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The role of TNF-α, IL-6, IL-10, and GDNF in neuronal apoptosis in neonatal rat with hypoxic-ischemic encephalopathy

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Abstract. – AIM: To examine the dynamic changes of TNF-α, IL-6, IL-10, and GDNF (glial cell-derived neurotrophic factor) in serum or brain tissues of neonatal rat with hypoxic-ischemic encephalopathy and to explore their roles in neuronal apoptosis.

MATERIALS AND METHODS: A total of 80 Wistar rats were randomly divided into the sham-operated (control) group and the hypoxia-ischemia (HI) group. To establish the hypoxic-ischemic encephalopathy (HIE) model, the pups from the HI group were subjected to left common carotid artery ligation followed by exposure to 8% O_2 and 92% N_2. The concentration of TNF-α, IL-6, IL-10, and GDNF in serum or brain tissues was measured by enzyme linked immunosorbent assay (ELISA). Neuronal apoptosis was examined by flow cytometry (FC). Statistical analysis was performed using the SPSS13.0 software.

RESULTS: We found that the neuronal apoptosis rate and the levels of TNF-α and IL-6 in rat with hypoxic-ischemic brain damage (HIBD) were significantly increased at 6 h, 24 h, 48 h, and 72 h after hypoxia compared to the control group ($p < 0.05$). We also found that the neuronal apoptosis rate was positively correlated with the levels of TNF-α and IL-6, and negatively correlated with IL-10 and GDNF.

CONCLUSIONS: In neonatal rats with HIE, the brain reaches its peak levels of damage by 24~72 h after the injury. Inflammatory cytokines such as TNF-α and IL-6 promote HIE-induced neuronal apoptosis, whereas IL-10 and GDNF antagonize it.

Key Words:
Hypoxia-ischemia encephalopathy, Brain damage, Neonatal rat, Cell apoptosis.

Introduction

Hypoxic-ischemic encephalopathy (HIE) is caused by a restriction in blood supply to tissues and a shortage of oxygen and glucose. It is one of the major threats to neonates in the perinatal period[1]. The incidence of HIE is around 1-80/1000, and 10-20% of HIE patients die in the neonatal period. Nearly one third of the survivors have defects in brain development, which creates burdens on families and society[2,3]. Therefore, it is of importance to study the mechanisms underlying hypoxic-ischemic brain damage (HIBD) and develop therapeutics for it.

In ischemic brain, activated astrocytes can synthesize and secrete a number of proteins including cytokines and neurotrophic factors which are important for the development and progression of ischemic brain damage. These secreted factors have opposite roles in neuronal apoptosis. On one hand, ischemia stimulates astrocytes to produce and secrete many inflammatory factors such as the transcription factor NF-κB, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)[4]. The inflammatory response to injury is the major cause for HIBD. TNF-α and IL-6 may contribute to injury-induced neuronal apoptosis and play important roles in the pathogenesis of HIE[5]. On the other hand, the astrocytes also increase the expression of some factors that can protect the cells in ischemia. For example, upregulation of interleukin-10 (IL-10), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) can delay the ischemia-induced damage to neurons[6,7]. In this study, we examined the dynamic changes of TNF-α, IL-6, IL-10, and GDNF in serum and brain tissues of neonatal rats with HIE and proposed that these factors may have different roles in HIBD-induced neuronal apoptosis. Thus, our results may provide new information to the development of therapeutics for HIE.

Materials and Methods

Animals

Experimental animals were provided by the Laboratory Animal Center, Xinxiang Medical

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University. A total of 80 Wistar rats (postnatal day 7, average weight: 12.23 ± 3.18 g) were randomly divided into the control group and the hypoxia-ischemia (HI) group. The pups from the HI group were subjected to left common carotid artery ligation followed by exposure to 8% O_2 and 92% N_2 to establish the HIE model. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Xinxiang Medical University.

Animal Model of HIE

All experiments were performed in accordance with the Institutional Animal Care guidelines. Rat model of HIE was established as previously reported[8,9]. Individual rat pups were anesthetized by diethyl-ether inhalation. Under deep ether anesthesia, the left common carotid artery was isolated, double ligated, and cut between the ligatures. After the surgical procedure, the animal was put to recover for 4-8 h. Then the pups were transferred into an enclosed and vented chamber and exposed to 8% O_2 that was balanced with 92% N_2 for 2-3 h. All animals showed symptoms of hemiplegia and anoxia after surgery.

