Biomolecules & Therapeutics, **18(1)**, 48-55 (2010)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2010.18.1.048

Inhibitory Effects of Phylligenin on the Proliferation of Cultured Rat Neural Progenitor Cells

Sung Hoon LEE[1,5], Hyo Sang GO[1], Chang Soon CHOI[1], Jae Hoon CHEONG[2], Sun-Young HAN[3], KiHwan BAE[4], Kwang Ho KO[1,*], and Seung Hwa PARK[5,*]

[1]Department of Pharmacology, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, [2]Department of Pharmacy, Sahmyook University, Seoul 139-742, [3]Pharmacology Research Center, Korea Research Institute of Chemical Technology, Daejeon 305-343, [4]College of Pharmacy, Chungnam University, Daejeon 301-747, [5]Center for Geriatric Neuroscience Research, IBST, and Research Institute of Medical Sciences,School of Medicine, Konkuk University, Seoul 143-130, Republic of Korea

(Received January 11, 2010; Revised January 19, 2010; Accepted January 19, 2010)

Abstract – Neural progenitor cells (NPCs) differentiate into astrocytes, neurons and oligodendrocytes, which is controlled by various factors in brain. Recent evidences suggest that small molecules modulating the proliferation and differentiation of NPCs may have therapeutic value as well as the potential use as chemical probes. Phylligenin is a lignan with anti-inflammatory activity that is isolated from the fruits of *Forsythia koreana*. We investigated effects of phylligenin on proliferation and differentiation of NPCs. Treatment of phylligenin decreased the number of proliferating NPCs in culture without effects on the differentiation and survival of neural cells such as neurons and astrocytes. To examine the mechanism of the decreased NPCs number, we performed cell cycle analysis. Proliferation of NPCs was decreased via G1-S transition block by phylligenin treatment, and it was mediated by the increase of p21 level. However, phylligenin did not induce apoptosis of NPCs as determined by TUNEL assay and PARP cleavage. We also found that viability of glioma cell lines such as C6 and U87MG glioma cells, but not that of primary neuron and astrocyte, was inhibited by phylligenin. These results suggest that phylligenin selectively inhibits proliferation of rapidly growing cells such as neural stem cells and glioma cells. Given that the possible role of brain tumor stem cells in the pathology of brain cancers, the inhibitory effects of phylligenin might be useful in the development of new therapeutic agents against brain cancers.

Keywords: Neural progenitor cells, Phylligenin, Proliferation, p21, Glioma

INTRODUCTION

Neural stem cell (NSC) is a type of adult stem cell found in CNS, which has multipotent and self-renewal property (Temple, 2001). Normal cells show symmetric division which divide into two daughter cells, but NSCs exhibit asymmetric division which divide into one neural stem cell and one daughter cell. The divided neural stem cell has unlimited proliferative potential and differentiate as cell fate progeny (Gage, 2000). NSCs are generated in subventricular zone (SVZ) of lateral ventricle and subgranular zone (SGZ) of hippocampal dentate gyrus (Conover and Notti, 2008). In rodents, SVZ NSCs migrate to olfactory bulb along the rostal migratory stream.

In adult subventricular zone, three forms of NSC, namely type B, C, and A have been suggested (Martino and Pluchino, 2006; Stiles and Rowitch, 2008). First, type B cell is called SVZ astrocyte, which has unlimited multi-potency for self renewal and proliferate in response to mitogen. Second, type C cell is called multipotent progenitor or transit amplifying cell, which are proliferative cells with only limited capacity for self-renewal that can differentiate into at least two different cell lineages. Type A cell is called lineage-specific precursor or progenitor cells that are restricted to one distinct lineage for example, neuron or astrocyte. Microglia originates not from stem cells but from monocytes.

Embryonic brain containing SVZ cells form a spherical

*Corresponding authors
Tel: +82-2-880-7848 (KH Ko), +82-2-2049-6322 (SH Park)
Fax: +82-2-885-8211 (KH Ko), +82-2-2049-6192 (SH Park)
E-mail: khk123@snu. ac.kr (KH Ko), seunghwa.park@kku.ac.kr (SH Park)

aggregate in culture, which is called neurosphere. Neurosphere cells can self-renew and can differentiate into three major cell types of CNS. Therefore, neurosphere provides relatively homogenous population of neural stem/progenitor cells (NSC/NPC) in high amount, which was also used in this study.

