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Overexpression of mitofusin 2 inhibits reactive astrogliosis proliferation in vitro

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HIGHLIGHTS

- Scratch injury and starvation-serum stimulation induce reactive astrogliosis.
- Mfn2 expression is down-regulated during the activation of astrocytes.
- Overexpression of Mfn2 inhibits astrocytes proliferation by cell-cycle arrest.

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ABSTRACT

Astrocytes become activated in response to central nervous system (CNS) injury, and excessive astrogliosis is considered an impediment to axonal regeneration by forming glial scar. Mitofusin 2 (Mfn2), a key protein in mitochondrial network, has been reported to negatively regulate cell proliferation. The present study aimed to explore whether reactive astrogliosis could be suppressed by Mfn2 overexpression. Scratch injury and starvation-serum stimulation models in cultured astrocytes were combined to address this issue. In scratch model, reactive proliferation status of damaged astrocytes was implicated by migration of high ratio of EdU(+) cells into lesion region and significantly increased expression of GFAP and PCNA. At meantime, Mfn2 expression was found to exert a down-regulated trend both in gen and protein levels. Pretreatment of cells with adenoviral vector encoding Mfn2 gene increased Mfn2 expression and subsequently attenuated injury-induced astrocytes hyperplasia, activation-relevant protein synthesis, cellular proliferation, eventually delayed wound healing process. Furthermore, Mfn2 overexpression markedly inhibited astrocytes proliferation induced by serum stimulation, by arresting the transition of cell cycle from G1 to S phase. Together, these in vitro results demonstrated that reactive astrogliosis can be effectively suppressed by up-regulation of Mfn2, which might contribute to a promising therapeutic intervention in CNS disease characterized by glia-related damage.

1. Introduction

Reactive astrogliosis is an important cellular response to central nervous system (CNS) injury, which is characterized by hypertrophy of cellular processes, cell proliferation and increased expression of intermediate filament proteins, such as glia fibrillary acidic protein (GFAP) [1]. Reactive astrogliosis may play both a beneficial and a detrimental role in CNS repair depending on the timing of astrocyte activation and the local environment [2]. Initially, it may protect the intact tissue from exposure to toxic elements in the infarct area and secrete neurotrophic factors to provide a permissive substrate for neuron regeneration [3]. However, at later stages post injury, reactive astrocytes result in glial scar formation that impedes axonal regrowth by constituting a physical as well as a biochemical barrier [4]. Thus, early regulation of excessive reactive astrocyte proliferation may inhibit glial scar formation to create a favorable environment for neuronal regeneration and thereby enhance CNS injury recovery.

Mitofusin 2 (Mfn2) is a protein that localizes to the mitochondrial outer membrane. Apart from its major function in mitochondrial network [5], Mfn2 also has a potential role in regulating cell proliferation [6]. Mfn2 has been reported to take part in various hyper-proliferative diseases such as atherosclerosis,

Abbreviations: CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Ad, adenovirus; GFP, green fluorescent protein; EdU, 5-Ethynyl-2′-deoxyuridine; GFAP, glial fibrillary acidic protein; PCNA, proliferating cell nuclear antigen; MAPK/ERK, Mitogen-activated protein kinases/extracellular-signal-regulated kinase.

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cardiac hypertrophy, diabetic nephropathy and neoplasms [7–10]. In these studies, Mfn2 exerts its anti-proliferative effect by acting as an effector molecule of Ras and Raf-1, resulting in the inhibition of Mitogen-activated protein kinases/extracellular-signal-regulated kinase (MAPK/ERK) signaling pathway.

On one hand, several lines of evidence have revealed the close correlation between the MAPK/ERK pathway and astrocyte proliferation [11]. On the other hand, Mfn2 is abundantly expressed in brain [12]. Therefore, the present study aims to determine whether Mfn2 is associated with reactive astrogliosis induced by serum stimulation and mechanical injury. If Mfn2 is indeed involved, whether adenovirus-mediated Mfn2 overexpression is capable of inhibiting reactive astrocyte proliferation and the potential mechanisms will also be explored.

2. Materials and methods

2.1. Astrocyte culture and identification

Cultures of astrocytes from neonatal SD rat cortex were prepared according to a standard procedure [13]. The cultures were maintained at 37 °C and 95% O_2/5%CO_2 in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 0.5 mg/ml penicillin/streptomycin. About 48–60 h in vitro maintenance, purification of astrocyte was undertaken by shaking. At the end of the two weeks culture, primary astrocytes were trypsinized and replated onto 35-mm dishes at 3×10^4 cell/cm^2 density or into 96-well cell plates at the concentration of 1×10^4 cell/well for following experiments.

