Review

Peptidylarginine Deiminases as Mediators of Microvesicular Release - Novel Therapeutic Interventions

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Abstract: Extracellular vesicle (EV) release, which occurs in most eukaryotic cells, has recently been associated with peptidylarginine deiminase (PAD)-driven protein deimination. Evidence points to the involvement of deiminated cytoskeletal proteins and changes in histone deimination. Both PADs and EVs are associated with various pathologies including cancers, autoimmune and neurodegenerative diseases. The elevated PAD expression observed in cancers may contribute to increase in EV shedding observed from cancer cells, contributing to cancer progression. Similarly, elevated PAD expression observed in neurodegenerative diseases may cause increased EV shedding and spread of neurodegenerative EV cargo, contributing to disease progression and pathologies. Pharmacological inhibition of PAD-mediated deimination using pan-PAD inhibitor Cl-amidine, reduced cellular EV release in prostate cancer cells, rendering them significantly more susceptible to chemotherapeutic drugs. Studies on models of central nervous system damage have demonstrated critical functional roles for PADs and neuroprotective effects using PAD inhibitors *in vivo*, while human neurodegenerative iPSC *in vitro* models showed evidence of increased protein deimination. Besides using refined PAD inhibitors to selectively manipulate EV biogenesis for novel combination therapies in cancer treatment, we also speculate how EV biogenesis could be targeted via the newly identified PAD-pathway to ameliorate neurodegenerative disease progression.

Keywords: Extracellular vesicles (EVs); Peptidylarginine deiminases (PADs); Chlor-amidine (Cl-Am); cancer; neurodegeneration; deimination; cytoskeleton; induced pluripotent stem cells (iPSCs); histone H3; epigenetics.

1. Introduction

EVs are intact, submicron vesicles and include exosomes (50-100 nm) and microvesicles (MVs, 100-1000 nm in size), which are released from the cell membrane of diverse cell types upon stimulation and/or apoptosis [1,2]. EVs play physiological roles as mediators of intracellular communication, such as transferring growth factors, microRNAs and enzymes between cells, and play roles in diverse processes such as differentiation, migration and angiogenesis [3-7]. EV release

depends on calcium ion influx, which occurs either through pores created by sublytic complement or stimulation of calcium channels such as P2X7 or calcium released by the endoplasmic reticulum through various calcium channels on activated cells [5]. This increase in cytosolic calcium results in cytoskeletal reorganisation, loss of membrane asymmetry, membrane blebbing and subsequent EV formation and release. The cytoskeletal rearrangement is known to be facilitated by the activation of various enzymes, including calpain, gelsolin, scramblase and protein kinase and the simultaneous inhibition of translocase and phosphatases [8]. In addition, recent discoveries on peptidylarginine deiminase (PAD)-mediated deimination of cytoskeletal proteins and histones have elucidated a novel crucial pathway in the facilitation of EV release [7]. As EVs are present in body fluids including blood, urine and cerebrospinal fluid, the identity of circulating EVs may serve as reliable biomarkers of pathophysiological processes [1,2,8,9]. EVs are emerging as novel therapeutic targets in treatment of disease as they have been shown to actively contribute to the progression of numerous pathologies including autoimmune diseases [8,10], cancers [7,11,12] and more recently neurodegenerative diseases [9,13-19]. Novel discoveries of mechanistic pathways involved, provide potential for the development of new diagnostic assays directed at identifying early stages of disease and response to therapy [1,2]. Peptidylarginine deiminases (PADs) are a family of tissue specific calcium activated enzymes that cause irreversible changes of protein-bound arginines into citrullines (Figure 1a) [20,21], resulting in protein misfolding and functional changes in target proteins (Figure 1b) [21-23]. Both cellular EV release and PAD-mediated protein deimination are calcium-dependent events that are elevated in a number of human diseases including cancer, autoimmunity and neurodegenerative diseases [10,24-31]. Addressing the putative interplay between PADs and EV biogenesis, we demonstrated in a recent prostate cancer study that PAD enzyme activation plays a functional role in EV biogenesis through deimination of cytoskeletal actins and nuclear histones [7]. The identification of this novel mechanistic pathway in EV biogenesis has revealed novel synergistic roles for PAD-mediated EV biogenesis in cancer progression.
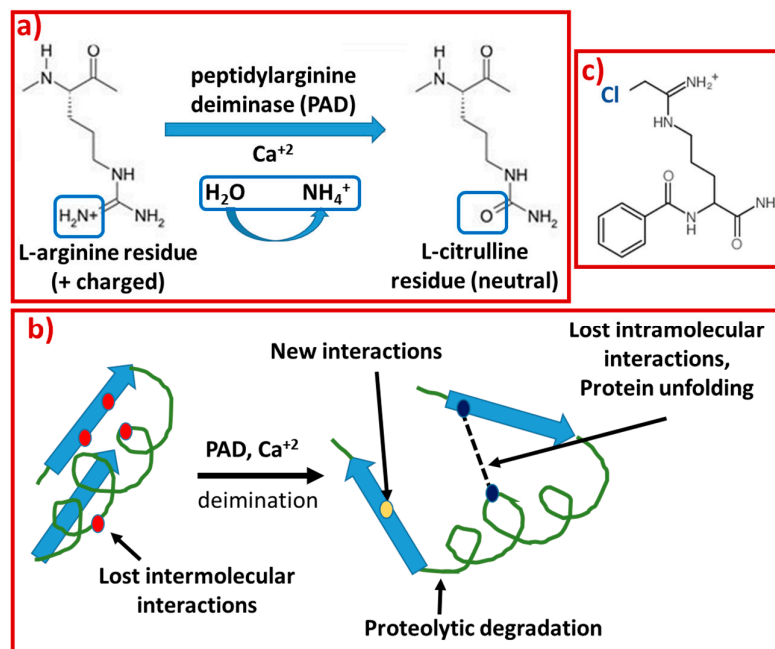

