated peptides by LC/MS/MS. Relative quantification of the degree to which a protein becomes post-translationally modified can be obtained using MS strategies that employ covalent labels containing unique isotopic signatures. One technology that allows this is termed isobaric Tagging for Relative and Absolute Quantification (iTRAQ) (Ross et al. 2004). The iTRAQ method introduces an isotopically unique chemical label onto lysine residues and peptide N-termini present within a protein digest. Most commonly, trypsin is used to prepare these digests. If there are four experimental samples within a given experiment, such as tissue extracts from four animals of different ages, each of these can be derivatized with a unique iTRAQ label. As peptides are sequenced, the MS/MS spectrum also allows relative quantification of the peptide within each of the four tissue preparations, based on the relative quantity of each of the four unique iTRAQ labels present within the iTRAQ region of the mass spectrum (i.e., the low-mass region from 114 to 117 amu). Quantitative data for individual peptide fragments can, in turn, be related to the overall expression level of the parent protein.

At this juncture, use of covalent isobaric labeling to quantify citrullination events is rare. Grant et al. (2007) performed a search for PTMs on an iTRAQ quantitative expression proteomics dataset (Liu et al. 2007) acquired from comparative analysis of differential protein expression in rats subjected to experimental autoimmune encephalopathy (Pitt et al. 2000; Brand-Schieber et al. 2005), which serves as a rodent model for multiple sclerosis. Of 21 deiminated peptides identified in this

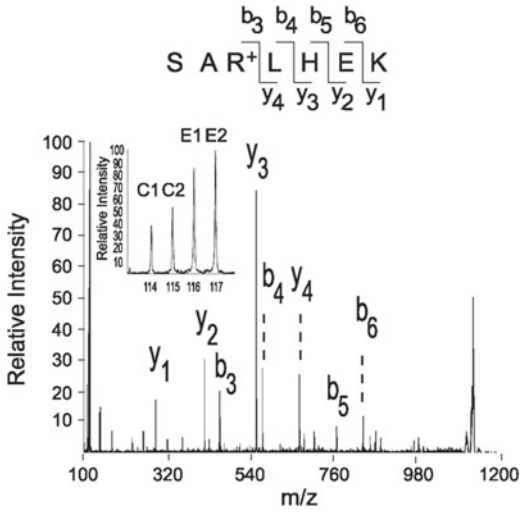

Fig. 19.2 MS/MS spectrum representing the peptide Ser/Thr kinase RIO3 [207–213]. The y- and b-ion series allow sequencing of the peptide and the localization of the citrullination site. The iTRAQ reporter region (*inset*) allows comparison of the level of this peptide in control tissue compared to levels in the spinal cords from a model of multiple sclerosis. (Reproduced from Grant et al. (2007), with permission from the American Chemical Society)

search, two were over-expressed, while another was observed at diminished levels. Citruillinated forms of both the Ser/Thr kinase RIO3 peptide [217–223] and glial fibrillary acidic protein [22–34] peptides were found over-expressed (iTRAQ ratios of 2.2 and 1.3, respectively). The citrullinated peptide ribophorin 1 [504–516] was found at diminished level, with an iTRAQ ratio of 0.74. The tandem mass spectrum for Ser/Thr kinase RIO3 peptide [217–223] is presented in Fig. 19.2, indicating the y- and b-ion series of peptides that were used to confirm the peptide sequence and localization of citrullination site.

The iTRAQ approach is powerful because it allows relative quantification of peptide levels between four sets of experiment, which may include a comparison of diseased to control tissue. For example, within the MS/MS spectrum in Fig. 19.2, the peptide sequence can be read from the y- and b-ion series. In the inset of this figure, the relative levels of an RIO3 peptide in control (C1, C2) tissue is compared to tissue harvested from a rat model of multiple sclerosis (E1, E2), which demonstrated a 2.2-fold increase in citrullination of the RIO3peptide [217–223] at position Arg219 in a rat model of multiple sclerosis. Quantification of the differential deimination during the progression of multiple sclerosis not only identifies potential therapeutic targets, but may also illuminate the antigenic processes that underpin autoimmunity.

False-positive results are always a concern in mass spectrometric analyses. To minimize false positives in this study, the iTRAQ data were first subjected to a database search performed with the GPS explorer software suite, using a precursor tolerance of 50 ppm and a MS/MS mass error tolerance of 0.3 Da. Peptide matches that possessed a confidence interval of less than 80 % were eliminated, then an in-house customized Perl script (Hu et al. 2006) was used to further eliminate any potential PTM peptides that may have generated an assignment during an analogous non-PTM search. To ensure the quality of the data, MS/MS spectra of

the deiminated peptides were inspected manually and were required to confirm unambiguous assignment of the citrullination event. Although the mass error of these deiminated peptides ranged from 9 to 20 ppm, this approach demonstrates how existing iTRAQ datasets can be mined to reveal quantitative information on PTMs in health and disease.

19.3.2.1 The Pros and Cons of iTRAQ Analysis

Differential protein expression analysis using iTRAQ requires a considerable investment in the iTRAQ chemistry, as well as instrument time. This procedure requires that tissue protein extracts must first be digested, usually with trypsin, before labeling with iTRAQ reagents. Considering that trypsin cleaves proteins after lysine or arginine, this can pose a problem in citrullination studies, where deimination of arginine can remove a normal trypsin digestion site. In this case, it might prove advantageous to utilize endoproteinase Lys-C for the digestion, bearing in mind that this adaptation would change the nature and length of the proteolyzed peptides. In general, the resultant peptides would be longer and carry more additional positive charges.

To reduce sample complexity, orthogonal chromatographic strategies are required to fractionate peptides, typically utilizing strong cation exchange chromatography (SCX) in the first dimension to generate 15–20 peptide fractions. Typically, ten of the SCX fractions, or more, are desalted using microchromatography strategies, often involving C18 microcolumns and then chromatographed further using C_{18} reversed-phase chromatography. MS analysis with the instrument of choice may then proceed. Despite the labor-intensive nature of iTRAQ analysis, it remains a powerful tool for identifying factors involved in disease progression, because protein expression in diseased tissue can be compared directly to that of control tissue.

iTRAQ analysis reports on all peptides identified in the study, not just peptides that display PTMs. Therefore it is up to the experimenter to employ database search strategies to detect potential citrullination sites, which may include manual analysis of MS/MS spectra to confirm them. This is exemplified in Fig. 19.2 for the Ser/Thr kinase RIO3 peptide [217–223], whose sequence was confirmed by hand. iTRAQ reagents are available as 4-plex and 8-plex sets, allowing up to eight experimental conditions to be assessed.

19.4 Neutral Loss Strategies

When generating peptide digests from complex biological tissues, it worthwhile to note that one cellular lysate digest might generate tens of thousands of peptides. High-abundance peptides are more apt to be sequenced at the expense of low-abundance peptides, and there are practical limitations to the number of ion signals

that can be subjected to MS/MS analysis within a given, initial mass spectrum. Deiminated peptides are, by their very nature, present in low abundance. Conveniently, peptides containing citrulline are usually subjected to a characteristic neutral loss fragmentation when ionized. A neutral loss is defined as a diminution in mass that does not affect charge of the parent ion. For a given analyte, it then becomes possible to make decisions about whether to invest time in generating peptide sequence data, once a citrullination-related neutral loss is observed. This strategy can save time, sample and instrument resources by focusing specifically on deiminated peptides.

An ion trap MS device is uniquely situated for dynamic data acquisition methods, such as neutral loss monitoring, because this instrument generates multistage MS/MS fragmentation (MS^n) spectra, which, for a peptide, translates to being able to collect MS^1, MS/MS (MS^2), and MS^3 spectra on a given peptide. First, molecular mass (MS^1) spectra are compared to MS/MS spectra in order to detect neutral loss fragmentations. If the MS/MS spectrum indicates an appropriate neutral loss event within a user-specified number of high-abundance ions, it will trigger the collection of MS^3 spectra, which can then provide peptide sequence information. However, if it does not, the MS device will continue to scan MS^1 and MS/MS spectra for the specified neutral loss, saving much time and sample.

A promising development in direct determination of deimination sites using dynamic data acquisition methods is identification of the neutral loss of isocyanic acid (HNCO) from the citrulline ureido moiety upon CID (Fig. 19.3a). Neutral loss analysis relies on the rearrangement of energetically activated citrulline, which results in the expulsion of isocyanic acid (HN=CO). This reaction results in loss of 43 Da from the citrulline precursor peptide, which is diagnostic for deimination. Using this strategy (Hao et al. 2009), employed a triple quadrupole linear ion trap (LIT) MS device to ultimately generate MS^3 spectra, thus describing the molecule that had resulted from the neutral loss of HNCO. Described in Fig. 19.3b, this workflow first required scanning of precursor ions (MS^1), followed by neutral loss fragmentation under low-energy CID, which resulted in MS^2 spectra. If a fragment was identified with the diagnostic loss of −43 Da, MS^3 spectra were then utilized to determine the peptide sequence of the fragment. In (Hao et al. 2009), this permitted identification of citrullination sites on recombinant nucleophosmin that had been deiminated in vitro. For example, a reported loss of 43 Da, which was readily apparent for a citrullinated precursor peptide, corresponded to the sequence [SIXDTPAK], where X represented citrullinated arginine. It is important to note that, to date, identification of citrulline through neutral loss analysis has only been successfully applied to peptides and recombinant proteins that were highly enriched or purified.

Realizing the advantages of using ETD to fragment polypeptides, Creese et al. (2011) utilized this neutral loss phenomenon to create a method that targeted peptides for supplemented activation ETD analysis, using an Orbitrap MS device. Neutral loss of isocyanic acid (−43 Da) was monitored from each of four synthetic citrullinated peptides that had been spiked into a trypsin digest of salivary proteins. In this case, however, the precursor ion was used to generate peptide sequence information, a process that did not require MS^3 analysis (Fig. 19.3c).

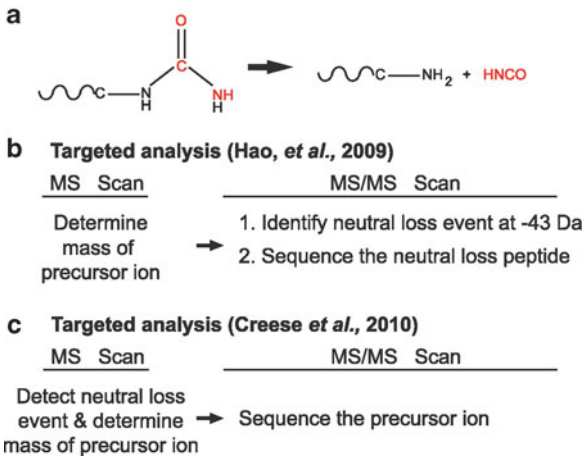

Fig. 19.3 Neutral loss of isocyanic acid from citrulline. (**a**) Neutral loss of isocyanic acid from arginine results in a mass deficit of 43 Da. (**b**) Workflow for neutral loss scanning with MS^3 analysis of the −43 Da fragment, according to the method of Hao et al. (2009). (Reproduced with permission from Springer.) (**c**) Workflow for neutral loss analysis according to the method of Creese et al. (2011). Note that MS^2 analysis is performed on the precursor ion. (Reproduced with permission from the Royal Society of Chemistry.)

Both alternating CID and ETD MS/MS spectra were acquired in this experiment. Intriguingly, the authors noted that, although four deiminated peptides could be identified manually in both the CID and ETD spectra, three were successfully identified by database search of the ETD data. In contrast, a database search of CID data identified only two of the citrullinated peptides, while capturing a significant number of non-deiminated peptides, which later proved to be either deamidated peptides or genuine false positives. The database search strategm utilized the SEQUEST algorithm and peptide assignment data was filtered utilizing Xcorr values that graded the extent to which an experimentally determined tandem mass spectrum agreed with the theoretical spectrum.

19.5 Derivatization Strategies Targeting Ureido Chemistry

The chemistry of the ureido group lends itself to selective derivatization. In early experiments, butanedione monoxime was used to derivatize citrulline, which proceeds through several reactive intermediates including an imidazoline (Butler and Hussain 1982; Senshu et al. 1995; Sugawara et al. 1998). Large and distinct enough to generate an antigen that is recognized by a unique set of commercial antibodies developed specifically for this purpose, this cyclic derivative is most often detected by

western blot (Senshu et al. 1992), although colorimetric applications exist (Knipp and Vasak 2000). Within MS workflows, the derivatization agents butanedione and phenylglyoxal are commonly employed, leading to characteristic mass additions that identify deimination events.

Derivatization of citrulline with 2,3-butanedione (diacetal) in the presence of antipyrine, under acidic conditions, leads to a mass addition of 238 Da per ureido group that can be observed, even using low-resolution MS instruments. Holm et al. (2006) first reported the mechanism of this reaction. Performed under strongly acidic conditions, the charge on the guanidine group is suppressed, thereby limiting electrophilic attack of protonated arginine. As a result, derivatization occurs only to citrullinated residues and not unmodified arginines. Having a characteristic absorption maximum at 464 nm, this product is readily detected spectrophotometrically.

Recently, De Ceuleneer et al. (2011) performed derivatization of model citrulline peptides with 2,3-butanedione under acidic conditions but in the absence of antipyrine. Although the reactions required longer incubation times, often overnight, derivatization reached 96 % in many cases. The resultant mass addition for each citrulline was 50 Da, a much smaller dm than that induced by the chemistry reported by Holm et al. (2006). This technique potentially may provide advantages, when quantifying deiminated and non-deiminated peptide pairs, using AMT or other proteomic strategies.

Citrulline-reactive beads (Tutturen et al. 2010) are a new technology that might provide the breakthrough needed to make identification of citrullination sites routine. Realizing that phenylglyoxal-derivatives can be used to specifically target deiminated peptides, Tutturen et al. (2010) created affinity beads that could be used to capture citrullinated peptides within myelin basic protein. Once a deiminated peptide was covalently bound to the beads, extensive washing was performed to remove nonspecifically bound peptides, greatly reducing sample complexity. The presence of a labile ester bond in the bead linker region allowed cleavage of the covalently modified citrulline residue for MS analysis (Fig. 19.4a). Deiminated peptides were released from the resin in the presence of a strong base. The resultant peptides, containing a carboxymethyl hydroxyphenyl-glyoxal-modified citrulline residue (Fig 19.4b), harbored a mass shift of 190.03 Da, which could be further localized to specific residues in MS/MS spectra. The authors provided proof of principle by demonstrating that several mono- and di-citrullinated peptides, each bearing the 190.03 Da modification, could be isolated and identified from a Lys-C digest of myelin basic protein.

19.5.1 The Pros and Cons of Derivatization Techniques

In general, the covalent derivatization techniques based on ureido group chemistry offer opportunities for uniquely derivatizing deiminated peptides and allow confirmation of the presence of citrulline in the absence of 5 ppm mass accuracy.

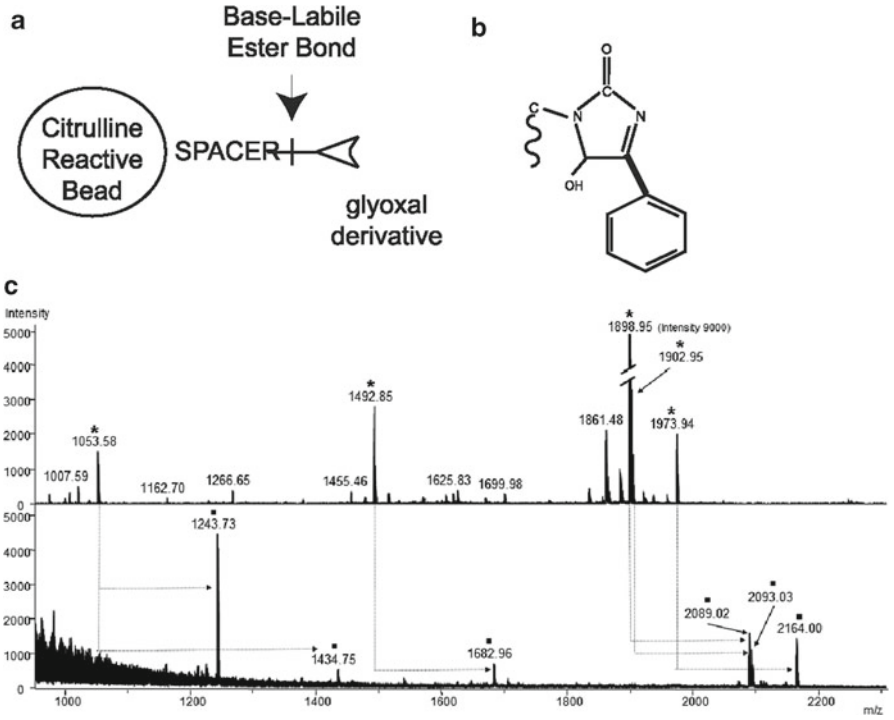

Fig. 19.4 Use of citrulline-reactive beads to isolate deiminated peptides. To generate beads, the cirulline-reactive phenylglyoxal moiety was conjugated to acrylamide-based beads through a base-labile linker (**a**). Reaction of the derivatized beads with deiminated peptides generates the intermediate (**b**). Enrichment of citrullinated peptides from an endoproteinase Lys-C digest before (*top*) and after (*bottom*) the enrichment are shown (**c**). Deiminated peptides observed before enrichment are marked by *asterisks*. Enriched, carboxymethylated hydroxyphenylgloxal-modified citrullinated peptides are marked by squares. (Modified from Tutturen et al. (2010), with permission from Elsevier, Inc.)

The resultant mass addition may be useful for identifying deiminated peptides within purified proteins or samples of reduced complexity, where the number of tryptic peptides, and their masses, is known. It is important that the derivatization reaction proceed to completion, or deiminated peptides that remained un-derivatized will contribute to inaccurate quantification.

In complex tissue preparations, the utility of derivatization techniques may be limited, due to the presence of a wide variety of peptides of varying mass. In this case, the citrulline-reactive bead method offers many advantages over other derivatization methods, including the ability to remove non-deiminated peptides from the beads, thus generating cleaner spectra with higher signal-to-noise ratios. It is of significant disadvantage to deimination researchers that citrulline-reactive beads are not yet commercially available.

19.6 Stable Isotope-Labeling Methods

Because deimination is defined as the replacement of NH with O, stable isotope labeling of citrullination sites with ^{18}O is one useful strategy for detecting deimination events. This process can be accomplished in the citrullination reactions in the presence of $H_2\,^{18}O$ and the appropriate PAD isoforms. This generates a characteristic isotope distribution pattern for deiminated peptides that reflects a +3 Da mass addition, compared to the original unmodified peptide. In this instance, ^{18}O is incorporated only upon citrullination of the peptide, resulting in a +2 mass addition. An additional 0.9848 Da must be added to account for deimination. Because this reaction is enzymatically catalyzed, ^{18}O incorporations result in a highly specific reaction.

This approach was utilized by Kubota et al. (2005) to examine the deimination of fibrinogen in vitro. For most of the peptides studied, the authors reported that visual inspection of the MS/MS spectrum often identified the site of citrullination, even when multiple arginines were present within a given peptide. Enzyme-catalyzed labeling of the deiminated peptide in 50 % $H_2\,^{18}O$ resulted in a characteristic labeling pattern that reported the presence of peptides containing either the ^{16}O-labeled citrulline or ^{18}O-labeled citrulline. This idealized +3 pattern is described in Fig. 19.5, which is an idealized spectrum modified from Kubota et al. (2005).

19.6.1 The Pros and Cons of Stable Isotope-Labeling Techniques

This approach has two distinct advantages. First, the unique isotope pattern generated in 50 % $H_2\,^{18}O$ simplifies manual inspection of the data. Secondly, the +3 mass addition is specific for the enzyme-catalyzed deimination event. One serious drawback to stable isotope-labeling approaches is the requirement that samples must be treated with PAD and ^{18}O in vitro. This prevents analysis of citrullination sites that have accumulated over time in diseased tissue. Furthermore, the experimenter must rely on accurate mass strategies to precisely identify citrullinated peptides and sequence them.

19.7 Summary

The study of the cause and effect relationships between peptide deimination and autoimmune disorders by MS is an emerging field that will benefit by improvements in proteomic techniques and also in MS instruments. Accurate mass strategies are rather straightforward to implement, although definitive assignment of citrullination sites relies on extremely precise mass measurements and/or neutral loss strategies.

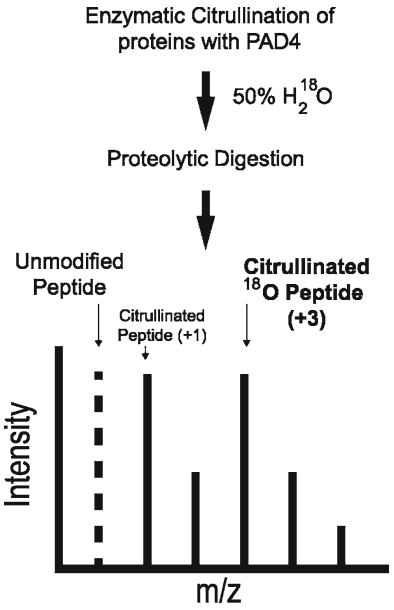

Fig. 19.5 Strategy for use of enzymatic ^{18}O labeling to visualize citrullination sites. Kubota et al. (2005) utilized PAD4-catalyzed ^{18}O labeling to identify deiminated residues. Full-length peptide is first treated with PAD enzyme in the presence of 50 % H_2 ^{18}O, then digested with a protease. Ultimately, when peptides are analyzed using mass spectrometry, a characteristic pattern emerges. A citrullinated peptide that received a ^{16}O oxygen atom carries a mass shift of +1 Da. However, a citrullinated peptide that received an ^{18}O oxygen atom displays a mass shift of +3 Da. (Modified from Kubota et al. (2005), with permission from John Wiley and Sons, Inc.)