Chemical modulators of NSC or NPC are a subject of great interest due to its potential for cure of neurological diseases (Rishton, 2008). Chemical entities capable of promoting differentiation of NPCs or eliminating NPCs are intriguing molecules in CNS drug development. Approaches directing the differentiation of NPCs to neurons can be applied for diseases caused by neuronal loss. Alzheimer's or Parkinson's disease can be examples requiring neurogenesis from NPCs. Chemical modulators inhibiting proliferation of NPCs can also be used to eliminate cells with stem-like properties such as brain tumor stem cells (Diamandis *et al*., 2009). As direct relationship between normal neural stem cells and brain tumor stem cells emerges, these chemical entities will have great value as chemical probes for brain cancer therapeutics. In 2007, Peter Dirks group reported small molecules inhibiting neurosphere proliferation, through chemical genetic screening (Diamandis *et al*., 2007). The identified compounds from the screen also had inhibitory effect on the cells enriched with brain tumor stem cells. Searching and characterizing such chemical probes will lead to better understanding of NSC biology and ultimately brain disease therapeutics.

Phylligenin is a lignan that is isolated from the fruits of *Forsythia koreana* and *Oropheae eneandra* Bl (Cavin *et al*., 1998; Lim *et al*., 2008). Phylligenin is reported to have anti-inflammatory activity by inhibiting prostaglandin E2 and nitric oxide production (Lim *et al*., 2008). Phylligenin also exhibited antioxidative and radical scavenging properties as determined by carotene bleaching and DPPH radical scavenging (Cavin *et al*., 1998).

In this report, we investigated the effect of phylligenin on rapidly proliferating NPCs using primary culture of rats. Phylligenin inhibited proliferation of NPCs, and it was mediated by cell cycle regulation. Phylligenin also repressed the growth of glioma cell lines, indicating its potential therapeutic value in brain cancer.

MATERIALS AND METHODS

Cell cultures

Neural progenitor cell culture was prepared from E16 embryo Sprague Dawley (SD) rat as reported previously (Benoit *et al*., 2001; Conti *et al*., 2005). In brief, cortices was dissociated into single cells by pipetting several times and passed through 40 μm cell strainer (BD bioscience, Franklin Lakes, NJ). Dissociated single cells were incubated with Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) supplemented with 20 ng/ml EGF (Millipore, Billerica, MA) and 10 ng/ml bFGF (Invitrogen) in CO2 incubator. The cells grew into floating neurosphere and then dissociated into single cells with trypsin-EDTA (Invitrogen) and re-grew into neurosphere in EGF and FGF containing media. This procedure was repeated once again and neurosphere colonies were dissociated again into single cells for further experiments.

Cells were differentiated by plating them onto poly-L-ornithine (Sigma, Saint Louis, MO)-coated plate with DMEM/F12 media containing 2% penicillin/streptomycin without addition of any growth factors or serum. The cells were allowed to differentiate for 10 days at 37°C in a humidified atmosphere CO_2 incubator. Media was changed every third day. C6 glioma cells and U87MG cells were obtained from American Type Culture collection (Manassas, VA) and cultured in DMEM/F12 supplemented with 10% FBS and 2% penicillin/streptomycin at 37°C in 5% CO_2 incubator (Shin *et al*., 2002).

Primary neuron culture was prepared from E16 embryo SD rat according to previously published procedure (Shin *et al*., 2001). In brief, cortices was dissociated into single cells by incubating with trypsin and the cells were incubated for 14 days with Neuro-basal medium (Invitrogen) supplemented with B27 in 5% CO_2 incubator. Rat primary astrocytes were prepared from postnatal days 2 SD rat as previously described (Shin *et al*., 2002). Cortices was dissociated into single cells by incubating with trypsin and the cells were incubated for 14 days with DMEM/F12 supplemented with 10% FBS in 5% CO_2 incubator.

Western blot

Western blot was performed according to previously published procedure (Shin *et al*., 2002). Separation of protein bands was performed using 8% SDS-polyacrylamide gel electrophoresis and proteins were electrically transferred onto nitrocellulose transfer membranes (Whatman, Hahnestraβe, Dassel, Germany). The membranes were blocked with polyvinyl alcohol diluted in water (1 μg/ml). Membranes were then incubated overnight with the antibody directed against Tuj-1 (1:5,000, Covance, Prinston, NJ), GFAP (1:2,000, Dako, Carpinteria, CA), Nestin (1:2000, Millipore), cyclin E (Santa Cruz, CA), or CDK2 (Santa Cruz). The membranes were washed three times with PBS-Tween (0.2% Tween-20) for 10 min each, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 2 hours at room tem-

perature. The membranes were developed with enhanced chemiluminescence solution (Amersham, Buckinghamshire, UK) according to manufacturer's instructions. The signal intensity of blots was analyzed using 'Image J' (National Institutes of Health, Bethesda, MD) software.

Immunofluorescence microscopy

NPCs or differentiated cells cultured on cover glass were fixed with 4% paraformaldehyde at 37°C for 15 min. The cells were treated with 0.3% Triton X-100 for 10 min at room temperature and blocked for 30 min with blocking buffer (1% BSA (Sigma), 5% FBS in PBS) at room temperature. The cells were incubated overnight at 4°C with primary antibodies against Tuj-1, GFAP, nestin or anti-BrdU (1:500, Abcam, Cambridge, UK). After washing three times with washing buffer (0.1% BSA, 0.5% FBS in PBS), secondary antibodies conjugated either TMRE (1:500, Molecular probes, Eugene, OR) or FITC (1:500, Molecular probes) were diluted in blocking buffer and incubated for 2 hours at room temperature. After washing three times with washing buffer, the cover glass were mounted in Vectashield (Vector, Burlingame, CA) and visualized with a confocal microscope (Leica, Wetzlar, Germany).

