·Basic Research·

Expression of Fas/FasL and the apoptosis in rat ischemia/reperfusion –induced retinal injury and effects of bFGF

Ying Zhao, Ying–Jun Niu, Zhan–Yu Zhou, Yun–Xia Gao, Hong–Yun Wang

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Department of Ophthalmology, the Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China

Correspondence to: Ying-Jun Niu. Department of Ophthalmology, the Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China. niuyingjun@yahoo.com.cn

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Abstract

• AIM: To explore the relationship between the expression of Fas/FasL and the apoptosis in retinal ischemia/reperfusion injury of rats, as well as the therapeutic effects of basic fibroblast growth factor (bFGF) on the ischemic retina.

• METHODS: The models of retinal ischemia/reperfusion injury were made by transiently elevating intraocular pressure (IOP). A total of 28 rats were divided into Normal Group and Operative Group. The latter was subdivided into 1, 6, 12, 24, 48 and 72 hours groups after reperfusion, in which the left eyes of the rats were in the ischemia/reperfusion groups and the right ones were in the treatment groups(bFGF intracameral injection). Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) method, and the expression of Fas/FasL was studied by strept avidin-biotin complex (SABC) immunohistochemistry.

• RESULTS: No positive cells were observed in the normal rats' retinae, but there were a significant number of TUNEL positive cells in 6-24 hours after transient ischemia followed by a decrease at 48 hours. The number of TUNEL positive cells reached a maximum at 24 hours after ischemia. The expression of Fas gradually increased as early as at 6 hours, reached a peak at 24 hours, then decreased at 48 hours. Similarly, the expression of Fas ligand was at peak in 24 - 48 hours in ganglion cell layer (GCL) and INL of retina. bFGF administered before reperfusion inhibited apoptotsis and ameliorated the tissue damage. It also diminished Fas and FasL expression in ischemic/reperfused retina.

• CONCLUSION: Retinal ischemia-reperfusion after transiently elevated IOP induced apoptosis of cells in the retina. Fas/FasL may have an important role in the early events of the apoptotic pathways. bFGF can rescue retinal ganglion cells from retinal ischemia/reperfusion injury through down-regulation of Fas and Fas ligand expression and may represent an important mechanism for therapeutic neuroprotection.

• KEYWORDS: retina; ischemia/reperfusion injury; apoptosis; fibroblast growth factor, basic; Fas/FasL

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INTRODUCTION

Retinal ischemia/reperfusion injury (RIRI) is a kind of common pathologic process, which damages visual function severely. Apoptosis is one of the most common kinds of patterns of the ganglion cells' death in RIRI. And Fas/FasL death-induced-pathway is the important pathway of apoptosis. To date, neither investigations about the expression of Fas and Fas ligand in RIRI nor its significance has been found home and abroad. Basic fibroblast growth factor (bFGF) is a kind of polypeptide growth factor possessing multifunctional biological activity, such as protection of neuron, promoting the growth of nerve and the anti-ischemic injury of neuron [1]. In this study, we explored the relationship between neuron apoptosis and the expression of Fas and Fas ligand in RIRI, as well as the therapeutic effects of bFGF on the ischemic retina.

MATERIALS AND METHODS

Animal Grouping A total of 28 healthy Wistar rats (body mass 250-300g, female 14 and male 14) were divided into Normal and Operative Groups randomly. In Operative Group, the left eyes were injected with saline into the vitreous cavity at 1 hour after ischemia (regarded as Ischemia/ reperfusion Group), and the right eyes were injected with bFGF into the vitreous cavity at the same time

(regarded as Treatment Group). The Operative Group was subdivided into groups of 1, 6, 12, 24, 48 and 72 hours according to the time after reperfusion.

Reagents Recombinating bFGF was purchased from Bioengineering Company of Jinan University. Rabbit-anti-rat Fas polyclonal antibody and rabbit-anti-rat FasL polyclonal antibody came from Santa Cruz biotechnology Co., USA. TUNEL kit, and strept avidin-biotin complex (SABC) immunohistochemistry kit was bought from Boster Bioengineering Company, Wuhan.

Animal Model The RIRI rat models were made by transiently elevating intraocular pressure (IOL) according to methods in literature 2.

Specimen Preparation At 1, 6, 12, 24, 48 and 72 hours after reperfusion, rats were killed immediately and eyeballs were extirpated, and then made into paraffin sections according to methods in literature 2.

TUNEL for *In Situ* Detection of Apoptotic Cells Paraffin sections were dewaxed, hydrated by a graded alcohol series gradually. Endogenous peroxidase was inactivated by covering the sections with 30mL/L H_2O_2 for 10 minutes at room temperature. After that, they were digested by protease for 5 minutes at room temperature. Sections were incubated with 20μL of terminal deoxynu cleotidyl transferase (TdT) in a moist chamber at 37℃ for 2 hours. Then 50μL biotin-anti-digoxin antibody was added on each section for 30 minutes at 37℃. Strept avidin-biotin complex(SABC) was added for 30 minutes at 37℃. The sections showed color by adding DAB and slightly counterstained by hematein, then dehydrated, hyalined, sealed and observed under the microscope. The reaction was terminated by transferring the slides to phosphate buffer solution (PBS) at room temperature for 15 minutes. As a positive control, sections of retina were exposed to 1g/L DNase I at room temperature for 15 minutes before nick end labeling. As a negative control, sections of retina were exposed to PBS in stand of TdT.

