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Neuronal ERCC6 mRNA expression in rat brain induced by a transient focal cerebral ischemia[1]

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KEY WORDS transient cerebral ischemia; DNA repair; reperfusion injury; messenger RNA; Northern blotting; complementary DNA; *in situ* hybridization; phosphopyruvate hydratase; glial fibrillary acidic protein

ABSTRACT

AIM: To study whether the excision repair cross-complementing group 6 (ERCC6) is involved in the neuronal pathophysiological process following cerebral ischemia-reperfusion injury. **METHODS**: A transient middle cerebral artery occlusion (MCAO) was used to induce cerebral ischemia-reperfusion injury in rat brain. Northern blot was used to check a specific signal for oligonucleotide probe. The expression of ERCC6 mRNA in the rat brain was observed by *in situ* hybridization. The specific cellular distribution of ERCC6 mRNA in the neuron or glia of the rat brain was analyzed by double staining combined with confocal laser scanning microscopic analysis. **RESULT**: The expression of ERCC6 mRNA in the penumbra area increased following ischemia and reperfusion with a time-dependent manner. ERCC6 was expressed on d 2, reached peak values on d 3, and kept high level even on d 14 of reperfusion following ischemia. Number of ERCC6 mRNA expressive cell in the penumbra area on d 1, d 2, d 3, d 7, d 14 of reperfusion following ischemia were (0 ± 0), (253 ± 56), (816 ± 355), (341 ± 185), $(128 \pm 95) \times 10^6$ cells/m^2, respectively. Confocal microscopic analysis showed that ERCC6 mRNA coexpressed with phosphopyruvate hydratase in the neurons and with glial fibrillary acidic protein (GFAP) in a few proliferation astrocyte glia. **CONCLUSION**: The expression of transcription-repair coupling factor ERCC6 mRNA in the neuron and glia was induced by ischemia-reperfusion injury.

INTRODUCTION

Cerebral ischemia could induce DNA damage, which might involve both apoptotic and nonapoptotic mechanisms[1]. DNA damage resulting from cerebral ischemia was observed as two forms, DNA single-strand breaks (SSB) and DNA double-strand breaks (DSB) in ischemic neurons[2]. Accumulation of free radicals is refered to as one of main mechanisms inducing neuronal DNA damage and causing delayed cell death[3,4]. Neuronal DNA repair system are considered to be able to correct most of lesions mainly through the activation of nucleotide excision repair (NER) pathway[5]. At least two protein complexes are involved in the first step of NER. One of these complexes has helicase activity, which may link DNA repair with DNA transcription[6]. The excision repair cross-complementing group 6 (ERCC6) may be a member of recently identified family of putative helicases[7]. Bioactivity analysis suggested that ERCC6 was a DNA-activated ATPase[8] and

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could bind RNA polymerase Ⅱ to guide nucleotide excision repair protein to the lesion regions, thereby link transcription to DNA repair[9]. ERCC6 is considered as a preferential repair gene, and with an ability to correct the repair defect of Cockayne's syndrome complementation group B (CS-B), a neuronal degenerative disease induced by gene repair defect. In the present study, we first observed the change of ERCC6 mRNA expression in the rat brain with a transient cerebral ischemia and further analyzed the cellular distribution of ERCC6 mRNA in the neuron and glia.

MATERIALS AND METHODS

Chemicals Digoxigenin oligonucleotide tailing labeled kit, DIG oligonucleotide detected kit, anti-DIG-fluorescein, monoclonal antibodies against glial fibrillary acidic protein (GFAP), positively charged nylon membranes, Tripure isolation reagent were purchased from Boehringer Mannheim. Antibodies against phosphopyruvate hydratase was purchased from ICN.

