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Wogonin induces differentiation and neurite outgrowth of neural precursor cells

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ABSTRACT

Wogonin is a flavonoid isolated from *Scutellaria baicalensis* root, and has multiple pharmacological effects, including anti-inflammatory, anti-oxidant, and anti-cancer effects. It is also neuroprotective in the brain under many stress conditions, but wogonin does not elevate neuronal cell survival. Thus, the mechanisms controlling the neuroprotective effect of wogonin are not clear. Neural precursor cells (NPCs), present in the hippocampus and subventricular zone of adult brains, replace damaged cells. In this study we investigated the biological functions underlying the neuroprotective effect of wogonin on NPCs. We initially examined survival of NPCs but found it was slightly reduced at concentrations higher than 2 μg/ml. When we explored differentiation of NPCs into neuronal cells, the number of differentiated cells expressing neurofilaments was increased remarkably (fourfold) in the hippocampal NPCs treated with wogonin. Wogonin maximally elevated the expressions of presynaptic protein, synapsin I and postsynaptic protein (PSD95) at a concentration of 0.7 μg/ml. Differentiated cells containing longer neurites were significantly increased in cortical NPCs, primarily cultured from rat E14 embryonic brain. Wogonin also promoted differentiation of NPCs into mature neurons in vivo. When transplanted into the adult rat hippocampus, NPCs differentiated into cells expressing NeuN, the mature neuron marker, by 4 weeks after transplantation. These data indicate that wogonin induces differentiation of NPCs both in culture and in vivo, and suggest that facilitation of NPC differentiation is a biological activity by which wogonin protects neurons in damaged brain.

1. Introduction

Wogonin is a flavonoid component of *Scutellaria baicalensis Georgi*. It has been reported to have multiple pharmacological effects, including anti-inflammatory, anti-oxidant, anti-convulsant, and anti-cancer effects [1–4]. Several groups have shown that wogonin has neuroprotective effects through inhibition of the inflammatory activation of microglia in brain injury models and in a focal cerebral ischemia rat model [1,3]. Wogonin also suppresses NADPH-induced lipid peroxidation [5] and the oxidative stress in primary cultured rat cortical cells and in human neuroblastoma SH-SY5Y cells [6,7]. Elevation of neuronal cell survival has not been observed in neuronal cells under normal conditions. In contrast, it has been reported that wogonin increased release of LDH and the reduct ion of MTT in primary cultured rat brain cells, suggesting that wogonin has detrimental effects on neurons [5,8]. Recent studies have shown that wogonin has a potential effect on cell cycle arrest in leukemic cells [9,10].

Recently, neural precursor cells (NPCs) have been reported in adult brains. These NPCs can self-renew and replace neurons in a damaged area in mammalian brains, including those of humans and rodents. For regeneration of damaged or diseased areas, promotion of NPC survival and differentiation into the neuronal cells are required in the damaged area and may be necessary for application of cellular therapeutic treatments. Because the potential biological effects of wogonin on NPCs have not yet been explored, we investigated the effects of wogonin on cell survival and differentiation in hippocampal precursor cells, HiB5 and cortical NPCs primarily cultured from rat E14 embryos. We also examined whether the NPCs treated with wogonin prior to transplantation into the adult rat brain can be efficiently differentiated into neuronal cells.

2. Materials and methods

2.1. Primary cultures of cortical precursor cells

Cortical NPCs were isolated from the rat E14 embryonic brain as described previously [11]. Time-pregnant Sprague–Dawley rats were purchased from Orient Co., Ltd., a branch of Charles River Laboratories (Gyunggi-do, Korea). Briefly, dorsal cortex were

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dissected from embryonic forebrain aseptically, dissociated mechanically in Ca^{2+}/Mg^{2+}-free HBSS (Invitrogen) and then plated at 19,000 cells/cm^2 on 35 mm dishes pre-coated with 15 μg/ml poly-L-ornithine and 1 μg/ml fibronectin (Sigma–Aldrich). Cells were cultured in serum-free N2 media supplemented with 10 ng/mL bFGF (Invitrogen) in 5% CO_2 for 3 days.

