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In Vitro Screening of Exogenous Factors for Human Neural Stem/Progenitor Cell Proliferation Using Measurement of Total ATP Content in Viable Cells

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One of the newest and most promising methods for treating intractable neuronal diseases and injures is the transplantation of ex vivo-expanded human neural stem/progenitor cells (NSPCs). Human NSPCs are selectively expanded as free-floating neurospheres in serum-free culture medium containing fibroblast growth factor 2 (FGF2) and/or epidermal growth factor (EGF); however, the culture conditions still need to be optimized for performance and cost before the method is used clinically. Here, to improve the NSPC culture method for clinical use, we used an ATP assay to screen the effects of various reagents on human NSPC proliferation. Human NSPCs responded to EGF, FGF2, and leukemia inhibitory factor (LIF) in a dose-dependent manner, and the minimum concentrations eliciting maximum effects were 10 ng/ml EGF, 10 ng/ml FGF2, and 5 ng/ml LIF. EGF and LIF were stable in culture medium without NSPCs, although FGF2 was degraded. In the presence of human NSPCs, however, FGF2 and LIF were both degraded very rapidly, to below the estimated minimum concentration on day 3, but EGF remained above the minimum concentration for 5 days. Adding supplemental doses of each growth factor during the incubation promoted human NSPC proliferation. Among other supplements, insulin and transferrin promoted human NSPC growth, but progesterone, putrescine, selenite, D-glucose, and lactate were not effective and were cytotoxic at higher concentrations. Supplementing with conditioned medium from human NSPCs significantly increased human NSPC proliferation, but using a high percentage of the medium had a negative effect. These findings suggest that human NSPC culture is regulated by a balance in the culture medium between decreasing growth factor levels and increasing positive or negative factors derived from the NSPCs. Thus, in designing culture conditions for human NSPCs, it is useful to take the individual properties of each factor into consideration.

Key words: Human neural stem/progenitor cell; ATP assay; Neuropshere; Culture condition

INTRODUCTION

For a long time it was believed that the function of the mammalian central nervous system (CNS) could not be restored once it was damaged (e.g., by injury, stroke, or disease). However, today, how to treat and regenerate the damaged CNS is a subject of intense study (4,17,26). One of the newest and most promising methods for treating intractable neurological diseases and the damaged mammalian CNS is the transplantation of ex vivo-expanded neural stem cells (NSCs); some studies have already reported encouraging results on the clinical use of human NSCs (3,4,12–14,20,27,31,32).

NSCs are defined as self-renewing and immature undifferentiated cells that are multipotent, meaning they have the capacity to differentiate into neurons, astrocytes, and oligodendrocytes (10,35). Usually, NSCs of either rodent or human origin are selectively expanded as free-floating aggregates termed "neurospheres" in serum-free culture medium containing fibroblast growth factor 2

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(FGF2) and/or epidermal growth factor (EGF). Each neurosphere contains a heterogeneous cell population, including immature neural cells (neural stem/progenitor cells; NSPCs) (7,11,15,22–24,27,31–34). Although human NSPCs can be isolated from both fetal and adult brain tissues (7,12–14,22,27,31,32), from a practical aspect it is very difficult to isolate a large enough portion of the initial tissue from a fetus for clinical use, and the use of large numbers of human donor fetal tissues is controversial for ethical reasons (14). To isolate adult NSPCs, especially the autologous CNS tissues, a large number of NSPCs from small biopsy specimens must be collected—also a difficult task (13,20,34). Moreover, human NSPCs proliferate more slowly than rodent NSPCs, making it even more difficult to expand human NSPCs ex vivo in a large scale for clinical use (14).

To transplant NSPCs, a large volume of uncontaminated and high-quality NSPCs must first be expanded from a small primary culture, which is expensive when human NSPCs are used and has been the rate-limiting step for early clinical applications (14,34,36). Therefore, to use human NSPCs clinically, it is very important to establish optimum conditions for their culture, so that their ex vivo expansion can be done on a large scale and at as low a cost as possible. Moreover, the culture conditions must maintain the NSPC heterogeneity and keep the culture free of pathogens, toxins, and other harmful agents (14,15,26,34,36). Here, to determine the most effective conditions for human NSPC culture that meet these criteria, we screened the effects of various reagents on human NSPC proliferation using an indirect measurement of viable NSPCs. The present findings provide useful information for establishing new culture methods for human NSPCs intended for clinical use, and contribute basic information for their clinical applications.

MATERIALS AND METHODS

Culture Media, Growth Factors, and Other Supplements

D-Glucose-free Dulbecco's modified Eagle's medium (DMEM/wo Glu) and Ham's F12 and DMEM/F12 (1:1) with 3.151 g/L (17.5 mM) D-glucose (DMEM/F12/Glu) were purchased from Sigma (St. Louis, MO). Human recombinant epidermal growth factor (hr-EGF), fibroblast growth factor 2 (hr-FGF2), and leukemia inhibitory factor (hr-LIF) were from Invitrogen Corp. (Carlsbad, CA), PeproTech Inc. (Rocky Hill, NJ), and Chemicon International Inc. (Temecula, CA), respectively. Human progesterone, insulin, transferrin, putrescine, and sodium selenite were purchased from Sigma. B27 supplement was purchased from Invitrogen Corp. All other materials were of the finest grade commercially available.

