Original Article

Inhibition on Apoptosis Induced by Elevated Hydrostatic Pressure in Retinal Ganglion Cell-5 via Laminin Upregulating β1-integrin/Focal Adhesion Kinase/Protein Kinase B Signaling Pathway

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Abstract

Background: Glaucoma is a progressive optic neuropathy characterized by degeneration of neurons due to loss of retinal ganglion cells (RGCs). High intraocular pressure (HIOP), the main risk factor, causes the optic nerve damage. However, the precise mechanism of HIOP-induced RGC death is not yet completely understood. This study was conducted to determine apoptosis of RGC-5 cells induced by elevated hydrostatic pressures, explore whether laminin is associated with apoptosis under pressure, whether laminin can protect RGCs from apoptosis and affirm the mechanism that regulates the process of RGCs survival.

Methods: RGC-5 cells were exposed to 0, 20, 40, and 60 mmHg in a pressurized incubator for 6, 12, and 24 h, respectively. The effect of elevated hydrostatic pressure on RGC-5 cells was measured by Annexin V-fluorescein isothiocyanate/propidium iodide staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and Western blotting of cleaved caspase-3 protein. Location and expression of laminin were detected by immunofluorescence. The expression of β1-integrin, phosphorylation of focal adhesion kinase (FAK) and protein kinase B (PKB, or AKT) were investigated with real-time polymerase chain reaction and Western blotting analysis.

Results: Elevated hydrostatic pressure induced apoptosis in cultured RGC-5 cells. Pressure with 40 mmHg for 24 h induced a maximum apoptosis. Laminin was declined in RGC-5 cells after exposing to 40 mmHg for 24 h. After pretreating with laminin, RGC-5 cells survived from elevated pressure. Furthermore, β1-integrin and phosphorylation of FAK and AKT were increased compared to 40 mmHg group.

Conclusions: The data show apoptosis tendency of RGC-5 cells with elevated hydrostatic pressure. Laminin can protect RGC-5 cells against high pressure via β1-integrin/FAK/AKT signaling pathway. These results suggest that the decreased laminin of RGC-5 cells might be responsible for apoptosis induced by elevated hydrostatic pressure, and laminin or activating β1-integrin/FAK/AKT pathway might be potential treatments to prevent RGC loss in glaucomatous optic neuropathy.

Key words: Apoptosis; Glaucoma; Hydrostatic Pressure; Laminin; Retinal Ganglion Cells

Introduction

Glaucoma is a progressive optic neuropathy characterized by degeneration of neurons due to loss of retinal ganglion cells (RGCs), with accompanying damage of the visual field over time.[1,2] The main risk factor for glaucoma is high intraocular pressure (HIOP), which causes the optic nerve and visual function damage.[3-6] Although the precise mechanisms of HIOP-induced glaucomatous optic nerve damage and RGC death are not yet completely understood, it is hypothesized that ameliorating or preventing optic nerve injury might be conducive to treating HIOP-induced death of RGCs.

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.179785

In vivo studies revealed the characteristic apoptosis of RGCs in response to elevated intraocular pressure (IOP)[7-9] and there has been some research on the direct effect of IOP on RGC apoptosis. Agar *et al*.[10] induced apoptosis in cultured

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Received: 16-10-2015 **Edited by:** Peng Lyu

How to cite this article: Li Y, Chen YM, Sun MM, Guo XD, Wang YC, Zhang ZZ. Inhibition on Apoptosis Induced by Elevated Hydrostatic Pressure in Retinal Ganglion Cell-5 via Laminin Upregulating β1-integrin/Focal Adhesion Kinase/Protein Kinase B Signaling Pathway. Chin Med J 2016;129:976-83.

RGCs subjected to elevated hydrostatic pressure (30 or 100 mmHg for 2 h) in a pressurized incubator, and other studies have confirmed the injuries of RGCs at several other pressures for different lengths time.[11-14] It is believed that the loss of RGCs in glaucoma occurs by apoptosis under the unbearable pressure. However, a pressure of 100 mmHg cannot be achieved *in vivo*, and the apoptosis coherency between time and pressure on RGCs has not yet been determined.