For immunostaining assay, NPCs were passaged using 0.05% trypsin–EDTA on 12 mm glass coverslips (Bellco) and grown in N2 media with bFGF for an additional 1 day. They were grown for 4 days more without FGF and with or without wogonin (0.1–0.7 μg/ml, Calbiochem) and PDGF-BB (20 ng/ml, Invitrogen). NPCs were immunostained with anti-neurofilament 200 antibodies (1:1000, Sigma–Aldrich, [12]).

2.2. Hippocampal precursor cell culture

Hippocampal precursor cells, HiB5 are immortalized by the temperature sensitive SV40 large T antigen [13]. They proliferate at a permissive temperature, 33 °C in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Hyclone), and express nestin, a stem cell marker.

For cell viability assay, HiB5 cells (8×10^3) were plated in serum containing media for 48 h at 33 °C and changed to serum-free N2 medium at a nonpermissive temperature of 39 °C, where they stop growing and only 30∼40% of them are able to survive without survival factor by 2 days. Wogonin (0.05–3 μg/ml) and PDGF (20 ng/ml) were added for 2 days and the cell viability was measured using an MTT assay, as previously described [14].

Immunoblotting assay was carried out as previously described [15]. The primary antibodies used were anti-neurofilament (1:1000, Covance), anti-PSD95 (1:500, Chemicon), anti-Synapsin Ia (1:200, Chemicon), and anti-β tubulin (1:1000, Sigma) antibodies.

2.3. Animals and transplantation of NPCs into the rat brain

Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85–23, revised 1985) and the Animal Care and Use Guidelines of Kyung hee University, Korea.

Male SD rats (250–270 g, Orient Co.) were anesthetized with Equithensin (2 ml/kg), aligned in the stereotaxic device (Stoelting). For identification within the host tissue, HiB5 cells were labeled by incubation with a fluorescence dye, DiI-C18-(3) (Molecular probes Inc.) one day before transplantation, followed by exposure to wogonin for 2 h prior to transplantation.

Two microliters of HiB5 cell suspension (at a density of 6.0×10^4 cells/μl) in saline containing 0.7% penicillin/streptomycin, 20 mM hepes (pH 7.2) and 0.5% glucose was transplanted

