Original Article
AQP 9 promotes optic nerve regeneration by regulating glucose metabolism

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Abstract: Introduction: Injury of optic nerve can give rise to axonal degeneration, and gradually lead to death of retinal ganglion cells (RGCs), which ultimately cause irreversible vision loss. It was suggested that one of the aquaporin (AQP) family members, AQP-9, is a critical factor in optic nerve regeneration. However, its mechanism is unknown. Methods: Here, we explored the repair and regeneration mechanism of injury optic nerve associated with glucose metabolism regulated by AQP-9 using the model rats of optic nerve crush by RT-PCR and western blot. Results: Our data indicated that the damage degree of retinal in siRNA-AQP-9 rats (13.20±2.24) was relatively severity and relatively light in rats treated with overexpression of AQP-9 (32.10±2.28). The treatment of siRNA-AQP-9 significantly reduced the number of surviving RGC cells in optic nerve of model rats and the treatment with overexpression of AQP-9 can restore the reduction. The overexpression of AQP-9 can also restore the the expression level of G6PC, GK and GOT1 in optic nerve and glucose production in model rats of optic nerve damage. Conclusions: AQP-9 can restore the optic nerve damage through the pathway related to gluconeogenesis gene, which may promote the glucose metabolism of optic nerve and repair of retinal ganglion cells. AQP-9 may be a promising new target for the treatment of optic nerve damage.

Keywords: Optic nerve crush, retinal ganglion cells, aquaporin (AQP), glucose metabolism

Introduction
Like other CNS tracts in higher vertebrates, the optic nerve cannot regenerate after traumatic injury. The poor regenerative capacity of injured nervous axons leads to permanent neurological deficits after optic nerve lesions [1]. A balanced growth stimulatory treatment will have to be combined with guidance factors and suppression of local growth inhibitory factors to obtain the full regeneration of optic nerve [1]. The optic nerve crush is a model of choice to study this phenomenon and to establish new regenerative strategies and potential treatments.

The weak intrinsic capacity of adult neurons to reactivate a growth program after injury was proposed to be an important impediment to axonal regrowth [2]. Indeed, after birth, intrinsic growth repressors, for example, of the Kruppel-like factor (KLF) transcription factor family, are upregulated in the developing retina, while growth-inducing transcription factors are downregulated in retinal ganglion cells and other central nervous system (CNS) neurons [3]. The presence of growth-repressing molecules in the CNS tissue and within the neurons, and the lack of adequate stimulation and growth responses by adult neurons are currently thought to be the predominant causes of axon regeneration failure.

Aquaporins (AQP), as a water channel, are channel-forming integral proteins [4]. Until now, more than 10 AQP have been found [5]. Reported studies have classified AQP family as two subgroups: One is aquaglyceroporins that transport water and glycerol; the other is AQPs that are selective water channels [6]. As a member of AQP water channel family, AQP 9 is an aquaglyceroporin permeable to water and non-charged solutes like lactate. Miki et al [7] had discovered that expression of AQP-9 could be
observed in retinal ganglion cells (RGCs) and that elevated intraocular pressure promotes death of neurons of retinal ganglion cell layer and reduces the expression of AQP-9 in rodents. Both optic nerve transection (ONT) and serum deprivation obviously reduced the expression of AQP-9 protein in RGCs and raised the rate of RGC death. After ONT, the expression of retinal AQP-9 gene also declined, which markedly increased apoptosis, the NAD (+)/NADH ratio, and ROS accumulation in the RGC-5 cells. These findings inferred that deficiency of AQP-9 adversely impacts survival of RGCs, at least partly due to decreased lactate transport as a substrate for energy and/or ROS scavenger [7].

Dynamics of aquaporin in the optic nerve AQP is a membrane protein that forms a water channel to facilitate water crossing the plasma membrane. AQP-4 was originally thought to be expressed in the optic nerve, but it is expressed only in the retrobulbar medullated region of the optic nerve and the expression of AQPs in the optic disc has not been detected. Astrocytes were observed to express AQP-9, because AQP-9 immunoreactivity was identical to that of glial fibrillary acidic protein. Elevated intraocular pressure substantially reduced AQP-9 expression in the optic nerve, whereas expression of AQP-4 was not changed in rat eyes. The same phenomena were also observed in the monkey eye with ocular hypertension as well as human eye with glaucoma. AQP-9 is an aquaglyceroporin that allows solutes such as lactate rather than water to cross the cell membrane. The astrocyte-to-neuron lactate shuttle hypothesis has been proposed, in which lactate transported from astrocytes is used by neurons as an energy substrate. Reduction of AQP-9 expression in the optic nerve head under elevated intraocular pressures might be closely related to the mechanisms of glaucomatous optic neuropathy (GON), the most common optic neuropathy [8]. These results suggest that AQP-9 is a critical factor in optic nerve regeneration. Recent studies suggest that aquaporins could be a promising drug target [9, 10].

