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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.

Both supernatants were pooled, dried in a concentrator and acidified with 0.1% trifluoroacetic. C-18 ZipTips were used to purify the samples according to the manufacture protocol. The samples were then dried and frozen at −20° till further analysis.

2.4.3. LC-ESI/MS for protein identification

Analysis of peptides was performed with a capillary LC-ESI-MS system consisting of a BioBasic C-18 precolumn (30 mm × 0.5 mm, Thermo Scientific) and a BioBasic C18 analytical column (150 mm × 0.5 mm, Thermo Scientific). The system was protected by an A 316 0.5 μm online precolumn filter (Upchurch Scientific, Washington, U.S.A.). As solvent delivery system, a Rheos Allegro HPLC Pump (Thermo Scientific) was used. The pump flow rate was 200 μl/min and reduced to a column flow of 10 μl/min by use of an M−472 graduated micro-split valve (Upchurch, Scientific). Using running buffer A (98% H_2O, 1.94% acetonitril, 0.06% methanol, 0.05% trifluoroacetic) and B (95% acetonitril, 3 %methanol, 2% H_2O and 0.05% trifluoroacetic), a linear gradient of 80 min was performed (0–47 min: 0–100 %B, 47–49 min: 100% B, 49–58: 100 %–0 % B, 58–80 min: 0 %B). Equilibration gradients of 30 min were run between samples by injecting 80% acetonitril to prevent sample-to-sample carry over. Mass spectra were obtained using an LTQ OrbitrapXL and were recorded in the "profile" mode to allow quantification in MaxQuant (Max Planck Institute of Biochemistry, Martinsried, Germany) with settings as described in previous studies (17).

2.4.4. Data processing

The mass spectra were used for protein identification and quantification with Maxquant with a fixed modification Carbamidomethylation. The tolerance in mass precision for MS/MS was 20 ppm and 0.5 Da. The protein and peptide false discovery rate were set of 0.01 and the minimum peptide length was 6 amino acids. The evaluation was implemented with Ingenuity Pathway Analysis Software to investigate biological networks and pathways. We included only proteins with a 2-fold changed expression in GFAP antibody treated cells to the analysis.

2.4.5. Identification of GFAP antibody binding partners

100 μg cell lysate was dissolved in 125 μl sample solution (2% w/v chaps, 8 M Urea, 2 M Thiourea, 0.8% v/v Bromphenol blue solution, 0.8% v/v Proteinase inhibitor cocktail, 1% v/v immobiline pH-gradient buffer 3–10 non-linear). After incubation (30 min, 4 °C) the cell lysates were applied to Immobiline dry-strips pH 3–10 non-linear (7 cm). After isoelectric focusing, the gel strips were equilibrated (6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% v/v glycerol, 2% w/v SDS, 0.002% v/v bromphenol blue) containing 2.5% w/v Dithiothreitol (12 min) and then in equilibrating buffer containing 3.2% w/v Iodacetamid (12 min) whilst gentle shaking. The second dimension (SDS-Page) was carried out using a 12% acrylamide gel. Gels were either stained with colloidal blue staining kit or electroblotted to nitrocellulose for western blotting. Blots were blocked in 4% non-fat-dry milk (1 h) and incubated with anti-GFAP antibody overnight. After washing, the blot was incubated with secondary antibody coupled with horseradish peroxidase (2 h). The spots identified by western blot, were excised in the corresponding gel and digested as described above. The peptides were deposited directly on a 386 MTP polished steel Maldi target (Bruker Daltonics, Bremen, Germany) and the matrix (20 mg alpha-cyano-4-hxdroxycinnamic acid, 60% acetonitril, 40% H_2O, 0.1% trifluoroacetic) was applied, after drying the samples. 3 spots with 2 μl peptide calibration standard mixed with 18 μl matrix were applied. The applied spots were measured with an Ultraflex II Maldi-Time of flight/Time of flight (TOF) MS (Bruker Daltonics). The identified interaction partner of the GFAP antibody was used to create an antigen microarray. The identified antigen was spotted on a nitrocellulose slide using an array-spotter (Scienion, Germany). The microarray slides was blocked with 5% bovine serum albumin in 0.5% Tween-PBS (1 h), washed three times with 0.5% Tween-PBS, subsequently incubated with anti-GFAP antibody overnight and incubated with anti-IgY secondary antibody coupled with FITC (2 h) after 3 wash steps. The spots were visualized with Leica fluorescence microscope using Lucia G/F software.

