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Research report

F-actin depolymerization accelerates clasmatodendrosis via activation of lysosome-derived autophagic astroglial death

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ABSTRACT

Clasmatodendrosis is an irreversible astroglial degenerative change, which includes extensive swelling and vacuolization of cell bodies and disintegrated and beaded processes. Since alteration in F-actin level influences on the formation of vacuoles/vesicles during exocytosis/endocytosis in astrocytes, we investigated whether F-actin polymerization involves clasmatodendrosis in the rat hippocampus following status epilepticus (SE). In the present study, vacuoles in clasmatodendrotic astrocytes showed LAMP-1 and LC3-II (a marker for autophagy) immunoreactivity. These findings reveal that clasmatodendrosis may be lysosome-derived autophagic astroglial death. Jasplakinolide (an F-actin stabilizer) infusion significantly decreased the size and the number of medium/large-sized vacuoles in each clasmatodendritic astrocyte accompanied by enhancement of phalloidin signals, as compared to vehicle-infusion. In contrast, latrunculin A (an F-actin-depolymerizing agent) infusion increased the size and the number of medium/large-sized vacuoles, which were dissociated adjacent to cell membrane. Therefore, our findings suggest that F-actin stabilization may inhibit lysosome-derived autophagic astroglial death during clasmatodendrosis.

1. Introduction

Astrocytes play a role in maintenance of homeostasis in the brain by regulating local ion concentrations, pH and clearance of neurotransmitters released into the synaptic cleft [12]. Furthermore, astrocytes release many neuroactive substances, such as chemical transmitters, cytokines, neuropeptides and growth factors [6,35]. Astroglial vacuolization is considered as irreversible coagulative necrotic changes [33,19,16]. This fundamental change of irreversibly injured astrocytes was first report by Alzheimer in 1910, which includes extensive swelling and vacuolization of cell bodies and disintegrated and beaded processes, and termed "clasmatodendrosis" by Cajal [29]. This irreversible astroglial changes is directly relevant to dysfunction of these cells.

Reactive astrocytes upregulate high glial fibrillary acidic protein (GFAP) and their cell bodies hypertrophy, and begin to proliferate, migrate and form glial scars [24,31,9]. In contrast to naive astrocyte, reactive astrocytes also up-regulate F-actin [3,13,1], since the F-actin is responsible for the motility and morphogenesis in astrocytes [11,7,8], which are important in the migration of the astrocytes and increased plasticity of the processes [36,10,26]. Since alteration in F-actin level influences on the formation of vacuoles/vesicles during exocytosis/endocytosis in astrocytes [14], the present study was thus designed to elucidate whether F-actin polymerization involves clasmatodendrosis in the rat hippocampus following status epilepticus (SE).

2. Materials and methods

2.1. Experimental animals and chemicals

This study utilized the progeny of Sprague-Dawley (SD) rats obtained from Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions (22 ± 2 °C, 55 ± 5% and a 12:12

Abbreviations: GFAP, glial fibrillary acidic protein; PBS, phosphate-buffered saline; SD, Sprague-Dawley; SE, status epilepticus.

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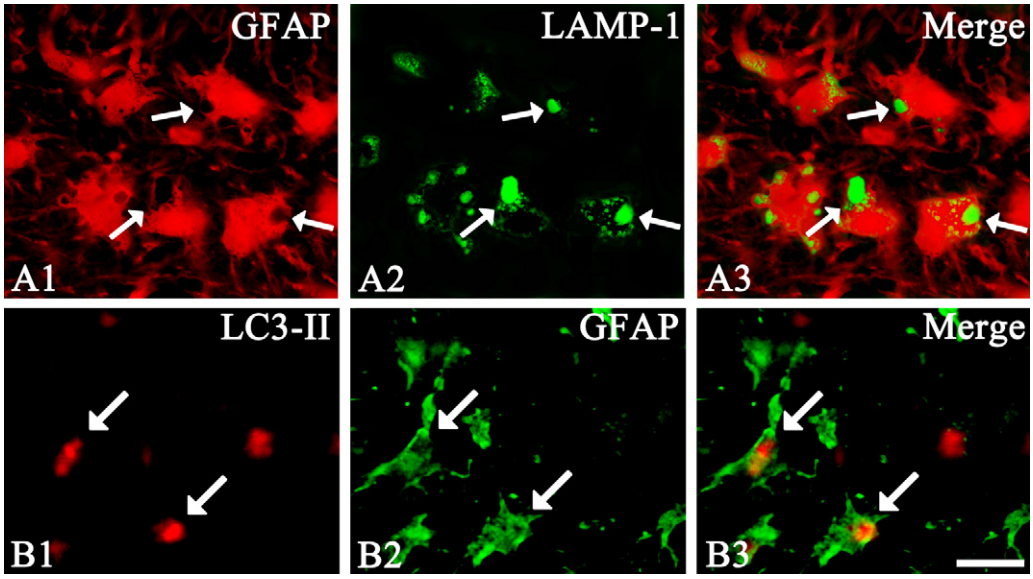

Fig. 1. Clasmatodendrosis is autophagic astroglial death. (A) Double staining for GFAP and LAMP-1. Vacuoles in astrocytes contain LAMP-1 immunoreactivity (arrows). (B) Double staining for LC3-II and GFAP. Vacuoles in astrocytes show LC3-II immunoreactivity (arrows). Bar = 25 μm.

light/dark cycle with lights). Procedures involving animals and their care were conducted in accord with our institutional guidelines that comply with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, 1996). In addition, all possible efforts were taken to avoid animals' suffering and to minimize the number of animals used at each stage of the experiment. All reagents were obtained from Sigma–Aldrich, except as noted.

