Neurobiology of Disease 32 (2008) 486–498

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Neuroprotection by cord blood stem cells against glutamate-induced apoptosis is mediated by Akt pathway

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ARTICLE INFO

Article history:
Received 18 April 2008
Revised 14 August 2008
Accepted 9 September 2008
Available online 30 September 2008

Keywords:
Cord blood stem cells
Cortical neurons
Glutamate excitotoxicity
NMDA receptors
Neuroprotection
Microarrays

ABSTRACT

The neurotransmitter glutamate mediates excitatory synaptic transmission in the brain and spinal cord. In pathological conditions massive glutamate release reaches near millimolar concentrations in the extracellular space and contributes to neuron degeneration and death. In the present study, we demonstrate a neuroprotective role for human umbilical cord blood stem cells (hUCB) against glutamate-induced apoptosis in cultured rat cortical neurons. Microarray analysis shows the upregulation of stress pathway genes after glutamate toxicity of neurons, while in cocultures with hUCB, survival pathway genes were upregulated. Real time-PCR analysis shows the expression of genes for NMDA receptors after glutamate toxicity in neurons. The neuroprotection of hUCB against glutamate toxicity is similar to the application of the glutamate receptor antagonist MK-801. Cocultures of hUCB protected neurons against glutamate-induced apoptosis as revealed by APO-BrdU TUNEL and FACS analyses. Immunoblot analysis shows that apoptosis is mediated by the cleavage of caspase-3 and caspase-7 in glutamate treated neurons. Cocultures with hUCB indicate the upregulation of Akt signaling pathway to protect neurons. Blocking of the Akt pathway by a dominant-negative Akt and using Akt-inhibitor IV, we confirm that the mechanism underlying hUCB neuroprotection involves activation of Akt signaling pathway. These results suggest the neuroprotective potential of hUCB against glutamate-induced apoptosis of cultured cortical neurons.

Introduction

Human umbilical cord blood is a highly promising source of cells for tissue regeneration. Umbilical cord blood is known as a rich source of hematopoietic stem cells. It is an abundant source for generation of stem cells, including mesenchymal stem cells and monocyte-derived stem cells. Human umbilical cord blood is now considered a valuable source for stem cell-based therapies (Sanberg et al., 2005). The widespread availability of human cord blood underlines the potential use of cord blood stem cells for clinical therapeutics. Cord blood as a source of stem cells has a number of significant advantages over other stem cell sources. It has unique advantages compared to other sources of stem cells including no ethical concerns, no risk to the donors and low risk of graft-versus-host disease.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and exerts its effects via several pre-synaptic and post-synaptic receptors with differing physiological properties. Active transport systems maintain a large glutamate gradient between the intracellular space and extracellular space, with intracellular concentrations in the millimolar range and extracellular concentrations in the low micromolar range (Attwell, 2000). Glutamate plays important roles in cellular processes underlying synaptic plasticity, neuronal development and excitation via the activation of glutamate receptors (Conn, 2003). However, in pathological conditions including epilepsy, traumatic brain injury, and ischemia, massive glutamate release reaching near millimolar concentrations in the extracellular space contributes to neuron degeneration and death (Katayama et al., 1990; Lee et al., 2000). High concentrations of glutamate have been shown to induce neuronal damage and cell loss *in vitro* using cultured neurons (Ankarcrona et al., 1995; Choi, 1987).

Apoptosis is an active process of cell death characterized by activation of endonucleases. Activation by cleavage of caspases, which are proteolytic enzymes, is a key step in the apoptotic process. The upstream signaling pathways leading to the assembly of the protein death complexes activated by caspases are matter of great interest. The involvement of glutamate receptors in promoting apoptotic cell

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

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doi:10.1016/j.nbd.2008.09.005

death has been reported in neural cells under *in vitro* conditions (Chen et al., 2001; Hirashima et al., 1999; Thomas and Mayle, 2000; Yu et al., 2003). In primary cortical neurons, glutamate-induced cell death involves upregulation of caspase 3 and its activation via a caspase-dependent pathway involving mitochondrial signaling. However, the mechanisms underlying endogenous neuroprotection remain unexplored. The present study is designed to demonstrate the neuroprotection of cord blood stem cells against glutamate excitotoxicity of cultured cortical neurons. Our data provides strong evidence for the protective effects of human umbilical cord blood stem cells (hUCB) against glutamate-induced injury to cortical neurons. Here, we report that glutamate excitotoxicity of cortical neurons upregulated N-methyl-D-aspartic acid (NMDA) receptors and increased apoptosis of neurons via caspase activation. We demonstrate that hUCB decreases caspases-3 and -7 activities and are responsible for activation of the Akt pathway and regulation of NMDA receptors, thereby giving neuroprotection to neurons.