The extracellular matrix (ECM) has a profound influence on individual cells and influences and/or controls several basic cellular processes such as adhesion, differentiation, survival, growth, and migration. Moreover, ECM structure and physical-chemical properties convey precise information to cells that profoundly influences their biology via interactions with cell surface receptors termed integrins.[15,16] Laminin is an ECM component believed to facilitate neuron attachment, regeneration, and survival.[15,17] It was reported that elevated IOP was significantly negatively related to the expression of laminin in the RGC layer in a rat model of glaucoma.[7] However, little is known about the relationship between laminin expression and pressure, and whether laminin is related to apoptotic cell death of RGCs in glaucoma.

Integrins participate in the regulation of the signaling pathways involved in growth, hypertrophy, survival, differentiation, migration, cell morphology, and apoptosis. They function as heterodimers, which consist of 18α and 8β subunits, and bind noncovalently to mediate cell-cell and cell-ECM interactions.[18-20] The β1-integrins, which bind to laminin, are expressed in several cell types.[21-23] They can sense mechanical stimuli, mediate mechanical force transfer across the cell surface and through their interconnections with focal adhesion kinase (FAK), and transduce into biochemical signals to regulate cell behavior.[24,25] β1-integrin-mediated FAK signaling contributes greatly to cell survival in the engagement of pathways such as phosphoinositide 3-kinase/protein kinase B (PI3-K/AKT). Although many studies have demonstrated that the signaling of β1-integrin/FAK/AKT could protect cells from apoptosis,[18,26] few investigations have focused on the pathway necessary for neuronal survival under pressure, especially for RGCs.

Although the origin and nature of RGC-5 cells are controversial, they might still be a tool in the hands of researchers who want to follow-up on some initial hypotheses that require a transformed retina cell line of neuronal origin.[27] RGC-5 cells constitute a widely used model for studying physiological and pathophysiological processes in retinal cells.[28] Therefore, we explored the effects of laminin on this cell line. To address the aforementioned issues, we determined which pressure level could cause RGC-5 cells death, whether laminin expression is changed under hydrostatic pressure *in vitro*, and whether laminin can contribute to the survival of RGC-5 cells through β1 integrin-FAK-AKT pathways.

Methods

Cell culture

RGC-5 cell lines (ATCC, USA) were cultured in high-glucose Dulbecco's medium Eagle's medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. The cells were passaged by trypsinization every two days. Routine testing confirmed that the cells were free of mycoplasma and viral contaminants during the entire study period.

Pressure system

To achieve stable and adjustable hydrostatic pressure, a customized pressure chamber equipped with an electronic regulator and a meter was designed to expose cultured RGC-5 cells to elevated hydrostatic pressure. For pressure experiment, the mixture of 95% O_2 and 5% CO_2 was pumped into the chamber. This arrangement provided a constant hydrostatic pressure within ± 0.1 mmHg and ranging from 0 to 100 mmHg. The experimental group was exposed to the pressure system and control group (0 mmHg) was incubated simultaneously in a conventional incubator at 37°C.

Flow cytometry

The percentage of normal nonapoptotic cells and apoptotic cells was measured by double supravital staining with Annexin-V and propidium iodide (PI) following manufacturer's instruction, using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (KeyGen, Nanjing, China). Briefly, cells were centrifuged, washed with cold phosphate-buffered saline twice, and then resuspended in 500 μl binding buffer. FITC-conjugated Annexin V (5 μl) and PI (5 μl) were added to each sample, and the mixture was incubated at room temperature in the dark for 15 min. The cells were then subjected to fluorescence-activated cell sorting (FACS) analysis (BD Facscalibur, BD Biosciences, Franklin Lakes, NJ, USA). During all FACS analyses, 10^5 events for each sample were analyzed. The percentage of apoptotic cells in each group was determined.

Viability assay

The activity of RGC-5 cells under hydrostatic pressure was detected by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells (1×10^5/well) were plated in 200 μl of minimal essential media in 96-well plates and treated at hydrostatic pressure incubator for 24 h. After incubation, 20 μl of MTT (5 mg/ml; Invitrogen, USA) reagent were added. The plates were incubated for 4 h and subsequently lysed using dimethyl sulfoxide (100 μl/well; Sigma, USA). Then, the optical density (OD) was determined at 490 nm using a Biotek Synergy Microplate Reader (Biotek, Winooski, VT, USA). Percentages of cell survival were calculated as follow: cell survival= (OD of treated group/OD of control group) ×100%.

