Lowered glucose suppressed the proliferation and increased the differentiation of murine neural stem cells in vitro

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Abstract Cerebral ischemia is known to activate endogenous neural stem cells (NSCs), but its mechanisms remain unknown. Since lowered glucose supply seems to mediate ischemic actions, we examined the effect of low glucose on NSC activities in vitro. Low glucose applied during the proliferation period diminished EGF-induced proliferation of NSCs without affecting subsequent differentiation, but low glucose directly exposed during the differentiation period facilitated the differentiation of NSCs into neurons and astrocytes. These findings suggest that low glucose facilitated NSC differentiation, but it diminished NSC proliferation. Moreover, the effect of low glucose may be dependent on the timing of application.

Keywords: Neural stem cell; Hypoglycemia; Proliferation; Differentiation; Ischemia

1. Introduction

Advance in neuroscience has led to a new understanding of the development and cell-lineage of mammalian brain cells and has revealed that neurons and glial cells are derived from common immature precursor cells called neural stem cells (NSCs) [1,2]. NSCs are defined as self-renewal and multipotential cells and have been recently found to exist not only in the developing brain, but also in the matured brain in mammals [3].

Cerebral ischemia is generally known to elicit a complex cascade of cellular events, leading to both acute and delayed death of a subset population of neurons in the central nervous system and severe brain functions in animal models as well as human beings [4]. Interestingly, recent lines of evidence have demonstrated that global and focal ischemia enhances the proliferation and neural differentiation of endogenous NSCs not only in continually neurogenic regions, such as the dentate gyrus and anterior subventricular zone [5–10], but also in sites where neurogenesis normally does not occur, such as the hippocampal CA1 area [11]. Nevertheless, the mechanism underlying ischemia-induced NSC activation has not been fully understood.

Among various incidents during ischemia, reductions in the supply of oxygen (hypoxia) and glucose (hypoglycemia) in the brain seem to be the main factors mediating ischemic brain damage. In response to hypoxia and hypoglycemia, however, neurons show different responses such as synaptic transmissions [12], neuronal death patterns [13] and gene expressions [14]. Recently, several studies have demonstrated that hypoxia enhances the proliferation of cultured NSCs and modifies their differentiative ability [15–17], whereas there have been no reports which examined the effect of hypoglycemia on the NSCs or progenitor cells. It is generally known that external glucose from plasma is the only fuel available in the central nervous system and that ischemia rapidly decreases the brain glucose concentration, which leads to various pathological events such as neuronal loss in the models both in vivo and in vitro [18]. Furthermore, the proliferation and differentiation of certain types of cells, such as hepatic oval cells and pancreatic β cells, have been proved to be dependent on the glucose concentration [19–21]. Therefore, to elucidate the mechanism underlying the ischemia-induced NSC activation, we tried to examine the effect of the glucose concentration on the proliferation and differentiation of NSCs by means of an in vitro NSC culture system, since the in vitro system can control the glucose concentrations and delineate the actions of extrinsic factors. Because cerebral ischemia has been reported to change the brain glucose concentration ranging from 0 and to 10 mM [22], we investigated the effect of glucose concentrations at 0, 3 and 10 mM. We also examined the effect of 36.4 mM glucose, the concentration of which is higher than the physiological glucose concentration in vivo but is used in traditional standard NSC cultures [23]. In addition, we also determined whether the effects of the glucose concentration are dependent on the timing of exposure, the proliferation or the differentiation period. Here, we show that low glucose incubations during the proliferation period drastically suppressed the proliferation of NSCs, but that low glucose incubations during the differentiation period increased the differentiation into neurons and astrocytes from NSCs.