Table 19.1 Methods for covalent labeling of citrulline

Labeling agent	dm (Da)	Reference
Butanedione + antipyrene	+238	Holm et al. (2006)
Butanedione	+50	De Ceuleneer et al. (2011)
Glyoxal derivatized beads	+116	Tutturen et al. (2010)
Stable isotope labeling	+3	Kubota et al. (2005)

Covalent derivatization strategies, summarized in Table 19.1, rely upon the addition of mass deiminated peptides, which may be small or large, depending on the chemistry of the labeling (i.e., stable isotope labeling vs. ureido group chemistry).

Regardless of the method employed, challenges remain in identification of protein citrullination in complex biological specimens, and the quantification and extent of deimination of these sites in diseased tissues. Yet, despite this, advances in instrumentation, combined with sophisticated sample enrichment techniques and detection strategies, have resulted in tangible progress in this field.

Rapid advances in the study of protein deimination by MS methods have much to offer clinical science. Identification of biomarkers that accurately predict an individual's risk for developing diseases like rheumatoid arthritis or multiple sclerosis is a powerful tool that, once developed, will transform diagnosis. Furthermore, the potential to diagnose these diseases at early stages could lead to improved patient outcomes. On a more general level, progress made in delineating citrullination sites that are relevant in these diseases has enhanced our understanding of these pathological processes at the molecular level and offer hope of devising improved medical therapies.

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Chapter 20
Homocitrulline: An Analog and Confounder Related to Citrulline

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Keywords Homocitrulline • Carbamoylation • Autoantibodies • Chemical modification • Cross-reaction • Monoclonal antibody F95

20.1 Introduction

A structural homolog to citrulline, homocitrulline, can be present in proteins and peptides as a product of posttranslational modification. Homocitrulline is formed nonenzymatically from lysine residues in the polypeptide chain by the action of cyanate. The latter compound is derived either from urea or, via a reaction catalyzed by the enzyme myeloperoxidase (MPO), from thiocyanate. The formation of homocitrulline is known as carbamoylation or carbamylation. In humans, this process takes place predominantly in two types of situations: uremia and inflammation. Smoking increases the circulating thiocyanate concentration and thus enhances carbamoylation of proteins.

The similarities between citrulline and homocitrulline are relevant in two ways. First, as structural analogs, they can act as confounding factors in assays aimed at

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specifically measuring just one of them. If such an assay is based on detecting ureido groups in proteins, it is likely that it cannot distinguish between citrulline and homocitrulline. If the presence of citrulline in tissues or in a protein is studied, it is imperative to avoid conditions that cause carbamoylation, in particular the use of high concentrations of urea. Secondly, immunization with proteins containing one of the amino acids can elicit antibodies that also recognize the other.

Experimentally produced antibodies against carbamoylated proteins have been shown to bind to synthetic cyclic citrullinated peptide (CCP), an antigen used to demonstrate the presence of anti-citrulline antibodies in patients with rheumatoid arthritis (RA). Anti-homocitrulline antibodies have also been found to be present in the sera of patients with RA and can also predict joint damage. These findings are particularly interesting, as smoking is the only environmental factor that has been associated with the development of RA (see Chap. 5) and the pathogenetic link has not been found so far.

20.2 Why Homocitrulline Is Important

Citrulline and homocitrulline (ε-carbamyl lysine) are two amino acids that both contain an ureido group (Fig. 20.1). In addition to similar structures, the two amino acids have common features in their biosynthesis and immunogenicity. In proteins, neither exists in a primary translation products. Their posttranslational formation mechanisms are different and the conditions that favor their respective formations are not identical; however, their close structural resemblance makes them potential

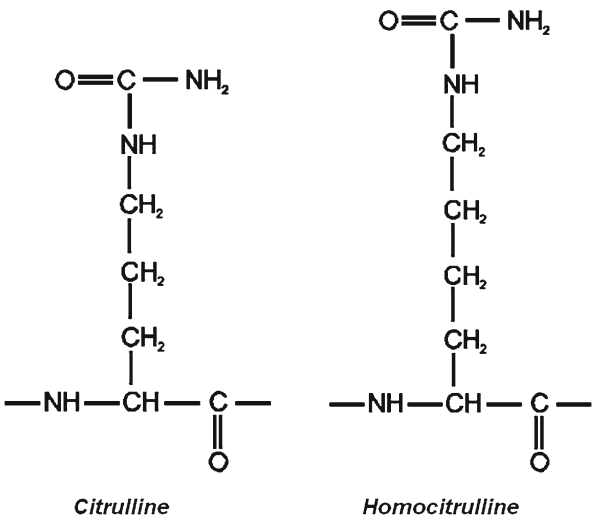

Fig. 20.1 Structures of citrulline and homocitrulline

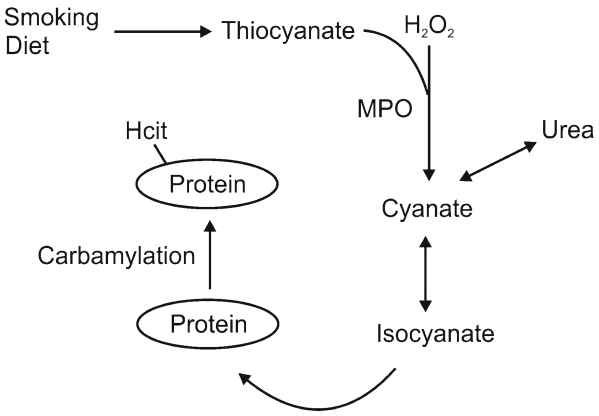

Fig. 20.2 Formation of homocitrulline by carbamoylation. *MPO* myeloperoxidase

confounders in assays designed to detect just one of them. Furthermore, these amino acids, when present in proteins, can induce antibodies that recognize them both.

Citrulline is formed from polypeptide-bound arginine by PAD enzymes (Chap. 1), while homocitrulline is formed from lysine in proteins via a spontaneous carbamoylation (also known as carbamylation) reaction that involves isocyanic acid, an isomer of cyanate. The latter compound, in turn, is derived either from the spontaneous decomposition of urea or from the oxidation of thiocyanate by the enzyme MPO (Fig. 20.2). Kidney failure with uremia leads to increased homocitrulline formation in tissue and plasma proteins. In addition, increased protein carbamoylation has been demonstrated in atherosclerosis and in inflammatory situations (Jaisson et al. 2011; Wang et al. 2007).

As hydrophilic, basic residues, arginine and lysine are often located on the surface of proteins. Thus, the loss of their charges caused by citrullination (also known as deimination) or carbamoylation (Kraus et al. 2001), respectively, distorts the protein structure (Kraus and Kraus 2001; Tarcsa et al. 1996), thus increasing its susceptibility to proteolytic degradation and can produce potential antigens (Mydel et al. 2010; Turunen et al. 2010). The antigenicity of peptide-bound citrulline is relevant in RA, as these patients have been shown to possess antibodies against deiminated proteins, even years before the clinical onset of the disease (Koivula et al. 2007; Nielen et al. 2004). The presence of such antibodies is also related to a more severe disease course (Rantapää-Dahlqvist et al. 2003). The antigenicity of homocitrullinated proteins is a more recent discovery, and, so far, the clinical significance of this modification in RA is not clear (Mydel et al. 2010). However, antibodies against homocitrullinated proteins can be induced in experimental animals (Turunen et al. 2010) and have also been found in the sera of RA patients, where they predict joint damage (Shi et al. 2011).

In this chapter, we review what is presently known about homocitrulline, in the contexts that are relevant for citrulline. However, it is beyond the scope of this chapter to provide a general review on the chemistry or biological functions of homocitrulline.

20.3 How Homocitrulline Is Formed in the Body

Spontaneous carbamoylation is a reaction between cyanate and a free amino group of an amino acid. Carbamoylation can affect all amino groups in proteins, but sometimes at different rates. In fact, the reaction for α-amino groups is 100 times faster than for the ε-amino group of lysine (Stark 1965). Also, the reaction is practically irreversible. Cyanate in tissues is derived from the chemical equilibrium reaction between urea, on one hand, and ammonium and cyanate ions, on the other hand (Fig. 20.2). Consequently, any urea-containing solution will eventually also contain cyanate. In the human body, the urea concentration in body fluids is particularly high in uremia, which is a state of accumulation of urea and other nitrogenous waste products due to kidney failure.

Because of its irreversible nature, carbamoylation leads to the accumulation of homocitrulline in proteins during aging and in various diseases (reviewed in Jaisson and Gillery 2010). In healthy individuals, the concentration of plasma cyanate is around 50 nmol/L, while in uremic patients, it increases by threefold (Nilsson et al. 1996). Even such a high cyanate concentration is significantly lower than the theoretical value, calculated from the corresponding urea concentration, and indicates that cyanate disappears by efficiently reacting with the available amino groups. In kidney failure, the large load of functionally altered carbamoylated proteins may further aggravate renal dysfunction (Kraus et al. 2001).

In addition to spontaneous carbamoylation, there is also another enzyme-assisted carbamoylation route. The enzyme MPO, presents in inflammatory cells, catalyzes the oxidation of thiocyanate to cyanate in the presence of hydrogen peroxide. As a result, the cyanate then becomes available for protein carbamoylation (Wang et al. 2007). Phagocytic cells, at least granulocytes and monocytes, contain MPO (reviewed in Klebanoff 2005), which is part of a defense mechanism against outside pathogens. MPO acts in the phagosome or, if the pathogen is too large to be ingested by the phagocytic cell, it is released into the extracellular space. An antimicrobial system that consists of MPO, halides (iodide, bromide, chloride) (Klebanoff 1968), or thiocyanate (Klebanoff et al. 1966), and hydrogen peroxide (H_2O_2) is effective against numerous organisms. In this reaction, the thiocyanate, which has been gained from diet or tobacco smoke, is turned to active cyanate. This reaction sequence links inflammation and smoking to protein carbamoylation (Wang et al. 2007).

20.4 Homocitrulline as a Confounding Factor in Deimination (Citrulline) Assays

Many methods for detecting citrulline rely on demonstrating the presence of a ureido group in the protein or tissue to be studied. Such methods should be regarded as detecting both homocitrulline and citrulline, unless higher specificity is rigorously proved and the reaction with homocitrulline excluded. One example of such a method is the chemical modification of a ureido group that involves the use of antipyrine and

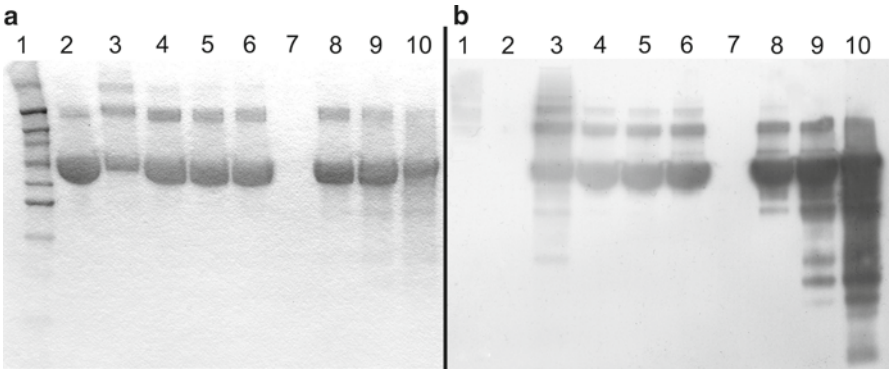

Fig. 20.3 Immunological cross-reaction between protein-bound citrulline and homocitrulline. SDS-polyacrylamide gel electrophoresis with subsequent blotting onto nitrocellulose membrane, chemical modification and immunostaining shows (**a**) protein staining (Coomassie) and (**b**) immunoreaction with polyclonal anti (modified)-citrulline antibody (Upstate Biotechnology/Millipore) (Senshu et al. 1992). The lanes for each reaction are molecular mass standard (*lane 1*), native human serum albumin (*lane 2*), PAD-treated human serum albumin (*lane 3*), and carbamoylated human serum albumin (*lanes 4–6* and *8–10*), under different conditions. *Lane 7* is blank. The KCNO treatment was carried out at 37 °C (*lane 4*: 2 h, *5*: 6 h and *6*: 17 h) and at 70 °C (*lane 8*: 2 h, *9*: 6 h and *10*: 17 h)

2,3-butanedione monoxime under acidic conditions, after which the modified amino acid is then detected immunologically. There is a commercially available antibody preparation designated as anti-citrulline (modified) antibody (Millipore; previously Upstate). As presented in Fig. 20.3, the antibody reacts with both citrulline and homocitrulline-containing proteins (Turunen et al. 2010; Shi et al. 2013). This is to be expected, as both amino acids contain identical ureido groups and the side chain of the homocitrulline residue is even longer than that of the citrulline residue. The results that have been obtained with this antibody should be interpreted with this possible cross-reaction in mind, paying particular attention to how probable it is that the conditions used favor the formation of homocitrulline by carbamoylation.

As discussed earlier, urea in solution is in equilibrium with cyanate, the latter of which readily carbamoylates the amino groups of proteins. We have treated human albumin with potassium cyanate under different conditions (Fig. 20.3) and found that, even at 37 °C, the degree of carbamoylation is significant. It seems that avoiding the use of urea when studying the presence of citrulline in tissues is really important. Urea is generally used for solubilizing proteins, such as from tissue samples, and it is not easy to find suitable replacements for it. When high concentrations (6–8 M) of urea are needed, the generation of cyanate is often inevitable. Removal of the cyanate by ion-exchange does not solve the problem completely, as the equilibrium between urea and cyanate is reestablished. However, the process can be controlled to some extent by using appropriate buffers and cyanate scavangers, such as Tris, glycine, ethanolamine, or diethylenediamine. In this respect, demonstrations of peptidyl-citrulline in tissue samples are in need of verification with more specific methods (Klareskog et al. 2006; Senshu et al. 1992).

20.5 Detection of Modified and Unmodified Citrulline and Homocitrulline in Hydrolysates

The chemical modification mentioned above can also be used for detecting free citrulline and homocitrulline in solution. The reaction between a ureido group-containing structure with 2,3-butanedione (also known as diacetyl) creates an adduct with a yellow/orange color. Absorbance of this color has been used for quantifying ureido group-containing molecules since the 1930s (Fearon 1939), but it can also be used as part of other techniques. If the modified protein is hydrolyzed, citrulline and homocitrulline can be separated on HPLC and detected (Turunen et al. 2010) as separate absorbance peaks at 464 nm (Fig. 20.4). The disadvantage of this method is that the sample is lost in the analysis and the location of citrulline or homocitrulline in the protein can only be determined by sample purification and identification before this analysis. Methods have been suggested for 2,3-butanedione-modified citrulline by mass spectrometry (De Ceuleneer et al. 2011), using the 50 mass shift as recognition tool, but the 2,3-butanedione reaction still remains common for all substituted ureas, including both citrulline and homocitrulline.

Classical methods for quantifying both citrulline and homocitrulline in proteins include amino acid analysis, either by ion-exchange chromatography or by high-performance liquid chromatography (HPLC), possibly coupled to tandem mass spectrometry, both after hydrolysis of the protein sample. Some loss of homocitrulline takes place during the hydrolysis, resulting in reappearance of lysine. These methods require the isolation of the protein in question, with subsequent hydrolysis, and are thus generally not used for demonstrating the presence of either citrulline or homocitrulline in complex tissue samples.

20.6 Citrulline and Homocitrulline as Immunogens

Citrullinated proteins can be used for raising antibodies in experimental animals, both in their native state and when citrulline has been chemically modified. Two immunological methods have been described for the detection of citrullinated proteins, respectively: a commercially available anti-(modified) citrulline rabbit antibody (Millipore; previously Upstate) (Senshu et al. 1992) and the monoclonal antibody F95 (Nicholas and Whitaker 2002). The former antibody recognizes citrulline in proteins after the amino acid has undergone a chemical modification by 2,3-butanedione monoxime and antipyrine in acidic conditions. The F95 antibody is against non-modified citrulline in proteins (see Chap. 14 for more details). As with any antibodies, verifying their specificity with respect to similar, homocitrulline-containing structures is essential. As shown in Figs. 20.3b and 20.5, both of these antibodies are able to react with carbamoylated (homocitrulline-containing) proteins, as well as with their citrulline-containing counterparts.

In rabbits, homocitrulline-containing proteins can be used to elicit antibodies that also recognize citrulline in proteins (Turunen et al. 2010). The immunogens

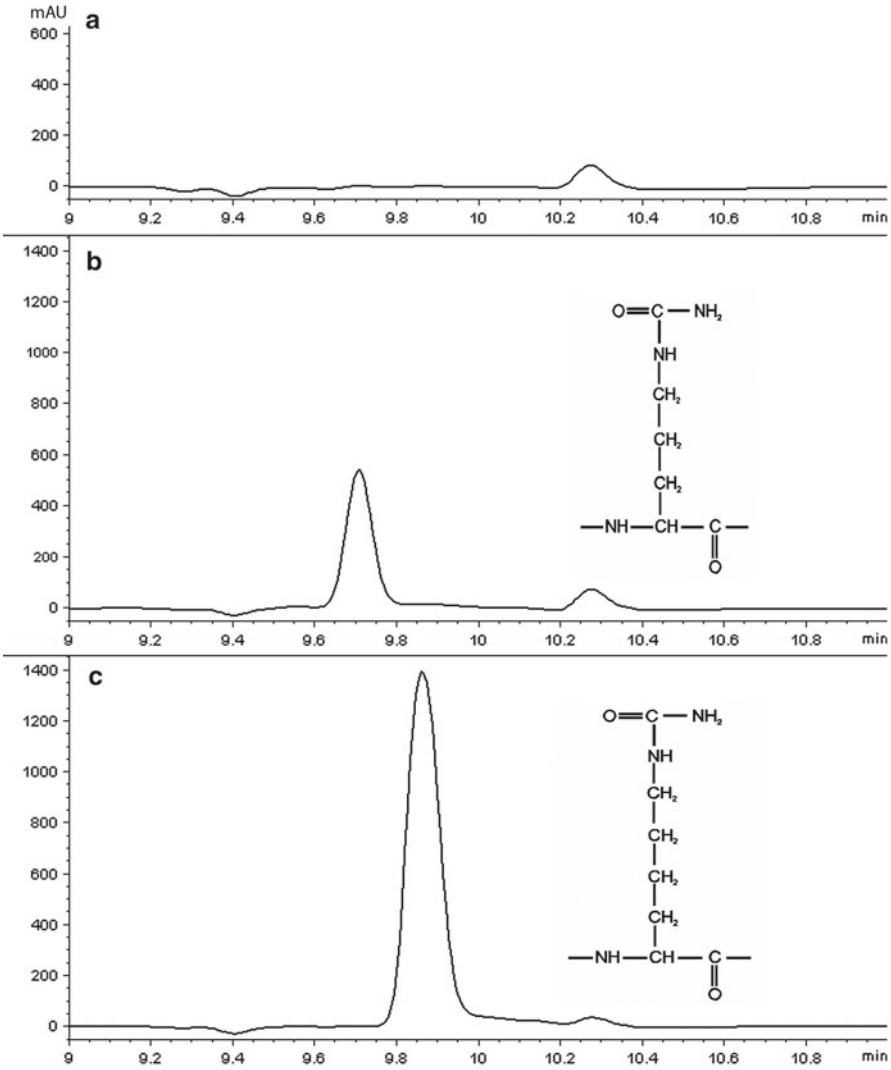

Fig. 20.4 Separation of modified citrulline and homocitrulline on HPLC. Hydrolyzed human serum albumin samples run on HPLC after chemical modification with 2,3-butanedione and antipyrine in acidic conditions. (**a**) Native albumin; (**b**) citrullinated albumin (PAD treated), together with the structure of citrulline; (**c**) homocitrullinated albumin (KCNO treated), together with the structure of homocitrulline. *X* axis = time in min, *Y* axis = absorbance at 464 nm

have been albumin and type I collagen or its peptide parts, and the antibodies produced also react with the CCP-2 antigen, an artificial cyclic peptide that has been designed for detecting anti-citrulline antibodies in humans with RA (Turunen et al. 2010). Furthermore, immunization with homocitrulline-containing peptides causes a T cell response in mice, similar to that known to be critical to the induction of autoimmune arthritis (Mydel et al. 2010).