Preparation of Brain Extracts

Neonatal pups were sacrificed at 6 h, 24 h, 48 h, 72 h, and 7 d after HI. Blood was taken from the heart and centrifuged at 2,000 rpm for 10 min. The supernatant was saved and used for the following assays. The brain was removed and extensively washed with ice-cold saline. Cortical tissue (left hemisphere) from individual pups was homogenized in ice-cold PBS (phosphate buffered saline), and then centrifuged at 2,500 rpm for 10 min at 4°C. The supernatant was collected and stored at –20°C.

Measurement of the Content of TNF-α, IL-6, IL-10, and GDNF

The content of TNF-α, IL-6, IL-10, and GDNF in serum or brain tissues were measured by ELISA using commercial kits purchased from Rapidbio Lab (Calabasa, CA, USA) for IL-6, Biosource (Chery Chase, MD, USA) for TNF-α and IL-10, and Adlitteram Diagnostic Laboratories Inc. (Chicago, IL, USA) for GDNF. Procedures were performed according to manufacturer's instruction.

Detection of Neuronal Apoptosis

Neuronal apoptosis was detected by flow cytometry (FC). The brain was rapidly decapitated and the forebrain was removed. Single cell suspensions were prepared by mechanical methods. Cells were labeled with Annexin V-FITC and subjected to FC to sort apoptotic cells.

Statistical Analysis

Statistical analysis was performed using the SPSS13.0 software (SPSS Inc., Chicago, IL, USA). Values were expressed as ± s. The *t* test was used to determine whether there is a significant difference between two group means. The relationship between two variables was analyzed by linear regression. Statistical significance was defined as $p < 0.05$.

Results

Changes of TNF-α, IL-6, IL-10, and GDNF Levels in Serum and Brain Tissues of Neonatal rats at Different Time Points After HI

As shown in Tables I and II, the levels of TNF-α and IL-6 were significantly upregulated at 6 h, 24 h, 48 h, and 72 h after HI in the HI group compared to that in the control group ($p < 0.05$). They peaked by 24 h, gradually decreased thereafter, and returned to normal by 7 d. Similar to TNF-α and IL-6, the levels of IL-10 and GDNF were also upregulated. However, they peaked by 48 h and returned back to normal by 7 d (Tables III and IV).

Changes of the Neuronal Apoptosis Rate in Neonatal Rats at Different Time Points After HI

We found that neuronal apoptosis rates were significantly higher in rats from the HI group at 6 h, 24 h, 48 h, and 72 h after HI than that in rats from the control group ($p < 0.05$, Table V). Moreover, they peaked by 24 h after hypoxia and are comparable to the control group by 7 d ($p > 0.05$, Table V).

The Correlation Between Neuronal Apoptosis rate and the Levels of Inflammatory Factors and Neurotrophic Factors

We analyzed the relationship between the neuronal apoptosis rate and the levels of TNF-α, IL-6, IL-10, and GDNF by a linear regression

Table I. Dynamic changes of TNF-α in serum or brain tissues of neonatal rat (ng/mL, $\bar{x} \pm s$).

Time	Serum		Brain tissue	
	Control	HI	Control	HI
6 h	14.21 ± 2.07	35.26 ± 4.65*	53.21 ± 7.43	97.32 ± 10.47*
24 h	16.35 ± 1.89	53.27 ± 6.21*,#	56.05 ± 6.37	157.29 ± 26.73*,#
48 h	17.24 ± 3.08	46.01 ± 5.73*	55.48 ± 5.22	106.25 ± 18.77*
72 h	16.83 ± 2.53	39.26 ± 4.15*	57.19 ± 3.24	73.78 ± 8.43*
7 d	13.00 ± 2.16	15.13 ± 3.08	60.17 ± 8.09	62.10 ± 7.22

Compared to control group $^{*}p < 0.05$; compared to 6 h, 48 h, 72 h subgroups $^{\#}p < 0.05$. HI: hipoxia-ischemia.

Table II. Dynamic changes of IL-6 in serum or brain tissues of neonatal rat (ng/mL, $\bar{x} \pm s$).

