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Full paper

GFAP antibodies show protective effect on oxidatively stressed neuroretinal cells via interaction with ERP57

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ABSTRACT

The pathogenesis of glaucoma, a common neurodegenerative disease, involves an immunologic component. Changes in the natural autoantibody profile of glaucoma patients were detected, showing not only up-regulated but also down-regulated immunoreactivities. In recent studies we were able to demonstrate that the antibody changes have a large influence on protein profiles of neuroretinal cells. Furthermore we could demonstrate neuroprotective potential of one of the down-regulated antibodies (γ-synuclein antibody). Anti-GFAP antibody is another antibody found down-regulated in glaucoma patients. Since GFAP expression is intensified in glaucomatous retina, the aim of this study was to detect the effect of GFAP antibodies on neuroretinal cells. This is realized with a viability-test as well as proteomic analysis of cells incubated with GFAP antibodies. Furthermore, possible interaction partners of the GFAP antibody in neuroretinal cells were identified by western blot, mass spectrometry and indirect immunofluorescence staining. We found that the GFAP antibody is able to protect cells from oxidative stress, which is due to changed protein expressions of the actin cytoskeleton. Furthermore we detected a cross-reaction of the antibody to endoplasmic reticulum resident protein 57 on the cell membrane, which seems to lead to a changed signaling in the cells triggering the protective effects.

1. Introduction

Glial fibrillary acidic protein (GFAP) is a cytoskeletal type III intermediate filament and was first isolated from multiple sclerosis plaques (1). Intermediate filament maintain cell stability as well as cell shape. GFAP additionally plays a role in the modulation of cell motility, proliferation, vesicle trafficking and interaction between astrocytes and neurons (2) and is a main component in astrocytes of the central nervous system. Following acute injury of the brain, but also progressive central nervous system degeneration, astrocytes are activated resulting in reactive gliosis. Activated astrocytes express enhanced GFAP levels (3) and exist in many neurodegenerative disorders such as Alzheimer's (4, 5) and Parkinson's disease (6). Furthermore, activated glial cells expressing increased GFAP levels were detected in glaucoma animal models (7) and human glaucomatous donor eyes (8). Studies also demonstrated reactive astrocytes in the optic nerve head of monkey glaucoma models (9). Glaucoma is a common neurodegenerative disease and one of the leading courses for blindness worldwide (10). It comprises a heterogeneous group of eye diseases, defined by a progressive loss of retinal ganglion cells, optic nerve degeneration and resulting visual fields defects (11). Although so frequent, the reason for its development is still unknown. A major risk factor is an elevated intraocular eye pressure, but the fact that 30% of glaucoma patients fail to demonstrate this symptom (12) reveals, that other pathogenesis factors are involved, such as immunological components. Complex analysis of sera derived from numerous glaucoma patients showed up-as well as down-regulated autoantibodies against alpha-foldrin (up-regulated) or αB-Crystallin and Vimentin (down-regulated) and additionally complex changes in the

Abbreviations: Hsp, heat shock protein; PBS, phosphate buffered saline; endoplasmatic reticulum resident protein 57, ERP57; glial fibrillary acidic protein, GFAP.

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antibody reactions against human optic nerve antigens (13–15). Furthermore, antibody reactions in the aqueous humour of glaucoma patients against antigens such as αB-Crystallin (down-regulated), Vimentin (down-regulated) and Hsp 70 (up-regulated) (15) were detected.

After incubating neuroretinal cells (R28) with glaucoma serum, we found significantly changed protein expression profiles of the cells in comparison to cells incubated with healthy serum (16). These changes mainly involved cell regulatory pathway proteins. We also found that antibodies in the serum of glaucoma patients had the largest significant impact on the detected protein expression changes (16). These results emphasize the hypothesis that changes of autoantibodies participate in glaucoma disease. Recently we showed that γ-synuclein antibody, down-regulated in glaucoma patients, has a protective effect on stressed neuroretinal cells (retinal ganglion cell line 5). This effect can be traced back to anti-apoptotic altered protein expressions in the mitochondrial apoptosis pathway (17). After demonstrating protective effects of γ-synuclein antibody the aim of this study was to analyze whether other antibodies, also down-regulated in glaucoma, possess similar properties, or if the cells possible react contradictory.

2. Material and methods

2.1. Reagents

2′, 7′-dichlorodihydrofluorescein-diacetate from Sigma–Aldrich (St. Louis, MO). Polyclonal antibody to myoglobin, rabbit anti-sheep IgG-H&L (FITC), mouse monoclonal antibody to endoplasmic reticulum resident protein 57 (ERP57), rabbit polyclonal secondary antibody to mouse IgG-H&L (TRITC), chicken polyclonal antibody to GFAP and rabbit polyclonal secondary antibody to chicken IgY-H&L (FITC) were from Abcam, (Cambridge, UK). BCA Pierce Protein Assay kit was purchased from Fisher scientific (Waltham, MA.). Goat anti-chicken IgY-Horseradish peroxidase was from Santa Cruz (California, U.S.A.).