Immunofluorescence

Cells were grown on 24-well plates during 24 h before hydrostatic pressure treatment (40 mmHg, 24 h). Then, they

were fixed with a paraformaldehyde solution (4%) before incubation with anti-laminin antibody (1:400, Abcam, UK) overnight at 4°C. After extensive washing, plates were incubated for 2 h at room temperature with green-fluorescent donkey anti-rabbit IgG (H+L) secondary antibody (1:500, Invitrogen, USA). 4',6-diamidino-2-phenylindole was used to stain the cell nuclei (blue). Finally, cells were observed under a fluorescence microscope (IX71, Olympus, Tokyo, Japan) and images were analyzed with Image-Pro Plus software (Image-Pro Plus 6.0, Media Cybernetics, Inc., Silver Spring, MD, USA).

Real-time polymerase chain reaction analysis

Total RNA was extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Trizol was joined according to 10 cm^2/ml proportion. About 1 μg total RNA from each sample was reverse-transcribed into cDNA with the Takara RT Kit in a total volume of 10 μl and stored at −20°C. Real-time polymerase chain reaction (PCR) was performed using a SYBR Premix Ex Taq II Reagent Kit (Takara, Japan). PCR reactions were performed in a 25 μl reaction mixture containing 12.5 μl SYBR Premix Ex Taq II (Takara, Japan), 2 μl primer pairs, and 2 μl cDNA samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used at the same time as an internal control. The primer sequences (forward/reverse) used were as follows: β1-integrin, 5'-CCTGTGACCCACTGC AAGGA-3'/5'-GGGATGATGTCGGGACCAGTA-3'; GAPDH, 5'- TGATTCTACCCACGGCAAGTT-3'/5'-TGATGGGTTTCCCATTCATGA-3'. Reactions were performed through the following conditions: 30 s at 95°C for initial denaturation, 5 s at 95°C followed by 40 cycles of denaturing, and extension for 30 s at 72°C. Experiments were repeated in triplicate. Data analysis based on measurements of the threshold cycle was performed using the $2^{-\Delta\Delta Ct}$ method.[29]

Western blotting analysis

Cells were harvested and lysed in radio immunoprecipitation assay (RIPA) lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) protease inhibitors after treatment. Protein samples (60 μg) were separated by different concentrations of sodium dodecyl sulfate-poly-acrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline Tween-20 (TBS-T, pH 7.6, Sigma-Aldrich, USA) at room temperature for 2 h. Immunoblots were incubated at 4°C overnight with primary antibodies specific for β1-integrin (1:1000, Abcam, UK), FAK (1:1000, CST, USA), phospho-FAK^{Tyr397} (1:1000, CST, USA), AKT (1:1000, CST, USA), phospho-AKT^{Ser473} (1:2000, CST, USA), cleaved caspase-3 (1:1000, CST, USA), and GAPDH (1:10,000, Proteintech Group, China). After washing with TBS-T, the membranes were incubated with anti-rabbit IgG secondary antibody (1:5000) for 2 h at room temperature with gentle shaking. After a final wash with TBS-T, membranes were reacted with electrochemiluminescence (ECL)-plus chemiluminescent detection horseradish peroxidase (HRP) reagents (Pierce ECL Western Blotting Kit, Thermo, USA), detected and quantified using an image analysis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel), and normalized to the reference bands of GAPDH. The densitometry of phosphorylated protein was normalized to its corresponding total protein for assessing protein activation.

Statistical analysis

All data were expressed as a mean ± standard deviation (SD) from the indicated number of independent experiments which were repeated at least three times. The overall statistical comparisons between multiple groups were performed by one-way analysis of variance (ANOVA). Comparison of two experimental conditions was evaluated using Student's *t*-test. A value of $P < 0.05$ was considered to represent statistical significance. All statistical analyses were performed with SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA).