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Abbreviations: NSCs, neural stem cells; DMEM, Dulbecco's modified Eagle's medium; BrdU, bromodeoxyuridine

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2. Materials and methods

2.1. Primary NSC culture

The NSCs were isolated and propagated by a neurosphere method developed by Reynolds et al. [24]. ICR mouse fetuses on embryonic day 15.5 (midnight of overnight mating is designated as embryonic day 0) were isolated from their mother under deep anesthesia with ether and placed into ice-cold 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (DMEM/F-12; Gibco-BRL, Gaithersburg, Md., USA). The ganglionic eminence tissues were carefully microdissected under a stereomicroscope and triturated with a commercial 1 ml plastic pipette to obtain a single cell suspension in N-2 medium (DMEM/F-12 supplemented with 36.4 mM glucose, 23 μg/ml insulin, 92 μg/ml transferrin, 55 μM putrescine, 27.5 nM sodium selenite, 20 nM progesterone and 50 U/ml penicillin–streptomycin) [23]. The viable dissociated cells at a density of 25×10^4 cells/5 ml in N-2 medium containing 20 ng/ml EGF (Invitrogen Corp., Carlsbad, CA, USA) were seeded into uncoated T25 culture flasks (Becton–Dickinson, Franklin Lakes, NJ) and were maintained in a humidified incubator at 37 °C with 95% atmospheric air/5% CO_2. Cultures were fed with 1.25 ml of fresh medium every other day and were kept incubated for 7 days to form enough primary neurospheres.

2.2. Experimental procedure

The effects of the glucose concentration on the proliferative activity of the NSCs were evaluated by three methods; (1) WST-8 assay [25], (2) the bromodeoxyuridine (BrdU) incorporation assay [25] and (3) the counting of neurospheres [16]. The single cell suspensions from primary neurospheres were prepared by a centrifugation (1200 rpm, 5 min) followed by a mechanical dissociation. They were seeded in the N-2 medium containing EGF (20 ng/ml) with various concentrations of glucose (0, 3, 10 or 36.4 mM) in a non-treated 96-well plate (Becton–Dickinson). Thereafter, the viable cell number, BrdU incorporation and the number of secondary neurospheres were measured on an appropriate day.

The effects of the glucose concentration on the differentiation of the NSCs were evaluated by fluorescence-based immunocytochemistry and ELISA [26]. In addition, we discriminated the timing of exposure to hypoglycemia (the proliferation or differentiation period), since a previous report noted that a facilitative action of hypoxia on NSC differentiation depended on the timing of exposure [16]. Briefly, the single cell suspensions from primary neurospheres were re-expanded in the N-2 medium containing EGF with various concentrations of glucose (0, 3, 10 or 36.4 mM) in a T25 culture flask. The resultant secondary neurospheres were mechanically dissociated and then differentiated for 7 days in the presence of 36.4 mM glucose by incubation in 1% FBS-containing N-2 medium lacking EGF on a Lab-Tek® Chamber Slide™ (Nunc, Inc., Naperville, IL, USA) pre-coated with 0.005% poly-L-ornithine (Sigma, St. Louis, MO, USA) for immunocytochemistry or in a 96-well plate (Becton–Dickinson) pre-coated with 0.0025% poly-L-ornithine for ELISA. On the other hand, the single cell suspensions from primary neurospheres formed in 36.4 mM glucose-containing medium were mechanically dissociated and differentiated for 7 days in the N-2 medium lacking EGF at various concentrations of glucose (0, 3, 10 or 36.4 mM), and the differentiative activity was evaluated by the same method as described above. We changed the mannose concentrations to keep the osmotic pressure of the culture medium at various glucose concentrations constant. We monitored the glucose level during the proliferation and differentiation period using GLUTESTACE R (SANWA KAGAKU KENKYUSHO CO., LTD, Nagoya, Japan), because it is possible that glucose level is decreased during experiments. During the proliferation period, actual glucose levels of 36.4, 10, 3 and 0 mM were measured to be 36.6, 11.4, 2.7 and 0 mM at the beginning of the experiment and 33.3, 10.8, 2.5 and 0 mM on 5th day, respectively. During the differentiation period, actual glucose levels of 36.4, 10, 3 and 0 mM were measured to be 36.3, 10.9, 2.4 and 0 mM at the beginning of the experiment and 33.6, 9.9, 2.2 and 0 mM on 7th day, respectively. Thus, the change in glucose level was not significant.