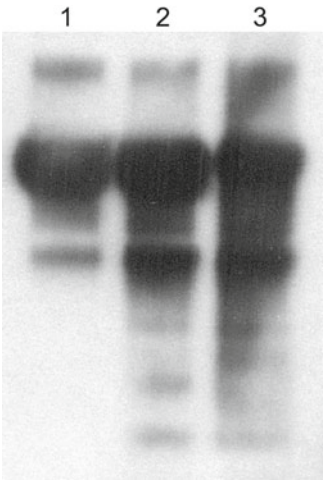

Fig. 20.5 Binding of protein-bound homocitrulline to monoclonal antibody F95 raised against a deca-citrullinated peptide (Nicholas and Whitaker 2002). The proteins have not been chemically modified before SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting. *Lanes 1–3* are human serum albumin carbamoylated with KCNO at 70 °C (*lane 1*: 2 h, *2*: 6 h and *3*: 17 h). No staining was seen with unmodified albumin

20.7 What Homocitrulline Has to Do with Citrulline In Vivo

The clinical significance of citrulline is largely the subject of this book. At the protein level, effects of carbamoylation are similar to those of citrullination. Lysine is often located on the surface of proteins and enhances its solubility in an aqueous environment. The loss of charge, caused by carbamoylation (Kraus et al. 2001; Kraus and Kraus 2001), distorts the protein structure (Kraus and Kraus 2001; Tarcsa et al. 1996) and results in a potentially antigenic structure (Mydel et al. 2010; Turunen et al. 2010). Antigenicity of carbamoylated proteins in experimental animals has been demonstrated (Turunen et al. 2010), and carbamoylation-dependent activation of T cells has been recorded (Mydel et al. 2010). In addition, interaction of carbamoylated albumin and collagen with immune responsive cells has also been shown (Jaisson et al. 2006, 2007). Carbamoylation is present in tissues of patients with chronic renal failure and in atherosclerosis (reviewed in Jaisson et al. 2011).

It is a relatively new finding that patients with RA also have antibodies against homocitrulline, in addition to those against citrulline, and such antibodies are also able to predict joint damage (Shi et al. 2011). Antibodies of these two specificities can be separated by immunoabsorption (Shi et al. 2013). The major assay used for detecting anti-citrulline antibodies in RA, that for anti-CCP, does not distinguish between these two specificities, as antisera that are experimentally produced to carbamoylated antigens, also readily bind to the CCP antigen (Turunen et al. 2010).

Smoking is an environmental factor that has a role in several diseases. In RA (see Chap. 5), it is the only environmental factor connected to citrulline antibodies in genetically susceptible individuals (Klareskog et al. 2006). The mechanism of the effect of smoking on the immune response against citrullinated proteins is not yet clear. However, the finding that antibodies against carbamoylated proteins are also present in RA sera and correlate with the disease outcome is interesting, considering

the carbamoylation route through MPO. The serum levels of thiocyanate are elevated in smokers (Rubab and Rahman 2006), who are also more prone to infections (reviewed in Huttunen et al. 2011).

As a result of these factors, the contribution of carbamoylation, as it relates to that of deimination in different disease states, still needs to be further explored. In this regard, new methods that can differentiate between citrulline- and homocitrulline-containing peptides will be needed in future studies.

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Chapter 21
Picking the PAD Lock: Chemical and Biological Approaches to Identify PAD Substrates and Inhibitors

Venkataraman Subramanian, Daniel J. Slade, and Paul R. Thompson

Keywords Deiminase • Citrulline • Cl-amidine • PAD inhibitor • Posttranslational modification • Rheumatoid arthritis • Ulcerative colitis • Inflammatory disease • Cancer

21.1 Introduction

Citrullination has emerged as an important posttranslational modification that is associated with a broad range of diseases including rheumatoid arthritis, cancer, ulcerative colitis, multiple sclerosis, glaucoma, and osteoarthritis (Jones et al. 2009). The presence of citrulline within proteins was first reported in 1958 (see the timeline outlined in Fig. 21.1), with the protein arginine deiminases (PADs) being identified as the enzymes catalyzing this PTM in 1977 (Rogers et al. 1977; Rogers and Simmonds 1958; Rogers and Taylor 1977). PAD activity was originally reported to be catalyzed by an unusual calcium-dependent enzyme that produced peptidylcitrulline in the protein trichohyalin in the inner root sheath during hair growth. Studies approximately 20 years later identified this isozyme to be PAD3 (Nachat et al. 2005a), which is now known to play a role in normal epidermal physiology through the deimination of intermediate filament proteins in hair follicles (Nachat et al. 2005a).

Further studies focused on delineating the substrates and reaction conditions of a PAD purified from the epidermis of newborn rats (Fujisaki and Sugawara 1981). These seminal works showed that L-arginine is not a PAD substrate and that the α-amino group of the L-arginine substrate must be in an amide linkage for it to be a substrate. This provided the groundwork for this enzyme family to be named the

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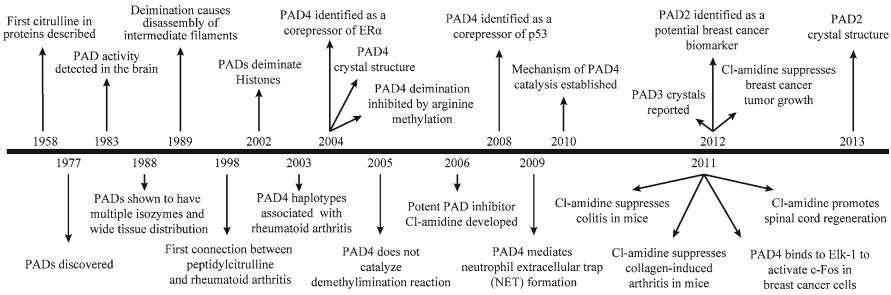


Fig. 21.1 Timeline of protein citrullination and PAD discoveries

protein (or peptidyl) arginine deiminases. The emergence of molecular biology was crucial for the identification and cloning of multiple PAD isozymes from a variety of mammals (Tsuchida et al. 1991; Watanabe and Senshu 1989), which ultimately provided the foundation for the discovery and characterization of the five human PADs (PAD1, 2, 3, 4, and 6). Worth noting is that while PAD6 is considered a family member by its homology to PADs 1–4, no enzymatic activity has been detected in vitro (Raijmakers et al. 2007), and no substrates for this enzyme have been reported.

Since the discovery 15 years ago that patients with rheumatoid arthritis produce autoantibodies targeting citrullinated proteins (Schellekens et al. 1998), the PADs have attracted significant interest, and it is now clear that these enzymes play fundamental roles in both normal tissue homeostasis and human disease. Although we will not discuss in detail the role of PADs in specific diseases, as they are thoroughly discussed elsewhere (Jones et al. 2009) and in other chapters, it is evident that both PADs 2 and 4 are potential therapeutic targets for a variety of inflammatory diseases and cancer. Instead, this chapter focuses on the mechanism of PAD catalysis, known PAD substrates, the development of PAD inhibitors, and how these studies have greatly enhanced our understanding of the roles the PADs play in the maintenance of human health and the progression of multiple diseases.

21.2 PAD4 Structural Organization

PAD4 is composed of two IgG-like domains and a C-terminal α/β-propeller domain that forms the active site (Fig. 21.2a) (Arita et al. 2004). The crystal structure of PAD4 revealed a head-to-tail dimer whose existence has been confirmed in vitro through sedimentation velocity experiments (Liu et al. 2011). Although the dimer is formed through ionic and hydrophobic interactions, PAD4 enzymatic activity is not drastically decreased when mutations creating a monomeric enzyme are introduced. Structures of the enzyme bound to calcium also demonstrated that this regulatory ion converts the substrate binding pocket from an open to closed conformation that decreases the solvent-accessible interior space by 100 % (Fig. 21.3). This conformational change results in a more rigid active site cleft that is presumably selective

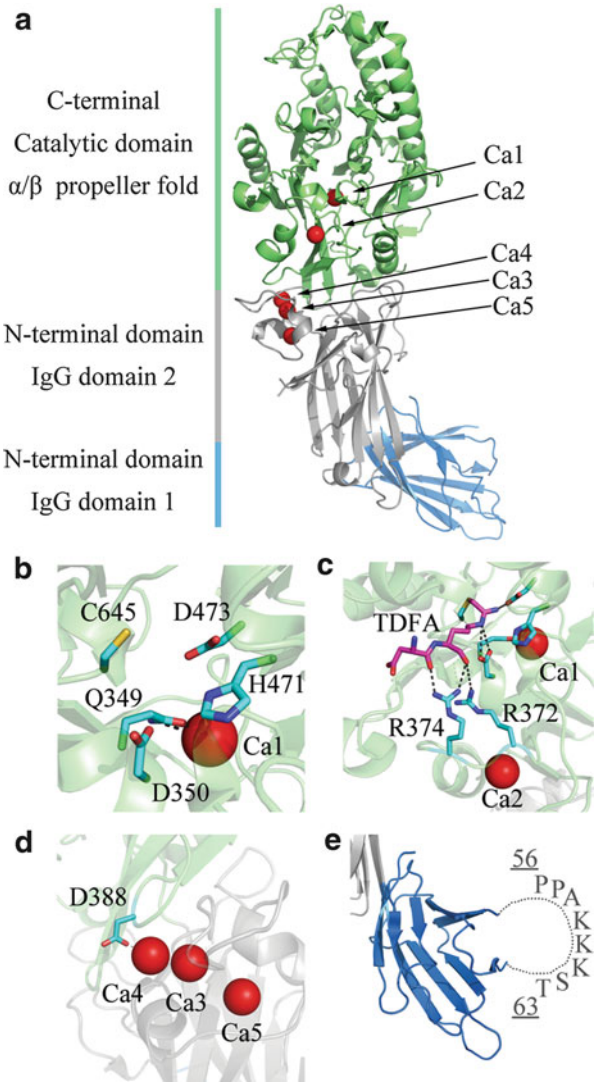


Fig. 21.2 PAD4 structural features. (**a**) PAD4 is composed of three domains and five calcium binding sites. (**b**) The Calcium 1 binding site (Ca1) is crucial for enzymatic activity and is located below the active site substrate binding pocket. (**c**) Ca2 is necessary to position R372 and R374 to bind substrate as shown here with the covalent inhibitor TDFA. (**d**) Ca3–5 are in close proximity, and Ca4 is coordinated by D388. This residue is connected to a loop responsible for Ca2 binding. (**e**) PAD4 contains a nuclear localization signal (NLS) located in IgG domain 1 from residues P56-T63. Figure created from PDB: 4DKT using Pymol

for substrates. Additionally, calcium binding triggers the movement of several active site residues, including Cys645, into positions that are competent for catalysis.

The structure of the PAD4·calcium complex (PDB ID: 1WD9) also revealed five distinct calcium binding sites in IgG-like domain 2 (Ca3–5) and in the C-terminal

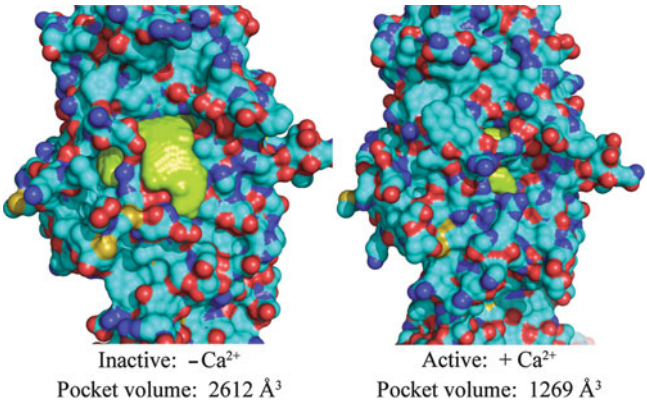


Fig. 21.3 Calcium-dependent volume of the PAD4 active site. The PAD4 active site undergoes drastic conformational changes upon the binding of calcium, including a 100 % reduction in the volume of the active site pocket. HOLLOW was used to create a model of the binding pocket of PAD4, and the image was created using Pymol

catalytic domain (Ca1–2) (Arita et al. 2004). Calcium 1 (Ca1) is positioned below the active site pocket and is coordinated by Gln349 (Fig. 21.2b). A major role for Ca1 is to properly position Asp350, an active site residue that is absolutely required for PAD4 activity, such that it occupies a conformation that is competent for substrate binding and catalysis. Ca2 is positioned below and in front of the active site binding pocket and is the major driving force for aligning a loop that contains Arg372 and Arg374, which are critical for binding substrates and inhibitors (Fig. 21.2c) (Arita et al. 2006). Ca3–5 are located in a cluster at the interface between the C-terminal catalytic domain and IgG-like domain 2. While these residues are 32–37 Å from the active site cysteine, they play a critical role in properly positioning Ca2, through the coordination of Ca4 to Asp388. Asp388 resides on a loop that positions residues involved in both substrate binding and the coordination of Ca2 (Fig. 21.2d). This intricate cross-talk between calcium ions that are positioned large distances from one another is a unique feature of the PADs, and reveals a regulatory mechanism to keep these enzymes inactive until the proper calcium concentration is achieved within the cell. Future studies that focus on the order of binding and affinity of each calcium within the PADs will broaden our knowledge of the regulatory role calcium plays.

Among the five PADs, PAD4 uniquely contains a monopartite nuclear localization signal (NLS; Fig. 21.2e) (Nakashima et al. 2002) and deiminates a number of nuclear proteins, including histone H3, histone H4, and ING4 (Guo et al. 2011; Guo and Fast 2011; Hagiwara et al. 2002; Wang et al. 2004). The NLS is comprised of residues 56–63 (PPAKKKST) and is located in the N-terminal Ig-like domain. Confirming its importance, mutation of the central lysine cluster inhibited localization of PAD4 to the nucleus (Nakashima et al. 2002). Although this motif is absent in PAD2, recent studies have conclusively demonstrated that a fraction of PAD2 is also nuclear and that it deiminates histone H3 (Cherrington et al. 2010, 2012). Recruitment of PAD2 to the nucleus may be due to a noncanonical NLS, or,

alternatively, PAD2 be transported into the nucleus by an unidentified interacting protein or receptor. Also present in the N-terminal IgG-like domain, clustered close to the NLS, are three residues mutated in a PAD4 haplotype that is associated with an increased risk of developing RA in Asian but not Caucasian populations (odds ratio = 1.14) (Burr et al. 2010; Gandjbakhch et al. 2009; Suzuki et al. 2003); the haplotype contains four mutations (S55G, A82V, A112G, and a silent polymorphism). Given their position, these residues may play a role in regulating PAD4 activity via effects on interacting proteins.

21.3 Catalytic Mechanism

Important insights into how the PADs citrullinate arginine residues has been gained in the past several years, and Fig. 21.4 describes the proposed catalytic mechanism, using PAD4 as the model (Knuckley et al. 2007). The PAD4 active site contains four residues that are absolutely required for activity (Knuckley et al. 2007):

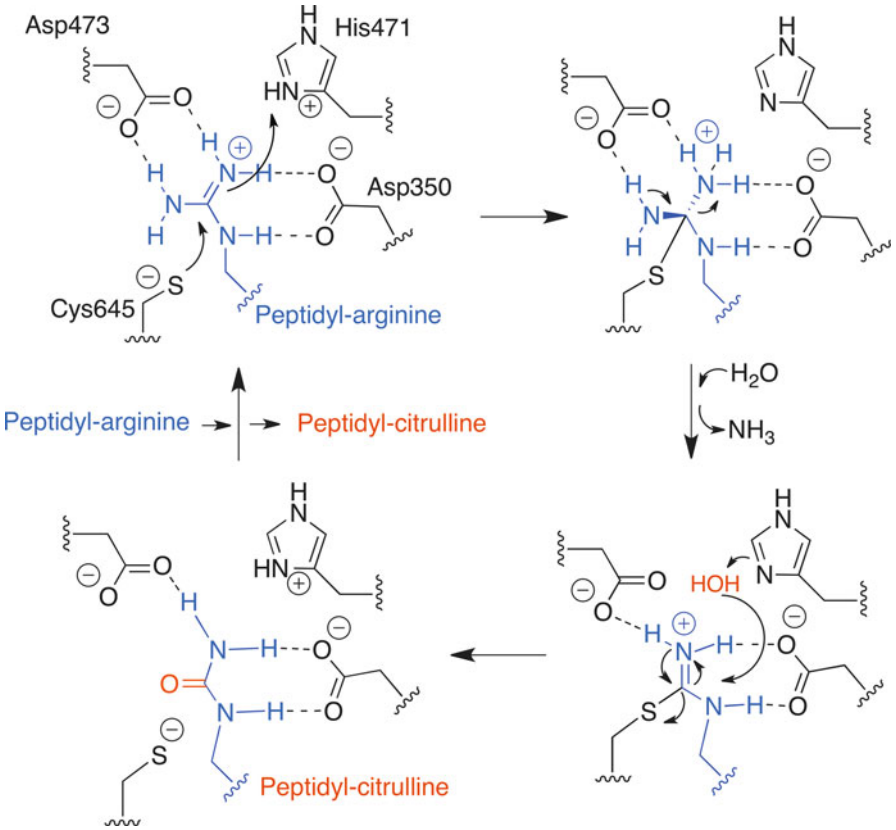


Fig. 21.4 Proposed reaction mechanism, using PAD4 as the model

Cys645, His471, Asp473, and Asp350. Detailed mechanistic studies with PAD4 show that the pK_a of Cys645 and His471 are ~8.2 and ~7.3, respectively, and correspond to the basic and acidic limbs of the pH rate profiles. This pattern is the reverse of what is traditionally expected based on their presumed roles in catalysis, i.e., that the acidic and basic limbs should correspond to the Cys645 thiolate and His471 imidazolium, respectively. Since they do not correspond to the norm, this type of pattern is typically referred to as a reverse protonation mechanism (Knuckley et al. 2007). One outcome of this mechanism is that only a fraction of the enzyme exists as the catalytically competent thiolate at the pH optimum, which likely protects the enzyme from inactivation by reactive oxygen species (ROS).

In the proposed mechanism, the Cys645 thiolate acts as the nucleophile, attacking the guanidinium group of arginine to form a tetrahedral intermediate that is stabilized by the donation of a proton from His471 in either a stepwise or concerted fashion. The protonated tetrahedral intermediate then collapses and releases ammonia while forming an *S*-alkylthiouronium adduct that is also seen in reaction mechanisms of the L-arginine deiminases, dimethylarginine dimethylaminohydrolase (DDAH), and the agmatine deiminases (Galkin et al. 2005; Jones et al. 2010; Stone et al. 2005a). His471 now acts as a general base to deprotonate a water molecule in preparation for attack of the *S*-alkylthiouronium species by a hydroxide ion, resulting in the formation of a second tetrahedral intermediate that collapses to form the final product citrulline. The resulting ureido group lacks the electrostatic and hydrogen bonding characteristics of the substrate guanidinium, which form favorable interactions with the two active site aspartates, and as a result the citrullinated product is released from the enzyme. The enzyme is then free to bind the next arginine residue for another round of catalysis.

21.4 Calcium Dependence

Structural and functional studies show that the PADs are calcium-dependent enzymes that require multiple ions to be active (Kearney et al. 2005). Five calcium binding sites were identified in the structure of PAD4, and the Hill coefficients range from 1.5 to 3.0, depending on the substrate, indicating that calcium binding occurs with a significant degree of cooperativity (Kearney et al. 2005). The reported calcium concentrations required for half maximal activity ($K_{0.5}$) are ~0.15–0.5 mM when performing in vitro enzymatic assays (Kearney et al. 2005; Raijmakers et al. 2007; Watanabe and Senshu 1989). This concentration is much higher than normal cellular calcium concentrations, suggesting that an auxillary regulatory mechanism acts to either raise calcium levels above normal or lower the concentration of calcium required for functional activity. For example, in mucosal cells, PADs are not activated until late in the differentiation process when the integrity of plasma membrane is compromised, allowing for an influx of calcium (Senshu et al. 1996). The addition of calcium ionophores to cells is also able to activate PADs, reiterating the need for higher than normal cellular calcium levels for enzyme activation

(Asaga et al. 1998). It is also likely that an unidentified posttranslational modification or interacting protein could lower the calcium dependence of the PADs. Such a mechanism would offer an explanation for the histone deiminating activity of the PADs.

21.5 Functional Effects of Protein Citrullination

When activated by calcium, the PADs will also citrullinate themselves. Although autodeimination of PAD4 was originally reported to inactivate the enzyme (Andrade et al. 2010), subsequent studies from the Thompson lab showed that autodeimination does not affect its enzymatic activity. Instead this PTM reduces protein–protein interactions with citrullinated histone H3, protein arginine methyltransferase 1 (PRMT1), and histone deacetylase 1 (HDAC1) (Slack et al. 2011c). Experiments also suggest that deimination acts as a precursor to protein cleavage because proteolysis of myelin basic protein (MBP) by cathepsin D is greatly enhanced upon citrullination, which is thought to be due to MBP adopting a more open conformation (Pritzker et al. 2000). While cathepsin D is not known to specifically cleave at citrulline residues, the discovery that bleomycin hydrolase proteolyzes deiminated filagrin at citrulline residues adds another possible dimension to the role of the PADs in protein modification and processing (Kamata et al. 2009). Other potential physiological roles of protein citrullination include alterations in kinase signaling (Rust and Thompson 2011) and protein–nucleic acid interactions because arginine residues are present in numerous kinase consensus sequences (Rust and Thompson 2011) and are critical for high affinity interactions between proteins and both RNA and DNA.

