

Full-length article

Antiapoptotic effect both *in vivo* and *in vitro* of A20 gene when transfected into rat hippocampal neurons¹Hong-sheng MIAO^{2,3}, Lu-yang YU⁴, Guo-zhen HUI², Li-he GUO^{2,5}² Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China;³ Department of Neurosurgery, First Hospital of Soochow University, Suzhou 215007, China**Key words**

A20 protein; tumor necrosis factor; primary hippocampal neurons; middle cerebral artery infarction; apoptosis

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Abstract

Aim: To evaluate the antiapoptotic effect of the A20 gene in primary hippocampal neurons both *in vivo* and *in vitro*. **Methods:** Primary hippocampal neurons in embryonic day 18 (E18) rats were transfected with the A20 gene by using the new Nucleofector electroporation transfection method. We then examined, whether A20 -neurons possessed anti-apoptotic abilities after TNF- α stimulation *in vitro*. A20-neurons and pcDNA3 -neurons were transplanted into the penumbra of the brains of rats that had been subjected to 90-min of ischemia induced by left middle cerebral artery occlusion (MCAO). **Results:** A20-neurons resisted TNF- α induced apoptosis *in vitro*. The apoptosis rate of neurons overexpressing A20 (28.46% \pm 3.87%) was lower than that in neurons transfected with pcDNA3 (53.06% \pm 5.36%). More A20-neurons survived in the penumbra both 3-d and 7-d after transplantation than did sham pcDNA3 neurons. **Conclusion:** The novel function of A20 may make it a potential targets for the gene therapy for neurological diseases.

Introduction

Uncontrolled gene expression and apoptosis result in the development of various diseases. Excessive apoptosis plays a role in the pathogenesis of AIDS and neurological diseases such as Alzheimer disease and stroke^[1].

The zinc finger protein A20 is a novel protein type in that it seems to have a dual activity, both as an inhibitor of NF- κ B activation and as an antiapoptotic molecule in some cell systems, but not in the case of human cervix carcinoma HeLa cells, lung epithelial A549 cell, or human hepatoma HepG2 cells^[2–6]. The reason why some cell lines are protected by A20 and others not are still unclear.

Little research work has been done on the functions of A20 in primary cultured neurons, although cell apoptosis is involved in many neurological diseases. The poor transfection efficiency resulting from the extreme resistance of cultured neurons to chemical transfection vectors has limited further research^[7].

In this study, we overexpressed A20 by means of electroporation gene transfer into neurons to investigate whether

A20 can inhibit TNF-induced apoptosis in primary cultured neurons both *in vivo* and *in vitro*.

Materials and methods

Cloning of the A20 gene Human umbilical vein endothelial cells were treated with TNF- α 1000 kU/L and cycloheximide 10 μ g/L for 4 h. Poly(A)⁺-mRNA was extracted with Trizol Reagents (Gibco) and identified by electrophoresis on a 1.2% agarose gel. cDNA was obtained by using reverse transcription-polymerase chain reaction (PCR). The PCR was performed with a sense primer (5'-AAC GAGCG-GTTCCGATGCC-CTGAG-3') and an anti-sense primer (5'-TGTCGCCTTCACCGTTC-CAGTT-3') using 30 cycles of 95 °C for 1 min, 62 °C for 1.5 min, 72 °C for 2.5 min. The pcDNA3 expression vector containing the CMV immediate early promoter was used for this study.

Analysis of A20 mRNA contents Isolation of total mRNA from the transfected neurons cultured *in vitro* or in the penumbra tissue was performed using Trizol reagent (Promega, Charbonnieres, France), followed by RT-PCR

using the following primers: forward 5'-CGGTACCGCACAA-TGGCTGAACAAGTCCTTCCT-3' and reverse 5'-CGTC-TAGAGTTAGCCATACATC-TGCTTGAAGT-3'. The PCR was run at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 62 °C for 1.5 min, and 72 °C for 2.5 min, with a 10 min final extension period at 72 °C. The house-keeping gene β -actin was used as a control.