2.3. WST-8 assay

The number of the viable cells was estimated by WST-8 assay with Cell Counting Kit-SF (Tesque Nacalai, Kyoto, Japan). WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) is metabolized by the mitochondrial enzyme NAD-dependent succinate dehydrogenase to form a colored formazan product [27–29]. The amount of formazan dye generated by the activity of the dehydrogenases in cells is known to be directly proportional to the number of living cells.

Briefly, the single cell suspensions of EGF-expanded NSCs were seeded in a non-treated 96-well plate (Becton–Dickinson) at a cell density of 5000 cells per well. After 1, 2, 3, 4 and 5 day incubation at various concentrations of glucose, 5 μl of the Cell Counting Kit solution was added to each well and incubated for an additional 5 h at 37 °C. The absorbance at 450 nm with a reference wavelength of 630 nm was measured by a microplate reader (Thermo Labsystems, Vantaa, Finland). By means of these experimental procedures, we obtained a good linear relationship between the net absorbance and the cell density (0–15 000 cells per well) (data not shown).

2.4. BrdU incorporation assay

The BrdU incorporation assay was performed with a Cell Proliferation ELISA BrdU kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the single cell suspensions of EGF-expanded NSCs were seeded in a well of a non-treated 96-well plate (Becton–Dickinson) at a cell density of 5000 cells per well. After 24 h incubation, the cells were labeled with a 10 μM of BrdU and incubated for an additional 8 h. After centrifugation at $300 \times g$ for 10 min, the labeling medium was removed by aspiration and the cells were dried. Thereafter, cellular DNA was denatured by adding FixDenat solution (Roche) for 30 min at room temperature. After removal of the FixDenat solution, anti-BrdU antibody conjugated with peroxidase solution was added to each well and incubated for 90 min at room temperature. After three washes with PBS, tetramethylbenzidine (TMB) solution with H_2O_2 (SUMILON, Tokyo, Japan) was added to each well and the cells were incubated for 60 min at room temperature. Immediately after stopping the total reaction by adding 2 N H_2SO_4 solution, the absorbance at 450 nm (reference wavelength 630 nm) was measured with a microplate reader (Thermo Labsystems). By means of these experimental procedures, we observed a good linear relationship between the net absorbance and the cell density (0–15 000 cells per well) (data not shown).

2.5. Neurosphere counting

The single cell suspensions of EGF-expanded NSCs were seeded in a non-treated well of 96-well plate (Becton–Dickinson) at a cell density of 5000 cells per well and then incubated for 5 days with EGF at various concentrations of glucose. A fixed area (10 mm^2) of the center of each well was converted into a digital image with a digital still camera (DSC-S70, SONY, Tokyo, Japan) and the number of neurospheres with a diameter of more than 60 μm was counted with a Scion Image Beta 4.02 (Scion Corporation, Frederick, MD).