Immunohistochemistry Paraffin sections were dewaxed, hydrated by a graded alcohol series gradually. They were covered with 30mL/L H_2O_2 for 10 minutes at room temperature and then digested by protease for 5 minutes at room temperature. Sections were added normal goat serum for 10 minutes at room temperature. Rabbit-anti-rat Fas polyclonal antibody and rabbit-anti-rat FasL polyclonal antibody were added in a moist chamber at 37℃ for 20 minutes. Then SABC was added for 20 minutes at room temperature; DAB was added to show color. The sections were slightly counterstained by hematein, then dehydrated, hyalined, sealed and observed under the microscope. As a positive control, the section of galactophore cancer replaced sections of retinae to detect Fas, and sections of stomach cancer substituted retinae sections to detect FasL. As a negative control, the sections were incubated with PBS instead of the primary antibody.

Results Observation Results were observed by microscope. TUNEL positive cells were identified by cytoplasms or nucleus stained yellow or yellow- brown. Fas/FasL positive cells were identified by cytomembranes or cytoplasms stained yellow or yellow-brown. Sections were scanned by image analysis system (OPTON VIDAS); four sights were chosen from each section and each sight had an area of 0.2mm×0.2mm. Every two sights were taken besides the optic nerve. Then the average positively-stained cell quantity was obtained. Apoptotic index(AI) is equal to the number of apoptotic cells/the number of total cells×100%.

Statistical Analysis The data were analyzed with SPSS software. Pair-matching t-test was used to compare the same time courses of two different groups; analysis of variance was used to compare the different time courses of the same group.

RESULTS

***In Situ* Detection of Apoptosis in Ischemic/Reperfused Retina** There were no apoptotic cells in normal retinal tissue. At 6 hours, there were a small quantity of yellow-stained cells in ganglion cell layer (GCL); at 12 hours, apoptotic cells increased significantly; the number of apoptotic cells reached the peak at 24 hours and all layers were yellow-stained; at 48 hours, the number of apoptotic cell decreased; and at 72 hours apoptotic cells also could be found. In the bFGF treated Group, Fas expressions changed similarly as those in the Ischemia Group. But there was a significant reduction in the number of apoptotic cells at 12, 24, 48 hours in bFGF Treated groups compared with Ischemia/reperfusion Group(Table 1). The negative control sections were not positively stained.

Expression of Fas There were no positive cells in normal retinal tissue. In the Ischemia Group, there were a small quantity of Fas positive expressions at 6 hours; at 12 hours, the expression was comparatively significant; the expression reached the peak at 24 hours; at 48 hours the number of apoptotic cell decreased; and at 72hours Fas expression obviously descended. In the bFGF Treated Group, Fas expressions changed similarly to those of the Ischemia Group except that the expression intensity obviously weakened at 6, 12 and 24 hours after reperfusion, as shown in Table 2. The negative control sections had no positively stained signs.

Expression of FasL In normal retinal tissue, there were low expressions in all layers. In Ischemia Group, FasL expression was comparatively significant at 12 hours, especially in NLC and RGCL. At 24 hours, the expression

reached the peak; at 48 hours the number of apoptotic cell decreased; and at 72 hours apoptotic cells could also be found. In the bFGF Treated Group, FasL expressions changed similarly to those of the Ischemia Group except that the expression intensity obviously weakened at 12, 24 and 48 hours after reperfusion, as shown in Table 3. The negative control sections had no positively stained signs.

DISCUSSION

RIRI is a common pathological process at ophthalmic clinic, such as glaucoma, central retinal artery occlusion (CRAO), ischemic retinal neuropathy, etc. In our study, TUNEL method can mark apoptotic cells *in situ*, which identified apoptosis during RIRI reliably and directly. The results showed that cell apoptosis mainly occurred in INL and GCL as early as at 6 hours after reperfusion and reached the peak at 24 and 48 hours after reperfusion. Moreover, in early days we found diversified death forms of retinal cells in RIRI by electron microscope, in which apoptosis was a main death form of retinal cells in RIRI. Those studies proved that cells died mainly through apoptosis in the RIRI and in accordance with the research by Buchi[3] and Kaneda *et al*[4]. Apoptosis is an active gene-directed process of cell suicide controlled by proapoptotic and antiapoptotic genes. The balance between proapoptotic and antiapoptotic factors ensured cells homeostasis and the unbalance could result in cell proliferation or apoptosis. Fas is a kind of proapoptotic factor and belongs to the tumor necrosis factor receptor family. FasL is the ligand of Fas receptor. When Fas binds with FasL, it will induce apoptosis of Fas-expression-cells[5]. The basal level of Fas in normal retinal tissue was low or below a detectable level. FasL expressed weakly in every retinal layer, which was related to mechanisms of ocular initial immune privilege [6]. Rosenbaum *et al* [7] found that Fas, FasL expression level was step-up in ischemia/reperfusion injury in rats' brain and the peak was during 24 and 48 hours after reperfusion. However, there were no reports about Fas/FasL expression in RIRI home and abroad. It was detected by immunohistochemistry that Fas/FasL expressions were markedly upregulated in GCL and NFL subjected to RIRI. The expression tendency of Fas, FasL and apoptosis was similar. There were a small quantity of Fas positive expressions at 6 hours; the expression reached the peak at 24 hours and 48 hours; the number of apoptotic cells decreased. FasL expression was comparatively significant at 12 hours. At 24 hours, the expression reached the peak. Then the number of apoptotic cells decreased, and at 72 hours apoptotic cells could also be found. From those we could find that RIRI caused an increase in Fas/FasL expression and was in accordance with TUNEL in time and sites. Our data suggested, therefore, that overexpression of Fas/FasL might result in apoptosis in retina.