The purity of astrocyte was identified by double-labeling with GFAP antibody (1:200, Sigma) and Hoechst 33342. The immunocytochemistry revealed that more than 95% of the cultured cells were GFAP-positive astrocytes.

2.2. Adenovirus infection and expression identification

Replication-defective adenovirus encoding the complete rat Mfn2 open reading frame (Ad-Mfn2) and the control adenovirus encoding the green fluorescent protein open reading frame (Ad-GFP) were constructed by BGI Tech (Shenzhen, China). Astrocytes were incubated with adenovirus at the indicated multiplicity of infection (MOI) of 30 pfu/cell for 24 h, then the virus-containing medium was changed to fresh complete growth medium to continue incubation. Ad-Mfn2 expression was detected by western-blotting, which showed that Mfn2 was significantly overexpressed after 48 h infection.

2.3. Starvation and serum stimulation assay

Cells were starved with serum-free DMEM for 24 h to achieve cell synchronization and treated by 10% FBS mitotic stimulation for 24 h or 48 h [14]. Control cell samples included cells nontreated and only treated with starvation.

2.4. Scratch injury model

Monolayer confluent astrocytes were scratched with sterile plastic pipette tips (100 μL) longitudinally and latitudinally every 0.5 cm at right angles to each other in 35 mm dishes. This process has been described to establish a reproducible model of 30–40% damage [15]. Immediately, the detached cells and debris were washed out with fresh medium. Scratched cultures were maintained for 12 h, 24 h and 48 h for following experiments.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The trend of Mfn2 mRNA expression during serum stimulation and scratch model was detected by RT-PCR. Total RNA were extracted from cultured astrocytes by using Trizol reagent (Invitrogen). RNA concentration and quantity were determined by ultraviolet spectrophotometry (absorbance at 260 nm/280 nm). RNA were reverse-transcribed with the Revertaid first strand cDNA synthesis Kit (Thermo Scientific), followed by cDNA amplification in a DNA thermal cycler. The primers were designed as follows:

Mfn2 sense primer: 5′-CTCAGGAGCAGCGGGTTTATTGTCT-3′ antisense primer: 5′-TGTCGAGGGACCAGCATGTCTATCT-3′, fragment size: 412 bp.
β-actin sense primer: 5′-GGAGATTACTGCCCTGGCTCCTA-3′ antisense primer: 5′-GACTCATCGTACTCCTGCTTGCTG-3′, fragment size: 151 bp.

The PCR products were separated by 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.6. Western blotting and quantification

Total proteins of each group were harvested and then centrifuged at 12,000 × *g* for 10 min at 4 °C and protein concentrations in the supernatant were detected using the BCA protein assay kit (Pierce). Protein samples were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 5% nonfat milk at room temperature and respectively incubated with primary antibodies overnight at 4 °C, which included Mfn2 (1:500, CST), GFAP (1:2000, Sigma), PCNA (1:1000, Abcam), Phospho-ERK1/2 (1:500, CST) and β-actin (1:1500, Santa Cruz). On the following day, the membranes were incubated with Odyssey secondary antibodies (780-conjugated goat anti-rabbit and anti-mouse IgG, 1:15,000) for 1 h and then visualized and quantitated by Odyssey IR imaging system (Li-COR Bioscience, USA), then expressed as the ratio of optical density (OD) from the tested proteins to that from actin.

2.7. Assessment of cell proliferation

To assess the proliferation of astrocytes, EdU staining was used [16]. Briefly, cells were incubated with culture medium in presence of 10 μM EdU (RiboBio) for 24 h. Cells were then fixed with 4% paraformaldehyde for 30 min. After naturalization with 2 mg/ml glycine and permeabilized with 0.2% triton X-100, cells were incubated with Apollo® staining solution for 30 min to chase the template of DNA. To label nuclei, cells were washed in 0.2% Triton X-100 for three times prior to incubation with Hoechst 33342 for 10 min. Finally cells were visualized and counted with a fluorescent microscope.

2.8. Wound healing assay

At 0 h, 12 h, 24 h and 48 h after scratch, astrocytes were fixed for immunofluorescence staining. The membranes of fixed cells were permeabilized with Triton X-100 (15 min, 0.2% in PBS), followed by incubation with 5% bovine serum albumin in PBS at room temperature for 1 h to block nonspecific antibody binding and incubated overnight at 4 °C with monoclonal mouse anti-GFAP (1:200, Sigma, USA). Photographs of cells were captured with a fluorescent microscope. Scratch areas before and after recovery were measured using Axiovision 4.1 software [17].