Possible Involvement of Caspases in Proliferation of Neocortical Neural Stem/Progenitor Cells in the Developing Mouse Brain

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Caspases are well-known enzymes that work as initiators and effectors of apoptosis. To elucidate the role of caspases in neurodevelopment, we sought to determine if caspases are involved in the proliferation of neural stem/progenitor cells (NPCs) in the developing mouse brain. Labeling with 5-bromo-2′-deoxyuridine (BrdU) from days 14 to 18 of pregnant mice revealed that the 18-d old fetus had many BrdU-positive cells in its brain. Double-labeling revealed that active caspase-3 was co-localized with these BrdU-positive cells in the neocortex, hippocampus, and subventricular zone of the fetal brain. Active caspase-3 was detected in cultures of NPCs derived from the neocortex of 15-d old fetuses during culture periods. Importantly, the pan-caspase inhibitor z-VAD-FMK was effective at completely inhibiting neurosphere formation by the NPCs. These results suggest the possibility that the caspase cascade is essential for the proliferation of neocortical NPCs in the developing mouse brain.

Key words caspase; neural progenitor cell; proliferation; developing brain

Neural stem/progenitor cells (NPCs) are defined by their ability for self-renewal and differentiation into the 3 major cell types, i.e., neurons, astrocytes, and oligodendrocytes, and they play an essential role in the development and maturation of the central nervous system. NPCs are present not only in the developing brain but also in the adult brain in different areas having neurogenic potential.1) The self-renewal of NPCs is regulated by a dynamic interplay between cell-intrinsic signals and cell-extrinsic signals from the microenvironment, in which the NPCs reside.2) A good understanding of the regulation of NPC proliferation would aid the development of therapeutic strategies for neurological disorders.

Apoptosis is an important biological process involved in a variety of developmental processes, acting in the control of cell numbers and in the removal of dying cells. A critical step is the activation of caspases that cleave numerous cellular proteins once activated, leading to alteration of cellular function. Recently, it has been proposed as an attractive hypothesis that dying cells in Drosophila actively induce the proliferation of their neighboring cells by secreting mitogens before they disappear.3) When apoptosis is initiated but the cells are still alive, they actively induce the proliferation of NPC cells by secreting mitogens. This is a process for the autonomous induction of compensatory proliferation through the control of the intracellular signaling pathway for apoptosis. To data, however, no evidence has been presented for the involvement of caspases in the proliferation of NPCs during development in mammals. Thus, the aim of the present study was to determine if caspase cascades contribute to the proliferative activity of NPCs in mammalian brain. To this end, we examined the effects of a caspase inhibitor on the proliferation of NPCs derived from the neocortex of the developing mouse brain.

MATERIALS AND METHODS

Immunostaining of the Fetal Brain The protocol used here met the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. A day-14 pregnant mouse was given 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg, intraperitoneally (i.p.)) every 24 h for 4 d (4 times) and then decapitated to obtain fetal brains. For fixation of the brains, the fetal heads were immersed in Bouin’s solution for 24 h. Their brains were then removed and stored in 70% ethanol at room temperature for 24 h. Post-fixed brains were embedded in paraffin and then cut as sagittal sections of 3–µm thickness with a microtome. Sections so obtained were subjected to immunostaining analysis using a rabbit polyclonal antibody against active caspase-3 (Merck Millipore Co., Billerica, MA, U.S.A.), and a rat monoclonal antibody against BrdU (Abcam, Cambridge, U.K.) as described previously.4,5) Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (IgG) antibody and Alexa Fluor 594-conjugated anti-rat IgG antibody (Life Technologies Inc., Carlsbad, CA, U.S.A.) were used as the secondary antibodies. Stained sections were viewed with an Olympus U-LH100 HG fluorescence microscope.

Culture of the NPCs and Proliferative Activity Assay

Neocortical NPCs were prepared by the floating culture method from day-14 fetal mice as described previously.6) In brief, the neocortex dissected from the fetal Std-ddY mouse, after which its cells were dissociated in 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) at room temperature for 12 min. The cells were then washed twice with Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 10% (v/v) fetal bovine serum and subsequently once with DMEM/F12 containing 0.6% (w/v) glucose, 15 mM sodium bicarbonate, 20 mM progestosterone, 30 mM sodium selenite, 60 mM putrescine, and 100 µg/mL apo-transferrin. They were next suspended in growth medium consisting of DMEM/F12 containing 0.6% (w/v) glucose, 15 mM sodium bicarbonate, 10 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factors, 20 mM progestosterone, 30 mM sodium selenite, 60 mM putrescine, 25 µg/mL insulin, and 100 µg/mL apo-transferrin. For formation of neurospheres, aliquots (0.5 mL) of the cell suspension (60000 cells/mL) were seeded into each well (1.9 cm²) of a culture plate (24 wells, Greiner Bio-One, Germany) and cultured for

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in vitro (DIV) in the growth medium with a half medium change every 3 d. The cultures were always maintained at 37°C in 95% (v/v) air/5% (v/v) CO₂.