Western blotting Protein was isolated from the transfected neurons and the penumbra of the MCAO rats using RIPA reagent 10 d after transfection. The samples were then resolved on a 15% SDS-PAGE gel and electrophoretically transferred onto poly-vinylidene difluoride membranes. The membranes were probed overnight at 4 °C with mouse monoclonal anti-human A20 antibody (1/2000, Oncogene). After being incubated with goat anti-mouse-HRP (1/5000) for 1 h at room temperature, antigens were revealed by enhanced chemiluminescence reaction buffer.

Cell culture Hippocampal neurons from embryonic day 18 were freshly prepared by hippocampal dissection. The C-shaped hippocampus was cut off and trypsinized for 20 min at 37 °C in a 5% CO₂ incubator. The cells were broken apart by pipetting with complete media and were then plated with a density of 3×10^8 cells/L in neurobasal medium supplemented with B27 (all materials from Gibco Invitrogen, Grand Island, NY).

Rat neuron Nucleofector transfection Transfection was accomplished using a Rat Neuron Nucleofector kit and the device from Amaxa. For each example, the prepared neurons were resuspended in Rat Neuron Nucleofector Solution to a final concentration of 1×10^6 – 6×10^6 cells/L. Then, 100 μ L of cell suspension was mixed with 1–3 μ g DNA (in 1–5 μ L H₂O or TE). Each nucleofection sample was transferred into an Amaxa certified cuvette. Insert the cuvette into the cuvette holder (Amaxa, Germany) and rotate the turning wheel clockwise to the final position. And tranfection program G-13 was initiated. The transfections efficiencies were evaluated and cell transplantation was carried out 3 d later.

Immunocytochemistry Three days after transfection, the wells were fixed with 4% paraformaldehyde for 15 min. Then the wells were incubated for 12 h at 4 °C with mouse anti-rat NF antibody (1:100, DAKO) and mouse anti-human A20 antibody (1:200, Oncogene), diluted in blocking buffer. The wells were incubated with rhodamine-labeled secondary antibodies (1/500, Kirkegaard Perry Laboratories, MD, USA) and FITC-labeled secondary antibodies (1/200, DAKO) in blocking buffer for 60 min at room temperature. Fluorescence staining was evaluated using a Leica fluorescent microscope.

Cell counting Neurons stained positively for A20 were counted on coded slides using an Olympus CAST Grid system (Denmark). The area of each culture well was delineated and a counting frame was randomly placed to mark the first area to be sampled. The frame was then systematically moved through the delineated area. The total number of neurons and A20-immunoreactive neurons was extrapolated from the data, and the transfection efficiency was evaluated.

For the experiments *in vivo*, 3 d and 7 d after transplantation, rats were anesthetized and perfused intracardially with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde dissolved in PBS (pH 7.4). Then, serial coronal brain cryostat sections were cut at 20 μ m thickness and every third section was used to count the surviving cells. To assess the survival of grafted Hoechst-stained neurons, a similar method was employed^[8].

Neurons cultured with TNF- α Three days after transfection, TNF- α was added to A20-neurons and pcDNA3-neurons to a final concentration of 2000 kU/L, and the cells were incubated at 37 °C in 5% CO₂ for 8 h. Then, the apoptosis rates were accessed by flow cytometry using a FACStar (Becton Dickinson) with excitation wavelength set at 585 nm.

Labeling of neurons for transplantation Seventy-two hours after transfection, Hoechst 33258 (Sigma) was added to each well to achieve a final concentration of 10 mg/L. One hour later, cells were rinsed 3 times with PBS to completely flush out excess fluorescence and the cells were then collected for transplantation.

Surgical procedure All animal experimental procedures were approved by the local animal protection authority. Twenty-four male Sprague-Dawley rats (250–300 g) were randomly allocated to four groups of equal size (group A–D). Rats in groups C and D were anesthetized with 10% chloralhydrate, a midline neck incision was made and a 30-mm long piece of 4–0 monofilament nylon suture was advanced from the external carotid artery (ECA) through the common carotid artery (CCA) and into the lumen of the internal carotid artery (ICA) until it blocked the origin of the middle cerebral artery (MCA). One and a half hours after the occlusion, the filament was withdrawn^[9]. The rats were allowed to recover and were monitored daily for behavioral and neurological deficits (motor weakness of extremities). The rats that did not show neurological deficits were excluded^[10].

