www.jkns.or.kr 10.3340/jkns.2008.44.6.375
J Korean Neurosurg Soc 44 : 375-381, 2008

Print ISSN 2005-3711 On-line ISSN 1598-7876
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Laboratory Investigation

Effect of Single Growth Factor and Growth Factor Combinations on Differentiation of Neural Stem Cells

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Objective : The effects on neural proliferation and differentiation of neural stem cells (NSC) of basic fibroblast growth factor-2 (bFGF), insulin growth factor-I (IGF-I), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) were assessed. Also, following combinations of various factors were investigated : bFGF+IGF-I, bFGF+BDNF, bFGF+NGF, IGF-I+BDNF, IGF-I+NGF, and BDNF+NGF.
Methods : Isolated NSC of Fisher 344 rats were cultured with individual growth factors, combinations of factors, and no growth factor (control) for 14 days. A proportion of neurons was analyzed using β-tubulin III and NeuN as neural markers.
Results : Neural differentiations in the presence of individual growth factors for β-tubulin III-positive cells were : BDNF, 35.3%; IGF-I, 30.9%; bFGF, 18.1%; and NGF, 15.1%, and for NeuN-positive cells was : BDNF, 34.3%; bFGF, 32.2%; IGF-1, 26.6%; and NGF, 24.9%. However, neural differentiations in the absence of growth factor was only 2.6% for β-tubulin III and 3.1% for NeuN. For β-tubulin III-positive cells, neural differentiations were evident for the growth factor combinations as follows : bFGF+IGF-I, 73.1%; bFGF+NGF, 65.4%; bFGF+BDNF, 58.7%; BDNF+IGF-I, 52.2%; NGF+IGF-I, 40.6%; and BDNF+NGF, 40.0%. For NeuN-positive cells : bFGF+IGF-I, 81.9%; bFGF+NGF, 63.5%; bFGF+BDNF, 62.8%; NGF+IGF-I, 62.3%; BDNF+NGF, 56.3%; and BDNF+IGF-I, 46.0%. Significant differences in neural differentiation were evident for single growth factor and combination of growth factors respectively (p<0.05).
Conclusion : Combinations of growth factors have an additive effect on neural differentiation. The most prominent neural differentiation results from growth factor combinations involving bFGF and IGF-I. These findings suggest that the combination of a mitogenic action of bFGF and post-mitotic differentiation action of IGF-I synergistically affects neural proliferation and NSC differentiation.

KEY WORDS : Neuron · Single growth factor · Combination of growth factors · Synergistic effect.

INTRODUCTION

It had been known that neurons could not be regenerated in the adult central nervous system (CNS). Neuron was taken for granted to be in the G0 phase in its mitotic activity[38]. However, more recently, neural stem cells (NSC) were isolated from the walls of the ventricular zone of the CNS and the dentate gyrus of the hippocampus in adult mammals[1,31]. The NSC are defined as undifferentiated cells that have the ability of self-renewal and potentiality to differentiate into neurons, astrocytes, and oligodendrocytes in the CNS. The NSC give rise to immature neurons that migrate along the rostral migratory stream to the olfactory bulb, where they differentiate and integrate as interneurons in the rat[11]. NSC are potentially valuable as therapeutic tools for Parkinson's disease[20], cerebral infarction[8], spinal cord injury[34], and many neurodegenerative diseases. Many attempts have been made to transplant NSC to remediate neurodegenerative diseases and CNS injuries in animal models; however, NSC which were transplanted differentiated into glial cells or remained undifferentiated[7]. Transplantation of only NSC fails to regenerate nervous systems[9]. Neuron cannot be regenerated despite the presence of NSC in the adult mammalian brain, because cell-extrinsic signals may inhibit differentiation of NSC to neurons[35]. Therefore, the micro-environment, including mitogens, hormones, genes, and stress is important in the proliferation, migration, survival, and differentiation aspects of neurogenesis[3,13]. However, the mechanism controlling NSC and progenitor cell proliferation remains only partial-

• Received : August 13, 2008 • Accepted : November 28, 2008
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ly understood.

Presently, harvested NSC were cultured with single growth factors and combinations of growth factors for 2 weeks and the cell characteristics were evaluated with the specific neural markers. We investigated effects of neural differentiation in each growth factor and combination.

