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Neural Stem and Progenitor Cells with High Proliferation Potential in Injured Areas in a Model of Neonatal Hypoxic-Ischemic Encephalopathy

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Abstract

Objective: Neonatal hypoxic-ischemic encephalopathy causes death and severe neurological sequelae including cerebral palsy, epilepsy, and mental retardation. Because hypothermia therapy is the only effective treatment for neonatal hypoxic-ischemic encephalopathy and its efficacy is limited in severe cases, a more effective treatment is needed. Neural stem and progenitor cells are important for neuroprotection and neuronal replacement following hypoxic-ischemic encephalopathy. The aim of this study was to confirm the presence of neural stem cells and the acceleration of their mitotic activity in injured areas in a model of neonatal hypoxic-ischemic encephalopathy.

Method: Hypoxic-ischemic encephalopathy was induced in rat pups by left common carotid artery occlusion followed by hypoxia. Following ischemic injury, brains were sectioned, and columns of tissue were excised from infarct areas for cell culture using the neurosphere assay. Cell clusters were analyzed by immunocytochemistry and reverse transcription polymerase chain reaction. Clustered cells were also tested for their ability to differentiate into neurons, astrocytes, and oligodendrocytes.

Results: Neurosphere-like cell clusters from infarct areas of ischemia-injured animals were dramatically increased compared to samples from sham-operated control animals. Clusters included cells immunopositive for bromodeoxyuridine, nestin, neural/glial antigen 2, β-III tubulin, glial fibrillary acidic protein, oligodendrocyte marker O4, vimentin, and ionizing calcium-binding adaptor molecule 1. Cell clusters also expressed mRNA for NANOG, (sex determining region Y)-box 2, octamer-binding transcription factor 3/4, and Rex1, similar to embryonic stem cells, and could be differentiated into neurons, astrocytes or oligodendrocytes that expressed mRNAs for nestin, β-III tubulin, glial fibrillary acidic protein, and myelin basic protein.

Conclusion: We demonstrate the presence of many neural stem and progenitor cells with enhanced proliferative potential in infarct brain areas of a rat model of neonatal hypoxic-ischemic encephalopathy. Activation of neural stem and progenitor cells in the infarct brain area may contribute to neuroprotection and regeneration.

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is the most common neonatal encephalopathy and can cause death and severe neurological sequelae such as cerebral palsy, epilepsy, and mental retardation. The frequency of neonatal encephalopathy in developed countries is estimated at 3 per 1000 live births and the frequency of HIE is 1.5 per 1000 live births [1]. At present, hypothermia therapy is the only effective treatment for HIE [2-4] and is commonly used worldwide. However, the efficacy of hypothermia therapy is limited in severe cases; thus, a more effective treatment based on inherent brain protective mechanisms is needed to supplement hypothermia therapy.

Recent progress in neural stem cell research suggests the potential use of neural regeneration treatment for hypoxic-ischemic brain injury. Continuous neurogenesis occurs in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus in the adult mammalian brain [5-7]. Neurogenesis can also be activated by stimuli such as hypoxia and ischemic injury [8]. Neurogenesis after ischemic brain injury occurs in three steps: (1) proliferation of neural stem and progenitor cells in the SVZ [9,10], (2) migration of neural stem and progenitor cells to the injured area [11,12], and (3) differentiation into mature neurons in the injured area [13,14]. In the adult brain, neurons differentiated from stem and progenitor cells integrate into the neural network at the ischemia-injured area and participate in the recovery of neurological function [10,15]. In HIE

models, however, increased proliferation of neural stem and progenitor cells in the SVZ and progenitor cell migration to injured areas are observed, but neuronal differentiation and survival are limited [16,17]. The poor viability of newborn neurons in HIE models may be caused by an unfavorable microenvironment and lack of appropriate trophic support. As support for this notion, erythropoietin treatment enhances revascularization to ischemia-injured areas after HIE induction and thereby promotes neuronal replacement [18,19]. However, the reason for the inability of neural progenitor cells to survive and differentiate in injured areas in HIE models is not fully understood. Because neural stem cells cannot be directly detected because of the lack of a specific marker, it is not clear how many stem cells migrate to injured areas after HIE induction.

Different organs throughout the body contain tissue-specific stem

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cells whose mitotic activity is stimulated by injury to promote tissue repair [18-24]. In adult stroke models, cells derived from ischemia-injured brain tissue form neurosphere-like clusters in neurosphere assays; these cells can be differentiated into neurons, astrocytes, and oligodendrocytes [25]. Because ischemia-injured areas in HIE models contain many neural progenitor cells [12], we hypothesized that we could isolate neural stem cells derived from ischemia-injured areas. The aim of this study was to confirm the presence of neural stem cells in injured areas after HIE induction and examine their mitotic activity and differentiation potential. Neural stem and progenitor cells are thought to migrate to injured brain areas to not only to compensate for neuronal loss but also to protect surviving neurons. The presence of neural stem cells with increased mitotic activity in ischemia-injured areas in HIE models would suggest that progenitor cells may be used for neuroprotection or neuronal replacement in the treatment of HIE.

