VEGF protects human cerebral hybrid neurons from in vitro ischemia

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Vascular endothelial growth factor (VEGF), the most potent angiogenic peptide, protects the neurons against experimental ischemia. However, its neuroprotective effect on human brain is unknown. The present study attempted to determine whether VEGF can protect human cerebral neurons in vitro. A1 human hybrid clonal neurons (human cerebral neuron + neuroblastoma cell) were exposed to hypoxia with glucose deprivation. Pretreatment with VEGF reduced the A1 cell death, and VEGF-2/Flik-1 and VEGF increased with a neuroprotective effect. However, the human neuroblastoma or neuroglioma cells failed to show these findings. Our results suggest that VEGF can protect human cerebral neurons from cell death after an ischemic insult in vitro, which is correlated to both increased expression of VEGF-2/Flik-1 and VEGF within the cells. NeuroReport 15:847–850 © 2004 Lippincott Williams & Wilkins.

Key words: Human hybrid neuronal cell line; Hypoxia; Neuroprotective; Vascular endothelial growth factor (VEGF); Vascular endothelial growth factor receptor-2 (VEGFR-2)

INTRODUCTION

Cerebrovascular disease is the most common life-threatening neurologic disease: stroke in humans is most often due to the occlusion of a cerebral blood vessel. The reduction of cerebral blood flow results in a lack of oxygen and nutrient transportation, which leads to tissue hypoxia and cell death. One potential mechanism for increasing oxygen availability is the induction of angiogenesis. The extent of newly formed vessels is probably an important factor in determining the improvement in the cerebral blood flow, culminating in the recovery and repair of neurons, and a reduction in the ischemic damage [1,2]. Among the factors modulating angiogenesis, the vascular endothelial growth factor (VEGF) is the most important regulator of vascular endothelial cell growth and differentiation [3]. In ischemic rat brain, expression of VEGF and its receptors increased both in the neurons and blood vessels [4]. In addition to this angiogenic function, VEGF also has a neurotrophic effect. Some neurotrophic factors can protect neurons from hypoxia or ischemic injury, and recent studies have shown VEGF to have a direct neuroprotective effect on in vitro ischemia in cultured animal hippocampal or cortical neurons [5–7]. It stimulates axonal outgrowth and promotes the survival of mouse superior cervical neurons, dorsal root ganglion neurons, and rat mesencephalic neurons in culture [8]. These effects are mediated through the main target receptor of VEGF, VEGF-2/Flik-1, or the phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway [7]. VEGF is induced by hypoxia in many cells both in vitro and animal models of ischemia [5,6,9–12].

VEGF has been considered as a potential treatment for stroke due to its angiogenic and direct neuroprotective action, but it is still unknown whether VEGF or its receptor is expressed in the neurons of the human brain. Therefore, this study investigated whether VEGF protects human cerebral neurons against in vitro cerebral ischemia, and whether this effect is associated with VEGFR-2/Flik-1 expression. In addition, we determined whether human cerebral neurons expressed VEGF in response to hypoxic injury [13].

MATERIALS AND METHODS

Cell culture experiments: The human cerebral–neuroblastoma hybrid cell line (A1) was generated by somatic fusion of human cerebral neurons isolated from a 14-week gestation fetus with neuroblastoma SK-SH-Sy5Y-TG4 cells. The A1 human hybrid neurons express neuron-specific markers such as MAP-2, neurofilament-L (NF-L), NF-M, NF-H, and β-tubulin III. The A1 hybrid neurons also express choline acetyltransferase (ChAT), glutamic acid decarboxylase (GAD), and tyrosine hydroxylase (TH), which...
suggests that they can differentiate into various subsets of neuronal types. Whole-cell patch clamp experiments showed neurophysiological evidence of sodium channel activity. In addition, depolarizing and hyperpolarizing voltage clamp steps evoked respective outward and inward K+ currents in these cells.

The A1 human hybrid neurons express various cytokines and cytokine receptors, which are similar to the parental human CNS neurons and different from the other parental neuroblastoma cell line, SK-SH-SY5Y neuroblastoma. This study suggests that the cells express the morphological, immunochimical, physiological, and genetic features of the human cerebral neurons and should serve as a valuable in vitro model for examining the biology, physiology, and pathology of human neurons in health and disease [14]. The other parental human neuroblastoma cell line, SK-SH-SY5Y was also tested in order to compare the A1 neuronal hybrid cells. Another human CNS origin H4 neuroglioma cell line was also evaluated. The cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U penicillin and 0.1 mg/ml streptomycin at 37°C in 5% CO2/95% air.

