Neuroscience 267 (2014) 135–146

ECTOPIC EXPRESSION OF HUMAN ANGIOPOIETIN-1 PROMOTES FUNCTIONAL RECOVERY AND NEUROGENESIS AFTER FOCAL CEREBRAL ISCHEMIA

Z. MENG,[a,b†] M. LI,[a†] Q. HE,[a] S. JIANG,[a] X. ZHANG,[a] J. XIAO[a] AND Y. BAI[a*]

[a] *Department of Cell Biology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China*

[b] *Clinical Laboratory of Shanxi Provincial People's Hospital, Taiyuan 030012, China*

Abstract—Neuropathologic processes such as cerebral ischemia can enhance neurogenesis. Angiopoietin-1 (Ang1) emerges as a critical regulator of physiological and pathological angiogenesis during embryonic and postnatal life. Although Ang1 could protect peripheral vasculature from vascular leakage following ischemic injury, the role of Ang1 in long-term neurological recovery after ischemic stroke remains elusive. This study aims to examine whether Ang1 overexpression via lentivirus-mediated gene transfer enhances neurovascular remodeling and improves functional outcome in a rat model of focal cerebral ischemia. Our results demonstrated that lentivirus-mediated Ang1 gene transfer led to improved neurological behavior and reduced infarction volume, and protected against blood–brain barrier (BBB) leakage in the ischemic rats. In addition, we revealed that these effects of Ang1 are related to the ability of Ang1 to increase vascular density and accelerate endogenous neuronal differentiation. These findings suggest that Ang1 is a promising agent for the treatment of cerebral ischemia.

Key words: angiogenesis, angiopoietin-1, cerebral ischemia, neurogenesis, neuronal differentiation, stroke.

INTRODUCTION

Functional neurological recovery is often incomplete in patients who survive a stroke (Davis et al., 1996; Pons, 1998; Ortega and Jolkkonen, 2013). Stroke patients with a higher density of blood vessels appear to have reduced morbidity and longer survival (Arenillas et al., 2007). Functional imaging of stroke patients showed increased cerebral blood flow and metabolism in the tissues surrounding focal brain infarcts (Marshall et al., 2009; Lin et al., 2010). These data suggest that the restoration of cerebral microvascular circulation is important for functional recovery after stroke. Late restoration of cerebral microcirculatory flow may involve increased collateral circulation and angiogenesis in the penumbra (Zhang et al., 2000; Zhang and Chopp, 2002; Nishimura et al., 2010). However, the substances responsible for inducing the formation of new blood vessels in ischemic brain remain to be identified.

Angiopoietin-1 (Ang1) plays an essential role in physiological and pathological angiogenesis during embryonic and postnatal life (Brindle et al., 2006; Al Sabti, 2007). Ang1 has been identified as a ligand for the receptor tyrosine kinase Tie-2 which expressed on endothelial cells and hematopoietic stem cells (Suri et al., 1996). Ang1 has little effect on the proliferation, but induces the migration, sprouting, chemotaxis, survival, and network formation of endothelial cells (Koblizek et al., 1998; Kim et al., 2000; Papapetropoulos et al., 2000; Takakura et al., 2000; Ng et al., 2011; Oubaha et al., 2012). Moreover, Ang1 is essential for vascular maturation and stabilization via endothelial attachment to extracellular matrices (Suri et al., 1998; Thurston et al., 1999, 2000). Ectopic Ang1 expression significantly enhanced neovascularization *in vivo* (Shyu et al., 1988).

Although Ang1 could protect peripheral vasculature from vascular leakage following ischemic injury, the role of Ang1 in long-term neurological recovery after ischemic stroke remains elusive. In this study, we investigated whether Ang1 overexpression enhances neurovascular remodeling and improves long-term functional outcome in a rat model of permanent focal cerebral ischemia. Recombinant lentiviral vector was used to deliver Ang1 to the brain and achieve efficient Ang1 expression over a long period. Our results showed that Ang1 overexpression confers improved neurological behavior and reduced infarction volume, and protects against blood–brain barrier (BBB) leakage. Furthermore, Ang1 overexpression increases vascular density and accelerates endogenous neuronal differentiation.

EXPERIMENTAL PROCEDURES

Animal treatment was in accordance with *the Guide for the Care and Use of Laboratory Animals of National*

*Corresponding author. Tel: +86-10-82801495.
E-mail address: baiyun@bjmu.edu.cn (Y. Bai).
† These authors contributed equally to this work.
Abbreviations: Ang1, Angiopoietin-1; BBB, blood–brain barrier; BrdU, bromodeoxyuridine; ELISA, enzyme-linked immunosorbent assay; EB, Evan's Blue; IMG, intussusceptive microvascular growth; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; SVZ, subventricular zone; TTC, triphenyltetrazolium chloride.

http://dx.doi.org/10.1016/j.neuroscience.2014.02.036
0306-4522/

Institutes of Health, and the experimental protocol was approved by the Animal and Ethics Review Committee at the Peking University Health Sciences Center (Beijing, China). Every effort was made to minimize animal suffering and reduce the number of animals used.

