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Protection of ischemic brain cells is dependent on astrocyte-derived growth factors and their receptors

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Abstract

An in vitro ischemia model (oxygen, glucose, and serum deprivation) is used to investigate the possible cellular and molecular mechanisms responsible for cerebral ischemia. We have previously demonstrated that supernatants derived from ischemic microglia can protect ischemic brain cells by releasing GDNF and TGF-β1. In the present study, we investigate whether products of ischemic astrocytes can also protect ischemic microglia, astrocytes, and neurons in a similar manner. Supernatants from ischemic astrocytes were collected after various periods of ischemia and incubated with microglia, astrocytes, or neurons individually, under in vitro ischemic conditions. The components responsible for the protective effects of astrocyte-derived supernatants were then identified by Western blot, ELISA, trypan blue dye exclusion, and immunoblocking assays. Results showed that under conditions of in vitro ischemia the number of surviving microglia, astrocytes, and neurons was significantly increased by the incorporation of the astrocyte-derived supernatants. Astrocyte supernatant-mediated protection of ischemic microglia was dependent on TGF-β1 and NT-3, ischemic astrocytes were protected by GDNF, and ischemic neurons were protected by NT-3. In addition, protein expression of TGF-β1 and NT-3 receptors in microglia, GDNF receptors in astrocytes, and NT-3 receptors in neurons was increased by in vitro ischemia. These results suggest that astrocyte-derived protection of ischemic brain cells is dependent not only on factors released from the ischemic astrocytes, but also on the type of receptor present on the responding cells. Therapeutic potential of TGF-β1, GDNF, and NT-3 in the control of cerebral ischemia is further suggested.

Keywords: In vitro ischemia; Astrocytes; Microglia; Neurons; TGF-β1; GDNF; NT-3

Introduction

Astrocytes represent the most common cellular element in the brain. They outnumber neurons by ten times and occupy about 1/3 volume of the cerebral cortex (Pope, 1978). These cells possess a high rate of metabolic activity and pleiotropic functions responsible for the regulation of brain microenvironment including ion content and extracellular neurotransmitter levels. Astrocytes are also indicated in a variety of neurological and immunological disorders including multiple sclerosis, Creutzfeldt-Jakob disease, Alzheimer's disease, AIDS, and cerebral ischemia (Baltrons et al., 2004; Lee et al., 2004a; Graft et al., 2004; Docagne et al., 1999; Siegel and Chauhan, 2000). In response to different types of stimulation, astrocytes are known to release various factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial-cell-line-derived neurotrophic factor (GDNF), transforming growth factor-β1 (TGF-β1), neurotrophin (NT), nitric oxide (NO), reactive oxygen species (ROS), interleukins (ILs) and tumor necrosis factor-α (TNF-α) (Tokumine et al., 2003; Mizuta et al., 2001; Ouyang and Giffard, 2004; Gabryel et al., 2004; Gubba et al., 2004; Guan et al., 2004; Yoo et al., 2003). Some of these factors (e.g. BDNF, NGF, GDNF, TGF-β1, and NT) promote growth of brain cells (Tokumine et al., 2003; Mizuta et al., 2001; Lee et al., 2004b; Lu et al., 2005), whereas NO, ROS, and TNF-α play a role in inflammation and/or apoptosis of brain cells (Ouyang and Giffard, 2004; Gabryel et al., 2004).

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Although relationship between cerebral ischemia and growth factors such as TGF-β1, GDNF, and NT-3 has been suggested by many groups, the clinical relevance or true therapeutic value of these factors in control of cerebral ischemia still remains to be established. Focal cerebral ischemia is known to up-regulate the expression of TGF-β1 and TGF-β receptors (TβRs: TβR I and TβR II) in perifocal neurons, astrocytes, endothelial cells, and macrophages (Ata et al., 1999; Frander et al., 1993). TGF-β1 can signal multiple cellular pathways to either promote cell growth or prevent apoptosis of most TGF-β1 responsive cells (Zhu et al., 2001, 2002, 2004; Docagne et al., 1999; Prehn et al., 1994). GDNF expression in neurons, astrocytes, and microglia is increased by ischemia and is neuroprotective (Miyazaki et al., 2001; Lee et al., 2004b; Abe and Hayashi, 1997; Kitaawa et al., 1998; Wang et al., 2002). There are four major receptor types (GFRα-1, -2, -3, and -4) involved in the action of GDNF family proteins. GDNF binds specifically to GFRα-1 and acts through MAPK and PI-3K/Akt pathway to either promote growth/differentiation or prevent apoptosis of GDNF responsive cells (Hou et al., 1996; Honda et al., 1999; Rosa et al., 1999; Soler et al., 1999; Olivier et al., 2001; Lee et al., 2004b). NT-3, a member of NGF family, also plays an important role in protecting neurons from ischemic damage (Zhang et al., 1999; Lindvall et al., 1992; Galvin and Oorschot, 2003). It is known that NT-3 by binding to tyrosin kinase C (trkC; a high-affinity receptor of NT-3) and/or p75 (a low-affinity receptor) (Cordon-Cardo et al., 1991; Chao and Hempstead, 1995) can recruit PI-3 kinase/Akt pathway and in turn rescue cortical neurons from apoptosis (Liot et al., 2004; Barnabe-Heider and Miller, 2003), through Bcl-2 expression, promote growth of oligodendrocytes (Saini et al., 2004), and down-regulate synthesis of microglial nitric oxide synthetase (Tzeng and Huang, 2003).