**In vitro ischemia:** All the ischemia experiments were performed with the cells incubated in a humidified hypoxic chamber. To induce hypoxia, the cultures were incubated in a 95% N2/4%CO2/1% air atmosphere at 37°C. Serum-free culture medium without glucose was used for glucose deprivation. Each cell line was seeded at a density of 1 x 10⁴ cells/ml in a 96-well plate for the viability measurements or in a 100 mm culture plate for the immunoblot assay. The cells were maintained in a plate at 37°C in 95% air/5% CO2 until they reached 70% confluence. After starving with DMEM containing 0.2% FBS for 24 h, the cultures were placed in normoxic or in hypoxic conditions with or without the recombinant VEGF (Calbiochem; 50 ng/ml) throughout the time course of the experiments (0-18 h). The cells were also tested in serum-glucose free conditions or hypoxia alone.

**Cell viability assay:** Cell viability was determined by an MTT assay. A stock solution of MTT (5 mg/ml in phosphate-buffered saline, pH 7.4) was freshly prepared and the cells were incubated for 4 h at a final concentration of 1 mg/ml. The samples on each plate were read on the ELISA reader with a reference wavelength of 570 nm. The results are expressed as a percentage of the absorbance at 570 nm, which is directly proportional to the number of living cells following the experimental ischemia.

**VEGF and VEGFR-2/Flk-1 immunoassay:** For immunoblot analysis, the cells cultured on the 100 mm plate were washed with 4°C phosphate-buffered saline (PBS) and collected. They were homogenized in a lysis buffer (100 mmol/l NaCl, 10 mmol/l Tris (pH 7.5), 1 mmol/l EDTA) to which the protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mmol/ml aprotinin) were freshly added. The protein concentrations were determined using the Bradford method (Bio-Rad, Richmond, CA). The protein extracts (30 μg) were separated by 7-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. They were blocked in 5% non-fat skim milk in TBS (0.15 M NaCl, 25 mM Tris–HCl, 25 mM Tris) for 2 h and then incubated at a 1:500 dilution overnight at 4°C with anti-VEGF (rabbit polyclonal antibodies, NeoMarkers), or anti-VEGFR-2/Flk-1 (rabbit polyclonal antibodies, Santa Cruz Biotechnology) antibodies. After washing three times in TBST (TBS + 0.5% Tween-20), the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase at a 1:5000 dilution for 1 h at room temperature. The immunoreactive bands were detected by enhanced chemiluminescence (ECL) with Kodak film.

**Statistical analysis:** A Mann-Whitney test was performed to examine the effects of VEGF on the cell viabilities under the control or hypoxic-ischemic conditions; p < 0.05 was considered significant. The experiment was repeated three times.

**RESULTS**

**Ischemia increased cell death in the A1 hybrid neuronal cells:** After ischemia, the A1 human hybrid neuronal cells showed a reduced viability over time. The A1 cells exhibited shrinkage with retracted or a loss of neurites (Fig. 1a) from 6 h. Between 6 h and 12 h viability was reduced to 50% of

**Fig. 1.** A1 human hybrid neuronal cells after in vitro ischemia. (a) At 6 h ~ 50% of AI cells appeared to be rounded and shrunken (large arrows) with retracted or beaded dystrophic neurites (small arrows). At 18 h only a few cells remained on the bottom of culture plate but most of them were shrunken. (b) MTT assay shows a decreasing pattern of A1 cell viability as the duration of ischemia increases. All values are mean ± s.e.m. *p < 0.05, Mann-Whitney test (n = 6). X axis denotes ischemia duration, Y axis denotes percentage cell viability comparing to the 0 time point. (c) Morphology of the human neuroblastoma cells is unchanged at 12 h compared to that of control. At 24 h these cells are clustered with the round and shrunken cell body but the neural processes appears to be intact.
control level (6 h ~ 55%; 12 h ~ 53%), and after 18 h 90% of the cells were dead (Fig. 1a,b). Cells incubated under hypoxia or glucose deprivation alone did not show a similar extent of cell damage. The parental human neuroblastoma cell line, SK-SH-Sy5Y did not show morphological evidence of ischemic damage at 12 h, but this began to show at 24 h (Fig. 1c). H4 cells showed decreased viability after ischemia, reduced to 50% of the level measured at 6 h (Fig. 2c).

VEGF protects A1 neuronal cells death from ischemic damage: In order to determine the protective effect of VEGF, the cells were also preincubated with VEGF. The addition of VEGF reduced cell death after 6, 12 and 18 h compared with A1 cells without the VEGF pretreatment. With VEGF, cell viability increased by 20% (6 h), 13% (12 h), and 7% (18 h) following ischemia (Fig. 2a,b). However, VEGF failed to protect H4 cells from ischemic injury (Fig. 2c).