Figure 1. Post-translational protein deimination. a) Protein deimination is a post-translational modification mediated by peptidylarginine deiminases in a calcium catalysed manner. Positively charged arginine residues are modified into neutral citrulline, with ammonia as a by-product. This results in: b) change in protein charge (loss of one positive charge per conversion), lost intermolecular interactions, proteolytic degradation, change in protein conformation and unfolding and change or loss in function [21]. c) Cl-Amidine [141] is the most effective pan-PAD inhibitor used to date.

As the use of the pan-PAD inhibitor Cl-Amidine (Figure 1c) significantly reduced EV biogenesis it is tempting to speculate how the manipulation of EVs via the PAD pathway may provide novel treatment options, not only for cancer progression, but also in other diseases associated with EVs, including neurodegenerative diseases. Previous studies on *in vivo* models of CNS damage showed that PAD-upregulation and increased protein deimination lead to vast neuronal death which was preventable by pharmacological PAD inhibition, significantly reducing neuroinflammatory responses and histone H3 deimination, which has implications in gene regulation [23,33-35]. In addition we have observed increased PAD expression during disease progression in tau mutation mouse models compared to age-matched controls [36]. Using iPSC neuronal models derived from fibroblasts from patients carrying neurodegenerative valosin containing protein *VCP*R155C and *VCP*R191Q mutations [37,38], as well as from patients carrying α-synuclein triplication [39] we have also observed significantly increased pan-protein deimination compared to control (non-mutation carrying) cultures, with significant changes in histone H3 deimination [36]. These studies are in accordance with the mounting evidence for significant roles for protein deimination in neurodegenerative diseases [40-45].   Roles identified for EVs in neurodegenerative disease progression so far include intercellular communication and neuroinflammation by transport of proteins, misfolded proteins, as well as mRNAs and miRNAs, which can be translated in recipient cells and affect gene regulation [46-48]. Unveiling the mechanisms of EV biogenesis and identifying pathways for modulation is thus pivotal for novel treatment options and interception in pathologies.

2. EVs in Cancer

Cumulative evidence implicates EVs in the pathogenesis of various diseases, including cancer, either directly or indirectly. Elevated EV levels in the blood from cancer patients compared to healthy individuals has been demonstrated by various investigators [49 - 51]. EV release has also been shown to aid tumour spread and survival as EVs transport various pathological growth factor receptors, soluble proteins and micro RNAs [5,6,8]. Interestingly, EV shedding from cancer cells also aids increased active drug efflux and thus contributes to their resistance to chemotherapeutic agents [11,52]. In addition, inhibition of microvesiculation has been shown to render cancer cells more susceptible to anticancer drug treatment [7,11] and to reduce the dose of anti-cancer drug docetaxel required to limit tumor growth *in vivo* [53].