Materials and Method

This article is partly adhered to current RIGOR guidelines [26,27]. 1) Blinding of the study: no blinded conduct of the experiment and no blinded assessment of koutcome. 2) Randomization: Animals should be assigned randomly to the Sham-operated control and HIE groups. 3) Power analysis and 4) Statistical analysis are performed in the Sham-operated control and HIE groups.

Animal model

Pregnant Wistar rats were purchased from Japan Charles River Co., Ltd. (Tsukuba, Japan). On postnatal day 7, each pup was subjected to a modified Levine procedure to induce HIE [28,29]. Briefly, after induction of anesthesia with isoflurane, the left common carotid artery of each pup was sectioned between double ligatures with 5-0 surgical silk. The pups were allowed to recover for 1 h and were then exposed to an additional 2 h of hypoxia (8% oxygen, 92% nitrogen) in a plastic container at 37C. Sham-operated control animals were treated identically, but did not undergo sectioning of the left common carotid artery or subsequent hypoxia. The experimental protocol was approved by the ethics committee for animal research at Kumamoto University

Confirmation of ischemic tissue damage

Three days after HIE induction, the animals' forebrains were removed under deep anesthesia with diethyl ether. Coronal sections (2-mm thick) were obtained -3 mm from the bregma and stained with 1% triphenyltetrazolium chloride (TTC; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to identify the ischemia-injured area [30]. Brain images were obtained using a stereoscopic microscope (SZ61-TRC-CK, Olympus, Tokyo, Japan).

Primary cell culture and determination of cell cluster number

Three days after HIE induction, 6 columnar tissue samples (1.4 mm in diameter) between -3 and -1 mm from the bregma were excised from the SVZ, cerebral cortex, and striatum of both the ischemic and non-ischemic hemispheres (Figure 1A, areas a-f). Each sample was passed through a 29-gauge needle to dissociate the cells. The resulting cell suspensions were incubated according to previously described neurosphere culture assay protocols [31-33]. Briefly, cell suspensions were placed in uncoated 6-well plates and incubated for 14 days at 37°C with 5% CO_2 with Neurobasal Medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing epidermal growth factor (EGF; 20 ng/mL; Sigma-Aldrich, St. Louis, MO, USA), basic fibroblast growth factor (bFGF; 20 ng/mL; R&D Systems, Inc., Minneapolis, MN, USA), N2 supplement (1%), B27 supplement (2%), Insulin-Transferrin-Selenium-X supplement (1%), and Glutamine-Penicillin-Streptomycin (1%; all from Thermo Fisher Scientific). To determine the peak of cell proliferation potential, total numbers of cell clusters exceeding 50 m in diameter were counted for 9 days using a phase contrast microscope (IX70, Olympus, Tokyo, Japan). In order to investigate the biological properties of the primary cell clusters derived from the ischemia-
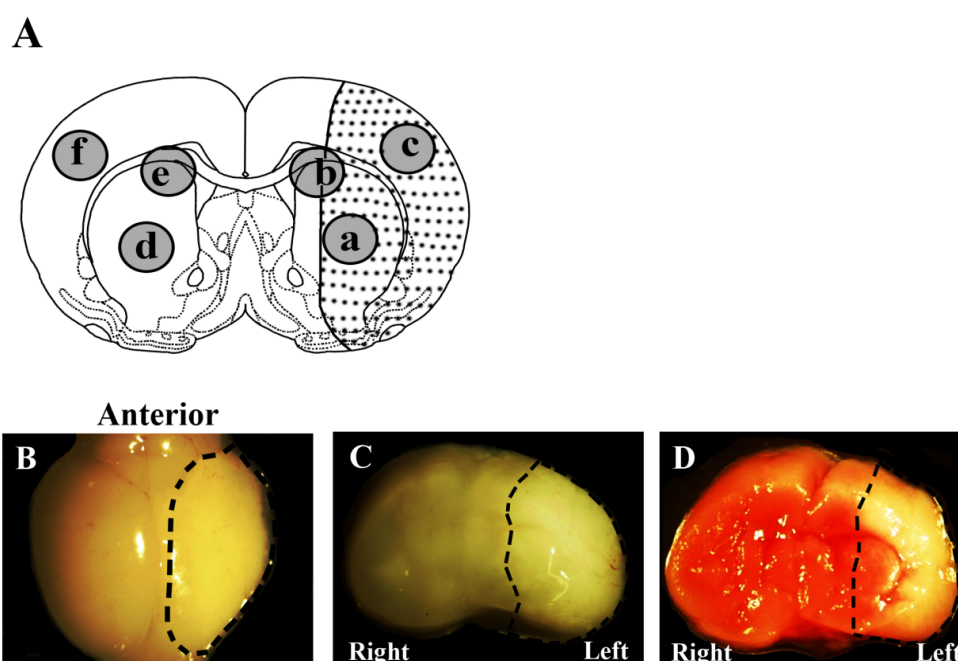
Figure 1: Rat model of neonatal hypoxic-ischemic encephalopathy (HIE) (A) The dotted area represents the infarct region. Areas a and c, but not b, are infarct regions. (B-D) Stereomicroscopic images of the brain of a Wistar rat 3 days after HIE induction. Dashed lines indicate the infarct area. (B) Whole brain; (C) coronal section; (D) triphenyltetrazolium chloride (TTC)-stained coronal section; infarct area is not stained.