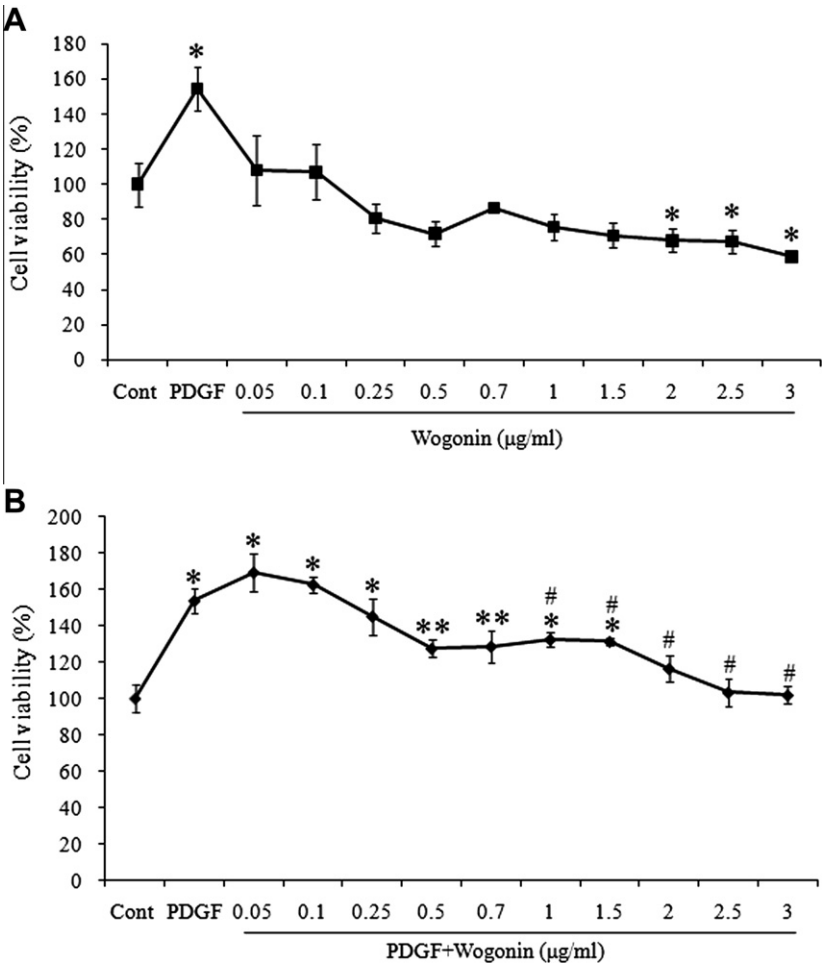

Fig. 1. Effect of wogonin on the cell survival of neural precursor cells, HiB5 and the differentiated HiB5 cells. (A) Wogonin (0.05–3 μg/ml) was added to hippocampal precursor cells, HiB5. PDGF-B (20 ng/ml) was added to elevate cell viability as a positive control. (B) Wogonin (0.05–3 μg/ml) was added to differentiated Hib5 cells induced by PDGF. PDGF (20 ng/ml) was added to N2 serum free medium for 2 days to induce differentiation. Cell viability was measured by the MTT assay and normalized to the control. Data from five independent experiments were subjected to statistical analysis. Shown are means ± S.E.M. $^{*}P < 0.05$ and $^{**}P < 0.005$ compared with the control. $^{\#}P < 0.05$ compared with the PDGF alone.