2.2. Cell culture

Retinal ganglion cell line 5 provided by Dr. Neeraj Agarwal, are mouse cells transformed with a ψ2E1A virus (18) and represent a neuronal precursor cell line (19). Studies show neuron like characteristics and a specific expression of neuronal proteins (20). The cells were grown in 75 cm^2 culture flasks in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin and 4% L-alanyl-L-glutamin and cultivated in a humidified incubator at 37 °C, and 5% CO_2.

2.2.1. Cell treatment with GFAP antibodies and different stress factors

Retinal ganglion cell line 5 cells were seeded in 24 well plates at a density of 45000 and grown overnight. After preincubation with different concentrations of polyclonal anti-GFAP antibodies [0.005 and 20 μg/ml (n = 4) and 0.1, 0.5, 1, 5, 10 μg/ml (n = 6)], the cells were incubated with 50 μM H_2O_2 for 1 h to induce oxidative stress. In order to detect the specificity of the results the experiments were also performed with different concentrations of rabbit polyclonal to myoglobin antibodies and either stressed with 50 μM H_2O_2.

2.2.2. Cell viability test

Cell viability was assessed with crystal violet staining. The cells were fixed with 3% paraformaldehyde (15 min) and rinsed with phosphate buffered saline (PBS). Subsequently the cells were stained with 0.1% crystal violet solution according to the protocol (17). The absorption was expressed as percentage of the control cells, which were only treated with H_2O_2. An unpaired student's t-test was used to compare the data obtained and was calculated with Statistica (StatSoft, U.S.A.). A p-value <0.05 is described as significant and $p < 0.01$ as highly significant.

2.2.3. Reactive oxygen species-test

To quantify reactive oxygen species we used 2′, 7′ dichlorodihydrofluorescein-diacetate. Intracellular esterase and reactive oxygen species convert non-fluorescence stain 2′, 7′ dichlorodihydrofluorescein-diacetate to fluorescent stain dichlorofluorescein. Cells were loaded with 10 mM 2′, 7′ dichlorodihydrofluorescein-diacetate in the incubation chamber according to the protocol (17) The absorption was expressed as a percentage of the control cells only treated with 50 μM H_2O_2. Reactive oxygen species-levels were normalized by measuring the viability of the cells in the same well. An unpaired student t-test was used to compare the data obtained (Statistica).

2.3. Immunocytochemical staining

Retinal ganglion cell line 5 were grown in μ-slide IV (Ibidi GmbH, Munich, Germany) overnight and subsequently washed with PBS. The cells were fixed with 3% paraformaldehyde (15min) and incubated with 0.25% Triton-X-100 in PBS (12 min). After 3 wash steps (PBS), the cells were treated with 1% bovine serum albumin (20 min). Then the cells were incubated with 2 μg/ml chicken polyclonal anti-GFAP antibodies overnight, gently washed 3 times with PBS and incubated with rabbit polyclonal secondary antibody to chicken IgY-H&L (FITC) (1.5 h). After washing (PBS), the cells were visualized with a Leica fluorescence microscope using Lucia G/F software. To investigate the antibody staining in living cells, the cells were treated with 15 μg/ml polyclonal anti-GFAP antibodies and washed with PBS. The cell membrane was visualized using wheat germ agglutinin and the cells were handled and visualized as described above. Immunocytochemical staining was also performed to visualize ERP57 and GFAP in non-permeabilized fixed cells. Cells were fixed as described above and incubated with 5 μg monoclonal anti-ERP57 antibody and 2 μg/ml polyclonal anti-GFAP antibody overnight. After washing, the cells were incubated with rabbit polyclonal secondary antibody to mouse IgG-H&L (TRITC) and rabbit polyclonal secondary antibody to chicken IgY-H&L (FITC).

2.4. Mass spectrometric analyses

2.4.1. Cell lysate preparation

For proteomic analysis, cells were grown in 60 × 15 mm cell culture dishes overnight and subsequently incubated with 1 μg/ml polyclonal anti-GFAP antibodies for 3 h. Control cells were incubated without antibodies. After washing with PBS they were detached from the cell culture dish with cell dissociation solution and lysed by freezing at −80 °C, adding 0.1% w/v Dodecyl-D-β- Maltoside and treatment in an ultrasonic bath for 1 min. After centrifugation, the supernatant was used for protein concentration determination by BCA Pierce Protein Assay kit.

2.4.2. SDS PAGE separation and in-gel digestion

Protein separation was performed via denaturing gel electrophoresis. Each lane was cut into 17 pieces, incubated with acetonitril and ammonium bicarbonate and dried in a concentrator. Then the pieces were tryptically digested overnight, based on a modified protocol of Shevchenkov (21) (0.7 μg Trypsin in 80% HPLC H_2O, 10% acetonitril, 10% ammonium bicarbonate). The supernatant was collected and remaining proteins were dissolved with extraction buffer (38% HPLC H_2O, 0.2% formic acid, 60% acetonitril) for 30 min.