2.6. Immunocytochemistry

The differentiated cells in a Lab-Tek® Chamber Slide™ (Nunc) were fixed in PBS containing 4% paraformaldehyde for 30 min at room temperature and they were then permeabilized by incubation with 100% methanol at −20 °C for 10 min. After removal of the methanol, the cells were incubated overnight with anti-TuJ1 antibody (1: 200; mouse monoclonal antibody, Sigma) and anti-GFAP antibody (1:80; rabbit polyclonal antibody, Sigma) in PBS containing 0.1% Triton X-100 and 3% normal goat serum at 4 °C. After removal of the primary antibody solution, the cells were washed with PBS three times and were incubated with two secondary antibodies (Alexa Fluoro® 488 goat anti-mouse IgG, 1:200 and Alexa Fluoro® 568 goat anti-rabbit IgG, 1:200, Molecular Probes Inc., Eugene, OR) and 1.25 μg/ml of Hoechst 33258 for a nuclear counter staining for 4 h at room temperature under light-shading conditions. After sequential washing with PBS and water, the slides were mounted with Perma-Fluor™ Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA, USA) and their fluorescent images were viewed under a microscope (LSM10, CarlZeiss). For the quantitative analysis, we counted numbers of TuJ1-positive cells (neurons) and GFAP-positive cells (astrocytes) in a fixed area (200×200 μm) of the center of each well under the fluorescent-microscope. The numbers of TuJ1- and GFAP-positive cells were counted by an observer without knowledge of treatment condition and were normalized to the total cell number (Hoechst33258-stained nucleus).

2.7. ELISA

Cells differentiated in the well of tissue culture treated 96-well plates (Becton–Dickinson) were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and they were then permeabilized by incubation with 100% methanol at −20 °C for 10 min. After removal of the methanol, the cells were incubated with one of the following primary antibodies in PBS containing 0.1% Triton X-100 and 3% normal goat serum overnight at 4 °C; anti-TuJ1 mouse monoclonal antibody (1:600, Sigma) or anti-GFAP rabbit polyclonal antibody (1:300, Sigma). After three washes with PBS, the cells were incubated with an appropriate secondary antibody (goat anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase, CHEMICON, California, USA; 1:7000) for 4 h. After three washes with PBS, cultures were added to TMB solution with H_2O_2 (SUMILON) for 60 min at room temperature. Immediately after terminating the reaction by adding 1N H_2SO_4 solution, the absorbance at 450 nm with a reference wavelength of 630 nm was measured by a microplate reader (Thermo Lab Systems).

2.8. Statistical analysis

Results are shown as the means ± S.E.M. Statistical difference was analyzed by one-way ANOVA followed by Dunnett's test. Results were considered to be significant when the P value was less than 0.05.

3. Results

3.1. Effect of lower glucose exposure during the proliferation period on the NSC proliferative activity

The incubation with EGF produced big colonies that were termed neurospheres. The cell viability for all the primary cultures examined was over 90% at the beginning of the experiment. As shown in Fig. 1A, the glucose concentration during the proliferation period did not affect the viable cell number of NSCs on day 1 and day 2. Exposure to lower glucose during the proliferation period, however, suppressed the increment in the number of viable cells on days 3, 4 and 5. On day 5, viable cells in 10 mM glucose medium were approximately 56% of those in 36.4 mM glucose and the lower glucose concentrations at 3 and 0 mM further suppressed the NSCs proliferation to 29% and 23%, respectively. We also found that the BrdU incorporation was decreased when cultures were exposed to a lower glucose concentration (Fig. 1B). The degree of BrdU incorporation at 0, 3 and 10 mM glucose concentrations was 64%, 66% and 81%, respectively (Fig. 1B). Furthermore, we determined whether the glucose concentration affects the ability to form secondary neurospheres. The continuous culture at various concentrations of glucose (0, 3 or 10 mM) significantly diminished the number of neurospheres compared with that at a high concentration of glucose (36.4 mM) (Fig. 1C).

3.2. Effect of lower glucose exposure during the proliferation period on the NSC differentiative activity

We evaluated the differentiative activity of the NSC, which had been increased at various glucose concentrations. Single cells grown at various concentrations of glucose medium (0, 3, 10 and 36.4 mM) for 5 days were suspended in the control medium (glucose 36.4 mM) with 1% FBS lacking EGF, and then plated onto a poly-L-ornithine coated Lab-Tek® Chamber Slide™ for immunocytochemistry and into a 96-well plate for ELISA. After 7 day incubation, approximately 50% of NSCs differentiated into GFAP-immunoreactive astrocytes and small portion (approximately 20%) of NSCs into TuJ1-immunoreactive neurons (Fig. 2A and B). However, the differentiation activity was not affected by low glucose exposure (0, 3, or 10 mM) during the proliferation period when evaluated by immunocytochemistry (Fig. 2B) and ELISA (Fig. 2C).