Transplantation surgery Immediately after MCAO, the rats' heads were immobilized in a stereotaxic frame. Either A20 or pcDNA3 neurons (3×10^5 cells) 2 μ L were injected

into the penumbra at the following coordinates: 1.00 mm rostral to bregma, 3.0 mm right of the midline, and 1.2 mm ventral from the dural surface, with the incisor bar set at zero. Another 12 normal rats serving as controls received identical grafts^[11]. Rats were divided into 4 groups: normal rats with A20-neurons (group A), normal rats with pcDNA3-neurons (group b), MCAO rats with A20-neurons (group C), MCAO rats with pcDNA3-neurons (group D).

Statistical analysis Data are expressed as mean±SD. Statistical analysis was performed by one-way ANOVA and the *t*-test, using statistical software package SPSS 10.0. Statistical significance was set at a level of *P*<0.05.

Results

Identification of primary cultured cells and evaluation of transfection efficiency The fact that most cells were immunoreactive for NF indicates that most cells were neurons (Figure 1). Seventy-two hours after gene delivery, neurons immunoreactive for A20 were counted as described above for quantitative determination of transfection effi-

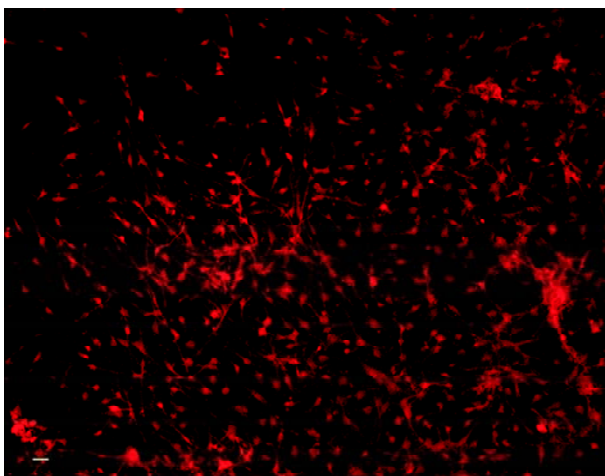


Figure 1. E18 hippocampal neurons tagged with anti-NF antibody on day 3 after plating (×100).

ciency (Figure 2). The percentage of neurons successfully transfected with A20 was 52.46%±5.26%.

Expression of A20 in neurons *in vitro* and after transplantation into MCAO brains Activation of A20 was detected 10 d after transfection by both RT-PCR (Figure 3A) and Western blot analysis (Figure 3B).

Induction of apoptosis by TNF-α *in vitro* The results of FACS analysis showed that after the proapoptotic insult with TNF-α, the apoptosis rate of neurons overexpressing

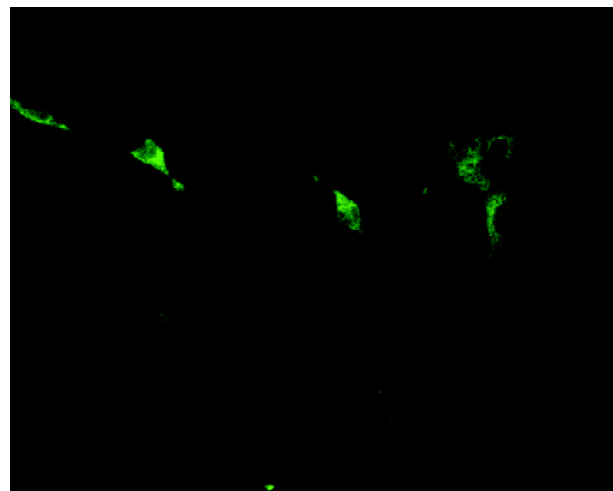


Figure 2. The immunocytochemical staining for protein A20 of the rat E18 primary hippocampal neurons 3 d after transfection (×100).