Rats Adult ♂ Sprague-Dawley rats weighing between 210 – 250 g (Experiment Animal Center of Shanghai Medical University, Grade Ⅱ, Certificate № 02-22-2) were housed under a 12-h light/dark cycle with free access to food and water. Rats were anesthetized with ip injection of chloral hydrate 300 mg·kg^{-1}. Arterial blood was collected to measure pH, pO_2, and pCO_2. Heating lamp and pad were used to maintain brain temperature measured by using a 23-gauge stainless thermocouple probe (Barnhart Co, USA) inserted into the temporalis muscle[10]. Rats within normal range of physiologic variables were subjected to a transient middle cerebral arterial occlusion (MCAO)[11]. The occlusion lasted 1 h. Sham-operated rats ($n = 4$) were subjected to the same operation but no nylon suture. Following MCAO, rats were killed on d 1, d 2, d 3, d 7, and d 14 (3 – 4 rats at each time).

Brain section Rats were anesthetized by chloral hydrate and intracardially perfused with saline, followed by 300 mL fixative of 4 % paraformaldehyde in phosphate-buffer saline 0.1 mol·L^{-1} (PBS, pH 7.2). Following the perfusion, brains were removed and postfixed in the same fixative overnight, then immersed in 20 % and 30 % sucrose solutions in PBS until sinking. Coronal brain sections were cut at 0.26 – 3.3 mm at bregma level on freezing microtome and stored at – 20 ℃ in cryoprotectant solution consisting of sucrose/ethylene glycol/PBS for histological study.

***In situ* hybridization** Oligonucleotide probe (41-mer) complementary to nucleotides sequence 4113 – 4153 of ERCC6 cDNA[5] was synthesized on an automated DNA synthesizer. The probe was labeled at 3′-end using DIG oligonucleotide tailing kit. Specificity of the probe was confirmed by Northern blot analysis and hybridization with sense probe.

A free-floating technique was employed to perform a nonradioactive *in situ* hybridization histochemical reaction. The sections were postfixed in 4 % paraformaldehyde for 10 min, washed in PBS 0.1 mol·L^{-1} three times at room temperature for 15 min each, and then treated with proteinase K 1 mg·L^{-1} at 37 ℃ for 15 min. Following two rinses with PBS 0.1 mol·L^{-1}, the sections were incubated with 0.25 % acetic anhydride in triethanolamine 0.1 mol·L^{-1} for 10 min, treated with 50 % formamide in 4 × saline sodium citrate (SSC) at 24 ℃ for 20 min, and incubated in humidified chamber at 37 ℃ for 20 h with hybridization buffer (50 % formamide, 5 × SSC, 2 % block reagent, 0.02 % SDS) containing digoxigenin-labeled antisense oligonucleotide probe at the final concentration of 0.25 mg·L^{-1}. After hybridization, sections were washed with 2 × SSC twice at 24 ℃ for 15 min each and in 0.1 × SSC twice at 42 ℃ for 15

min each, then incubated in alkaline phosphatase-labeled anti-digoxigenin antibody (diluted 1∶5000) at 24 ℃ for 4 h and treated with alkaline phosphates substrate solution, contained nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), in dark for 6 h to develop color. Finally, the hybridized sections were mounted on glass slides, dehydrated through absolute alcohol, cleared in xylem, and cover-slipped.

RNA isolation and Northern blotting The total RNA of rat brain was isolated using the Tripure isolation reagent. RNA sample (30 μg) was separated by electrophoresis through 1 % agarose-formaldehyde gel and transferred onto positively charged nylon membrane. The filter was hybridized with a 3′-end labeled oligonucleotide probe at 42 ℃ in 50 % (V/V) formamide, 5 × SSC, 0.1 % *N*-lauroyl-sarcosine, 0.02 % SDS for 20 h, washed twice with 2 × SSC, 0.1 % SDS and twice with 0.1 × SSC, 0.1 % SDS at 68 ℃, incubated in alkaline phosphatase-labeled anti-digoxigenin antibody (diluted 1∶5000) for 4 h and treated with alkaline phosphates substrate solution, containing NBT and BCIP, in dark to develop color. The Northern blotting showed that only one band corresponding to the molecular weight of ERCC6 was hybridized.