3. Results

3.1. Effect of GFAP antibodies on stressed retinal ganglion cell 5

The effects of GFAP antibodies were determined by viability and reactive oxygen species-tests. We detected significant increased cell viability of up to 9%, when preincubating the cells with 0.1 ($p < 0.05$), 0.5 ($p < 0.05$), 1 ($p < 0.01$) μg/ml GFAP antibodies and additionally stressing them with H_2O_2 in comparison to the control cells only treated with H_2O_2 (Fig. 1). The concentrations ranging from 0.5 to 5 μg/ml GFAP antibodies also showed significantly decreased reactive oxygen species-levels of up to −9% ($p < 0.05$) in H_2O_2 stressed cells (Fig. 1). No significant effect was found, when the cells were incubated with lower concentrations of the

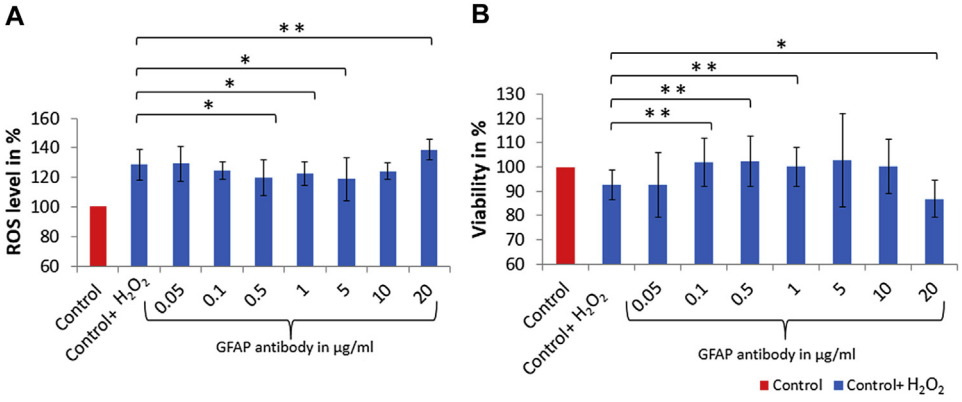

Fig. 1. Reactive oxygen species-level and viability of GFAP antibody treated and stressed cells. Reactive oxygen species-production was measured using 2′, 7′-dichlorodihydrofluorescein-diacetate. The viability test was performed with crystal violet. (*=$p < 0.05$; **$p < 0.01$). **A**: Retinal ganglion cell line 5 cells were preincubated with different concentrations of GFAP antibodies for 3 h and additionally stressed with 50 μM H_2O_2 for 1 h. Significant decreased reactive oxygen species-production was measured, when the cells were preincubated with 0.5–5 μg/ml GFAP antibodies in comparison to stressed cells. **B**: Increased high significant and significant cell viability was demonstrated after the cells were preincubated with 0.1–1 μg/ml GFAP antibodies and additionally stressed with H_2O_2.

antibodies. The highest concentrations of 20 μg/ml GFAP antibody show a significant decreased viability of −6% and a highly significant increased reactive oxygen species-level of 10% in comparison to untreated cells.

To validate the specificity of the protective effect of GFAP antibodies, the experiment was also performed with anti-myoglobin antibodies as control antibodies. We could indicate no significant change of viability when preincubating cells with different concentrations of myoglobin antibodies and additionally stressing with H_2O_2 in comparison to untreated stressed cells (data not shown).

3.2. Mass spectrometric analysis

To investigate GFAP antibodies effects on the protein expression of retinal ganglion cell line 5, proteomic analyses of cells after preincubation with GFAP antibodies in comparison to untreated cells were performed. Overall we identified 486 proteins and 102 significant protein alterations with intensity differences of ≥2 fold increase or decrease (Supplement File I). These changed proteins were analyzed with Ingenuity Pathway Analysis and classified into 31 significant canonical pathways. Among these pathways we found the actin cytoskeleton signaling pathway to be of particular interest. We indicated 7 associated proteins differently expressed, namely actin beta, profilin1, Talin 1, Vinculin, Spectrin alpha (up-regulated) and actin regulated protein 2, Cofilin 1 (down-regulated), all involved in regulating the actin cytoskeleton organization. (Fig. 2).