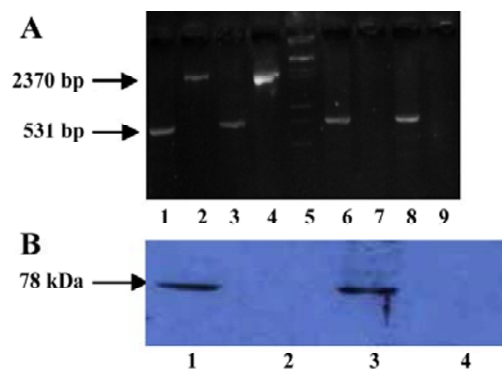


Figure 3. A) RT-PCR showed the expression of A20 in neurons 10 d after transfection both *in vivo* (lane 2) and *in vitro* (lane 4). Neurons transfected with pcDNA3 were chosen as the negative control both *in vivo* (lane 7) and *in vitro* (lane 9). The β-actin was selected as intrinsic standard (lanes 1, 3, 6, 8). B) Western blot analysis showed the expression of protein A20 in neurons 10 d after transfection both *in vivo* (lane 1) and *in vitro* (lane 3). Neurons transfected with pcDNA3 were chosen as the negative control both *in vivo* (lane 2) and *in vitro* (lane 4).

A20 (28.46%±3.87%) was lower than that in neurons transfected with pcDNA3 (53.06%±5.36%) (*P*<0.05, Figure 4A, 4B).

A20 protected the transplanted neurons from apoptosis in the penumbra of the MCAO brain Cells derived from the hippocampus of E18 Sprague-Dawley rats were transfected with A20 or pcDNA3 and grafted into the apoptotic zone (penumbra) of MCAO rats, and at an identical position in normal rats. Grafts were identified *in vivo* 3 d and 7 d after transplantation by staining using the Hoechst 33258 stain. A large number of cells implanted into the nor-

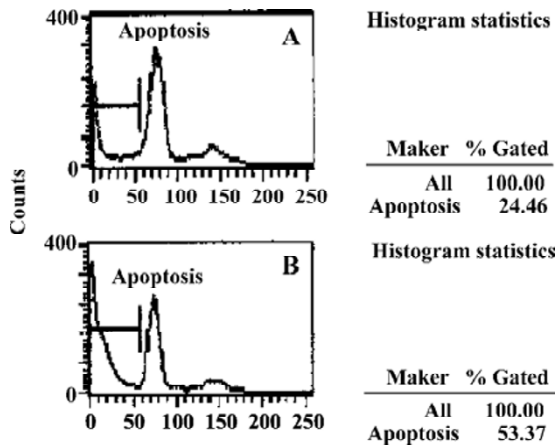


Figure 4. The apoptosis rate of the cultured neurons at 3 d after transfected with A20 under TNF- α stimulus (B). The neurons transfected with native pcDNA3 was as the control group (A).

mal rat brain survived, with no significant difference in the cell survival rate between the A20 group and the pcDNA3 group on d 3 or d 7 ($P > 0.05$). On the other hand, a great deal of grafted cells died when transplanted into the apoptotic zone of the ischemic brain. In this case, neurons with A20 that were grafted into MCAO rats had a greater survival rate compared to pcDNA3 cells both on d 3 and d 7 ($P < 0.05$, Figure 5A-5H, 6).

Discussion

A20 is encoded by a primary response gene which was originally identified as a TNF-inducible gene in human umbilical vein endothelial cells^[12]. It seems to have a dual activities, serving not only as an inhibitor of NF- κ B activation, but also as an antiapoptotic gene in some cell systems. Primary cells that are taken directly from the body are morphologically and physiologically similar to the parent tissue. Gene transfer using primary cells is a much better way to mimic the *in vivo* situation. This method permits much more precise medical and scientific conclusions than when genes are transferred into degenerated cell lines^[14].

In our research, we used rat primary hippocampal neurons, which form an important *in vitro* model for the research of many central nervous system diseases (such as brain ischemia and Parkinson disease) and for studying routine events in most parts of the brain. And a newly developed electroporation method known as Nucleofector technology was employed in the study of the rat primary hippocampal neurons. It is a physical method that is suitable for the transfection of nonphagocytic and nonproliferating cells, such as

peripheral leukocytes and stem cells, which are usually refractory to chemical transfection vectors. In addition, it is free of biocontaminants and does not induce immune reactions^[15]. The results of immunocytochemical analysis indicated that Nucleofector technology obtained a $52.46\% \pm 5.26\%$ transfection efficiency for the A20 gene, which is the highest transfection efficiency reported so far involving nonviral gene transfer of primary cell cultures. This high rate of efficiency is important for advanced functional experiments. RT-PCR and Western blotting analysis further proved the continuous expression of A20 proteins both *in vitro* and *in vivo* 10 d after transfection.

