Peptidylarginine deiminases and extracellular vesicles: prospective drug targets and biomarkers in central nervous system diseases and repair

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Abstract

Peptidylarginine deiminases are a family of calcium-activated enzymes with multifaceted roles in physiological and pathological processes, including in the central nervous system. Peptidylarginine deiminases cause post-translational deimination/citrullination, leading to changes in structure and function of a wide range of target proteins. Deimination can facilitate protein moonlighting, modify protein-protein interaction, cause protein dysfunction and induce inflammatory responses. Peptidylarginine deiminases also regulate the biogenesis of extracellular vesicles, which play important roles in cellular communication through transfer of extracellular vesicle-cargo, e.g., proteins and genetic material. Both peptidylarginine deiminases and extracellular vesicles are linked to a number of pathologies, including in the central nervous system, and their modulation with pharmacological peptidylarginine deiminase inhibitors have shown great promise in several *in vitro* and *in vivo* central nervous system disease models. Furthermore, extracellular vesicles derived from mesenchymal stem cells have been assessed for their therapeutic application in central nervous system injury. As circulating extracellular vesicles can be used as non-invasive liquid biopsies, their specific cargo-signatures (including deiminated proteins and microRNAs) may allow for disease "fingerprinting" and aid early central nervous system disease diagnosis, inform disease progression and response to therapy. This mini-review discusses recent advances in the field of peptidylarginine deiminase and extracellular vesicle research in the central nervous system, focusing on several central nervous system acute injury, degeneration and cancer models.

Key Words: central nervous system; citrullination/deimination; COVID-19; ectracellular trap formation; extracellular vesicles; glioblastoma; neurodegeneration; peptidylarginine deiminases; regeneration

Introduction

Peptidylarginine deiminases (PADs) are a family of calcium-activated enzymes, with five isozymes identified in human (PAD1, PAD2, PAD3, PAD4 and PAD6), which play multiple roles in physiological and pathological processes, including in the central nervous system (CNS) (Moscarello et al., 1994; Lange et al., 2011, 2014; Nicholas et al., 2014; Ishigami et al., 2015; Caprariello et al., 2018; Faigle et al., 2019; Sancandi et al, 2020). PADs cause post-translational modification of arginine to citrulline (deimination/citrullination), reducing net charge and increasing hydrophobicity of target proteins, leading to changes in protein structure and consequently protein-protein interactions. The different PAD isozymes display tissue-specific expression, with PAD2, PAD3 and PAD4 being the predominant isozymes of the CNS, and PADs furthermore differ in preference for target proteins. Intrinsically disordered proteins are most susceptible for deimination, alongside beta-sheets, and the position of the arginine also plays a role as arginines sitting next to aspartic acid residues are most prone to citrullination/deimination, while arginines flanked by proline or next to glutamic acid residues are rarely deiminated (György et al., 2006; Alghamdi et al., 2019). Deimination can affect a wide range of target proteins, ranging from mitochondrial, cytoplasmic, cytoskeletal and nuclear proteins (e.g., histones) and PADs are furthermore involved in extracellular vesicle (EV) release (Kholia et al., 2015; Lange et al., 2017a). The consequential modification of protein structure, caused by deimination, can lead to changes in protein-protein interactions and also facilitate protein moonlighting, a process which allows the same protein to carry out multiple autonomous and often unrelated functions and therefore contribute to multifaceted use of the same protein according to circumstantial requirements, both temporally and spatially, including in developmental processes, normal physiology and in pathological processes (Jeffrey et al., 2018). Deimination can though also cause protein denaturation and loss of protein function, and result in the generation of neo-epitopes, which can contribute to inflammatory and chronic inflammatory responses (György et al., 2006; Mondal and Thompson, 2018; Alghamdi et al., 2019). This mini-review discusses recent advances in the field of peptidylarginine deiminase and extracellular vesicle research in the central nervous system, highlighting research from our group on acute injury, neurodegeneration and brain cancer models.