Induction of VEGFR-2/Flk-1 in A1 neuronal cells following ischemia: We next examined the expression of VEGFR-2/Flk-1, one of the main target receptors for VEGF. VEGFR-2/Flk-1 expression in A1 cells was increased 1.6-fold after 6 h and by 1.5-fold after 12 h compared with the control level (Fig. 2b). At 18 h the VEGFR-2/Flk-1 protein level began to decrease. At 3 h, when the VEGFR-2/Flk-1 expression was not increased, VEGF pretreatment did not alter the viability of A1 cells (Fig. 2b). In the H4 neuroglioma cells, VEGFR-2/Flk-1 induction was not observed at these time points (Fig. 2c).

Ischemia induced VEGF expression in A1 neuronal cells: An immunoblot was performed to examine whether A1 neuronal cells could express neuronal VEGF. Ischemia increased the VEGF immunoreactivities between 6 h and 12 h by 1.6-fold. This then decreased after 18 h (92% vs baseline). However, at 3 h no increased VEGF immunoreactivity was observed (Fig. 2d). The VEGF protein presented as a major band of ~43 kDa, which is consistent with dimeric VEGF. The ischemia did not increase VEGF expression in H4 neuroglioma cells (Fig. 2d).

**DISCUSSION**

This study investigated whether VEGF protects human cerebral neurons against ischemic injury. Exogenous VEGF has a neuroprotective effect against ischemic injury in this human neuronal cell line, associated with VEGFR-2/Flk-1 expression. The neuroprotective effect obtained from this study corresponds with previous reports [5-8]. In addition, this human cerebral neuron line could express VEGF in response to ischemic injury in vitro. This result has limited relevance to the ischemic human brain, but enabled us to indirectly gain information from clonal human CNS neurons.

Earlier studies on human neurons have been in primary cultures of cells from embryonic/fetal brain [15]. However, obtaining a sufficient number of human neurons is difficult. The immortalized A1 hybrid neurons overcome this problem and the observed findings from the A1 neuronal cells, but not from the parent neuroblastoma cells, are...
attributed to human cerebral neurons. The A1 hybrid neuronal cells should serve as a valuable in vitro model for investigating the biology, physiology, and pathology in similar way to human CNS neurons [14]. Exogenous VEGF had a neuroprotective effect on the A1 neuronal cells that underwent ischemic injury in vitro. The neuroprotective effect of VEGF has been suggested to result from the rescue of glutamate-induced excitotoxicity through the VEGFR-2/Flk-1 or the phosphatidylinositol 3-kinase(Pi3-K)/Akt signaling pathway [5,6,16]. Previous reports have shown an up-regulation of VEGFR-2/Flk-1, which is the target receptor for VEGF, when cells are exposed to hypoxia in vitro [17]. In our experiments, the effect of VEGF on the A1 neuronal cell viability was mainly observed between 6 h and 18 h, with up-regulation of VEGFR-2/Flk-1 between 6 h and 12 h. VEGF was ineffective at 3 h, when VEGFR-2/Flk-1 induction is undetectable. The protective effect of VEGF on the H4 human neuroglioma cells was not observed, nor did this cell line express VEGFR-2/Flk-1. Overall, these results suggest that the protective effect of VEGF on the A1 neuronal cells is associated with VEGFR-2/Flk-1 receptor expression. Further involvement of excitotoxicity or the phosphatidylinositol 3-kinase(Pi3-K)/Akt signaling pathway remain to be investigated [5,6,16].

Thus A1 human cerebral hybrid neurons showed VEGF expression in response to ischemia in vitro. The underlying pathophysiology of VEGF expression in these cells is unknown. Hypoxia in vitro is a strong inducer of VEGF expression in many cells, including astrocytes, endothelial cells and hepatic cells, as well as in rat models of ischemia [4–8]. In the developing cerebral cortex, the role of neuronal VEGF is both paracrine and autocrine signaling in the maintenance of the neurons and endothelia in the CNS [18]. In our experiments, VEGF expression corresponded to the VEGFR-2/Flk-1 immunoreactivities over the experimental time period. Whether the coincident neuronal VEGF expression affects protection of A1 cells remains unknown. The possibility of a neuroprotective effect by paracrine or autocrine signaling will require further clarification.

In conclusion, the present study shows VEGF can protect human neuronal A1 cells from cell death after an ischemic insult, and that this protection is correlated with both increased expression of the VEGFR-2/Flk-1 receptors and increase in VEGF within the cells.

REFERENCES

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