Effects of Ginsenoside Rg_1 on Neural Progenitors Proliferation *in Vitro* and *in Vivo*

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Abstract

We have already known, neural progenitor cells exist not only in the developing brain, but in certain spots in adult CNS in mammals, so it will be of great value to find out some compounds which can interfere these cells proliferation ability. In this research, we observed that ginsenoside Rg1 can not only enhance neural progenitors' proliferation ability in vitro, but increase neurogenesis in adult mouse dentate gyrus in vivo. Firstly, we set up neural progenitor cells' culture system from embryonic rats' hippocampus and prove their feature through immunocytochemistry. Then by using MTT assay , we found that when growing with ginsenoside Rg1 (0.5~2.5 μmol/l), the progenitor cells' survival rate nearly doubled, furthermore, we proved that this increase was due to the increment of cell proliferation through ^{3}H-thimidine incorporation assay, hence, we drew the first conclusion: ginsenoside Rg1 has the ability to stimulate neural progenitor cells' proliferation in vitro; in order to observe this compound's effect in vivo, we devised the following experiment: after administering ginsenoside Rg1 (5, 10 mg/kg, once a day) intraperitoneally for two weeks, we examine the number of BrdU positive cells in the dentate gyrus of mice, and found that Rg1 could increase the number of proliferation cells significantly in vivo. From these studies, we are quite sure about Rg1's effects on the proliferation ability of neural progenitor cells both in vitro and in vivo, certain targets of the compound and its underlying mechanisms are in progress.

Key words : ginsenoside Rg1, neural progenitor cells, proliferation, ^{3}H-thimidine, BrdU

Introduction

Being one of the hottest fields in neuroscience, stem cells in CNS have generated increased attention because they may offer a brand new prospective for the therapy of disorders in nervous

system. Here ,we may have two choices, firstly, we can isolate NSCs from fetal or adult brain, expand and engineer them in vitro and transplant them back to the injured brain; secondly, we can induce endogenous NSCs to proliferate and differentiate in vivo to achieve self-repair. In the last decade, both subfields have attracted a large number of investigators and both have promising results, especially in rodent[1-5]. But when it comes to human being, the first group met with several difficulties ranging from ethical concerns to limited supply, so it will be of great value to find out some compounds which can interfere progenitor cells proliferation ability in vivo.

Ginseng has been used as a tonic drug in chinese traditional medicine for over two thousand years. It has been proved to have various pharmacological actions on central nervous system. Ginsenoside Rg1 is one of the most important active ingredients in ginseng and shares many of its effects, such as facilitating learning ,memory and alleviating age related decline of functions. In this study, we explored Rg1's effects on neural progenitor cells' proliferating ability both in vitro and in vivo and got exciting results. These effects of this compound has important implications for its antiaging and nootropic actions.

Materials and Methods

Progenitor cell culture

The hippocampus was dissected from embryonic day 16(E16) to E18 rat embryo, minced into small fragments and incubated in 0.1% trypsin for 30 min at 37°C, after being triturated with glass Pasteur pipettes of narrowing diameter, the dissociated cells were centrifuged and resuspended in growing medium containing DMEM/F12 (1:1) medium supplemented with penicillin, streptomycin, glutamine and the synthetic mixture B27 (Life Technologies), in the presence of 20 ng/ml basic FGF (bFGF; Promega). The density of cells was maintained at $4\sim5\times10^5$/ml. Half of the medium was replaced every 3~4 days with appropriate fresh medium. For long time culture, neurospheres were collected after 6 or 7 days by centrifugation, dissociated with 0.1% trpsin and 0.02% EDTA, and resuspended in the same growing medium described above. The number of viable cells in each culture was determined by the Trypan blue exclusion technique.

Immunocytochemistry

Immunocytochemistry for nestin: Intact and healthy spheres were grown on poly-l-lysine-coated glass slides in growing medium supplemented with 1% fetal bovine serum overnight. In

the following day, the slides were rinsed with PBS and put into 4% PFA for at least 30 minutes. nestin monoclonal antibody (Chemicon) was diluted 1:100 and incubated with the cells for 2 hours at 37°C, then, the antigen was visualized either with FITC-anti-mouse-IgG(Zymed) under fluo-microscope or with SP kit (Zymed), which was developed using 3,3,diaminobenzidine (DAB) as chromogen. Meanwhile, the attached spheres also were detected for cell-specific antigens of mature neural cells: mouse monoclonal anti-β-Tubulin-(1:400) for neurons, mouse monoclonal anti-GFAP (1:400) for astrocytes and rabbit anti-Gal (1:50) for oligodendrocytes (all from Sigma). The protocol of immunostaining was similar to that of nesin immunolabelling. For oligodendrocyte staining, cells were stained for the cell-surface specific antigen Gal-C without permeabilization.