21.6 PAD Tissue Distribution and Substrate Specificity

While each of the five PADs is highly homologous to one another, their individual function and cellular distribution in the human body is highly varied. (Table 21.1 contains a list of experimentally identified PAD substrates along with a functional overview of each protein.) When the substrates in Table 21.1 are analyzed by Interactive Pathway Analysis (unpublished results, Fig. 21.5), no single category of protein function or disease stands out. These results are not surprising as PADs have a very broad tissue distribution, and classical methods to identify PAD substrates by primary sequence or function have proven difficult. Nevertheless, the results strengthen previous data linking PADs to inflammatory diseases and cancer and provide further evidence that the substrates the PADs are deiminating are in fact involved in disease onset and progression. Even as the number of known PAD substrates increase, our understanding of what makes a protein a good substrate is severely lacking because consensus sequences cannot be identified for the PADs. Nevertheless, studies with peptide substrates suggest that amino acids directly flanking arginines can influence citrullination with the most significant effect being

Table 21.1 Summary of PAD substrates and their function

Substrate	Function	In vivo	In vitro	Reference
PAD1				
Filaggrin	Keratin intermediate filament aggregation	*x*		Nachat et al. (2005b)
Keratin K1	Differentiation of epithelial tissue. Associated with K10	*x*		Vossenaar et al. (2003)
Keratin K10	Differentiation of epithelial tissue. Associated with K1	*x*		Vossenaar et al. (2003)
PAD2				
β/γ-Actin	Cell motility, structure, and integrity	*x*		Darrah et al. (2012)
Histone H3	Core component of nucleosomes with roles in transcriptional regulation	*x*		Cherrington et al. (2010); Zhang et al. (2012)
IKKγ	Serine kinase in the NF-κB signaling pathway	*x*		Lee et al. (2010)
Vimentin	Class-III intermediate filaments found in various non-epithelial cells	*x*		Hojo-Nakashima et al. (2009)
MBP	Formation and stabilization of the myelin membrane in the CNS	*x*		Vossenaar et al. (2003); Wood et al. (2008)
GFAP	Astrocyte-specific type-III intermediate filament	*x*		Ishigami et al. (2005)
ENO1	Glycolytic enzyme that downregulates the c-myc protooncogene	*x*		Jang et al. (2012)
ENO2/NSE	Neuron survival. Can form a heterodimer with ENO1	*x*		Jang et al. (2012)
Fibrinogen	Precursor to fibrin and cofactor in platelet aggregation	*x*		van Beers et al. (2010)
CXCL10	Chemotactic for monocytes and T-lymphocytes	*x*		Loos et al. (2008)
IL-8/CXCL8	Chemotactic factor that attracts neutrophils, basophils, and T-cells	*x*		Proost et al. (2008)
CXCL5	Involved in neutrophil activation	*x*		Mortier et al. (2010); Proost et al. (2008)
CCL17	Chemotactic factor for T-lymphocytes but not monocytes or granulocytes	*x*		Proost et al. (2008)
CCL26	Chemotactic for eosinophils and basophils. Binds to CCR3	*x*		Proost et al. (2008)
PAD3				
Filaggrin	Keratin intermediate filament aggregation	*x*		Nachat et al. (2005b)
Trichohyalin	Intermediate filament-associated protein of the hair follicle	*x*		Nachat et al. (2005a)
S100A3	EF hand calcium binding protein involved in differentiation	*x*		Kizawa et al. (2008)
PAD4				
ING4	HBO1 complex protein with histone H4-specific acetyltransferase activity	*x*		Guo et al. (2011); Guo and Fast (2011)
Histone H3	Core component of nucleosomes with roles in transcriptional regulation	*x*		Wang et al. (2004)
Histone H4	Core component of nucleosomes with roles in transcriptional regulation	*x*		Wang et al. (2004)
Histone H2A	Core component of nucleosomes with roles in transcriptional regulation	*x*		Hagiwara et al. (2005)

p300	Histone acetyltransferase. Regulates transcription by chromatin remodeling	*x*		Lee et al. (2005)
RPS2	Ribosomal protein of the 40s subunit		*x*	Guo et al. (2011)
NPM1/B23	Diverse properties, including histone chaperoning and assembly	*x*		Hagiwara et al. (2002)
Elk-1	E twenty-six (ETS)-like transcription factor 1	*x*		Zhang et al. (2011)
PRMT1	Mono and asymmetric dimethylation of arginine in multiple proteins		*x*	Guo et al. (2011)
Fibrinogen	Precursor to fibrin and cofactor in platelet aggregation	*x*		van Beers et al. (2010)
Lamin C	Type V intermediate filament formation and transcriptional regulation	*x*		Tanikawa et al. (2012)
Antithrombin	Serine protease inhibitor that regulates the blood coagulation cascade		*x*	Chang et al. (2005); Ordonez et al. (2009)
IL-8/CXCL8	Chemotactic factor that attracts neutrophils, basophils, and T-cells	*x*		Proost et al. (2008)
CXCL5	Involved in neutrophil activation	*x*		Proost et al. (2008)
CCL17	Chemotactic factor for T-lymphocytes but not monocytes or granulocytes	*x*		Proost et al. (2008)
CCL26	Chemotactic for eosinophils and basophils. Binds to CCR3	*x*		Proost et al. (2008)
CIRP	Cold-inducible RNA-binding protein		*x*	Guo et al. (2011)
EBP2	Probable rRNA-processing protein		*x*	Guo et al. (2011)
FEZ	Fasciculation and elongation protein zeta 1		*x*	Guo et al. (2011)
Fau	Ubiquitin-like protein FUBI		*x*	Guo et al. (2011)
HNRPA1	Heterogeneous nuclear ribonucleoprotein A1		*x*	Guo et al. (2011)
PSTB4	Proteasome subunit beta type 4		*x*	Guo et al. (2011)
UFC	Ufm-1-conjugating enzyme 1		*x*	Guo et al. (2011)
PAD6				
Unknown	PAD6 is enzymatically inactive when tested in vitro	*NA*	*NA*	NA
Rabbit PAD				
TNF-α	Inflammatory cytokine involved in immune cell regulation	*x*		Moelants et al. (2013)

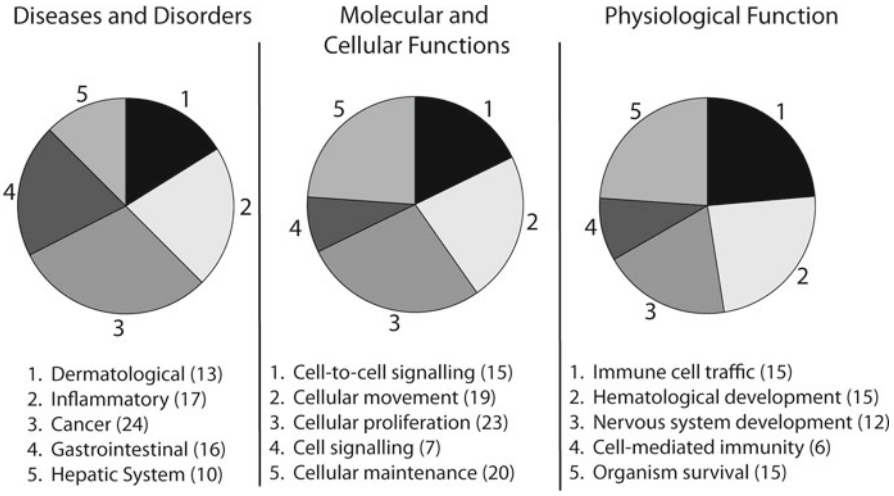


Fig. 21.5 Classification of identified PAD substrates in the top five categories based on Interactive Pathway Analysis. The number next to the description of the disease or function corresponds to the number of proteins out of 38 known substrates, e.g., 24 of the 38 PAD substrates have a connection to cancer under the Diseases and Disorders chart

a Proline-Arginine-Proline sequence that is unable to be deiminated (Nomura 1992). However, most accessible arginines can be modified by the PADs (Knuckley et al. 2010a; Stensland et al. 2009). It is likely that the lack of sequence specificity for target arginines means that other factors, e.g., interacting proteins, are key factors in targeting the PADs to the appropriate protein substrates.

21.7 PAD1

In epidermal keratinocytes, PAD1 helps hydrate the stratum corneum, the outermost layer of the epidermis, and maintain the cutaneous barrier, which protects the underlying tissue from infection and other outside stresses (Nachat et al. 2005b). Within the epidermis, PAD1 is responsible for the deimination of keratins K1 and K10, and also the keratin-associated protein filaggrin (Senshu et al. 1995, 1999b; Senshu et al. 1996). Deimination of these filament-forming proteins leads to unfolding and reduced flexibility of keratin, ultimately promoting cornification of the epidermis (Ishida-Yamamoto et al. 2002; Tarcsa et al. 1996).

Limited studies also suggest that PAD1 plays a role in female reproduction as this isozyme is expressed in an estrogen-dependent manner during the middle stage of pregnancy in mouse decidual cells (Arai et al. 1995; Terakawa et al. 1991). This would fit with the discovery that PAD2 plays a role in reproduction through the deimination of histones in canine mammary tissue (Cherrington et al. 2010).

Further studies are needed to identify PAD1 substrates in the uterus, and its possible role in human reproduction. As of yet there is no direct link to altered PAD1 citrullination leading to disease progression as is seen with PAD2 and PAD4.

21.8 PAD2

Recent data indicates that dysregulated PAD2 activity contributes to the onset or progression of several diseases including multiple sclerosis, breast cancer, and rheumatoid arthritis (Cherrington et al. 2012; Foulquier et al. 2007; McElwee et al. 2012; Musse et al. 2008). This isozyme has the widest tissue distribution with expression being detected in the brain, spinal cord, spleen, pancreas, bone marrow, uterus, macrophages, and several secretory glands (Vossenaar et al. 2003). PAD2 substrates are quite diverse and include proteins crucial for cell structure, immunological responses, and gene transcription. For example, when transfected into HL-60 cells, PAD2 was the only PAD isozyme capable of citrullinating β/γ-actin, and, interestingly, once citrullinated, this protein was recognized by RA autoantibodies (Darrah et al. 2012). PAD2 also citrullinates actin in a mouse model of glomerular Bowman's capsule, and citrullinated actin levels are increased in obstructive nephropathy (Feng et al. 2005). How or whether actin citrullination regulates its activity is not known, but it may be involved in regulating the cytoskeleton and cell motility. Vimentin, another component of the cytoskeleton and a candidate autoantigen in RA, is also a PAD2 substrate. Vimentin deimination, which occurs in human leukemia THP-1 cells during macrophage differentiation, disrupts vimentin filament formation, and could play a role in monocyte physiology and maturation (Hojo-Nakashima et al. 2009). Citrullinated fibrinogen (α and β chains) is also frequently found in the synovium of RA patients (van Beers et al. 2010). While it has not been tested, deimination by the PADs could lead to structural changes in fibrinogen or fibrin that could lead to changes in blood clotting during wound healing.

Within the past 5 years PAD2 has been linked to immunoregulation through the deimination of several chemokines. Citrullinated CXCL10 was the first immune modulating protein reported to be functionally modified by citrullination, and the data show that this PTM reduced chemoattraction, cell signaling, and heparin binding (Loos et al. 2008). The PAD2 catalyzed citrullination of CXCL8 (IL-8) also alters its ability to influence tissue inflammation, and increased the ability of CXCL8 to induce egress of neutrophils from the bone marrow and into the blood (Loos et al. 2009; Proost et al. 2008). Changes in chemokine function have also been linked to a loss in immune function as citrullination of CXCL12 reduced the antiviral potency of this chemokine; citrullinated CXCL12 does not prevent infection of lymphocytic MT-4 cells by HIV-1 (Struyf et al. 2009).

Classic studies identified the existence of an enzyme in the mammalian brain capable of producing citrullinated proteins (Kubilus and Baden 1983). This enzyme was later identified as PAD2, and its major substrate was found to be MBP

(Vossenaar et al. 2003; Wood et al. 2008). White matter isolated from patients with multiple sclerosis (MS) shows increased levels of citrullinated MBP, and higher amounts correlate with increased PAD2 expression, which in turn is associated with a hypomethylated PAD2 promoter (Mastronardi et al. 2007). Overexpression of PAD2 in mice also leads to increased MBP citrullination, which is associated with decreased myelination in the central nervous system (Musse et al. 2008). More recently, PAD2 was also shown to be overexpressed in peripheral blood mononuclear cells (PBMCs) of MS patients (Calabrese et al. 2012). Just as was seen in white matter from the brain, overexpression is associated with promoter demethylation. Since PBMCs also express MBP, defective regulation of PAD2 outside of the blood brain barrier could also contribute to MS pathology.

Recent studies demonstrated that PAD2 is present in the nucleus of canine mammary epithelial cells, and higher levels are associated with citrullinated histones and the epigenetic control of gene transcription (Cherrington et al. 2010). Further studies suggest that PAD2 plays a role in breast cancer because PAD2 mRNA and protein expression levels correlate with HER2/ERBB2 expression in multiple breast cancer cell lines (McElwee et al. 2012). Further strengthening the link between dysregulated PAD2 activity and breast cancer is the demonstration that stimulation of estrogen receptor α (ERα) positive cells with 17β-estradiol (E_2) promotes the PAD2-dependent citrullination of chromatin-associated histone H3 at arginine 26 (H3R26) (Zhang et al. 2012). PAD2 was also shown to be recruited to promoters where it directly interacts with ERα after E_2 stimulation. This interaction results in H3R26 citrullination and causes chromatin decondensation and transcriptional activation.

21.9 PAD3

Historically, the catalytic activity of PAD3 was the first reported when citrulline containing proteins were detected in hair follicles (Rogers and Simmonds 1958). Subsequently, this citrullinated protein was found to be trichohyalin (Kanno et al. 2000). PAD3 expression is limited to the epidermis; more specifically to the inner and outer root sheath of hair follicles (Kanno et al. 2000). The full-length clone was obtained from human epidermal keratinocytes, even though no detectable PAD3 protein is present in these cells. Since PAD3 citrullinates trichohyalin, this isozyme likely plays a role in normal tissue homeostasis in the epidermis and the differentiation of hair follicles (Nachat et al. 2005a). Like PAD1, a substrate for PAD3 is filaggrin, and deimination by PADs lowers its affinity for keratin, leading to its dissociation from the matrix and subsequent proteolysis (Senshu et al. 1996). Filaggrin degradation is critical for generating the amino acid component of Natural Moisturizing Factor (NMF). PAD3 also citrullinates Arg51 in S100A3, which is a member of the S100 family of calcium binding proteins, and this PTM promotes homotetramer assembly (Kizawa et al. 2008). Additional studies of PAD3 are limited; however, the crystallization of PAD3 and initial X-ray diffraction data to 2.95 Å resolution was recently reported (Unno et al. 2012).

21.10 PAD4

PAD4 is the best characterized member of the PAD family and is the only member for which an X-ray crystal structure has been reported (Arita et al. 2004). The role of PAD4 in protein citrullination and the subsequent production of anti-citrullinated protein antibodies (ACPA) are well documented in multiple reviews and are not discussed at length in this chapter (Jones et al. 2009; Raptopoulou et al. 2007; Trouw and Mahler 2012). PAD4 was first cloned from a rat epidermal cDNA library, and it is 51 and 54 % identical to PADs 2 and 3 (Yamakoshi et al. 1998). The human enzyme was subsequently cloned from HL60 granulocytes (Nakashima et al. 1999). Since the substrate specificity differed from rat PAD4, this isozyme was originally annotated as PAD5. Genome sequencing efforts, however, ultimately showed that they were orthologs and PAD5 was reannotated as human PAD4. Subsequent studies showed that PAD4 is highly expressed in healthy immune cells such as macrophages and granulocytes, as well as in several malignant but not benign cancerous tissues including breast carcinomas and colorectal adenocarcinomas (Chang et al. 2009). Within cells, PAD4 is localized to cytoplasmic granules and the nucleus. While PAD4 expression is predominantly intracellular, it has also been detected in the sera of patients with RA and cancer (Chang et al. 2009; De Rycke et al. 2005). At the time of writing this chapter, more than 20 protein substrates have been identified for PAD4 (see Table 21.1), including histones H2A, H3, and H4. Histones H2A and H4 are both citrullinated at arginine 3 in the N-terminal Ac-SGRGK sequence that is conserved between both proteins (Hagiwara et al. 2005). PAD4 also deiminates H3 at arginines 2, 8, and 17, and citrullination of these residues is associated with altered gene expression (Cuthbert et al. 2004; Li et al. 2008; Nakashima et al. 1999; Wang et al. 2004; Yao et al. 2008; Zhang et al. 2011). Other substrates involved in the regulation of gene transcription include ING4, p53, ELK1, p300, p21, and CIP1 (Guo and Fast 2011; Li et al. 2008; Tanikawa et al. 2009; Yao et al. 2008; Zhang et al. 2011). PAD4 also citrullinates nucleophosmin (B23, NPM1) (Hagiwara et al. 2002), a nuclear protein that acts as a histone chaperone and aids nucleosome assembly, at Arg197 and modification of this site initiates its translocation from nucleoli to the nucleoplasm (Tanikawa et al. 2009). Although methylation and citrullination appear to be reciprocally regulated and PAD4 was reported to convert methylated arginine residues to citrulline (Wang et al. 2004), it is unlikely that the latter reaction is physiologically relevant because methyl-arginines are quite poor PAD substrates as they are processed at rates that are 150- to 1,000-fold slower than for an unmodified arginine (Cuthbert et al. 2004; Hidaka et al. 2005; Kearney et al. 2005; Raijmakers et al. 2007; Thompson and Fast 2006).

21.11 PAD4 in NETosis, Immune Regulation, and the Brain

Neutrophils make up 75 % of the white blood cells in circulation and are a first-line defense against bacteria through the release of proteases, ROS production, and direct phagocytosis of pathogens (Borregaard 2010). A unique and recently

characterized immunological response of neutrophils is the formation of neutrophil extracellular traps (NETs), which result when granulocytes extrude their DNA in a web or net-like structure to capture bacteria, fungi, and protozoa in a process called NETosis (Leshner et al. 2012; Rohrbach et al. 2012). The extruded DNA is decorated with antimicrobial peptides and proteins, including neutrophil elastase (NE) (Papayannopoulos and Zychlinsky 2009), lactoferrin (Papayannopoulos and Zychlinsky 2009), and histones (Parseghian and Luhrs 2006). It is thought that one reason neutrophils initiate this alternative pathway is to kill pathogens such as fungi and helminthes that are too large to phagocytose. While the normal physiological role of NET formation is to respond to invading pathogens, several diseases such as sepsis, thrombosis, and systemic lupus erythramatosis (SLE) have reported non-pathogenic induction of NET formation (Kessenbrock et al. 2009; Logters et al. 2009). PAD4 activity and histone hypercitrullination is required for chromatin decondensation, expulsion of DNA, and release of antimicrobial proteins, and both occur in response to a variety of extracellular stimuli including LPS, LTS, or TNFα (Jones et al. 2009; Li et al. 2010; Wang et al. 2009). NET formation is calcium dependent, and is suppressed by the addition of the PAD inhibitor Cl-amidine, indicating that deimination by PAD4 is a key step in NETosis (Li et al. 2010; Wang et al. 2009). While increased PAD activity is generally associated with disease progression, NETosis is a case where PAD4 activation could be beneficial during bacterial infection. Like PAD2, PAD4 plays a role in regulating the human immune system through the deimination of chemokines IL-8/CXCL8, CXCL5, CCL17, and CCL2, which are activated during infection. There is cross-talk between PAD4 citrullination of chemokines and NET formation because IL-8 activates NETosis, although activation is dependent on the cell culture conditions (Marcos et al. 2010).

Also like PAD2, PAD4 expression is elevated in the brain of multiple sclerosis patients and animal models of demyelinating diseases and correlates with an increase in histone H3 citrullination. Moreover the nuclear translocation of PAD4 in normal-appearing white matter (NAWM) of MS patients is increased in response to TNFα. Interestingly, TNFα is deiminated by rabbit muscle PAD, although the functional effects of this PTM are not clear (Mastronardi et al. 2006; Moelants et al. 2013). PAD4 has been further characterized as playing a role in MS through citrullination of H3 arginine 8, leading to decreased binding of HP1α to H3, and increased transcriptional activation of cytokines and the onset of inflammation (Sharma et al. 2012). This inflammation could lead to cross-talk with PAD2, which deiminates MBP and causes MS-like symptoms.

21.12 PAD6

PAD6 was the last isozyme to be discovered and cloned, and is also the least well-characterized member of this enzyme family. Although PAD6 shows strong sequence homology to the other PADs, it lacks the enzymatic activity that the other four isozymes possess (Raijmakers et al. 2007). Since PAD6 retains all four active

site catalytic residues (Asp351, His472, Asp474, Cys667, or Cys669) it is not completely clear why this isozyme lacks activity. Nevertheless, several differences exist between PAD6 and PAD4 including the mutation of the motif surrounding the catalytic cysteine (Cys645 in PAD4 and Cys667 or Cys669 in PAD6) which is mutated from $H^{644}CGT^{647}$ in PAD4 to $C^{667}ACA^{670}$ in PAD6. This indicates that the active site cysteine of PAD6 has been shifted by one residue compared to the other PADs and could explain the lack of enzymatic activity. Alternatively, mutation of one or more of the residues required for calcium binding could explain the lack of activity. The residues in PAD6 that are mutated (from Asp or Glu in PAD4) are Ala158, Thr169, Ala354, Ala374, Gly389, and Met412. Sequence alignments of the five PAD family members also reveal that PAD6 contains multiple uncharacterized insertions that are unique to this protein and make it the largest PAD at 686 amino acids. The roles of these insertions are not known but may be responsible for the role of PAD6 in embryonic development and reproduction (see below).