Time	Serum		Brain tissue	
	Control	HI	Control	HI
6 h	108.23 ± 12.07	138.34 ± 15.08*	137.30 ± 18.47	169.35 ± 17.37*
24 h	110.25 ± 9.35	167.27 ± 13.29*,#	140.85 ± 15.38	237.34 ± 30.10*,#
48 h	112.54 ± 11.81	160.87 ± 18.36*	142.68 ± 20.07	189.55 ± 28.12*
72 h	110.47 ± 13.04	146.24 ± 19.05*	136.32 ± 15.21	147.03 ± 15.02*
7 d	109.38 ± 10.56	120.32 ± 13.76	134.04 ± 14.35	138.48 ± 12.48

Compared to control group $^{*}p$ 0.05; compared to 6 h, 48 h, 72 h subgroups $^{\#}p < 0.05$.

Table III. Dynamic changes of IL-10 in serum or brain tissues of neonatal rat (ng/mL, $\bar{x} \pm s$).

Time	Serum		Brain tissue	
	Control	HI	Control	HI
6 h	27.21 ± 3.26	43.27± 6.03*	72.18 ± 8.97	128.93 ± 10.37*
24 h	28.34 ± 2.08	68.83 ± 8.47*	74.56 ± 9.10	159.25 ± 23.04*
48 h	26.53 ± 4.71	72.05 ± 7.36*,#	68.25 ± 8.31	166.35 ± 14.26*,#
72 h	26.20 ± 1.90	36.30 ± 5.29*	75.27 ± 5.19	103.08 ± 9.54*
7 d	24.03 ± 3.78	32.34 ± 4.59	70.56 ± 6.36	82.18 ± 11.40

Compared to control group $^{*}p$ 0.05; compared to 6 h, 48 h, 72 h subgroups $^{\#}p < 0.05$.

Table IV. Dynamic changes of GDNF in serum or brain tissues of neonatal rat (ng/mL, $\bar{x} \pm s$).

Time	Serum		Brain tissue	
	Control	HI	Control	HI
6 h	13.25 ± 0.37	25.24 ± 0.28*	34.26 ± 2.44	49.49 ± 3.02*
24 h	12.97 ± 0.28	28.87 ± 0.63*	33.08 ± 5.37	55.28 ± 4.84*
48 h	13.17 ± 0.58	37.88 ± 1.06*,#	35.37 ± 2.56	63.17 ± 4.56*,#
72 h	14.03 ± 0.22	24.20 ± 0.85*	34.05 ± 3.66	54.56 ± 3.05*
7 d	13.25 ± 0.64	23.71 ± 0.55	35.00 ± 1.89	47.88 ± 2.7

Compared to control group $^{*}p$ 0.05; compared to 6 h, 48 h, 72 h subgroups $^{\#}p < 0.05$.

method. The levels of TNF-α, IL-6, IL-10, and GDNF were defined as the independent variable. We found that the apoptosis rate was positively correlated with the levels of TNF-α and IL-6, and negatively correlated with the levels of IL-10 and GDNF (Table VI).

Table V. Neuronal apoptosis rate in neonatal rats after HI (%, $\bar{x}$ ± s).

Group	6 h	24 h	48 h	72 h	7 d
Control	0.73 ± 0.04	0.75 ± 0.06	0.70 ± 0.05	0.67 ± 0.02	0.74 ± 0.12
HI	4.27 ± 0.21*	13.02 ± 0.33*	8.26 ± 0.56*	3.26 ± 0.82*	0.89 ± 0.07

Compared to control group *p 0.05.

Discussion

Hypoxic-ischemic brain damage can result from many causes, of which inflammatory response to the injury is a major one. After ischemia, the expression of adhesion molecules and the activation of astrocytes indicate that a cascade of inflammatory response is activated. The upregulation of inflammatory factors and the activation of proteases are responsible for early damage to neurons and to the blood-brain barrier in ischemic tissues[10]. TNF-α and IL-6 are important inflammatory factors. TNF-α is mainly secreted by activated macrophages, and its overexpression is toxic to cells[11]. It plays critical roles in HI-induced neutrophil infiltration and tissue damages. It can also increase permeability of endotheliocyte and induce the expression of other inflammatory factors[12]. Matrix metalloproteinases (MMP) can be activated by TNF-α which damages the blood-brain barrier and increases its permeability, leading to the swelling and degeneration of neurons and glial cells. The dead cells release neurotoxicity factors such as arachidonic acid metabolites, excitatory amino acids, and oxygen free radicals, which promotes neuronal apoptosis[13]. Furthermore, TNF-α can also increase the expression of Bcl-2 (B-cell lymphoma 2)[14]. Thus, the upregulation of TNF-α suggests that neurons may undergo apoptosis in ischemic brain. IL-6 is a glycoprotein containing 184 amino acids. In the brain, IL-6 is mainly synthesized in glial cells, and can induce the aggregation of inflammatory cells in lesion area and increase the release of oxygen free radicals from neutrophils. It can also cooperate with other inflammatory factors such as TNF-α to cause calcium overload and cell death[15].