Cell proliferation was assessed in terms BrdU incorporation into cells during the culture period. Cells were exposed to 0.1 µM BrdU for 12 h and then centrifuged at 300 × g for 10 min. After removal of the medium, the BrdU level in the cells remaining in the dish was determined by using a Cell Proliferation ELISA kit according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). For proliferation activity, cell viability also was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.7)

Immunoblot Analysis and Immunostaining of the Neurospheres Cell lysates were prepared from the neurospheres and subjected to immunoblot assays by using primary antibodies against α-fodrin (BIOMOL International, Exeter, U.K.), caspase-3/CPP32 (BD Transduction Laboratories, San Jose, CA, U.S.A.), and active caspase-3 as described previously.9)

Neurospheres were kept on poly-l-lysine-coated dishes for 2h and then fixed with 4% paraformaldehyde for 15 min. Thereafter they were immunostained with rabbit polyclonal antibody against active caspase-3 or mouse monoclonal antibody specific for nestin (Merck Millipore Co., Billerica, MA, U.S.A.) as primary antibodies and the Alexa-Fluor 568-conjugated anti-mouse IgG antibody or Alexa Fluro 488-conjugated anti-mouse IgG antibody (Life Technologies Inc., Carlsbad, CA, U.S.A.) as secondary antibodies, and viewed with an Olympus U-LH100HG fluorescence microscope.6)

RESULTS

To determine whether active caspase-3 was located within mitotic cells including stem cells and neural progenitor cells in the brain of the fetal mouse, we first performed double labeling for the detection of BrdU and active caspase-3 in the brain of the fetal mice obtained from a day-18 pregnant mouse that had been treated with BrdU once a day for 4 d starting on day 14 (Fig. 1). Large numbers of BrdU-labeled cells were found in the neocortex, hippocampus, and subventricular zone.
of various brain regions (Fig. 1a). Some of these BrdU-labeled cells were localized with active caspase-3 in these regions (Fig. 1b). Most of these active caspase-3-positive cells in these regions had incorporated BrdU in these regions.

We next examined the expression of active caspase-3 in NPCs derived from the neocortex of fetal mouse. \(\alpha\)-Fodrin is a known substrate for both caspase and calpain, with the \(\alpha\)-fodrin fragment of 150 kDa being indicative of caspase and calpain activities; that of 145 kDa, due to calpain activity; and that of 120 kDa, due to caspase activity. To evaluate the activity of endogenous caspase-3 in the NPCs in culture, we tested the proteolysis of \(\alpha\)-fodrin by utilizing an epitope-specific antibody for determining the products of \(\alpha\)-fodrin degraded by caspase-3 (Fig. 2a). At 2 DIV, the 120-kDa degradation product formed by caspase-3 was found in remarkable quantity found in the NPCs; and the level of this caspase-dependent product in the NPCs significantly increased at 6 DIV. However, calpain-dependent product was not affected during the culture periods tested. Figure 2b shows the results of immunoblot analysis using antibodies against whole caspase-3 or active caspase-3. The level of active caspase-3 in the NPCs dramatically increased up to 6 DIV, whereas whole caspase-3 level was not significant changed throughout the culture period. Immunostaining of the neurosphere revealed that active caspase-3 was detected in almost of NPCs at 6 DIV, which were stained with nestin, a marker for NPC (Fig. 2c).

We next examined the effect of z-VAD-FMK on the growth of neurospheres. At first, cells were harvested for subsequent replating and culturing for 6 DIV in the growth medium in the presence or absence of z-VAD-FMK for assessment of cell proliferation done by performing the MTT assay and by measuring BrdU incorporation. The results of MTT assay revealed that the proliferation of NPCs was markedly attenuated by z-VAD-FMK (Fig. 3a). In addition, z-VAD-FMK at 25 \(\mu\)M led to significant reduction to 40±12% of the control in BrdU incorporation by the cells at 4 DIV (Fig. 3b). Treatment with z-VAD-FMK led to a dose-dependent decrease in the number of neurospheres formed (Fig. 3c). In addition, this assay revealed that treatment with z-VAD-FMK at 25 \(\mu\)M significantly decreased the number of the neurospheres in the size range (\(\mu\m)) of 50–100 and 100–150 (Fig. 3d).
DISCUSSION

Under these conditions in the current study, the essential importance of our findings is that activation of caspase-3 cascades played a critical role in the proliferation of NPCs in the developing mouse brain. In the present study, evidence for this proposition comes from our findings that active caspase-3 was co-localized with BrdU in newly-generated cells in the fetal brain and that the caspase inhibitor completely blocked NPC proliferation. As caspase-3 is activated by death signals, it may be possible that dying cells of NPCs have active caspase-3. In the present study, however, the finding that immunoreactivity for active caspase-3 in the neurospheres cultured found in almost of the cells allows us to propose that caspase-3 was activated in surviving cells rather than dying cells in developing NPCs.

Some previous reports indicated the involvement of caspase signaling in compensatory cell proliferation in developing Drosophila; i.e., as compensation for accidental injury to developing tissues, dying cells induce the proliferation of their neighboring cells by secreting mitogens before they disappear. In addition, antagonism of an endogenous and crucial inhibitor of caspase in Drosophila embryos is known to be required for mitogen expression and stimulation of cell growth. This is evidence for the requirement of caspases in compensatory cell proliferation in Drosophila embryos. However, our finding that active caspase-3 was detected within BrdU-incorporating cells or in cultured NPCs derived from fetal brain indicates that caspase signaling itself is activated during proliferation of NPCs in the developing mouse brain. Thus, we propose that caspase-dependent proliferation is not compensatory cell proliferation at least in the developing brain of mouse, and that caspase signaling is involved in proliferation of NPCs in the developing brain.

In the addition to the case of Drosophila, dying tumor cells have been shown to use caspase signaling to stimulate the growth of the surviving tumor cells. In the tumor cells, a downstream effector of caspase-3 was identified as prostaglandin E2, which potently stimulate the proliferation the surviving tumor cells. Studies focusing on finding downstream factors of caspase signaling that enhance cell proliferation remain to be done in the future.

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