Recently, we found that overexpression of AQP-9 could restore the rats of optic nerve damage, then promoting repair of retinal ganglion cells. However, the mechanism was unknown. The aims of this study were to explore the repair and regeneration mechanism of injury optic nerve associated with glucose metabolism and to evaluate the possible options for the treatment on optic nerve injury related to AQP-9.

Materials and methods

Animals

All animal procedures complied strictly with the guidelines of the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Visual Research, and were carried out according to approved protocols by the Board of Animal Welfare, The Military General Hospital of Beijing PLA, Beijing, China.

There were 40 adult male SD rats, weight 280-320 g, examination of eye and fundus was normal. Rats was divided by a random number table method: there were 10 rats of control group, 10 rats of model group (treated with PBS buffer), 10 rats of AQP-9 group (treated with overexpression of AQP-9) and 10 rats of siRNA-AQP-9 group (treated with siRNA-AQP-9) for 14 days.

Model preparation of optic nerve damage and treatment: After intraperitoneal injection of 50 mg/kg ketamine anesthesia, optic nerve was fully exposed. Then the optic nerve was clamped by reverse tweezers behind the eyes for 0.5~1.0 mm, incomplete injury of optic nerve was achieved. Then rats of AQP-9 group and siRNA-AQP-9 group received an i.v. injection at 250 mg/kg body weight one time every day [11], the rats of model group were injected with PBS on the same way. After treatment for 14 days, rats were executed, and then optic nerve and retinal was separated, respectively.

siRNA

In vivo ready synthetic siRNA were purified by high-performance liquid chromatography and purchased from Santa Cruz Technologies Inc. (Santa Cruz, CA, USA). The most efficient sequence, sc-42372 were used, which can knock-down their specific targets in vivo. Sc-37007 was used as scrambled control siRNA.

SiRNA/IVF2.0 complex preparation

Invivofectamine 2.0 (Invitrogen, Carlsbad, CA) was applied following the manufacturer’s protocols. We diluted stock siRNA solution with the
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| Table 1. The primers for RT-PCR |
|---|---|
| Gene | Primers |
| AQP-9 | Upstream 5’-ATAGCAGCGAACAGGGAATG-3’ |
| | Downstream 5’-CGTTCATAAGGGCGT-3’ |
| G6PC | Upstream 5’-TCCATTCTCAGCTTCA-3’ |
| | Downstream 5’-CAGCTGGCCCTAAGAGATA-3’ |
| GK | Upstream 5’-CCACTTTGGAATGGGATAA-3’ |
| | Downstream 5’-GCCATAGATCTCAGAAACTGC-3’ |
| GOT1 | Upstream 5’-GGTGAGAGTCTCTCCAGTCA-3’ |
| | Downstream 5’-CGGAGACCTCGAGAACGA-3’ |

decomplexation buffer at 1.5 mg/ml (250 μl final) and subsequently mixed with Invivofectamine 2.0 reagent (250 μl). Then the mixture was incubated at 50°C for 30 minutes followed by dialysis against PBS for 2 hrs in a Spectra/Por Float-A-Lyzer G2 8-10 KD (Spectrum Medical Laboratories, Rancho Dominguez, CA). For i.v. injections, we adjusted the concentration of siRNA with PBS at 0.25 mg/ml or 0.5 mg/ml respectively. A total of 0.1 mg of siRNA was injected per model mouse for the in vivo experiments [11].

Plasmid constructs and DNA purification, injection

The plasmids of overexpression of AQP-9 were constructed and sequenced according to standard techniques. The CMV plasmids were obtained from Clontech [12]. The AQP-9 group received one of the CMV-AQP-9 plasmid DNA via the tail vein. We resuspended pellets of prepared overnight bacterial cultures with Glucose/Tris/EDTA (GET) and added fresh 0.2 M NaOH/1% SDS solution for alkaline lysis. Neutralization was performed with 3 M potassium acetate solution (pH 5.5) and then DNA was precipitated using 0.6 vol isopropanol. Resuspending the DNA pellet in TE buffer with 1 g of CsCl per ml of solution, the complex was transferred with addition of ethidium bromide to a 12 ml ultracentrifuge tube and centrifuged with a speed of 79000 rpm for 13.5 hrs. Subsequently, plasmid DNA was extracted from the DNA band collection to remove ethidium bromide with an equal volume of NaCl-saturated isopropanol. Then Plasmid DNA was separated out with 150 mM NaCl [11].