2.2. Seizure induction

Male SD rats (9–11 weeks old) were treated with pilocarpine (380 mg/kg, i.p.) at 20 min after atropine methylbromide (5 mg/kg, i.p.). Following pilocarpine injection, animals showed acute behavioral features of status epilepticus (including akinesia, facial automatisms, limbic seizures consisting of forelimb clonus with rearing, salivation, masticatory jaw movements, and falling). Diazepam (10 mg/kg, i.p.) was administered 2 h after onset of SE and repeated, as needed. Age-matched animals ($n = 5$) were used as normal control.

2.3. Intracerebroventricular drug infusion

Four weeks after SE, rats were divided into three groups ($n = 5$, respectively): Vehicle (0.1% DMSO/saline, v/v), latrunculin A (an F-actin-depolymerizing agent, 2 μg/ml in vehicle), jasplakinolide (an F-actin stabilizer, 2 μg/ml in vehicle) treated animals. The dosage of each compound was determined as the highest dose that did not affect seizure threshold in the preliminary study. Animals were anesthetized (Zolretil, 50 mg/kg, I.M. Virbac Laboratories, France) and placed in a stereotaxic frames. For the osmotic pump implantation, holes were drilled through the skull for introducing a brain infusion kit 1 (Alzet, USA) into the right lateral ventricle (1 mm posterior; 1.5 mm lateral; −3.5 mm depth; flat skull position with bregma as reference), according to the atlas of Paxinos and Watson [27]. The infusion kit was sealed with dental cement and connected to an osmotic pump (1007D, Alzet, USA). The pump was placed in a subcutaneous pocket in the dorsal region. Animals received 0.5 μl/h of vehicle or compound for 1 week [32,28,20].

2.4. Tissue processing

Six week after SE, animals were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) under urethane anesthesia (1.5 g/kg, i.p.). The brains were removed, and postfixed in the same fixative for 4 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, the entire hippocampus was frozen and sectioned with a cryostat at 30 μm and consecutive sections were contained in six-well plates containing PBS. For stereological study, every sixth section in the series throughout the entire hippocampus was used in some animals [19].

2.5. Immunohistochemistry

Sections were first incubated with 3% bovine serum albumin in PBS for 30 min at room temperature. Brain tissues were incubated in mixture of mouse anti-GFAP IgG (Chemicon, USA, diluted 1:200)/phalloidin (Fluka, USA, diluted 1:100) mouse anti-GFAP IgG/rabbit anti-LAMP-1 IgG (abcam, USA, diluted 1:100), mouse anti-GFAP IgG/rabbit anti-LC3-II IgG (Abgent, USA, diluted 1:100), mouse anti-GFAP IgG/rabbit anti-GM130 IgG (Santa Cruz, USA, diluted 1:200), rabbit anti-GFAP/mouse anti-PLPP/CIN antibody [15] overnight at room temperature. After washing three times for 10 min with PBS, sections were also incubated in a mixture of FITC- or Cy3-conjugated secondary antisera (1:200, Amersham, USA) for 1 h at room temperature. Sections were mounted in Vectashield mounting media (Vector, USA). For negative control, the rat hippocampal tissues were incubated with only the secondary antibody without primary antibody. All negative controls for immunohistochemistry resulted in the absence of immunoreactivity in any structure (data not shown). All images were captured using an AxioImage M2 microscope and AxioVision Rel. 4.8 software, or Bio-Rad MRC 1024 Confocal Microscope System (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547, USA). Figures were mounted with Adobe PhotoShop 7.0 (San Jose, CA). Manipulation of the images was restricted to threshold and brightness adjustments to the whole image [16–18].

2.6. Stereology and morphometry

The hippocampal volumes (V) were estimated according to the formula based on the modified Cavalieri method: $V = \Sigma a \times t_{nom} \times 1/ssf$, where a is area of the region of the delineated subfield measured by AxioVision Rel. 4.8 software, t_{nom} is the nominal section thickness (of 30 μm in this study), and ssf is the fraction of the sections sampled or section sampling fraction (of 1/6 in this study). The subfield areas were delineated with a 2.5× objective lens. The volumes are reported as mm^3 [5,22]. The optical fractionator was used to estimate the cell numbers. The optical fractionator (combination of performing counting with the optical dissector, with fractionator sampling) is a stereological method based on a properly designed systematic random sampling method that by definition yields unbiased estimates of population number. The sampling procedure is accomplished by focusing through the depth of the tissue (the optical dissector height, h; of 15 μm in all cases for this study). The number of each cell type (C) in each of the subregions is estimated as: $C = \Sigma Q^- \times t/h \times 1/asf \times 1/ssf$, where Q^- is the number of cells actually counted in the dissectors that fell within the sectional profiles of the subregion seen on the sampled sections, and Asf is the areal sampling fraction calculated by the area of the counting frame of the dissector, a(frame) (of 50 μm × 50 μm in this study) and the area associated with each x, y movement, grid (x, y step) (of 250 μm × 250 μm in this study) {asf = [a(frame)/a(x,y step)]}. The diameter/number of vacuoles in astrocytes was also measured by AxioVision Rel. 4.8 software. Stereology and morphometry were performed by two different investigators who were blind to the classification of tissues [19].

2.7. Quantification of data and statistical analysis

For quantification of immunohistochemical data, images of each section on the monitor were captured (15 sections per each animal). The mean gray value and its standard deviation were obtained from the selected images using Adobe PhotoShop v. 8.0. Each image was normalized by assessing the mean gray value. After regions were outlined, 10 areas/rat (500 μm^2/area) were selected from the hippocampus and the gray values were measured. Intensity measurements were represented as the mean number of a 256 gray scale (The University of Texas Image Tool program V. 3.0 and AxioVision Rel. 4.8 software). Values of background staining