injured areas in the HIE model, differentiation was induced by omitting EGF and bFGF in a laminin-coated dish for 7 days and confirmed by immunocytochemistry as described previously [34].

Secondary cell culture

To investigate the biological properties of cell clusters derived from ischemia-injured areas in the HIE model, secondary cell culture using the neurosphere culture protocol was performed as described previously [32]. Cell clusters derived from ischemia-injured areas of the cortex were collected at the peak of cell proliferation potential, and single-cell suspensions were prepared by isolating 1 or 2 cells in 10 µl volumes. These single-cell suspensions were placed in 96-well plates with 100 µl of medium containing EGF and b-FGF, as previously described. After 8 h, wells containing single viable cells were marked, and the number of wells containing cell clusters was recorded for 30 days. On alternate days, half of the growth medium was exchanged for fresh medium. A differentiation study was also performed on these secondary cultures by omitting EGF and bFGF.

MTT cell proliferation assay

The yellow tetrazolium MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Wako Pure Chemical Industries) is reduced by metabolically active cells, in part by mitochondrial dehydrogenase enzymes, to generate reducing equivalents such as NADH and NAPDH. The resulting intracellular purple formazan can be solubilized and quantified using a spectrophotometer. The MTT cell proliferation assay measures the cell proliferation rate. The linear relationship between cell number and signal produced has been established, thus allowing accurate quantification of cell proliferation rate [35].

Cell suspensions were prepared from SVZ samples isolated from sham-operated controls and the ischemic and non-ischemic hemispheres of HIE model animals 3 days after HIE induction. Each suspension was prepared at a density of 10,000 cells/100 µL, plated in 96-well plates, and cultured using the above neurosphere culture protocol. On cell culture day 4, 10 µL of MTT solution (2.5 mg/mL) was added to each well, and the plates were incubated at 37°C for 3 h to allow mitochondrial uptake of MTT. An equal volume (100 µL) of acidified isopropanol was then added to each well in order to induce cytolysis, and the absorbance at 570 nm was measured for each well.

Immunocytochemistry

Cell clusters were attached to glass slides using cytospin preparations. After fixation with 4% paraformaldehyde and blocking with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour, sections were incubated with primary antibodies at 4°C overnight followed by the appropriate secondary antibodies for 1 hour at room temperature. Primary antibodies included the following: chicken anti-nestin (1:2000; Aves Labs, Inc., Chicago, IL, USA), mouse anti-β-III-tubulin (1:100; Merck Millipore, Inc., Darmstadt, Germany), chicken anti-microtubule-associated protein 2 (MAP2; 1:1000; Aves Labs), rabbit anti-glial fibrillary acidic protein (GFAP; 1:100; Epitomics, Inc., Burlingame, CA, USA), mouse anti-neural/glial antigen 2 (NG2; 1:100; Abcam, Inc., Cambridge, MA, USA), mouse anti-oligodendrocyte marker O4 (O4; 1:100; Merck Millipore), chicken anti-vimentin (1:1000; Aves Labs), and rabbit anti-ionizing calcium-binding adaptor molecule 1 (Iba1; 1:500; Wako Pure Chemical Industries). Secondary antibodies used were Alexa Fluor 488 or 594 anti-mouse, rabbit, or chicken (all 1:2000; Thermo Fisher Scientific). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific).