Table 1 Comparison of apoptotic index between Treatment Group and Ischemia Group (%, n =4)

Time course	Ischemia group	Treatment group	t	P
1h	2.4 ± 0.7	2.1 ± 0.6	2.931	0.061
6h	6.9 ± 0.2 [a]	6.7 ± 0.2 [a]	2.611	0.080
12h	11.3 ± 0.8 [a]	8.0 ± 0.7 [a]	5.595	0.011
24h	29.1 ± 2.1 [a]	22.2 ± 1.5 [a]	7.508	0.005
48h	16.5 ± 0.6 [a]	13.8 ± 1.4 [a]	5.362	0.013
72h	5.8 ± 0.5 [a]	4.8 ± 0.7 [a]	2.777	0.069

Note: the comparison of each time course to its prior time course within a group [a] P<0.05

Table 2 Changes of Fas expressions of different time courses after RIRI (positive cells/mm^2, n =4)

Time course	Ischemia group	Treatment group	t	P
1h	8.7 ± 0.7	8.4 ± 0.6	3.000	0.058
6h	189.2 ± 3.0 [a]	183.4 ± 4.6 [a]	3.954	0.029
12h	476.9 ± 19.0 [a]	352.9 ± 12.4 [a]	7.037	0.006
24h	1050.9 ± 55.1 [a]	870.5 ± 18.7 [a]	9.327	0.003
48h	662.1 ± 3.0 [a]	648.0 ± 7.9 [a]	3.052	0.055
72h	84.3 ± 6.4 [a]	82.9 ± 6.6 [a]	2.996	0.058

Note: the comparison of each time course to its prior time course within a group [a] P<0.05

Table 3 Changes of FasL expressions of different time courses after RIRI (positive cells/mm^2, n =4)

Time course	Ischemia group	Treatment group	t	P
1h	46.1 ± 0.5	45.0 ± 0.5	2.901	0. 062
6h	54.8 ± 1.1 [a]	52.4 ± 0.6 [a]	2.961	0. 059
12h	374.9 ± 7.7 [a]	353.9 ± 6.7 [a]	4.569	0. 020
24h	995.1 ± 18.8 [a]	901.0 ± 5.82 [a]	11.715	0. 001
48h	778.9 ± 9.4 [a]	664.8 ± 14.4 [a]	9.870	0. 002
72h	183.4 ± 5.3 [a]	176.5 ± 4.1 [a]	2.483	0. 089

Note: the comparison of each time course to its prior time course within a group [a] P<0.05

We found that bFGF intravitreous injection reduced the quantity of apoptotic cells in RIRI. In Treatment Group, there was statistical difference compared with ischemia groups at 12, 24 and 48 hours after reperfusion. Meanwhile, bFGF depressed the levels of Fas and FasL expression significantly. In Treatment Group, the quantity of Fas positive cells decreased at 6, 12, 24 and 48 hours after reperfusion and FasL decreased at 12, 24 and 48 hours after reperfusion compared with Ischemia Groups. It suggested that bFGF's therapeutic effect on RIRI could partly contribute to its inhibition to apoptosis by its inhibition to Fas/FasL expression. Our early study[8] has proved that bFGF

decreased the loss of neuron and ameliorated thin NFL in RIRI by decreasing intracellular calcium and free radical. Yue *et al* [9] found that both increased intracellular calcium and accelerated free radical production could induce Fas expression and apoptosis. However, calcium antagonist and antioxidant can decrease Fas expression obviously. Those studies indicated that bFGF might inhibit apoptosis in RIRI by inhibiting Fas/FasL directly and/or inhibiting increased intracellular calcium, and accelerate free radical production to decrease Fas/FasL expression indirectly.

In conclusion, our results demonstrated that RIRI resulted in cell apoptosis in the rat retina and overexpression of Fas/FasL induced RGC apoptosis. Meanwhile, bFGF inhibited Fas/FasL expression, and then inhibited apoptosis in RIRI. It may represent an important mechanism for bFGF therapeutic neuroprotection in RIRI.

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