Results

Elevated hydrostatic pressure induces retinal ganglion cell apoptosis

Flow cytometry analysis was used to detect RGC-5 cell survival at different time with 0, 20, 40, and 60 mmHg [Figure 1a]. The histogram indicates that a pressure of 40 mmHg for 24 h induced maximum apoptosis, 7.63% ± 1.02% in early apoptosis and 17.93% ± 1.66% in advanced mortality apoptosis ($P < 0.001$ vs. 0, 20, and 60 mmHg group for 24 h; $P < 0.001$ vs. 40 mmHg group at 6 and 12 h) [Figure 1b]. MTT cell proliferation assay was performed to assess the activity of RGC-5 cells treated with hydrostatic pressure. Cell viability was as follows: 88.67% ± 5.40% (20 mmHg), 52.70% ± 2.00% (40 mmHg), and 80.62% ± 7.91% (60 mmHg). Apparently, the MTT results showed that the cell survival rate decreased at 40 mmHg for 24 h ($P = 0.002$ vs. 20 mmHg, and $P = 0.010$ vs. 60 mmHg) [Figure 1c]. Moreover, we analyzed the expression of cleaved caspase-3, which was significantly increased with 40 mmHg for 24 h ($P < 0.001$ vs. 0, 20, and 60 mmHg) [Figure 1d and 1e]. These results suggested that there was a much greater injury to RGC-5 cells with 40 mmHg for 24 h than that was seen in other experimental groups.

Laminin expression and function in cells exposed to 40 mmHg pressure

In cultured RGC-5 cells, laminin was localized to the cell membranes using immunofluorescence (IF) [Figure 2a]. Moreover, IF staining revealed a marked change of laminin in the pressure group compared with the normal group. The fluorescent intensity of laminin was less prominent in the 40 mmHg for 24 h group compared to the control group ($P = 0.010$) [Figure 2b]. To determine whether laminin plays a protective role in RGC-5 cells under elevated hydrostatic pressure, cells incubated and pretreated with 10 μg/ml laminin were exposed to 40 mmHg for 24 h. Apoptosis was detected by Annexin-V and PI staining and Western blotting of cleaved caspase-3 proteins. Surprisingly, flow cytometry analysis showed that survival was arrested in the 40 mmHg plus laminin (40 + LM) group ($P < 0.001$ vs. control group, $P < 0.001$ vs. 40 mmHg alone) [Figure 2c and 2d], and Western blotting revealed that expression of cleaved caspase-3 was