To identify newborn cells in the cell clusters, dissociated cells derived from the ischemia-injured area of the cortex were incubated for 96 h with 5-bromo-2'-deoxyuridine (BrdU; 10 µM; Nacalai Tesque, Inc., Kyoto, Japan). After attachment and fixation of the cell clusters as described above, the clusters were denatured with 2 N HCl for 30 minutes at 37°C and incubated with 5% BSA in PBS for 1 hour followed by mouse anti-BrdU antibody (1:100; Merck Millipore) at 4°C overnight. The cells were then incubated with Alexa Fluor 488 anti-mouse and visualized using a laser scanning confocal microscope (FLUOVIEW FV500, Olympus, Tokyo, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell clusters using an RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA). cDNA was synthesized from the purified RNA using the ThermoScript RT-PCR System (Thermo Fisher Scientific). cDNA was amplified under the following conditions: denaturation for 40 s at 94°C; annealing for 40 s at 52–60°C (primer-specific); and extension for 1 min at 72°C (35 cycles) [36-41]. Primer sequences were as follows:

NANOG forward, TCTCCTCCGCCTTCCTCT and NANOG reverse, TTGCCTCTGAAACCTATCCTTG (amplicon size: 204 bp; annealing temperature: 53°C); (sex determining region Y)-box 2 (Sox2) forward, GGGCTCTGTGGTCAAGTC and Sox2 reverse, TAGTCGGCATCACGGTTT (amplicon size: 435 bp; annealing temperature: 60°C); octamer-binding transcription factor 3/4 (Oct3/4) forward, AGGCAGGAGCACGAGTGGA and Oct3/4 reverse, CGAAGCGGCAGATGGTTGT (amplicon size: 264 bp; annealing temperature: 59°C); Rex1 forward, AAATCATGACGAGGCAAGGC and Rex1 reverse, TGAGTTCGCTCCAACAGTCT (amplicon size: 350 bp; annealing temperature: 52°C); Kruppel-like factor 4 (KLF4) forward, CAGACCTGGAAAGTGGTGG and KLF4 reverse, ACCTGTGTTGCCCGCAGCC (amplicon size: 283 bp; annealing temperature: 52°C); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, CCTCTGGAAAGCTGTGGCGT and GAPDH reverse, TTGGAGGCCATGTAGGCCAT (amplicon size: 430 bp; annealing temperature: 56°C); nestin forward, CAGGCTTCTCTTGGCTTTCTGG and nestin reverse, TGGTGAGGGGTTGAGGTTTGT (amplicon size: 431 bp; annealing temperature: 56°C); β-III-tubulin forward, TGCGTGTGTACAGGTGAATGC and β-III-tubulin reverse, AGGCTGCATAGTCATTTCCAAG (amplicon size: 240 bp; annealing temperature: 55°C); GFAP forward, ACCTCGGCACCCTGAGGCAG and GFAP reverse, CCAGCGACTCAACCTTCCTC (amplicon size: 141 bp; annealing temperature: 59°C); myelin basic protein (MBP) forward, CAAGGTACCCTGGCTAAAG and MBP reverse, CCCAGCTTAAAGATTTTGG (amplicon size: 372 bp; annealing temperature: 55°C); β-actin forward, CTAAGGCCAACCGTGAAAAGATGAC and β-actin reverse, TGGGTACATGGTGGTGCCACCAGAC (amplicon size: 587 bp; annealing temperature: 57°C).

