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Reactive protoplasmic and fibrous astrocytes contain high levels of calpain-cleaved alpha 2 spectrin

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ABSTRACT

Calpain, a family of calcium-dependent neutral proteases, plays important roles in neurophysiology and pathology through the proteolytic modification of cytoskeletal proteins, receptors and kinases. Alpha 2 spectrin (αII spectrin) is a major substrate for this protease family, and the presence of the αII spectrin breakdown product (αII spectrin BDP) in a cell is evidence of calpain activity triggered by enhanced intracytoplasmic Ca^{2+} concentrations. Astrocytes, the most dynamic CNS cells, respond to micro-environmental changes or noxious stimuli by elevating intracytoplasmic Ca^{2+} concentration to become activated. As one measure of whether calpains are involved with reactive glial transformation, we examined paraffin sections of the human cerebral cortex and white matter by immunohistochemistry with an antibody specific for the calpain-mediated αII spectrin BDP. We also performed conventional double immunohistochemistry as well as immunofluorescent studies utilizing antibodies against αII spectrin BDP as well as glial fibrillary acidic protein (GFAP). We found strong immunopositivity in selected protoplasmic and fibrous astrocytes, and in transitional forms that raise the possibility of some of fibrous astrocytes emerging from protoplasmic astrocytes. Immunoreactive astrocytes were numerous in brain sections from cases with severe cardiac and/or respiratory diseases in the current study as opposed to our previous study of cases without significant clinical conditions that failed to reveal such remarkable immunohistochemical alterations. Our study suggests that astrocytes become αII spectrin BDP immunopositive in various stages of activation, and that spectrin cleavage product persists even in fully reactive astrocytes. Immunohistochemistry for αII spectrin BDP thus marks reactive astrocytes, and highlights the likelihood that calpains and their proteolytic processing of spectrin participate in the morphologic and physiologic transition from resting protoplasmic astrocytes to reactive fibrous astrocytes.

1. Introduction

Astrocytes, originally thought to be the structural bedrock of the CNS, are the most dynamic among various CNS cells. Now recognized as playing a critical role in the development of the CNS, astrocytes also support the homeostatic balance of neurotransmitters and ions in the CNS microenvironment, and contribute to synaptic plasticity and the regulation of blood flow in the CNS. Astrocytes are coupled with other astrocytes through gap junctions (Giaume and Venance, 1998), and secondary intracellular calcium elevations are noted in astrocytes due to an activation of adjacent neurons (Bernardinelli et al., 2011). The glioneuronal relationship is so intimate that human protoplasmic astrocytes each harbor as many as two million synapses (Oberheim et al., 2009).

Astrocytes are extremely sensitive to various noxious stimuli, and they readily transform themselves to reactive cells upon stimulation (Sofroniew, 2014). This transformation is partially mediated by increased intracytoplasmic Ca^{2+} concentration (Du et al., 1999, Colangelo et al., 2014) accompanied by gliotransmitter release. These events may presage hyperexcitability, excitotoxicity and neurodegeneration (Agulhon et al., 2012). During astrocytic activation, calpain, a calcium dependent protease, may be triggered to participate in this reactive process. A major substrate of calpain is αII spectrin (Harris et al., 1988) although other cytoskeletal proteins and cellular enzymes are also among the targets of calpain (Wang and Yuen, 1994).

Spectrin is a highly conserved cytoskeletal and membrane scaffolding protein that provides stability and organization to the cell membrane and contributes to many cellular processes including membrane biogenesis, membrane remodeling, signal transduction, and the maintenance of membrane organization and viability (De Matteis and Morrow, 2000; Fletcher et al., 2015; Machnicka et al., 2012; Morrow et al., 1997; Stankewich et al., 2011). In astrocytes it has been demonstrated that αII spectrin can serve as a molecular bridge between Ca^{2+} signaling complexes in the ER and microdomains in the plasma membrane (Lencesova et al., 2004). Seven spectrin genes are recognized in humans; two encode alpha spectrins (αI and αII); five encode beta spectrins (βI-βV) (Bennett and Healy, 2009; Stankewich et al., 2010,

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Table 1
List of cases with clinical history.