MTT assay

The viability of cells were measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. NPCs were incubated for 20 min with 500 μg/ml MTT reagent (Sigma) in the dark. After incubation, medium was removed and the formazan dye was extracted using 100% ethanol. The absorbance at 590 nm was read using a microplate reader (Spectrafluor, Tecan, Switzerland).

BrdU incorporation

Proliferation of NPCs was determined using cell proliferation ELISA, BrdU (colorimetric) kit (Roche, Mannheim, Germany) according to manufacturer's instructions. After treated with phylligenin, NPCs were grown in 96-well plate and incubated for 24 hours with EGF, bFGF and 10 μM of BrdU labeling solution. BrdU labeling solution was removed, and cells were fixed for 30 min at room temperature. Fixative was washed away and 100 μl of anti-BrdU solution was added for 90 min. After washing with PBS for three times, colors were developed using anti-BrdU-POD solution and incubated for 10-30 minutes at room temperature. After the color development, the reaction was stopped with 1N HCl (100 μl/well) and the absorbance was read using microplate reader at 450 nm. In some cases, proliferation of NPCs was determined using BrdU immunofluorescence. After phylligenin treatment, cells were incubated for 24 hours with 10 μM of BrdU labeling solution. After removal of BrdU labeling solution, immunostaining against BrdU was performed as described above.

Flow cytometry

Cell cycle characteristic of NPCs was analyzed by FACS. NPCs were plated on 6 well plate and were incubated with phylligenin for 48 hours in the presence of EGF and bFGF. Plated single cells were trypsinized and resuspended in PBS with 1% FBS. Samples were centrifuged at 3,000 rpm for 5 min and supernatant was removed as completely as possible without disturbing the pellet. Resuspended cells were fixed with 70% ethanol in PBS and incubated overnight at 4°C. After centrifugation at 3,000 rpm for 5 min, supernatant was removed and cells were incubated with 50 μg/ml propidium iodide (Sigma), 100 μg/ml ribonuclease A (Sigma) in 500 μl PBS. Cells were kept at 37°C, protected from the light for 30-40 min prior to analysis. The incubated cells were analyzed with BD FACS Caliber (BD Biosciences).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The commercially available death detection kit (Invitrogen) was used to detect DNA fragmentation. Cultured NPCs on cover glass (Fisher Scientific, Pittsburgh, PA) were washed with PBS and fixed for 20 min in 4% paraformaldehyde solution at room temperature and blocked for 30 min with blocking buffer (1% BSA, 5% FBS in PBS) at room temperature. The cells were incubated with the TUNEL reaction mixture (20% reaction buffer, 1.5% TdT enzyme, 16% BrdU in 50 μM dH_2O) for 90 min at 37°C and washed 3 times with PBS. Samples were labeled with Alexa Flour 488 dye-conjugated anti-BrdU antibody for an additional 30 min. After washing and mounting with Vectashield, samples were visualized with a confocal microscope (Leica).

Statistical analysis

Data are expressed as the mean ± standard error of mean (S.E.M.) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Newman-Keuls test as a *post hoc* test and a p-value < 0.05 was considered significant.