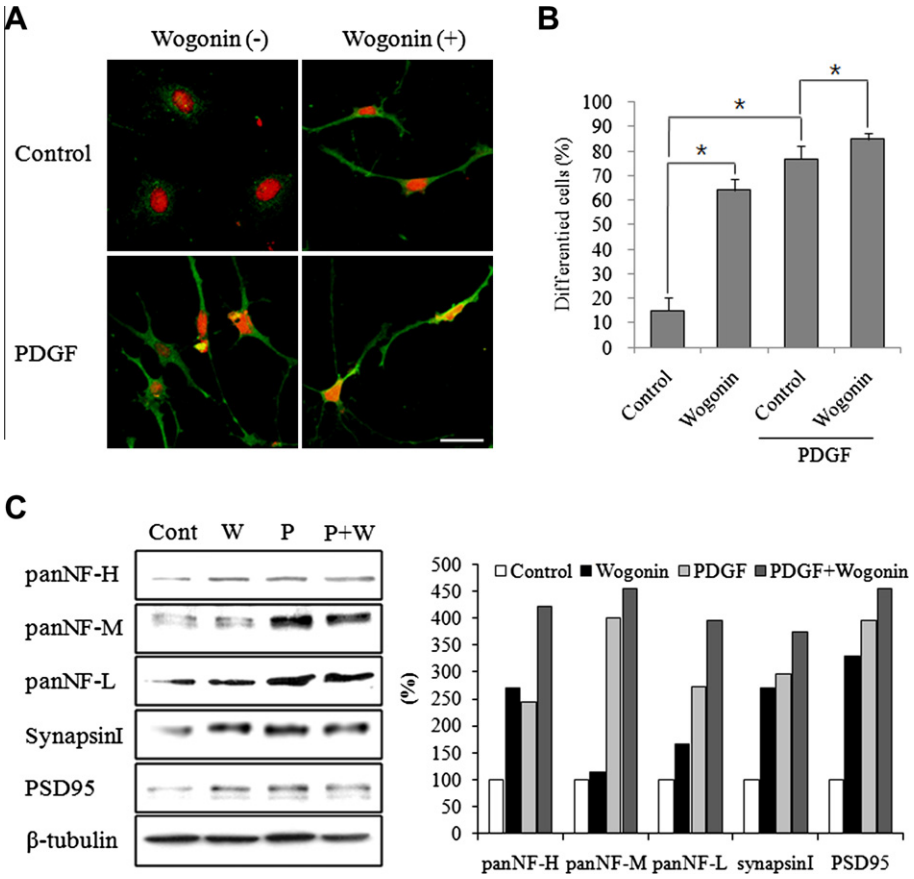

Fig. 2. Promotion of the differentiation of HiB5 cells by addition of wogonin. (A) The differentiation of HiB5 cells into neuronal cells was assessed by immunocytochemical staining with anti- pan-neurofilament antibodies (green). Differentiation of HiB5 cells was induced in the presence of wogonin (0.7 μg/ml) and/or PDGF for 2 days. PDGF (20 ng/ml) was added as an inducer of differentiation. Nucleus was counterstained with propidium iodide (red). Scale bar = 50 μm. (B) Quantification of pan-neurofilament immunoreactive cells. Data from five independent experiments were subjected to statistical analysis. Shown are means ± S.E.M. $^{*}P < 0.05$ compared with the control. (C) Increased expression of neuronal marker proteins by addition of wogonin and/or PDGF. Differentiation of HiB5 cells was induced with and without PDGF (20 ng/ml) for 2 days and analyzed by immunoblotting analysis. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

1.4 mm lateral to midline, 4.3 mm posterior to bregma, and 2.2 mm below the dura using automatic nano-microinjector (Stoelting) at a pressure of 10 psi as described previously [13,15].

For immunohistochemical analysis, the brains were removed following perfusion and immersion fixed with 4% paraformaldehyde, and then cryo-sectioned through the coronal plane at a thickness of 35 μm. After immunostaining as previously described [16], the brain slices were scanned under a confocal laser scanning microscope.

2.4. Quantification of data and statistical analysis

Immunoreactive cells which had neurites longer than double the cell body width were counted from 9–12 confocal microscopic fields (the area = 211.6 mm^2). The statistical analyses of the data were performed by one-way analysis of variance and $P < 0.05$ was taken as significant.

3. Results

3.1. Cell viability of either neural precursor cells or mature cells was not increased by wogonin

To investigate whether wogonin facilitates survival of neural precursor cells (NPCs) compared to the differentiated mature neurons, we measured cell viability of hippocampal precursor cells, HiB5. HiB5 cells were isolated from an embryonic day-16 rat hippocampus, where progenitors of pyramidal cells begin to proliferate [17]. We cultured HiB5 cells in serum containing medium for 48 h and changed to serum-free N2 medium to evaluate viability of NPCs. We added PDGF BB as a positive control for survival and differentiation, one day after changing to N2 media. We have previously shown that PDGF induces the differentiation of HiB5 precursor cells into neuronal marker expressing cells with neurite structures [18]. Wogonin (0.05–3 μg/ml) was added to the N2 medium for 2 days and cell viability of both proliferating and differentiated HiB5 cells was measured using an MTT assay (Fig. 1). The viabilities of both HiB5 cells were slightly higher at wogonin concentrations of <0.1 μg/ml but were reduced at concentrations between 0.25–3 μg/ml to 68.06 ± 6.5% for proliferating HiB5 and to 75.32 ± 7.3% for differentiated HiB5 cells. These results suggest that wogonin did not act as a survival factor for either proliferating NPCs or the differentiated neuronal cells.