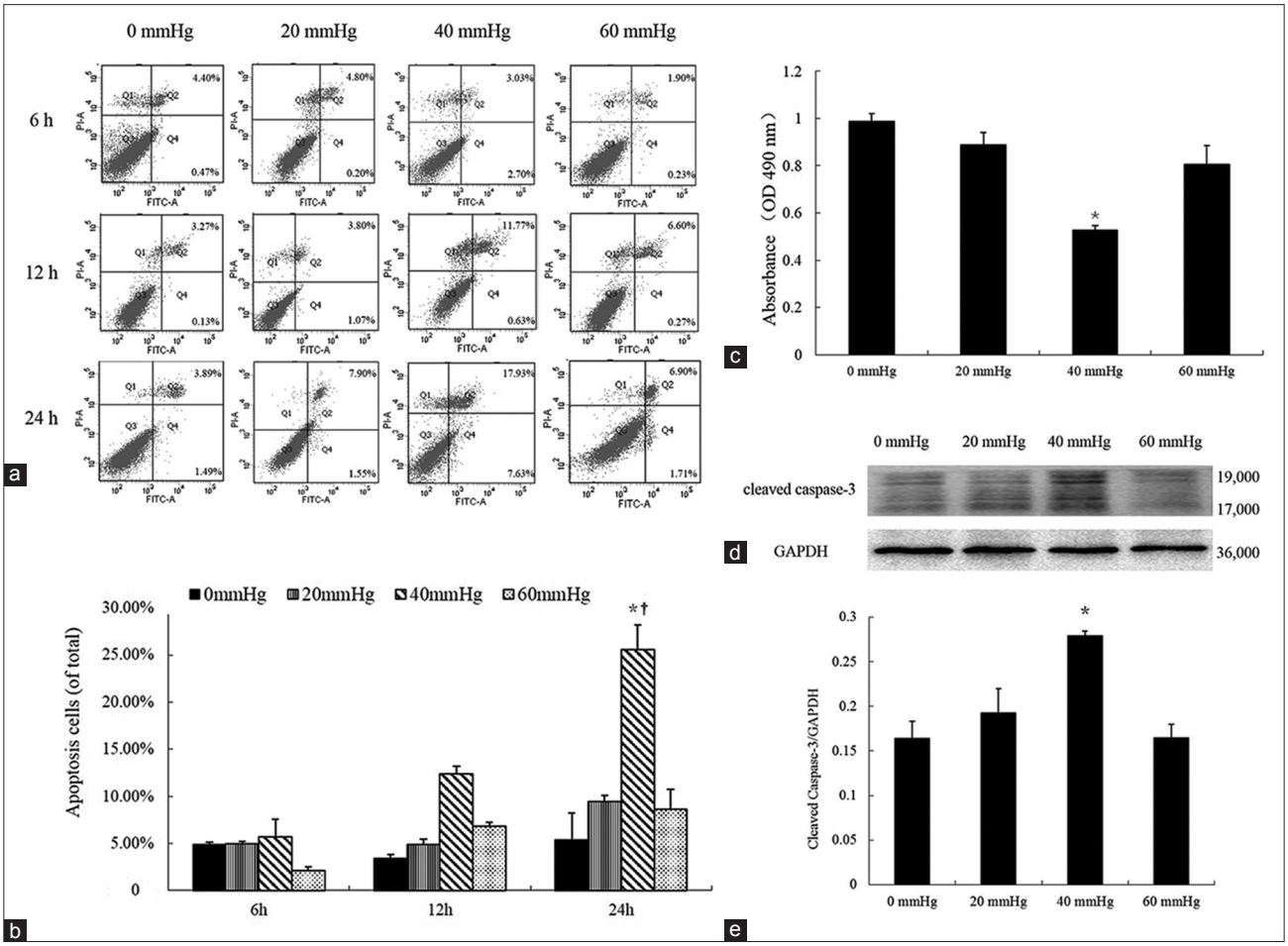

Figure 1: Hydrostatic pressure regulated survival of retinal ganglion cell-5 cells. (a) Flow cytometry of cell apoptosis probed by Annexin V binding (horizontal: FITC-A) and propidium iodide exclusion (vertical: Propidium iodide-A). (b) Percentages of apoptotic cells. *$P < 0.001$ vs. 0, 20, and 60 mmHg group for 24 h; †$P < 0.001$ vs. 40 mmHg group at 6 and 12 h. (c) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide measured the activity. *$P = 0.002$ vs. 20 mmHg, and $P = 0.010$ vs. 60 mmHg. (d) Western blotting analysis for the cleaved caspase-3 and glyceraldehyde-3-phosphate dehydrogenase. (e) The relative expression of cleaved caspase-3. *$P < 0.001$ vs. 0, 20, and 60 mmHg group. FITC: Fluorescein isothiocyanate; OD: Optical density; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

diminished after preconditioning with laminin [Figure 2e and 2f] ($P = 0.016$ vs. 40 mmHg alone).

Laminin modulated the expression of β1-integrin, phospho-FAK, and phospho-AKT in elevated hydrostatic pressure-induced retinal ganglion cells

To assess whether β1-integrin/FAK/AKT pathway was responsible for the anti-apoptosis of RGC-5 cells pretreated with laminin under elevated hydrostatic pressure, we measured levels of β1-integrin and phosphorylation of FAK and AKT by RT-PCR and Western blotting. The level of β1-integrin mRNA was decreased in the 40 mmHg alone ($P < 0.001$ vs. control group). Elevated hydrostatic pressure with laminin resulted in enhanced levels of β1-integrin expression compared to the 40 mmHg alone ($P < 0.001$) [Figure 3a]. The result was similar to the outcome of β1-integrin protein expression ($P = 0.015$ vs. control group, $P < 0.001$ vs. 40 mmHg alone) [Figure 3b and 3c]. Phosphorylation of FAK and AKT was increased in RGC-5 cells exposed to 40 mmHg with laminin for 24 h compared to the 40 mmHg alone group (phospho-FAK, $P = 0.005$ vs. control group, $P = 0.001$ vs. 40 mmHg alone; phospho-AKT, $P = 0.002$ vs. control group, $P < 0.001$ vs. 40 mmHg alone) [Figure 3d and 3e].

Discussion

In this study, we confirmed that elevated hydrostatic pressure can cause apoptosis in RGC-5 cells *in vitro*. The present study systematically evaluated the correlation between time and pressure and the apoptotic effect. We found that laminin was reduced significantly with 40 mmHg for 24 h and that it can protect RGC-5 cells against apoptosis induced by elevated pressure through the β1-integrin/FAK/AKT pathway.