While no enzymatic activity or substrates have been identified for this isozyme, PAD6 still plays an important role in female fertility through the formation of novel oocyte-restricted fibrous structures known as cytoplasmic lattices (CPL) (Kan et al. 2011). It was shown that tubulin associates with the PAD6/CPL complex and is responsible for proper microtubule-mediated organelle positioning in oocyte development. PAD6 colocalizes with another protein known as Mater, which also plays a role in CPL formation and is essential for female fertility (Kim et al. 2010). Given its important role in this process, it is unsurprising that PAD6 null mice are infertile and do not progress past the two cell stage. During oocyte maturation, PAD6 also interacts with the 3-monooxygenase/tryptophan 5-monooxygenase activation protein family (YWHAZ; also known as 14-3-3 ζ) and undergoes a drastic change in phosphorylation which could be a means of regulating PAD6 activity (Snow et al. 2008).

21.13 Probes to Detect Protein Citrullination

A major challenge to understanding the role of the PADs in human health and disease has been the inability to easily and accurately identify new substrates. This is due to a number of factors, including (1) the small 1 Da mass increase that occurs upon citrullination is not easily discriminated by most mass spectrometers; (2) because unlike with phosphorylation, citrullinated proteins are not readily enriched; and (3) since it is a hydrolytic reaction, azide or alkyne containing alternative substrates cannot be used as orthogonal reagents to facilitate the isolation of modified proteins. In response to this challenge, we and others have taken advantage of the chemoselective reaction that occurs between ureas and glyoxals under acidic conditions. The first success with this approach was the development, by Senshu and colleagues, of an antibody that specifically recognizes citrullinated proteins that have been chemically modified with a ureido group adduct formed by reaction of citrulline with 2,3-butanedione monoxime, antipyrine, and $FeCl_3$ in a strong acid solution (Senshu et al. 1999a). Although this approach has played an incredible role in pushing

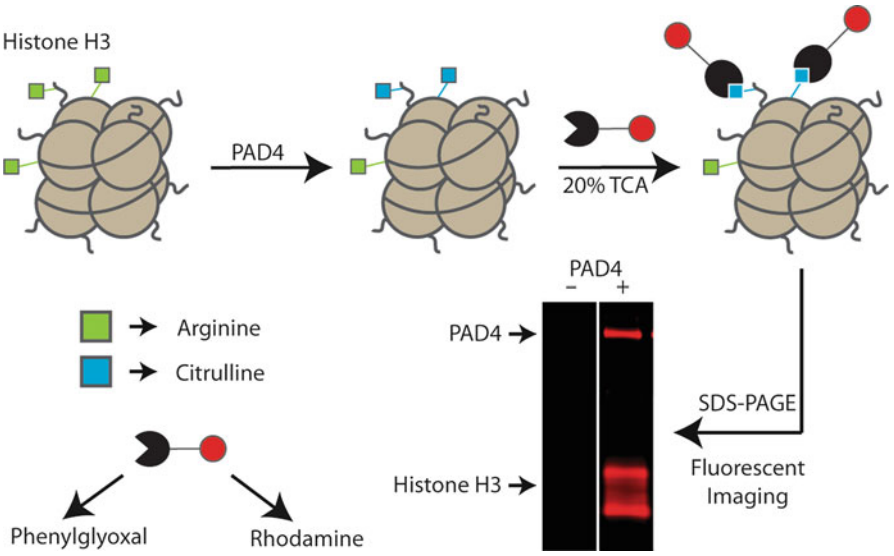


Fig. 21.6 Application of Rh-PG in detecting protein citrullination

the PAD field forward, it suffers from a number of limitations, including sensitivity, the need for long incubation times, and the adducts generated are not readily detected by MS methods. Additionally, this antibody is no longer commercially available, which obviously limits its current utility. Although Proost and colleagues recently described the development of an antibody raised against a citrullinated peptide modified by 2,3-butanedione and antipyrine that shows promise in ELISA-based assays of PAD activity (see below). A second important development was the work of Fleckenstein and colleagues who characterized the adducts formed upon reacting citrullinated proteins with a variety of glyoxal-containing compounds (Holm et al. 2006; Tutturen et al. 2010). In one such approach, the authors synthesized a chemically cleavable phenylglyoxal derivative on PL-DMA resin and demonstrated that it could be used to isolate citrullinated proteins (Tutturen et al. 2010). While this technology represents a vast improvement over existing technologies, it suffers from a number of limitations, including the fact that it cannot be used to visualize proteins. Additionally, the phenyllglyoxal was derivatized through a *para* hydroxy group, which in our hands is a rather low yielding reaction, likely due to the electronics of the system negatively impacting the nucleophilicity of the *para* hydroxyl.

Nevertheless, this work inspired our efforts to develop a chemical probe that could detect protein citrullination. Taking advantage of the higher reactivity of the meta position, we synthesized a meta azide substituted phenylglyoxal that was subsequently reacted with alkyne-tagged rhodamine to afford rhodamine-phenylglyoxal (Rh-PG) (Bicker et al. 2012). To validate its utility, we demonstrated that Rh-PG can be used to detect femtomole amounts of citrullinated histone H3 and autodeiminated PAD4 (Fig. 21.6). Key advantages of Rh-PG include throughput

(labeling and analysis is completed in ~3 h) and ease of quantification (fluorescence intensity is readily quantified on a flatbed fluorescence scanner). The versatility of Rh-PG is further illustrated by our demonstration that it can be used to determine the steady-state kinetic parameters for a variety of protein substrates and detect the presence of disease-associated biomarkers. In the latter example, serum samples from a mouse model of ulcerative colitis that had either been treated with the PAD inhibitor Cl-amidine or vehicle control were labeled with Rh-PG, and we observed statistically significant reductions in total protein citrullination in the mice treated with Cl-amidine. Moreover, this analysis identified five distinct proteins that showed a marked difference in citrullination levels, as a function of disease severity. Importantly, for several of these proteins, higher citrullination levels correlated with a number of metrics of disease severity, including colon length and inflammation score. Although the full scope and significance of Rh-PG is currently unknown, we predict that it will be a powerful chemical probe that will enable the detection of citrullinated protein biomarkers for other diseases where PADs play a role.

21.14 PAD Inhibitor Development

Given that the links between dysregulated PAD activity and RA drove our initial interest in developing inhibitors for these enzymes, we briefly describe those links. The first link was the determination, by Schellekens and colleagues, that RA serum autoantibodies recognize citrullinated proteins, and that the presence of these autoantibodies is the most specific diagnostic marker of RA (Girbal-Neuhauser et al. 1999; Schellekens et al. 1998, 2000). The second link was the demonstration that citrullinated proteins are present in the RA synovium and that there is a direct correlation between the levels of citrullinated proteins and disease severity in the formative stages of RA (Lundberg et al. 2005). In 2003, as we began to work in this area, Suzuki et al. published a seminal paper that identified mutations in *PADI4* (the PAD genes are referred to as *PADI*s) as being associated with a small, yet statistically significant, increased risk of developing RA in the Japanese population (Suzuki et al. 2003). Although this association has not been observed in all European populations, it has been replicated in several Asian communities (Barton et al. 2004; Jones et al. 2009; Lee et al. 2007). Since PAD4 is expressed primarily in cells of hemopoetic origin, this paper strongly suggested that this isozyme was principally responsible for citrullinating proteins in the joints of RA patients and the subsequent generation of anti-citrullinated protein autoantibodies (ACPAs). Although these studies prompted our initial focus on PAD4, subsequent studies now suggest that PAD2, which is expressed in macrophages and mast cells, also plays a role because increased levels of both PAD2 and PAD4 are present in the synovial joints of RA patients (Foulquier et al. 2007). Given this evidence, we set out to develop inhibitors targeting the PADs.

21.15 Development of Cl-Amidine

PADs are members of the amidinotransferase superfamily of enzymes which catalyze hydrolytic and group transfer reactions on substrates that are structurally related to arginine (Linsky and Fast 2010; Shirai et al. 2001; Tocilj et al. 2005). Although the PADs modify a number of protein substrates (see above), they also modify a number of small molecule peptidyl-arginine mimetics, including benzoyl L-arginine (BA), benzoyl L-arginine ethylester (BAEE), and benzoyl L-arginine amide (BAA), which have historically been used as mechanistic probes of PAD catalysis. Among these compounds, BAA is the best small molecule substrate for PAD4 and is deiminated with comparable kinetics to known protein substrates. For example, the k_{cat}/K_m for BAA is actually higher than the value obtained for histone H4 ($k_{cat}/K_{m(BAA)} = 11{,}000$ vs. $k_{cat}/K_{m(\text{histone H4})} = 10{,}000$) (Kearney et al. 2005; Slack et al. 2011c). Given the comparable kinetics, these data suggested that BAA could be used as a scaffold on which to develop a PAD4 inhibitor.

Additional considerations driving our thoughts about inhibitor development came from our mechanistic studies of PAD catalysis. As described above, the PADs hydrolyze arginine residues to form citrulline via a covalent intermediate that arises from nucleophilic attack by the Cys645 thiolate (PAD4 numbering) on the substrate guanidinium (Kearney et al. 2005; Knuckley et al. 2007). Given the similarities in the catalytic mechanisms employed by the PADs and cysteine proteases, and the great success in developing irreversible inhibitors for these enzymes (Powers et al. 2002), we were inspired to develop irreversible inhibitors targeting the PADs.

Although we initially considered replacing the guanidinium group in BAA with a fluoromethylketone-like warhead, we quickly abandoned that idea because such a compound would lack the electrostatic and hydrogen bonding interactions between the guanidinium and the two active site aspartates, Asp350 and Asp473, which are critical for catalysis (Knuckley et al. 2007). Additionally, we and others showed that methylated arginines are extremely poor substrates for the PADs (Cuthbert et al. 2004; Hidaka et al. 2005; Kearney et al. 2005), despite an earlier report (Wang et al. 2004), and we quickly recognized that the ideal electrophile, or warhead, must be similar in size to the substrate guanidinium, which the fluoromethylketone is not. Instead, we hypothesized that a haloacetamidine (see Fig. 21.7) would be the ideal replacement because it is relatively isosteric, it is positively charged at neutral pH,

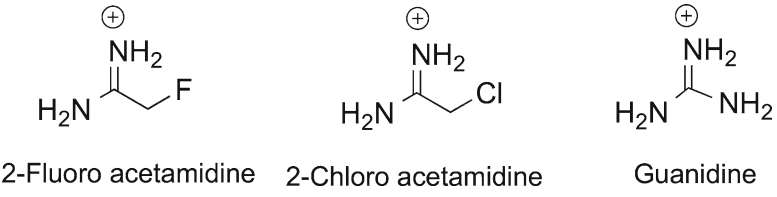
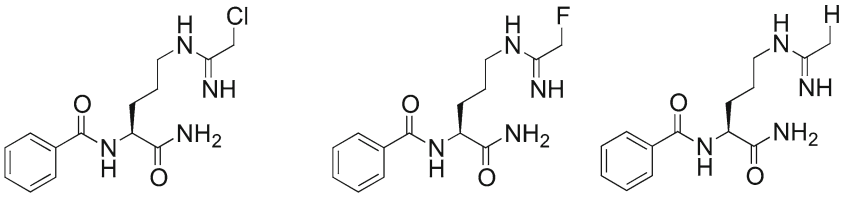


Fig. 21.7 Structures of PAD-targeted warheads versus the guanidinium group



Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	K_I= 3.2 ± 0.9 mM (PAD4)
PAD1	37000	0.4	PAD1	2800	1.1	
PAD2	1200	11	PAD2	380	8.0	
PAD3	2000	7.0	PAD3	170	18	
PAD4	13000	1.0	PAD4	3000	1.0	

Fig. 21.8 Structures of the first highly potent PAD inhibitors: Cl-amidine, F-amidine, and H-amidine

and it maintains most of the hydrogen bonding network (3 out of 4 hydrogen bonds) between the guanidinium and Asp350 and Asp473. Based on these considerations, and using BAA as our initial scaffold, we developed F-amidine as the first highly potent and bioavailable PAD inhibitor ($k_{inact}/K_I = 3{,}000\ M^{-1}\ min^{-1}$; Fig. 21.8) (Luo et al. 2006c). Around the same time, Fast and colleagues demonstrated that 2-chloroacetamidine, the chloro warhead alone, could inactivate PAD4 ($k_{inact}/K_I = 35\ M^{-1}\ min^{-1}$) (Stone et al. 2005b).

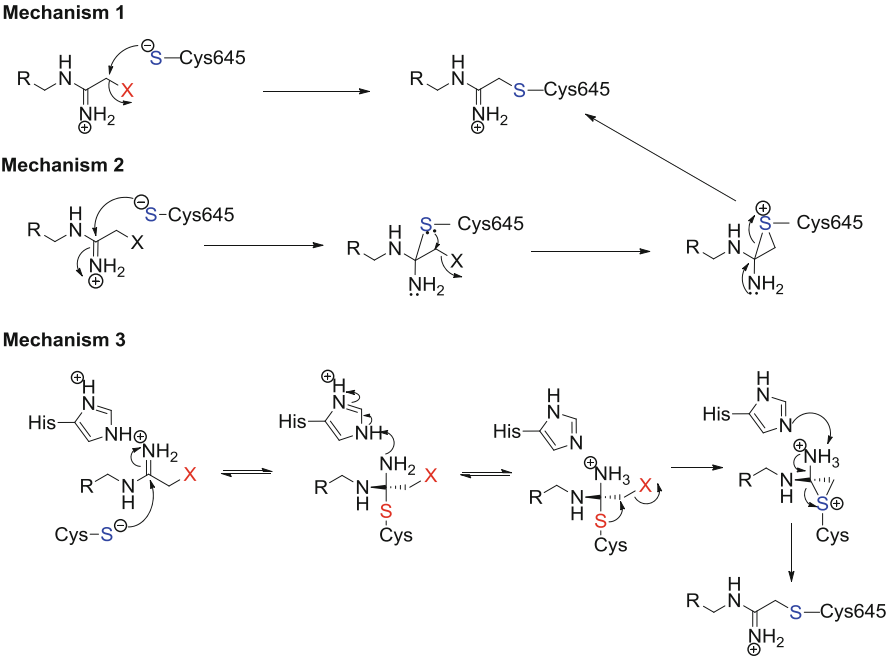
Initial elaboration of F-amidine focused on examining the importance of the halide, as well as side chain length to inhibitor potency. The results of these studies showed that replacement of the fluoro group with a hydrogen atom led to a substantial loss in potency, and the compound, H-amidine (Fig. 21.8), is a reversible competitive inhibitor of PAD4 ($K_i = 3.2 \pm 0.9$ mM). In contrast, replacement with a chloro group increased potency by three- to fourfold ($k_{inact}/K_I = 13{,}000\ M^{-1}\ min^{-1}$). The enhanced potency of Cl-amidine is likely due to the higher leaving group potential of the chloro group (Luo et al. 2006a). As expected, increasing or decreasing the length of the side chain by one methylene unit, led to a substantial loss in potency, indicating that proper positioning of the warhead with respect to the active site cysteine is critical for effective enzyme inactivation (Luo et al. 2006a).

The development of F-amidine and Cl-amidine was important for the field, and these compounds, particularly Cl-amidine, have been widely used to study the normal and pathophysiological roles of these enzymes (Luo et al. 2006a, c). For example, Cl-amidine was used to establish: (1) that PAD4 acts as a transcriptional corepressor for p53 (Li et al. 2008; Yao et al. 2008); (2) that PAD4 is a transcriptional coactivator for ELK1 (Zhang et al. 2011); (3) that PAD4 activity is required for Neutrophil Extracellular Trap formation (Li et al. 2010; Wang et al. 2009); and (4) that PAD2 is a transcriptional coactivator of the estrogen receptor (McElwee et al. 2012).

21.16 Mechanism of Inactivation

Irreversible inhibitors typically fall into one of three classes: (1) affinity labels, which simply react with the free enzyme in a bimolecular reaction; (2) quiescent affinity labels, which first bind the enzyme to form an E·I complex that can either dissociate to regenerate the free enzyme or react with a nucleophile to effect inactivation; and (3) mechanism-based inactivators, which bind the enzyme reversibly, and then undergo a partial reaction to generate a reactive species that then covalently modifies the enzyme. To establish that F- and Cl-amidine are irreversible PAD4 inhibitors, the enzyme was first incubated separately with an inhibitor to generate the PAD4·Inhibitor complex, and then exhaustively dialyzed to remove any unbound inhibitor and the residual activity measured. The fact that no activity was recovered after this treatment established that both compounds are irreversible inhibitors (Luo et al. 2006a, c). To begin to differentiate between the aforementioned types of irreversible inhibitors, the kinetics of inactivation were then examined by monitoring product formation as a function of time in the absence and presence of increasing concentrations of an inhibitor. For both F- and Cl-amidine, but not H-amidine, the progress curves were curved in the presence of inhibitor, indicating a time-dependent decrease in enzyme activity, consistent with an irreversible mode of inhibition. From each of these curves, one can extract a pseudo-first order rate constant of inactivation, which is then plotted against inhibitor concentration. If the resultant plot is linear, it indicates that the inhibitor is an affinity label, whereas if the plot is hyberbolic, it indicates that the compound forms an initial encounter complex, that is, an E·I complex, and is either a quiescent affinity label or mechanism-based inactivator. With both F- and Cl-amidine, the plots were hyperbolic indicating that these compounds form an E·I complex before reacting with the enzyme (Luo et al. 2006a, c). Importantly, this analysis also provides information on k_{inact}, the maximum rate of inactivation, and K_I, the concentration of inhibitor that yields the half maximal rate of inactivation. Note that K_I is generally not a dissociation constant and therefore should not be used as a measure of affinity for an irreversible inhibitor. The best measure of potency and selectivity is the second order rate constant k_{inact}/K_I.

Based on this data and our knowledge of the PAD4 active site and catalytic mechanism, we initially proposed two possible inactivation mechanisms (Scheme 21.1). The first involves the direct displacement of the halide through an S_N2 type mechanism (Mechanism 1). The second mechanism begins with the attack of the nucleophilic thiolate on the amidinium carbon to form a tetrahedral intermediate. The sulfur then undergoes an intramolecular nucleophilic halide displacement reaction that generates a three membered sulfonium ring, which ultimately collapses to form a stable thioether linkage; see Mechanism 2 in Scheme 21.1. Support for either mechanism comes from cocrystal structures of PAD4 bound to F- and Cl-amidine (Fig. 21.9), which show clear electron density for a bond between the Cys645 sulfur atom and the ω-carbon of F- and Cl-amidine.



Scheme 21.1 Possible mechanisms of PAD inactivation by haloacetamidine-based inhibitors

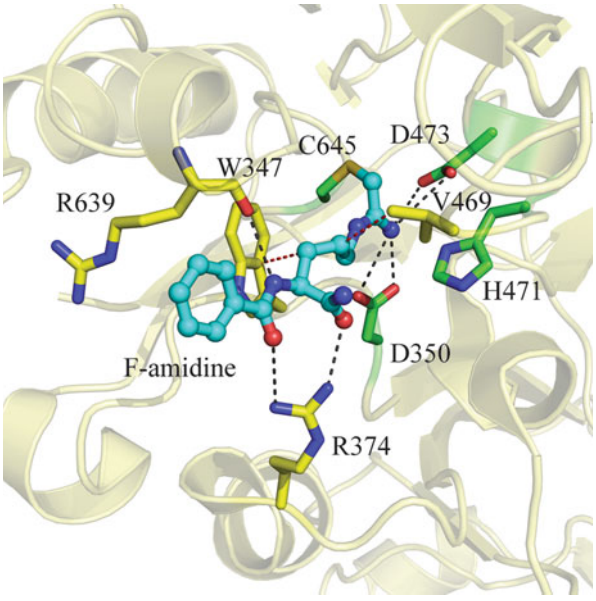


Fig. 21.9 Structure of the PAD4 active site covalently complexed with F-amidine (cyan ball and stick). Active site residues are *green*, and residues that only participate in substrate binding are in *yellow*. Electrostatic interactions are shown as *gray dashed lines*, and hydrophobic interactions are shown as *red dashed lines*. Figure created from PDB: 2DW5 using Pymol

While Mechanism 1 may be the most intuitive, Mechanism 2 is analogous to the mechanism by which the fluoromethylketones inactivate the cysteine proteases (Drenth et al. 1976; Kreutter et al. 1994; Powers et al. 2002), and accounts for the rather poor leaving group potential of the fluoride in F-amidine. To provide support for the latter mechanism, we examined the effect of pH, in both H_2O and D_2O, on the rates of F-amidine, Cl-amidine, and 2-fluoroacetamidine inactivation (Knuckley et al. 2010b). The results of these studies revealed that the pH rate profiles are bell shaped for both k_{inact} and k_{inact}/K_I, with 2 pK_a values that are similar to those obtained for substrate catalysis; as such, they likely correspond to Cys645 and H471. The bell-shaped k_{inact}/K_I pH rate profiles also indicate that these residues contribute to "inactivator capture," that is, all steps up to and including the first irreversible step of the reaction, and that one residue, likely Cys645, must be deprotonated and one residue, likely His471, must be protonated for efficient enzyme inactivation. Given these results, we modified our inactivation mechanism (Mechanism 3) to account for the requirement for general acid catalysis. In this mechanism, proton transfer to the developing tetrahedral intermediate stabilizes the intrinsically unstable thiohemiaminal intermediate, thereby promoting the intramolecular halide displacement reaction.