Lentivirus construction and production

For the construction of Ang1 expression lentiviral vector, cDNA encoding human Ang1 (Regeneron Pharmaceuticals, Tarrytown, NK, USA) was inserted into SwaI and PacI sites of lentivirus-TG006 plasmid, forming recombinant Ang1 lentiviral vector (Fig. 1A). 293T cells were plated at 6×10^6 cells per 100-mm dish and incubated overnight, and transfected with Ang1 lentiviral vector or empty lentivirus-TG006 vector (12 mg) and packaging plasmids MD.G (4 mg) and CMVDR8.91 (16 mg) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Viral supernatants were harvested on two consecutive days 24 h after transfection. A total of 30 ml of supernatant was typically harvested per virus. The supernatants were filtered through a 0.45-μm syringe filter (Millipore, Billerica, MA, USA), and loaded into a Backman Centrifugal Filter (Beckman, California, USA) to be concentrated approximately 10,000-fold by centrifugation at 20,000 rpm for 2 h at 4 °C. Viral concentrates were stored at −80 °C.

Lentivirus infection

Briefly, Sprague–Dawley male rats (weight 180–230 g) were placed in a stereotactic frame (David Kopf Instrument, Tujunga, USA) under anesthesia, with reference to Paxinos and Watson's Rat Brain map (Paxinos et al., 1980), 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, a burr hole was drilled 2.5 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. A 10-μl Hamilton syringe was slowly inserted into the right lateral ventricle (4.5 mm ventral to the surface of the skull). Five microliters of viral suspension containing 2×10^9 particles was injected into the right hemisphere. The rats were injected intraperitoneally with 3 ml of sterile 25% mannitol in 0.9% saline per 100 g body weight 15 min before intracerebral vector injection. On 3, 7, 10, 14 and 21 days after injection, animals were anesthetized and killed by an overdose of sodium pentobarbital then subjected to intracardiac perfusion of ice-cold 4% paraformaldehyde in PBS. The brain was removed, post-fixed, cryoprotected in 30% sucrose in PBS and embedded in TissuTek. Cryostat sections (20 μm) were collected on the slides, and then GFP expression was analyzed by immunofluorescence staining.

Infection efficiency of Tie2 siRNA

The siRNA of rat Tie2 was purchased from Thermo Fisher Scientific, Inc. (Rockford, IL, USA) (Xu et al., 2012). Non-silencing siRNA that had no sequence homology to any known rattus genes was used as the control. To test if the siRNAs could be successfully transfected into the brain tissues following intracerebroventricular injection, a fluorescence conjugated siRNA (Santa Cruz, Texas, USA; sc-36869) was used immediately after MACO in the pilot experiments. At 24 h after injection, the animals were anesthetized and sacrificed by intracardiac perfusion of ice-cold 4% paraformaldehyde in PBS. The brain was removed, post-fixed, cryoprotected in 30% sucrose in PBS and embedded in TissueTek. Cryostat sections (20 μm) were collected on slides and observed under a fluorescence microscope. siRNA was injected one week before the lentivirus infection.

Animal stroke model

The animals were fasted overnight with free access to water. The body weight for each rat was calculated prior to surgery. Anesthesia was induced with ketamine (80 mg/kg IP) followed by atropine at a dose of 0.1 mg/kg. A heating pad and a heating lamp were used to maintain rectal temperature between 36.5 °C and 37.5 °C. Rats were intubated, and respiration was maintained with a small animal respirator (Harvard Apparatus). Rats were subjected to middle cerebral artery (MCA) occlusion as previously described (Longa et al., 1989) with some modifications. Briefly, the right common carotid, internal carotid and external carotid arteries were surgically exposed. The external carotid artery was then isolated and coagulated. A 4–0 nylon suture with silicon was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. The mean arterial blood pressure, heart rate, arterial blood gases, and blood glucose levels before, during, and after ischemia were analyzed. After 2 h of MCA occlusion (MCAO), the suture was carefully removed to restore blood flow, the neck incision was closed and the rats were allowed to recover. The body temperature was carefully monitored during the postoperative period until the animal had completely recovered from the anesthetic. After the experiment, animals were housed individually until euthanized. Following surgery, all animals had free access to food and water.

Behavioral tests

A set of behavioral tests was performed 1 day before MCAO (as baseline values) and 14 days after MCAO by an investigator blinded to the experimental groups.

Corner test

Corner test was used to detect sensorimotor and postural asymmetries (Zhang et al., 2002). Rats were placed between the two angled boards facing the corner. When entering deep into the corner, the vibrissae on both sides were stimulated simultaneously. The rats would then rear forward and upward, and then turn back to face the open end. The nonischemic animals would turn back from either left or right randomly. The ischemic animals would preferentially turn toward the ipsilateral side. The number of turns taken to each side was recorded from 10 trials for each test. Turning