3. PADs in Cancer

In patients suffering from malignant tumours, the overexpression of the PAD2 and PAD4 isozymes has been reported in blood and tissues [54-56]. PAD4, which is commonly found in the nucleus [57,58], and the only isozyme that contains a classic nuclear localisation signal, has been shown to translocate to the nucleus in response to tumour necrosis factor alpha (TNFα) upregulation [59]. PAD4 acts as a transcriptional co-regulator for various factors including p53, p300, p21 and ELK1 and via deimination of the N-terminal tails of various histone proteins [60-62].   PAD4 plays a role in apoptosis as it regulates p53 gene activity during DNA damage by acting as a co-mediator of gene transcription and epigenetic cross talk with histone deacetylase 2 (HDAC2) [63]. PAD4 is also co-localised with cytokeratin (CK), an established tumour marker which occurs in various isoforms, some of which are deiminated [64]. The deiminated CK isoforms become resistant to caspase-mediated cleavage, contributing to the disruption of apoptosis in cancer tumours [64]. PAD4 also acts as a cofactor in epidermal growth factor-mediated target gene activity, activating the expression of proto-oncogene c-fos [62], interacting with p53 and influencing the expression of its target genes [60,61,65,66]. PAD4 is also linked with oestrogen receptor target gene activity via histone tail deimination [67]. In spite of lacking a classic nuclear translocation site such as found in PAD4, both PAD2 and PAD3 have also been localized and detected in the nucleus [32,33,56,68]. In cancer cells, PAD2, which is the most widely expressed isozyme in the body [20], has been shown to deiminate histone H3 and play a role in gene regulation [30,56,69,70].

4. The interplay of EVs and PADs in Prostate Cancer

We have recently shown novel roles for PADs in the biogenesis of EVs in prostate cancer and effective inhibition of EV biogenesis using pharmacological PAD-inhibition [7,71]. It was shown that PAD2 and 4 were present at significantly elevated levels in metastatic prostate PC3 cancer cells compared to a control benign (PNT2) prostate cell line [7]. Upon stimulating cells to microvesiculate, the nuclear translocation of PAD2 and particularly PAD4 was observed in the metastatic PC3 cancer cells, but prevented when the cells were pre-treated with PAD-inhibitor prior to stimulating the cells for EV release [7]. PAD4 has previously been shown to regulate histone arginine methylation levels in the nucleus [67]. It may be postulated that the PAD-mediated EV release cascade causing translocation of PADs to the nucleus also causes upregulation in TNFα as part of the inflammatory response, and as TNFα is implicated in nuclear translocation of PAD4 [59,72] this may cause in turn additional EV shedding. The pan-PAD inhibitor Cl-Am had a dose-dependent inhibitory effect also on EV release in normal prostate control cell lines (PNT2) [7], indicating that PAD activity impacts cell communication via this pathway also in non-cancerous cells [7]. Which of the PAD isozymes is the main player in EV release is currently under investigation as the PADs may well be collectively involved with different, albeit equally important roles. Essentially, a novel mechanistic pathway for PADs in EV biogenesis via the deimination of cytoskeletal actin and histones, which can be selectively targeted using pharmacological PAD inhibition, has been identified in cancer [7].