3.3. GFAP antibody binding partner in retinal ganglion cell line 5

To determine GFAP expression and GFAP antibody binding indirect immunofluorescence staining was performed. Permeabilized fixed cells showed cytoplasmatic staining in the area of the nucleus (Supplementary Fig. 1) whereas the antibody bound to the cell membrane in non-permeabilized cells (Fig. 4B). We could not detect antibody internalization or binding in living cells preincubated with GFAP antibodies. Western blot and Maldi analysis were used to identify possible interaction partners. Western blot analysis visualized two GFAP antibody binding sites. One spot was identified at a molecular weight of 49 kDa, which correlates with the molecular weight of GFAP and the second spot was seen at a molecular weight of 56 kDa. Maldi analysis identified the second spot as ERP57, which was confirmed in a microarray approach showing GFAP antibody binding to the antigen ERP57 (Fig. 3B). The expression of ERP57 on the cell membrane and also around the nucleus of non-permeabilized and permeabilized cells was shown in immunocytochemical staining. Double staining of ERP57 and GFAP of non permeabilized cells showed the same staining pattern on the cell membrane, thus we assume that GFAP antibody binds to ERP57 on the cell membrane of the cells (Fig. 4).

4. Discussion

These studies show a dose response effect of the GFAP antibody, with protective effects in the range from 0.1 to 5 μg/ml, lower concentrations do not show any effect, higher concentrations show negative effects on the cells. In contrast to the long lasted opinion that autoantibodies are auto-aggressive like in Myasthenia gravis (22, 23), previous studies could also show the protective potential of autoantibodies. In Alzheimers' disease autoantibodies against Aβ, which are also found down-regulated in the patients, convey a protective effect by inhibiting oligomerization of Aβ peptides (24, 25). In addition to the protective effects of the GFAP antibody on neuroretinal cells, our group was also able to show the protective potential of γ-synuclein antibodies, down-regulated in glaucoma patients (17). Proteomic analysis showed that the actin cytoskeleton pathway is effected by the GFAP antibody. The actin cytoskeleton, which is the main microfilament in axons, consists of dynamic assembly and disassembly filaments and is essential for a lot of biological functions such as mechanic cell stabilization, cell motility, vesicle trafficking, endocytosis, intracellular organization and apoptosis (26). The actin dynamic is regulated by different actin regulated proteins like Profilin, Cofilin and actin regulated protein 2/3, which were down-regulated in GFAP antibody treated cells and Vinculin, Talin, alpha Spectrin, up-regulated in GFAP antibody treated cells. Next to mechanic cell stability, studies could show, that actin dynamics are also involved in apoptosis regulation. Cytochalasin d, which prevents polymerization of f-actin, can induce caspase 3 mediated apoptosis in t-lymphocytes (27) whereas Phalloidin, a f-actin stabilization drug, prevents nuclear fragmentation by apoptosis in cisplatin treated kidney cells (28). Cells are dependent on actin dynamics and changes can affect apoptosis signaling pathways demonstrated by the fact that actin and actin regulated proteins participate in the mitochondrial apoptosis pathway (26). Direct influence of actin and actin regulated proteins on the mitochondrial apoptosis pathway has also