Retina dissection, staining and imaging

Rats were anesthetized with Avertin (Sigma-Aldrich) and sacrificed with CO₂. We enucleated eyes, then fixed them with 4% paraformaldehyde for 1 hr, and dissected them to isolate the retina carefully. Subsequently, the retinas were stained overnight by fluoresceinated Griffonia Simplicifolia Isolectin B4 (Invitrogen; 1:100 dilution) with 1 mM CaCl₂. After washes with PBS for 2 hrs, retinas with the photoreceptor side down were put onto Superfrost/Plus microscope slides (Fisher Scientific) using SlowFade Antifade reagent (S2828; Invitrogen). Images at 5× magnification on a Zeiss AxioObserver were obtained. Z1 microscope was merged to obtain entire images of retina vasculature by AxioVision 4.6.3.0 software. Estimates of RGC counts were obtained using a previously published model which combines estimates of RGC numbers from SAP sensitivity thresholds and retinal nerve fiber layer (RNFL) thickness measurements with spectral domain optical coherence tomography (SDOCT).

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from optic nerve through the RNeasy Mini kit (Qiagen, Valencia, CA). Then, with SuperScriptIII reverse transcriptase (Life Technologies), the reverse transcriptions were performed according to the manufacturer’s instructions. The amplification was carried out by ABI 7500 Fast Real-Time PCR System (Life Technologies) as follows: a hot start at 95°C for 5 min was followed by 40 cycles: denaturation at 95°C for 15 s, annealing of the primers (Table 1) at 60°C for 30 s and elongation at 72°C for 30 s. Results were normalized to GAPDH. The mean value three independent experiments were calculated as the final data.

Western blot analysis

The optic nerve cells lysate was prepared using a lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The concentration of extracted protein was measured by Bradford assay by Coomassie Plus Protein Reagent (Thermo Scientific, Rockford, IL). All protein samples were incubated with primary antibodies like anti-AQP-9, G6PC, GK or GOT1 respectively (Santa Cruz Biotechnology, Santa Cruz, CA), followed with secondary horseradish peroxidase-conjugated antibodies (GE Healthcare, Piscataway, NJ). The protein bands were visualized by Enhanced Chemilumine-
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Detection of glucose production

Glucose production of optic nerve was determined using the glucose oxidase colorimetric method. Retina ganglion cells were prepared and cultured in gluconeogenic medium [13] for 4 hrs. All relevant experiments were repeated at least three times.

Statistics

All statistical analyses were performed by software SPSS 13.0. Data were presented as mean ± SD. For numerical variable, t-test or analysis of variance (ANOVA) was performed. We used Kruskal-Wallis ANOVA to compare medians for groups not normally distributed. \( \chi^2 \) test was done to analyze qualitative data. \( P \) value <0.05 was considered statistically significant.

Results

Rat model of optic nerve crush

The wound healing of rats was well after incision without inflammatory response. The cornea of model rats was transparency with no traumatic cataract. There was no inflammatory reaction in vitreous and no blood in vitreous cavity, no bleeding and detachment in retinal after surgery. There was mydriasis of eyes after surgery, diameter 2~4 mm, direct light reflex (-), indirect light reflex (+), which was relative affer-
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ent papillary defect (RAPD). These evaluation indexes indicated that the model of optic nerve crush was successful.

Morphology changes of retina

The ganglion cell layer and the inner and outer nuclear layer of retina in rats of normal control group from the outside to the inside were arranged densely and parallel (Figure 1A). At 14 days after optic nerve crush, the cell nuclei of ganglion cell layer have scattered distribution, shrank nuclei volume, chromatin condensation and margination (Figure 1B). The inner and outer nuclear layer was thinning, and cells were arranged in disorder. There were more obviously changes in model rats treated with si-AQP-9 group, the count of cell nuclei of ganglion cell layer decreased obviously, and cells arranged rarefaction, more condense nuclei could be seen. The inner and outer nuclear layer was more thinning (Figure 1C). However, there were no marked changes in model rats treated with AQP-9. At 14 days after optic nerve crush, the count of cells in each layer decreased lightly. Moreover, there was not chromatin margination of nucleus in each layer, and cavitation on ganglion cells accompanied with infiltration of glial cell. The count of cells in inner and outer nuclear layer decreased significantly (Figure 1D). The damage degree in model rats treated with AQP-9 was relatively light, and in model rats treated with si-AQP-9 was relatively severe.