Although F-amidine, Cl-amidine, and 2-fluoroacetamidine all display the same bell-shaped pH rate profiles, 2-chloroacetamidine does not. For this compound, the rates of inactivation increase to a limiting value as the pH is raised and the observed pK_a is consistent with the one assigned to Cys645. In other words, there is no requirement for general acid catalysis, and the rate of inactivation increases as the concentration of the thiolate increases. Based on these differences, inactivation by 2-chloroacetamidine likely proceeds via an S_N2 mechanism (Mechanism 1), whereas F-amidine, Cl-amidine, and 2-fluoroacetamidine likely inactivate PAD4 via Mechanism 3. So why don't F-amidine, Cl-amidine, and 2-fluoroacetamidine proceed through Mechanism 1? For Cl-amidine, the most likely explanation is that the steric constraints imposed by the extended amino acid structure, enforce a conformation of the warhead that favors Mechanism 3. In contrast, for 2-fluoroacetamidine, which lacks these steric constraints, the poor leaving group potential of the fluoride explains the requirement for the intramolecular halide displacement reaction. For F-amidine, a compilation of the two factors is likely operative.

Consistent with the above conclusions are the following observations. First, the rates at which F- and Cl-amidine inactivate PAD4 are similar, despite a 10^3 to 10^4-fold difference in leaving group potential, which is inconsistent with an S_N2 displacement. Second, Cl2-amidine, whose side chain is one methylene unit shorter, is an irreversible inhibitor, whereas F2-amidine, its fluoro-containing analog, is a reversible inhibitor, suggesting that the warhead in F2-amidine cannot be properly positioned for nucleophilic attack on the amidinium carbon due to the shorter side chain, whereas the warhead in Cl2-amidine likely undergoes an S_N2 reaction due to its positioning and higher reactivity (Luo et al. 2006a).

21.17 Cl-Amidine Reduces Disease Severity in Mouse Models of RA

Given the above-described links between dysregulated PAD activity and RA, we set out to examine whether our inhibitors could reduce disease severity in the murine collagen-induced arthritis (CIA) model of RA. Initial unpublished studies examined the effect of both F- and Cl-amidine in this model. Briefly, male DBA/1j mice were injected at day 0 in the tail (i.d.) with a mixture of type II collagen (CII) and Complete Freund's Adjuvant (CFA), and then boosted at day 15 with CII and Incomplete Freund's Adjuvant. The mice were treated daily (i.p.) with 1, 10, and 100 mg/kg/day of F-amidine, Cl-amidine, or a vehicle control for 56 days starting at day 0. The results of these studies demonstrated that at the highest dose of Cl-amidine, disease incidence decreased from 100 to 20 %. Lower doses were less effective in reducing incidence, but did delay arthritis onset. Of particular note is the fact that the severity score of the Cl-amidine-treated mice that did develop arthritis was very low. Although F-amidine did not decrease disease incidence, the highest doses were able to delay disease onset and modestly decrease disease severity, although the differences were not statistically significant. While the effects of F-amidine are less marked than those obtained with Cl-amidine, they are consistent with their relative potencies; Cl-amidine is ~fourfold more potent that than F-amidine towards PAD4 in vitro. Differences in F-amidine versus Cl-amidine may also reflect the relatively greater ability of Cl-amidine to penetrate cells or the relevant tissue sites.

Since we only obtained clinical disease measures from these studies, and not serum or histological markers of disease, these studies were repeated in collaboration with Michael Holers. For these latter studies, DBA/1j mice were injected at day 0 with a mixture of CII and CFA, and then boosted at day 21 with CII and CFA. The mice were treated daily (i.p.), starting on day 0, with 1, 10, and 50 mg/kg/day concentrations of Cl-amidine or a vehicle control for the next 35 days. F-amidine was not tested here because it lacked efficacy in the studies described above. The results confirmed that Cl-amidine reduces disease severity, by up to 55 %. Although less efficacious in this model, the most likely reason for these differences is the use of CFA in the booster shot, which causes a more severe form of arthritis with a quicker rate of onset (Willis et al. 2011). Nevertheless, mice treated with Cl-amidine showed reduced bone damage, inflammation, and pannus formation (up to a ~30 % reduction), as well as decreased Complement deposition, epitope spreading, and production of autoantibodies to mouse CII. A statistically significant reduction in protein citrullination within the arthritic limbs of mice treated with Cl-amidine was also observed, consistent with the efficacy of Cl-amidine being due, at least in part, to the inhibition of PAD activity (Willis et al. 2011). This result was quite gratifying because it represented the *first* demonstration that a PAD inhibitor could decrease disease severity in an animal model of disease.

To determine whether Cl-amidine shows efficacy in the effector phase of the disease, we also evaluated the collagen antibody-induced arthritis (CAIA) model (Willis et al. 2011). In this model, the initial antigen recognition phase of the immune response is bypassed by administering Arthrogen, a cocktail of monoclonal antibodies that target CII. LPS is then given 3 days later, which results in rapid disease onset. For these studies, DBA/1 J mice were dosed with the same concentrations of Cl-amidine, or vehicle control, 7 days before the Arthrogen injection, and this treatment continued for 10 days after disease induction. In contrast to the results from the CIA models, Cl-amidine treatment had no effect on disease activity or joint damage in this model, suggesting that PAD inhibition does not alter the effector phase of the disease.

In total, given that Cl-amidine reduces epitope spreading from bovine to mouse CII, and limits the generation of antibodies targeting a number of citrullinated antigens, these data suggest that Cl-amidine derives its efficacy from its ability to inhibit the generation of ACPAs by decreasing protein citrullination. Although a reasonable hypothesis, recent data linking PAD activity to the regulation of gene transcription, apoptosis, and NET formation, suggest that the efficacy of Cl-amidine may be due to a combination of factors, including effects on antigen generation, inhibition of NET formation (Li et al. 2010; Wang et al. 2009), and altered gene transcription (Cuthbert et al. 2004; Li et al. 2008; Slack et al. 2011b; Yao et al. 2008; Zhang et al. 2011), which can alter the activation of T and B cells.

21.18 Cl-Amidine Reduces Disease Severity in a Mouse Model of Colitis

As our RA efficacy studies were ongoing, we also initiated a collaboration with Lorne Hofseth at the University of South Carolina to study the roles of the PADs in inflammatory bowel disease (IBD), and in particular ulcerative colitis (UC). This collaboration was premised on the finding that a PAD4 haplotype is associated with an increased risk of developing UC in the Japanese population (OR = 1.722) (Chen et al. 2008). We subsequently confirmed that PADs 2 and 4 are overexpressed in human UC and the murine dextran sodium sulfate (DSS) model of UC (Chumanevich et al. 2011). Given these results, we examined whether Cl-amidine could decrease disease severity in this model. For these studies, mice were treated either prophylactically or after the onset of disease. In both cases, we observed a significant reduction in disease severity, as measured by effects on body weight, colon length, and histological scores. For the mice treated after disease onset (treatment began after 1 week on DSS), the results are most impressive because Cl-amidine was provided orally, suggesting that Cl-amidine successfully transits the stomach and reaches the target organ, that is, the colon, where it inhibits PAD activity. Interestingly, the systemic oral bioavailability of Cl-amidine is quite poor (Bicker et al. 2012), suggesting that the observed efficacy of Cl-amidine is principally due to the inhibition of PAD enzymes present in the mouse colon (Chumanevich et al. 2011).

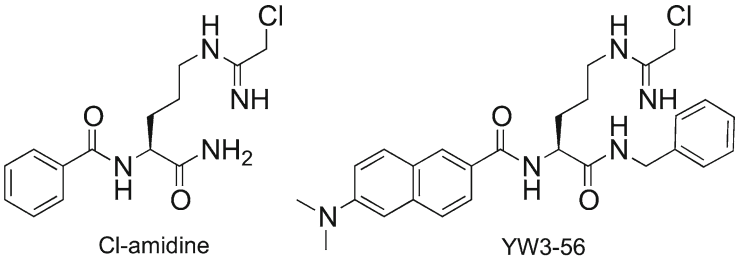
Since ACPA are not observed in UC, the mechanism by which Cl-amidine decreases disease severity is less clear in this model. Nevertheless, Cl-amidine does induce apoptosis of TK6 cells, a lymphoblastoid cell line, in vitro, and we observed increased apoptosis of inflammatory cells in the colons of Cl-amidine-treated mice. In contrast, there is no increase in apoptosis of normal colon epithelium cells, suggesting that the observed efficacy is due in part to the preferential apoptosis of activated immune cells (Chumanevich et al. 2011). Although a reasonable explanation, other factors are also likely at play and may contribute to the observed efficacy. For example, NET formation is associated with UC (Savchenko et al. 2011) and its inhibition could explain the decrease in disease severity.

21.19 Cl-Amidine Reduces Disease Severity in Tumor Xenografts

While a role for the PADs in inflammation is clear, less is known about how these enzymes contribute to tumorgenesis. Nevertheless, links between dysregulated PAD activity and cancer have emerged in the last several years (Jones et al. 2009). These links include the observations that PAD4 is overexpressed in multiple carcinomas (Chang et al. 2009), PAD4 is overexpressed in the blood of patients with malignant tumors (Chang et al. 2009), serum levels of citrullinated antithrombin are elevated, which may alter thrombin activity (Chang et al. 2005), and PAD4 is a transcriptional corepressor for p53 and inhibition of PAD4 activity with Cl-amidine induces the expression of a number of p53 target genes (Li et al. 2008; Yao et al. 2008). These genes include *p21*, a cell cycle inhibitor, as well as *GADD45* and *PUMA*, which binds to anti-apoptotic Bcl2 family members and inhibits their activity, thereby promoting apoptosis (Li et al. 2008; Yao et al. 2008).

Although these data support a role for PAD4 in tumorgenesis, recent studies, particularly those from the Coonrod lab, suggest that PAD2 is also involved in cancer. For example, *PADI2* is highly expressed in multiple breast cancer cell lines (Blick et al. 2010; Mackay et al. 2009), and *PADI2* is one of 29 genes defining a HER2+ gene expression signature in invasive primary breast tumors (Bertucci et al. 2004). RNA seq analysis also showed that *PADI2* expression is the 12th most correlated gene with HER2 across more than 55 breast cancer cell lines, suggesting that PAD2 plays an important role in HER2 biology (McElwee et al. 2012). Given these findings, the Coonrod lab examined PAD2 expression in the MCF10AT tumor progression series, which are used as a model of cells progressing from normal to metastatic breast cancer. The cell lines in this series include MCF10A (normal), MCF10AT (hyperplastic), MCF10DCIS.com (ductal carcinoma in situ (DCIS) with necrosis), and MCF10CA1 (invasive/metastatic breast cancer) (Dawson et al. 1996; Heppner and Wolman 1999; Miller et al. 2000).

The results showed that PAD2 expression increases with aggressiveness and that high levels of PAD2 correlate with increased expression of HER2 (McElwee et al. 2012). Given that PAD2 expression was highest in the MCF10DCIS.com cells,



Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity
PAD1	37000	0.4
PAD2	1200	11
PAD3	2000	7.0
PAD4	13000	1.0

Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	IC_{50} (μM)	Selectivity
PAD1	ND	ND	ND
PAD2	ND	~2.5	~1
PAD3	ND	ND	ND
PAD4	ND	1-5	~1

Fig. 21.10 Structure of Cl-amidine versus YW3-56, a Cl-amidine analog with improved cellular efficacy

we xenografted this cell line onto nude mice and examined the effect of Cl-amidine on tumor growth. For these studies, the tumors were allowed to grow for ~2 weeks to ~200 mm^3, at which point treatment with Cl-amidine (50 mg/kg/day) or vehicle (PBS) control was initiated. Cl-amidine was delivered by i.p. injection for the next 14 days. These studies revealed that Cl-amidine decreases tumor volume by three-fold. Histological analysis of the tumors showed an advanced, potentially invasive, tumor phenotype for the vehicle controle group. By contrast, tumors in the Cl-amidine-treated mice had a relatively benign appearance, with an intact basement membrane and decreased leukocyte invasion. In total, these findings suggest that PAD inhibitors will possess utility as anticancer therapeutics. Consistent with the above statement is the fact that PAD inhibition, by either F- or Cl-amidine decreases the growth of multiple cell lines (e.g., HL60, HT29, TK6, U2OS, MCF7, MCF10AT, MCF10DCIS.com, and MCF10CA1) in both standard culturing conditions and in tumor spheroid assays (Causey et al. 2011; Chumanevich et al. 2011; McElwee et al. 2012; Slack et al. 2011b; Wang et al. 2012).

The Wang group also recently described the development of a Cl-amidine analog, YW3-56 (Fig. 21.10), which shows efficacy in a mouse sarcoma model (Wang et al. 2012). YW3-56 differs from Cl-amidine at the N- and C-termini, which are replaced with 6-(dimethylamino)-2-naphthoic acid and benzylamine. Although YW3–56 is roughly equipotent to Cl-amidine in vitro, its cellular efficacy is dramatically improved (the EC_{50} is ~5 μM vs. 100–200 μM for Cl-amidine), likely due to the increased hydrophobicity of the compound. Similar to Cl-amidine, YW3–56 decreases the growth of S180 mouse sarcoma xenografts by 50 % at 10 mg/kg and to 25 % at 5 mg/kg when used in combination with the histone deacetylase inhibitor SAHA at 2.5 mg/kg (Wang et al. 2012).

Although the mechanisms through which PAD inhibitors decrease cell growth are not fully established, effects on multiple signaling pathways are likely because

PAD inhibitors are known to exert effects on cell growth, differentiation, apoptosis, and autophagy. For example, we showed that F- and Cl-amidine promote the differentiation of HL60 cells into granulocytes (Slack et al. 2011b). Additionally, Cl-amidine treatment of MCF10DCIS.com cells leads to an S phase arrest coupled with increased apoptosis (McElwee et al. 2012). Increased apoptosis upon Cl-amidine treatment is also observed with TK6 cells, a lymphoblast cell line, and in vivo in the DSS model of colitis (Causey et al. 2011; Chumanevich et al. 2011). YW3-56 increases sestrin expression via effects on p53-dependent transcription, which ultimately leads to the inhibition of mTORC1 and the induction of autophagy (Wang et al. 2012). Although many of the observed effects of PAD inhibitors are likely attributable to increased p53 signaling (by inhibiting the corepressor activity of PAD4), other mechanisms are likely operative because HL60 cells are p53 negative but respond to PAD inhibitors by inducing the expression of the cell cycle inhibitor p21 (Slack et al. 2011b). Effects on HER2 and ER signaling may also explain the efficacy of Cl-amidine in the MCF10DCIS xenografts because this isozyme is highly expressed in this cell line, and PAD2 is known to activate the expression of both HER2 and ER regulated genes (McElwee et al. 2012).

21.20 PAD Inhibitor Discovery

Although Cl-amidine has been widely adopted, and successfully used in both cellular and animal studies, it suffers from a number of limitations, including a lack of selectivity among the PADs, relatively low potency, the potential for polypharmacology, a short in vivo half life, and limited bioavailability. Given these considerations, we and others have adopted a number of strategies to develop/identify PAD inhibitors that overcome these limitations, including target-based approaches, high-throughput screening (HTS), and traditional medicinal chemistry on the Cl-amidine scaffold. Below we highlight each of these areas.

21.21 Historical Efforts

Prior to our entry into the PAD field, Moscarello and colleagues identified the microtubule stabilizing drug Taxol as a weak inhibitor of bovine brain PAD (Fig. 21.11; IC_{50} = ~5 mM) (Pritzker and Moscarello 1998). Based on the fact that the Taxane ring alone does not inhibit PAD activity, the authors suggested that the "side arm," *N*-α-benzoyl-(2R,3S)-3-phenylisoserine, was principally responsible for the observed inhibition. This is likely the case because benzoyl-(2R,3S)-3-phenylisoserine ethyl ester is a weak reversible PAD4 inhibitor (IC_{50} = ~3 mM; unpublished data). After demonstrating that Taxol inhibits PAD activity, Moscarello examined its efficacy in a spontaneously demyelinating model of multiple sclerosis (Moscarello et al. 2002). Although remyelination was observed, it is difficult to

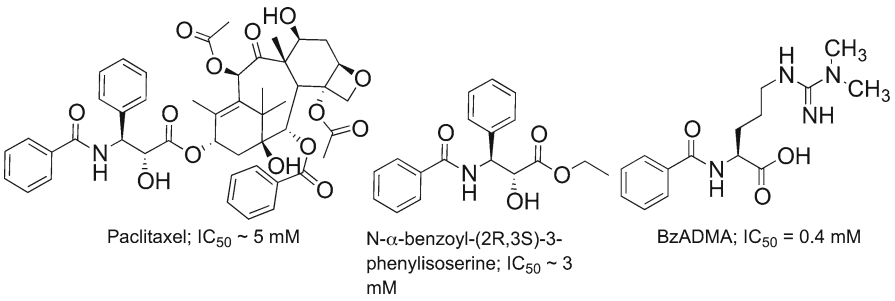


Fig. 21.11 Structures of selected reversible PAD inhibitors

separate the effects of inhibiting PAD activity from the microtubule stabilizing effects of Taxol on cell cycle progression. Michiyuki Yamada, who originally cloned PAD4 and who deserves much credit for binging the PAD field to the forefront, also identified benzoylated asymmetrically dimethylated arginine (BzADMA) as a second PAD4 inhibitor; however, the in vitro efficacy of this compound is also quite weak (IC_{50} = ~0.4 mM) (Hidaka et al. 2005).

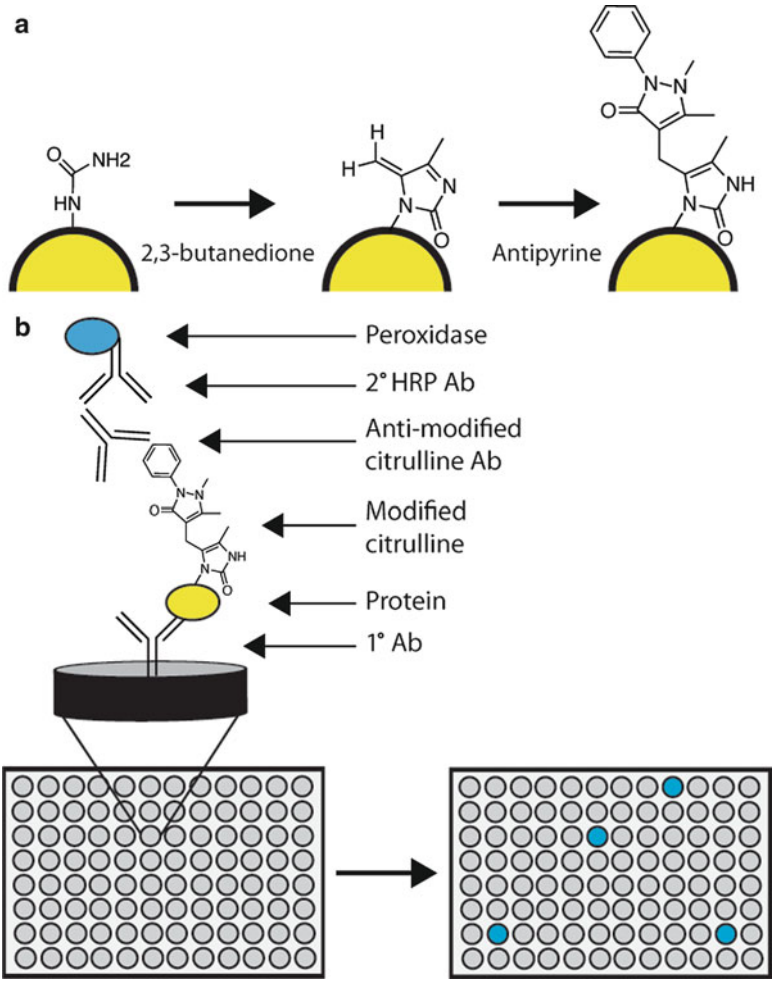
21.22 Novel Assays to Identify PAD Inhibitors

One of the key challenges for PAD inhibitor discovery is that the principle assays used to detect and quantify protein citrullination are generally not amenable to HTS. For example, the COLDER assay requires strong acids (i.e., H_2SO_4 and H_3PO_4) and toxic reagents (i.e., diacetylmonooxime) that are used to derivatize the ureido group of citrulline and thereby generate a chromophore that absorbs light at 540 nm (Kearney et al. 2005; Knipp and Vasak 2000). Although glutamate dehydrogenase can be used to monitor PAD activity by coupling ammonia production to the reductive amination of α-ketoglutarate in the presence of NADH (oxidation of NADH leads to an increase in the absorbance at 340 nm) (Liao et al. 2005), the required coupling enzyme limits its utility in HTS assays because of the need to deconvolute hits targeting the coupling enzyme versus the PAD isozyme under study.

