·Basic Research·

Expression of caspase-9 affected by AG on retina of rats with chronic IOP elevation

Qing-Zhu Nie, Qian Sha, Ying-Shuang Wang, Dong-Mei Gui, Zhi-Li Liu, Dian-Wen Gao

Department of Ophthalmology, Shengjing Hospital Affiliated to China Medical University, Shenyang 110004, Liaoning Province, China

Correspondence to: Qian Sha. Department of Ophthalmology, Shengjing Hospital Affiliated to China Medical University, Shenyang 110004, Liaoning Province, China. nieyjin@126.com
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Abstract

• AIM: To study caspase-9 expression on rat retina in the process of chronic elevation of IOP and the changes with the application of amino guanidine (AG), thus to investigate the potential protective function of AG to rat retina with chronic elevation of IOP.

• METHODS: Immunohistochemistry, RT-PCR and Western blot were used to observe retinal morphology and the expression of caspase-9 at different time points of rat with chronic IOP elevation, both affected and not affected by the application of AG.

• RESULTS: Compared with control group, as time passed retina of experimental group gradually had detectable morphological changes. On 21st day of chronic IOP elevation, retinas became thinner and the quantity of retinal ganglion cells (RGCs) decreased; caspase-9 expression increased, consistent with the morphological changes. The group using AG presented relatively smaller morphology changes and less expression of caspase-9.

• CONCLUSION: Apoptosis-related gene caspase-9 plays a part in the process of chronic IOP elevation; AG protects retina by down-regulating expression of caspase-9.

• KEYWORDS: retina; chronic IOP elevation; apoptosis; amino guanidine; caspase-9

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INTRODUCTION

Intraocular hypertension is a major risk factor of glaucoma injury. Nowadays, the treatment of glaucoma is mainly based on the decrease of IOP with medicine or operation. However, a simple decrease of IOP is not enough to prevent the progressive neural injuries caused by glaucoma [1]. Therefore, there might be also some other factors working. At present, glaucoma is considered as a multi-factor controlling disease and the final common pathway is the apoptosis of RGCs, while the mechanisms of how all these factors induce apoptosis of RGCs and the regulations of apoptosis genes are still unclear. Therefore, it is very important to investigate how these apoptosis-related genes are involved in the apoptosis of RGCs at different levels and how they are regulated, so that we can provide better treatment for patients. Caspase is a family of intracellular cysteine endopeptidases, which is the most important apoptosis-related gene found by now [2]. It is classified into two groups based on their primary constructions and the size of N-terminal pro-domain: (1) Promoter: caspase triggers on DNA fragmentation during apoptosis. (2) Effector: caspase induces apoptosis directly by enzymolysis to substrate protein. Caspase-9 is a promoter. Amino guanidine (AG) is an inhibitor of induced type of nitricoxide synthase, which is safe, effective and extensively applied in the research of myocardial ischemia and cerebral anoxia but comparatively less used in glaucoma investigation. Neufield and his coworkers reported that AG is an optic nerve protector of rat model of chronic IOP elevation, but the mechanisms need further investigation. This experiment is designed to explore the connection between the expression of caspase-9 and RGCs in chronic IOP elevation, and to testify whether AG is involved in the regulation of caspase-9 playing the role of neuroprotection. This experiment will be performed at levels of gene and protein. It will investigate apoptosis-related genes and the changes of its protein expression in normal retinal tissue and retinal tissue of IOP elevated rats with and without AG application. We performed this experiment to reveal the mechanisms of optic nerve injury and protection for glaucoma to some degree, and we look forward to applying all our findings to

clinical treatment. Hopefully, we can find the best neuro-protectant in this multiple pathway system and provide a new idea for the treatment of glaucoma.

MATERIALS AND METHODS

Materials

Laboratory animals Fifty male Wistar rats, weighing between 200-300g, were supplied by Experiment Animal Department of China Medical University. The animals and experimental conditions followed laboratory animal regulations of State Science and Technology Commision. The animals were randomly divided into 3 groups, which were 10 in blank control group (20 eyes), 30 in chronic IOP elevated group (60 eyes), and 30 in chronic IOP elevated with AG application group (60 eyes).

Reagents of immunohistochemistry Protein antibody: rabbit anti-rat caspase-9 (Wuhan Boster Biotechnology Co., Ltd), SABC kit (Wuhan Boster Biotechnology Co., Ltd) were pure.

