Neuroscience Letters 545 (2013) 107–111

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Dual immunofluorescence study of citrullinated proteins in Alzheimer diseased frontal cortex

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HIGHLIGHTS

- Increased deiminated proteins were seen in astrocytes of Alzheimer frontal cortex.
- Increased deiminated proteins were seen in Alzheimer brain blood vessels.
- Increased deiminated proteins were seen in Alzheimer amyloid and neuritic plaques.
- Deiminated proteins were seen in nuclei of phosphorylated tau-labeled neurons.

ARTICLE INFO

Article history:
Received 15 March 2013
Accepted 5 April 2013

Keywords:
Amyloid beta
Citrulline
Deimination
Glial fibrillary acidic protein
Peptidyl arginine deiminase
Tau

ABSTRACT

Deimination is a post-translational modification of proteins in which selected arginine amino acids are enzymatically converted to citrullines. Using dual-color immunofluorescence, the present study is the first to examine the frontal cortex of patients with Alzheimer's disease (AD) *versus* age-matched controls with an established monoclonal antibody (F95) against peptidyl-citrulline moieties. In AD specimens, a number of new findings were discovered, including evidence for deiminated proteins in extracellular plaques, the walls of large blood vessels, the nuclei of selective neurons immunoreactive for phosphorylated tau and numerous reactive astrocytes concentrated around extracellular plaques, ventricular surfaces and at the interface between the gray and white matter of the cortex. Although the identities of these citrullinated proteins remain largely unknown, the present study adds to the growing number of locations in which deiminated proteins may be found in the brains of patients with AD.

1. Introduction

Deimination is a calcium-mediated, post-translational modification in which arginine amino acids in selected proteins are converted to citrullines through the peptidyl arginine deiminase (PAD) family of enzymes. In the central nervous system, it was previously believed that the vast majority of protein deimination takes place in glial cells using the PAD-2 isozyme [2–4,34], yet a number of studies now provide evidence that proteins in neuronal cells may also be deiminated [1,3,4,18,26,27]. Our laboratory has shown that both myelin basic protein and glial fibrillary acidic protein (GFAP) are deiminated under non-pathologic conditions in rodent [27] and human brains [30]. However, there is mounting evidence to suggest that deimination is increased in various pathologic conditions affecting the nervous system, including glaucoma [7], prion disease [15,16], hypoxia [3,32], peripheral nerve injury [18], spinal cord neural regeneration [19], Parkinson's disease [26], Marburg disease [36], multiple sclerosis [23,24,29] and experimental autoimmune encephalomyelitis [12,28,31].

In Alzheimer's disease (AD), increased PAD-2 and deiminated protein immunoreactivities were shown in hippocampal astrocytes of human AD patients compared to non-demented elderly controls [14]. Using an antibody that recognizes peptidyl-citrulline moieties after they have been chemically modified, along with 2-dimentional gel electrophoresis and mass spectrometry, the first deiminated proteins identified as being increased in AD hippocampus were GFAP and vimentin [14]. In a more recent study using a different anti-citrulline antibody, penta-tricopeptide repeat domain-2 (PTCD2) was identified as another protein present in the citrullinated form in AD brains [1]. Additionally, both PAD-2 in astrocytes and PAD-4 in neurons were reportedly seen in AD hippocampus and entorhinal cortex [1]. In contrast, our laboratory

Abbreviations: AD, Alzheimer's disease; PAD, peptidyl arginine deiminase; GFAP, glial fibrillary acidic protein; PTCD2, penta-tricopeptide repeat domain-2; PBS, phosphate buffered saline; Aβ, amyloid beta peptide; P-tau, phosphorylated tau; HRP, horseradish peroxidase; DAB, diaminobenzadine; TSA, tyramide signal amplification; FITC, fluorescein isothiocyanate.

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http://dx.doi.org/10.1016/j.neulet.2013.04.028

used a well-established monoclonal antibody (referred to as F95) against peptidyl-citrulline [30] in the present study. The results of these experiments suggest that the number of deiminated proteins in AD brains should be expanded further.

2. Materials and methods

Formalin-fixed, paraffinized samples of frontal cortex were obtained post-mortem from the Brain Donation Program Bank associated with the Alzheimer's Disease Research Center at the University of Alabama at Birmingham (UAB). All patients that provided brain tissue did so with written, pre-mortem, informed consent in accordance with the Declaration of Helsinki. A total of 13 blocks were immunohistochemically examined, consisting of 5 age-matched controls without AD and 8 patients with a pre-mortem clinical diagnosis of AD that was verified post-mortem using established histopathological criteria [11].

These samples were cut (5 μm) with an Microm HM 355S sliding microtome, adhered to plus-coated glass slides (Fisher) and deparaffinized in Citrisolv clearing agent (Fisher) and isopropanol. These sections were then prepared for antigen retrieval using citrate buffer (pH 6.0) steaming, followed by oxidation with 3% hydrogen peroxide. They were then incubated in auto-fluorescence eliminator solution (Sigma) containing Sudan black [6], followed by washes in 70% ethyl alcohol and phosphate buffered saline (PBS; pH 7.2). Any specimen still containing auto-fluorescence subsequently was not used.