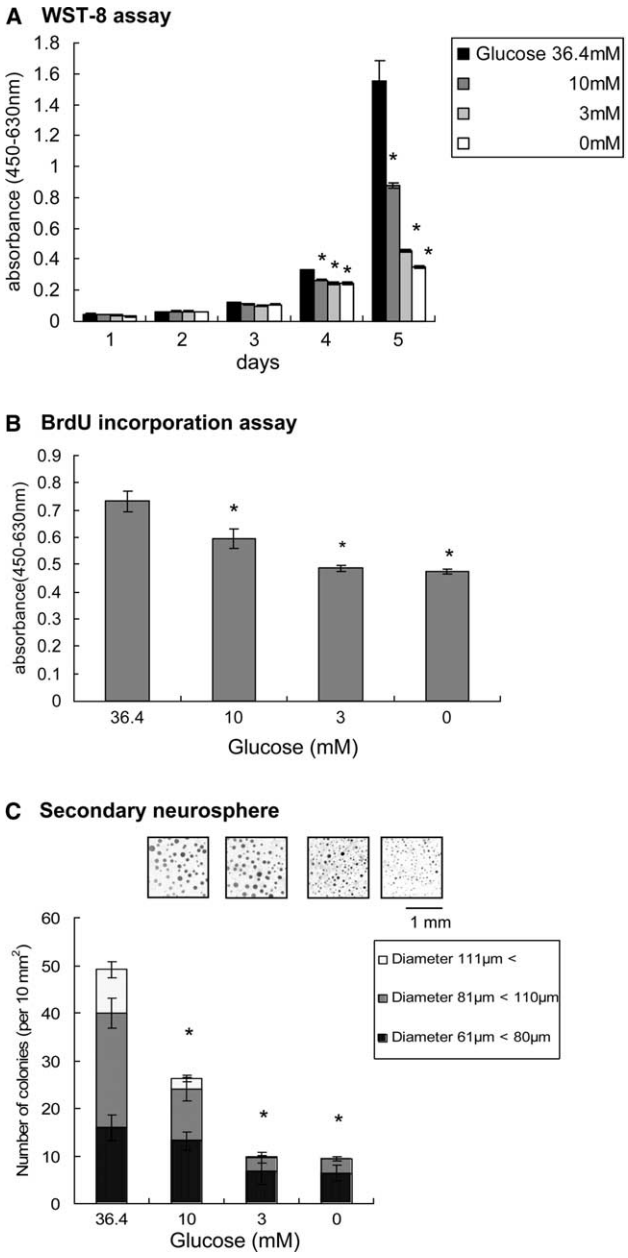

Fig. 1. Low glucose culture during the proliferation period diminished the proliferative activity of the NSCs. NSCs dissociated from the primary neurospheres were cultured at various concentrations of glucose (0, 3, 10 and 36.4 mM) in the presence of EGF. Relative values of viable NSCs and their DNA synthesis were measured by WST-8 assay on days 1–5 (A) and BrdU incorporation assay on day 1 (B), respectively. (C) The neurospheres formed under various glucose concentrations on day 5. Representative photographs and the numbers of neurospheres with small (61–80 μm), medium (81–110 μm) and large (>111 μm) diameters are shown. The continuous culture with low glucose medium significantly produced smaller neurospheres than those under high glucose medium. The asterisk indicates the significant difference from high glucose (36.4 mM) ($P < 0.05$; one-way ANOVA followed by Dunnett's test). $n = 6$.