Double hybridization- and immunofluorescein staining Following hybridization, the sections were rinsed with 0.1 × SSC, incubated with anti-DIG-fluorescein diluted 1∶250 at 37 ℃ for 1 h, rinsed with PBS 0.01 $mol \cdot L^{-1}$, incubated at 37 ℃ for 1 h with mouse monoclonal antibodies against GFAP diluted 1∶200 or with rabbit polyclonal antibodies against phosphopyruvate hydratase diluted 1∶80. Sections were incubated at 37 ℃ for 1 h with anti-mouse IgG-rhodamine diluted 1∶20 or anti-rabbit IgG-rhodamine diluted 1∶20. Double staining was examined using a confocal laser scanning microscope (Leica TCS NT).

Data analysis and statistics The expressive cells for ERCC6 mRNA in ischemic core (caudate putamen) and penumbra area (parietal cortex) were counted[12]. The number of cells in a section was given by an average from 3 countings in each area. Data were expressed as $\bar{x} \pm s$ and analyzed using one-way ANOVA followed by unpaired *t*-test.

RESULTS

Time-course of ERCC6 mRNA expression following a temporal MCA occlusion By *in situ* hybridization analysis, ERCC6 mRNA was barely detected on d 1 of reperfusion following ischemia in both the ischemic core and penumbra area. In the penumbra region, the expression of ERCC6 mRNA increased on d 2 and reached peak values on d 3 of reperfusion, started to decrease on d 7 and still kept high level even on d 14 of reperfusion following ischemia. The expression of ERCC6 mRNA in the ischemic core, where most cells destined to die, was also induced on d 3 of reperfusion, but the induction in the ischemic core was much less than that in the penumbra region where most of the cells survived. No significant ERCC6 mRNA expressive cells in the brain was seen in the sham-operated group (Fig 1, Tab 1).

Tab 1. ERCC6 mRNA expression after cerebral ischemia-reperfusion injury. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* sham-operated group. $^dP > 0.05$, $^eP < 0.05$ *vs* 3-d ischemia-reperfusion group.

Area	Ischemic area	Penumbra, $\times 10^6$ cells/m^2
Sham (*n* = 4)	0 ± 0	0 ± 0
1 d (*n* = 4)	0 ± 0	0 ± 0
2 d (*n* = 3)	28 ± 25^{ad}	235 ± 56^{be}
3 d (*n* = 3)	37 ± 36^{b}	816 ± 355^{b}
7 d (*n* = 3)	21 ± 37^{ad}	341 ± 185^{be}
14 d (*n* = 3)	6 ± 11^{ad}	128 ± 95^{be}