MATERIALS AND METHODS

Cell suspension

Fisher 344 male or female rats weighing 170-190 g were deeply anesthetized with isofluran inhalation (5 Mac) for 10 min. The animals were decapitated and the whole brains were removed. The harvested brains were transported in a cold phosphate buffer solution (PBS). Two coronal cuts were made in the areas between the rhinal fissure and the hippocampus. The resulting tissue chunk was laid on its posterior surface, two parasagittal cuts were made just lateral to the lateral ventricles, and one horizontal cut was made at approximately the level of the corpus callosum[35,36]. Neurospheres were made by dissociating the central rectangular piece of tissue containing the lateral ventricles. The tissues were minced (1 mm^3) with a knife and digested in a solution of papain (2.5 U/mL; Worthington, Freehold, NJ) dissolved in Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. The cells and tissue fragments were passed serially through ascending gauge needles (16 G, 18 G, 20 G, and 24 G), and washed three times with Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) (DMEM-FBS). The whole digested tissue was suspended in DMEM-FBS and filtered through a sterile 50 μm nylon mesh. The Percoll solution was made by mixing nine parts of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) to one part 10X PBS (Irvine Scientific, Santa Ana, CA). The cell suspension was then fractionated by high-speed centrifugation (20,000 × g, 30 min, 18°C) in the presence of blue and red marker beads. The cell fractions between the red and blue markers were harvested and washed free of Percoll by three or more rinses in DMEM-FBS[21].

Cell culture

After isolation of NSC from the subventricular zone, the cells were cultured with single growth factors (bFGF, IGF-I, BDNF, and NGF), combinations of two factors (bFGF +IGF-I, bFGF +BDNF, bFGF +NGF, IGF-I +BDNF, IGF-I +NGF, and BDNF +NGF), or in the absence of growth factor (control) for 14 days. The cells fractionated through the Percoll gradients were washed free of Percoll and plated in flasks (Fisher Scientific, Pittsburgh, PA) or wells of multi-well dishes (Fisher Scientific) that had been coated with polyornithine/laminin (Sigma-Aldrich). For samples treated using a single growth factor, cells were allowed to attach over 24 h to the glass slides in DMEM/F-12 (1 : 1) containing 10% FBS (Gibco, Carlsbad, CA). The culture medium consisting of DMEM/F-12 (1 : 1) supplemented with either N2 supplement (Life Technologies, Gaithersburg, MD) and 20 ng/mL recombinant human FGF-2 (Roche Diagnostics, Indianapolis, IN), recombinant mouse IGF-I (R & D Systems, Minneapolis, MN), recombinant human BDNF (R & D Systems), or recombinant rat β-NGF (R & D Systems) was changed every 48 h for 14 days. For samples treated with a combination of two growth factors, the cells were allowed to attach as just described. The medium was supplemented with N2 supplement and 10 ng/mL bFGF +10 ng/mL IGF-I, 10 ng/mL bFGF +10 ng/mL BDNF, 10 ng/mL bFGF +10 ng/mL NGF, 10 ng/mL IGF-I +10 ng/mL BDNF, 10 ng/mL IGF-I +10 ng/mL NGF, or 10 ng/mL BDNF +10 ng/mL NGF. The medium was changed every 48 h for 14 days. A dish devoid of growth factor was used as a control.

Immunostaining

The culture media was removed after the cells have been cultured on polyornithine/laminin-coated glass chamber slides for 14 days. The cells were fixed with 4% paraformaldehyde in PBS for 10 min, rinsed three times (5 min each wash) with PBS and treated as follows. The cells were first pre-incubated with PBS containing 5% pre-immune donkey serum (PBS-DS) for 30 min and then incubated with primary antibodies for the cell surface markers; NeuN (Chemicon, Temecula, CA), β-tubulin-III (Covance, Berkeley, CA), O4 (Chemicon), and glial fibrillary acidic protein (GFAP; DAKO, Carpinteria, CA) in PBS-DS overnight at 4°C. The cells were then washed three times, 10 min each, with PBS. Washed cells were then incubated for 2 h with secondary donkey anti-mouse IgG (Jackson Immuno Reseach, West Grove, PA; Cy-2, green color), and anti-rabbit IgG (Jackson Immuno Reseach; Cy-3, red color) diluted 1 : 200 in PBS-DS. The cells were then washed with PBS three times. The final wash contained 10 ng/mL 4,6-diamindino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) in PBS, which was used as a fluorescent counterstain for the cell nuclei. Samples were added to 100 mM Tris, pH 8.5, containing 25% glycerol, 10% polyvinyl alcohol (Air Products and Chemicals, Allen town, PA), 2.5% 1,4-diazobicyclo-[2.2.2]- octane (Sigma-Aldrich), a drop was placed on a slide, a coverslip was added, and counted as described below.