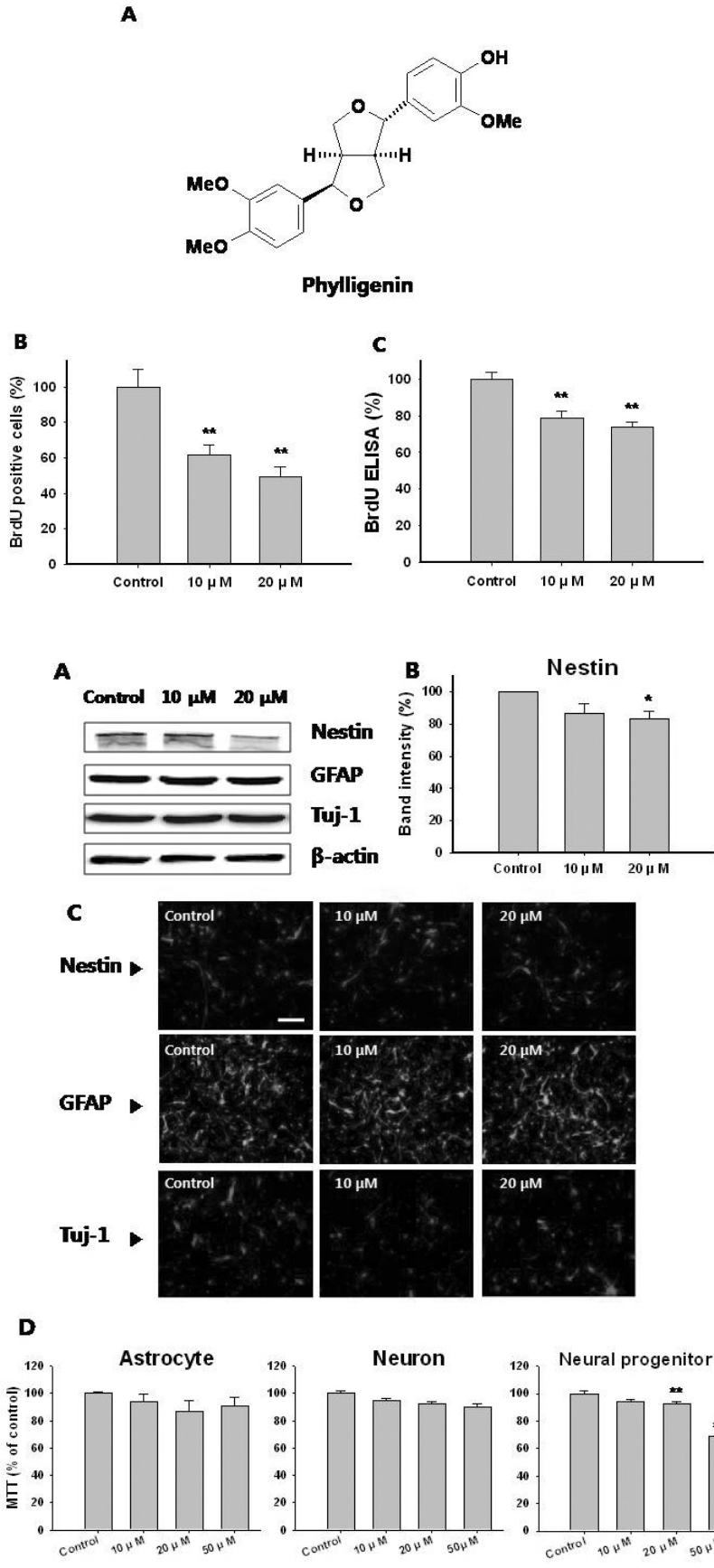

Fig. 1. (A) Chemical structure of phylligenin. Effect of phylligenin on NPCs. (B) NPCs were treated with phylligenin for 24 hours and incubated with BrdU labeling solution. Labeled cells were immunostained and visualized as described in Materials and Methods. BrdU positive cells were counted in immunostained cells at randomly selected fields. Data were mean ± S.E.M of six independent experiments. (C) BrdU incorporation was measured by ELISA (n=3). Data were mean ± S.E.M. $^{**}p < 0.01$ compared with controls.

Fig. 2. Effect of phylligenin on NPCs differentiation and cell viability. NPCs were treated with phylligenin for 24 hours and subject to differentiation for 5 days. Expression of specific markers for NPC (nestin), astrocytes (GFAP), and neuron (Tuj-1) in differentiated NPCs were examined using Western blot (A, B) and immunocytochemistry (C). Scale bar: 100 μm. n=3. (D) Cell viability of NPCs, mature astrocyte or neuron was determined by MTT analysis 24 h after phylligenin treatment. Data were mean ± S.E.M. of six independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

Effect of phylligenin on the growth of NPCs

During the search of chemical modulators affecting proliferation of NPCs, phylligenin was found to inhibit NPC proliferation. Treatment of phylligenin, a lignan found in *Forsythia koreana* (Fig. 1A), resulted in the decrease of NPC proliferation as determined by BrdU incorporation. Incorporation of BrdU was measured by way of both counting BrdU positive cells (Fig. 1B) and BrdU ELISA (Fig. 1C) as described in Materials and Methods. 10 μM and 20 μM of phylligenin reduced BrdU incorporation concentration- dependently from 78% to 73% (Fig. 1C).

Effect of phylligenin on the differentiation of NPCs

NPCs were subjected to differentiation for five days following phylligenin pretreatment. Expression level of differentiation marker proteins was measured using Western blot and immunostaining (Fig. 2). Nestin is a marker of stem cells, and the level was decreased by phylligenin treatment. Quantification of Western blot signal showed concentration-dependent and statistically-significant reduction of nestin level by 14% (Fig. 2B). Immunofluorescence staining also exhibited reduced number of cells expressing nestin (Fig. 2C). The results are consistent with the growth inhibition (Fig. 2B, C) of NPCs by phylligenin.