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Peptidylarginine Deiminases in Central Nervous System Injury and Disease

PADs have been reported to be associated with numerous neurodegenerative diseases (Nicholas et al., 2014; Ishigami et al., 2015; Lange et al., 2017b; Jang et al., 2018; Faigle et al., 2019), CNS injury, regeneration and repair (Lange et al., 2011, 2014; Lazarus et al., 2015; Attilio et al., 2017), as well as cancers of the CNS (Kosgodage et al., 2018; Uysal-Onganer et al., 2020). Furthermore, PADs have been described as a key regulator of EV release (Kholia et al., 2015; Kosgodage et al., 2018; Uysal-Onganer et al., 2020), which not only plays roles in normal cell communication, but also is implicated in a number of chronic diseases, including cancer, in neurodegeneration and in regenerative pathways (Lange et al., 2017a; Sancandi et al., 2020; Uysal-Onganer et al., 2020). PAD-mediated effects on microRNA expression have also been identified in CNS degeneration and brain cancer (Sancandi et al., 2020; Uysal-Onganer et al., 2020). PAD-related pathways therefore offer novel avenues in targeting a range of CNS diseases, as well as being important players in CNS repair and regeneration (**Figure 1**).

Roles of Peptidylarginine Deiminases in Central Nervous System Development and Neurodegeneration

Roles for PADs in CNS development have been studied to some extent, particularly with relation to myelin basic protein during early development (Moscarello et al., 1994) and in the CNS in lower vertebrates by our group, indicating roles in ontogeny for tissue remodeling of nervous tissue in the spinal cord, brain and eye (Magnadottir et al., 2018). More emphasis has though been on research on pathological roles for PADs in the CNS, both in relation to acute injury and chronic conditions. Initially, PADs and associated protein deimination in the CNS was mainly investigated in relation to multiple sclerosis (MS), including assessment of PAD-mediated contribution to auto-inflammatory responses, which is an ongoing topic in MS research by several groups and may play major roles in MS (Caprariello et al., 2018; Faigle et al., 2019). Research on citrullination/deimination expanded to other areas, finding increasing evidence for significant roles of PADs in a number of other neurodegenerative diseases including Alzheimer's disease (AD), frontotemporal dementia, prion diseases, amyotrophic lateral sclerosis and Parkinson's disease (PD) (Nicholas et al., 2014; Ishigami et al., 2015; Lange et al., 2017b; Jang et al., 2018). Many of the initial studies focused on post-mortem sample analysis, with a few target proteins for deimination being identified, including glial fibrillary acidic portein, while further understanding of pathways regulated by deimination required investigation. *In vitro* approaches by our group, using induced pluripotent stem cell neuronal models of patient derived fibroblasts carrying various mutations for frontotemporal dementia, amyotrophic lateral sclerosis and PD, furthermore confirmed that deimination patterns, as assessed by deiminated protein bands present in western blot analysis, were modified in these induced pluripotent stem cell models (Lange et al., 2017b), in accordance with increased deimination observed in previous studies using post-mortem samples from patients with AD, amyotrophic lateral sclerosis and PD. Most recently, our group carried out an *in vivo* study using a rat model of pre-motor PD, identifying PADs and protein deimination as hitherto unrecognized players in these early stages of PD. The study reported significantly increased deimination in the brain vasculature, changes in deiminated protein pathways relating to neurodegenerative pathways in plasma, in plasma EVs, as well as changes in circulating EV numbers and EV-microRNA cargo (Sancandi et al., 2020).