5 Deiminated Target Proteins Involved in EV Biogenesis

The mechanism of EV release is regulated by either influx of Ca^{2+} that is released by the ER or through Ca^{2+} that enters the cell through pores generated by sublytic complement [8] or through the various calcium channels [73,74] on activated cells. Besides leading to calpain-mediated cleavage of a variety of cytoskeletal actin filaments [75], Ca^{2+} influx also causes downstream PAD-activation, contributing to reorganisation of the cytoskeleton, thereby facilitating EV release [7]. Both β- and F-actin stress fibres play important roles in the redistribution of the actin-cytoskeleton during the EV formation process. This happens through the activation of Rho/Rho-associated kinase (ROCK) pathways during apoptosis and thrombin stimulation [76]. In PC3 cells the presence of deiminated β-actin increased in cells that were stimulated for EV release while pre-treatment with PAD-inhibitor markedly decreased β-actin deimination [7]. Beta actin, one of six different human actin isoforms, is a cytoskeletal protein involved in cell structure and integrity, cell migration and movement [77]. This provides evidence for the importance of PAD-mediated deimination of target proteins that are involved in cytoskeletal rearrangement, such as β-actin, actin alpha 1 and glyceraldehyde-3-phosphate dehydrogenase, as an essential step for successful EV biogenesis. Upon PAD upregulation the deimination of cytoskeletal actin filaments facilitates the cytoskeletal reorganisation necessary for successful EV release while PAD inhibition prevents this deimination, rendering cytoskeleton less flexible and significantly impacts effective EV formation and shedding (Figure 2). Deiminated β- and γ-actins have indeed also previously been detected in sera and synovial fluid from RA patients [78,79] and been identified as a substrate for PAD2 in ionomycin-activated neutrophils [80].
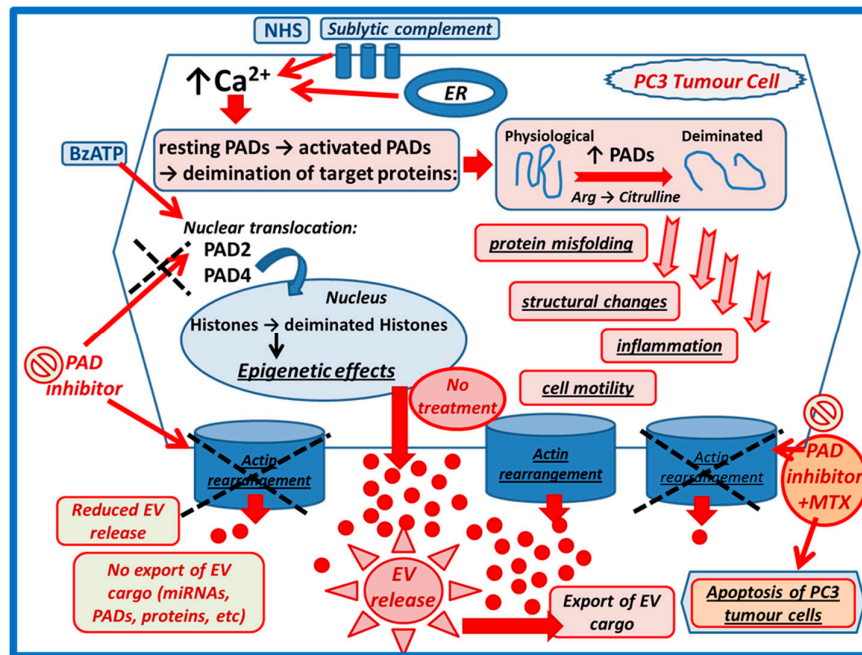
Figure 2. PADs in Extracellular vesicle biogenesis and the potential therapeutic application of PAD inhibitors in anti-cancer therapy. PAD2 and PAD4 are cytosolic enzymes, which during the course of cells being stimulated to microvesiculate (e.g. using BzATP stimulation of P2X7 channels or normal human serum (NHS) as a source of sublytic complement), by raised intracellular calcium levels, results in PAD enzymes (PAD2 and PAD4) translocating to the nucleus or remaining partially in the cytosol (PAD2). These PAD isozymes play a potential novel role in the biogenesis of EV release. This may happen by influencing actin-cytoskeleton cleavage and actin rearrangement and/or by nuclear histone deimination. The pharmacological inhibition of PADs abrogates the release of EVs, and when combined with the anticancer drug methotrexate (MTX), works synergistically to induce increased cytotoxic effects and apoptosis of PC3 tumour cells. [Based on 7]

