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Original article

Yokukansan enhances the proliferation of B65 neuroblastoma

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ABSTRACT

Yokukansan, a traditional Japanese herbal medicine, has been considered to be a novel alternative treatment for several neurological diseases such as neurodegenerative disorders, as well as neurosis, insomnia, and behavioral and psychological symptoms in Alzheimer's disease. Moreover, it has been shown that yokukansan has antidepressant-like and pain-relieving effects in animal models. Recently, several studies have shown that yokukansan has a neuroprotective effect. In this study, we focused on whether or no yokukansan influences cell proliferation related to cell-cycle progression by using B65 neuroblastoma cells derived from monoaminergic neurons. Under treatment with yokukansan, the proliferation rate of B65 neuroblastoma cells significantly increased in a dose-dependent manner. In particular, a proliferative effect was observed after treatment with yokukansan for 48 h and 72 h. Moreover, among seven medicinal herbs that comprise yokukansan, both *Bupleuri Radix* and *Glycyrrhize Radix* also enhanced the proliferation of B65 neuroblastoma cells. We assessed the effect of yokukansan on p44/42 mitogen-activated protein kinase (MAPK) phosphorylation in B65 neuroblastoma cells, and found that yokukansan increased p44/42 MAPK phosphorylation after treatment for 48 h. In contrast, neither *Bupleuri Radix* nor *Glycyrrhize Radix* altered the level of p44/42 MAPK phosphorylation, although they did increase cell proliferation. Our findings suggest that yokukansan has a cell-proliferative due to both *Bupleuri Radix* and *Glycyrrhize Radix*, and this is unrelated to the p44/42 MAPK signaling cascade.

1. Introduction

Yokukansan is a traditional Japanese herbal medicine. It originated from the traditional Chinese herbal medicine Yi-Gan San, which was modified to create a unique Japanese herbal medicine called “Kampo”. Yokukansan is composed of seven medicinal herbs: *Atractylodis lanceae Rhizoma* (ALR), *Poria* (PR), *Cnidii Rhizoma* (CR), *Uncariae Uncis Cum Ramulus* (UR), *Angelicae Radix* (AR), *Bupleuri Radix* (BR) and *Glycyrrhize Radix* (GR). Since ancient times, this herbal medicine has been used to treat patients with symptoms such as nervousness, short temper, irritability and sleeplessness in infants and young children.

Recently, clinical evidence regarding the effectiveness of yokukansan has been reported. In particular, it has been demonstrated that yokukansan can improve dementia and several psychiatric conditions.[1] Several clinical studies have shown that the administration of yokukansan could ameliorate the symptoms of behavioral and psychological symptoms of dementia (BPSD) in

Abbreviations: BPSD, Behavioral and psychological symptoms of dementia; ALR, *Atractylodis lanceae Rhizoma*; PR, *Poria*; CR, *Cnidii Rhizoma*; UR, *Uncariae Uncis cum Ramulus*; AR, *Angelicae Radix*; BR, *Bupleuri Radix*; GR, *Glycyrrhize Radix*; MAPK, Mitogen-activated protein kinase; 5-HT, Serotonin; SSRI, Selective serotonin reuptake inhibitor.