Case	Age	Sex	PIH	Clinical Conditions
1	71	F	24	Severe COPD, Aortic dissection with cardiac tamponade
2	28	F	17	Pneumonia with massive pulmonary fibrosis
3	81	F	18.3	Acute myocardial infarction with severe ASCVD
4	31	F	7.1	AIDS, Pulmonary hypertension, Status epilepticus
5	12	F	24	Delayed death after drowning
6	44	M	17	Non-Hodgkin's lymphoma with congestive heart failure
7	56	M	19	AIDS, Pneumonia

Note: PIH: Postmortem interval hours, COPD: Chronic obstructive pulmonary disease, ASCVD: Arteriosclerotc cardiovascular disease, AIDS: Acquired immune deficiency syndrome.

2011). The most abundant and ubiquitous form in the brain is αIIβII spectrin. The functional unit of spectrin is typically a heterodimer, although homopolymeric forms composed of just a beta spectrin have been detected (Papal et al., 2013; Pumplin, 1995). Spectrins of one form or another are associated with many membrane compartments in the cell, and are most abundant on the plasma membrane (De Matteis and Morrow, 2000). The genetic or acquired loss of spectrin underlies several neurologic and hematologic disorders (Berghs et al., 2001; Dick et al., 2012; Gallagher et al., 1997; Stankewich et al., 2010, 2011; Tohyama et al., 2015).

The αII subunit of spectrin is exquisitely sensitive to calpain-mediated proteolysis, resulting in the production of alpha II spectrin breakdown product (αII spectrin BDP), and the presence in a cell of αII spectrin cleaved between Tyr_{1176} and Gly_{1177} is a reliable marker of calcium-activated calpain action (Harris et al. 1989). This action is also regulated at the substrate level by calmodulin (Harris and Morrow, 1990) and by tyrosine phosphorylation (Nedrelow et al., 2003; Nicolas et al., 2002). Through such proteolytic modification of membrane-associated proteins, calpains appear to play a central role in neuronal physiology and pathology, including processes involved with synaptic remodeling, memory and long-term potentiation. Excessive activation of calpains also results in cell death (Glantz and Morrow, 1996; McGinn et al., 2009; Nath et al., 1996; Vanderklish and Bahr, 2000), and based on the interaction between αII spectrin and calpains, tissue degradation is monitored in clinical specimens (Sun et al., 2015).

In the present study, we use an affinity-purified epitope-specific antibody that recognizes the novel COOH-terminus created at Tyr_{1176} in αII spectrin by calpain cleavage (Harris et al., 1988; Glantz et al., 2007) to monitor this event in astrocytes in human brain sections. We find that this cleavage is a sensitive marker of astrocyte activation, making it both a useful reagent for the detection of such occurrences, as well as establishing a close temporal relationship between αII spectrin proteolysis and glial activation.

2. Materials and methods

Included for this study are the cerebral cortex and subcortical white matter from seven postmortem brains from two men and five women. All samples were obtained at autopsy under institutional Human Investigation Committee approval. The list of patients is shown in Table 1.

After removal, brains were fixed in 4% buffered formaldehyde for 2 weeks before sampling. Blocks of the cerebral gray and white matter from formalin-fixed brains were dehydrated and embedded in paraffin. For immunohistochemistry, six micrometer-thick paraffin sections of the brain were deparaffinized and subjected to antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 3 min, followed by cooling to RT and rinsing. The sections were covered with 2% normal goat serum for 30 min, then incubated overnight with affinity purified rabbit anti-αII spectrin BDP antibody (1:500 dilution in 1% BSA, 1.0μg/ml) at 4 °C. The antibody used for this study was a polyclonal cleavage-specific anti-αII-spectrin BDP antibody prepared against the peptide CQQEVY that recognizes only the calpain cleavage product of αII spectrin encompassing amino acids 1–1176 (Glantz et al., 2007). Sections were rinsed with tris-buffered saline (TBS) and incubated with biotinylated goat anti-rabbit antibody (K & P Labs, Gaithersburg, MD, #71–00-30) for one hour. They were rinsed with TBS, followed by incubation with streptavidin-alkaline phosphatase complex (K & P Labs, Gaithersburg, MD, #71–00-45) for one hour. Immunoreactivity of the tissue was evaluated after treatment with neutral red (Vector, Burlingame, CA, #SK-5100). Most sections were counter-stained with hematoxylin.