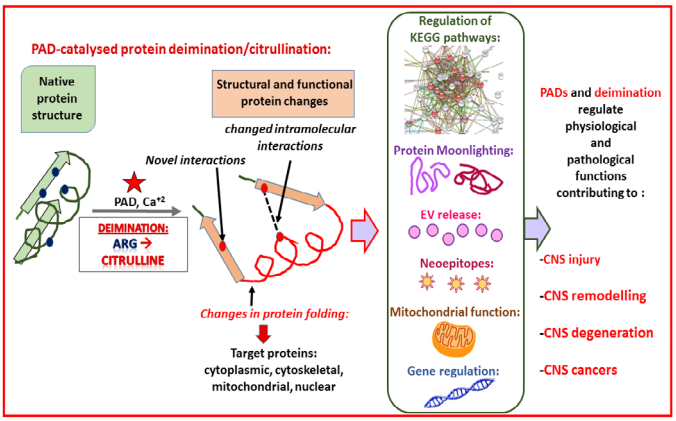

Figure 1 | **Schematic view of PAD-mediated processes in CNS pathologies.** PADs are calcium catalyzed enzymes that cause conversion of ARG to citrulline, causing post-translational protein deimination and downstream pathways relating to CNS pathologies. Deimination causes changes in target protein structure and function, contributing to protein moonlighting and release of EVs. Depending on target proteins, deimination can affect gene regulation, mitochondrial function and generate neo-epitopes, contributing to inflammatory, including chronic inflammatory, responses. As deimination changes protein-protein interactions, this can influence protein networks in a range of CNS pathologies, as indicated by regulation of KEGG pathways via this post-translational modification. All the processes shown have been identified in various CNS pathologies by our group, including acute CNS injury and associated remodeling and repair, to neurodegenerative disease and in CNS cancer. ARG: Arginine; CNS: central nervous system; EV: extracellular vesicles; KEGG: kyoto encyclopedia of genes and genomes; PADs: peptidylarginine deiminases.

Relevance of Peptidylarginine Deiminases and Extracellular Vesicles as Biomarkers

Deimination signatures in plasma and plasma-EVs, or in EVs isolated from other biofluids, may serve as liquid biopsies for biomarkers for detection of disease, disease progression and for developing intervention strategies at early stages of disease. Such signatures of deimination/citrullination may therefore provide considerable information. Given that PADs are a key modulator of EVs, which are membrane bound vesicles released from cells and carry protein and genetic cargo from the cell of origin and are therefore critical both in cellular communication as well as in disease progression, the roles for PADs and EVs may be closely linked in many pathologies. Furthermore, PAD-activation and resulting protein deimination, alongside EV biogenesis, are both calcium-dependent processes. Indeed, the "citrullinome" of selected neurodegenerative diseases, including MS and AD has for example recently been described to some degree by other groups (Gallart-Palau et al., 2016; Faigle et al., 2019), albeit not in EVs. Our group has recently identified that deiminated protein targets differ in plasma *versus* plasma-EVs in a rat model of early pre-motor PD, shedding light on EV-mediated contributions to early changes in neurodegeneration and associated citrullinome "fingerprinting" (Sancandi et al., 2020). Importantly, deimination signatures of both plasma and plasma-EVs identified in pre-motor PD were found to mirror early deimination changes observed in the brain-vasculature. Such deimination has not previously been described in pre-motor PD brains, until in our recent study, and was prominent in the brain vasculature in cortex, hippocampus and white matter, while deiminated histone H3 levels were significantly elevated in the dentate gyrus and cortex. While histone H3 is involved in gene regulation, it also contributes to extracellular trap formation (ETosis), which can act as a double edged sword in immunity by contributing to damage of own surrounding tissue. Upon analysis of deiminated proteins isolated from plasma and circulating plasma-EVs