6. PAD Inhibitors in Synergy with Chemotherapeutic Drugs

Cytotoxic effects of PAD inhibitors (Cl-Am and F-Am) have been shown on several cancer cell lines (HL60, MCF-7 and HT-29), while the viability of normal cells was not affected [31,81]. When using PAD inhibitors in synergy with chemotherapeutic agents such as for example doxorubicin, increased cancer cell cytotoxicity was observed [82]. In addition, PAD2 enzymatic activity has been shown to be inhibited by paclitaxel, which is used to treat neoplasms such as breast and ovarian cancers [83]. The novel pathway of PAD-mediated EV release in this context was first described in PC3 prostate cancer cells [7]. Depending on target protein preference of PAD2 and PAD4, EV release may occur via both cytoskeletal and epigenetic pathways. The different PAD isozymes have indeed demonstrated distinct substrate preferences, with PAD4 showing more restrictive substrate specificity compared to PAD2 [80,84-86]. While PAD4 prefers sequences with highly disordered conformation, PAD2 has a broader sequence specificity [87], which might partly be reflected by the broader tissue expression of PAD2 [20]. For example, PAD4 has been shown to be the main isozyme to deiminate histone H3, while PAD2 deiminates β- and γ-actins [80]. The selectivity of PAD inhibitors and combinatory application with chemotherapeutic agents is thus of great interest. In PC3 cells, Cl-Am and Methotrexate (MTX) exerted synergistic cytotoxic effects on PC3 cells compared to MTX alone and significantly reduced EV biogenesis and release [7]. This radical reduction in EV shedding prevents the cancer cells from carrying out active drug efflux through EV shedding, and thus sensitizes them to chemotherapeutic drugs (Figure 2). Accordingly, other studies performed in our laboratories show that neoplastic cells are rendered more sensitive to cancer drugs when EV biogenesis is inhibited [11,71]. We have also shown that EV inhibition via a calpeptin inhibitor or

siRNA limits tumour growth in vivo, emphasizing the important role of EV generation and manipulation for tumour growth [53].

7. EVs and PADs in the Spread of Tumorigenesis

EVs play various roles in cellular cross talk and act as carriers of active molecules that can have an effect at sites distal from the origin of EV release [1,2]. Both PAD2 and PAD4 isozymes are expressed in patients with malignant tumours and PAD expression is elevated in cancers, which in turn may cause increased EV release that contributes to cancer progression [7]. Intriguingly, the presence of PAD4 has been reported in the plasma of cancer patients without evidence for how it reaches that location [64]. Indeed, tumour cells may increase EV release in a PAD-dependent mechanism through which PAD enzymes are packaged in EVs and carried into plasma where they then deiminate target proteins (such as anti-thrombin) and aid in the spread of cancer indirectly. In the same vein, PAD enzymes may be packaged in EVs from non-cancerous cells in response to inflammation and elevated TNFα levels [7]. It can be postulated that EVs play similar roles in autoimmune and neurodegenerative diseases, explaining the unknown mechanism by which PAD molecules gain access to the extracellular space for example in rheumatoid arthritis (RA) where extracellular proteins such as collagen and fibrinogen are deiminated [88-90]. Another method by which PAD4 has been shown to be released from cells to generate deiminated antigens is neutrophil extracellular trap formation (NETosis) [91,92]. It may well be possible that PAD isozymes carried in EVs from different cells differ between physiological and disease states and vary between diseases. PADs exported in EVs in this manner may thus play previously unidentified and important roles in cancer, autoimmune and neurodegenerative diseases.

8. PADs in Central Nervous System (CNS) damage and Neuroprotective Effects of PAD Inhibitors

Previous work in two animal models of acute CNS damage has demonstrated that pharmacological pan-PAD inhibition is neuroprotective in vivo [32-34]. Administration of one dose of Cl-Am (80 mg/kg) straight after injury and up to two hours post-injury, in a chick-model of spinal cord damage, significantly reduced neuronal cell death, tissue loss and histone H3 deimination, compared to non-treated control injuries [32]. In two different mouse models of neonatal hypoxic ischaemic encephalopathy (HIE), firstly where permanent left carotid occlusion was followed with severe hypoxia (8% oxygen for 60 min), or secondly with 30 min hypoxia in combination with infection (as mimicked by LPS stimulation), one dose of Cl-Am (30 mg/kg) straight after hypoxia, or 10 min after LPS stimulation and again straight after hypoxia, significant reduction was observed in microglial activation, histone H3 deimination and cell death in all affected brain regions [33]. Interestingly, EV release has previously been associated with cerebral hypoxia induced by acute ischaemic stroke [93,94] while increased protein deimination has also been detected in the pathology of traumatic brain injury [95]. The fact that dampening of neuroinflammation and neuroprotective effects are achieved in vivo, using pharmacological PAD inhibition, and that this effect is translatable between CNS injury and animal models, is indeed promising for effective application also in other cases of neuronal damage. The significance of PAD-mediated EV release and contribution of EV shedding in acute CNS damage is currently under investigation in our laboratories.

