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Neuronal PAD4 expression and protein citrullination: Possible role in production of autoantibodies associated with neurodegenerative disease

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A B S T R A C T

Peptidyl arginine deiminases (PADs) catalyze a post-translational protein modification reaction called citrullination, where arginine is converted to citrulline. This modification has been linked to the pathogenesis of autoimmune diseases including rheumatoid arthritis (RA). More recently, several studies have suggested that Alzheimer's disease (AD), a devastating neurodegenerative disorder, may have an autoimmune component. In the present study, we have investigated the possibility that expression of PADs and protein citrullination plays a role in the production of brain-reactive autoantibodies that may contribute to Alzheimer's-related brain pathology. Here, we report the selective expression of the PAD isoforms, PAD2 and PAD4, in astrocytes and neurons, respectively, and the concomitant accumulation of citrullinated proteins within PAD4-expressing cells, including neurons of the hippocampus and cerebral cortex. Expression of PADs and citrullinated proteins is prominent in brain regions engaged in neurodegenerative changes typical for AD pathology. Furthermore, we also demonstrate that the pentatricopeptide repeat domain2 (PTCD2) protein, an antigen target of a prominent AD diagnostic autoantibody, is present in a citrullinated form in AD brains. Our results suggest that disease-associated neuronal loss results in the release of cellular contents, including citrullinated proteins, into the brain interstitium. We propose that these citrullinated proteins and their degradation fragments enter into the blood and lymphatic circulation, and some are capable of eliciting an immune response that results in the production of autoantibodies. The long-term and progressive nature of AD and other neurodegenerative diseases results in chronic exposure of the immune system to these citrullinated products and may drive the continual production of autoantibodies.

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1. Introduction

Citrullination (also called deimination) is a post-translational protein modification catalyzed by PADs that involves conversion of the amino acid arginine to citrulline within proteins [1–5]. The involvement of citrullinated proteins in the pathogenesis of a number of autoimmune diseases has been well established [2,5–9]. For example, protein citrullination has been directly linked to the generation of autoantibodies contributing to RA [2,3,5,10–14]. Although the exact circumstances and underlying purpose of PAD activation and consequent protein citrullination within cells are not fully understood, the specific autoimmune response to citrullinated proteins is now recognized to be a key component of RA [3,5,8,14,15]. In addition, the production of autoantibodies targeting citrullinated proteins has been implicated in many other autoimmune diseases such as multiple sclerosis (MS), psoriasis, sporadic Creutzfeld–Jakob disease (CJD), Parkinson's disease (PD) [2,5,7,16–21] and AD [22].

AD is a devastating and progressive neurodegenerative disorder characterized pathologically by the neuronal accumulation of beta-amyloid peptides, the appearance of amyloid plaques and neurofibrillary tangles and reactive gliosis within the brain parenchyma [23–27]. A study demonstrating the co-localization of citrullinated proteins and PAD2 in the hippocampus of AD brains [22] has raised the possibility that citrullinated proteins are somehow involved in AD pathogenesis. In previous studies, we have demonstrated the ubiquitous presence of brain-reactive autoantibodies in human sera [28] and have provided evidence that the chronic binding of

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these autoantibodies to selected neurons in the brain may contribute to AD pathogenesis, especially in brain regions with compromised blood–brain barrier (BBB) integrity [29]. More recently, we have also demonstrated the presence of numerous autoantibodies in the serum, some of which are useful as biomarkers for detection and diagnosis of this disease as well as Parkinson's disease [30,31]. However, despite their widespread presence in the blood, the origin and purpose of these autoantibodies are still unknown.

We have hypothesized that protein citrullination plays a role in the generation of autoantibodies during the pathogenesis of specific neurodegenerative diseases such as AD. In the present study we report the selective expression of the PAD isoforms, PAD2 and PAD4, and the concomitant accumulation of citrullinated proteins within neurons of the cerebral cortex and hippocampus, two brain regions that are well-known to be particularly vulnerable to AD pathology [32–34]. Since citrullination alters the overall charge distribution within a protein, potentially modifying tertiary structure, many citrullinated proteins in these PAD-expressing cells may be in a non-native conformation that is potentially immunogenic, especially if they are released from the cell and thus become accessible to immune surveillance [1,5,35,36]. In support of this scenario, we demonstrate here that the target antigen of an autoantibody that we have shown to be a useful diagnostic biomarker for both AD and Parkinson's disease [30,31], the PTCD2 protein, is often present in its citrullinated form in the AD brain. Taken together, these results support the notion that at least some of the disease-associated autoantibodies [29–31] present in human sera are generated as a consequence of the production and release of citrullinated proteins and their fragments, most likely from damaged and/or dying cells, in regions of pathology [29].