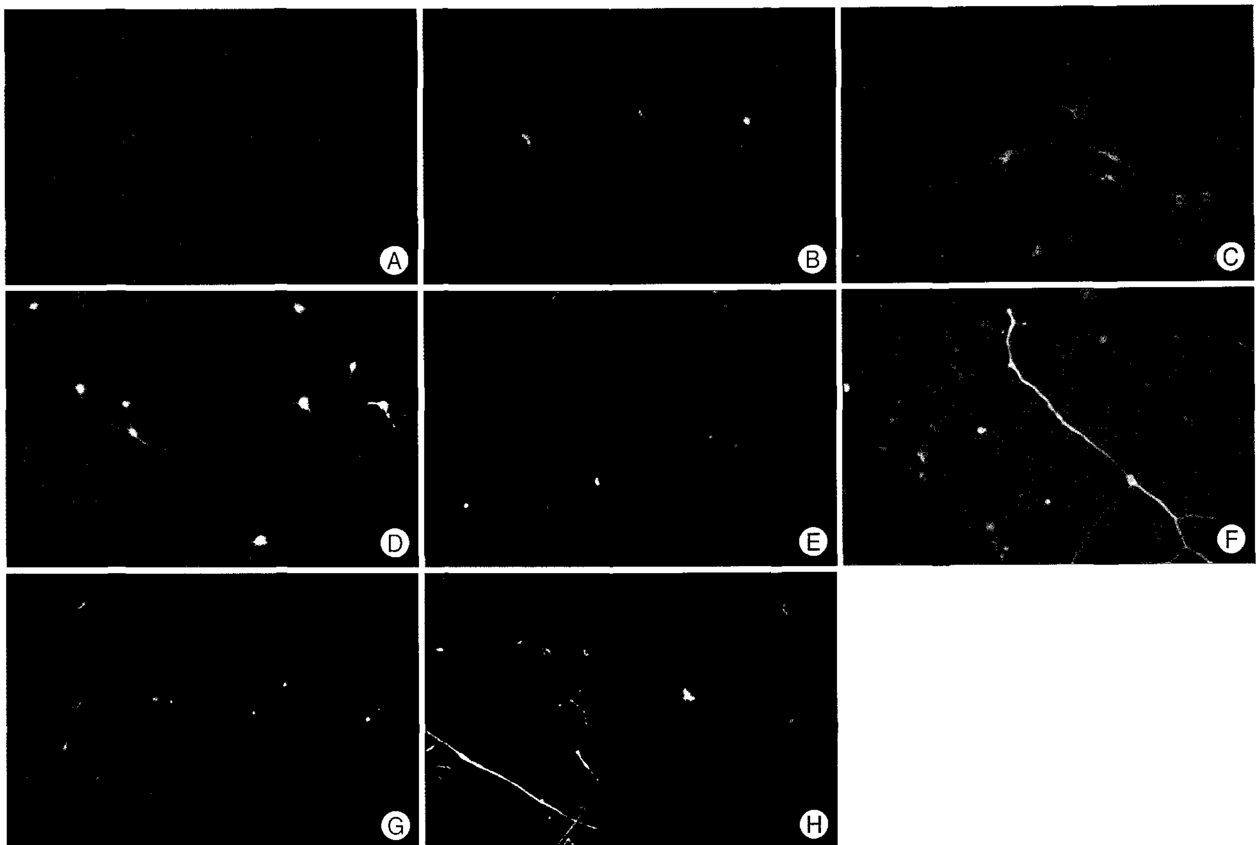

Fig. 1. Immunocytofluorescence for expressions of neuron specific markers (β-tubulin III, NeuN) in the neurogenic stimulation with brain-derived neurotrophic factor (A, E), basic fibroblast growth factor (B, F), insulin growth factor-I (C, G) and nerve growth factor (D, H) for 14 days respectively. Immunocytofluorescence micrographs show β-tubulin III-positive cells (A, B, C, D) and NeuN-positive cells (E, F, G, H). Magnification, 200X.