from pre-motor PD animals, compared with control animals, KEGG pathways identified for deiminated proteins in the pre-motor PD model were linked to AD, PD, Huntington's disease, prion diseases, as well as for oxidative phosphorylation, thermogenesis, metabolic pathways, *Staphylococcus aureus* infection, gap junction, platelet activation, apelin signaling, retrograde endocannabinoid signaling, systemic lupus erythematosus and non-alcoholic fatty liver disease. These findings indicate PAD-mediated regulation of these pathways in neurodegeneration, both in plasma and via plasma-EVs. Identification of deimination signatures, which in the pre-motor PD model were detectable at early disease stages before motor symptoms, therefore offers a novel non-invasive blood-test which can also mirror the observed early brain vascular changes and reveals blood markers relating to neurodegenerative disease (Sancandi et al., 2020). As early diagnosis in neurodegenerative diseases is of pivotal importance, and biomarkers are scarce, the findings in the pre-motor PD model offer such novel markers and also pave the way for similar investigation in other neurodegenerative diseases, as deimination pathways in the PD model were also found to have some common factors with AD, prion disease, and Huntington's disease (Sancandi et al., 2020). In AD, deimination targets have been identified to be sex related (Gallart-Palau et al., 2016) and such sex differences do warrant further exploration in other models of CNS disease. The role for PADs as regulators of EV release, alongside their roles in a number of neurodegenerative diseases including via the regulation of EVs and modulation of EV-cargo, highlights that this pathway may furthermore play critical roles to the contribution of neurodegenerative disease spread and progression via the prion-theory of EV-mediated distribution of misfolded proteins and other EV-cargo. In addition, PADs can contribute to neuroinflammation through the generation of deiminated neo-epitopes, via modification in gene-regulation caused by histone deimination, as well as histone H3-mediated ETosis.

Lessons from Animal Models

In animal models of acute CNS injury, crucial roles for PADs have been revealed by us in spinal cord injury and associated regenerative capacity (Lange et al., 2011), as well as in neonatal hypoxic-ischemic encephalopathy (HIE) and related brain repair mechanisms (Lange et al., 2014). In the spinal cord, target cells of deimination were identified to be glia, neurones and oligodendrocytes, while detection of deiminated proteins by immunohistochemical analysis, in neonatal HIE, including for pan-deiminated proteins (F95 antibody) and deiminated histone H3, was prominent in hippocampus and cortex, which include main areas affected by this type of insult (Lange et al., 2014). Studies by other groups have assessed PADs and deimination in traumatic and blast brain injury and reported that deimination of specific proteins was selective with respect to cell type and brain region, and may also contribute to autoimmune dysfunction in chronic pathology following head injury, including blast exposure (Lazarus et al., 2015; Attilio et al., 2017). Our studies on a pre-motor PD rat model have furthermore revealed a new brain pathology at this early stage of PD, with significant increase in deiminated proteins detected in the brain vasculature, as well as circulating deiminated proteins in plasma and plasma-EVs relating to neurodegenerative pathways (Sancandi et al., 2020). In addition to using EVs as biomarkers, therapeutic approaches for using mesenchymal stromal/stem cell (MSC) derived EVs for aiding CNS repair have also been assessed (Ophelders et al., 2016; Dabrowska et al., 2019; Kodali et al., 2019), including by our group (Sisa et al., 2020). We showed that MSC application reduced neuroinflammation and provided significant neuroprotection in multiple brain regions and improved behavioral outcomes following severe HIE insult in a neonatal mouse model (Sisa et al., 2020). There is indeed a great interest in the potential of MSC-EVs for immunomodulation and tissue regeneration (Varderidou-Minasian and Lorenowicz, 2020) and such strategies also provide scope for future in-depth studies in relation to CNS diseases and repair.

Peptidylarginine Deiminases and Extracellular Vesicles in Central Nervous System Cancer

A recent focus of our research on PADs in the CNS has also been on cancers of the CNS, in particular glioblastoma multiforme (GBM), the most aggressive adult brain cancer with poor prognosis. We have reported differences in deiminated protein targets according to GBM cell lines, including differences in deimination of proteins involved in cancer progression and invasion. Furthermore, we have shown significant roles for PADs in the regulation and modulation of EV release profiles and on changes in EV-mediated microRNA transport (Kosgodage et al., 2018; Uysal-Onganer et al., 2020). We identified that PAD isozyme-specific expression varies between GBM cell lines, and that this correlates with different protein deimination signatures found between the GBM cell lines, therefore reflecting a fingerprint "citrullinome", depending on brain cancer subtype. Importantly, these findings point to a significant contribution for PADs to the known heterogeneity of GBM tumors. Analysis of deiminated proteins identified that pathways for deiminated proteins relating to cancer, metabolism and inflammation differed between the two GBM cell lines; this included HIF-1-, thyroid hormone synthesis-, viral-, necroptosis-, phagosome-, pentose phosphate-, pyruvate metabolism-, central carbon metabolism- and interleukin-17-KEGG pathways (Uysal-Onganer et al., 2020). Furthermore, we identified that the different PAD isozymes (PAD2, PAD3 and PAD4) differently regulated EV release between GBM cell lines, as well as modulating EV microRNA-cargo relating to oncogenic signatures, and also differently affected GBM cell invasion (Onganer et al., 2020). Interestingly, we also found that the citrullinome of GBM cell lines differed depending on whether they were derived from male or female patients, although low experimental numbers need to be considered (Kosgodage et al., 2018; Uysal-Onganer et al., 2020). To what extent sex differences contribute to GBM heterogeneity, and the associated citrullinome, remains therefore to be further investigated in more GBM cell lines, including primary patient cell lines from different GBM tumors, also accounting for sex.