Materials and methods

Isolation and primary culture of cortical neurons

Primary cultures of cortical neurons were prepared from embryonic day 18 Sprague–Dawley rats following Brewer et al. (1993) and Brewer and Price (1996). The cerebral cortices were collected (from Brain Bits, Springfield, IL) and triturated gently (8–10 times) in cold Hibernate E medium plus 1× B27 supplement (Invitrogen, Carlsbad, CA), 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA). After the tissue settled, the Hibernate E medium was aspirated, and the tissue was triturated for 1 min in 0.1% trypsin in a Ca^{2+}/Mg^{2+}-free phosphate-buffered saline solution supplemented with glucose (1.5 mM), after which trypsin was inactivated by addition of soybean trypsin inhibitor (0.1 mg/mL). The mixture was transferred into Hibernate E medium containing 20 U/mL DNase (Promega) in 0.2× reaction buffer (Promega), and the cells were centrifuged at 200 ×*g* for 1.5 min. The supernatant was quickly aspirated, and the cells were resuspended in 1 mL of neurobasal medium (Invitrogen, Carlsbad, CA) plus glutamate (25 μM), 0.5 mM L-glutamine, 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA), 1× B27 supplement, and 5 mM sodium pyruvate. Once in suspension, the cells were diluted in 3 mL of the same medium without pyruvate (initial plating medium), and the number of viable cells was determined by trypan blue exclusion. Cells were plated at 0.25×10^4 cells/μL on poly-D-lysine-coated (50 μg/mL) 24-well plates for toxicity experiments or 100 mm culture dishes (1.0×10^5 cells/μL) for immunoblots in neurobasal media containing serum and B27 serum-free supplement (Invitrogen, Carlsbad, CA), 0.5 mM L-glutamine (Mediatech Inc.-Fisher, Hanover Park, IL), and 1% penicillin–streptomycin. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere, and all experiments were performed after 12–15 days in culture. Cultures were fed every 3 days with fresh medium. All experiments were performed 12–15 days after culture in neurobasal medium containing 2% antioxidant-free B27 supplement. Under these culture conditions, only neuronal cells survive. These cultures were comprised of approximately 90–95% neuronal cells, as estimated by immunocytochemical staining performed according to the manufacturer's instructions with rabbit anti-neurofilament 200 kDa (NF200) (Chemicon, Temecula, CA), mouse anti-myelin basic protein (BD Biosciences, Franklin Lakes, NJ), rabbit anti-Glial fibrillary acidic protein (GFAP) (Abcam, Cambridge, MA).

Isolation and culture of hUCB

After obtaining informed consent, human umbilical cord blood was collected from healthy volunteers according to a protocol approved by the Institutional Review Board. Human umbilical cord blood was enriched by sequential Ficoll density gradient purification. Next, we selected cells using $CD44^+$markers. The nucleated cells were suspended at a concentration of 1×10^5/μL in neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA), 0.5 mM L-Glutamine (Mediatech Inc.-Fisher, Hanover Park, IL), and plated in 100 mm culture dishes. The cells were incubated for 3 days and the non-adherent cells were removed with medium replacement. After the cultures reached confluency, the cells were lifted by incubation with 0.25% trypsin and 1 mM ethylene diamine tetraacetic acid (EDTA) at 37 °C for 3 to 4 min. Cells were diluted at a ratio of 1:2 or 1:3 and replated and cultured at 37 °C in an incubator with a 5% CO_2 atmosphere. For co-culture experiments, hUCB and cortical neurons were cultured at a ratio of 1:4.

Immunocytochemistry

Cultured cortical neurons were checked for purity of neuronal population by immunocytochemistry. They were characterized with antibodies against neural and oligodendroglial markers. For this, cultured cells plated in 2-well chamber slides were rinsed twice with phosphate buffered saline (PBS), and then fixed in 4% paraformaldehyde. After additional PBS rinses, cells were blocked with 0.1 M PBS with 1% bovine serum albumin (BSA) for 1 h. Primary antibodies specific for neurons and oligodendrocytes were used to label the plated cells. Primary antibodies (1:100 dilutions) specific for neurons-rabbit anti-NF200 (Chemicon, Temecula, CA), for oligodendrocytes-mouse anti-myelin basic protein (MBP) (BD Biosciences, Franklin Lakes, NJ), for astrocytes-rabbit anti-GFAP (Abcam Cambridge, MA) were diluted in 0.1 M PBS containing 1% BSA and applied overnight at 4 °C. Texas-Red conjugated anti-mouse or anti-rabbit secondary antibodies were diluted 1:200 in 0.1 M PBS containing 1% BSA and applied individually for 1 to 2 h at room temperature. Before mounting, the cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI). The cells were observed using a fluorescence microscope (Olympus IX71, Olympus, Melville, NY) and/or a confocal microscope (Olympus Fluoview, Olympus, Melville, NY) and photographed.

Glutamate treatment of cortical neurons and co-cultures

To ensure sensitivity to glutamate, we used 12- to 15-day old cultures composed of >95% neurons (Ivenshitz and Segal, 2006; White and Reynolds, 1995). Cultures were exposed to glutamate in a Locke solution (134 mM NaCl, 25 mM KCl, 4 mM $NaHCO_3$, 5 mM HEPES, 2.3 mM $CaCl_2$, 5 mM glucose) for 60 min and in the presence of 10 μM glycine (Ankarcrona et al., 1995). Neuronal cell death, measured as trypan blue uptake or formation of apoptotic nuclei, was negligible in control cultures. In experiments with MK-801 (10 μM) (Sigma, St. Louis, MO), and Akt inhibitor IV (10 μM) (Calbiochem, Gibbstown, NJ), the inhibitors were added 60 min before the glutamate treatment and during the entire length of the experiment.

Evaluation of cell death by LDH assay

Glutamate toxicity was evaluated by measuring lactate dehydrogenase (LDH) activity released in the media 24 h after glutamate exposure using the CytoTox96 non-radioactive assay (Promega, Madison, WI). The experiment was performed as per manufacturer's instructions and quantitated by measuring wavelength absorbance at 490 nm. The experiment was performed three times ($n=3$) with 3 wells per condition each time.

Measurement of cell viability by MTT assay

Cell viability following glutamate exposure was examined in single cultures and co-cultures. This assay is based on the ability of active mitochondrial dehydrogenase to convert dissolved (3-(4, 5-