GFAP and Tuj-1 are markers for astrocytes and neurons, respectively. The level of GFAP and Tuj-1 was not changed by phylligenin treatment as determined by Western blot and immunofluorescence (Fig. 2A, C). Consistent with the decreased BrdU incorporation (Fig. 1B, C), 50 μM phylligenin decreased NPCs proliferation as determined by MTT analysis (Fig. 2D). In contrast, when primary cultures of astrocytes and neurons were exposed to phylligenin up to 50 μM, the viability of cells was not changed significantly (Fig. 2D). These data indicate that phylligenin inhibits neither differentiation process nor the viability of fully differentiated brain cells. Only proliferation of NPCs was affected by phylligenin treatment.

Effect of phylligenin on the cell cycle and apoptosis

To investigate the inhibitory mechanism on NPC proliferation, we examined the effects of phylligenin on cell cycle distribution. Plated single cell was incubated for 24 hours with phylligenin and cell cycle profile was determined by FACS analysis. In control NPCs, G_0/G_1 phase cells constitute 61% of total cells, and S phase cells were 22% and M phase cells were 17%. Phylligenin treatment induced G_1 phase arrest of NPCs. Cell population in S phase and G_2/M phase was decreased by 23 ± 7% and 20 ± 5%, respectively (Fig. 3B, C). And slight increase of cells in G_0/G_1 phase was observed with phylligenin treatment (Fig. 3A). Expression of cyclin E, cyclin-dependent kinases (CDKs), and CDK inhibitor (CKI) p21 was examined by immunoblot analysis (Fig. 3D). Dose-dependent increment of p21 level was observed by phylligenin stimulation. Phylligenin, however, did not induce change of CDK2, CDK4, and cyclin E expression. These results suggest that phylli-

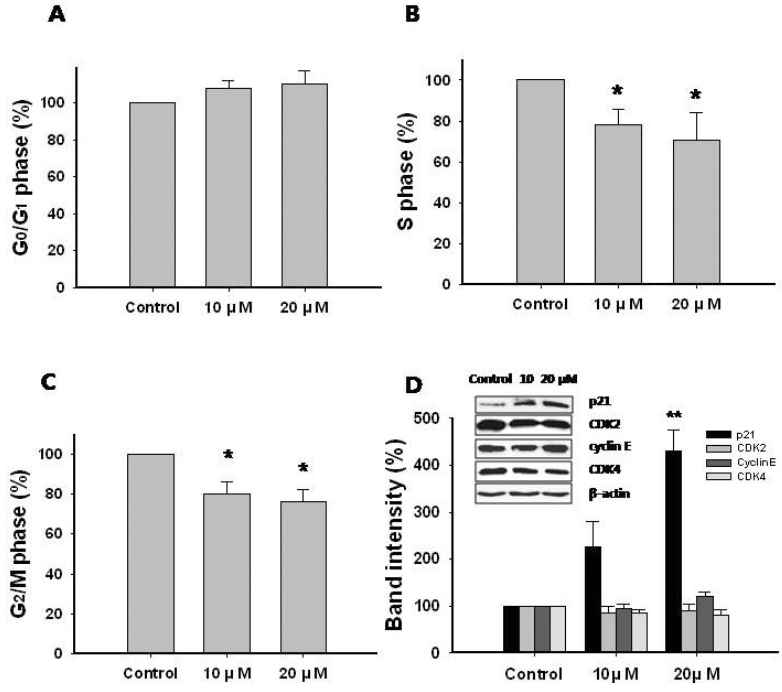

Fig. 3. Effect of phylligenin on cell cycle characteristics of NPCs. NPC cells were treated with phylligenin for 24 hours and stained with propidium iodide. Then cells were analyzed using FACS as described in Materials and Methods. Changes of NPC numbers by phylligenin in G_0/G_1 phase (A), S phase (B) and G2/M phases (C) were expressed as percent of control. (D) Western blot analyses of cell cycle regulators. Data were mean ± S.E.M. *$p < 0.05$ compared with controls. n=3.