Reagents of RT-PCR M-MLV reverse transcriptase (Promega, USA), Oligo (dT) (Promega, USA), Heat resistant Taq DNA polymerase (Takara Biotechnology (Dalian) Co., Ltd), TaKaRa RNA PCR (Takara Biotechnology (Dalian) Co., Ltd), PCR primer: designed by primer Express 3.0 based on GeneBank.

Reagents of western blot N, N'-methylene bisacrylamide (BBI, USA), N, N, N, N-TEMED (Fluka, USA), sodium dodecylsulphate (SDS), DTT (Sigma, USA), NC filter (Amersham, UK), and Normal goat serum (Wuhan Boster Biotechnology Co., Ltd) were also provided.

Methods

Rat model of chronic IOP elevation The rats were anesthetized by 100g/L chloral hydrate 0.3mL for every 100g body weight with intraperitoneal injection. Bulbar conjunctiva was cut and two superficial venous tributaries were burnt (signs of successful burn: episclera venous blood flow disappeared on the distal end of the burnt point; distension and darkness of the vessels near corneoscleral limbus). Bulbar conjunctiva was reset with TobraDex drops and paste as eyedrop application. IOP was also measured with TONO-PEN Ⅱ, and the measurement time points were before the operation, half an hour after the operation, at the 7^{th}, the 14^{th}, the 21^{st}, and the 28^{th} day. IOP that was 40% beyond preoperative value (9-16mmHg) mean that modeling was successful. To group with AG application, AG (Sigma, USA) was given 1 day ahead of modeling with 1g in 1L of their drinking water, but the total amount will be under 150mg/(kg•d). The modeling procedure was all the same.

Immunohistochemistry After sampling, fixation, dehydration and paraffin imbedding were performed according to the instruction of the kit. Positive cells were those with yellow or brownish-yellow granules deposited in cytoplasm or nuclei. We selected five discontinued high power fields from each section to assess the expression intensity with etaMorph/BX51 microgram analytical system through data analysis of the determination of integrated OD of positive cells.

RT-PCR Total RNA was extracted from retinal tissue; then reverse transcription product of 1μg total RNA was amplified (reaction conditions: 95℃ 5 minutes, 94℃ 30 seconds, 54℃ 30 seconds, 72℃ 40 seconds, total 35 cycles, 72℃ 10 minutes). Caspase-9 primer (sense: 5'-ACC ACA GTC CAT GCC ATC AC-3'; antisense: 5'-TCC ACC ACC CCT GTT GCT GTA-3'), length of product was 452bp. Internal control GAPDH primer (sense: 5'-TTT AAG CTC TCA GAA GAC ATG – 3'; antisense: 5'-TGT TGA AGT ACA GAC AGT ACC CCC A-3'), length of product was 363bp. Optical density of PCR product was detected by AGE and relative absorbance ratio was calculated to internal control.

Western-blot Retinal tissue was cut up and was added with 100mL cytolysis solution, cell protein was extracted to carry out SDS-PAGE electrophoresis and then was transmembraned to nitrocellulose filter. Acted with first and second antibody of anti-caspase-9, labeled by horseradish peroxidase (HRP) and then DAB coloration was performed.

Statistical Analysis The results were analyzed by SPSS 13.0 and presented with mean±standard deviation (mean±SD). The comparison between medication group and non-medication group was analyzed by Student's t-test, while the comparison between model group and control group was analyzed by one-way ANOVA test.

RESULTS

Change of RGCs and Thickness of Nerve Fiber Layer in Rats with Chronic IOP Elevation On 21^{st} day after modeling, the retina became thinner and the number of RGCs decreased; however, the change of medication group was relatively mild. Among three groups, at least two of them had distinctive difference according to the analysis by Levene homogeneity test for variance (P=0.137>0.05), which mean homogeneity of variance. Inter-group multiple

comparison test indicated that, in terms of thickness comparison, compared with control group the variances of other two groups both had statistical significance. Thickness comparison between medication group and un-medication group had statistical significance(Table 1).

Distribution and Intensity of Expression of Caspase-9 by Immunohistochemistry It is illustrated in Figure 1. Control group: only trace quantity of positive expression was detected in layer of ganglion cells in the retina of normal rats. Immune positive cells had yellow brown nuclei or cytoplasm stained; Un-medication model group: expression increased at the 3rd day, and at the 7th and 14th day it was the highest. Bodies of ganglion cells were big and round while bodies of microglia were long and thin. Besides ganglion cell layer, there was also positive expression in inner plexiform layer and inner nuclear layer. There was a small quantity of expression in ocular cones and rod cells. On the 28th day, a certain amount of nuclei was yellow- brown stained in cells mentioned above. Medication group: change of time course was similar to un-medication model group, but with less extent. IOD values which indicated expression intensity of caspase-9 in rat retinas from model groups were shown in Table 2.