Cell counting and analysis

Immunofluorescent positive cells in each well were counted using an inverted fluorescent microscope. β-tubulin III and NeuN are neuron cell surface makers, O4 is a oligodendroglia marker, and GFAP is an astroglial cell marker. Neurons were defined as β-tubulin III- or NeuN-positive cells. The percentage of immunfluorescent neurons was analyzed as neural specific marker (NeuN or β-tubulin-III)-positive cells in comparison to all nucleated cells. All experiments were repeated 10 times and counted at least five times blindly in each well.

The statistical significance of differences between each mean of a single growth factor, combination of growth factors, and group were evaluated by one-way ANOVA with $p < 0.05$ considered significant.

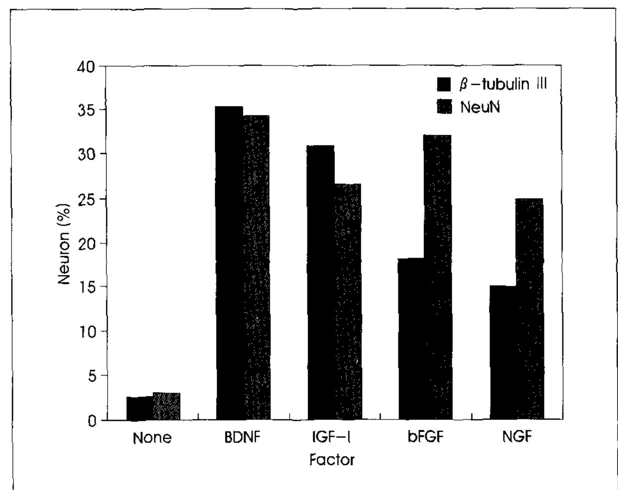

Fig. 2. Neuronal outgrowth and survival of neural stem cells in response to brain-derived neurotrophic factor, insulin growth factor-I, basic fibroblast growth factor, and nerve growth factor. Neurogenically stimulated cells were cultured for neural cell analysis with β-tubulin III and NeuN for 14 days. Significant differences in neural differentiation with single growth factor were apparent (β-tubulin III, p=0.032; NeuN , p= 0.039).

RESULTS

Neural differentiations were demonstrated by the expression of neural markers in neurons treated with single growth factors. For β-tubulin III-positive cells, the following percentages were observed : BDNF, 35.3%; IGF-I, 30.9%; bFGF, 18.1%; and NGF, 15.1%. For NeuN-positive cells the following percentages were observed : BDNF, 34.3%; bFGF, 32.2%; IGF-1, 26.6%; and NGF, 24.9% (Fig. 1, 2). However, neurons were rarely evident when growth factor