FACS analysis demonstrated that the A20 could protect the primary culture neurons from TNF-induced apoptosis *in vitro*. The mechanism of action has not yet been completely clarified. Some researchers speculate that A20 might interfere with TNF-R-associated death domain (TRADD) binding to TNF-R1 and might, thus, negatively regulate TNF induced cytotoxicity^[6].

Most of the evidence supporting the role of apoptosis in neuronal cell death comes from studies using animal models of global or focal cerebral ischemia. In the focal ischemia models, which create a condition emulating human strokes, transient ischemia followed by reperfusion is often associated with massive induction of apoptosis-like cell death^[16]. After reversible middle cerebral artery occlusion in adult rats, both TNF and TRAIL proteins are expressed in the apoptotic areas of the post-ischemic brain (the penumbra), thus providing conditions for inducing apoptosis. TRAIL mRNA levels increase in response to ischemia and reperfusion, reaching a maximum after 3 d. In contrast to TRAIL, TNF- α mRNA is not consistently upregulated in the ischemic hemisphere. TNF- α mRNA exhibits a first peak after 24 h, followed by a decline after 3 d, and a second rise after 5 d^[17]. Furthermore, in focal cerebral ischemia, NF- κ B is activated and promotes cell death^[17,18]. So when cultured neurons are injected into this zone, apoptosis is expected to occur. Cell counting of surviving neurons in the present study confirmed this speculation. Higher levels of cell death were observed in the case of both A20-neurons and pcDNA3-neurons when grafted into MCAO rats than when grafted into normal rats. This result proves that apoptosis-inducing circumstances definitely exist in the penumbra following transient cerebral ischemia. On the other hand, among the two groups of MCAO rats, more A20-neurons survived than the sham group pcDNA3-neurons. This result indicates that A20 protein can protect the neurons from the cytotoxicity associated with the ischemic zone. Some further points should be made. First, the transfection efficiency was approximately 50%, and neu-