Human NSPCs

Approval to use human fetal neural tissues was obtained from the ethical committees of both Osaka National Hospital and the National Institute of Advanced Industrial Science and Technology. Tissue procurement was in accordance with the declaration of Helsinki and in agreement with the ethical guidelines of the European Network for Transplantation (NECTA) and the Japan Society of Obstetrics and Gynecology (12,14,19). Human NSPCs were isolated from three human fetal forebrain tissues of 7, 9, and 10 weeks gestational age (GW), which had been obtained from routine legal terminations performed at Osaka National Hospital, and the cells were cultured using the neurosphere culture technique as previously reported (12,14,19). Briefly, human NSPCs were routinely grown using DMEM/F12/Glu-based defined medium supplemented with hr-EGF (20 ng/ml), hr-FGF2 (20 ng/ml), hr-LIF (10 ng/ml), heparin (HP; 5 μg/ml; Sigma) (6), B27 supplement, 15 mM HEPES, penicillin (100 U/ml; Invitrogen), streptomycin (100 μg/ml; Invitrogen), and amphotericin B (250 ng/ml; Invitrogen) (human neurosphere growth medium) (6,14,18,27). Viable single cells at a density of $2–4 \times 10^6$ cells/15 ml were seeded into uncoated T75 culture flasks and incubated at 37°C in 5% CO_2/95% air. Half the volume of the culture medium was replaced by fresh growth medium every 7 days. For passaging, every 14–21 days, neurospheres were dissociated to single cells by digestion with 0.05% trypsin/0.53 mM EDTA (Invitrogen) at 37°C for 20 min, and resuspended in 50% fresh growth medium plus 50% neurosphere conditioned medium (12,14,19).

Cell Viability Assay

The proliferation of human NSPCs was estimated by measuring the total ATP content in viable cells (CellTiter-Glo™ Luminescent Cell Viability Assay; Promega, Madison, WI), as reported previously (8,14,21). Briefly, single-cell suspensions were prepared from neurospheres by enzymatic dissociation with 0.05% trypsin followed by passage through a 40-μm nylon mesh (Cell Strainer; Becton-Dickinson, Franklin Lakes, NJ). The cells were then seeded into two 96-well microplates at a density of 1×10^5 cells/ml in the various culture mediums to be examined and incubated at 37°C in 5% CO_2 for the designated number of days. For the ATP assay, 75 μl of CellTiter-Glo™ Reagent was added to each well of the microplate, and the plate was incubated for 30 min at room temperature. The luminescence signals were then analyzed using a detection system for chemiluminescence (Wallac 1420 ARVOSX; Perkin-Elmer). The proliferation rates were calculated from the difference in luminescence signals between days 1 and 6, and were represented as a percentage.

Effects of Growth Factors and Other Supplements on Human NSPC Proliferation

The effects of individual reagents (growth factors, supplements, glucose, etc.) on human NSPC proliferation (6 days of culture) were examined using the ATP assay; for example, cultures treated with different doses of EGF (0, 2, 5, 10, 20, and 50 ng/ml) plus LIF (10 ng/ml), different doses of FGF-2 (0, 2, 5, 10, 20, and 50 ng/ml) plus LIF (10 ng/ml), and different doses of LIF (0, 1, 2, 5, 10, and 20 ng/ml) plus EGF (20 ng/ml) and FGF2 (20 ng/ml) (7,15) were examined. The effect of additional supplements on human NSPC proliferation was determined keeping the concentration of growth factors constant (20 ng/ml hr-EGF + 20 ng/ml hr-FGF2 + 10 ng/ml hr-LIF) and varying the concentration of a supplement: insulin (0, 0.5, 5, 10, 50, 100, and 500 μg/ml), progesterone (0, 0.2, 2, 20, 200, and 2000 nM), transferrin (0, 10, 100, 500, and 1000 μg/ml), putrescine (0, 0.1, 1, 10, 100, and 1000 μM), or selenite (0, 3, 15, 30, 300, and 3000 nM) (23,36). To examine the effect of D-glucose, DMEM/F12(1:1) containing different concentrations of D-glucose (10, 15, 17.5, 35, 52.5, and 87.5 mM) was prepared by mixing DMEM/wo Glu, Ham's F12, and additional D-glucose to obtain the above-mentioned final concentrations. The effect of lactate (0, 0.0015, 0.045, 0.45, and 4.5% w/v) was also investigated using human neurosphere growth medium. The effects of human NSPC conditioned medium (CM) on NSPC proliferation were examined for 6 days of culture with various amounts of CM (0%, 20%, 40%, 60%, 80%, and 100%) in fresh medium. The effects of supplementing the human NSPC culture with additional doses of growth factor during the 6-day culture period were also examined. To elevate the target dose of each growth factor in the culture medium, the appropriate amounts of each growth factor were added to the culture medium according to one of the following schedules: five times (i.e., once each day), twice (on days 2 and 4), or once (on day 3). The cell proliferation rates for each culture protocol were determined on day 6 using the ATP assay (14).