IOP is a critical risk factor for visual impairment with RGCs in glaucoma onset or progression.[30-32] It is believed that experimental animal models of glaucoma are not convenient to research the injury of RGCs in connection with IOP alone. Therefore, we chose a hydrostatic pressure culture system in order to find more associations between cells and IOP. Our findings are supported by the studies of Agar *et al*.[10] and Fragoso *et al*.,[13] which demonstrated

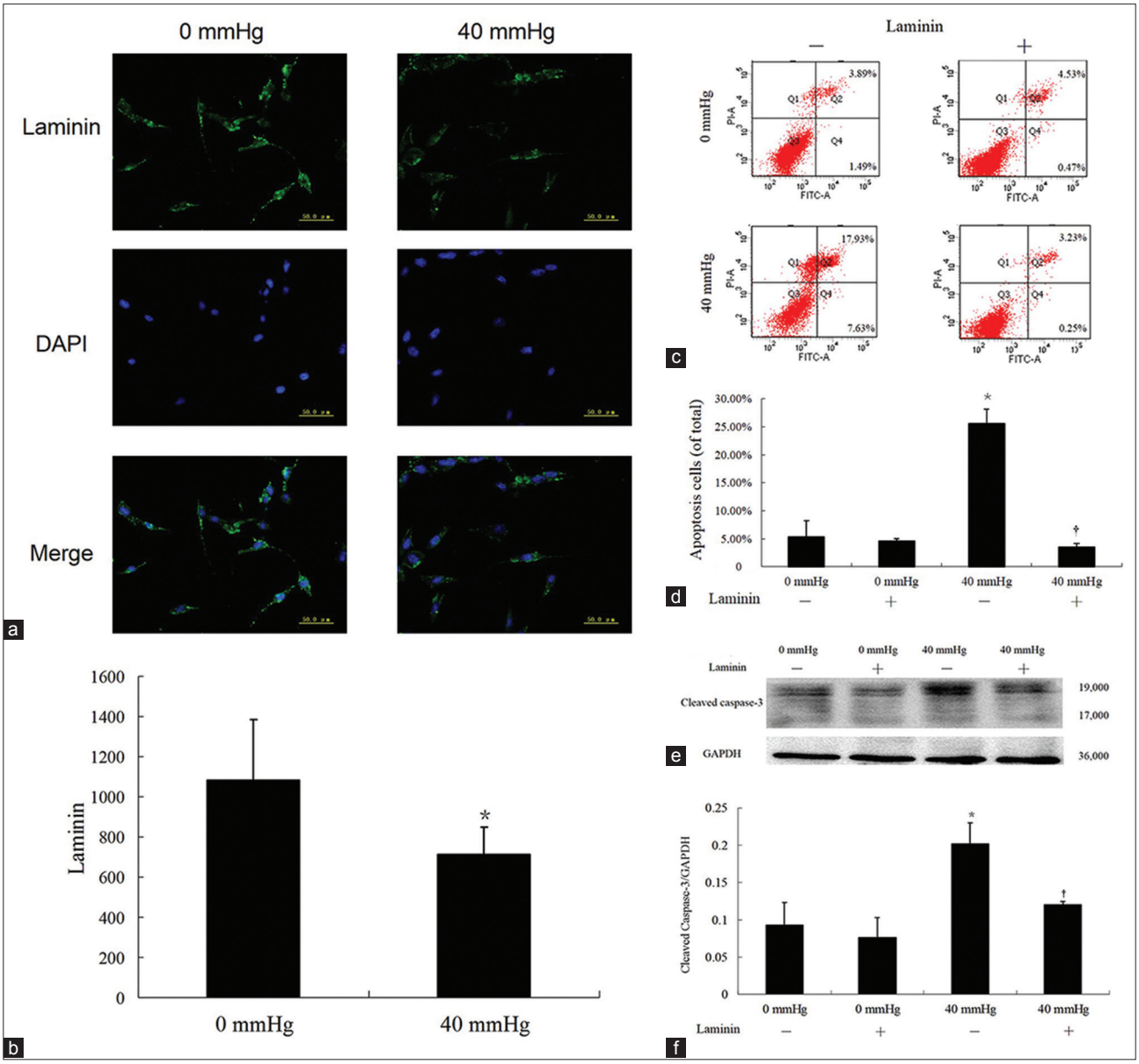

Figure 2: The expression and role of laminin in retinal ganglion cell-5 cells. (a) Representative immunofluorescence images of laminin. Membranes were stained green with anti-laminin antibody, and nuclei were stained blue with 4',6-diamidino-2-phenylindole (original magnification, ×400). Scale bar = 50 μm. (b) The level of laminin was assessed by immunofluorescence. *P = 0.010. (c) Flow cytometry was assessed to apoptosis. (d) Percentages of apoptotic cells. *P < 0.001 vs. control group. †P < 0.001 vs. 40 mmHg alone. (e) Western blotting analysis for the cleaved caspase-3 and glyceraldehyde-3-phosphate dehydrogenase. (f) The relative expression of cleaved caspase-3. *P < 0.001 vs. control group. †P = 0.016 vs. 40 mmHg alone. DAPI: 4',6-diamidino-2-phenylindole; FITC: Fluorescein isothiocyanate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