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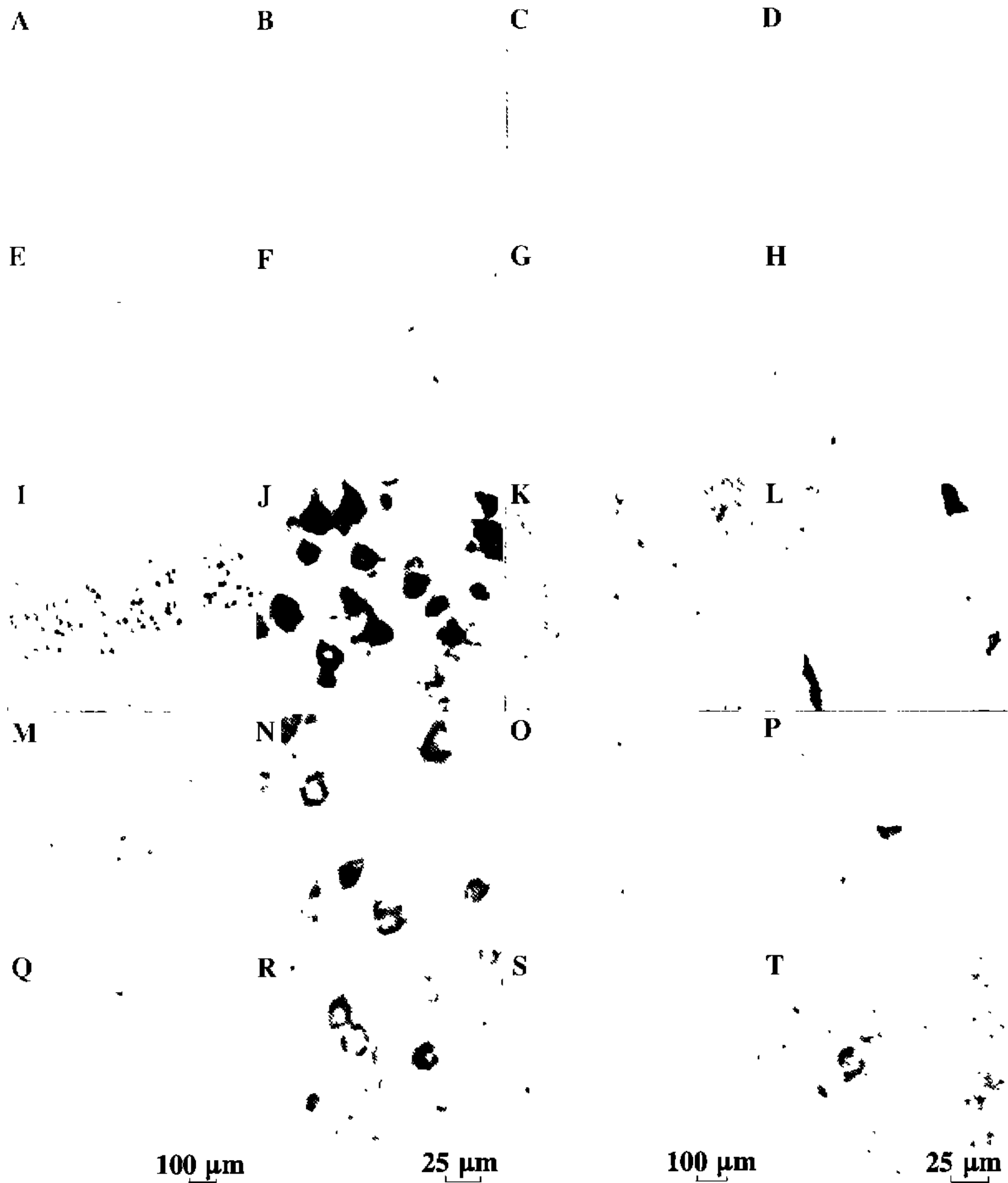

Fig 1. ERCC6 mRNA expression. A, E, I, M, Q, C, G, K, O, S: ×40; B, F, J, N, R, D, H, L, P, T: ×200. Sham-operated (A – D) and on d 1 (E – H), d 3 (I – L), d 7 (M – P), and d 14 (Q – T) of reperfusion following 1-h MCAO.

Cellular localization of ERCC6 mRNA The brain section on d 3 of reperfusion following ischemia was double stained ERCC6 mRNA with phosphopyruvate hydratase or GFAP and analyzed with confocal laser scanning microscope. ERCC6 mRNA was revealed with a secondary

antibody conjugated with fluorescein (green), while phosphopyruvate hydratase or GFAP was revealed with rhodamine-conjugated secondary antibody (red). The image was taken in a single focal plane. Yellow-orange staining after superimposition of the fluorescein and rhodamine image were detected in the most neuron in the penumbra and in a few glia in the ischemic core. (Fig 2).

DISCUSSION

In the present study, we first revealed that ERCC6 mRNA could express in both neuron and astrocyte glia of rat brain following a cerebral ischemia. These results suggest that ERCC6 may be involved in the neuronal pathophysiological process following the cerebral ischemia.

As we known, damaged DNA could stimulate the activity of DNA repair system in the cells. Theoretically, if the repair function can be activated enough to repair damaged DNA during the ischemia, the cells will be rescued and keep alive. ERCC6 is a gene participating in the preferential repair and has correction in repair defect of CS-B[7]. Although the functions of ERCC6 are not quite clear, it is confirmed that ERCC6 gene is essential for the preferential repair[13,14] and mutations in the ERCC6 gene in Cockayne's syndrome group B abolish preferential repair[15]. In the present study we observed that the expression of ERCC6 mRNA was markedly induced in the brain following the ischemia. High expression of ERCC6 was observed in the penumbra region where most of the cells survived although some degree of discretion, which could be diminished by increasing the number of experimental animal. Putting together, it seems that induction of ERCC6 mRNA expression resulting from reperfusion following ischemia may reflect a possibility of neuronal DNA repair activation. Of course, the exact function for the expression of inducible ERCC6 mRNA should be illustrated.