Table VI. Correlation between neuronal apoptosis rate and the levels of TNF-α, IL-6, IL-10, and GDNF.

Items	r	t	p
Serum TNF-α	0.435	2.34	< 0.05
Brain TNF-α	0.864	4.06	< 0.05
Serum IL-6	0.572	3.27	< 0.05
Brain IL-6	0.738	3.85	< 0.05
Serum IL-10	-0.529	3.04	< 0.05
Brain IL-10	-0.638	3.65	< 0.05
Serum GDNF	-0.426	2.27	< 0.05
Brain GDNF	-0.811	3.98	< 0.05

After HIBD, activated glial cells also secrete some neurotrophic factors such as IL-10 and GDNF. GDNF belongs to the TNF-α superfamily and is one of the most effective neurotrophic factors isolated from glial cells[16]. In brain ischemia, GDNF has protective effects on neurons. A number of studies have demonstrated that GDNF can reduce the infarct size and alleviate encephaledema in brain ischemia by preventing neuronal apoptosis[17,18]. As an anti-inflammatory cytokine, IL-10 inhibits the secretion of IL-1, IL-6, and TNF-α from leukocytes and glial cells, which suppresses the aggregation of neutrophils and reduces the production of chemokines[19]. IL-10 can also reduce the secretion of pro-inflammatory cytokines and inhibits the activation of inflammatory cells. Moreover, IL-10 can protect the brain by directly reducing neuronal apoptosis[20].

In this study, we examined the changes of TNF-α, IL-6, IL-10, and GDNF in neonatal rats with HIBD. We found that inflammatory factors (TNF-α and IL-6) and anti-inflammatory factors (IL-10 and GDNF) were both upregulated at 6 h, 24 h, 48 h, and 72 h after HI. The levels of TNF-α and IL-6 peaked by 24 h, which is a typical feature of inflammatory response. However, the peak levels of IL-10 and GDNF appeared by 48 h, suggesting a different effect of two factors to neurons. Furthermore, our findings showed that neurons started to die at 6 h after brain injury and reached the peak damage by 24 h. The time course of TNF-α and IL-6 expression was similar to that of neuronal apoptosis, suggesting that TNF-α and IL-6 may promote neuronal death. On the other hand, the delayed expression of IL-10 and GDNF suggests that these two factors may antagonize neuronal apoptosis in brain ischemia.

During brain injury, the cell membranes of neurons and glial cells are damaged, leading to the release of TNF-α, IL-6, IL-10, and GDNF to the extracellular spaces. These factors enter the blood through the damaged blood-brain barrier. Therefore, the content of TNF-α, IL-6, IL-10, and GDNF in serum can be measured. Because the changes of these factors in serum are similar to that in the brain, the levels of these factors in serum are correlated with the severity of brain damage.