Expression of Caspase-9 mRNA Detected by RT-PCR Expression of caspase-9 mRNA was enhanced with the extension of time. The strongest expression was on 7th and 14th day, and expression decreased after that (Figure 2). Levene variance homogeneity test, P =0.179 (>0.05), showed homogeneity of variance. One-way ANOVA analysis, P <0.05, showed among the expressions of caspase-9 mRNA at different time points, there was at least one value significantly different from other time point values; time point multiple comparison (Turky method) test showed that except 3rd day IOP elevated group, values of each group had statistical difference compared with control group. Expression of caspase-9 mRNA in medication model group was obviously lower than that in un-medication model group by t test (P <0.05), which suggested that it decreased significantly. Relative absorbance ratios of caspase-9 mRNA expressions in rat retinas of various groups were shown in Table 3.

Expression of Caspase-9 Protein Detected by Western Blotting Expression of caspase-9 protein was enhanced with the extension of time. The strongest expression was on 7th and 14th day, and decreased thereafter (Figure 3). Levene variance homogeneity test, P =0.153(>0.05), which showed homogeneity of variance. One-way ANOVA analysis, P<0.05, which showed among the expressions of caspase-9 protein at different time points, there was at least one value significantly different from other time point values; time point multiple comparison (Turky method) test showed that except 3rd day IOP elevated group, values of each group had statistical differences compared with control group. Expression of caspase-9 protein of medication model group, compared with un-medication model group by t test, P<0.05, which showed it decreased significantly. Relative absorbance ratios suggesting caspase-9 protein expressions in rat retinas of various groups were shown in Table 4.

Table 1 Thickness change of retina in rat of chronic IOP elevation with and without AG application (mean±SD, μm, n=10)

Group	n	Thickness of layer of ganglion cells + nerve fiber layer	
		21d	28d
Control group	10	27.5±9.7[b]	25.7±6.4[b]
Un-medication model group	10	17.4±5.8[a,b]	16.6±7.1[a,b]
Medication model group	10	22.3±7.3[a]	20.4±5.5[a]

[a]P <0.05 *vs* control group, [b]P <0.01 *vs* medication model group

Table 2 Expression intensity (IOD) of caspase-9 in rat retina of each model group (mean±SD, n=5)

Group	IOD	
	Un-medication model group	Medication model group
3d	11.46±3.06	8.87±3.14
7d	37.37±4.49	24.11±2.71[b]
14d	38.73±3.34	26.56±3.26[b]
21d	31.22±3.09	22.37±2.11[b]
28d	26.66±2.03	21.14±3.73[a]

[a]P<0.05, [b]P<0.01

Table 3 IDV ratio of expression of caspase-9 mRNA in retina of rats from each group (mean±SD, n=5)

Group	IDV/IDV	
	Un-medication model group	Medication model group
3d	0.181±0.021	0.179±0.014
7d	0.412±0.030	0.294±0.031[b]
14d	0.438±0.045	0.295±0.019[b]
21d	0.389±0.019	0.267±0.022[b]
28d	0.349±0.042	0.201±0.017[b]

[b]P<0.01

Table 4 IDV ratio of expression of caspase-9 protein in retina of rats from each group (mean±SD, n=5)

Group	IDV/IDV	
	Un-medication model group	Medication model group
3d	0.140±0.011	0.121±0.019
7d	0.408±0.021	0.343±0.016[b]
14d	0.425±0.045	0.333±0.027[a]
21d	0.375±0.030	0.301±0.010[a]
28d	0.352±0.023	0.299±0.040[a]