Statistical analysis

Data were analyzed using the Shapiro-Wilk normality test. Normalized results are presented as the mean ± standard error (SE) in Figure 2. Statistical comparisons among groups (HIE ipsilateral and contralateral hemispheres and sham-operated animals) were performed using the Games-Howell test or Mann-Whitney U test in SPSS version 17.0 (IBM, Chicago, IL, USA). *P* values <0.05 were considered statistically significant.
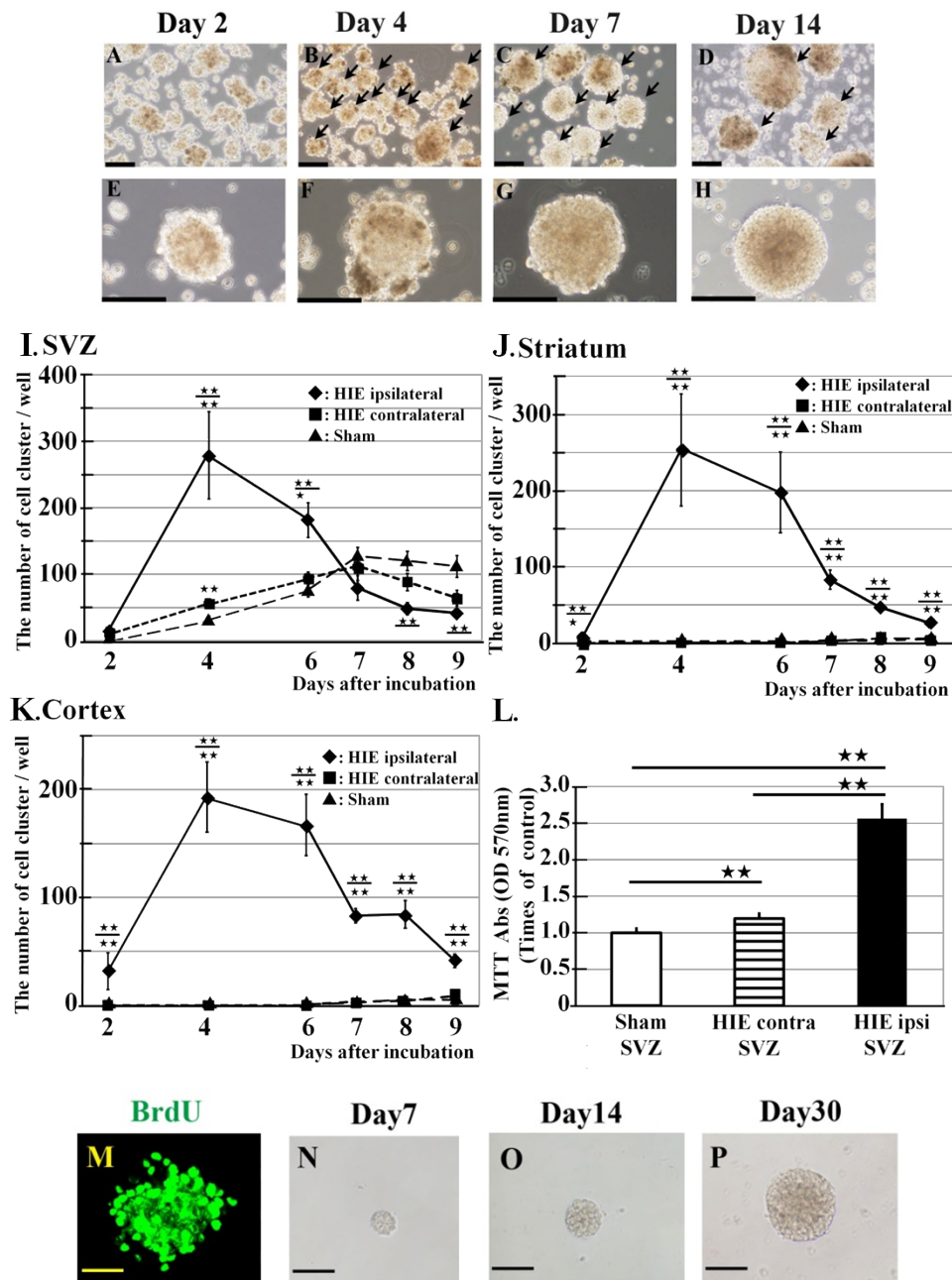
Figure 2: Formation of neurosphere-like cell clusters by cells harvested from HIE model animals. (A-H) Cells obtained from the infarct cortex form cell clusters in neurosphere culture assays that increase in diameter over time. Images were obtained after 2, 4, 7, and 14 days in culture. Scale bars: 200 μm (A-D) and 100 μm (E-H). (I-K) Numbers of cell clusters greater than 50 μm in diameter in neurosphere cultures derived from areas a-f (see Figure 1) on culture day 9. (I) Subventricular zone (SVZ); (J) striatum; (K) cortex. HIE ipsilateral and contralateral areas: n=6; sham-operated: n=6. Statistical comparisons among groups (1. sham-operated 2. HIE contralateral side 3. HIE ipsilateral side) were performed using the Games-Howell test or Mann-Whitney U test. Data are shown as mean ± SEM. ★★:1 vs. 3, ★★ or ★:1 vs. 2 or 2 vs. 3. (L) Proliferation of cells in the SVZ of sham-operated animals and the contralateral (HIE contra) or ipsilateral (HIE ipsi) SVZ of HIE model animals, as measured by the MTT assay. (I-L) ★ $P<0.05$ and ★★ $P<0.01$. (M-P) Proliferating cell in the D4 cell clusters incorporated BrdU and formed secondary neurosphere-like cell clusters. (M) Proliferating cells stained for BrdU (L: green) observed in the D4 cell cluster. (N-P) Secondary neurosphere-like cell cluster formed from a single cell of the D4 cell clusters after 7, 14, and 30 days in culture. Scale bars: 100 μm.