21.23 Antibody-Based Assays

Given these issues, we and others developed several new medium and high-throughput PAD activity assays. For example, Proost and colleagues developed an antibody-based assay that detects citrulline residues chemically modified with 2,3-butanedione and antipyrine (Moelants et al. 2011). The antibody was raised against a peptide (YAGCit*LLTK-NH_2) in which the citrulline residue is chemically modified by these two reagents under acidic conditions. The assay, which has been formatted to 96-well microtiter plates, can be used in either a Sandwich ELISA or

ELISA-based mode (Moelants et al. 2011). For the former (Scheme 21.2) citrullinated proteins are derivatized with 2,3-butanedione and antipyrine, dialyzed to remove the reactants, and then incubated in 96-well plates that are coated with an antibody that targets the protein of interest. Subsequently, the anti-modified citrulline antibody is added and its presence quantified with a peroxidase coupled secondary antibody. In the ELISA-based format, which can be used to directly measure PAD activity, peptide-coated microtiter plates are first incubated with a PAD, and then citrulline residues generated by the enzyme are derivatized in place, the plates washed, and citrullinated peptides detected with the anti-modified citrulline antibody and a peroxidase coupled secondary antibody. Using this assay, one can readily detect changes in PAD activity as a function of added inhibitor, as demonstrated in a recent publication (Bozdag et al. 2013; Moelants et al. 2011).



Scheme 21.2 (**a**) Modification of citrulline residues in proteins with butanedione and antipyrine. (**b**) ELISA with modified proteins using an anti-modified citrulline antibody

21.24 Gel-Based Competitive ABPP Assay

A second assay format that overcomes the technically challenging aspects of traditional PAD assays is the competitive ABPP-based assay described by Knuckley et al. (Knuckley et al. 2008). In this assay, a PAD is incubated with and without an inhibitor in the presence of rhodamine-conjugated F-amidine (RFA), a PAD-targeted Activity Based Protein Profiling (ABPP) reagent developed by the Thompson lab (Luo et al. 2006b) (Fig. 21.12a). RFA is classified as an ABPP because it only modifies the active forms of the PADs. Here, RFA competes with an inhibitor for binding the enzyme active site (Fig. 21.12b). If the inhibitor binds, RFA is displaced and the PAD is not labeled by RFA. In contrast for weak inhibitors, RFA binds to the enzyme and covalently modifies the active site cysteine. In this assay, protein labeling is visualized by SDS-PAGE and changes in fluorescence are directly monitored *in gel* using a fluorescence scanner. Although the assays themselves are readily automated, the only bottleneck is SDS-PAGE. Nevertheless, 20–25 samples/gel are easily processed, thereby enabling medium throughput. Note that an additional benefit of this assay comes from the fact that it is possible to quantify changes in fluorescence intensity, and thereby provide a semiquantitative readout of inhibitor potency (Knuckley et al. 2008). Additionally, the assay is readily "tuned" to identify both weak and strong inhibitors by altering the concentration of RFA.

As an initial test of this assay format, Knuckley et al. used it to determine whether a number of Disease Modifying Anti-Rheumatic Drugs (DMARDs) derived their efficacy from their ability to inhibit PAD4 (Knuckley et al. 2008). These compounds included 5-aminosalicylic acid, azathioprine, azithromycin, clindamycin, leflunomide, methotrexate, minocyline, sulfamethoxazole, sulfapyridine, and trimethoprim. We focused on the DMARDs because in many cases the molecular mechanisms by which they exert efficacy are incompletely understood. Streptomycin was also tested in this assay because it contains two guanidinium groups and represented a possible alternative substrate. Using this assay, Knuckley et al. rapidly identified minocycline and streptomycin as PAD4 inhibitors; the other compounds showed either no or very weak inhibition at millimolar concentrations of compound

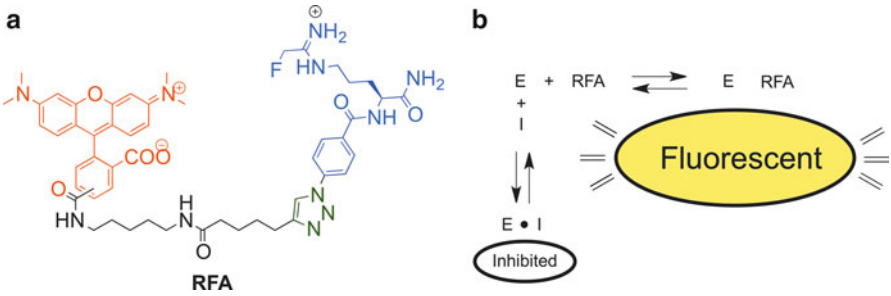
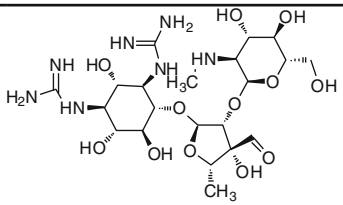
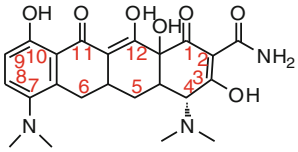
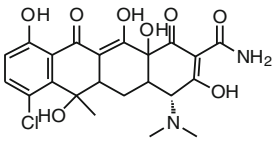


Fig. 21.12 (**a**) Structure of rhodamide-conjugated F-amidine (RFA). (**b**) Mode of action for RFA-based readout assay where RFA competes with an inhibitor for binding the enzyme active site

Table 21.2 Inhibition constants for streptomycin, minocycline, and chlortetracycline

Compound	Structure	Inhibition mechanism	K_{is} (mM)	K_{ii} (mM)
Streptomycin		Competitive	0.56 ± 0.17	NA
Minocycline		Mixed	0.18 ± 0.06	0.63 ± 0.15
Chlortetracycline		Mixed	0.54 ± 0.53	0.11 ± 0.01

NA not applicable

(IC_{50} ≥2.5 mM) (Knuckley et al. 2008). Importantly, similar trends were observed in the IC_{50} and fluorescence intensity data, helping to validate this assay format.

Since minocycline showed the highest potency among the DMARDs, we developed a structure–activity relationship (SAR) by determining IC_{50} values for several tetracyclines, including tetracycline, chlortetracycline, and doxycycline. Of these compounds, chlortetracycline showed the highest potency (IC_{50} = 100 μM); it is ~sixfold more potent than minocycline (IC_{50} = 620 μM). Kinetic studies with the three most potent compounds, that is, streptomycin, minocycline, and chlortetracycline, showed that the these compounds are reversible PAD4 inhibitors (Table 21.2). The fact that streptomycin is a competitive inhibitor indicates that it binds to the active site, whereas minocycline and chlortetracycline are mixed inhibitors, suggesting that they bind distal to the active site, or perhaps an alternative conformation. Since we have been unable to obtain structures of PAD4 bound to these compounds, it is difficult to comment on the molecular basis of the enhanced potency of chlortetracycline versus minocycline and tetracycline. Nevertheless, a key difference between these compounds is the presence of a chloro group at the 7 position versus the dimethylamine in minocycline (see Table 21.2) and it is tempting to speculate that the increased affinity is due to the electron withdrawing effects of the chloro group altering the electronics of the ring system. Since most DMARDs are relatively poor PAD4 inhibitors, their efficacy is unlikely to derive from their ability to inhibit this enzyme. Nevertheless, the identification of the tetracycline scaffold as a potential pharmacophore, suggests that it could be further elaborated to develop inhibitors with enhanced potency and selectivity.

21.25 Fluorescence Polarization ABPP (FluoPol-ABPP)-Based HTS Assay

The third assay is a fluorescence polarization ABPP (FluoPol-ABPP)-based HTS assay (Fig. 21.13). The assay format is similar in design to the competitive ABPP assay described above except that the readout is FluoPol (Knuckley et al. 2010c). The biophysical basis of the screen involves monitoring the time-dependent increase in FluoPol that occurs upon labeling of PAD4 with RFA; FluoPol increases because the RFA–PAD4 complex rotates slowly, relative to free RFA, and therefore emits highly polarized light when excited with plane polarized light. By contrast, the fast rotation of free RFA results in the emission of depolarized light. In the presence of an inhibitor, this effect is mimicked (because the rate of RFA labeling is decreased), and the FluoPol signal is decreased.

The utility of this assay was first demonstrated in a pilot screen of 2,000 compounds from the NIH Validation Set at The Scripps Research Institute in La Jolla, CA (Pubchem AID 463073). From this screen, we identified 10 compounds that demonstrated greater than 30 % inhibition of PAD4. Seven of these compounds were available, including streptonigrin (Fig. 21.13b), an aminoquinone-containing antitumor antibiotic produced by *Streptomyces flocculus*, and tosyl phenylalanyl chloromethyl ketone (TPCK), a known cysteine protease inhibitor. Their ability to inhibit PAD4 was confirmed using our gel-based competitive ABPP assay as a secondary screen. Of the compounds tested, streptonigrin showed the strongest inhibition of PAD4 labeling by RFA, whereas TPCK was one of the weakest (Knuckley et al. 2010c).

Characterization of the mechanism of inhibition revealed that streptonigrin is an irreversible PAD inhibitor. Plots of k_{obs} versus streptonigrin concentration are hyperbolic, consistent with the formation of an initial encounter complex that then reacts to afford the inactivated enzyme. Given that streptonigrin is not a substrate analog, this result suggests that it is a quiescent affinity label. From this analysis, values for k_{inact}, K_I, and k_{inact}/K_I were obtained: 3.7 ± 0.5 min^{-1}, 8.5 ± 4.3 μM, and

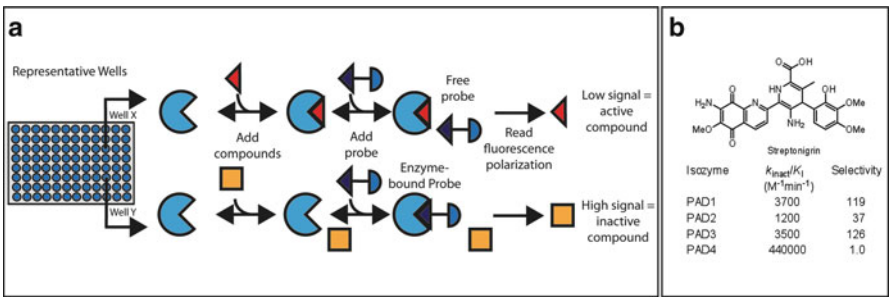


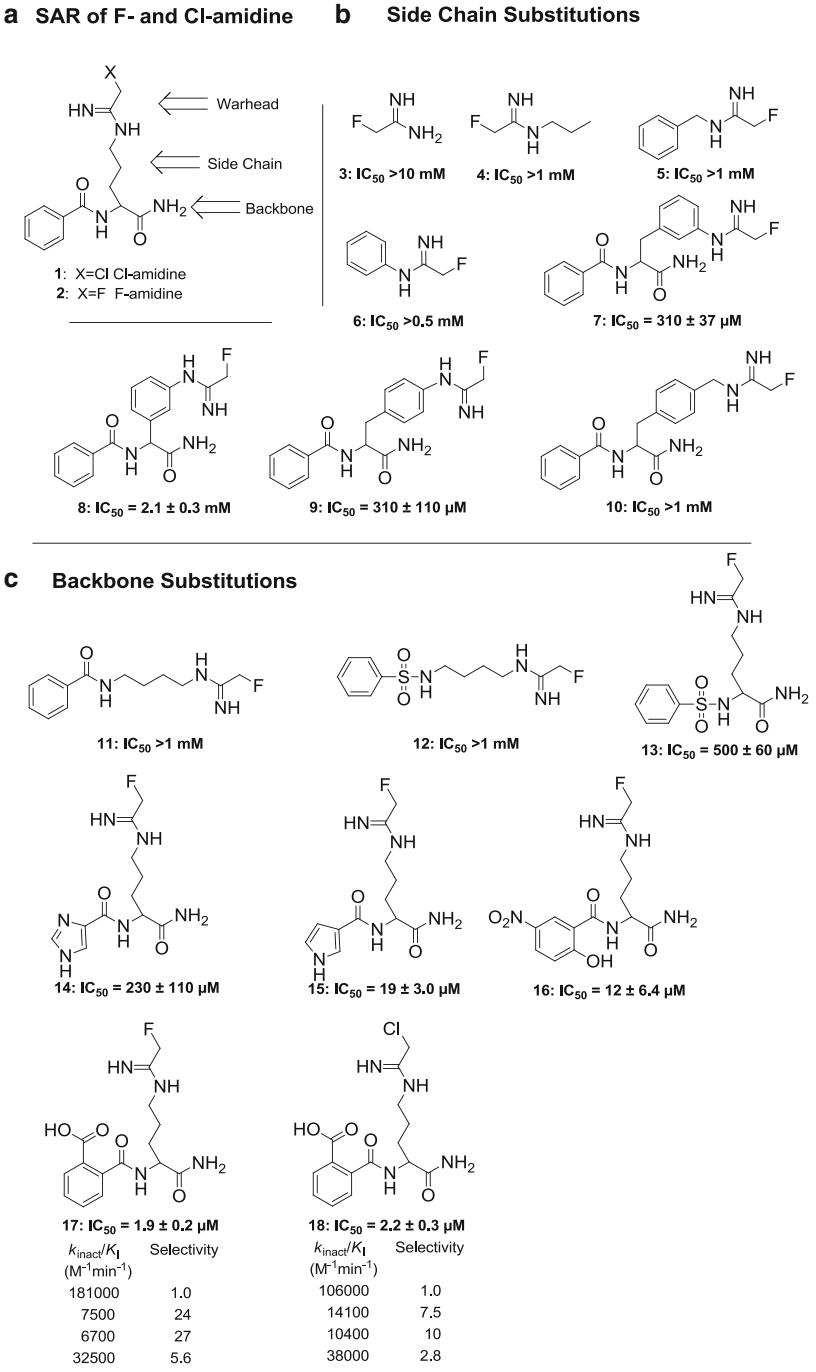
Fig. 21.13 Fluorescence polarization (FluoPol)-ABPP HTS assay. (**a**) Assay design where inhibitors are screened and positive hits result in low signal due to competition with the probe. (**b**) Streptonigrin was found to be a PAD4 selective inhibitor using the FP-ABPP HTS assay

4.4×10^5 M^{-1} min^{-1}, respectively. The k_{inact}/K_I is ~33-fold higher than Cl-amidine, making streptonigrin one of the most potent PAD4 inhibitors described to date. Similar analyses on the other active PAD isozymes revealed that streptonigrin selectively targets PAD4 by more than 37-fold (Fig. 21.13b). Although we do not yet know the residue on PAD4 that is modified by streptonigrin, the fact that substrate protects against inactivation suggests that an active site residue, possibly Cys645, reacts with the aminoquinone ring of streptonigrin to effect inactivation.

Streptonigrin also dose dependently reduces the citrullination of histone H3 in both MCF7 and HL60 cells stimulated with estrogen and LPS, respectively. The EC_{50} values are in the 10–100 nM range versus 100–200 μM for Cl-amidine. The enhanced cellular potency of streptonigrin likely reflects both its higher in vitro potency (33-fold vs. Cl-amidine) and bioavailability; streptonigrin is neutral and highly hydrophobic which is expected to promote cellular uptake. Given the fact that streptonigrin is a highly potent PAD4 inhibitor, and that PAD4 is overexpressed in multiple cancer cell lines and tumors (Chang and Han 2006; Chang et al. 2009), it is tempting to speculate that the antitumor activity of this compound (Beall et al. 1994, 1995; Oleson et al. 1961; Reilly and Sugiura 1961; Teller et al. 1961) is due in part to its ability to inhibit PAD4. While this seems likely, the fact that streptonigrin affects numerous cellular processes (e.g., DNA replication, respiration, and replication) suggests that it is unlikely to represent a useful chemical probe of PAD4 function or even a targeted therapeutic. Nevertheless, these studies highlight the power of this FluoPol-ABPP-based HTS assay for identifying PAD4 inhibitors. Studies to identify inhibitors from larger libraries are currently ongoing.

21.26 Elaboration of the Cl-Amidine Scaffold

To develop inhibitors with enhanced potency, selectivity, and bioavailability, we also developed a SAR for F- and Cl-amidine (Causey et al. 2011). Structurally, these compounds can be divided into three parts: (1) the warhead, (2) the side chain, and (3) the backbone (Fig. 21.14a). Initially, we examined the contribution of the side chain to inhibitor potency by comparing the relative potency of 2-fluoroacetamidine, the warhead alone, to 2-fluoro-*N*-propylacetamidine (**4**) (Fig. 21.14b), that is, the propylamine modified warhead, which mimics the side chain of F-amidine. Given that both compounds are relatively weak PAD4 inhibitors with IC_{50} values that are at least 100-fold worse than F-amidine, the results indicate that the side chain contributes minimally to the potent inhibition observed with F-amidine. Nevertheless, the presence of a highly conserved tryptophan (Trp347 in PAD4) lining the wall of the side chain binding channel (Fig. 21.9) suggested that enhanced potency could be achieved by replacing the aliphatic side chain of F-amidine with an aromatic moiety, that is, pi–pi interactions between the two groups would enhance potency by providing additional binding energy for the initial encounter complex. As a first test of this hypothesis, we replaced the propylamine with either benzylamine (**5**: $IC_{50} > 1$ mM) or aniline (**6**: $IC_{50} > 500$ μM). Although still weak inhibitors, the higher potency of



k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity
181000	1.0
7500	24
6700	27
32500	5.6

k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity
106000	1.0
14100	7.5
10400	10
38000	2.8

Fig. 21.14 Selected F- and Cl-amidine analogs. (**a**) SAR study on F-amidine. (**b**) Side chain modified F-amidine analogs. (**c**) Backbone modified F-amidine analogs

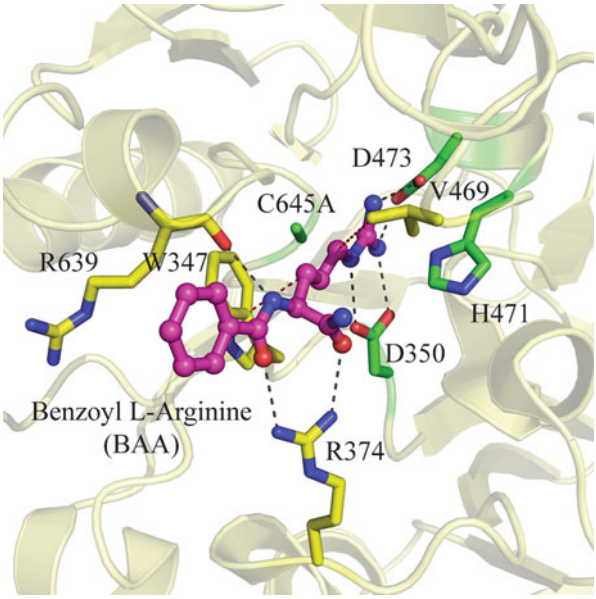


Fig. 21.15 Structure of the PAD4 C645A mutant active site covalently complexed with Benzoyl L-Arginine Amide (BAA) (magenta ball and stick). Active site residues are *green*, and residues that only participate in substrate binding are in *yellow*. Electrostatic interactions are shown as *gray dashed lines*, and hydrophobic interactions are shown as *red dashed lines*. Figure created from PDB: 1DWA using Pymol

the aniline prompted us to directly test our hypothesis and substitute the side chain with a variety of aromatic moieties. For these studies, a variety of phenylalanine and phenylglycine derivatives were synthesized, i.e., compounds **7**–**10**. None, however, were more potent than F-amidine. The lack of potency is likely caused by an inability to properly position the warhead for nucleophilic attack by Cys645 as a result of the steric constraints imposed by the presence of the backbone.

Given that side chain substitutions did not enhance potency, we next focused on developing a SAR for the backbone. Initial efforts, exploring the importance of the C-terminal carboxamide, revealed that this group is critical for inhibitor potency because the version of F-amidine lacking the C-terminal carboxamide, compound **11** (Fig. 21.14c), is a weak PAD4 inhibitor (IC_{50} > 1 mM). Substitution of the benzoyl-carboxamide for the sulfonamide (**12**) did not improve potency (IC_{50} > 1 mM), despite the introduction of a second potential H-bond acceptor. Since the decreased potency of the sulfonamide may also relate to its tetrahedral geometry, versus the trigonal planar geometry of the carboxamide in F-amidine, we replaced the benzoyl-carboxamide with a sulfonamide in the context of F-amidine. This compound (**13**) also showed limited potency. In retrospect, given the structure of PAD4 bound to BAA (Fig. 21.15), these results are unsurprising because the two backbone carbonyls form H-bonds with the guanidinium group of R374, and our

inhibition data suggests that these two groups synergistically enhance the potency of F-amidine (see Figs. 21.9 and 21.15).