Differentiation assay: Individual neurosphere was dissociated mechanically with a fire-polished Pasteur pipet in 1.5 ml differentiation medium (DMEM/F12, 2%B27, 1%FBS, without bFGF), then, every 500l cell-suspension was plated in one well of 24-well plate containing poly-l-lysine-coated glass slides, half the medium was changed every 2 or 3 days, after 8 days differentiation, cell types were analyzed with the cell type-specific antibodies described above.

MTT assay

This assay was carried out in 96-well plates. In general, dissociated cells were seeded at 40Kcells per 100 μl of apropriate medium, following some days incubation, 25 μl MTT (5 mg/ml) was added to each well and the incubation continued for 4 hours at 37°C, then, 100 μl 20%SDS (sodium didecyl sulfate solution) was added and the plate was incubated at 37°C for a further 22 hours. The optical density was read at 540 nm on an ELISA plate reader.

Growing characters of neural progenitors: dissociated hippocampal cells were plated in DMEM/F12, DMEM/F12+B27 or DMED/F12+B27+bFGF medium respectively, after growing in the incubator for 3 or 6 days, the cells OD value were determined according to the protocol described above.

Rg1's effect on the progenitors

Rg1 with purity of 98% was provided by the department of organic chemistry . Bethune medical university. Dissociated cells from the secondary spheres were seeded with Rg1 (0.5~2.5 μM) or PBS in growing medium, On the 6th day, MTT was added in the medium and the OD value was read.

^{3}[H]Thymidine incorporation

Dissociated cells from the secondary spheres were seeded at a density of 4×10^{5}/ml/well in 24-well plate in growing medium (with ginsenoside Rg1 (0.5~2.5 μmol/l) and allowed to grow for 7 days, in the third day, half the medium was exchanged with fresh growing medium with appropriate Rg1, then, in the sixth day, 0.5 μCi/ml of ^{3}[H]Thymidine was added to each well and the assay was carried out 24 hours later. After being rinsed 3 times with PBS, the incorporated ^{3}[H]Thymidine was solubilized using 0.4 M NaOH for 1 hour at 37°C. This solution was then added to 4 ml of scintillation cocktail and counted using a scintillation spectrometer.

Animals and BrdU labelling

Adult male Kunming mice (24~28 g body weight) were used in the present study. The animals were given Rg1 (5, 10 mg/kg) or saline intraperitoneally once a day for 2 weeks (n=5), on the 14th day, 2 hours after the last drug administration, the mice received 100 mg/ml BrdU (sigma) intraperitoneally and were perfused transcardially with 4% paraformaldehyde in 0.01 M PBS (pH=7.4) within 24 hours.

Immunohistochemistry

After perfusion with 4% PFA, the brains were removed, cut into coronal blocks containing the hippocampus, immersion-fixed in the same fixative, then after being embedded in paraffin, coronal sections (12 μm) were cut on a microtome.

For BrdU immunolabeling, the sections were incubated in 0.1 M citrate buffer (pH=6.0) for 10min at 95°C, rinsed, incubated in 3% H_2O_2 for 10 min, rinsed, digested with trpsin (0.1%) for 10~20 min at 37°C, rinsed, denatured in 2 N HCL for 30 min, rinsed, blocked in 5% normal goat serum for 30 min, and incubated in mouse monoclonal antibody against BrdU (1:100; Zymed) at 4°C for 24 hours, the sections were then incubated with ingredients of SP Kit (commercial available; Zymed) according to manufacturer's protocol, and developed using DAB as chromogen.

As to data analysis. Every 10th coronal section was selected from each brain for a total 6~8 sections from the middle area of the hippocampus. Each microscope image was digitized, BrdU-labeled nuclei were counted, the density of BrdU immunoreactive cells in each section was calculated by dividing the number of BrdU-positive nuclei by the area of the DG (in mm^2). These values were averaged to obtain a mean density value of BrdU-positive cells per mm^2 for each

subject. Differences between the mean values from each treatment group were analyzed using one way AVONA.