2. Materials and methods

2.1. Human brain tissues

Entorhinal cortex and hippocampal brain regions from sporadic AD [$n = 26$, age range yrs = 70–96, average age = 80.6 yrs] and age-matched control [$n = 29$, age range yrs = 44–93, average age = 74 yrs] with postmortem interval <24 h were selected for this study. The AD brain tissues used in the study matched the criteria defined by the National Institute on Aging and the Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of AD. The age-matched control tissues used exhibited minimal AD-associated pathology. Tissues were obtained from the Harvard Brain Tissue Resource Center (Belmont, MA), Slidomics (Cherry Hill, NJ), Analytical Biological Services Inc. (Wilmington, DE) and Cooperative Human Tissue Network (Philadelphia, PA). The formalin-fixed tissues were processed using routine histological procedures while the frozen brain tissues were maintained at −80 °C. Five micrometer thick tissue sections were cut and mounted onto Superfrost® Plus microslides (VWR International, West Chester, PA).

2.2. Antibodies

Amyloid-beta 1-42 (Ab42) antibody was obtained from Millipore (Temecula, CA) (polyclonal, Cat. No. AB5078P, dilution = 1:50). Antibodies to PAD4 (polyclonal, Cat. No. ab38772, dilution = 1:600), PAD2 (polyclonal, Cat. No. ab56928, dilution = 1:150) and citrulline (polyclonal, Cat. No. ab6464, dilution = 1:1000) were obtained from Abcam (Cambridge, MA). Antibodies to glial fibrillary acidic protein (GFAP) and human leukocyte antigen-DR (HLA-DR) were also obtained from Millipore and used at dilutions recommended by the manufacturer.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin-embedded tissue sections. Briefly, paraffin-embedded tissues were first deparaffinized using xylene and subsequently rehydrated. Antigenicity was enhanced by heating the sections in a citrate buffer for 4 min at low heat. Sections were cooled for 10 min and then dipped in 3.0% hydrogen peroxide for 10 min to remove endogenous peroxidase activity. After washes with distilled water and phosphate-buffered saline (PBS), sections were blocked for 30 min using an appropriate dilution of sera in which secondary antibody was generated. Sections were then treated for an hour with primary antibody at room temperature and washed twice with PBS. Subsequently, sections were treated with an appropriate dilution of biotin-labeled secondary antibody for 30 min, rinsed in PBS, treated with avidin–peroxidase complex (Vectastain ABC Elite, Vector Laboratories Inc., Foster City, CA) for 30 min and again washed with PBS. The sections were finally visualized with 3-3-diaminobenzidine (DAB) [Dako, Carpinteria, CA, DAB chromogen system, Code K3468] and briefly stained with hematoxylin. Sections were then dehydrated in increasing concentrations of ethanol, cleared in xylene and mounted in Permount. Control sections were treated with nonimmune serum instead of primary antibody or with secondary antibody only. A Nikon FXA microscope and Nikon DXMI1200F digital camera were used to examine and photograph the samples.

2.4. Total protein extraction

Ice-cold RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), protease inhibitor cocktail (0.5 ml per gm of brain tissue, Sigma, St. Louis, MO) and PMSF (phenylmethanesulfonylfluoride, 1 mM) were added to a pre-cooled Dounce glass homogenizer. From each cortical tissue sample, small sections from different areas of the available tissue were dissected, weighed, added to the pre-cooled homogenizer and subsequently homogenized. The homogenized tissue sample was left on ice for 30 min and centrifuged at 14,000 rpm at 4 °C for 15 min. The supernatant thus separated was used as the total protein fraction. Total protein concentration was estimated using the Bradford assay.

2.5. Western blotting

To study the expression and citrullination of PTCD2, western blotting was carried out using the Mini PROTEAN 3 System (165-3302, BioRad, Hercules, CA). Total brain protein samples were first separated by running on 12% SDS-polyacrylamide separating gels and then was normalized based on β-actin expression. Normalized samples were then run on the gel at 115 V alongside PageRuler™ Prestained Protein Ladder Plus (SM 1811, Fermentas, Glen Burnie, MD). Separated proteins were transferred to Hybond-ECL Nitrocellulose Membrane (GE Healthcare Amersham™ Hybond™ – ECL) for 45 min at 100 V. After transfer, blots were treated with blocking solution [5% non-fat dried milk prepared in PBS–Tween20 (PBS-T, Sigma, St. Louis, MO)] for an hour at room temperature and then probed with primary antibody overnight at 4 °C. After rinsing thoroughly with PBS-T, blots were treated with peroxidase-conjugated secondary antibody for 1 h at 4 °C. After several washes with PBS-T and distilled water, blots were developed using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo scientific/Pierce, IL, USA). To probe with another set of antibodies, the blot was stripped using stripping buffer (100 mM 2-mercaptoethanol, 2% (W/V) SDS, 62.5 mM Tris–HCl, pH 6.7) for 40 min at 50 °C. After thorough washes with PBS-T, blots were

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