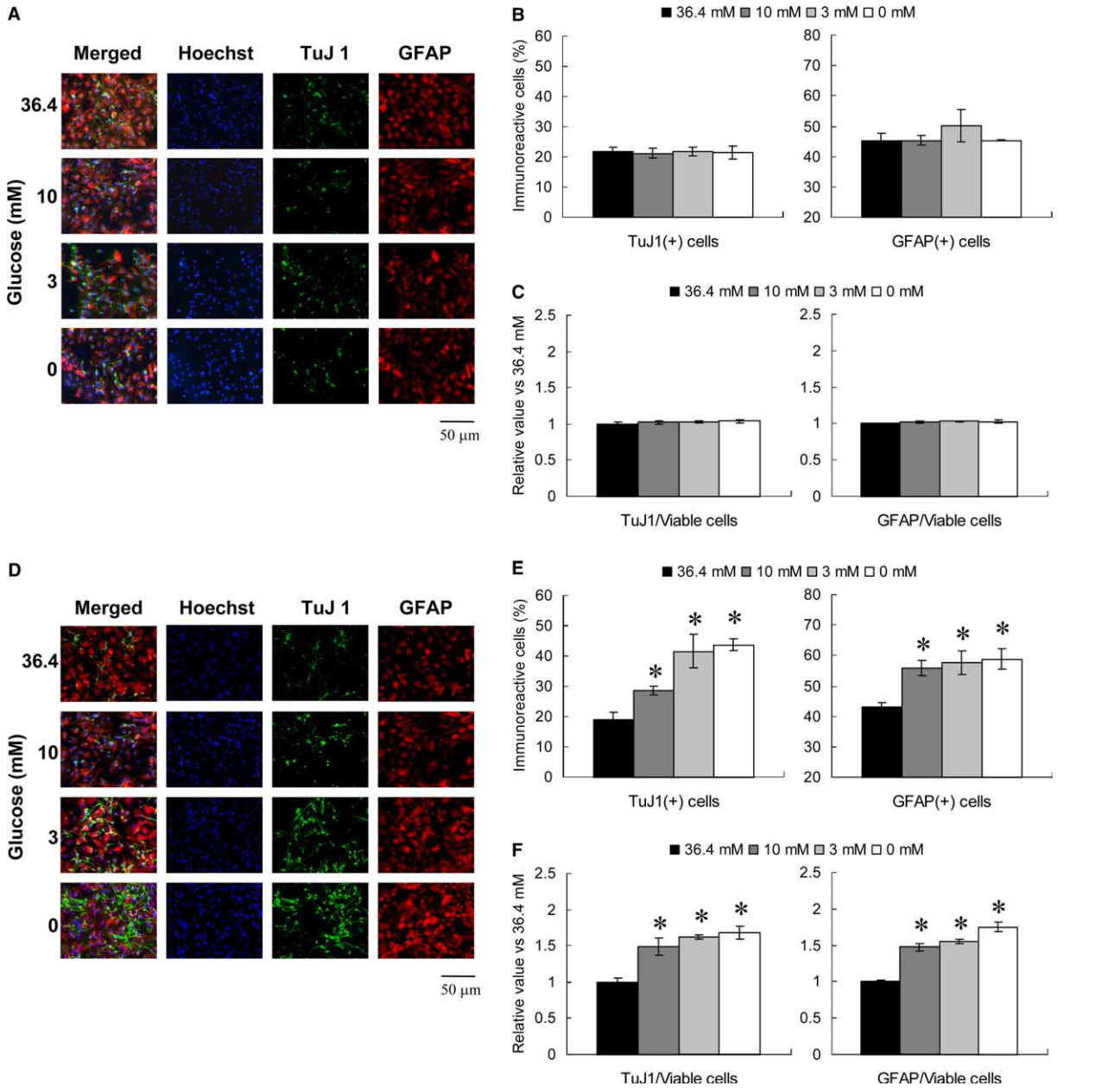

Fig. 2. The effects of low glucose in the proliferation (A)–(C) and differentiation (D)–(F) period on the differentiative activity of the cultured NSCs. (A)–(C) NSCs dissociated from the secondary neurospheres, which had been expanded in EGF-containing medium at various concentrations of glucose (0, 3, 10 and 36.4 mM) or (D)–(F) NSCs dissociated from the primary neurospheres, were differentiated in 1% FBS-containing medium lacking EGF. Seven days after the treatment, the cells were fixed with 4% paraformaldehyde and were processed for immunocytochemistry and the representative immunofluorescence images (A and D) and the quantified data of immunoreactive cell proportion (B and E) are shown. Otherwise, the fixed cells were processed for ELISA (C and F). The glucose concentrations during the proliferation period did not affect the differentiative activity of the NSCs, while low glucose culture during the differentiation period drastically increased the differentiation of NSCs into both neurons and astrocytes. The asterisk indicates the significant difference from high glucose (36.4 mM) ($P < 0.05$; one-way ANOVA followed by Dunnett's test). $n = 6$.

3.3. *Effect of low glucose exposure during the differentiation period on the differentiation of NSCs*

In the next series of experiments, we examined the effect of low glucose exposure during the differentiation period on the differentiative activity of the NSCs, because it is possible that effects of low glucose exposure are dependent on the timing of exposure (the proliferation or differentiation period). When the cells were differentiated in high glucose medium (36.4 mM) for 7 days, differentiation of NSCs into GFAP-immunoreactive astrocytes and TuJ1-immunoreactive neurons (Fig. 2D upper 4 photographs) was observed, although approximately 50% of differentiated cells were astrocytes. In low glucose medium (0, 3, and 10 mM), however, NSCs differentiated into many more neurons and astocytes than those in high glucose medium (36.4 mM) (Fig. 2D and E). This hypoglycemia-induced facilitation of NSC differentiation was dose-dependent, namely,

many more neurons and astrocytes were produced when the glucose concentration was lower (Fig. 2E and F). Thus, low glucose exposure during the differentiation period resulted in NSCs differentiated into neurons or astrocytes, whereas low glucose exposure during the proliferation period failed to affect the differentiative activity.

4. Discussion