9. EVs in Neurodegenerative Diseases

EVs are increasingly being associated with neurodegenerative disease progression and pathologies [96-99]. In the CNS, EVs have been shown to be produced by several cell types including neurones, microglia, oligodendrocytes, astrocytes and embryonic neural stem cells [9] and play important roles in the development and function of the nervous system [100]. Roles for EVs in neurodegenerative disease progression include intercellular communication and neuroinflammation by transport of proteins, misfolded proteins, as well as mRNAs and miRNAs, which can be translated in recipient cells and affect gene regulation [46-48]. In Amyotrophic Lateral Sclerosis (ALS), EVs

have for example been shown to export misfolded mutant SOD1 [101,102]; in relation to ALS and Frontotemporal dementia (FTD) to export TDP-43 [15,103]; and there is increasing evidence emerging for critical roles for miRNAs in the pathogenesis of FTD-ALS [104,105]. In tauopathies EVs have been shown to export phosphorylated tau [106,107]; in Parkinson's disease (PD) they export α-synuclein and LRRK2, [19,108-110]; and in Alzheimer's disease (AD) they export Aβ [111,112]. All of these proteins form aggregates involved in the disease pathologies [113]. As EVs have the capability to travel further via the blood or cerebrospinal fluid, misfolded proteins may spread via this pathway in a prion-like manner [114-119]. In addition, functional effects of such protein transport has been indicated for Aβ, which progressively accumulates in EVs with age, while the β-site cleavage of APP has been reported to occur inside EVs [120]. Also, the phosphorylation of tau differs in exosomes compared to total cell lysates, indicating functional consequences for its seeding capability [106]. In AD neuroinflammation has been linked to circulating TNFα [121-123], which causes nuclear translocation of PADs [59,72], and to neutrophil extracellular trap formation [124], which is PAD4-dependent [23,72]. In addition, in PD, α-synuclein induces TNF-α containing exosomes from microglia [110] while TNF-α has been shown to promote EV shedding from endothelial cells [125]. In the light of this increasing evidence for crucial roles of EVs in neuroinflammation, and the transfer and spreading of neurodegenerative protein aggregates alongside other proteins, enzymes, mRNAs and miRNAs, the mechanisms of EV biogenesis and routes of modulation are pivotal.

10. Protein Deimination in Neurodegenerative Diseases

The evidence for critical roles of PADs in various neurodegenerative diseases is mounting [40-45]. In many cases where protein deimination has been associated with neurodegenerative diseases, including multiple sclerosis (MS) [126-129], AD and PD, studies have mainly focused on histological analysis of post mortem human samples. AD post mortem human brain samples display increased protein deimination [41-43;130-132] and deiminated proteins are present in amyloid-containing areas in amyloid-precursor-protein / presenilin1 (APP+PSEN1) transgenic AD mouse models [31,133]. In AD patients, β-amyloid has been shown to be deiminated [31,43]. In hippocampal lysates from AD patients, glial fibrillary acidic protein (GFAP), an astrocyte-specific marker protein, and vimentin were identified as deiminated proteins and the deimination of GFAP was shown to be PAD2 specific [134]. In vitro studies demonstrated that amyloid peptides bind to PAD2, resulting in catalytic fibrillogenesis and formation of insoluble fibril aggregates [135]. In PD brain samples, increased levels of total protein deimination and deimination-positive extracellular plaques were observed [40]. Mutated α-synuclein protein, resulting in misfolding, has been related to increased protein deimination [31]. Amyotrophic lateral sclerosis (ALS) spinal cords show increase in deiminated proteins [31] and Creutzfeldt Jacob Disease (CJD) brain samples indicate roles for deiminated enolase [136]. In AD brains, PTCD2 protein, an antigen target of an AD diagnostic autoantibody, is present in a deiminated form [137]. There are thus indications that disease-associated autoantibodies are generated due to the production and release of deiminated proteins and deiminated protein fragments, which may be released from damaged cells in regions of pathology [137,138]. In AD cortex and hippocampus, both PAD2 and PAD4 were shown to be expressed in cerebral cortex and hippocampus, the brain regions most vulnerable to AD pathology, with PAD2 localized in activated astrocytes and PAD4 selectively expressed in neurones. PAD4 expression was shown to co-localize with amyloid-β-42 in pyramidal neurones in cerebral cortex and in hippocampal large hilar neurones of the hippocampus, which were also surrounded by activated astrocytes and microglia [137]. These neurones contained cytoplasmic accumulations of deiminated proteins. Also, the release of deiminated proteins from necrotic neurones was thought to cause an increased exposure of deiminated neuronal proteins to the immune system. In addition, the continual return of cerebrospinal fluid to the circulation via the arachnoid villi, containing modified deiminated proteins and protein fragments, was suggested to be a key step in the ongoing pathology due to generation of autoantibodies [137].