[a]P<0.05, [b]P<0.01

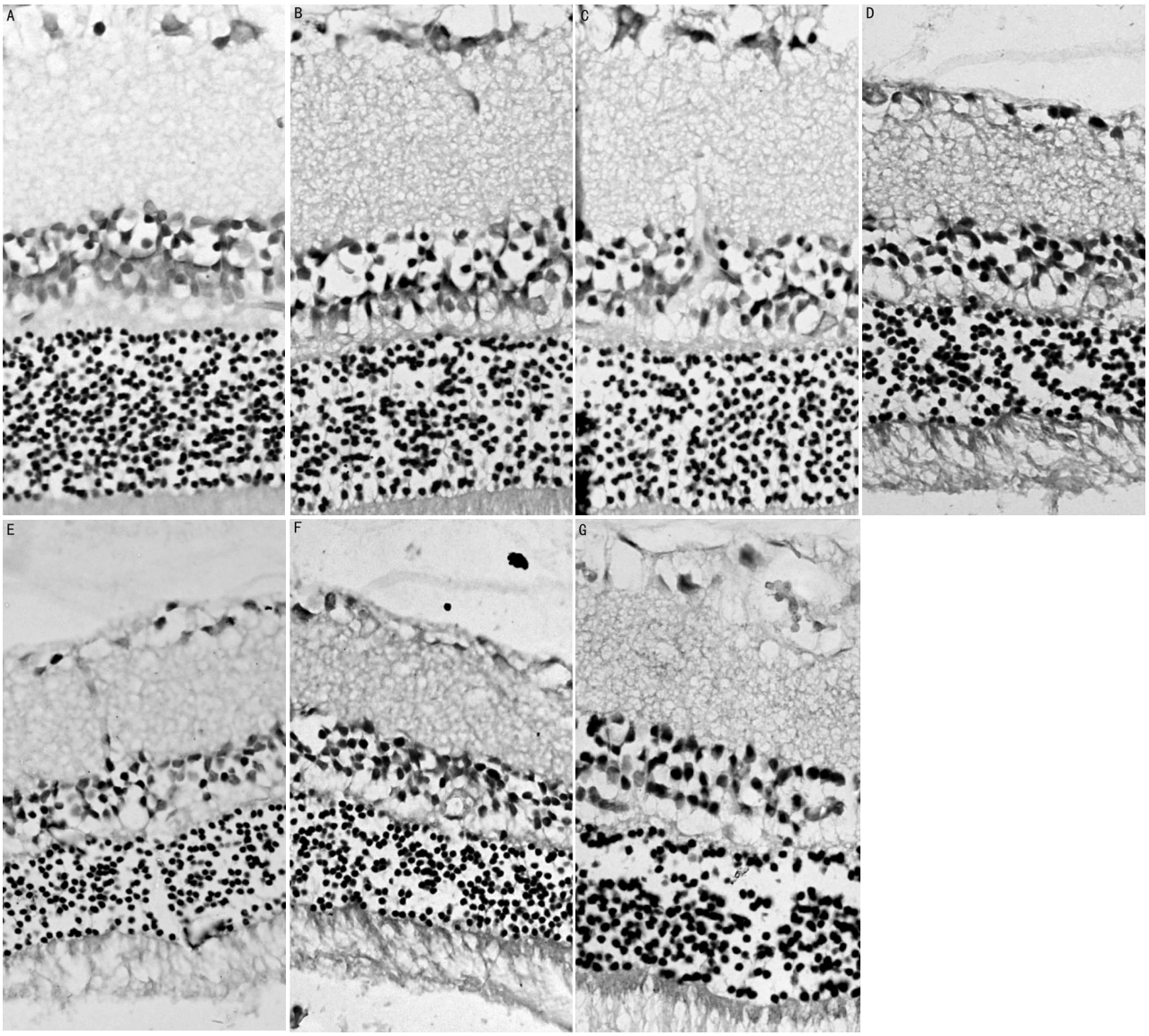

Figure 1 Intensity and distribution of caspase-9 protein expression of control group and model groups A: control group; B: the 7th day after IOP elevation; C: the 14th day after IOP elevation; D: the 28th day after IOP elevation; E: the 7th day AG+IOP elevated; F: the 14th day AG+IOP elevated; G: the 28th day AG+IOP elevated

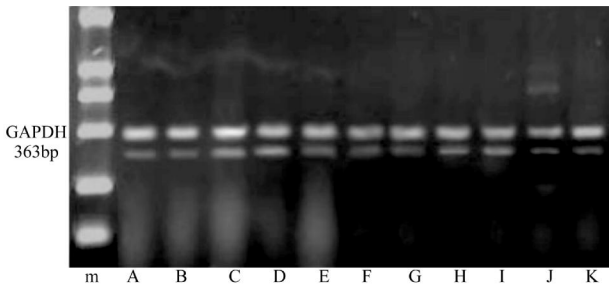

Figure 2 Expression of caspase-9 mRNA in retina of rats with chronic IOP elevation A: control group; B: the 3rd day after IOP elevation; C: the 7th day after IOP elevation; D: the 14th day after IOP elevation; E: the 21st day after IOP elevation; F: the 28th day after IOP elevation; G: the 3rd day AG+IOP elevation; H: the 7th day AG+IOP elevation; I: the 14th day AG+IOP elevation; J: the 21st day AG+IOP elevation; K: the 28th day AG+IOP elevation