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of EGF, FGF-2, and LIF in the culture medium during human NSPC culture were examined for 6 days using ELISA. Every 24 h, 500 μl of the cell culture supernatants was obtained from three independent T75 culture flasks, and after the particles were removed with a 0.22-μm filter, the supernatants were diluted with DMEM-F12/Glu medium (1:50 and 1:100 for EGF estimation, 1:25 and 1:50 for FGF2 estimation, 1:10 and 1:20 for LIF estimation). The level of EGF, FGF2, and LIF was then measured in the diluted samples using the quantitative sandwich enzyme immunoassay, according to the manufacturer's instructions (EGF, Human ELISA Kit, Quantikine; FGF basic, Human ELISA Kit, Quantikine; and LIF Human ELISA Kit, Quantikine; R&& Systems, Inc.). The optical densities of each well were measured at 450 nm using a microplate reader (Benchmark Microplate Reader; BIO-RAD).

Statistical Analysis

Statistical analyses were performed using analysis of variance (ANOVA) or the Mann-Whitney *U* test.

RESULTS

Effects of EGF, FGF2, and LIF on Human NSPC Proliferation

We first evaluated the effects of EGF, FGF2, and LIF on human NSPC proliferation, using the ATP assay. In the presence of 10 ng/ml LIF, the human NSPC proliferation rate peaked at 10 ng/ml of EGF and FGF individually, but higher concentrations of EGF and FGF did not increase the proliferation rate any further (Fig. 1A, B). Human NSPCs also showed maximum proliferation with LIF at a concentration of 5 ng/ml in the presence of 20 ng/ml EGF and FGF2 (Fig. 1C). These findings indicated a dose–response relationship between human NSPC proliferation and the concentration of EGF, FGF2, and LIF, with maximum human NSPC proliferation at 10 ng/ml EGF, 10 ng/ml FGF2, and 5 ng/ml LIF.

Variation of the EGF, FGF2, and LIF Concentration in the Medium During NSPC Culture

To determine the concentrations of EGF, FGF2, and LIF in the culture medium during human NSPC culture, each growth factor was assessed using ELISA. The concentrations (ng/ml) of EGF in culture medium without human NSPCs were 21.6 ± 0.1 (day 0), 21.2 ± 0.1 (day 1), 21.1 ± 0.1 (day 2), 21.1 ± 0.1 (day 3), 21.0 ± 0.1 (day 4), 20.7 ± 0.1 (day 5), and 20.7 ± 0.1 (day 6) (Fig. 2A), which showed a slight but statistically significant decrease over the 6 days of culture ($p < 0.01$, ANOVA), but suggested that EGF is fairly stable for 6 days in culture medium. In contrast, the concentration of EGF in culture medium with human NSPCs decreased more rapidly: 22.6 ± 0.2 (day 0), 21.3 ± 0.1 (day 1), 18.0 ± 0.1 (day 2), 14.5 ± 0.1 (day 3), 13.3 ± 0.1 (day 4), 10.2 ± 0.1 (day 5), and 7.40 ± 0.10 (day 6) (Fig. 2A), suggesting the utilization of EGF by human NSPCs. The concentration (ng/ml) of FGF2 in culture medium without human NSPCs showed a statistically significant decrease: 20.2 ± 0.1 (day 0), 19.5 ± 0.03 (day 1), 18.7 ± 0.1 (day 2), 17.9 ± 0.1 (day 3), 16.8 ± 0.1 (day 4), 15.4 ± 0.1 (day 5), and 14.5 ± 0.1 (day 6) (Fig. 2C), and the concentration of FGF2 in culture medium with viable human NSPCs decreased faster: 20.2 ± 0.1 (day 0), 15.5 ± 0.2 (day 1), 12.5 ± 0.2 (day 2), 9.98 ± 0.14 (day 3), 7.95 ±