Our present results clearly show that exposure to low glucose influences the proliferative and differentiative activity of NSCs in an exposure-timing-dependent manner. When low glucose was applied to NSCs during the proliferation period, it diminished the EGF-induced proliferation, but failed to influence NSC differentiation elicited by 1% FBS. On the other hand, low glucose exposure during the differentiation period drastically enhanced neurogenesis and astrogliogenesis from NSCs. Thus, the glucose level was proved to be a crucial factor for self-renewal and multipoietic activity of NSCs. To the best of our knowledge, this is the first report demonstrating that glucose levels affect the proliferation and differentiation of NSCs. Because glucose concentrations in the brain as well as in peripheral organs change dynamically under physiological and pathological conditions such as feeding, ischemia and diabetes, it is important to pay attention to glucose concentrations when we evaluate the proliferation and differentiation abilities in vivo as well as in vitro.

It has been shown that cultured NSCs derived from murine embryonic brains are propagated by incubation in EGF-containing and serum-free medium and begin to differentiate into neurons and astrocytes in incubation in low serum (for example, in 1% FBS-containing medium without EGF) [1]. By means of this NSC culture system, we were able to examine the effect of low glucose on NSC activities during the proliferation and differentiation periods separately, so that we demonstrated that the effect of low glucose on the NSC differentiation during the proliferation period is different from that during the differentiation period. When NSCs were exposed to low glucose during the proliferation period, it drastically depressed NSC proliferation without affecting the subsequent differentiation elicited by 1% FBS. On the other hand, the differentiation of NSCs into neurons and astrocytes was significantly facilitated when NSCs were incubated in 1% FBS-containing medium at low glucose concentrations. These observations suggest that low glucose concentrations differently influence the proliferation and differentiation mechanisms composed of a number of signal and transcriptional factors [30], depending on the time of low glucose exposure. From the present results, we cannot explain why low glucose promoted NSC differentiation when applied during the differentiation period but not during the proliferation period. It was reported that, in PC12 cells, the glucose consumption changes during neural differentiation by nerve growth factor treatment [31]. It was also reported that there is a switch in the expression of glucose transporter isoforms during myogenic differentiation. These reports suggested that the glucose-requirement and glucose-uptake change during cell differentiations [32]. Therefore, the change in glucose requirement and glucose-uptake during the NSC differentiation may underlie the timing-dependent-effect of hypoglycemia.