Peptidylarginine Deiminase Inhibitors

A range of PAD inhibitors have been developed in the previous decade by a number of laboratories for assessment in chronic disease pathologies relating to PADs, including MS, colitis, rheumatoid arthritis and cancers (Mondal and Thompson, 2019). PAD inhibitors tested in relation to CNS disease and repair in our group have mainly focused on the pan-PAD inhibitor Cl-amidine, as well as PAD-isozyme specific inhibitors against PAD2 (AMF30a), PAD3 (Cl4-amidine) and PAD4 (GSK-199), and these pharmacological PAD inhibitors have all been developed by the Thompson laboratory (UMASS Medical School; Mondal and Thompson, 2019). For this purpose, we have carried out a number of both *in vitro* and *in vivo* studies to assess the effects of the PAD inhibitors in relation to different CNS pathologies. Pan-PAD inhibitor Cl-amidine was shown by us to significantly reduce spinal cord damage *in vivo* in a chick model, significantly reducing cell death,

reducing histone H3 deimination and promoting spinal cord regeneration (Lange et al., 2011). Similar protective effects for Cl-amidine were thereafter confirmed in an *in vivo* acute murine CNS injury model of neonatal HIE, also in combination with LPS stimulation, which mimics combinatory bacterial infection with HIE. Following pan-PAD inhibitor treatment, significantly reduced neuronal tissue loss and neuronal cell death, alongside reduced microglial activation and reduction in histone H3 deimination was observed in the brains of Cl-amidine treated HIE animals, compared with sham brains (Lange et al., 2014). In relation to our studies on brain cancers, human *in vitro* models of GBM revealed that pan-PAD inhibitor Cl-amidine modulated EV release and EV-cargo differently, depending on GBM cell lines used, but overall changed EV-cargo to anti-oncogenic signatures. Such effects were also assessed in combination with temozolomide, the main chemotherapeutic agent for GBM, and found to sensitize cells to temozolomide treatment. Furthermore we found that Cl-amidine modulated post-translational deimination levels of histone H3, as well as deimination of prohibitin, a multifaceted protein with roles in mitochondrial architecture and housekeeping (Kosgodage et al., 2018). In a subsequent study, we assessed PAD2, PAD3 and PAD4 specific inhibitors in the same GBM cell lines and showed that these had selective effects on the different GBM cell lines, which also were shown to differ correspondingly in their proportional expression of the different PAD isozymes. The PAD isozyme-specific inhibitors showed cell-line specific effects on GBM invasion ability, selective effects on the modulation of EV release and a shift to more anti-oncogenic signatures of EV-cargo, including the reduction of the pro-oncogenic micro-RNA21, reduction in the hypoxia-related micro-RNA210 and elevation in micro-RNA126, which has been shown to have a positive correlation with improved outcomes in GBM. Therefore, variability in PAD isozyme-specific expression, identified in the different GBM cell lines, may lay the foundations for novel personalized treatment approaches, using selective PAD-isozyme inhibitors, according to GBM subtype.