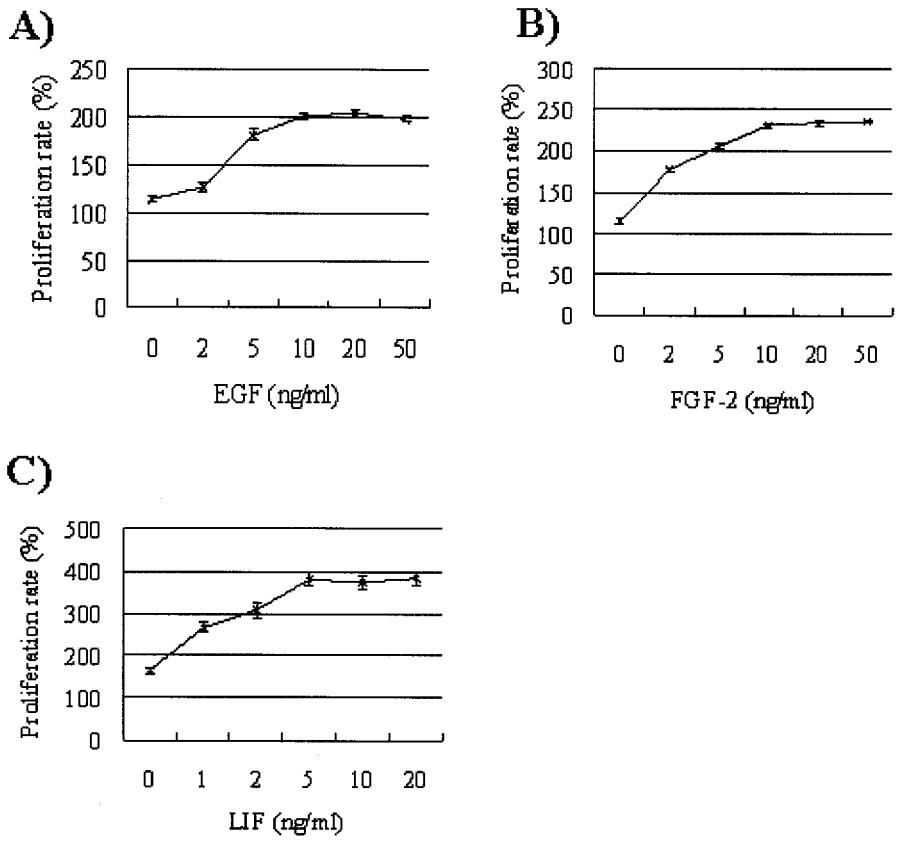

Figure 1. Effects of hr-EGF, hr-FGF2, and hr-LIF on human NSPC proliferation. The proliferation rate of human NSPCs with various concentrations of (A) hr-EGF and (B) hr-FGF2 in the presence of 10 ng/ml hr-LIF. (C) The proliferation rate of human NSPCs with various concentration of hr-LIF in the presence of both 20 ng/ml hr-EGF and 20 ng/ml FGF2. All data represent the difference in luminescence signals between days 1 and 6 of culture, shown as a percentage ($n = 8$, mean ± SEM).

0.13 (day 4), 5.93 ± 0.14 (day 5), and 5.35 ± 0.15 (day 6), with statistical significance ($p < 0.01$, ANOVA) (Fig. 2C). These results suggested that, unlike EGF, FGF2 was unstable in cell-free culture medium and was rapidly decreased in the medium containing viable human NSPCs, dropping below 10 ng/ml after 3 days (Fig. 2C). The concentration (ng/ml) of LIF in culture medium without human NSPCs showed a slight but statistically significant decrease: 9.67 ± 0.04 (day 0), 9.44 ± 0.05 (day 1), 9.50 ± 0.04 (day 2), 9.44 ± 0.06 (day 3), 9.36 ± 0.08 (day 4), 8.61 ± 0.06 (day 5), and 8.72 ± 0.03 (day 6) (Fig. 2E), while the concentration of LIF in medium containing human NSPCs decreased more rapidly: 9.58 ± 0.06 (day 0), 8.08 ± 0.11 (day 1), 5.71 ± 0.41 (day 2), 4.43 ± 0.07 (day 3), 2.84 ± 0.03 (day 4), 1.78 ± 0.03 (day 5), and 1.51 ± 0.02 (day 6), with statistical significance ($p < 0.001$, ANOVA) (Fig. 2E). These findings indicated that, like EGF, LIF was relatively stable in the culture medium without viable cells but in the presence of human NSPCs its concentration decreased rapidly, dropping below 5 ng/ml after 3 days (Fig. 2E).

Effects of Adding Supplemental EGF, FGF2, or LIF on Human NSPC Proliferation During the 6-Day Culture Period

To examine the effect of adding growth factor supplements to replace the growth factors lost from the medium during culture with NSPCs, we added each growth factor individually with different frequencies and measured the cellular proliferation. Compared with the 6-day culture to which no further EGF supplements had been added, the proliferation rate of human NSPCs that received supplemental EGF during the 6-day culture period was significantly increased: 104.3 ± 0.4% (2.5 ng/ml added five times), 104.3 ± 0.4% (5 ng/ml added five times), 103.7 ± 0.4% (2.5 ng/ml added twice), 103.5 ± 0.6% (5 ng/ml added twice), 103.6 ± 0.5% (5 ng/ml added once), and 102.9 ± 0.8% (7.5 ng/ml added once)

