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Neurobiology of Disease

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Neurobiology of Disease 24 (2006) 345–356

Bcl-2 enhances neurogenesis and inhibits apoptosis of newborn neurons in adult rat brain following a transient middle cerebral artery occlusion

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Received 26 March 2006; revised 7 July 2006; accepted 17 July 2006
Available online 25 September 2006

To determine whether Bcl-2 could influence adult neurogenesis and prevent apoptosis of newborn neurons, we injected Bcl-2 expressing plasmid into the lateral ventricle of rat brain immediately following a 30-min occlusion of the middle cerebral artery (MCAO). We found that Bcl-2 increased neural progenitor cells ($BrdU^+$-DCX^+) in the ipsilateral striatum, newborn immature neurons ($BrdU^+$-$Tuj\text{-}1^+$) and newborn mature neurons ($BrdU^+$-$MAP\text{-}2^+$) in the ipsilateral striatum and frontal cortex at 1 to 4 weeks following MCAO. Bcl-2 overexpression promoted development of newborn neurons into GABAergic and cholinergic neurons in the ipsilateral striatum. Moreover, Bcl-2 significantly decreased the apoptosis of newborn neurons, determined by double staining of Tuj-1 and activated caspase-3 ($Tuj\text{-}1^+$-$Casp^+$). These results indicate that overexpression of Bcl-2 in adult rat brain enhances neurogenesis and survival of newborn neurons. Increasing neurogenesis and preventing the death of newborn neuron may be a strategy to aid in the repair of adult brain after stroke.

Keywords: Anti-apoptotic gene; Overexpression; Stroke; Adult neurogenesis; Brain repair

Introduction

Neurogenesis in the adult brain persists in at least two regions, the subgranular zone (SGZ) and the subventricular zone (SVZ) (Abrous et al., 2005). Although a low level of neurogenesis occurs in the healthy, uninjured brain, it is significantly enhanced following pathological events (Parent, 2003). Cerebral ischemia can stimulate proliferation and differentiation of neural progenitor cells in both the SGZ (Liu et al., 1998) and the SVZ (Zhang et al., 2001). Ischemia-induced neurogenesis is also seen in regions that are not a direct source of neurogenic precursors, including the hippocampal CA1 area (Nakatomi et al., 2002), cerebral cortex (Jiang et al., 2001) and striatum (Arvidsson et al., 2002; Nadareishvili and Hallenbeck, 2003), implying that newborn neurons can migrate toward damaged regions and replace injured neurons. Adult neurogenesis as a treatment to replace dying neurons in the injured brain is therefore a potentially significant treatment scheme for brain injury. Attention has been given to the study of factors that have the potential of enhancing neurogenesis in the adult brain, including growth factors, exercise and exposure to an enriched environment (Parent, 2003; Abrous et al., 2005). Unfortunately, the majority of newborn neurons in the adult brain do not persevere. Within 6 weeks of their birth, there is a 50% loss of newborn neurons in the adult olfactory bulb (Petreanu and Alvarez-Buylla, 2002) and dentate gyrus (Dayer et al., 2003). There is evidence that the loss of newborn neurons is caused by apoptosis. It is found a large number of cells positive for dUTP-nick end labeling (TUNEL) (Biebl et al., 2000) or caspase-3 (Bingham et al., 2005) in the same area containing bromodeoxyuridine (BrdU) immunoreactivity. Following ischemic stroke, 80% of newborn striatal neurons die from 2 to 6 weeks after the stroke event (Arvidsson et al., 2002). Although the mechanisms underlying apoptosis of newborn neurons are unclear, knockout of the pro-apoptotic bcl-2 family gene *bax* in mice increases the survival of newborn adult hippocampal neurons, suggesting that endogenous Bax participates in apoptosis of recently born adult neurons (Sun et al., 2004).

Bcl-2, an inhibitor of apoptosis in neuronal and glial cells, is highly expressed in the embryonic and developing mammalian brain (Abe-Dohmae et al., 1993; Ferrer et al., 1994). In the adult brain, however, it is restricted to progenitor rich-regions (Bernier and Parent, 1998; Bernier et al., 2000). Increasing evidence suggests that, in addition to its anti-apoptotic properties, Bcl-2 has an important function in cell differentiation and growth. Cultured

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Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2006.07.012

sensory neurons isolated from the trigeminal ganglia of *bcl-2*-deficient mouse embryos extend axons more slowly than neurons from wild type mice (Hilton et al., 1997). On the other hand, cultured retinal ganglion neurons from *bcl-2*-overexpressing transgenic mice extend axons at a greater rate than the ganglion neurons from wild type mice (Chen et al., 1997). In vivo studies also indicate that Bcl-2 overexpression enhances retinal axon regeneration after optic-tract transaction (Chen et al., 1997) and increases axonal growth of transplanted fetal dopaminergic neurons in the rat striatum (Holm et al., 2001).

Augmenting neuronal replacement by enhancing the survival and maturation of endogenous progenitors is a potentially useful treatment following cerebral ischemia. The neuroprotective effects of Bcl-2 against ischemic brain injury are due to its anti-apoptotic effect; however, it is not known whether Bcl-2 has any influence on ischemia-induced neurogenesis. We therefore used MCAO model to induce transient focal brain ischemia. Following MCAO, neurogenesis was enhanced and apoptosis did occur in newborn neurons. In addition, the injection of Bcl-2 expressing plasmid into the lateral ventricle enhanced neurogenesis and the survival of newborn neurons in adult rat brains after MCAO.