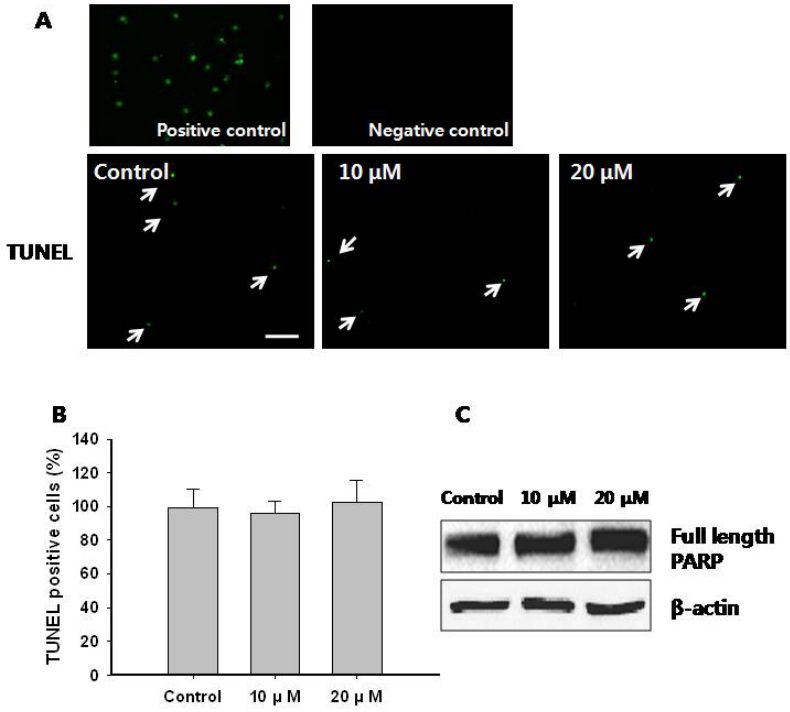

Fig. 4. Effect of phylligenin on the apoptosis of NPCs. NPCs were treated with phylligenin for 24 hours and apoptosis was measured using TUNEL assay (A, B). In (A), white arrows indicate cells undergone apoptosis. TUNEL positive cells were counted in immunostained cells at randomly selected fields. Quantitative analysis of TUNEL positive cells was shown in (B). Data were mean ± S.E.M. of ten independent experiments. Positive cells and negative cells were determined with fixed human lymphoma cell line. Scale bar: 100 μm. In (C), the level of full length of PARP was determined by Western blot.

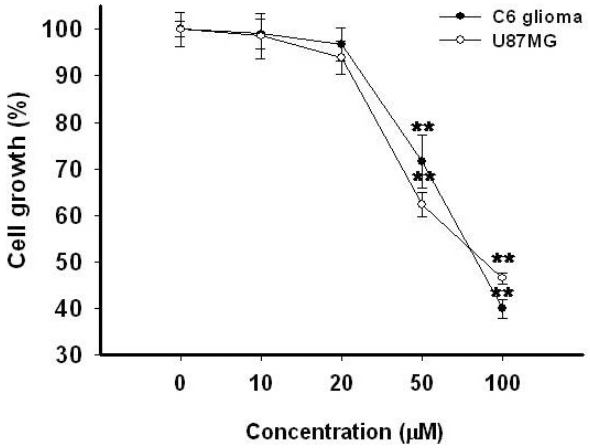

Fig. 5. Effect of phylligenin on the proliferation of glioma cell lines. Viability (cell growth) of C6 rat glioma cells (black circle) and U87MG human glioblastoma cells (white circle) was measured using MTT assay. Data were mean ± S.E.M. $^{**}p < 0.01$ compared with controls. n=5.

genin inhibit G_1 phase progression through up-regulating CKI p21 (Sherr and Roberts, 1999).

In contrast, phylligenin did not affect apoptosis of NPCs, as determined by TUNEL assay (Fig. 4A, B) and full-length PARP expression level (Fig. 4C). TUNEL positive cells were less than 10% of total cells in control group, and the number of positively stained cells was not changed by phylligenin treatment. Expression level of full-length PARP was examined and no change was observed (Fig. 4C). Thus, phylligenin does not seem to induce PARP cleavage, another marker for apoptosis. Taken together, phylligenin inhibits proliferation of NPCs not by apoptosis, but by cell cycle arrest.

Effect of phylligenin on the growth of glioma cell lines

Finally, we investigate the effect of phylligenin on the viability of two glioma cell lines using MTT assay (Fig. 5). The proliferation of C6 rat glioma and U87MG human glioblastoma cell line was inhibited by phylligenin. The cell growth was decreased up to 46 ± 1% in C6 cells and 38 ± 3% of control level in U87MG cells. These results implicate that phylligenin exhibits anti-tumor activity probably through repressing stem cell-like population in glioma cell lines (Singh *et al*., 2003).

DISCUSSION

In this study, we examined the effects of phylligenin on the growth of NPCs. We found that the proliferation of NPC is inhibited by phylligenin without effects on the differentiation of NPC as well as the viability of already differentiated cell (Fig. 1 and 2). Given that no effect was observed in mature astrocytes or neurons by phylligenin treatment, this study indicates phylligenin is selective only to rapidly proliferating cells.

Although the proliferation of NPCs was decreased by

phylligenin, the total number of differentiated neuron and astrocytes remains same as compared with control suggesting the differentiation of neural cells from NPCs was increased by phylligenin, thereby offset the effects of phylligenin on the decrease of total NPCs number. The selective effects of phylligenin on NPCs proliferation without affecting the number of differentiated neural cells might be beneficial for the potential use of phylligenin for therapeutic purposes.

Reduced proliferation can result from inhibited cell cycle progression or increased apoptosis. As shown in Fig. 3 and Fig. 4, phylligenin inhibited NPC proliferation through cell cycle regulation without affecting apoptotic cell death of NPCs. This is consistent with the observation that phylligenin only affect rapidly proliferating cells. Phylligenin induced G1 phase arrest by upregulating CKI p21 protein (Fig. 3). Induction of p21 inhibits activity of cyclinE/CDK2 complexes, resulting in block of G_1-S transition (Weinberg and Denning, 2002). It remains to be elucidated the mechanism how phylligenin increases p21 level in NPCs.