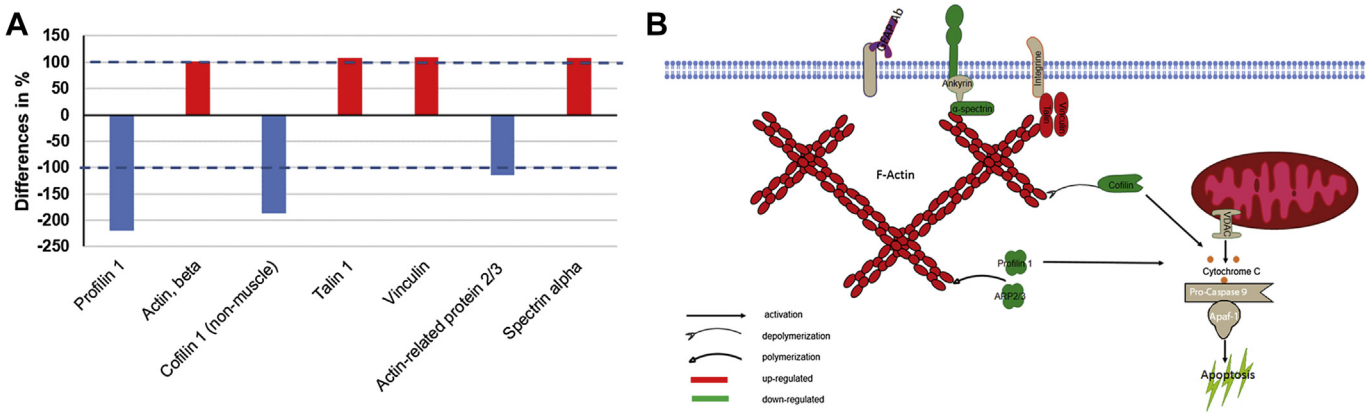

Fig. 2. Regulation of actin cytoskeleton associated proteins. Cells were preincubated with 1 μg/ml GFAP antibodies and subsequently lyzed, tryptically digested. Protein analyses was performed via capillary LC-ESI-MS system. **A**: Regulation of actin cytoskeleton proteins. Quantifications were realized with MaxQuant. The fold changes were calculated in comparison to untreated control cells. The dashed line highlights the relevant regulated proteins (>2fold changed expression). **B**: Overview of changed proteins of the actin cytoskeleton constructed by means of biocarta (www.biocarta.com). The red colored proteins demonstrate that the protein is up-regulated and the green colored that they are down-regulated.

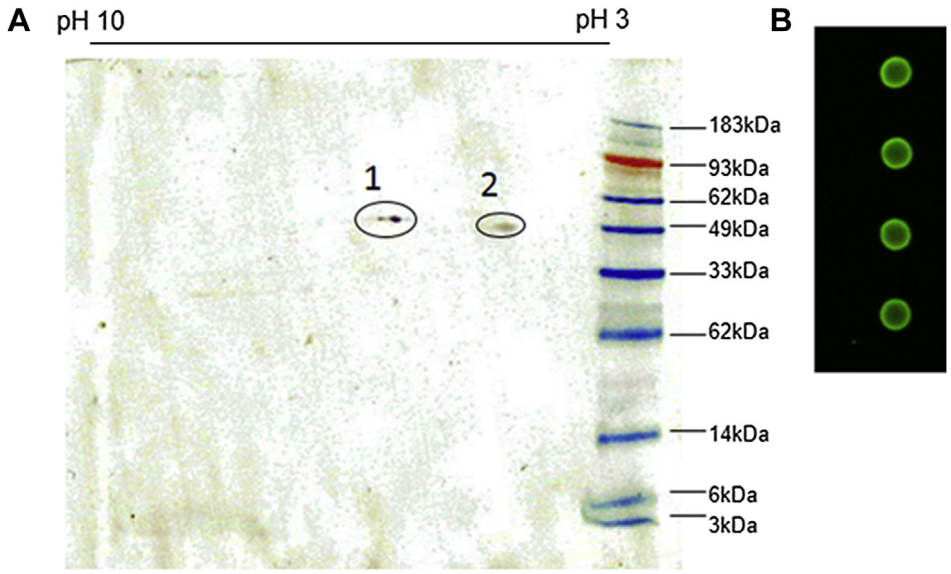

Fig. 3. Identification of GFAP antibody interaction partners via western blot and microarray. **A**: 2-D gel electrophoresis with cell lysate was performed. The proteins were electro blotted to nitrocellulose and incubated with anti-GFAP antibody overnight. The identified spots by western blot were excised in the corresponding gel, digested and identified with Ultraflex II Maldi-TOF/TOF MS. Spot 1 was identified as ERP57 and spot 2 was identified as GFAP. **B**: Antigen microarray show the binding of GFAP antibody to ERP57.

been demonstrated. Actin regulates the opening state of voltage dependent anion channel in the fungus Neurospora crassa (29). Voltage dependent anion channel is located on the outer mitochondrial membrane and participates in energy balance regulation as well as the release of pro-apoptotic factors such as cytochrome c. Actin regulated proteins e.g. Cofilin, which is altered in GFAP antibody treated cells, also is involved in mitochondrial apoptosis. Cofilin promotes depolymerization of actin and is involved in G-actin recycling. After induction of apoptosis, dephosphorylated Cofilin translocates from the cytosol to the mitochondria and can induce cytochrome c release (30). Over-expression of Gelsolin, which belongs also to the actin regulated proteins, inhibits apoptosis in Jurkat cells induced through several mitochondrial target drugs (31). Gelsolin also has a protective effect on neuronal cells by preventing the loss of mitochondrial membrane potential and activation of caspase-3 (32). All these findings verify the participation of the actin cytoskeleton and actin regulated proteins on apoptosis pathways especially on mitochondrial apoptosis.