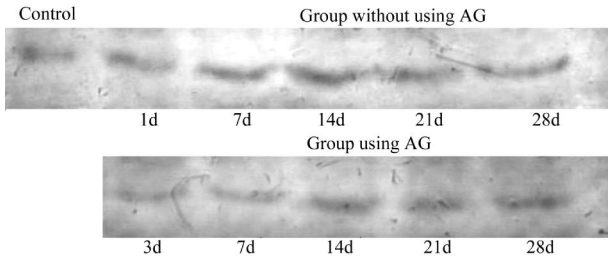

Figure 3 Expression of caspase-9 protein in retina of rat with chronic IOP elevation

DISCUSSION

In China, most animal models for glaucoma research are ischemia-reperfusion models, which have disadvantages for observation of retina protection, while reports about morphological changes under chronic IOP elevation are rare.

This investigation approached the protection of AG to retina suffering from glaucoma through observing the expression of apoptosis-related gene caspase-9 and its change with application of AG, in which chronic IOP elevation modeling method was generally accepted overseas. Studies indicate that there could be multiple factors involved in the mechanism of retinal injury due to IOP elevation, but there is only one outcome, which is apoptosis of RGCs [1]. Some researchers [2,3] suggested that patients with glaucoma lost their sights though their photoreceptors were still functional because of chronic progressive death of RGCs. Apoptosis of RGCs in chronic IOP elevation aggravates with the extension of time, which indicates this modeling method can be used in the research on retina protection of chronic IOP elevation.

Biological Characteristics of Caspase Family Caspase[4] is a family of intracellular cysteine endopeptidases. They play a key role in inflammation and mammalian apoptosis. Fourteen proteases of this kind have been identified till now, which are numbered Caspase 1-14 in the order they were found out. They are divided into two classes based on the lengths of their N-terminal prodomains. (1) Promotor caspase: have long prodomains and also named long prodomain caspase, including caspase-1, -2, -4, -5, -8, 9 and -10, which trigger on DNA fragmentation during apoptosis. (2) Effector caspase: including caspase-3, -6, -7, -14, have short prodomains, which induce apoptosis directly by enzymolysis to substrate protein. Caspase family extensively exists in all kinds of cell tissues of mammals, as well as in ocular region. Caspases can be detected in photoreceptor cells[5,6], RGCs[7] and so on, among which caspase-3 is found out to have high degree of similarity with the product of cell death gene ced-3, which is principal medium triggering RGCs death. When positioning active caspase-3 on RGCs of rat whose axons had been cut off, it was found that its active fragment p20 coexisted with TUNEL and its proteoclastic activity increased [8]. Caspase-9 is an important activator of caspase-3, bax/bcl-2 dependent cytochrome C released from mitochondrion to cytoplasm and combined with Caspase activator Apaf-1 to induce formation of oligomerization complex, mobilize and activate pre-caspase-9. Caspase-9 is formed and activates caspase-3 and initiates apoptosis in the end.

Influence of AG on Expression of Caspase According to results of immunohistochemical method in this investigation, there was only trace amount expression of caspase-9 in normal rat retina, which was localized in ganglion cell layer. As time went by, positive reaction cells were found in inner plexiform layer, inner nuclear layer and ocular cones and rod cells, which demonstrated the expression of caspase-9 was enhanced, and reached the peak on the 14th day, then decreased to a relatively stable level. Detections of caspase-9 mRNA by RT-PCR and caspase-9 protein by Western blotting both shared similar change course with results of immunohistochemical method, which indicated that it was a continuous procedure of apoptosis-related factor caspase-9 from transcription to translation to protein. All the above manifested with the existence of chronic IOP elevation the expression of caspase-9 increased and the injury to RGCs was continuous. Therefore, the retina protection to patients suffering from chronic IOP elevation should be performed in a long term. Besides, another phenomenon was observed: expression of caspase-9 in ocular cones and rod cells during 7-14 days of chronic IOP elevation, which gave us a better explanation of early phase decrease of color vision and contrast sensitivity in chronic IOP elevation.

With the application of AG, the degree of retina thinning diminished compared with un-medication model group ($P<0.05$), and the expression of caspase-9 in retina of rat with chronic IOP elevation was significantly inhibited. Immuno-histochemical method, RT-PCT and Western blotting showed respectively that caspase-9 IOD, mRNA and expression of protein were all inhibited and through statistical analysis the differences were significant($P<0.05$). The results mentioned above indicated AG as a relatively specific inhibitor of iNOS, could inhibit directly or indirectly the expression of caspase-9 to protect retina in chronic IOP elevation.

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