PADs are thus expressed in neurones residing in brain regions that are engaged in neurodegenerative pathological changes and inflammatory changes such as reactive astrogliosis and

microglial migration and invasion. Although some target proteins have been described, most deiminated proteins remain to be identified. We have, using proteomic analysis of deiminated proteins in the injured CNS [32,33,36], identified several proteins with neurodegenerative implications including neuroinflammation, perivascular drainage of Aβ, and the SNARE proteins, which are involved in EV release [139,140]. In pilot studies, using iPSC neuronal models derived from fibroblasts from patients [37] carrying valosin-protein containing mutations VCPR155C and VCPR191Q we have observed significantly increased pan-protein deimination compared to control (non-mutation carrying) neurones, with significant increases in histone H3 deimination in VCPR155C carrying neurones [36]. We also observed similar changes in pan-protein and histone H3 deimination in iPSC derived neurones from patients carrying α-synuclein triplication [39] compared to control neurones [36]. The predominantly expressed isozyme observed was PAD4 while PAD2 was also expressed, albeit at lower levels [36]. Evidence for increased PAD expression with progression of neurodegenerative disease was obtained by analysis of whole genome microarrays from mouse models carrying TAU and APP+PSEN1 mutations. Significant increase of PADI2 transcription was found in cortex and hippocampus in both mutants with disease progression compared to age matched controls [36]. Figure 3 summarizes the proposed interplay of PADs and EVs in neurodegenerative disease pathologies. The contribution of PAD-mediated EV shedding in selected neurodegenerative mutations is currently under study using human iPSC models in our laboratories.
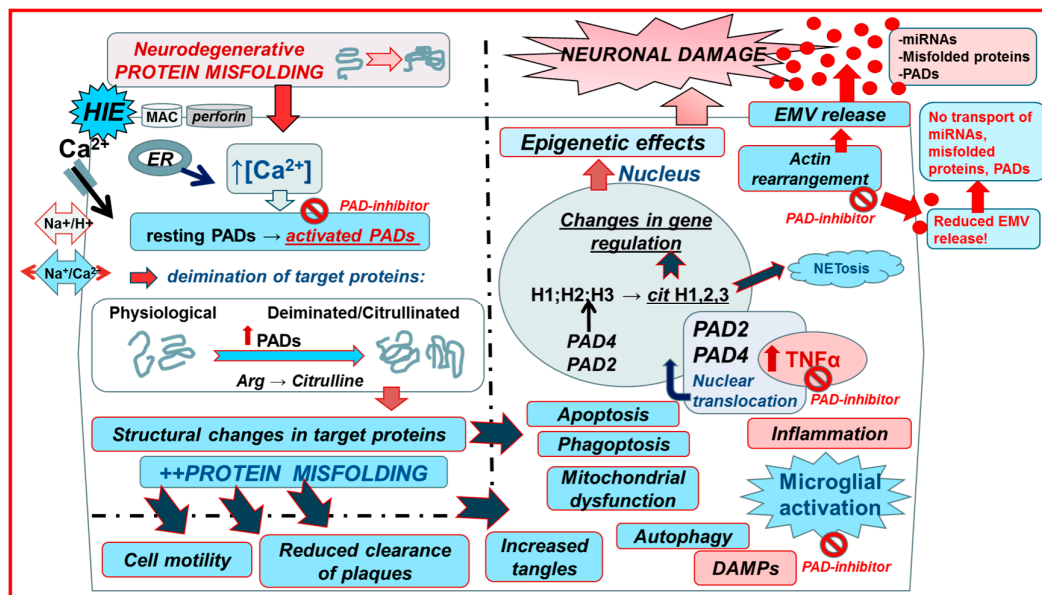