These studies propose, that the dynamic transformation of the actin cytoskeleton plays an important role in stress response of cells. Disorganization of the cytoskeleton could be observed in a glaucoma rat model with increased intraocular pressure. Control animals showed a consistent distribution of f-actin in the nerve fascicle, whereas a diffuse distribution could be observed in animals with an increased intraocular pressure (33). Mass spectrometric analyses are not suitable to detect structural changes such as polymerziation or depolymerization of the actin cytoskeleton, but they clearly show changes in the protein expression of actin cytoskeleton proteins.

The immunocytochemical staining showed binding of the antibody in the cytosol located around the nucleus inside the permeabilized cells and on the membrane of non-permeabilized cells. We detected the interaction of the antibody with GFAP and ERP57. The hypothesis, that antibody-antigen binding is monospecific, has changed since many monoclonal antibody were found to bind to unrelated self and foreign antibodies (34, 35). So-called polyreactive antibodies are able to bind to different antigens due to conformational changes in the antigen-binding pocket (35). These polyreactive natural antibodies are produced by polyreactive B-cells as a part of their physiological function (36). As postulated by Notkins, polyspecificity is not exclusive for natural antibodies, but rather a general property of antibodies (35). Possibly the GFAP antibodies are polyreactive and able to bind unrelated antigens such as ERP57. ERP57 is a thiol oxidoreductase and belongs to the protein disulfide isomerase family. All members of the family are present in the endoplasmatic reticulum and participate in proper protein folding. They catalyze oxidation, reduction and isomerization of disulfide bonds. There is evidence that some members of the protein disulfide isomerase family have a different cellular location and the function differs from those found in the endoplasmic reticulum lumen (37). Whilst the function of ERP57 in the endoplasmic reticulum is well understood, the physiological function in the nucleus or the cell membrane, is not understood very well. In common with our findings protein disulfide isomerase has been found on the cell surface of different cell types such as hepatocytes (38), endothelial cells (39) and neuroblastoma NG108–15 cells (40). ERP57 possesses an important role in signal transduction processes from the cell membrane to the cytoplasm. The involvement of ERP57 in signal transducer and activator of transcription 3 signaling pathway was also demonstrated (41). As yet, there is no direct link between plasma membrane associated ERP57 and actin cytoskeleton signaling, but studies were able to show the association of ERP57 and actin as well as Vimentin in the nucleus. The permeabilized cells did not show typical GFAP staining which was not surprising, considering the cells to be neuronal rather than glial origin. The protective effect is much more triggered through the binding on ERP57, localized on the cell membrane.

We hypothesize that GFAP antibody binding on ERP57 of the cell membrane leads to alterations of the actin cytoskeleton pathway and therefore leads to a protective effect of H_2O_2 stressed cells. The negative effect of high GFAP antibody concentrations could be a result of a permanent blockage of ERP57. We could detect the GFAP antibody binding on the cell membrane on fixed cells. In comparison, in living cells preincubated with GFAP antibody, no staining could be visualized. It is known that ERP57 catalyzes oxidation, reduction and isomerization of disulfide bonds and therefore it could also remodel disulfide bonds of the GFAP antibody. This could lead to a change of the GFAP antibody epitope preventing the detection by immunocytochemical staining.