3.2. Wogonin induced neuronal differentiation of hippocampal precursor cells

We next explored whether wogonin is able to promote differentiation of the surviving HiB5 cells. We chose concentrations of wogonin, (0.1–2 μg/ml) for differentiation experiments, based on results from the viability assay. After HiB5 cells were treated with or without wogonin in serum-free N2 medium for 2 days, a presynaptic protein, synapsin I and postsynaptic protein, PSD95 as well as a neuron marker, neurofilaments were analyzed using

an immunoblotting assay. The marker proteins were all increased by treatment with wogonin (Supplementary Fig. 1). Neurofilament expression was highest at a concentration of 0.7 μg/ml among other concentrations (0.1 and 2 μg/ml), while synapsin I and PSD95 gave similar results at concentrations of 0.1 and 0.7 μg/ml. The expression of these proteins, however, was reduced at wogonin concentration of 2 μg/ml. Thus, we chose a wogonin concentration of 0.7 μg/ml, (2.46 μM), as the optimal concentration for further experiments.

We then examined differentiation of HiB5 cells by immunocytochemical assay using anti-neurofilament antibodies (green), and the nuclei were counterstained with propidium iodide (red, 1 μg/ml). In N2 medium, untreated HiB5 cells showed a poor rate of differentiation. With addition of wogonin (0.7 μg/ml), many surviving HiB5 cells showed morphological changes characteristic of neuronal cells, such as neurite outgrowths (Fig. 2). Wogonin also up-regulated the expression of neurofilaments, the marker for differentiated neuronal cells. Wogonin increased the number of neurofilament expressing cells remarkably (0.7 μg/ml, 64.01 ± 4.8%, >4.3-fold) compared to the control (14.89 ± 5.3%, Fig. 2B), suggesting that wogonin alone can induce neuronal differentiation of hippocampal precursor cells.