Results

Cell cluster formation by cells derived from ischemia-injured areas in HIE model rats

Three days after HIE induction, the ischemia-injured area was observed as a white focal region in the ischemic hemisphere; this area was confirmed as the infarct area using TTC staining (Figures 1B-1D). Equal volumes of liquefied tissues were aspirated from the ischemia-injured area using a 29G needle at 3, 5, and 7 days after HIE induction, then extracted and isolated using a 29G needle for subsequent cell culture using the neurosphere assay. The largest numbers of cell clusters

from ischemia-injured areas were observed 3 days after HIE induction. The ischemia-injured hemisphere included more nestin- and/or GFAP-positive cells than the contralateral side 3 days after HIE induction (Supplemental Figure). Therefore, we extracted cells from injured tissue 3 days after HIE induction in subsequent experiments.

On cell culture day 2, some cell clusters were visualized in cultures derived from the ipsilateral (ischemic) cortex (area c in Figure 1A); these clusters showed increases in cell number and cluster diameter over the next 2 days. On culture day 4, some cell clusters showed neurosphere-like morphology (Figures 2B and 2F, black arrows). The diameters of neurosphere-like cell clusters increased with the adhesion of each cluster through culture day 14 (Figures 2C, 2D, 2G and 2H). The number of neurosphere-like cell clusters diminished with cluster adhesion (Figures 2B-2D).

Change in the number of cell clusters

To demonstrate changes in the number of cell clusters, clusters over 50 μm in diameter were counted under a microscope on cell culture days 2, 4, 6, 7, 8 and 9. Few cell clusters in cultures derived from the SVZ of sham-operated animals could be detected on culture day 2, and the number of clusters gradually increased through culture day 7 (127.33 ± 13.04 clusters/well, n=12; Figure 2I, closed triangles). Few cell clusters could be detected in cultures derived from the cerebral cortex and striatum of sham-operated animals on each observation day (Figures 2J and 2K, closed triangles).

Cells derived from the ipsilateral cortex, striatum, and SVZ of HIE model rats formed clusters on culture day 2 (Figures 2I-2K, closed diamonds). The numbers of cell clusters derived from each of these regions dramatically increased and peaked at culture day 4 (SVZ: 279.17 ± 65.76 clusters/well; cerebral cortex: 193.33 ± 32.49 clusters/well; striatum: 253.83 ± 73.74 clusters/well; n=6 for each) and then continuously decreased through culture day 9 (Figures 2I-2K, closed diamonds).

Cell cluster numbers in cultures derived from the contralateral SVZ of the HIE group were very similar to those of the sham-operated group (Figure 2I, closed squares). The small numbers of cell clusters in cultures derived from the contralateral cortex and striatum were also very similar to those of the sham-operated group (Figures 2J and 2K, closed squares).

Increased proliferative capacity of cells derived from the SVZ of HIE model rats

To determine the reason for the observed increase in cell clusters derived from the ischemic hemisphere of HIE model animals, MTT assays were performed to measure the mitotic activity of cells derived from the SVZs of sham control animals and the ipsilateral and contralateral hemispheres of HIE model animals. Absorbance values were normalized to those of the sham-operated control group. The mean (± SE) normalized absorbance values were 1.00 ± 0.06 (n=12) for sham-operated animals and 2.56 ± 0.20 (n=6) and 1.19 ± 0.07 (n=6) for the ipsilateral and contralateral hemispheres, respectively, of HIE model animals. The mitotic activity of cells derived from the ipsilateral hemisphere was significantly greater than that of cells derived from the contralateral hemisphere and sham-operated controls ($P<0.001$ for both comparisons).

Properties of D4 cell clusters derived from infarct cortex

To confirm whether cell clusters derived from the ischemia-injured cerebral cortex have properties of neural stem cells, we analyzed clusters derived from ischemia-injured areas after 4 days in culture (D4 cell clusters). To investigate the proliferation potential of the clustered cells, we labeled the D4 cell clusters with BrdU and detected BrdU positive cells in the D4 cell clusters (D4 cell clusters, Figure 2M).

Next, to investigate the self-renewal capacity of cells derived from the ischemic cortex, single cells isolated from D4 cell clusters were placed in single wells of a 96-well plate and cultured using the neurosphere culture protocol. These cells divided and formed clusters, the size of which increased with a morphological pattern very similar to that of a neurosphere (Figures 2N-2P) [31,32].

We performed immunocytochemical analyses to assess the potential of cells in the D4 clusters to differentiate into neurons, astrocytes, and oligodendrocytes. D4 cell clusters included cells positive for the neuronal progenitor cell markers nestin and β–III-tubulin, the astrocyte and astrocyte progenitor cell markers GFAP and vimentin, and the oligodendrocyte progenitor cell markers O4 and NG2 (Figures 3A-3F). Moreover, some cells expressed the activated microglial marker Iba1 (Figure 3G). To clarify the genetic identity of cells in the undifferentiated D4 clusters, RT-PCR analyses were performed. D4 cell clusters expressed mRNA for NANOG, Sox2, Oct3/4, and Rex1, but not KLF4 (Figure 3H).