Results

Proliferation of bFGF-responsive embryonic hippocampal progenitors and formation of neurospheres

Dissociated embryonic hippocampal cells were plated into 5 ml medium in 25 ml flask (Greiner, Germany). In control medium (growing medium minus bFGF), cells began to die soon after being planted, meanwhile, the surviving cells did not exhibit any proliferation; In growing medium, however, although a large number of cells died during the first 2 days, the surviving cells became hypertropic and began to divide (2 DIV; Fig. 1A); their progeny attached to each other tightly (3DIV; Fig. 1B) and formed a number of spherical cluster of cells, which became bigger and bigger with time going on (5 DIV & 7 DIV; Fig. 1C, 1D). Every 6~7 days, the spheres were dissociated into single cells and replated, new generation of spheres would come into being within 6~8 days.

On 3 days in vitro (DIV), the OD value of cells growing in D/F, D/F+B27 and D/F+B27+bFGF are 0.23±0.01, 0.38±0.01, 0.51±0.02; and on 6DIV, they are 0.31±0.04, 0.32±0.03, 1.04±0.23 respectively (Fig. 2). The data indicated that ① at beginning, B27 exerted as a trophic or survival factor, but with time going on, its effects became more and more trivial, and even indistinguishable on 6DIV; ② the OD value of cells in bFGF medium was higher than others both on 3DIV

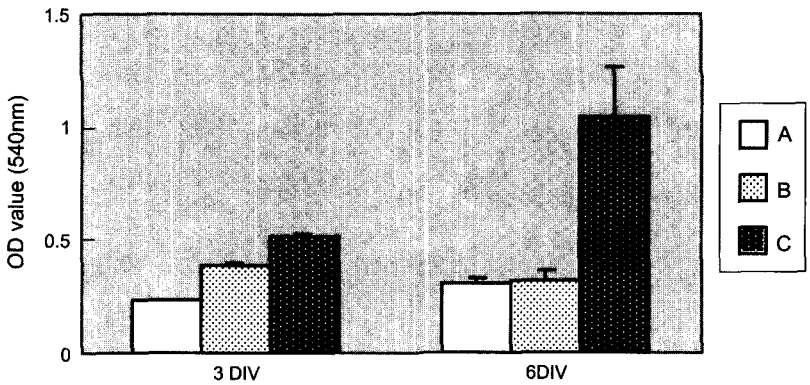

Fig. 2. Effects of B27 supplement and bFGF on neural progenitors (n=8). A : DMEM/F12 (1:1), B : DMEM/F12+B27, C : DMEM/F12+B27+bFGF. *P<0.01 vs Control.

and 6DIV, especially on 6DIV, which was 2 times higher than others. Taking together, we suggested that most cells in the growing medium were bFGF-responsive, in other words, it was this growth factor---bFGF which stimulated or supported cell-dividing.

Proliferating cells within neurospheres were found to be undifferentiated, as they were negative for markers of both mature neurons (β-Tubulin-III) and glia (GFAP for astrocytes and GalC for oligodendrocytes), however, virtually all of the cells in the shperes expressed the neuroepithelial antigen nestin[8] (Fig. 3A-3C) suggesting that only undifferentiated cells from the embryonic rat cortex proliferate in response to bFGF.

Differentiation potential of hippocampal neurosphere cells

When individual sphere was exposed to a substrate in differentiating medium, it rapidly attached and many cells migrated out from the core of sphere and formed a monolayer around the plated sphere (Fig. 4A & 4B). Continual growth of the neurospheres suggested self renewal was occurring, but did not determine the phenotypic potential of these cells. To assess this, Cells from a single sphere were processed for indirect immunocytochemistry. The results suggested that the cells were immunopositive to cell type specific antibodies for the 3 main phenotypes of nervous system, β-Tuj-III for neurons (Fig. 4C), GFAP for astrocytes (Fig. 4D) and Gal for oligodendrocytes (Fig. 4E & 4F). That is to say, cells within a single sphere have potential to differentiate into 3 different kinds of neural cells-----another character of neural stem cells or neural progenitors.