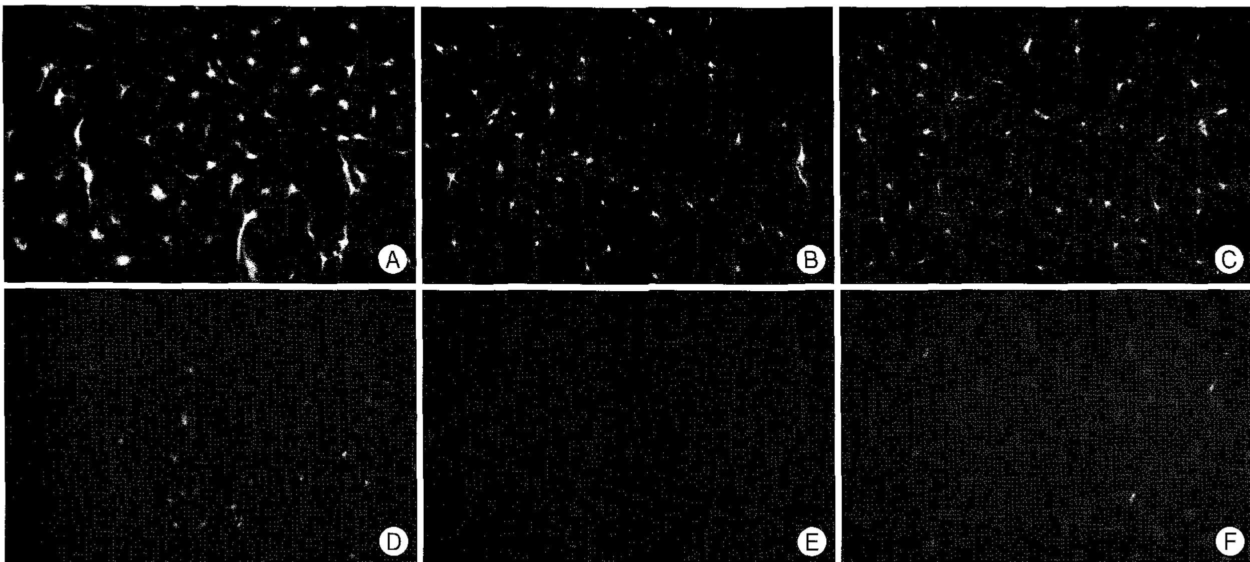

Fig. 3. Immunocyotofluorescence determined expression of neuron specific markers (β-tubulin III, NeuN) in the 14 day neurogenic stimulation by basic fibroblast growth factor (bFGF)+insulin growth factor-I (A, D), bFGF+brain-derived neurotrophic factor (BDNF) (B, E), and BDNF+nerve growth factor (C, F). Immunocytofluorescence micrographs demonstrating β-tubulin III-positive cells (A-C) and NeuN-positive cells (D-F). Magnification, 200X.

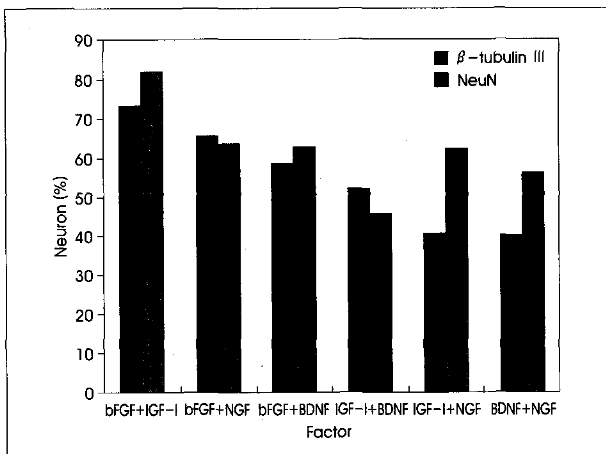

Fig. 4. Neuronal outgrowth and survival of neural stem cells in response to basic fibroblast growth factor (bFGF)+insulin growth factor-I (IGF-I), bFGF+brain-derived neurotrophic factor (BDNF), bFGF+nerve growth factor (NGF), IGF-I+BDNF, IGF-I+NGF, and BDNF+NGF. Neurogenically stimulated cells were cultured for neural cell analysis with β-tubulin III and NeuN for 14 days. Significant differences in neuronal positive cells were apparent with each group (β-tubulin III, p=0.023; NeuN, p=0.018).

was absent (β-tubulin III, 2.6%; NeuN 3.1%). Significant differences were evident in neural differentiation with single growth factors (β-tubulin III, p=0.032; NeuN, p=0.039).

Neural differentiations were also demonstrated when the growth factor combinations were used. For β-tubulin III-positive cells, the following results were obtained : bFGF+IGF-I, 73.1%; bFGF+NGF, 65.4%; bFGF+BDNF, 58.7%; BDNF+IGF-I, 52.2%; NGF+IGF-I, 40.6%; and BDNF+NGF, 40.0%. For NeuN-positive cells, the following results were obtained : bFGF+IGF-I, 81.9%; bFGF+NGF, 63.5%; bFGF+BDNF, 62.8%; NGF+IGF-I, 62.3%; BDNF+NGF, 56.3%; and BDNF+IGF-I, 46.0% (Fig. 3, 4). Significant differences in neural differentiation were evident (β-tubulin III, p=0.023; NeuN, p=0.018). All combinations of growth factors produced significantly more prominent neural proliferation and differentiation effect than any single growth factor.