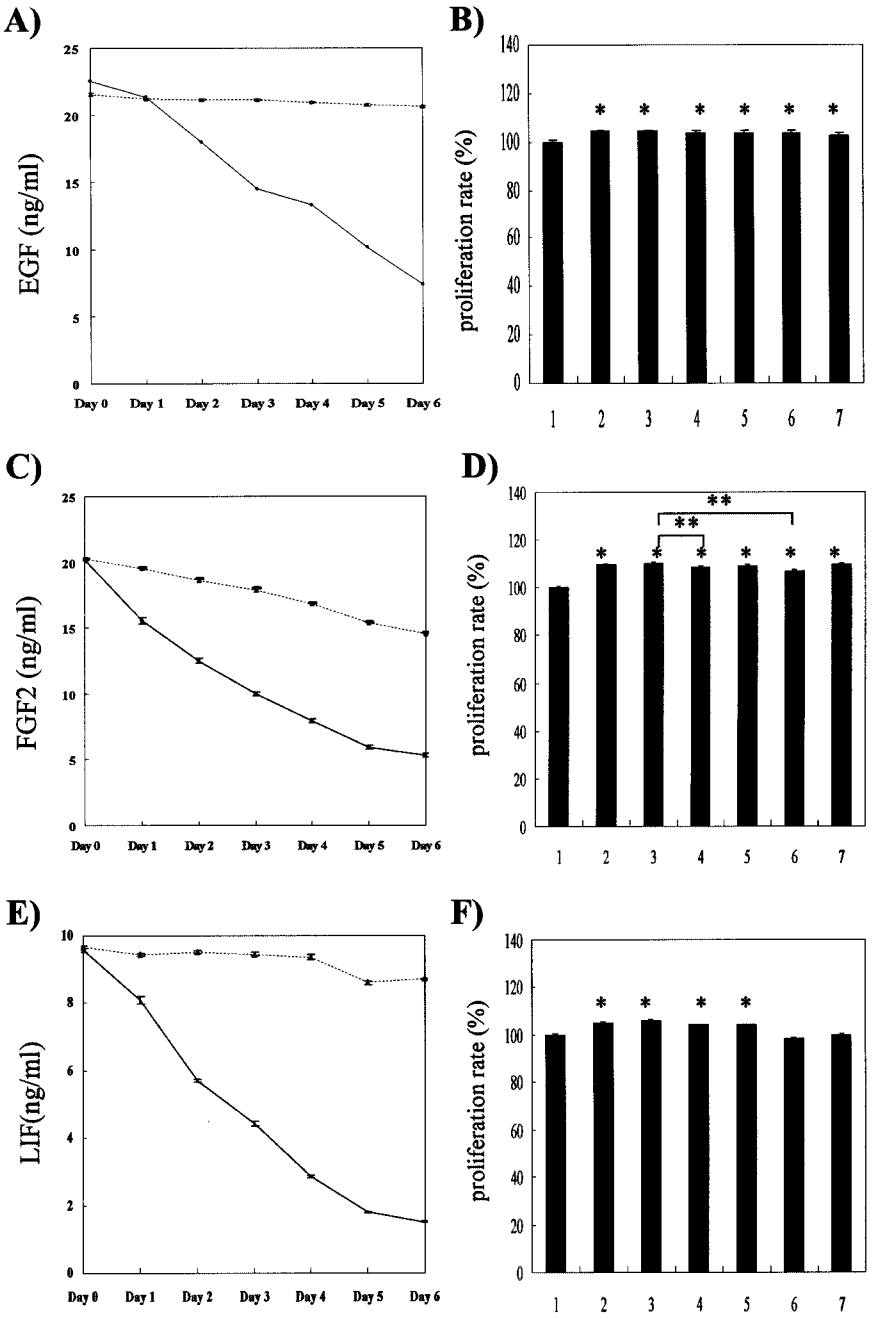

Figure 2. Concentrations of EGF, FGF2, and LIF in the culture medium for 6 days of culture and effects of additional supplements of each growth factor during the culture period on human NSPC proliferation. (A, C, E) The concentration of each growth factor in human NSPC culture medium over a 6-day culture period was determined by ELISA. Solid lines, with cells; broken lines, without cells. (B, D, F) Effects of additional supplements of each growth factor on human NSPC proliferation as measured by the ATP assay. (A) EGF concentration ($n = 12$, mean ± SEM; $p < 0.01$, ANOVA). (B) Effect of adding supplemental EGF during the 6-day culture period ($n = 16$, mean ± SEM; $*p < 0.01$, Mann-Whitney U test). (C) FGF2 concentration in human NSPC culture medium during the 6-day culture period ($n = 12$, mean ± SEM; $p < 0.01$, ANOVA). (D) Effect of adding supplemental FGF2 during the 6-day culture period ($n = 16$, mean ± SEM; $*p < 0.01$, $**p < 0.05$; Mann-Whitney U test). (E) LIF concentration in human NSPC culture medium for 6 days of culture ($n = 12$, mean ± SEM; $p < 0.01$, ANOVA). (F) Effect of adding supplemental LIF during the 6-day culture period. ($n = 16$, mean ± SEM; $*p < 0.01$, Mann-Whitney U test). (B, D) 1: no additions, 2: 2.5 ng/ml added every day (5 times), 3: 5 ng/ml added every day (5 times), 4: 2.5 ng/ml added on days 2 and 4 (twice), 5: 5 ng/ml added on days 2 and 4 (twice), 6: 5 ng/ml added on day 3 (once), and 7: 7.5 ng/ml added on day 3 (once). (F) 1: no addition, 2: 1 ng/ml added every day (5 times), 3: 2 ng/ml added every day (5 times), 4: 2 ng/ml added on days 2 and 4 (twice), 5: 5 ng/ml added on days 2 and 4 (twice), 6: 3 ng/ml added on day 3 (once), and 7: 6 ng/ml added on day 3 (once).

(Fig. 2B). Although the highest increase in proliferation was observed when EGF was added five times at 2.5 or 5 ng/ml, no significant differences were observed among the six protocols.

For FGF2, further supplements increased the human NSPC growth to 109.6 ± 0.6% (2.5 ng/ml added five times), 110.1 ± 0.6% (5 ng/ml added five times), 108.4 ± 0.6% (2.5 ng/ml added twice), 108.9 ± 0.5% (5 ng/ml added twice), 107.2 ± 0.5% (5 ng/ml added once), and 109.6 ± 0.4% (7.5 ng/ml added once) (Fig. 2D). Among these protocols, the most effective methods were the addition of 5 ng/ml FGF2 five times, 2.5 ng/ml five times, 5 ng/ml twice, and 7.5 ng/ml once, with the highest proliferation rates seen with five additions of 5 ng/ml (Fig. 2D). The other two protocols were significantly less effective than the most effective addition of FGF2 (Fig. 2D). These findings suggested that the addition of supplemental FGF2 during the 6-day culture period effectively improves the proliferation rate of human NSPCs.