that hydrostatic pressure could lead to RGC-5 cell death using 100 mmHg to imitate acute HIOP *in vitro*. Actually, 100 mmHg was not realistic for glaucoma patients. In our study, we initially examined different pressure-time course settings of elevated hydrostatic pressure-induced apoptosis in cultured RGCs to determine the top rate of apoptosis. Injuries at four pressure levels (0, 20, 40, and 60 mmHg) were measured at 6, 12, and 24 h. The results of Annexin V-FITC/PI staining, MTT and cleaved caspase-3 expression indicated that maximum injury occurred at 40 mmHg and that there was less apoptosis in RGC-5 cells at 60 mmHg than at 40 mmHg. Osborne *et al*.[33] also investigated that elevated hydrostatic pressure did not cause necrotic cell death in human organotypic retinal cultures under 60 mmHg for 24 h. It might be related to the fluctuating pressure speed. One previous paper has investigated the effects of hydrostatic pressure on retinal explants.[34] The research exposed rat retinal explants to raised hydrostatic pressure and showed a loss of RGC viability, but only when the pressure was increased very rapidly. Moreover, we supposed that some protective signaling pathways were more intensively activated in RGC-5 at 60 mmHg than at 40 mmHg, and we are currently investigating this issue.

As an important ECM component, laminin is critical for the ECM scaffold with which other protein components interact.[15] We found that laminin was located on membranes

