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G-CSF protects human cerebral hybrid neurons against in vitro ischemia

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Abstract

Granulocyte colony-stimulating factor (G-CSF) protects neurons against experimental focal cerebral ischemia. However, its neuroprotective effect on human brain is unknown. We sought to determine whether G-CSF can protect the human cerebral neurons in vitro. Human cerebral-neuroblastoma hybrid cell line (A1) was exposed to oxygen and glucose deprivation with or without G-CSF. G-CSF promoted cell survival and decreased cytotoxicity effectively at 25 ng/ml. G-CSF reduced early apoptotic (annexin V+/PI−), and late apoptotic or necrotic (annexin V+/PI+) cells, and decreased active caspase-3 immunoreactivity. G-CSF could protect human cerebral neurons following in vitro ischemia.

Keywords: G-CSF; Ischemia; Neuronal death; Neuroprotection; G-CSFR; STAT3

Ischemic stroke is a consequence of impairment of the blood supply to the brain, which leads to tissue hypoxia and cell death [6]. The strategies that can preserve neurons and promote blood flow have been attempted, and neurotrophin families have shown protective effects on neurons from hypoxia or ischemic injury [1,8,13,14,21,23,24].

Granulocyte colony-stimulating factor (G-CSF), a 20-kDa protein, is a member of the cytokine family of growth factors. G-CSF induces hematopoietic stem cell mobilization [9,19], and activates endothelial cell proliferation [2]. G-CSF exerts its activity via a receptor (G-CSFR) of the hematopoietin receptor superfamily [3]. The binding of G-CSF to its receptor has been reported to evoke signal transduction by activating the receptor-associated Janus family tyrosine kinases (JAK) and signal transducer and activator of transcriptor (STAT) proteins in hematopoietic cells [10]. Activated STAT translocates to the nucleus and regulates specific target gene expression, which allows cells to proliferate, differentiate and mobilize or to obtain enough trophic support to survive [11]. G-CSFR is also expressed in neurons or glial cells [22], suggesting the possibility of broader physiological role in the nervous system. Recent studies showed that G-CSF could protect neurons against experimental focal cerebral ischemia and STAT3 is upregulated in neurons of the penumbra, which in turn may mediate antiapoptotic effects [22,23,25]. However, its neuroprotective effect on human brain is unknown. The current interest in G-CSF as one of the few growth factors approved for clinical use led us to investigate the possible role of G-CSF on the human brain. We determined whether G-CSF protected human cerebral neurons from in vitro ischemia.

All experimental procedures were approved by the Care of Experimental Committee of Seoul National University Hospital and by institutional review board for the human cell use. The human cerebral-neuroblastoma hybrid cell line (A1, provided by Seung U. Kim) 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 the morphological, immunochemical, physiological, and genetic features of the human cerebral neurons [8,15,18]. The parental human neuroblastoma cell line, SK-SH-SY5Y was also tested in order to compare the A1 neuronal hybrid cells. The cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

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bovine serum (FBS), 100 U penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO_2/95% air. Fresh medium was supplied every 2 days.

All ischemia experiments were performed with the cells incubated in a humidified hypoxic chamber (Bactron 1.5, Sheldon Manufacturing Inc., Oregon, USA). A1 cells were dissociated by trypsinization, plated at a density of 1×10^4 cells/ml in a 96-well plate or in a 100 mm culture plate, and incubated at 37 °C with 95% air/5% CO_2 for 48 h. For ischemic injury, cells were washed three times with phosphate-buffered saline (PBS), supplied with DMEM without glucose and sodium pyruvate, and incubated at 37 °C with humidified 1% room air/5% CO_2/94% N_2 (oxygen glucose deprivation: OGD). The cells were also tested in serum glucose free conditions or hypoxia alone. During OGD, the human recombinant G-CSF (Kirin pharmaceuticals, Tokyo, Japan) was added to cultures at various concentrations (0–100 ng/ml).

Cell viability was assayed by MTT absorbance and by counting cells on photomicrographs of 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). For MTT assays, cultures were incubated with a stock solution of MTT (5 mg/ml in PBS, pH 7.4, Sigma) at 37 °C for 4 h at a final concentration of 1 mg/ml, and absorbance at 570 nm was measured in solubilized cells on the ELISA reader. For cell counting, the number of intact DAPI-stained nuclei in five 200× microscope fields per well (at the 3-, 6-, 9-, and 12-o'clock positions and in the center) was recorded. In both cases, results were expressed as a percentage of values obtained in control cultures not treated with G-CSF. The media was also harvested for lactate dehydrogenase (LDH) release assay. LDH activity was measured by a Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, USA), which is based on the enzymatic conversion of a tetrazolium salt into a red formazan product according to the manufacturer's instructions, and absorbance was read at 490 nm immediately thereafter. The results were expressed as percentage of peak LDH release obtained on complete cell lysis by 0.9% Triton X-100.