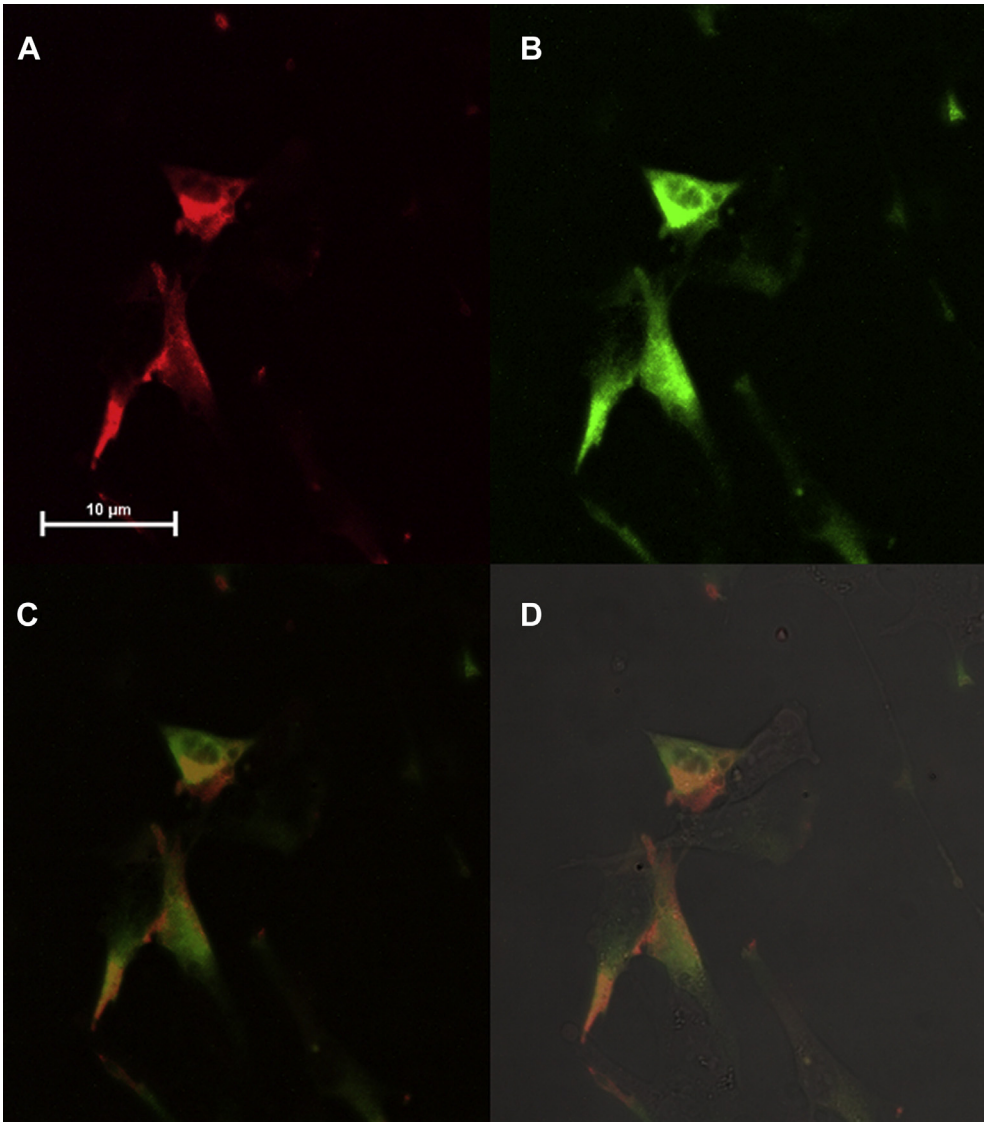

Fig. 4. Binding of GFAP antibody on ERP57 on non permeabilized cells revealed by immunocfluorescence. Cells were fixed, blocked and stained with chicken anti-GFAP antibody and rabbit anti-ERP57 antibody and then stained with a secondary antibody to chicken IgY-H&L conjugated with FITC and a rabbit polyclonal secondary antibody to mouse IgG-H&L (TRITC). Magnification 40x. **A**: Anti-ERP57 (red). **B**: Anti-GFAP (green). **C**: Merged fluorescence image. **D**: Merged fluorescence image with bright light microscopy.

5. Conclusion

In summary, immunocytochemical staining as well as Maldi TOF/TOF and microarray analyses could show, that GFAP antibody binds to ERP57 on the cell membrane, and that GFAP antibody incubation leads to a protective effect on H_2O_2 stressed neuroretinal cells. The protective effect could be traced back to altered proteins of the actin cytoskeleton pathway. These results show that an antibody not only has to be destructive but also can have protective effect on cells. This gives initial hints of the function of down-regulated antibody in the pathogenesis of glaucoma. We hypothesize that the down-regulation of autoantibodies in glaucoma patients results in a loss of protective regulatory functions, but additional studies have to observe the detailed function of GFAP antibody in the context of glaucoma.

We believe that these results show the potential for using protective autoantibodies to support glaucoma therapy and inhibiting the loss of retinal ganglion cells, e.g. as personalized therapy.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2014.12.019

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