Phylligenin is one of the lignans isolated from *Forsythia koreana* and *Orophea enneandra* BL, which is a small tree distributed throughout Korea as well as Java, Indonesia (Cavin *et al*., 1998). Eudesmin and epieudesmin were isolated together with phylligenin in *Orophea enneandra* BL, and they have similar chemical structures with phylligenin. Phylligenin, eudesmin, and epieudesmin all share antifungal activity against *Cladosporium cucumerinum* (Cavin *et al*., 1998). Thus, it will be interesting to investigate if eudesmin and epieudesmin have activity against NPC proliferation. Also it would be worthwhile to investigate whether further chemical modification of phylligenin improves its efficacy.

Brain tumor stem cells (BTSCs) are present as a small portion of brain cancer cell population and implicated in the maintenance of brain tumors (Vescovi *et al*., 2006). Increasing evidences suggest that property of BTSCs is similar to NPCs (Singh *et al*., 2004; Vescovi *et al*., 2006). Additionally, the risk of brain tumor was dependent on the size of SVZ where NPC were generated (Diamandis *et al*., 2007; Diamandis *et al*., 2009). It is estimated that risk of brain cancer was increased depending on the number of NPCs which is augmented by either increased survival or proliferation of transplanted NPCs. Traditional therapeutics are not very effective to BTSCs, thus it is presumed that BTSCs are the cause of drug resistance and relapses for brain tumor treatment. Therefore, development of agents targeting BTSCs is intriguing approach for eradicating brain tumors. NPCs and BTSCs share similar stem-like properties given that BTSCs have multipotency and self-renewal ability. Chemical modulators effective in NPCs may exhibit similar effects to BTSCs. Diamandis et al. reported that chemicals identified from screening for effects on neurosphere proliferation also inhibited proliferation of brain tumor cells enriched with cancer stem cells (Diamandis *et al*., 2007). In this study, phylligenin exhibited growth-inhibitory effect to C6 glioma and U87MG glioblastoma cells lines. C6 and U87MG cells are reported to contain stem-like cell population (Qiang *et al*., 2009; Zhou *et al*., 2009). These results implicate that phylligenin have a potential to be developed for anti-cancer drugs presumably through BTSC inhibition.

Screening effort to identify chemical entities regulating NPCs has been focused mainly on chemicals promoting neurogenesis. The first chemical modulator discovered as an inducer of neuronal differentiation from adult NPC origin was neuropathiazol (Warashina *et al*., 2006). Discovery of another small molecules inducing neurogenesis were followed (Saxe *et al*., 2007; Kim *et al*., 2008; Schneider *et al*., 2008). It is known that haloperidol induces neuron-specific differentiation in mouse model (Diamandis *et al*., 2009). In addition, bone morphogenic proteins (BMP) inhibit brain cancer by inducing differentiation of BTSCs (Piccirillo *et al*., 2006). Small molecules inhibiting proliferation, however, were not reported as much as the ones promoting neurogenesis. Only Diamandis *et al.* reported chemical probes repressing proliferation of neurospheres (Diamandis *et al*., 2007), which might acts via neurotransmission pathways. To our knowledge, this study is the first publication reporting chemical inhibitor of NPC cell cycle progression. Neuromodulator or small molecule which regulates proliferation or differentiation of NPC might be useful as a therapeutic agent against brain cancer and adjuvant during NPCs transplantation. With in depth mechanistic studies focusing on the biological properties as a stem cell regulator, chemical probes like phylligenin will be very useful in brain cancer therapeutics as well as NPC biology.

ACKNOWLEDGMENTS

This work was supported by Konkuk University (S.H. Park).

REFERENCES

Benoit, B. O., Savarese, T., Joly, M., Engstrom, C. M., Pang, L., Reilly, J., Recht, L. D., Ross, A. H. and Quesenberry, P. J. (2001). Neurotrophin channeling of neural progenitor cell differentiation. *J. Neurobiol.* **46**, 265-280.

Cavin, A., Potterat, O., Wolfender, J. L., Hostettmann, K. and Dyatmyko, W. (1998). Use of on-flow LC/1H NMR for the study of an antioxidant fraction from Orophea enneandra and isolation of a polyacetylene, lignans, and a tocopherol derivative. *J. Nat. Prod.* **61**, 1497-1501.

Conover, J. C. and Notti, R. Q. (2008). The neural stem cell niche. *Cell Tissue Res.* **331**, 211-224.

Conti, L., Pollard, S. M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q. L., Cattaneo, E. and Smith, A. (2005). Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS. Biol.* **3**, e283.