The further addition of LIF to human NSPC culture also significantly increased the proliferation rate, to 104.8 ± 0.5% (1 ng/ml added five times), 105.8 ± 0.7% (2 ng/ml added five times), 104.1 ± 0.5% (2 ng/ml added twice), and 104.1 ± 0.5% (5 ng/ml added twice) (Fig. 2F). Although no significant differences were observed among these four protocols, the proliferation rates of human NSPCs caused by the other two protocols—98.5 ± 0.3% (3 ng/ml added once) and 99.9 ± 0.7% (6 ng/ml added once)—were not significantly different from the control (Fig. 2F). These findings suggested that frequent supplements of LIF (every 1 or 2 days) produced a small beneficial effect on NSPC proliferation.

Effects of Additional Supplements on Human NSPC Proliferation

To determine the individual effects of insulin, progesterone, transferrin, putrescine, and selenite on NSPC proliferation, human NSPCs were cultured with various concentrations of the above-mentioned supplements plus the three growth factors. Human NSPCs showed dose-dependent growth in response to insulin from 0 to 50 μg/ml ($p < 0.001$, ANOVA) (Fig. 3A) and transferrin from 0 to 1000 μg/ml ($p < 0.001$, ANOVA) (Fig. 3B), while progesterone, putrescine, and selenite had no remarkable effect on human NSPC growth (Fig. 3C–E). These findings indicated that insulin and transferrin had growth-promoting activity for human NSPCs, but the other three supplements were not effective.

Effects of D-Glucose and Lactate on Human NSPC Proliferation

We also investigated the effects of D-glucose and lactate on human NSPC proliferation and found no dose–response effect of D-glucose from 5 to 17.5 mM, but concentrations above 35 μg/ml showed cytotoxic effects (Fig. 3F). Like D-glucose, lactate did not have any growth-promoting effect on human NSPCs, and at higher concentrations (0.045% and above) it was toxic (Fig. 3G). These findings indicated that neither of these compounds had a dose–response effect on human NSPC proliferation; rather, at higher doses they were toxic, resulting in cell death.

Effects of CM on Human NSPC Proliferation

Finally, we examined the effects of CM on human NSPC proliferation. Compared with CM-free culture medium, the proliferation rates of human NSPCs were significantly higher in the presence of CM: 127.5 ± 0.7% (20% CM), 110.9 ± 1.0% (40% CM), 106.5 ± 0.8% (60% CM) (Fig. 4). However, at higher concentrations of CM, the human NSPC proliferation rates decreased, to 92.1 ± 1.0% (80% CM) and 82.1 ± 1.0% (100% CM), with statistical significance (Fig. 4). These findings indicated that supplemental CM can significantly promote human NSPC proliferation, although high doses of CM inhibit their growth.

DISCUSSION

The culture of NSCs as floating cell aggregates called neurospheres in serum-free medium containing EGF and/or FGF2 was originally reported by Reynolds et al. (23,24) and, in general, this method or a modified form of it has been used for human NSPC culture ever since (6,7,13,14,19,20,22,27,31,32). One of the greatest improvements to this method was the addition of LIF, which permitted the long-term culture of human NSPCs (7,14). However, the optimal concentrations of each growth factor for human NSPC proliferation had not been determined (7,15). Therefore, we first examined the dose–response relationship between human NSPC proliferation and the concentration of EGF, FGF2, and LIF by measuring the viable cells with an ATP assay. Compared with manual cell counting, the advantage of the ATP assay is that it can detect the proliferation rate of slowly growing human NSPCs within the intact neurospheres with higher sensitivity and reproducibility (14). Moreover, unlike manual counting, it does not require the dissociation of the neurospheres, so cells can be counted, quickly, accurately, and reproducibly (14).

Human NSPC growth showed a dose–response relationship with EGF, FGF2, and LIF, and the minimum concentration of each growth factor exerting the maximum effect was 10 ng/ml of EGF, 10 ng/ml of FGF2, and 5 ng/ml of LIF, indicating the minimum concentration of each growth factor that could be used for the effective proliferation of human NSPCs. In most culture protocols for human NSPCs, the initial concentrations