Given that substitutions of the side chain and two backbone carbonyls were not tolerated, we envisioned that higher potency could be achieved by altering the identity of the benzyoyl group. Since structures of PAD4 bound to histone H3 and H4 tail mimics indicated that the majority of contacts between the enzyme and substrate are through the substrate backbone, we focused on substitutions that would mimic an extended peptide chain. Towards that goal, the phenyl group was initially replaced with an imidazole (**14**), hypothesizing that the NH moiety would act as an amide bond isostere and provide an additional H-bond that would drive an increase in potency. However, this substitution was poorly tolerated ($IC_{50} = 230 \pm 110$ μM). Reasoning that the decreased potency may be due to the imidazole being protonated under the assay conditions and decreasing binding via charge–charge repulsions, we synthesized the pyrollidine (**15**), as the higher pK_a renders this moiety neutral while retaining the potential H-bond donor. Although **15** is not more potent than F-amidine, the fact that these two compounds are equipotent confirmed that the decreased potency of the imidazole containing compound is due to a charge–charge repulsion.

Since these findings suggested the presence of a positively charged residue in proximity to the benzoyl group, we hypothesized that the installation of a negatively charged group would enhance inhibitor potency. Initially, we synthesized the *para*-nitrophenol derivative (**16**), rationalizing that the nitro group would depress the pK_a of the hydroxyl, such that it is negatively charged at neutral pH. Consistent with our hypothesis, we observed an ~twofold increase in potency ($IC_{50} = 12.1 \pm 6.4$ μM). Following up on these results, we installed a carboxylate ortho to the backbone amide to generate *o*-F-amidine (**17**). Importantly, the added carboxyl group increased potency by ~tenfold (1.9 ± 0.21 μM). The chloro derivative of this compound was also synthesized, and this compound, denoted *o*-Cl-amidine (**18**), is ~threefold more potent than Cl-amidine ($IC_{50} = 2.2 \pm 0.3$ μM). The lack of a similar increase in potency likely relates to an inability to properly position the warhead for reaction with the active site cysteine.

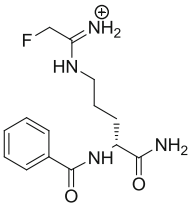
Given the increased potency, we further characterized their mode of inhibition and showed that like the parent compounds, *o*-F- and *o*-Cl-amidine irreversibly inhibit PADs 1–4. Based on k_{inact}/K_I, *o*-F-amidine is approximately 65-, 20-, 40-, and 11-fold more potent than F-amidine (Fig. 21.14). Similar increases in potency are observed with *o*-Cl-amidine. The compounds also selectively inhibit PAD1. For example, the k_{inact}/K_I values obtained with *o*-Cl-amidine are eight-, ten-, threefold higher for PAD1 than for PADs 2, 3, and 4, respectively, and *o*-F-amidine preferentially inhibits PAD1 by 24-, 27-, and sixfold versus PADs 2–4. Structural and mutagenesis studies suggest that the enhanced potency of *o*-F- and *o*-Cl-amidine is due to an interaction between the ortho carboxylate and the indole nitrogen of Trp347 (Fig. 21.15).

The bioavailability of *o*-F- and *o*-Cl-amidine was also examined and the results indicated that the compounds decrease histone citrullination and induce apoptosis of TK6 lymphoblastoid cells to a greater extent than Cl-amidine, indicating that

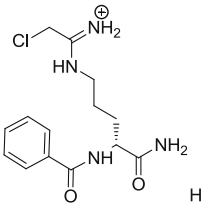
the improved in vitro potency is at least partially paralleled in cell culture. Further increases in cellular potency may be possible by generating pro-drugs that mask the negative charge of the carboxylate and promote cellular uptake.

21.27 D-Amino Acid-Based PAD Inhibitors

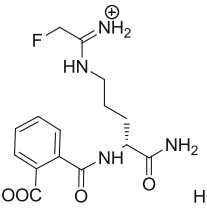
Given that Cl-amidine is built on a trypsin substrate scaffold, that is BAA, we considered the possibility that the relatively short in vivo half life (see below) may be due in part to hydrolysis of one or both of the peptide bonds in the molecule. Since the use of D-amino acids in place of their L-amino acid counterparts, is known to increase the stability of peptide-based compounds (Adessi and Soto 2002; Miller et al. 1995; Tugyi et al. 2005; Werle and Bernkop-Schnürch 2006), we synthesized and tested eight D-amino acid-based Cl-amidine analogs (Fig. 21.16).



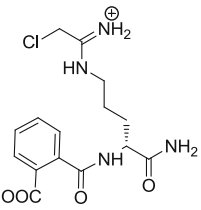
D-F-amidine



D-Cl-amidine

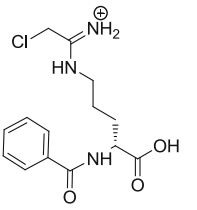


D-*o*-F-amidine

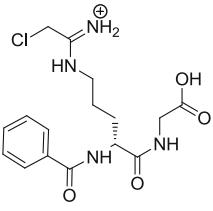


D-*o*-Cl-amidine

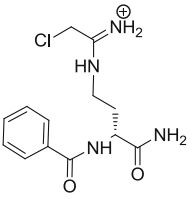
Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity
PAD1	1220	1	13500	1	12100	1	125	1
PAD2	160	8.0	270	50	340	36	95	1.3
PAD3	3	400	65	210	3	4000	3	42
PAD4	130	9	1400	10	50	240	30	4.2



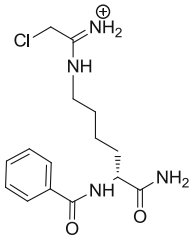
D-Cl-amidine-OH



D-Cl-amidine-Gly



D-Cl2-amidine



D-Cl4-amidine

Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity
PAD1	230	1	190	1.3	150	1	110	1
PAD2	160	1.7	240	1	60	2.5	90	1.2
PAD3	60	3.8	40	6.0	40	3.8	100	1.1
PAD4	40	5.8	15	16	40	3.8	60	1.8

Fig. 21.16 Structures of D-series compounds with their k_{inact}/K_I values and fold selectivities

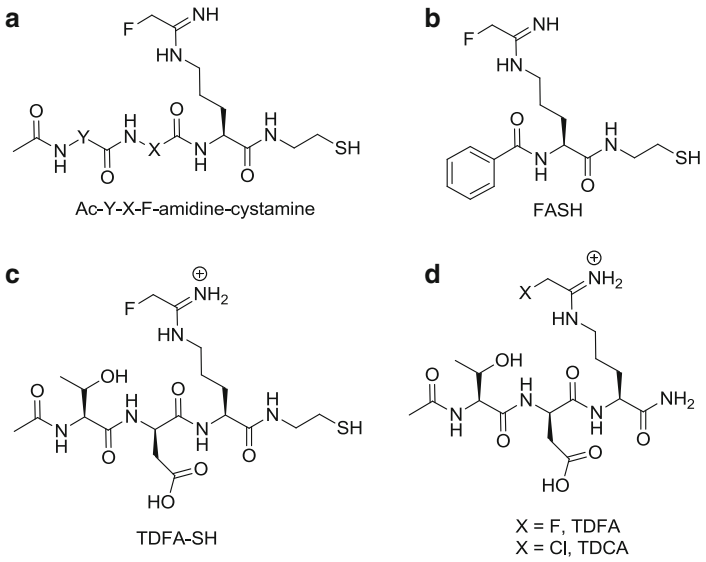
Although most of the compounds are rather poor inhibitors of all the isozymes, D-Cl-amidine and D-*o*-F-amidine are potent PAD1-selective compounds. Note that the D- denotes the altered stereochemistry. For example, D-*o*-F-amidine inhibits PAD1 ($k_{inact}/K_I = 12{,}100\ M^{-1}\ min^{-1}$) with 36-, 4,000-, and 240-fold selectivity versus PADs 2–4, whereas D-Cl-amidine ($k_{inact}/K_I = 13{,}500\ M^{-1}\ min^{-1}$) is 50-, 210-, and tenfold selective. As with the L-stereoisomers, these compounds are irreversible inhibitors. Interestingly, the potency of D-*o*-Cl-amidine ($k_{inact}/K_I = 125\ M^{-1}\ min^{-1}$) is significantly less than D-*o*-Cl-amidine ($k_{inact}/K_I = 12{,}100\ M^{-1}\ min^{-1}$) towards PAD1, despite the higher leaving group potential of the chloride. Although unexpected, this result is not without precedent as Thr-Asp-F-amidine (TDFA) is more potent and selective than Thr-Asp-Cl-amidine (TDCA) (Jones et al. 2012). Note that since these compounds prevent labeling of the enzymes with rhodamine-conjugated Cl-amidine (RCA), an active site directed ABPP, they likely inactivate the PADs similarly to the L-versions, that is, they modify the active site cysteine.

Given that D-Cl-amidine and D-*o*-F-amidine are the best compounds from this series, and because they preferentially inhibit PAD1, we examined their effect on MDA-MB-231 cells, a triple negative breast cancer cell line that overexpresses PAD1 (Bicker et al. 2012). Treatment of these cells with either L-Cl-amidine or D-Cl-amidine reduced both cell viability and cell number at 200 and 400 μM of compound, with a coincident induction of caspase 3 activity, suggesting that the reduction in viable cells is due to an induction of apoptosis; caspase 3 activity increases during apoptosis (Alnemri et al. 1996). Although more selective in vitro, D-*o*-F-amidine showed only small effects on cell viability, cell number, and apoptosis. While the reasons for the lack of an effect are unclear, the altered stereochemistry and the presence of the negatively charged carboxylate may limit cellular uptake.

In total, the fact that D-Cl-amidine and D-*o*-F-amidine are potent and selective PAD1 inhibitors suggests that it will be possible to develop inhibitors with improved potency and bioavailability using a D-amino acid scaffold. Additionally, while D-*o*-F-amidine shows only modest cell killing activity, it would be facile to convert this compound into a PAD1-selective ABPP that can be used to isolate PAD1 from cells expressing this isozyme and use it to identify the posttranslational modifications and binding proteins that regulate its activity in response to a variety of stimuli. Finally, the higher selectivity of D-Cl-amidine for PAD1 suggests that it will be a useful probe of in vivo PAD1 activity because its use will minimize off target effects.

21.28 Targeted Chemical Libraries

In an effort to identify a PAD4-specific inhibitor (Jones et al. 2012), we used parallel solid phase synthesis to generate a 264 member fluoroacetamidine containing peptide library (Fig. 21.17). The library was synthesized on Tentagel resin, using a cystamine linker that is readily cleaved with DTT. Tentagel is used as the resin because its polystyrene core is grafted to polyethylene glycol, which provides it with dynamic properties in both aqueous and organic solvents. Key advantages of



e

TDFA

Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity
PAD1	1700	15
PAD2	500	52
PAD3	400	65
PAD4	26000	1.0

TDCA

Isozyme	k_{inact}/K_I ($M^{-1}min^{-1}$)	Selectivity
PAD1	21000	1.1
PAD2	300	80
PAD3	920	26
PAD4	24000	1.0

Fig. 21.17 Structures of FASH and TDFA, TDCA, and TDFA-SH. (**a**) General structure of cystamine containing F-amidine library. (**b**) Structure of cystamine F-amidine (FASH). (**c**) Structure of TDFA-cystamine (TDFA-SH). (**d**) Structures of TDFA and TDCA. (**e**) Selectivities and potencies of TDFA and TDCA

this approach include the fact that the library is readily synthesized using standard solvents and can then be cleaved into an aqueous buffered solution to facilitate in vitro testing, thereby obviating the need for harsh cleavage conditions and purification of each library member.

The basic design of the library is Ac-Y-X-F-amidine-cystamine, where Ac is an acetylated N-terminus and Y and X are diversity elements (Fig. 21.17a). The diversity elements provide complementary polar, ionic, hydrophobic, and aromatic interactions with PAD4. Following library synthesis and side chain deprotection, individual library members were cleaved from the resin with HEPES buffered DTT, and their ability to inhibit PAD4 rapidly evaluated. Cystamine-modified F-amidine (FASH; Fig. 21.17b) was synthesized in parallel and used as a reference to titrate the amount of the library required to yield >50 % inhibition. Compounds that were more potent than FASH were considered hits. The top ten hits were resynthesized, purified and IC_{50} values determined. The most potent compound had the sequence

Ac-Thr-Asp-F-amidine-SH ($IC_{50} = 1.5 \pm 0.3$ μM) (Fig. 21.17c). Resynthesis of the C-terminal carboxamide version yielded Thr-Asp-F-amidine (TDFA; Fig. 21.17d), whose IC_{50} value (2.3 ± 0.2 μM) is almost identical to that obtained for Ac-Thr-Asp-F-amidine-SH. This result indicates that the improved potency is not due to the amino-thiol linker used to generate the library. The chloro containing analog, Thr-Asp Cl-amidine (TDCA), was also synthesized and its IC_{50} value (3.4 ± 0.5 μM) is comparable to that obtained for TDFA.

Further characterization of TDFA and TDCA demonstrated that these compounds are irreversible PAD4 inhibitors that show a significant degree of selectivity for PAD4. For example, based on differences in k_{inact}/K_I (Fig. 21.17e), TDFA preferentially targets PAD4 by at least 15-fold over PAD1 and 52- and 65-fold over PADs 2 and 3, respectively. TDCA shows similar selectivity trends versus PADs 2 and 3, but is an equipotent inhibitor of PADs 1 and 4. Although the molecular basis for the altered selectivity is unclear, based on our experience with these and other compounds, the identity of the warhead likely alters the conformation of the initial encounter complex, with consequent effects on affinity that ultimately impact the overall potency and selectivity of the compounds.

A structure of the PAD4·TDFA complex, determined to 2.98 Å resolution, showed clear electron density for the thioether linkage between Cys645 and the amidine warhead of TDFA. While the Ac-Thr fragment of TDFA was not resolved in this structure, electron density for the Asp was detected. With the caveat that this is the structure of a dead-end complex, the enhanced potency of TDFA is likely due to favorable interactions between the side chains of Gln346, Arg374, and Arg639, and the side chain carboxylate of the Asp in TDFA (Fig. 21.18). Given that the TDFA IC_{50} values are increased by 1.9-, 5.2-, and 2.9-fold for the Gln346Ala ($IC_{50(TDFA)} = 4.4 \pm 1.7$), Arg374Gln ($IC_{50(TDFA)} = 12 \pm 2.0$), and Arg639Gln ($IC_{50(TDFA)} = 6.6 \pm 0.8$) mutants, the increased potency and selectivity of TDFA is likely the result of favorable interactions between Arg374 and Arg639. The roles of these residues are consistent with the enhanced selectivity because R374 is conserved in PADs 1 and 4 and R639 is unique to PAD4. The residue corresponding to Arg374 is glycine in PADs 2 and 3 and alanine in PAD6, whereas in PADs 1 and 3, Arg639 is a leucine, a phenylalanine in PAD2, and a glutamate in PAD6.

A unique feature of irreversible inhibitors is that they are readily converted into activity-based proteomic probes or ABPPs that can be used to both isolate the targets of the inhibitors as well as determine the specific conditions under which the enzyme is active (Weerapana et al. 2007). Given these considerations, our experience in developing such reagents (Luo et al. 2006b; Obianyo et al. 2011; Slack et al. 2011a), and the selectivity of TDFA, we synthesized a PAD4-selective ABPP by appending the N-terminus of TDFA with a biotin moiety. Biotin was installed at this position because this region of TDFA does not interact with PAD4. The utility of biotin-TDFA was validated by selectively labeling PAD4, by 18-fold, in a mixture of both PADs 2 and 4.

TDFA is also bioavailable (uptake likely occurs through a tripeptide transporter) and inhibits global histone H3 citrullination in HL60 granulocytes, a PAD4 overexpressing cell line, with EC_{50} values in the 10–100 nM range (vs. 100–200 μM for

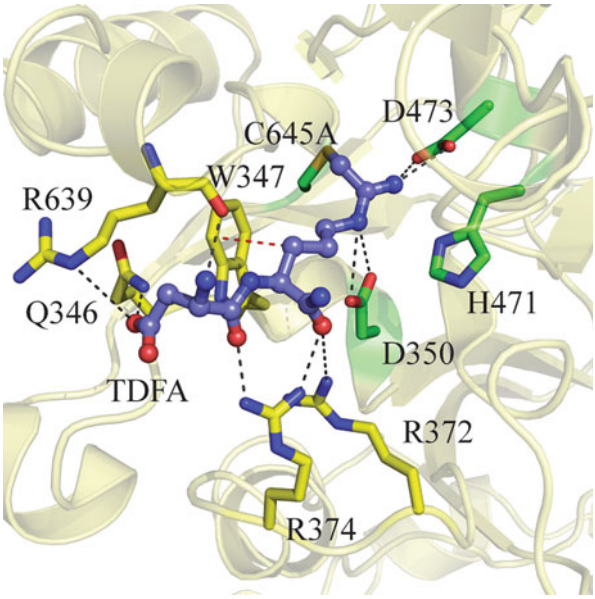


Fig. 21.18 Structure of the PAD4 active site covalently complexed with TDFA (purple ball and stick). Active site residues are *green*, and residues that only participate in substrate binding are in *yellow*. Electrostatic interactions are shown as *gray dashed lines*, and hydrophobic interactions are shown as *red dashed lines*. Figure created from PDB: 4DKT using Pymol

Cl-amidine). TDFA also inhibited histone citrullination at two PAD4 target genes, p21 and OKL38 (Li et al. 2008; Slack et al. 2011b; Yao et al. 2008), with an EC_{50} value in the 1 and 10 μM range. These results were obtained using chromatin immunoprecipitation (ChIP) assays on chromatin obtained from MCF-7 cells. The dissimilar EC_{50} values are most likely due to the time-dependent degradation of TDFA because different incubation times were used for the histone citrullination (30 min) and ChIP experiments (48 h) (Jones et al. 2012). Although cellular studies demonstrating the biological utility of TDFA and biotin-TDFA are so far limited, recent unpublished data indicate that this compound inhibits PAD4-dependent gene transcription, chromatin compaction, and NET formation, suggesting that it will ultimately be a useful probe of PAD4 biology.

21.29 Pharmacokinetics

An important consideration in the development of any therapeutic is the pharmacokinetic and pharmacodynamic properties of the compound. This is the case because such information can guide decisions on dosing (e.g., once vs. twice per day), delivery route (oral vs. i.p.), and maximum tolerable dose (MTD; i.e., the highest

dose that does not cause weight loss or overt toxicity). Additionally, this information can ultimately be used to identify what functional groups should be modified to improve in vivo efficacy. In the case of Cl-amidine, we determined the MTD, its stability in liver microsomes, and its in vivo half life. In parallel studies, these values were also determined for D-Cl-amidine to determine if the D-amino scaffold improved the parameters enumerated above.

For Cl-amidine and D-Cl-amidine, the MTDs are 75 mg/kg and >150 mg/kg per day when dosed orally, suggesting that these compounds are generally well tolerated. Interestingly, the MTD for Cl-amidine appears to be lower when delivered orally because we observed no signs of toxicity when mice were dosed i.p. with Cl-amidine at 100 mg/kg/day for 56 days in the CIA model (Willis et al. 2011). The fact that the MTD is higher for D-Cl-amidine suggests either that the D-analog has fewer off targets or that Cl-amidine metabolism produces a toxic metabolite that is not readily generated from D-Cl-amidine.

The results of our pharmacokinetic studies with Cl-amidine and D-Cl-amidine were also quite informative. For example, when Cl-amidine is dosed at 10 mg/kg i.v., the compound is rapidly degraded/eliminated and is undetectable after 2 h. When delivered by i.p. at the same dose, the in vivo lifetime is slightly longer (4 h vs. 2 h), but the maximum concentration (Cmax) is an order of magnitude lower. This result indicates that the majority of the compound is eliminated even before it reaches the blood stream. The pharmacokinetics of D-Cl-amidine are improved slightly. For example, 10–20 nM of D-Cl-amidine was still detectable in serum at the 4 h time point when dosed at a fourfold lower concentration by either i.v. or i.p., suggesting the altered stereochemistry delays its elimination, possibly via effects on proteolysis of either the N- or C-terminal amide bonds. At least partially consistent with the short in vivo lifetimes are the short half lives of Cl-amidine (37 min) and D-Cl-amidine (33 min) in murine hepatic microsome stability assays, which are a commonly used as initial predictors of drug clearance properties (Baranczewski et al. 2006). The short half life and rapid elimination of the compound may partially explain the limited toxicity of Cl-amidine because these properties limit its ability to nonspecifically react with off targets that could potentially cause toxic side effects.

21.30 Conclusions and Future Perspectives

In the last 10–15 years, protein citrullination has come a long way since it was once considered an obscure PTM with no known functions. It is now clear that this PTM plays a key role in eukaryotic cell signaling, gene regulation, and NET formation. Furthermore, it is evident that aberrant protein citrullination is a hallmark of cancer and virtually all inflammatory diseases, and that PAD-targeted inhibitors can decrease disease severity in many, if not all, of these diseases. Undoubtedly, the development of chemical probes targeting the PADs played a key role in moving the PAD field forward. Although the future of the PAD field is bright, we have only

reached the tip of the iceberg and several key challenges remain, including a deeper understanding of the normal and pathophysiological roles of the enzymes. The future development of isozyme-specific inhibitors will further facilitate our understanding of the specific roles of the individual isozymes, and the recent development of the phenylglyoxal-based probes will facilitate the identification of the specific proteins targeted by these enzymes, and thereby fully unlock the PADlock, and determine the full scope of protein citrullination in human physiology.

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