Figure 3. Mechanisms of PADs in CNS injury and Neurodegenerative Pathologies and the Use of PAD-inhibitors. Upon CNS injury (hypoxic ischaemic encephalopathy, HIE), Ca^{2+} entry is facilitated via the reversal of the Na^+/Ca^{2+} exchanger due to over activation of the Na+/H+ exchanger (NHE). Ca^{2+} entry can also be facilitated due to membranolytic pathways including the complement membrane attack complex (MAC) and perforin [based on 35 and 36]. Increased cytosolic Ca^{2+} triggers the neurotoxic cascade, which includes activation of the Ca^{2+} dependent PAD enzymes. Neurodegenerative disease mutations cause protein aggregation and impaired calcium buffering, which activates the downstream PAD-cascade. Both in CNS acute injury and neurodegeneration, PAD activation causes protein deimination and further protein misfolding, affecting cell motility, autophagy, phagoptosis, mictochondrial function, leading to neurotoxic events. Deiminated neo-epitopes and leakage of deiminated proteins from dying cells contribute to neuroinflammation that in turn upregulates TNFα, which causes nuclear translocation of PADs, leading to histone deimination and formation of neutrophil extracellular traps (NETosis). PAD-mediated cytoskeletal protein deimination and histone deimination contribute to EV release, resulting in export of misfolded proteins, miRNAs, enzymes (including PADs) and other EV cargo that can contribute to pathologies. PAD-inhibitior Cl-Amidine targets PAD activation and reduces deimination of target

proteins and neuroinflammatory responses [32,33,36]. Cl-Amidine also significantly reduces EV shedding via histone deimination and by affecting cytoskeletal rearrangement [7], resulting in decreased transport of noxious EV cargo.

11. Conclusions

Recent studies have shown novel roles for PAD2 and PAD4 in the biogenesis of cellular microvesiculation [7]. EV release can be modulated using pharmacological PAD inhibitors to render metastatic prostate PC3 cancer more susceptible to chemotherapeutic drugs. PADs may affect EV biogenesis directly through deimination of cellular actins and other cytoskeletal components, facilitating cytoskeletal rearrangement for effective EV biogenesis, or indirectly through histone deimination via nuclear PAD translocation. Increased EV shedding from cancer cells, facilitated in part by upregulated PAD expression, also acts as a protective mechanism of cancer cells to expel chemotherapeutic drugs. Functional roles for PADs in CNS damage, and neuroprotective pharmacological intervention in vivo, have recently been established and provide evidence for crucial roles of PAD-mediated mechanisms in neuroinflammation [32-35]. Pilot studies using human iPSC neuronal models, derived from patients' fibroblasts carrying neurodegenerative disease mutations, show increased PAD activity and PAD-mediated deimination that will also affect downstream EV release [36]. These neurodegenerative iPSC models will be useful functional tools for continuing studies on effects of PAD-mediated EV shedding, and the efficacy of pharmacological modulation of PAD-mediated mechanisms to ameliorate neurodegenerative disease pathologies. While Cl-Am [141] remains the most used experimental inhibitor to date, the therapeutic potential and generation of second generation and selective isozyme-specific PAD inhibitors is receiving ever increasing attention [72,82,142-146]. Ongoing work aims at dissecting the roles of individual PAD isozymes in EV biogenesis, to identify further deiminated key target proteins, assess the epigenetic impact of histone deimination, and to evaluate disease-specific EV cargo. The use of targeted isozyme-selective PAD inhibitors to modulate EV release pose as promising combinatory therapies for various pathologies including cancers, autoimmune and neurodegenerative diseases.

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
CJD	Creutzfeldt Jacob Disease
CK	Cytokeratin
Cl-Am	Chlor-amidine
CNS	Central nervous system
EV	Extracellular vesicle
FTD	Frontotemporal dementia
GFAP	Glial fibrillary acidic protein
HDAC2	Histone deacetylase 2
HIE	Hypoxic ischaemic encephalopathy
iPSC	Induced pluripotent stem cell
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MAC	Membrane attack complex
MS	Multiple sclerosis
MV	Microvesicle
NET	Neutrophil extracellular trap
PAD	Peptidylarginine deiminase
PC3	Prostate cancer cell line
PD	Parkinson disease
PNT2	Control benign prostate cell line
PSEN1	Presenilin 1

RA   Rheumatoid arthritis
ROCK   Rho/Rho-associated kinase
SNARE   SNAP (Soluble NSF Attachment Protein) REceptor
SOD1   Superoxide dismutase 1
TDP-43   TAR DNA-binding protein 43
TNFα Tumour necrosis factor alpha
VCP   Valosin containing protein

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