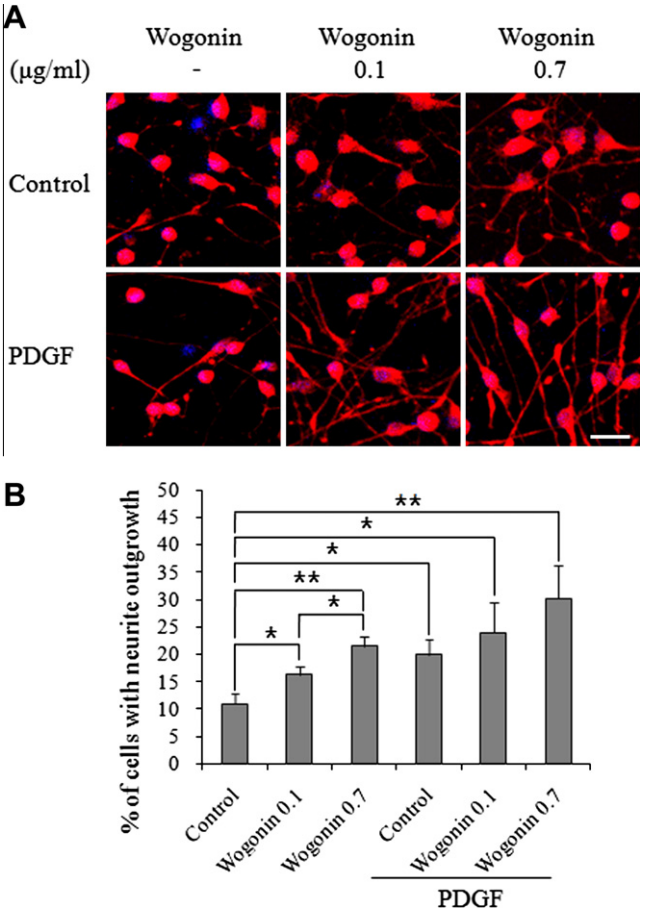

Fig. 3. Neurite outgrowth of cortical precursor cells primarily cultured from the rat E14 embryo, promoted by wogonin. (A) The differentiation of cortical precursor cells was assessed by immunocytochemical staining with anti-neurofilament 200 antibodies (red). Neurite outgrowth was induced in the presence of wogonin (0.1–0.7 μg/ml) and/or PDGF in serum-free N2 medium for 4 days. Nucleus was counterstained with DAPI (blue). Scale bar = 20 μm. (B) Quantification of neurofilament 200-immunoreactive cells with neurites longer than twofold the cell body width. Data from three independent experiments were subjected to statistical analysis. Shown are means ± S.E.M. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with the control. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Most of the surviving HiB5 cells treated with PDGF (76.77 ± 5.6%) as a positive control differentiated into neuronal cells, producing neurite outgrowths and neurofilament protein (Fig. 2A). When the differentiation of HiB5 cells was previously induced by PDGF, wogonin slightly but significantly increased their differentiation rate (~11% more than cells treated with PDGF-alone; 0.7 μg + PDGF, 84.97 ± 2.5%). Dendritic spine like structures were more evident in the elongated neurite of the differentiated cells under treatment of both PDGF and wogonin.

When we compared the expression of neuron markers, the heavy chains, middle chains, and light chains of neurofilaments were all elevated by treatment with wogonin (0.7 μg/ml, Fig. 2C). Expressions of synaptic proteins, synapsin I and PSD95 were also up-regulated (2 to 3-fold by Image J analysis), indicating formation of more synapses. While PDGF addition also increased expression of all neurofilament chains, especially the middle chain, there was small further increase upon addition of both PDGF and wogonin. The upregulation of synaptic proteins was also a little, but not additive suggesting that wogonin and PDGF may share a common pathway for promoting differentiation of NPCs.

3.3. Wogonin promoted neuronal differentiation of cortical precursor cells cultured primarily from the rat embryonic brains

To further analyze the effect of wogonin on differentiation of NPCs, we cultured cortical NPCs isolated from the rat E14 embryos and evaluated cell numbers with elongated neurite. NPCs were cultured in N2 medium with bFGF (10 ng/ml) for 3 days and then grown for 4 days without bFGF and with or without wogonin. Upregulation of neurofilaments was confirmed in cortical NPCs by immunostaining with anti-neurofilament 200 antibodies (Fig. 3). The cells with longer neurite than double the cell body width were counted from 9 to 12 confocal microscopic fields and the resulting numbers were expressed as the percentage of differentiation. The percentage of differentiated cells was increased more than twofold in the wogonin treated groups (0.1 μg/ml, 16.29 ± 0.8%; 0.7 μg/ml, 21.46 ± 0.9%) compared with control (10.96 ± 0.9%). The neuronal differentiation was also evident in the cells treated with PDGF (19.91 ± 1.6%). When the differentiation was induced with both wogonin and PDGF (0.1 μg + PDGF, 23.9 ± 3.2%; 0.7 μg + PDGF, 30.3 ± 2.7%), the percentage of differentiated cells were, however, was only slightly increased compared to that for cells treated with PDGF alone, and the difference was not statistically significant. These results suggest that there is not a strong synergistic effect between PDGF and wogonin in cortical NPCs as well as hippocampal NPCs.

3.4. Wogonin promoted neuronal differentiation of NPCs in vivo when transplanted into the adult rat brain

To investigate whether wogonin facilitates the differentiation of NPCs in vivo as well as in cultured cells, HiB5 cells treated with or without wogonin were transplanted into the adult rat brain. For identification within the host tissue, HiB5 cells were labeled with a fluorescence dye, DiI-C18-(3), followed by exposure to wogonin for 2 h prior to transplantation. We transplanted HiB5 cell suspension into the medial aspect of the dorsal hippocampal formation, above the CA1 subfield using stereotaxic device and microinjector [13,15].