For double immunostaining, the above procedure was performed in new slides for αII spectrin BDP immunohistochemistry as described above without counter-staining. Slides were rinsed in TBS for two hours, and were incubated with monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Sigma, Saint Louis, MO, #G3893, 1:400 dilution) overnight. The slides were rinsed in TBS for five minutes twice, which was followed by incubation with streptavidin peroxidase (Vector, Burlingame, CA, #SA-5004). Slides were washed in TBS for 5 min twice, and were treated with Vector SG solution (Vector, Burlingame, CA, #SK-4700) for three minutes. The slides were rinsed in water and were dehydrated before cover-slip mounting.

For immunofluorescent study, tissue in paraffin was prepared for antigen retrieval as above and incubated with the primary antibodies to αII spectrin-BDP and GFAP diluted in 2%BSA/0.1% saponin. After an overnight incubation at 4 °C, tissue was washed and treated with anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa dyes diluted 1:1000 for 1 h at room temperature rabbit and mouse secondary antibodies conjugated to the Alexa flours 488 and 594, respectively, for 1 h. To visualize nuclei, tissue was incubated with Hoechst dye (1:5000) (Invitrogen) for 10 min. Target or counterstained molecules were visualized and captured with an Olympus AX70 fluorescent microscope with a Hamamatsu digital camera. Image acquisition was processed using OpenLab software (Improvision Inc., Lexington, MA).

For negative controls of paraffin sections, tissue sections were incubated with normal rabbit serum in place of the primary antibody, and the rest of procedures were the same as above.

3. Results

The mean age of the patients was 46.1 years, ranging from 12 to 81 years. All seven patients experienced fulminant clinical courses terminally that were associated with considerable cardiac and/or respiratory complications (Table 1). The mean postmortem interval before fixation of the brain in formalin was 18.1 h, ranging from 7.1 h to 24 h.

Paraffin sections of the cerebral cortex and white matter revealed frequent αII spectrin BDP immunopositive astrocytes on single conventional immunohistochemistry (Fig. 1), whereas double immunohistochemistry for αII spectrin BDP and GFAP disclosed astrocytes with mixed reactions on both Conventional double immunohistochemistry and immunofluorescent study (Fig. 2).

3.1. Immunohistochemistry for αII spectrin BDP

Scattered cells in the cortex showed αII spectrin BDP immunopositive primary processes emanating from their perikarya. The primary processes were also decorated with delicate, fuzzy

Fig. 1. Immunohistochemistry for αII spectrin BDP in the cerebral cortex and white matter. A. The cerebral cortex with a typical protoplasmic astrocyte (arrow) and two transitional astrocytes (arrowheads), the latter showing larger nuclei and a varying degree of loss of tuft-like processes (Bar = 50um). B. The subpial region of the cortex is occupied by fibrous astrocytes with sparse tapering processes (Bar = 50um). C. Fibrous astrocytes are present in the white matter (Bar = 50um). D through I. Various forms of astrocytes are stained with αII spectrin BDP (Bar = 25um). Classical protoplasmic astrocytes (D, E) as well as transitional astrocytes still with remnants of original tufts (F-H) are seen. What appears to be a fibrous astrocyte is surrounded by faintly visible, speckled remnants of tufts (I). Arrows (D, G, H) show neurons partially or entirely hugged by astrocytic processes.