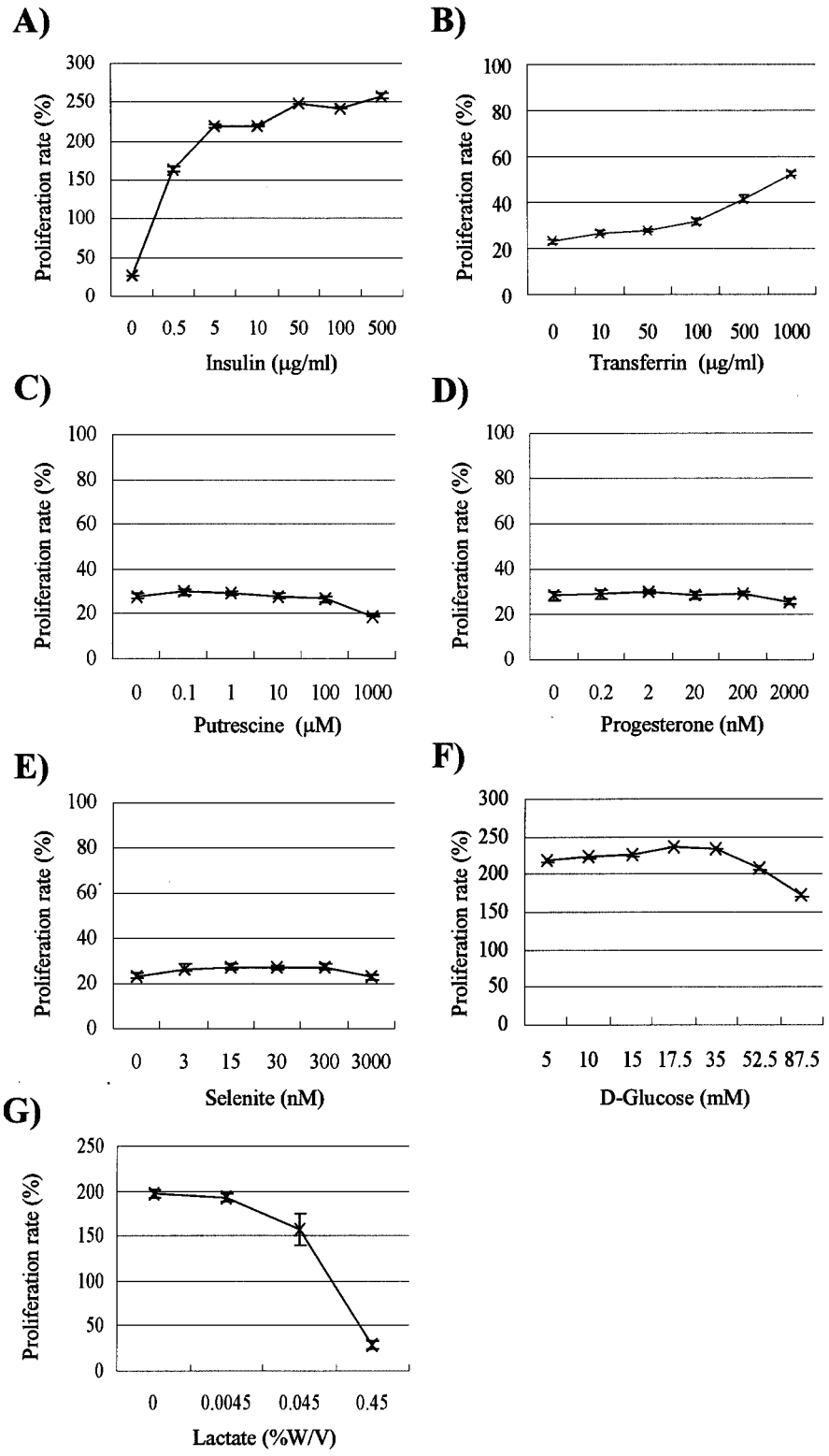

Figure 3. Effects of various compounds on human NSPC proliferation. (A–E) Human NSPCs were cultured using the neurosphere method in culture medium containing a single supplement at varied concentrations and the three growth factors at fixed concentrations (20 ng/ml hr-EGF, 20 ng/ml hr-FGF2, 10 ng/ml hr-LIF) for 6 days. (A) Insulin (μg/ml), (B) transferrin (μg/ml), (C) putrescine (μM), (D) progesterone (nM), (E) selenite (nM). Effect of (F) D-glucose (mM) and (G) lactate (% w/v) on human NSPC growth ($n = 8$, mean ± SEM).

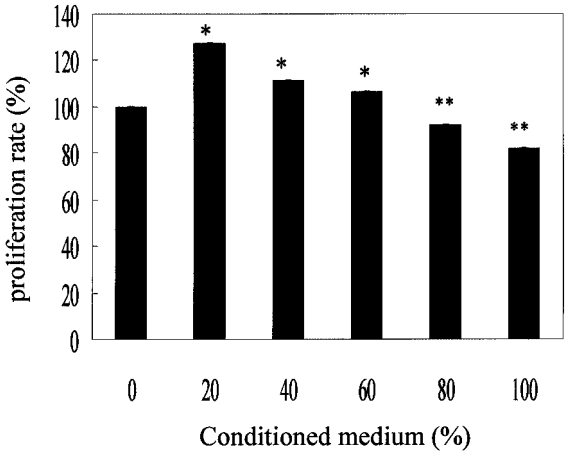

Figure 4. Effects of conditioned medium (CM) on human NSPC proliferation. Human NSPCs were cultured with various concentrations of human NSPC CM for 6 days ($n = 32$, mean ± SEM) with fresh medium. Proliferation rates of human NSPCs were significantly higher (*$p < 0.01$, Mann-Whitney U test) or lower (**$p < 0.01$, Mann-Whitney U test) compared with the control.

of the growth factors are: EGF (10–20 ng/ml), FGF2 (10–20 ng/ml), and LIF (5–10 ng/ml) (6,7,13,14,19,20, 22,27,31,32), which are up to twice as high as our estimated minimum concentrations. We therefore suggest that our estimated minimum concentrations are sufficient for the effective growth of human NSPCs, and would decrease the cost of the culture system.