The clusters of cells at the injection sites started to migrate 1 week after transplantation. After 4 weeks in vivo, most dil stained cells just dorsal to CA1 region were migrating laterally along hippocampal alveus and a portion of the cells were perpendicularly migrating down from the alveus towards the pyramidal layers. To examine the expression of mature neuron markers in the transplanted cells, we immunostained the brain slices with anti-NeuN antibodies (1:1000; Chemicon), which recognize mature neurons

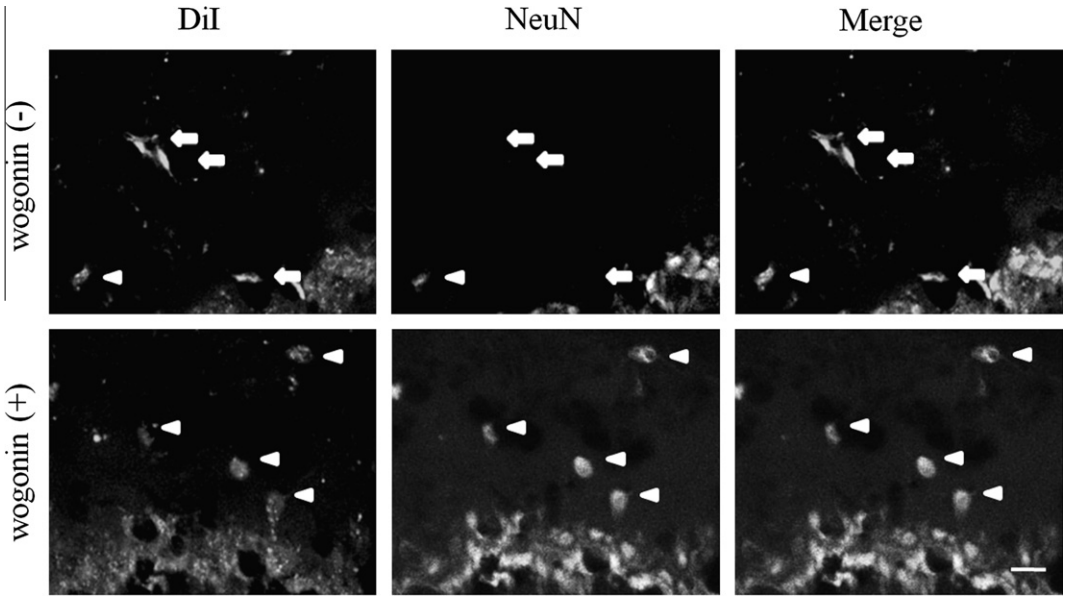

Fig. 4. Wogonin treated NPCs were differentiated into NeuN positive neurons in the adult rat brain. HiB5 cells (total 1.2×10^5 cells) were pre-incubated with or without wogonin (0.7 μg/ml for 2 h), labeled with DiI-C18-(3) (DiI, red), and then transplanted into the dorsal hippocampus of the adult rat brain. Brain slices were immunostained with mature neuron marker, NeuN (FITC, green) 4 weeks after transplantation and images were photographed under a confocal laser scanning microscope. DiI-positive neurons co-expressing NeuN and DiI-positive cells negative for NeuN are indicated by arrowhead and arrow, respectively. Scale bar, 10 μm.

and scanned under a confocal laser microscope (Fig. 4). DiI stained HiB5 cells were primarily found in the hippocampal CA1 subfield. The NeuN-positive mature neurons among the diI stained cells were found more in the brain slices from the rats transplanted with wogonin treated HiB5 cells than untreated cells. This observation indicates that wogonin accelerated the differentiation of NPCs into neuronal cells in the adult rat hippocampus.

4. Discussion

Wogonin has often been reported to have the opposite effects of apoptosis induction in tumor cells and inhibition of neural cell death in injured conditions [6,7,19,20]. Wogonin is found to have neuroprotective effects in brain injury models [1] and in excitotoxic neuronal damage [6]. We have also reported that administration of *S. baicalensis* extract including wogonin, baicalin and baicalensis improves learning and memory in ibotenic acid induced model rats and the extract increases cell survival and cholinergic neurons [15].