Diamandis, P., Sacher, A. G., Tyers, M. and Dirks, P. B. (2009). New drugs for brain tumors? Insights from chemical probing of neural stem cells. *Med. Hypotheses* **72**, 683-687.

Diamandis, P., Wildenhain, J., Clarke, I. D., Sacher, A. G., Graham, J., Bellows, D. S., Ling, E. K., Ward, R. J., Jamieson, L. G., Tyers, M. and Dirks, P. B. (2007). Chemical genetics reveals a complex functional ground state of neural stem cells. *Nat. Chem. Biol.* **3**, 268-273.

Gage, F. H. (2000). Mammalian neural stem cells. *Science* **287**, 1433-1438.

Kim, S. J., Son, T. G., Park, H. R., Park, M., Kim, M. S., Kim, H. S., Chung, H. Y., Mattson, M. P. and Lee, J. (2008). Curcumin stimulates proliferation of embryonic neural progenitor cells and neurogenesis in the adult hippocampus. *J. Biol. Chem.* **283**, 14497-14505.

Lim, H., Lee, J. G., Lee, S. H., Kim, Y. S. and Kim, H. P. (2008). Anti-inflammatory activity of phylligenin, a lignan from the fruits of Forsythia koreana, and its cellular mechanism of action. *J. Ethnopharmacol.* **118**, 113-117.

Martino, G., and Pluchino, S. (2006). The therapeutic potential of neural stem cells. *Nat. Rev. Neurosci.* **7**, 395-406.

Piccirillo, S. G., Reynolds, B. A., Zanetti, N., Lamorte, G., Binda, E., Broggi, G., Brem, H., Olivi, A., Dimeco, F. and Vescovi, A. L. (2006). Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. *Nature* **444**, 761-765.

Qiang, L., Yang, Y., Ma, Y. J., Chen, F. H., Zhang, L. B., Liu, W., Qi, Q., Lu, N., Tao, L., Wang, X. T., You, Q. D. and Guo, Q. L. (2009). Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. *Cancer Lett.* **279**, 13-21.

Rishton, G. M. (2008). Small molecules that promote neurogenesis in vitro. *Recent Pat. CNS Drug Discov.* **3**, 200-208.

Saxe, J. P., Wu, H., Kelly, T. K., Phelps, M. E., Sun, Y. E., Kornblum, H. I. and Huang, J. (2007). A phenotypic small-molecule screen identifies an orphan ligand-receptor pair that regulates neural stem cell differentiation. *Chem. Biol.* **14**, 1019-1030.

Schneider, J. W., Gao, Z., Li, S., Farooqi, M., Tang, T. S., Bezprozvanny, I., Frantz, D. E. and Hsieh, J. (2008). Small-molecule activation of neuronal cell fate. *Nat. Chem. Biol.* **4**, 408-410.

Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501-1512.

Shin, C. Y., Choi, J. W., Jang, E. S., Ju, C., Kim, W. K., Kim, H. C., Choi, C. R., and Ko, K. H. (2001). Dehydroepiandrosterone inhibits the death of immunostimulated rat C6 glioma cells deprived of glucose. *Brain Res.* **922**, 267-275.

Shin, C. Y., Choi, J. W., Ryu, J. R., Ko, K. H., Choi, J. J., Kim, H. S., Lee, J. C., Lee, S. J., Kim, H. C. and Kim, W. K. (2002). Glucose deprivation decreases nitric oxide production via NADPH depletion in immunostimulated rat primary astrocytes. *Glia* **37**, 268-274.

Singh, S. K., Clarke, I. D., Hide, T. and Dirks, P. B. (2004). Cancer stem cells in nervous system tumors. *Oncogene.* **23**, 7267-7273.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J. and Dirks, P. B. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res.* **63**, 5821-5828.

Stiles, C. D. and Rowitch, D. H. (2008). Glioma stem cells: a midterm exam. *Neuron* **58**, 832-846.

Temple, S. (2001). The development of neural stem cells. *Nature* **414**, 112-117.

Vescovi, A. L., Galli, R. and Reynolds. B. A. (2006). Brain tumour stem cells. *Nat. Rev. Cancer* **6**, 425-436.

Warashina, M., Min, K. H., Kuwabara, T., Huynh, A., Gage, F. H., Schultz, P. G. and Ding, S. (2006). A synthetic small molecule that induces neuronal differentiation of adult hippocampal neural progenitor cells. *Angew. Chem. Int. Ed. Engl.* **45**, 591-593.

Weinberg, W. C. and Denning, M. F. (2002). P21Waf1 control of epithelial cell cycle and cell fate. *Crit. Rev. Oral Biol. Med.* **13**, 453-464.

Zhou, X. D., Wang, X. Y., Qu, F. J., Zhong, Y. H., Lu, X. D., Zhao, P., Wang, D. H., Huang, Q. B., Zhang, L. and Li, X. G. (2009). Detection of cancer stem cells from the C6 glioma cell line. *J. Int. Med. Res.* **37**, 503-510.