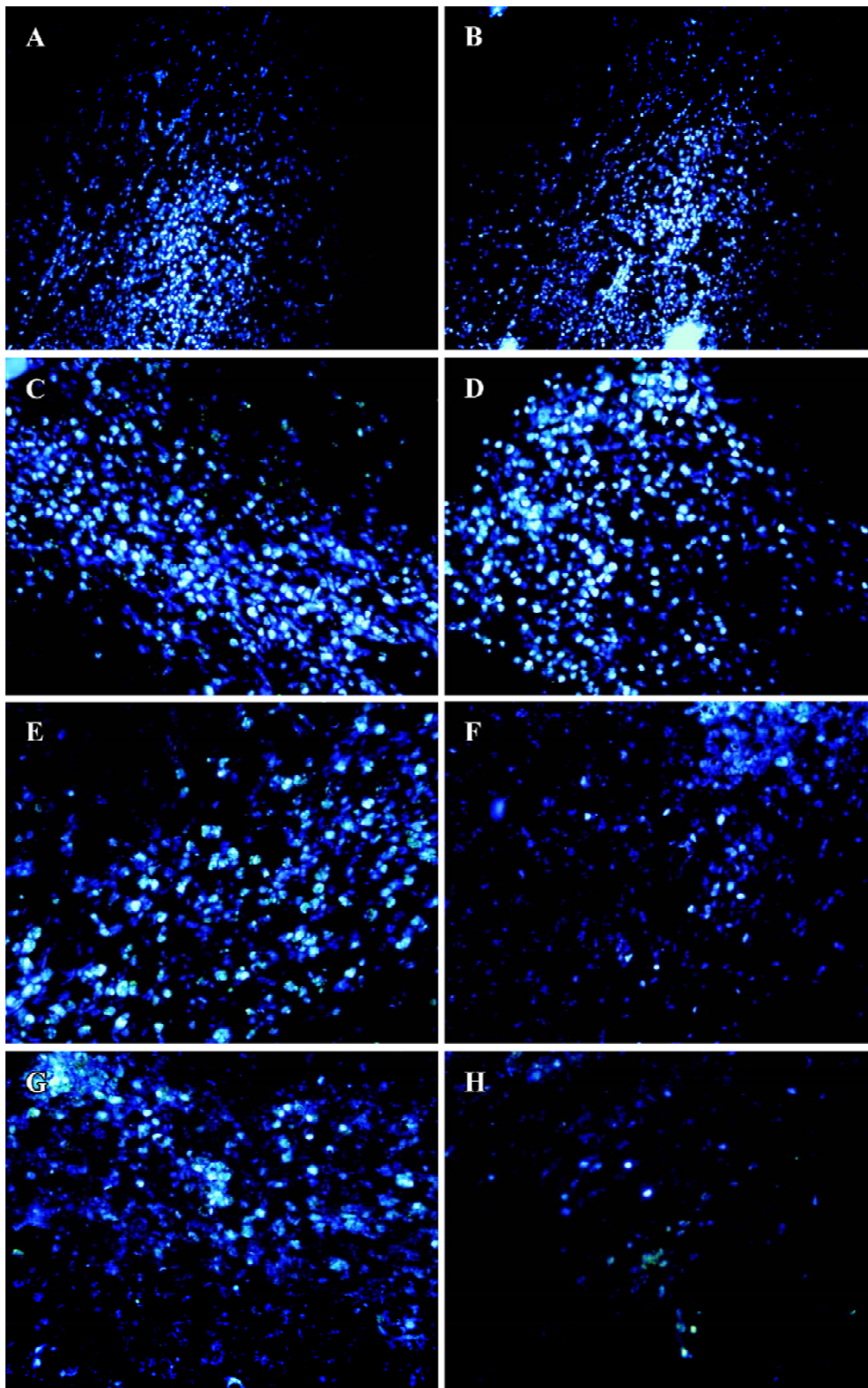


Figure 5. Immunostained 20- μ m cryosections of surviving neurons from Sprague-Dawley rats subjected to MCAO and subsequently reperfused for different lengths of time ($\times 200$). Hoechst reactive neurons which had been transfected with A20 and pcDNA3 were seen scattered throughout the penumbra at 3 d after A20-neurons (E) and pcDNA3-neurons (F) transplanted to the penumbra of MCAO rats, and 7 d after A20-neurons (G) and pcDNA3-neurons (H) transplanted to the penumbra of MCAO rats. There were corresponding controls to normal rats at 3 d after A20-neurons (5A) and pcDNA3-neurons (B) transplanted to the normal rats, and 7 d after A20-neurons (C) and pcDNA3-neurons (D) transplanted to the normal rats.

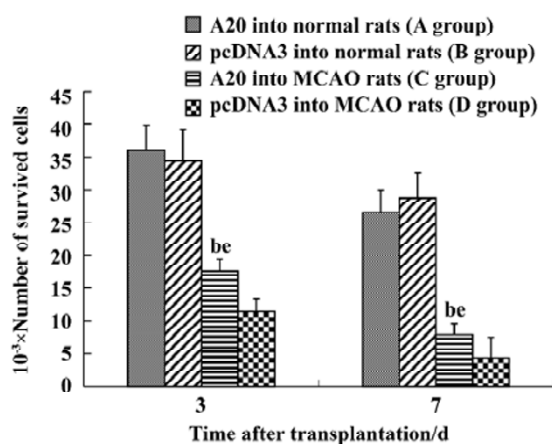


Figure 6. Number of the surviving cells at 3 d and 7 d after transfected neurons were transplanted into the brains of MCAO rats and normal rats. $n=3$. Mean \pm SD. ^b $P<0.05$ vs A group. ^c $P<0.05$ vs D group.

rons that did not express the A20 protein would likely be induced to undergo apoptosis. Second, the transplanted neurons were influenced not only by TNF and TRAIL, but also by multiple other toxic factors, such as the CD95-ligand, starvation, etc. Thus 7 d after transplantation into the ischemic zone, the fact that large number of A20-neurons nevertheless died indicates that the function of A20 protein can only partly and not completely protect the neurons *in vivo*.

In conclusion, our study showed that the zinc finger protein A20 was an effective neuroprotective agent that improved the survival of both cultured and grafted embryonic hippocampal neurons, which suggests that the A20 may have therapeutic potential in gene therapy for diseases of the central nervous system.

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