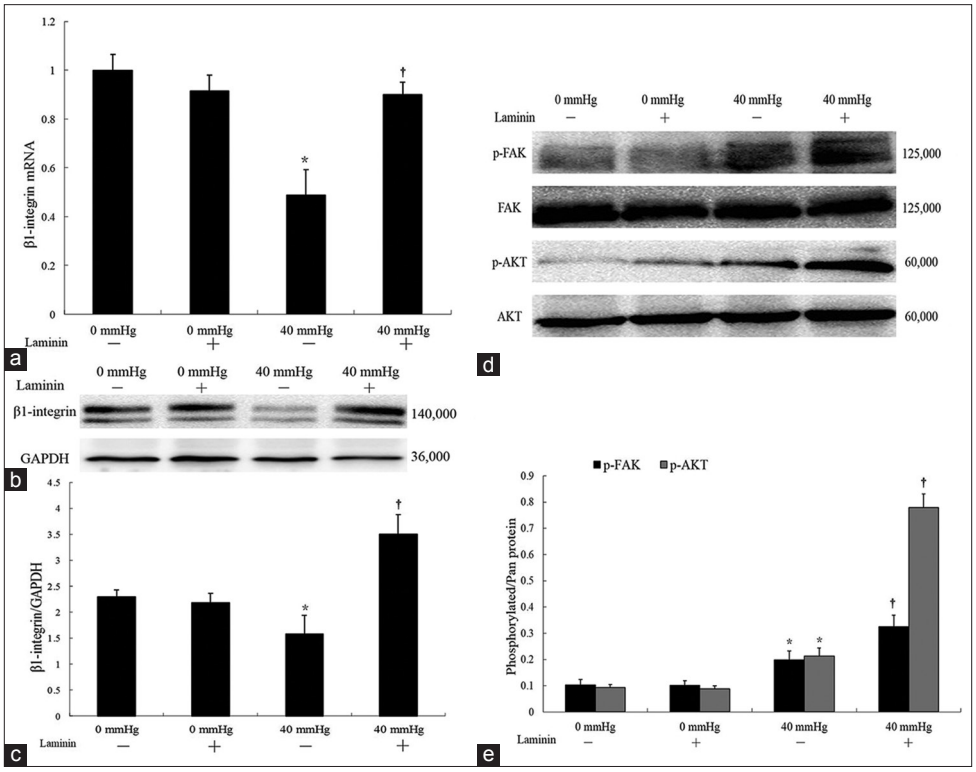

Figure 3: The alternation of β1-integrin/focal adhesion kinase/protein kinase B pathway after treated with laminin under 40 mmHg for 24 h. (a) Real-time polymerase chain reaction detected the level of β1-integrin mRNA. *$P < 0.001$ vs. control group, †$P < 0.001$ vs. 40 mmHg alone. (b) Western blotting analyzed β1-integrin protein. (c) The relative protein expression of β1-integrin. *$P = 0.015$ vs. control group, †$P < 0.001$ versus 40 mmHg alone. (d) Western blotting determined the phosphorylation of focal adhesion kinase and protein kinase B expression. (e) The relative expression of the phosphorylation of focal adhesion kinase and protein kinase B. *$P < 0.01$ vs. control group, †$P < 0.01$ vs. 40 mmHg alone. p-FAK: Phospho-focal adhesion kinase; p-AKT: Phospho-protein kinase B; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

and was reduced in cells after exposure to 40 mmHg for 24 h. This finding is consistent with the study by Guo *et al*.[7] which showed a decrease in laminin deposition with elevated IOP in an experimental rat glaucoma model. Because laminin might provide survival signals through interactions with cellular integrins, the reduction or lack of laminin might lead to the interruption of cell-ECM communication-making cells more susceptible to apoptosis.[35]

Integrins bind to ECM and require kinase proxies to enact signal transduction following their activation.[18,36] The laminin signal is generally received through the β1-integrin receptor on the cell surface. Recently, Santos *et al*.[26] showed that β1-integrin expression was reduced in the period after ischemia and that stimulating β1-integrins enhanced RGC survival *in vitro*. Similarly, our data showed that the level of β1-integrin mRNA and protein was decreased parallel to the reduction in laminin on RGC-5 cells with 40 mmHg for 24 h. Interestingly, after pretreatment with laminin, the level of β1-integrin mRNA and protein was increased significantly under 40 mmHg for 24 h, in line with the reduction in apoptosis. This proved that laminin-β1-integrin plays a role in the survival of RGC-5 cells against elevated pressure. In addition, it also suggested that β1-integrin is a critical mediator of cell survival signaling.

β1-integrin signaling occurs primarily through the recruitment and activation of the tyrosine protein kinase FAK, which acts as both a direct mechanosensor and also downstream of mechanical signaling events.[37] FAK activates downstream signaling cascades, including the regulation of PI3K, which stimulates phosphorylation of AKT at Ser-473.[38] It has been reported that the PI3K/AKT pathway mediates axon growth and protein synthesis.[39] Our results indicated that there was a slight increase in the phosphorylation of FAK and AKT in RGC-5 cells under 40 mmHg for 24 h. The report is parallel with that of Park *et al*.,[40] which demonstrated that the level of PI3K/AKT was activated after optic nerve axotomy. Our observation was also supported by the fact that AKT was activated in hypoxic primary cultured RGCs.[41] We speculated that the activation of FAK and AKT might be a manifestation of a self-defense mechanism against elevated hydrostatic pressure in RGC-5 cells. Previous studies have shown that β1-integrins translate RGC adhesion to laminin into survival signals mediated by FAK and associated with AKT activation in a rat model of retinal ischemia-reperfusion injury.[26] Similarly, we found that the phosphorylation of FAK and AKT was increased, evidently accompanying RGC-5 cells survival after treatment with laminin at 40 mmHg. In the future, it might be interesting to research the specific mechanism of the FAK/AKT pathway in laminin-β1-integrin-mediated survival signaling in RGC-5 cells after elevated hydrostatic pressure.

In conclusion, our findings demonstrate that there is severe damage in RGC-5 cells exposed to 40 mmHg for

24 h. The decreased laminin might be in charge of RGC-5 cell apoptosis, and it exerts a neuroprotective effect against injury originated by pressure on RGC-5 cells. The β1-integrin/FAK/AKT pathway plays an important role in cell survival *in vitro*. Although there are some limitations, such as some views should be demonstrated by an animal model of glaucoma, why 60 mmHg hydrostatic pressure could protect RGC-5 from apoptosis and other pathways might be associated with laminin against apoptosis, this study offers a foundation for future research. It is believed that laminin or upregulation of the β1-integrin/FAK/AKT pathway might lead to novel therapeutic agents for reducing visual field loss and blindness in patients with glaucoma.

Financial support and sponsorship

This work was supported by a grant from the National Natural Science Foundation of China (No. 30070798).

Conflicts of interest

There are no conflicts of interest.

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