Recent in vivo studies suggested that NSCs in the brain are activated in response to cerebral ischemia, which resulted in the production of new neurons [5–7,9,33–37]. This line of evidence demonstrated that ischemia leads to NSC proliferation, NSC migration to injury sites and NSC differentiation into neurons. It was also reported that, in the anesthetized rat, the extracellular concentration of glucose in the brain in situ is maintained at a level as low as 2.4 mM, which is similar to the glucose concentration (3 mM) used in the present study [22]. In their report, ischemia elicited by carotid compression caused a rapid fall in the external glucose concentration to undetectable levels [22]. We examined the effect of hypoglycemia in this physiological concentration range, so that in vitro observations in the present study may explain in vivo observations in their studies. Thus, the present study showed that the reduction in the glucose concentration from 3% to 0% facilitated the NSC differentiation. Low glucose, therefore, may be attributed to ischemia-induced NSC activations. It should be noted that, in the present study, the facilitation of neural differentiation by low glucose was observed within 1 week (because the differentiation period in the present study was 7 days). However, there are many reports demonstrating that newborn matured neurons appeared several weeks after ischemia in the adult neurogenerative regions, such as SVZ [10] and dentate gyrus [9]. On the other hand, when CRMP-4 [7] was used as a neuronal marker, differentiated neurons appeared within several days after ischemia. Thus, enhanced differentiation by low glucose in vitro may reflect the early process of neural differentiation soon after ischemia in vivo. The pathophysiology of ischemic brain damage is characterized by cell death in the core region and gliogenesis and neurogenesis in the penumbra region around ischemic area. Therefore, two actions of low glucose on the NSC activity, namely suppressed proliferation and increased differentiation, may contribute the cell death in the core and gliogenesis and neurogenesis in the penumbra region, respectively. We also showed that glucose concentrations higher than physiological concentrations (10 and 36.4 mM) affected NSC activities: the proliferation was activated and the differentiation was inhibited. Taken together, to activate the differentiation of NSCs in in vitro systems, we should use medium with high glucose during the proliferation period and medium with low glucose during the differentiation period, although these concentrations are not physiological in intact animals.

In addition to hypoglycemia, hypoxia is an another important incident mediating the ischemic actions and both hypoxia and hypoglycemia additionally or synergically may contribute to acute and delayed neuronal death [4]. Recent studies demonstrated that hypoxia enhances the proliferation of cultured NSCs and modifies their differentiative ability in in vitro models [16,17]. In their studies, hypoxia enhanced the proliferation of NSCs [16], which is in contrast to the effect of hypoglycemia on NSCs as shown in the present study. Furthermore, they demonstrated that exposure to lowered oxygen during the differentiation period does not affect the neuronal differentiation from NSCs, but that exposure to lowered oxygen during the proliferation period increases the differentiation into neurons [16]. From their studies and our present study, it can be concluded that the neuronal differentiations are initiated only when hypoxia is applied during the proliferation period or hypoglycemia is applied during the differentiation period, so that the critical period of NSC

differentiation caused by hypoxia was also proved to be different from that caused by hypoglycemia. Therefore, a simple energy failure may not account for the effect of ischemia on NSC functions.

In summary, our study provides evidence that glucose is an important regulator of neurogenesis. Higher glucose activated the proliferation but inhibited the differentiation of NSCs, whereas lower glucose inhibited the proliferation but activated the differentiation of NSCs.

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