Future Treatment Approaches with Peptidylarginine Deiminases and Extracellular Vesicles as Targets

Future approaches for clinical PAD inhibitor treatment, both aimed at modulating total deimination via pan-PAD inhibitors, or a narrower range of deimination targets using PAD isozyme-specific inhibitors, still requires further refinement and optimization of PAD-inhibitor treatment. This also refers to testing in more translatable animal models for clinical studies relating to CNS pathologies. The use of deimination signatures in EVs are furthermore a newly identified liquid biopsy tool for early PD diagnosis, prior to motor symptoms, and offers a promising new non-invasive blood test, which can reflect pathological deimination changes in the brain vasculature, indicative also of changes in brain glymphatics. The development of such PAD-related biomarkers in other neurodegenerative diseases is still a relatively unexplored area that warrants more research, based on the clear indications that PADs and deimination are critical factors in neuroinflammation and disease progression. The role for modulation of EV-release and EV-cargo also remains a relatively understudied field (Lange et al., 2017a; Sancandi et al., 2020). Furthermore, the application of MSC-derived EVs is an increasingly growing field of interest and has been shown great promise in a number of CNS injury models (Ophelders et al., 2016; Dabrowska et al., 2019; Kodali et al., 2019; Sisa et al., 2020). Roles for PADs and associated pharmacological manipulation in acute CNS injury also requires further in depth investigation, alongside refined assessment of application at different time windows in relation to different CNS injury scenarios. This also includes effects on PAD-mediated modulation of EVs and identification of EV-specific signatures, which yet have to be assessed in relation to PAD-mediated mechanisms in acute CNS injury and may therefore provide useful information for processes contributing to CNS insult and repair. Furthermore, understanding of the contribution of PADs in pathways relating to chronic neuropathologies in the wake of a primary insult, including those resulting from traumatic and blast head injury, as well as following viral infection also needs to be better understood and may aid the development of novel biomarkers and treatment approaches. In brain cancers, such as GBM, roles for PADs have only recently been identified and furthermore EV regulation in GBM is a current topic of focus, where PAD-mediated EV regulation has only recently been described by our group (Kosgodage et al., 2018; Uysal-Onganer et al., 2020). Therefore, further understanding of PADs and PAD isozyme-specific regulation of protein deimination and downstream processes, including EV release and EV-cargo, warrants further exploration for the development of reliable biomarkers and for effective treatment strategies in GBM, also offering tailored treatment for this heterogeneous brain cancer.

Furthermore, in the current SARS-CoV-2 pandemic, a wide range of unexplained neurological conditions has received increased attention. This includes acute cerebrovascular disease, stroke, encephalopathy, encephalitis, CNS vasculitis and acute neuropathies such as Guillain-Barré syndrome (Ellul et al., 2020; Varatharaj et al., 2020). This has prompted a call out for the need of studies identifying underlying mechanisms (Varatharaj et al., 2020). PADs have recently been assessed by our group in SARS-CoV-2 infected patient biopsies from multiple organs, indicating roles for the different PAD isozymes in the multifaceted symptoms and co-morbidities in COVID-19 (Arisan et al., 2020). Due to the central roles for PADs in the CNS, as discussed in this mini-review, their involvement in SARS-CoV-2, as well as other viral infection related neurological disease symptoms, may be of considerable importance for further investigation, including in relation to longer term neurological outcomes in COVID-19 patients.

Summary and Conclusions

In summary, roles for PADs and downstream processes, including post-translational deimination and modulation of EV release and EV signatures, are of pivotal importance in relation to CNS acute injury, neurodegenerative diseases and CNS cancers. Modulation of PADs, via selective PAD inhibitors, holds much promise for future therapies, both for intervention in acute CNS injury and longer-term chronic neuro-inflammatory conditions, as well as PAD-mediated mechanisms in CNS cancers. Roles for PADs in viral infections which lead to neurological symptoms, including in COVID-19, also need further investigation. The application of MSC-EVs for aiding repair in the injured CNS is also an increasingly attractive treatment strategy option, requiring further in depth investigation for efficient implementation. The use of deimination signatures in biofluids and associated EVs, including from plasma and serum, can furthermore provide reliable non-invasive tests with information aiding early CNS disease diagnosis, reveal information on disease progression and response to therapy.

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