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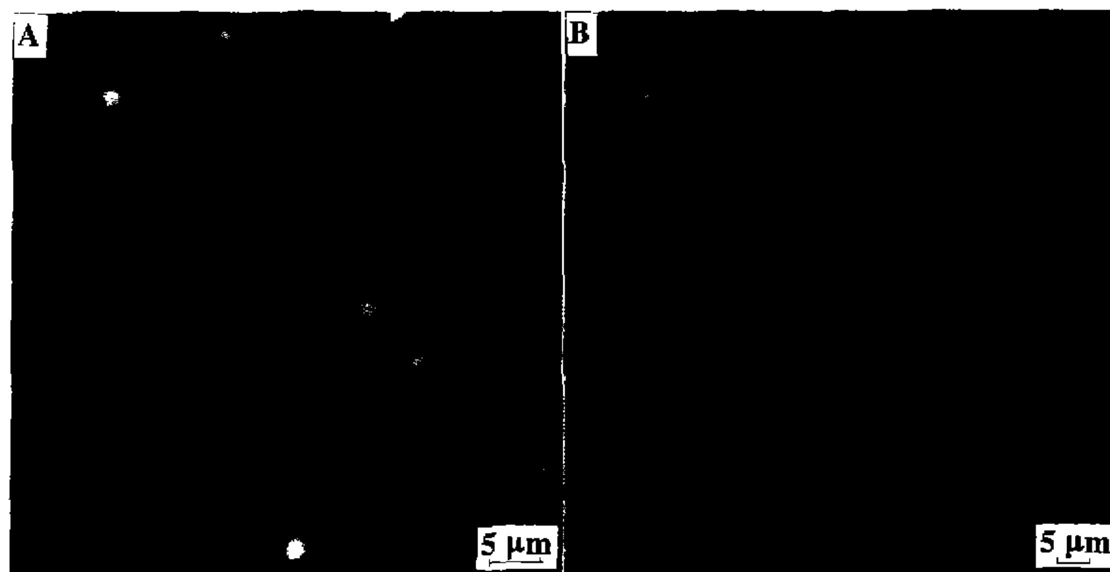

Fig 2. Simultaneous detection of ERCC6 mRNA and phosphopyruvate hydratase (A) or GFAP (B) on d 3 of reperfusion following 1-h MCAO. ERCC6 mRNA was detected using *in situ* hybridization histochemistry (green). Phosphopyruvate hydratase and GFAP were detected by using immunohistochemistry (red). Yellow color was produced by overlapping of green and red.

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脑缺血诱导大鼠神经细胞上 ERCC6 mRNA 的表达[1]

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关键词　暂时性脑缺血；DNA 修复；再灌注损伤；信使 RNA；RNA 印迹；互补 DNA；原位杂交；磷酸丙酮酸水合酶；神经胶质纤维酸性蛋白

目的：观察缺血再灌注损伤对转录修复耦联因子(ERCC6)mRNA 表达水平的影响． **方法**：在大鼠大脑中动脉栓塞模型上结合 Northern 杂交，原位杂交和共聚焦激光扫描显微镜的方法观察脑内 ERCC6 mRNA 的表达，并进行细胞定位分析． **结果**：缺血再灌注损伤后，在缺血中心及边周区的 ERCC6mRNA 表达，2 天时开始增加，3 天达峰值，7 天开始下降． ERCC 6 mRNA 主要在缺血侧的神经元上表达，少数在星型胶质细胞上表达． **结论**：缺血侧神经元和神经胶质细胞上 ERCC6 mRNA 表达增加，这提示了损伤后神经细胞的 DNA 自身修复能力被增强．

(责任编辑　李　颖)