To analyze the patterns of neuronal death or neuroprotection, flow cytometry using annexin V-FITC/propidium iodide (PI) labeling was used. For flow cytometry, floating and adherent cells were collected, washed, and resuspended in cold binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) to a final concentration of 1×10^6/ml. Aliquots of 1×10^5 cells were incubated with 5 μl of annexin V-FITC (Pharmingen) and 5 μg/ml of PI (Sigma). After 15 min at room temperature, 400 μl of binding buffer was added before flow cytometric analysis. For each sample, 10^4 cells were analyzed on a FACS II flow cytometer (Becton Dickinson and Company, NY, USA). FITC and PI fluorescences were passed through 520 and 630 nm bandpass filters, respectively.

Cells (5×10^3) were plated on 12 mm round Aclar plastic coverslips previously coated with 10 μg/ml polylysine and housed in 35 mm dishes. Cell cultures were processed for immunocytochemistry as described previously [4,12]. Anti-G-CSFR (1:500, BD Biosciences) and anti-active caspase-3 polyclonal antibodies (1:500, Pharmingen) were used for the primary antibodies. Cy3-conjugated anti-rabbit IgG antibody (1:300, Jackson Immunoresearch) was used for the secondary antibody. DAPI was used to counterstain nuclei in each experiment. The colocalization was analyzed using a laser scanning confocal microscopy with a Bio-Rad MRC 1024 (argon and krypton). Active caspase 3-positive cells in culture were counted in five fields per well (center and at 3, 6, 9, and 12 o'clock). Results were expressed as a percentage of the number of intact DAPI-stained nuclei obtained in the same fields.

Western blotting was used to determine the expression of G-CSFR on A1 cells and subsequent activation of STAT proteins following G-CSF treatment. The cells cultured on the 100 mm plate were washed with 4 °C 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, 1 mmol/ml aprotinin) were freshly added. The protein concentrations were determined using the Bradford method (Bio-Rad, Richmond, CA, USA). The protein extracts (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. They were blocked in 5% non-fat dry milk in TBS (0.15 M NaCl, 25 mM Tris–HCl, 25 mM Tris) for 2 h and then incubated overnight at 4 °C with anti-G-CSFR (1:500, BD Biosciences), anti-STAT3 (1:1000, Cell Signaling Technology), and anti-phospho-STAT3 (1:1000, Cell Signaling Technology) 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. Coimmunodetection of β-actin (Santa Cruz, USA) was performed to ensure equal gel loading. The blots were developed with enhanced chemiluminescence (Pierce), digitally scanned (GS-700, Bio-Rad) and analyzed (Molecular analyst®, Bio-Rad).

All data in this study are presented as mean ± standard deviations. Data were analyzed using repeated measures of analysis of variance or Mann–Whitney *U* test. Two-tailed probability value of <0.05 was considered significant.

Exposure of A1 cells to OGD decreased neuronal viability over time. The A1 cells exhibited shrinkage with a loss of neurites from 3 h. Between 6 and 12 h, viability was reduced to 50% of control level (3 h: ∼71%, 6 h: ∼52%, 12 h: ∼50%), and after 18 h, 85% of the cells were dead. 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 until 12 h.

MTT and LDH outcomes were obtained from A1 cell cultures under OGD for 6 h with a various concentration of G-CSF. A G-CSF concentration of 25 ng/ml was maximally effective in reducing cell death, leading to an increase of approximately 20% in MTT absorbance, consistent with a significant increase in the number of viable cells in culture (Fig. 1D, E; $p = 0.01$, $n = 3$). At higher G-CSF concentrations, the protective effect tapered off. In addition, OGD induced a significant cytotoxicity from A1 cell cultures (48%), as measured in LDH release assay. G-CSF reduced the cytotoxicity, at optimal concentrations (5 ng/ml: 35%, 25 ng/ml: 28%; Fig. 1F; $p = 0.002$, $n = 3$). The addition of G-CSF (25 ng/ml) also increased cell viability by