Although growth factors are used from the beginning of the culture, we did not know their stability (the remaining concentration) in the medium during human NSPC culture, which could be very important for human NSPC growth. Therefore, we examined the variation in concentration of each growth factor in the medium during human NSPC culture and the effect of adding supplements of each growth factor during the 6-day culture period. We found that in the absence of human NSPCs, both EGF and LIF were stable in the culture medium for 6 days at 37°C in 5% CO_2, but FGF2 was degraded, while in the presence of human NSPCs both FGF2 and LIF were degraded very rapidly, to below our estimated minimum concentration on day 3, but EGF remained over 10 ng/ml for 5 days. When the growth factors were adjusted to maintain their initial concentration in the culture medium, we found that adding EGF once during the 6-day culture period was sufficient to increase human NSPC proliferation, but in the case of FGF2, frequent or high-dose supplements were more effective. For LIF, frequent addition to the culture medium seemed to be important to improve NSPC proliferation, although this property may reflect the lower initial concentration of LIF required for maximum effect compared with the minimum concentrations of EGF and FGF2. Thus, the growth factors in the human neurosphere growth medium during human NSPC culture are regulated differently, and it is useful in designing culture conditions to take the individual properties of each growth factor into account.

In addition to growth factors, the effects of other supplements on human NSPC proliferation were evaluated. Reynolds's DMEM/F12-based serum-free medium was supplemented with growth factors and insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) (5,23). We found that among these five compounds, only insulin and transferrin in the presence of EGF, FGF2, and LIF improved the proliferation of human NSPCs. Insulin has been reported to promote the growth of cultured cells under serum-free conditions (5), and insulin-like growth factor-I (IGF-I) is key in the regulation of murine NSC proliferation; neither EGF nor FGF2 can induce murine NSC proliferation in its absence (2). In addition, Aberg et al. reported that the peripheral infusion of IGF-I increases progenitor cell proliferation and selectively induces neurogenesis in the adult rat hippocampus (1). Therefore, signal transduction by insulin or IGF-I may play a very important role in human NSPC proliferation. Transferrin, a β_1 globulin and major iron transporter protein in the blood, is reported to be a necessary element in cell proliferation (5), and the present findings also showed its growth-promoting role in human NSPCs. In contrast to insulin and transferrin, the other three compounds did not show any significant effect on the proliferation or survival of human NSPCs. Although several reports show a cell proliferation effect of progesterone, putrescine, or selenite (5,9,25,29,36), in our human NSPC culture they did not have a significant effect; however, they may play other important roles. For example, the growth-promoting activity of insulin on human NSPCs might be boosted by the combination of progesterone, putrescine, selenite, and transferrin. A dose–response effect of insulin from 0 to 500 μg/ml on human NSPC proliferation was also observed (Mori and Kanemura, unpublished data); thus, we think that of these supplements, insulin is the most important for human NSPCs proliferation.

In the presence of growth factors, human NSPCs showed good proliferation in DMEM/F12/Glu medium supplemented with either B27 or N2 without significant difference from medium lacking these supplements, but B27 and N2 supplement caused some morphological differences in the human neurospheres; cells cultured with N2 exhibited some adhesive tendency (Mori and Kanemura, unpublished data), suggesting that the existence of super oxide dismutase, retinal acetate, tocopherol acetate, corticosterone, T3, or other factors in the B27 or

different concentrations of any of the five key defined supplements (insulin, progesterone, transferrin, putrescine, and selenite) might change the morphology of human NSPCs. Therefore, using large numbers of different compounds in human NSPC cultures for clinical applications would change the cell heterogeneity and make the cells unsafe for clinical use. Considering this important point, we suggest that the optimal defined supplements for human NSPC culture for clinical use would be based on N2 with the adjustment of any of the five key components of N2 as necessary or the minimum amounts of any new supplements to N2. Although several groups have used DMEM/F12 medium containing more than 0.6% D-glucose (almost 33 mM) for human or rodent NSPC culture (7,27,31), the present findings suggest that such a high level of D-glucose is not important for human NSPC proliferation, and in fact, might be cytotoxic.

NSPCs secrete various cytokines and other factors into the culture medium [e.g., IL-1α, IL-1β, IL-6, TGF-β1, TGF-β2, TNF-α, glycosylated cystatin C (CCg), and stem cell-derived neural stem/progenitor cell supporting factor (SDNSF)] (16,28,30), and some of these NSPC-derived factors can promote NSPC proliferation. We have also identified several other factors in human NSPC CM that might be involved in their proliferation (Kanemura and Nakagawa, in preparation). In agreement with Morshead et al., who reported that neurospheres begin to arise in the absence of growth factors after repeated passaging (18), our present results suggested a positive effect of the NSPC CM on human NSPC proliferation, which might be due to previously reported or undefined novel factors in the CM that are derived from the NSPCs themselves. However, in addition to these positive factors, the CM seems to contain some negative factors for human NSPC proliferation (e.g., lactate), whose concentration increases with the duration of culture (Nakagawa and Kanemura, unpublished data). Because we showed that increasing concentrations of lactate had cytotoxic effects on human NSPCs, the withdrawal of lactate from the NSPC CM should promote human NSPC proliferation.

Cultured human NSPCs obtained using the present protocol were heterogeneous cell aggregates containing NSCs and various progenitor cells, and with this method it was impossible to control the growth of specific cell types. Different types of immature cells may show different responses to various exogenous factors, and the NSPCs within neurospheres are thought to vary widely in their proliferative activity and degree of differentiation. With this issue in mind, we are presently investigating the relationship between the culture medium (including growth factors, supplemental factors, and other conditions) and the degree of heterogeneity of human neurospheres. Although much work needs to be done to determine the effects of culture conditions on heterogeneity, the present findings provide useful information for establishing the appropriate culture methods for human NSPCs for clinical use.

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