RGCs number

The accounts of surviving RGCs cells of model rats were significantly decreased compared with control group (P<0.01). Compared with control group, at AQP-9 group, the number of surviving RGCs cells decreased slightly (P>0.05), and was much more than in model group and siRNA-AQP-9 (P<0.01). But, at siRNA-AQP-9 group, the mRNA expression level of AQP-9 decreased obviously compared with the control group (P<0.01) and was lower than model group (P<0.05). The data are presented as means ± SD from three independent experiments. *P<0.05, **P<0.01; P value was generated using Kruskal-Wallis ANOVA.

Figure 2. The surviving RGCs numbers were restored by AQP-9. The numbers of the surviving RGCs cells in retina of model rats were significantly reduced compared with control (P<0.01). The numbers of surviving RGCs cells in the AQP-9 group were comparable with control (P>0.05), but were significantly more than model and siRNA-AQP-9 group (P<0.01). The numbers of surviving RGCs cells in the siRNA-AQP-9 group significantly decreased compared with control (P<0.01), as well as model group (P<0.05).

Figure 3. The mRNA level of AQP-9 in optic nerve. The mRNA expression level of AQP-9 in optic nerve of model rats decreased significantly compared with control group (P<0.01). At AQP-9 group, the mRNA expression level of AQP-9 decreased slightly compared with the control group (P>0.05), and was much more than in model group and siRNA-AQP-9 (P<0.01). But, at siRNA-AQP-9 group, the mRNA expression level of AQP-9 decreased obviously compared with the control group (P<0.01) and was lower than model group (P<0.05). The data are presented as means ± SD from three independent experiments. *P<0.05, **P<0.01; P value was generated using Kruskal-Wallis ANOVA.

When compared with control group, the mRNA expression level of AQP-9 (Figure 3) and gluco-
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The mRNA expression level of gluconeogenesis related genes G6PC, GK and GOT1 in optic nerve of model rats decreased significantly compared with control group ($P<0.01$). At AQP-9 group, the mRNA expression level of G6PC decreased slightly compared with the control group ($P>0.05$), and was much more than in model group and siRNA-AQP-9 ($P<0.01$). But, at siRNA-AQP-9 group, the mRNA expression level of G6PC decreased obviously compared with the control group ($P<0.05$) and was lower than model group ($P<0.05$). The data are presented as means ± SD from three independent experiments. *$P<0.05$, **$P<0.01$; $P$ value was generated using Kruskal-Wallis ANOVA.

The protein expression level of AQP-9, G6PC, GK and GOT1 in optic nerve
eogenesis related genes G6PC (Figure 4), GK (Figure 5) and GOT1 (Figure 6) in optic nerve of model rats decreased significantly ($P<0.01$). At AQP-9 group, the mRNA expression level of AQP-9, G6PC, GK and GOT1 decreased slightly in comparison with control group ($P>0.05$), and was much more than in model group and siRNA-AQP-9 ($P<0.01$). But, at siRNA-AQP-9 group, the mRNA expression level of AQP-9, G6PC, GK and GOT1 decreased obviously ($P<0.01$) and was lower than model group ($P<0.05$). It inferred the mRNA level of AQP-9, G6PC, GK and GOT1 could be restored by the treatment with overexpression of AQP-9 in rats with optic nerve damage, but reduced by siRNA of AQP-9.
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The amounts of glucose production in optic nerve cells in model rats decreased obviously compared with control group (P<0.01). At AQP-9 group, the amounts of glucose production decreased slightly in comparison with control group (P>0.05), and was much more than in model group and siRNA-AQP-9 (P<0.01). But, at siRNA-AQP-9 group, the amounts of glucose production decreased obviously versus control group (P<0.01) and was lower than model group (P<0.05) (Figure 8). It indicated that the amounts of glucose production could be restored by the treatment with overexpression of AQP-9 in rats with optic nerve damage, but reduced by siRNA of AQP-9.

Discussion

AQP9 enhances glucose production in optic nerve

The amounts of glucose production in optic nerve cells in model rats decreased obviously compared with control group (P<0.01). At AQP-9 group, the amounts of glucose production decreased significantly compared with control group and was lower than model group (Figure 7). It indicated that the protein expression level of AQP-9, G6PC, GK and GOT1 could be restored by the treatment with overexpression of AQP-9 in rats with optic nerve damage, but reduced by siRNA of AQP-9.

Discussion

AQP9, an intrinsic protein, belongs to integral plasma membrane channel proteins permeated passively by some small, uncharged solutes and water [13, 14]. Based on their highly conserved dual asparagine-proline-alanine (NPA) boxes, AQPs have been identified. It has modest identity to differentiate conventional AQPs from another owing to their hydrophobic NPA box-like repeats and conserved six transmembrane domains [15-17]. The NPA structure of AQPs was essential for the formulation of water channel and transmembrane movement of water [14]. In accordance with the expression in most tissues, AQPs may be involved in sorts of physiological and pathophysiological processes. AQP-9 is a critical factor in optic nerve regeneration.