Conclusions

Our results showed that neuronal apoptosis rate was positively correlated with the content of TNF-α and IL-6 in serum and brain tissues, and negatively correlated with that of IL-10 and GDNF. TNF-α and IL-6 may promote neuronal apoptosis and aggravate brain injury, whereas IL-10 and GDNF can prevent neuronal apoptosis and promote the repair of the cells. Thus, drugs that can increase the expression of neurotrophic factors or suppress the expression of inflammatory factors may be effective treatments for neonatal IHE.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Boggio PS, Coutinho de Macedo E, Pascual-Leone A, Tormos Muñoz JM, Schwartzman JS, Fregni F. Neuromodulation in hypoxic-ischemic injury. Brain Stimul 2009; 2: 179-181.
2) Kaur C, Foulds WS, Ling EA. Hypoxia-ischemia and retinal ganglion cell damage. Clin Ophthalmol 2008; 2: 879-889.
3) Aly H, Hamed Z, Mohsen L, Ramy N, Arnaoot H, Lotfy A. Serum amyloid A protein and hypoxic ischemic encephalopathy in the newborn. J Perinatol 2011; 31: 263-268.
4) Vasiljevic B, Maglajlic-Djukic S, Gojnic M, Stankovic S, Ignjatovic S, Lutovac D. New insights into the pathogenesis of perinatal hypoxic-ischemic brain injury. Pediatr Int 2011; 53: 454-462.
5) Cojocaru IM, Cojocaru M, T n sescu R, Iliescu I, Dumitrescu L, Silosi I. Expression of IL-6 activity in patients with acute ischemic stroke. Rom J Intern Med 2009; 47: 393-396.
6) Carlsson Y, Leverin AL, Hedtjärn M, Wang X, Mallard C, Hagberg H. Role of mixed lineage kinase inhibition in neonatal hypoxia-ischemia. Dev Neurosci 2009; 31: 420-426.
7) Cheng H, Huang SS, Lin SM, Lin MJ, Chu YC, Chih CL, Tsai MJ, Lin HC, Huang WC, Tsai SK. The neuroprotective effect of glial cell line-derived neurotrophic factor in fibrin glue against chronic focal cerebral ischemia in conscious rats. Brain Res 2005; 1033: 28-33.
8) Kumral A, Ozer E, Yilmaz O, Akhisaroglu M, Gokmen N, Duman N, Ulukus C, Genc S, Ozkan H. Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. Biol Neonate 2003; 83: 224-228.
9) Taniguchi H, Andreasson K. The hypoxic-ischemic encephalopathy model of perinatal ischemia. J Vis Exp 2008; 19: 955.
10) Vasiljevic B, Maglajlic-Djukic S, Gojnic M, Stankovic S, Ignjatovic S, Lutovac D. New insights into the pathogenesis of perinatal hypoxic-ischemic brain injury. Pediatr Int 2011; 53: 454-462.
11) Nawashiro H, Tasaki K, Ruetzler CA, Hallenbeck JM. TNF-alpha pretreatment induces protective effects against focal cerebral ischemia in mice. J Cereb Blood Flow Metab 1997; 17: 483-490.
12) Ashdown H, Joita S, Luheshi GN, Boksa P. Acute brain cytokine responses after global birth hypoxia in the rat. J Neurosci Res 2008; 86: 3401-3409.
13) Aly H, Khashaba MT, El-Ayouty M, El-Sayed O, Hasanein BM. IL-1beta, IL-6 and TNF-alpha and outcomes of neonatal hypoxic ischemic encephalopathy. Brain Dev 2006; 28: 178-182.
14) Silveira RC, Procianoy RS. Interleukin-6 and tumor necrosis factor-alpha levels in plasma and cerebrospinal fluid of term newborn infants with hypoxic-ischemic encephalopathy. J Pediatr 2003; 143: 625-629.
15) Liu J, Ginis I, Spatz M, Hallenbeck JM. Hypoxic preconditioning protects cultured neurons againsthypoxic stress via TNF-alpha and ceramide. Am J Physiol Cell Physiol 2000; 278: 144-153.
16) Katsuragi S, Ikeda T, Date I, Shingo T, Yasuhara T, Ikenoue T. Grafting of glial cell line-derived neurotrophic factor secreting cells for hypoxic-ischemic encephalopathy in neonatal rats. Am J Obstet Gynecol 2005; 192: 1137-1145.
17) Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Ishii K, Kobune M, Hirai S, Uchida H, Sasaki K, Ito Y, Kato K, Honmou O, Houkin K, Date I, Hamada H. Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. Mol Ther 2005; 11: 96-104.
18) Miyazaki H, Okuma Y, Fujii Y, Nagashima K, Nomura Y. Glia cell line-derived neurotrophic factor protects against delayed neuronal death after transient forebrain ischemia in rats. Neurosci 1999; 89: 643-647.
19) Katsuragi S, Ikeda T, Date I, Shingo T, Yasuhara T, Mishima K, Aoo N, Harada K, Egashira N, Iwasaki K, Fujiwara M, Ikenoue T. Implantation of encapsulated glial cell line-derived neurotrophic factor-secreting cells prevents long-lasting learning impairment following neonatal hypoxic-ischemic brain insult in rats. Am J Obstet Gynecol 2005; 192: 1028-1037.
20) Youn Y, Kim SJ, Sung IK, Chung SY, Kim YH, Lee IG. Serial examination of serum IL-8, IL-10 and IL-1ra levels is significant in neonatal seizures induced by hypoxic ischemic encephalopathy. Scand J Immunol 2012; 76: 286-293.