However, baicalin and baicalein from *S. baicalensis Georgi*, but not wogonin, can scavenge free radicals in cultured SH-SY5Y cells [5]. Wogonin is not effective at promoting survival of mature neurons [21] but is cytotoxic at high concentrations [8]. Our study also shows that wogonin does not promote survival of NPCs or mature neurons at concentrations up to 1 μg/ml and decreases survival at high concentrations (1–3 μg/ml). Therefore, based on the these observations, wogonin does not appear to elevate cell survival in mature neurons or NPCs under normal conditions and the neuroprotective effects of wogonin are not caused by increased neuronal cell survival.

Our observation showed that wogonin is able to induce the neuronal differentiation of hippocampal NPCs without addition of co-factors and efficiently promoted neurite outgrowth of cortical NPCs cultured primarily from the rat embryonic brains, as well as in hippocampal NPCs. Since many studies using SH-SY5Y neuroblastoma cells showed a neuroprotective effect of wogonin under stress conditions, we also tested the effect of wogonin on cell differentiation. Wogonin promoted the differentiation of SH-SY5Y cells and initiated the regrowth of their short neurites (Supplementary data). This result implies that wogonin is a differentiation inducer and may bind to target molecules to activate factors that transduce signals for neuronal differentiation.

Zhang and his colleagues investigated the mechanism of growth arrest induced by wogonin in human U937 leukemic cells and reported that wogonin stimulates phosphorylation of protein kinase C (PKC) δ, up-regulation of p21 proteins, and down-regulation of cyclin D1/cyclin-dependent kinase (CDK) 4 and p-Rb, which leads to induction of G1 phase arrest and granulocytic differentiation of U937 leukemic cells [9,10]. Rottlerin, an inhibitor of PKCδ, abolished the upregulation of p21, implying that phosphorylation of PKCδ induced upregulation of p21 proteins. PKCδ isoforms have been implicated its role in neuronal differentiation and neurite outgrowth in retinal ganglion cells [22] and in retinoic acid-induced neuronal differentiation of human NT2 cells [23]. Our previous experiments showed that PKC isoforms play a role in neuronal differentiation, and inhibition of PKCδ by rottlerin blocked the differentiation but not the survival of neural precursor HiB5 cells induced by PDGF ([14]; Lim and Kwon, data not shown). Taken together, wogonin may induce neuronal differentiation and cell cycle arrest probably by activating signal molecules, such as PKCδ and p21. The direct target molecules of wogonin are under investigation. Efficient differentiation to neurons may facilitate cell survival, regeneration and neuroprotection because differentiated neurons could form synapses to target cells and neurons require target-derived factors from the synapses for their survival.

High concentrations of wogonin unexpectedly decreased cell survival of both NPCs and differentiated mature neurons. Furthermore, wogonin, at concentrations of 0.7 μg/ml, additionally increased the differentiation rate of hippocampal precursor cells induced by PDGF more than that of cells treated with PDGF alone, while the same concentration of wogonin decreased the cell survival of differentiated cells. This implies that wogonin may regulate the survival and differentiation of HiB5 precursor cells via different pathways.

Endogenous adult NPCs are found in two areas of the adult mammalian brain, the subventricular zone and the subgranular

zone of the hippocampus. We transplanted HiB5 cells into the hippocampus, where endogenous NPCs develop and migrate. Previous transplantation studies using the HiB5 cell line in vivo [13,24] have shown that the immortalized cells behave as multipotent neural progenitors after transplantation into the developing and adult brains. These results indicate that the cell fate of transplanted precursors into both neuronal and glial cells may depend on microenvironmental signals in the mature brain. In our experiments, the number of NeuN-positive mature neurons was higher in rats that were transplanted with wogonin treated hippocampal precursor cells than with untreated cells (Fig. 4). This result implies that the effect of wogonin on differentiation of NPCs into neuronal cells does not depend on the environment of the mature adult brain. This also suggests that wogonin may promote neuronal differentiation of endogenous NPCs in damaged and diseased areas of adult brain and thereby facilitate regeneration of neurons.

In conclusion, wogonin induces differentiation of NPCs into neuronal cells and this may be a mechanism underlying the effect of wogonin on neuroprotection.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.098.

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