Focusing on the repair and regeneration mechanism of injury optic nerve associated with AQP-9, AQP9 overexpression or knockdown was induced in rat model of optic nerve crush. We found that there were more obviously changes in model rats treated with si-AQP-9 group, the count of cell nuclei of ganglion cell
layer decreased obviously, and cells arranged rarefaction, more condense nuclei could be seen. The inner and outer nuclear layers were more thinning. But, there were no marked changes in model rats treated with AQP-9. Moreover, the damage degree in model rats treated with AQP-9 was relatively lighter, and in model rats treated with si-AQP-9 was relatively more severe. Furthermore, the number of surviving RGCs cells was counted; it was significantly reduced in retina of model rats compared with control group. Moreover, the number of surviving RGCs cells at AQP-9 group decreased slightly compared with the control group, and was much more than in model group and siRNA-AQP-9. But, the number of surviving RGCs cells at siRNA-AQP-9 group decreased obviously compared with the control group and was lower than model group. It indicated that the optic nerve damage could be restored by the treatment with overexpression of AQP-9 in rats with optic nerve damage, but the damage could be enhanced by siRNA of AQP-9.

As shown recently, relevant studies have established two models related to human metabolism and have identified their potential clinical utility [18-20]. When blood glucose levels decrease because of vigorous exercise or fasting, followed by activated gluconeogenesis in cells, thereby glucose is produced to restore glucose homeostasis. Induction of gluconeogenesis by a hormonal stimulus was achieved through various complicated reactions. Some essential enzymes, such as fructose-1, 6-bisphosphatase (F1, 6BPase), phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase (PC) and so on, were involved in these reactions. Thence for sake of an effective induction of gluconeogenesis, regulation of the genes expression encoding these enzymes is important [21].

The aims of this study were to explore the association with glucose metabolism and the methods for treatment on optic nerve injury related to AQP-9. For sake of comprehensive definition of metabolic pathways related to the role of AQP 9 on the repair and regeneration mechanism of injury optic nerve, the expression of gluconeogenic gene was examined. We performed the research of glucose metabolism and the methods for treatment on optic nerve injury related to role of AQP 9 in enhancing gluconeogenesis. Then our examination manifested that AQP9 may regulate the related genes expression of gluconeogenesis. The mRNA and protein expression level of AQP-9 and gluconeogenesis related genes G6PC, GK and GOT1 in optic nerve of model rats decreased significantly compared with control group. At AQP-9 group, the mRNA expression level of AQP-9, G6PC, GK and GOT1 decreased slightly compared with the control group, and was much more than in model group and siRNA-AQP-9. But, at siRNA-AQP-9 group, the mRNA expression level of AQP-9, G6PC, GK and GOT1 decreased obviously compared with the control group and were lower than model group. It indicated that the mRNA expression level of AQP-9, G6PC, GK and GOT1 could be restored by the treatment with overexpression of AQP-9 in rats with optic nerve damage, but reduced by siRNA of AQP-9. Consistent with these above results, the amounts of glucose production could be restored by the treatment with overexpression of AQP-9 in rats with optic nerve damage, but reduced by siRNA of AQP-9.

Our study demonstrated that AQP-9 could restore the rats of optic nerve damage throughout the pathway related to gluconeogenesis gene G6PC, GK and GOT-1, then promoting the glucose metabolism of optic nerve and repair of retinal ganglion cells. AQP-9 may be as a promising new target for the treatment of optic nerve damage.

The retina and optic nerve are parts of the central nervous system. Since the central nervous system damage is irreversible, injuries to the optic nerve, such as those caused by trauma, inflammation, and ocular hypertension, lead to retinal ganglion cell (RGC) loss which causes permanent vision loss or blindness. Until now, no drug therapy has been developed for effective treatment of optic nerve injury. Therefore, a neuroprotective medicine that can delay or prevent RGC loss after optic nerve injury is needed [22].

Our study examined the neuroprotective effect of AQP-9 on RGCs and investigated the role of glucose synthesis after optic nerve crush (ONC) injury in mice. In conclusion, AQP-9 may be as a promising new target for the treatment of optic nerve damage. Treatment with AQP-9 will be likely to regenerate optic nerve as well as a promising choice for prevention of severe injury
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following diseases of the optic nerve or surgery.

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Disclosure of conflict of interest

None.

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References