A partial proportion of neural-positive cells were labeled with GFAP or O4. The data of co-labeling neural-positive cell were summarized in Table 1. As aforementioned, GFAP and O4 are markers of glial cells. Both of them also attributed neural primitive cell/progenitor cell[3,35]. Although the cultured cells are not purified neural stem cells, it can conclude that a large part of these cultured cells are progenitor/stem cells[21].

DISCUSSION

Improving the activity of NSC is important to increase the therapeutic potential of NSC to several diseases and experimental models. The primary concern of NSC transplant therapy ensures that many NSC as possible differentiate into functional neurons. The present study was prompted by the need to create appropriate micro-environments for neural differentiation in NSC transplantation. Here, we investigated the effects of growth factors on proliferation and differentiation of NSC.

Neurotrophins are a group of neurotrophic factors that play an essential role in neural development, differentiation, survival, regeneration, and function in both the central and

Table 1. Co-labeling cells expressed with neural markers after 14 days in culture (%)

	β-tubulin III+GFAP	β-tubulin III+O4	NeuN+GFAP	NeuN+O4
BDNF	13.2	13.2	19.6	11.3
IGF-I	15.0	9.8	15.3	10.4
bFGF	23.7	15.1	22.8	20.6
NGF	8.5	5.6	14.4	7.5
bFGF+IGF-I	60.5	44.7	73	60.2
bFGF+NGF	40.1	31.9	55.6	40.9
bFGF+BDNF	30.3	22.5	40.4	20.0
BDNF+IGF-I	42.6	35.0	35.6	22.2
IGF+NGF	28.5	20.4	43.5	32.1
BDNF+NGF	19.8	23.3	26.8	23.4

peripheral nervous system. This neurotrophin family consists of NGF, BDNF, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6)[27]. The effects of neurotrophins are mediated by an interaction with specific cell surface receptors[19]. NGF supports the survival and growth of peripheral sympathetic and primary sensory neurons, and also induces a variety of effects in CNS cells including activation of gene expression[28], promotion of axons[12], dendritic branching[24], reduction of neuronal loss after injury, and the chemotrophic guidance of axons[18,25]. BDNF not only promotes cell differentiation, but offers strong neuroprotection that can be crucial for the survival of the stem cell in the CNS after implantation in vivo[9]. BDNF is extensively distributed in the developing and mature nervous system, and plays the most significant role in neural cell development, survival, and repair[32]. Kirschenbaum and Goldman reported that 35% of new neurons survived at 22 days in cultures exposed to BDNF in contrast to other neurotrophins (NGF, NT-3), which rarely affected neuronal viability[22]. There are differences of neural differentiation and proliferation in neurotrophins based on differences of distribution and affinity of receptor on the cell surface. Although BDNF supports the survival and proliferation of new neurons, this growth factor affects neither precursor mitosis nor initial commitment to neuronal lineage. BDNF does, however, affect the restricted differentiation, maturation, and survival of post-mitotic neuron[2,15].

bFGF has a much broader range of effects and supports the survival of a variety of neurons from different regions of the brain. It is well-known that bFGF is a necessary factor for maintaining proliferative multipotent precursors in vitro[16,35]. bFGF is a mitogen that induce multipotent stem cells to proliferate and stimulate committed neuronal precursors to produce neurons or astrocytes. NSC can differentiate into neurons or glial cells in the presence of bFGF in a dose-dependent manner. For example, neurons were differentiated at low concentration (0.1 ng/mL) of bFGF in rat embryonic NSC, while both neurons and astrocytes differentiate at high concentration (10 ng/mL)[13]. Infused bFGF also acts as a mitogen in the rat ventricle[26].