In earlier studies, we have used an in vitro ischemia model (oxygen-, glucose-, and serum-deprived culture condition) to demonstrate that GDNF and TGF-β1 are responsible for microglia-derived protection of ischemic astrocytes and microglia, respectively (Lee et al., 2004b; Lu et al., 2005). Using this model, cellular and molecular mechanisms responsible for cerebral ischemia can be targeted and pre-screened in vitro. We utilized this experimental model to investigate whether astrocytes could also provide similar protection to other brain cells (microglia, astrocytes, and neurons) in response to in vitro ischemia and the possible molecular mechanisms. In this study, astrocytes-derived supernatants were collected at various times of ischemia; molecular components and protective effects of these supernatants were then evaluated by Western blot, ELISA, trypan blue dye exclusion and immunoblocking assays. Expression of GFRα-1, TβR-1 and trkC in various ischemic brain cells was also analyzed to further understand the molecular specificity involving astrocytes-derived protection of various ischemic brain cells. Present results demonstrate that, in response to in vitro ischemia, astrocytes could protect other brain cells from ischemic damage in a growth factor- and receptor-specific manner. Furthermore, therapeutic potential of TGF-β1, GDNF, and NT-3 in control of cerebral ischemia is also suggested.

Materials and methods

Enrichment of glial and neuronal cultures

Enriched glial cultures (microglia and astrocytes) were prepared from postnatal day 1 newborn Sprague–Dawley (SD) rat brains. Briefly, the cerebral cortices were collected and the attached meninges were removed aseptically. Brains were dissociated by trituration in ice-cold HBSS buffer (H-2387, Sigma) containing 2.7 mM $CaCl_2$, 0.5 mM EDTA, 1 mM NaH_2PO_4, 10 units/ml papain, and 5 units/ml DNase I. Cells were then grown in Costar T-75 tissue culture flasks (pre-coated with 0.03 mg/ml poly-D-lysine) with an initial seeding density of 7×10^6 cells/flask. The culture medium used was glucose (4500 mg/l) containing DMEM (D-5648, Sigma) supplemented with 10% FBS, 3.7 g/l $NaHCO_3$, 100 units/ml, and 100 μg/ml of a mixture of penicillin and streptomycin (P/S). The medium was replenished on the second and fourth days after seeding and was changed every 3 days thereafter for 2 weeks. On day 14, DMEM/F12 medium (Gibco, #12400) was added into a mixed glial culture, microglia and astrocytes were separated on the next day by shaking the culture flask at 220 rpm for 5 h. The suspended microglia and settled astrocytes were collected separately and reseeded into 12-well tissue culture plates at 4×10^5 cells/well. Microglia and astrocytes were enriched in DMEM/F12 and DMEM (D-5648, Sigma), respectively, for an additional 24 h. The purity of these two isolated cells was about 95%, which was confirmed by pre-staining the cells with anti-CD11 and anti-GFAP antibodies, respectively.

Enriched neuronal culture was prepared from day 18 SD rat embryo brains according to the procedures described above with minor modifications. Briefly, dispersed brain cells from the cortex in ice-cold HBSS buffer were seeded into a 12-well tissue culture plate at 4×10^5 cells/well and enriched in DMEM 5648 supplemented with 1% B-27 and 10 mM glutamate for 5 days before the test. Purity of the enriched neurons was 95%, which was confirmed by pre-staining the cells with anti-MAP-2 antibodies.

In vitro ischemic treatment

Microglia, astrocytes and neurons were cultivated in a glucose- and serum-free DMEM (D5030, Sigma) supplemented with 1% non-essential amino acids and antibiotics (P/S). The culture plates were put into an anoxic chamber containing 95% N_2 and 5% CO_2 and incubated for various times (0, 2, 4, or 6 h) in a 37°C incubator. During this incubation, cells actually grew in an environment similar to the in vivo ischemic condition, we therefore defined this type of treatment as in vitro ischemia.

Evaluation of astrocytes-derived protection of various ischemic brain cells

Supernatants from astrocytes culture (at a density of 6.4×10^6 cells/dish) were collected at different times (0, 2, 4, and 6 h) of ischemia and centrifuged at 500×*g* to remove the cellular debris. After centrifugation, supernatants were transferred equally into three wells (1 ml/well), each well containing 4×10^5 brain cells (microglia, astrocytes, or neurons), and incubated for either 2 h