Materials and methods

Animals and reagents

Adult male Sprague–Dawley rats (220–250 g) from Shanghai Experimental Animal center, Chinese Academy of Sciences, were used in the study. The Medical Experimental Animal Administrative Committee of Shanghai approved all experiments. All efforts were made to minimize animal suffering and reduce the number of animals used.

Cresyl violet and diaminobenzidine (DAB) were purchased from Sigma (St. Louis, MO, USA). Fluoro-Jade B, guinea pig polyclonal anti-doublecortin (DCX), mouse monoclonal anti-βIII tubulin (Tuj-1), rabbit polyclonal anti-microtubule-associated protein-2 (MAP-2) and mouse monoclonal anti-choline acetyltransferase (ChAT) were obtained from Chemicon (Temecula, CA, USA). Mouse monoclonal anti-nestin and rabbit polyclonal anti-Bcl-2 were purchased from BD Pharmingen (San Jose, CA, USA). Rabbit polyclonal anti-p17 fragment of the activated caspase-3 was purchased from Abcam (Cambridge, UK). 5′-Bromodeoxyuridine (BrdU), mouse monoclonal anti-BrdU, anti-mouse and rabbit IgG-Rhodamine were obtained from Roche Applied Science (Mannheim, Germany). Mouse monoclonal anti-green fluorescent protein (GFP), goat polyclonal anti-doublecortin (DCX), rabbit polyclonal anti-67-kDa glutamic acid decrarboxylase (GAD_{67}) and anti-mouse, guinea pig and rabbit IgG-fluorescein isothiocyanate (FITC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). VECTASTAIN® ABC-alkaline phosphatase Kits (anti-mouse and rabbit IgG), VECTASTAIN® ABC-peroxidase Kits (anti-mouse, rabbit and goat IgG) and Vector® Blue were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). An anti-mouse IgG-FluoroLink™Cy™5 (Cy5) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Animal experimental protocols

Transient MCAO

Rats were anesthetized with 10% chloral hydrate (360 mg/kg, i.p.), and arterial blood samples obtained via femoral catheter were collected to measure pO_2, pCO_2 and pH with an AVL 990 Blood Gas Analyzer (AVL Co., Graz, Austria). The rectal temperature was maintained at 37±0.5°C during surgery and MCAO via a temperature-regulated heating lamp (Cao et al., 2002a,b; Sugiura et al., 2005). Rats with physiological variables within normal ranges were subjected to transient focal cerebral ischemia induced by left MCAO as previously described (Yang et al., 2002). Briefly, a 4-0 nylon monofilament suture with a rounded tip was introduced into the internal carotid artery through the stump of the external carotid artery and gently advanced for a distance of 22 mm from the common carotid artery bifurcation to block the origin of the middle cerebral artery for 30 min. Withdrawal of the suture restored MCA blood flow. Rats were then subjected to plasmid injection.

Plasmid administration

The cDNA encoding human *bcl-2* was inserted into the pEGFP-N1 plasmid between the constitutively active cytomegalovirus promoter (pCMV) and the enhanced green fluorescent protein (EGFP) reporter gene. Following MCAO, rats were divided into Bcl-2 (pEGFP-N1-Bcl-2 plasmid) and control (pEGFP-N1 empty plasmid) groups. The plasmid mixture (plasmids 5 μg/2 μl and 1 μl lipofectamine) were stereotaxically delivered into the ipsilateral lateral ventricle (coordinates from bregma: AP-0.8 mm, ML 1.4 mm, DV-3.6 mm from the pial surface) (Paxinos and Watson, 1986). After recovering from anesthesia, rats were returned to their cages and given ad libitum access to food and water.

BrdU administration

BrdU can incorporate into DNA synthesis during the S phase of cell cycle and into DNA lesion sites during DNA repair. Because DNA repair mostly occurs within hours and cell proliferation mostly occur days after stroke, we intraperitoneally injected BrdU (50 mg/kg body weight once daily, freshly prepared) into rats at 4–6 days after MCAO based on the protocol reported by Arvidsson et al. (2002) to label proliferating cells.

Tissue preparation

Rats were sacrificed at different time points as indicated in Fig. 2A with an overdose of 10% chloral hydrate and transcardially perfused with 0.9% saline solution followed by 4% ice-cold phosphate-buffered paraformaldehyde (PFA). The brains were then removed and post-fixed in 4% PFA for 12 h and then immersed sequentially in 20% and 30% sucrose solutions in 0.1 M phosphate buffer (pH 7.4) until they sank. Coronal sections were cut on a freezing microtome (Jung Histocut, Model 820-II, Leica, Germany) at a thickness of 30 μm at 1.60 to 0 −4.80 mm from bregma and stored at −20°C in cryoprotectant solution. Sections at 1.60 to −4.80 mm from bregma were used for cresyl violet staining, and sections at 1.0 to 0.48 mm from bregma were used for Fluoro-Jade B or immunohistochemical staining.

Measurement of infarct volume

Coronal sections were cut serially (30 μm thick at 420 μm intervals) from PFA fixed brains and stained with cresyl violet to identify viable cells. Infarct areas were measured by an image processing and analysis system (Q570IW, Leica, Germany) using a 1.25× objective. Infarct volume, expressed as a percentage of whole-brain volume, was calculated by integration of infarct area on each brain section along the rostral–caudal axis (Yang et al., 2002).