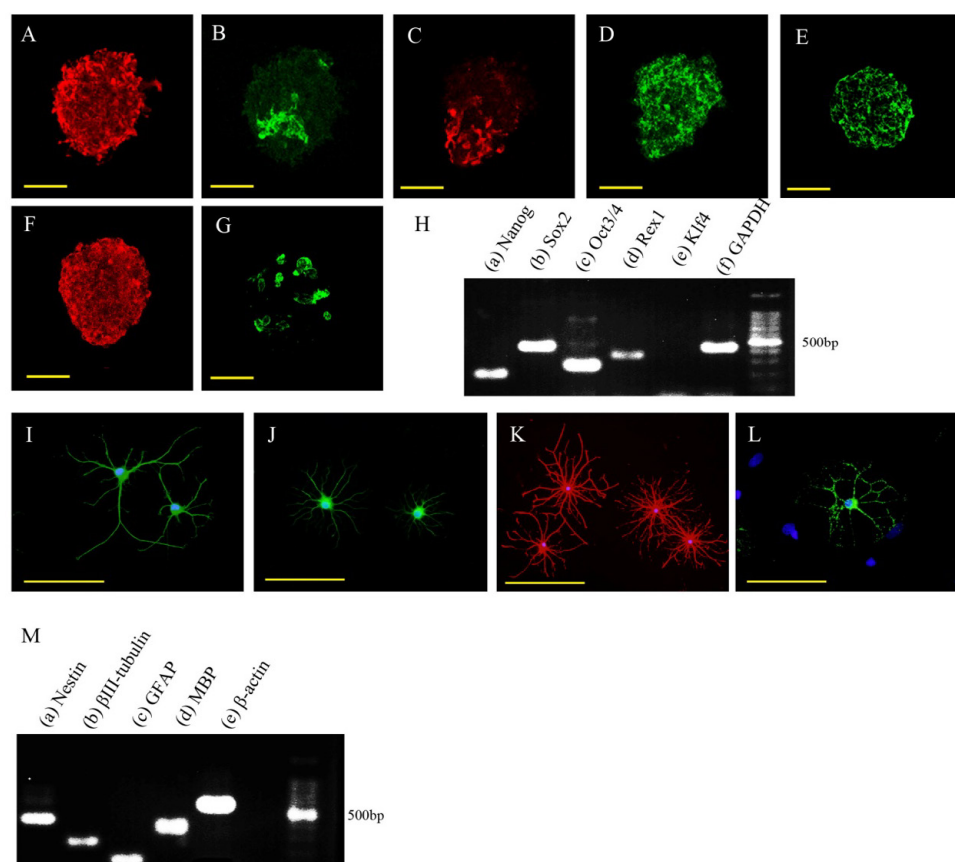## Differentiation of D4 cell clusters derived from the infarct cortex

Differentiation was induced in D4 cell clusters by omitting EGF and bFGF from the culture media. Immunocytochemistry was performed using antibodies against β-III-tubulin, MAP2, GFAP, and O4, which are specific markers for neuronal progenitor cells, mature neurons, astrocytes, and oligodendrocytes, respectively (Figures 3I-3L). Expression of mRNAs for nestin, β-III-tubulin, GFAP, and MBP in the differentiated D4 cell clusters was confirmed by RT-PCR (Figure 3M).

Discussion

In this study, we demonstrate that infarct brain areas in HIE model rats contain cells that can proliferate and form neurosphere-like clusters. In addition, these cells can differentiate into neurons, astrocytes, and oligodendrocytes. To our knowledge, this is the first report showing that endogenous neural stem and progenitor cells can be isolated from ischemia-injured brain areas of HIE animal models.

Neural stem and progenitor cells have been reported in the SVZ of the neonatal brain and are increased by hypoxic-ischemic injury [42]. We demonstrated that cells obtained not only from the ipsilateral SVZ in HIE model animals but also from the infarct cortex and striatum could form cell clusters that increased in diameter over time. Moreover, we observed that different types of cells formed clusters and that clusters fused with each other and enlarged (Figure 2). This phenomenon was similar to the formation of neurospheres [43]. Therefore, we conclude that many cells similar to neural stem and progenitor cells exist in infarct brain areas of HIE model animals.