IGF-I and IGF-I receptors are present throughout the CNS during embryogenesis, and their mRNAs are discretely localized in certain neuronal populations[4,6]. The presence of IGF-I and receptor suggests that this factor may play important a role in neurogenesis. Neurons are generated in a dose-dependent manner upon culture of NSC by IGF-I[2]. However, IGF-I has no mitogenic action in contrast with bFGF, and restrictively affects post-mitogenic differentiation[2,3]. In particular, IGF-I promotes the differentiation of the same neuronal precursors as does BDNF, with both utilizing tyrosin kinase receptors[23,29], activating p21ras as part of their signal transduction[33,37], and supporting the survival of mesencephalic dopamine and motor neurons[5,30]. Differentially, BDNF presents neurons containing GABA while IGF-I presents neurons containing amino-acid transmitters[2,14].

In our study, each growth factor that was examined affected neural differentiation in NSC cultures over 14 days. Variations in differentiation were apparent depending on the growth factor used. Neural presentation was most prominent using BDNF. However, use of a single growth factor consistently produced a low rate of neural differentiation (15%-35%).

Presently, we observed that combinations of growth factors consistently promoted more neural differentiation than any single growth factor. The combinations of growth factors had an additive effect on NSC differentiation. In particular, the combination of bFGF and IGF-I synergistically affected neural proliferation and differentiation of NSC, producing the most prominent neural differentiation noted in this study. Arsenijevic et al. suggested that IGF-I plays an important role with bFGF in neurogenesis based on the fact that IGF-I receptors are expressed in germinal regions that colocalize with FGF-receptor[3]. Moreover, Drago et al. reported that IGF-I acted as a survival factor for an FGF-2 responsive progenitor cell that give rise to neurons[10]. We considered that the combination of a potent mitogenic action of bFGF and post-mitotic differentiation of IGF-I has a synergistic effect on neural proliferation and differentiation of NSC.

Subsequently, bFGF+NGF and bFGF+BDNF displayed additive effects and quite prominent effects on neural proliferation and differentiation. We found that more neu-

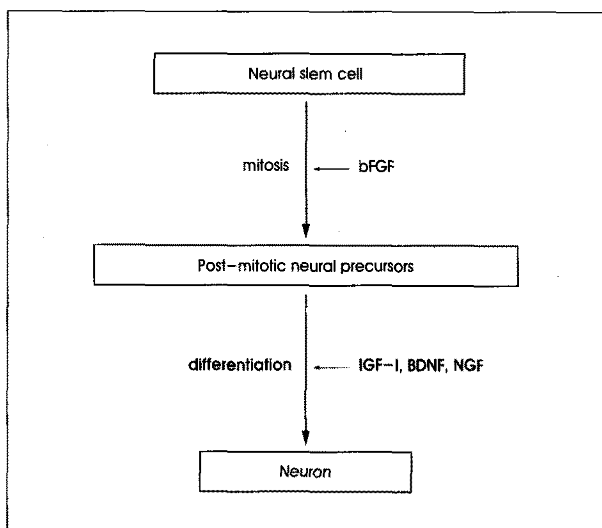

Fig. 5. Schematic pathway for neurogenesis by neural stem cells in vitro. Basic fibroblast growth factor induce neural stem cells to proliferate. Insulin growth factor-I, brain-derived neurotrophic factor, and nerve growth factor act to induce the differentiation of postmitotic neuronal precursors.

rons were differentiated and proliferated in the presence of combinations of factors, which regulated mitosis (bFGF) and post-mitotic processes (IGF-I, NGF, BDNF; Fig. 5). The result of the present study also demonstrated that combination of BDNF and NGF displayed an additive effect and presented more neuron on neural differentiation. Hanson et al. reported that additive effects were found concerning survival in short-term culture of spinal motor neuron when multiple neurotrophic factors were combined[17].

CONCLUSION

Combinations of growth factors produce a prominent effect on neural proliferation and differentiation than any single factor. Presently, neural differentiation was most prominently presented in the presence of bFGF+IGF-I. These findings suggest that the combination of the mitogenic action of bFGF and the influence on post-mitotic differentiation of IGF-I synergistically affects neural proliferation and differentiation of NSC.

• Acknowledgements
The authors wish to acknowledge the financial support of the Catholic Institute of Cell therapy Basic Science Programs Foundation made in the program year of 2007.

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