All sections were then placed in a blocking solution containing 3% BSA and 5% non-fat powdered skim milk in PBS with 0.3% Triton X-100 and then incubated overnight at 4 °C in 1:1000 dilutions of blocking solution containing the IgM F95 monoclonal antibody prepared from mouse ascites [18], or IgG mouse monoclonal antibodies against human amyloid beta peptide (Aβ) 1-42 (Signet), GFAP (Millipore), phosphorylated tau (P-tau; Millipore) or the neuronal marker NeuN (Pierce). The next day, sections processed only for F95 immunoperoxidase staining were washed with PBS, and then incubated in Immunopure Peroxidase Suppressor (Pierce) for 30 min followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Southern Biotechnology Associates, Inc.) diluted 1:50 in PBS with 0.3% Triton X-100 for 1 h at 37 °C. The sections were then washed with PBS and placed in 0.1 M acetate buffer (pH 6.0). The slides were then incubated in a nickel-intensified diaminobenzadine (DAB) solution consisting of 0.035% DAB, 2.5% nickel ammonium sulfate and 0.01% hydrogen peroxide in acetate buffer for 5–15 min until immunoreactive structures stained black, with the optimum signal to noise ratio determined visually. Controls were performed in similar sections by eliminating the primary or secondary antibody.

For dual-color immunofluorescence, similar sections were instead incubated for 1 h at 37 °C in a 1:1000 dilution of blocking solution containing HRP-conjugated donkey secondary antiserum (Jackson ImmunoResearch). Tyramide signal amplification (TSA) was then employed (TSA Plus Fluorescence System, Perkin Elmer) utilizing a 30-min incubation of TSA Plus Cy3 (1:500), resulting in F95 immunolabeled structures appearing red. In contrast, Aβ, GFAP, NeuN and P-tau immunofluorescence utilized a 30-min incubation of TSA Plus FITC (fluorescein isothiocyanate; 1:500), which appeared green.

All slides were then coverslipped with Vectashield® (Vector) and examined with a Nikon E-800 microscope. Digital images of this material were transferred to a Dell computer using a super-cooled SPOT™ color video camera (Diagnostic Instruments, Inc.). Adobe Photoshop CS software was used to save and merge the fluorescent images, as well as to optimize the contrast and brightness of the histological structures without altering the content.

3. Results

Using nickel-intensified DAB as the chromogen and F95 as the primary antiserum, immunoreactive staining for citrullinated proteins in the frontal cortex of AD patients showed numerous patches of extracellular staining reminiscent of amyloid plaques (Fig. 1 A and B). Often surrounding these structures were cells with the morphology of reactive astrocytes (Fig. 1B). However, similar F95-stained cells were also found concentrated at the border between the gray and white matter (Fig. 1C) and no similar F95 staining was seen in age-matched controls (Fig. 1D).

Using dual-color immunofluorescence, F95 staining for deiminated proteins was often co-localized with immunoreactivity for human Aβ 1-42 in extracellular plaques (Fig. 2A) and within reactive astrocytes that were adjacent to small intraparenchymal blood vessels (Fig. 2B). Although F95 and Aβ immunoreactivities were also both observed in larger extraparenchymal blood vessels, little if any co-localization was seen in these structures (Fig. 2C).

When F95 immunofluorescence in AD frontal cortex was combined with that of GFAP (Fig. 3A and B), NeuN (Fig. 3C and D) or P-tau (Fig. 3E and F), additional observations were made. Evidence for deiminated GFAP in astrocytes was often seen (Fig. 3A and B), but the co-localization of F95 and GFAP was especially concentrated around extracellular plaques and at the ventricular surface (Fig. 3B). Reactive astrocytes containing deiminated protein were also concentrated in the AD frontal cortex just deep to the cortical ribbon (Fig. 3C and D). Although theses cells and Neu-N positive neurons were closely adjacent, they primarily remained in separate cellular populations. Although in most instances deiminated proteins in AD frontal cortical cells were primarily seen in astrocytes, few exceptions were sometimes observed. For example, F95 immunoreactivity was occasionally seen in the cytoplasm (Fig. 3A) and nuclei (Fig. 3B and F) of cells that were not astrocytes. Using dual-color immunofluorescence of F95 and P-tau, no convincing co-localization was seen between these two immunoreactivities, although deiminated proteins appeared to be present in astrocytes surrounding neuritic plaques that also contained deiminated proteins (Fig. 3E). However, occasionally neurons that expressed immunofluoresence for P-tau in the cytoplasm, also expressed F95 immunoreactivity for deiminated proteins in their nuclei (Fig. 3F).

4. Discussion

Including this report, there are now three studies examining deimination in AD brains and their collective findings are summarized in Table 1. The first experiments analyzed AD hippocampus *versus* age-matched controls [14] and utilized a rabbit IgG against peptidyl-citrulline after being chemically modified [33]. Often referred to as the "mod-cit" antibody, this immunoglobulin is commercially available (Upstate/Millipore). A second more recent study [1] examined both hippocampus and entorhinal cortex of AD patients and age-matched controls using another polyclonal IgG antibody against gluteraldehye-conjugated citrulline that is also commercially available (Abcam). However, using another, well-established IgM monoclonal antibody (referred to as F95) that reacts with multiple synthetic and naturally citrullinated proteins [30], the present study is the first to describe the anatomical locations of deiminated proteins in the frontal cortex of AD patients.

Although previous reports have shown that increased PAD-2 [1,14] and protein deimination [14] are seen in large reactive astrocytes of human AD brains, the present study confirms these findings, but also provides further information regarding the location of these cells. For example, astrocytes containing deiminated proteins were observed around areas containing amyloid plaques [1,14], but were also quite prominent at the junction between