In cultures derived from infarct areas in HIE model rats, cell clusters greater than 50 μm in diameter were dramatically increased after 4 days in culture. This observation suggests that the proliferative capacity of neural stem and progenitor cells in infarct areas is increased by hypoxic-ischemic injury. Neural stem cells propagate and double approximately every 20 h in neurosphere culture assays [44]. Mori et al. [45] reported that when neural stem and progenitor cells were seeded at a density of 1×10^4 cells/well, the number of neurospheres greater than 30 μm in diameter rapidly increased over 72 h in culture.

Figure 3: Characterization of D4 cell clusters derived from the infract cortex area and differentiation of D4 cell clusters. (A-G) Immunocytochemistry of D4 cell clusters. (A) nestin; (B) neural/glial antigen 2 (NG2); (C) glial fibrillary acidic protein (GFAP); (D) β-III-tubulin; (E) oligodendrocyte marker O4; (F) vimentin; (G) ionizing calcium-binding adaptor molecule 1 (Iba1). Scale bars: 50 µm. (H) RT-PCR analyses showing expression of (a) NANOG, (b) (sex determining region Y)-box 2 (Sox2), (c) octamer-binding transcription factor 3/4 (Oct3/4), and (d) Rex1 in D4 cell clusters. Expression of KLF4 (e) was not observed. (f) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control. (I-L) Immunocytochemistry of differentiated cells from D4 cell clusters. (I) β-III-tubulin (green); (J) microtubule-associated protein 2 (MAP2; green); (K) GFAP (red); and (L) O4 (green). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars: 50 µm (I, J, and L) and 100 µm (K). (M) RT-PCR analyses showing expression of (a) nestin, (b) β-III-tubulin, (c) GFAP, and (d) myelin basic protein (MBP) in differentiated D4 cell clusters. (e) β-actin internal control.

The neurospheres then increased in diameter but decreased in number. Similar to their results, our study also showed that the number of cell clusters in cultures derived from infarct areas rapidly increased over 4 days in culture.

Proliferation of neural stem and progenitor cells in the SVZ *in vivo* is stimulated by hypoxic conditions [46]. Here, the MTT assay demonstrated that cells derived from the ipsilateral SVZ of HIE model rats increased at a rate 2.56 times greater than that of cells derived from the SVZ of sham-operated animals. A previous study reported that the induction of hypoxia-inducible transcription factor-1 alpha by hypoxia enhanced the proliferation of neural stem and progenitor cells [47]. Therefore, humoral factors such as hypoxia-inducible transcription factor-1 alpha may contribute to the enhanced proliferation of cells derived from the ipsilateral SVZ of HIE model animals. In addition, Deierborg et al. reported that mitogenic factors secreted from activated microglia, which migrate to injured brain areas, enhanced the proliferation potential of neural stem and progenitor cells [48]. In our neurosphere assay, cells obtained from infarct areas were cultured with activated microglia. This method may contribute to the accelerated formation and proliferation of cell clusters in cultures obtained from HIE model rats.

Similar to cells obtained from infarct brain areas in our HIE model, cells derived from the infarct cortex in a mouse model of adult middle carotid artery dissection form cell clusters in neurosphere assays [25]. Nakagomi et al. reported that the number of cell clusters greater than 80 µm in diameter increased over 14 days in culture, and the number of clusters observed on day 14 was 8 times greater than on that on day 2 [25]. We analyzed cell clusters greater than 50 µm in diameter; the number of cell clusters increased by 36 times in the ipsilateral SVZ, by 30 times in the infarct striatum, and by 6 times in the infarct cortex from culture day 2 to day 4. Secondary neurosphere-like cell clusters formed at a rate of 15 wells per 1000 wells in our study, which is 3 times greater than that found by Nakagomi et al. using an adult infarction model. These increases in the formation of primary and secondary cell clusters may be due to differences in species, age, and disease model.

D4 cell clusters expressed markers of 3 neural lineages, as demonstrated by immunocytochemistry, and expressed mRNAs for NANOG, Sox2, Oct3/4, and Rex1. These mRNAs are expressed in embryonic stem cells, and several studies have demonstrated the expression of NANOG and Sox2 in neural stem cells; however, expression patterns of Oct 3/4 and Rex1 varied in these reports [44,49,50]. Suslov et al. [51] reported that differentiation states and

gene expression patterns of neurosphere cells differed depending on the type and size of the neurosphere and the position of the cell within the neurosphere.

In conclusion, we demonstrate the presence of numerous neural stem and progenitor cells with enhanced proliferative potential in ischemia-injured brain areas of a rat model of HIE. Activation of neural stem and progenitor cells in infarct brain areas may contribute to neuroprotection and regeneration. Therefore, neural stem and progenitor cells may represent an important treatment option for HIE.

Acknowledgement

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