Ginsenoside Rg1 potentiates the division of free floating hippocampal progenitors

In order to establish whether Rg1 modulated the proliferating ability of neural progenitors, the compound was added into growing medium throughout a generation of neurospheres. We found that Rg1 (0.5~2.5 μmol/l) doubled the OD value of growing cells, but there was no evidence for

Table 1. Rg1s effects on neural progenitors survival and proliferation

	OD value (at 540 nm; n=10)	^{3}[H] thymidine incorporation (cpm; n=5)
Control	0.29±0.08	3014.1±936.4
Rg1 0.5 μM	0.59±0.15*	3730.8±861.0*
Rg1 1.0 μM	0.60±0.12*	11039.9±4344.9*
Rg1 2.5 μM	0.65±0.13*	9638.5±1503.8*

*P<0.01 verus Control.

dose-effect relation (Table 1).

Although MTT assay is a good indicator of cell survival, it does not give information of cell proliferation. In order to assess the rate of cell proliferation under various conditions, ^{3}[H]Thymidine incorporation assay was carried out. The same concentration of Rg1 was shown to significantly potentiate the proliferation ability of neural progenitors in vitro (Table 1).

Ginsenoside Rg1 increase BrdU positive cells in mouse dentate gyrus

It is now accepted that neurogenesis continued throughout individuals life in mammals, 2 anatomical regions : the subventuricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampus are particularly enriched with neurogenic activity. In order to establish Rg1s effect on neural progenitors in vivo, we observed the number of dividing cells in mouse dentate gyrus.

BrdU (bromodeoxyuridine, Sigma), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used to label proliferating cells in vivo. The morphology of proliferating cells is depicted in Fig. 5C, 5D. In both Rg1-treated and control mouse, BrdU-labeled cells were darkly stained and irregularly shaped, appeared frequently in clusters of two or more, and were localized within the subgranular zone (the border between the granule cell layer and the hilus) and throughout the hilus After 2 weeks' drug administration (5 mg/kg or 10 mg/kg daily), the density of proliferating cells in dentate gyrus increased from 25.9±14.2/mm^2 to 54.2±24.0/ mm^2 and 84.9±28.2/ mm^2 respectively (Fig. 5A & 5B, Fig. 6).

Discussion

The results presented here show that the E16-18 rat hippocampus contains progenitors that proliferate in response to bFGF; ginsenoside Rg1 can not only enhance neural progenitors' proliferation ability in vitro, but increase neurogenesis in adult mouse dentate gyrus in vivo.

Mltipotent neural progenitors exist in embryonic rat cortex

Stem cells are defined as undifferentiated cells, which can not only display high proliferative potential, but generate a wide variety of differentiated progeny, whereas progenitors refer to cells with restricted potential for proliferation and differentiation as well. As far as the cells in our culture system were concerned, we prefer to call them neural progenitors for the following reasons :

① with existence of bFGF, the cells can proliferate and form neurospheres, but the spheres can only be passaged a limited period of time; ② cells within neurospheres are immunopositve for nestin; ③ a single sphere can differentiate into 3 main kinds of neural cells.

Rg1 has the ability to increase neural progenitors proliferation both in vitro and in vivo

It is now accepted that neurogenesis lasts life long in mammals' brains, this function may allow the brain to respond to environmental demands such as increased intellectual challenge and brain injury. In other words, neurogenesis may underlie the plasticity of brains. Recently, several studies of rodents suggested that the proliferation of neural progenitor cell (NPC) was reduced in association with age-related cognitive decline[9], and that suppression of NPC proliferation could impair learning and memory[10]. Just a little while ago, Haughey et al.[11] reported that amyloid beta-peptide, a self-aggregating neurotoxic protein thought to cause AD, could impair neurogenesis in the SVZ of adult mice, meanwhile, it could suppress NPC proliferation and differentiation.The author suggested that the adverse effect of Abeta on neurogenesis contributed to the depletion of neurons and the resulting cognitive deficits in AD. Taking together, we hypothesize that damage of neurogenesis may be the elementary reason for function-declines in aging and neurodegenerative diseases. In this regard, it is of considerable interest that any compound has the ability to increase neurogenesis.

Ginsenoside Rg1 is one of the ingredients of ginseng, many researches suggested that the former shared most of the latter's anti-aging and nootropic functions. We found previously that Rg1 could accelerate protein synthesis, increase brain weight and cortex thickness, potentiate synapse plasticity in rodent's brain[12], meanwhile, the compound has the ability to ameliorate learning and memory impairment induced by Abeta[13]. On the basis of these works, we observed Rg1's effects on neural progenitors in vitro & in vivo and got the exciting results described above. To our knowledge, this is the first time to discover a compound has the ability to promote neural progenitor cell's proliferation.

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