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Alzheimer's disease.[2,3] Moreover, yokukansan could also improve neuropsychiatric symptoms associated with Parkinson's diseases, including hallucinations, anxiety and apathy without severe adverse events or worsening of Parkinsonism.[4] According to the clinical studies of yokukansan mentioned above, yokukansan may have multiple components that are effective at treating various central nervous system diseases. As a mechanism of the ameliorative effect against various central nervous system diseases, it is has been considered that yokukansan has neuroprotective effects. Several studies have demonstrated that yokukansan has a neuroprotective effect against glutamate-induced excitotoxicity in cultured cells,[5,6] and this neuroprotective effect involves the modification of gene expression of cystine/glutamate antiporter system Xc^-.[7] We also previously reported that yokukansan showed a neuroprotective effect against cytotoxicity induced by corticosterone on mouse hippocampal neurons.[8] In contrast, it has been demonstrated, albeit in an animal experiment, that yokukansan had antidepressive and antinociceptive effects on behavioral despair and acetic acid-induced writhing in mice.[9] Another animal behavioral experiment revealed that yokukansan showed anxiolytic effects against experienced aversive stress in rat.[10] Based on this evidence, it is reasonable to consider that yokukansan might contain some components that can improve nervousness, depression and anxiety disorder. Actually, several studies have stated that yokukansan contains components that act as agonists or antagonists against several receptors.[11,12] It has been reported that the anxiolytic or antidepressant effects of yokukansan in rats that have been exposed to experienced-aversive stress might be caused via serotonin 5-HT_{1A} receptor agonism.[10,13] Therefore since 5-HT_{1A} agonist has an antidepressant-like effect, the antidepressant-like effect induced by yokukansan may be due to stimulation of 5-HT_{1A} receptor. Moreover, chronic stress decreased the number of neural stem cells in the subventricular zone, and an antidepressant, such as selective serotonin reuptake inhibitor (SSRI), attenuated the corticosteroid- or chronic stress-induced decrease in the viability and proliferation of neuroblastoma or neural stem cells.[14,15] Furthermore, it has been suggested that the finding that 5-HT_{1A} agonists increased cell proliferation in the adult central nervous system may be related to antidepressive mechanisms.[16] These various findings suggest that some component(s) in yokukansan, which has an antidepressive effect, may enhance cell proliferation which is related to cell-cycle progression in neurogenesis or cell survival.

In this study, we investigated whether or not yokukansan has a cell-proliferative effect and the mechanism that underlies this effect by using B65 neuroblastoma cell derived from monoaminergic neurons.

2. Materials and methods

2.1. Materials

Yokukansan is composed of seven dried medicinal herbs: 4.0 g of ALR, 4.0 g of PC, 3.0 g of CR, 3.0 g of UR, 3.0 g of AR, 2.0 g of BR and 1.5 g of GR. These herbs are registered in the Pharmacopeia of Japan ver. 16. The powdered water extracts of yokukansan and each individual medicinal herb used in this study were manufactured according to previously reported,[8,17] and supplied by Tsumura & Co. (Tokyo, Japan). A three-dimensional high-performance liquid chromatography (3D-HPLC) profile of representative batches of yokukansan and each individual medicinal herb which were provided by Tsumura & Co. are shown in Fig. 1. All other materials for experiments, including reagents, drugs, antibodies and media were purchased from the companies indicated in each description.

2.2. Cell culture

B65 neuroblastoma (DS Pharma Biomedical, Osaka, Japan) is a rat ethyl nitrosourea-induced tumor cell line. Briefly, B65 neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with a high glucose concentration (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biowest, Nuaillé, France), 100 u/mL penicillin and 100 μg/mL streptomycin (Wako, Osaka, Japan) in a humidified incubator under a 95%/5% mixture of air and CO_2. Cells were generally passaged every 5 days.

2.3. Cell viability assay

The proliferation of B65 neuroblastoma cells was analyzed by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] assay using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Once cells became confluent, they were plated into 96-well microplates at a density of 5×10^4/mL (5×10^3/well). To apply yokukansan or each individual medicinal herb to B65 neuroblastoma cells, they were first suspended in culture media. The solution was then centrifuged for 5 min at 15,000 × *g* to remove insoluble residue. To avoid denaturing the effective component, the solution of cells dissolved in culture media was prepared immediately before every experiment. After centrifugation, the supernatant was used in all experiments. At 24, 48 and 72 h of culture with yokukansan, the individual medicinal herbs or chemical compounds, the cells were incubated with WST-8 solution at 37 °C for 2 h. The spectrophotometric absorbance of WST-8-formazan produced by dehydrogenase activity in living cells was measured at a wavelength of 450 nm using a VersaMax (Molecular Devices, Tokyo, Japan). Statistical analysis was performed by using absolute absorbance values.

2.4. Western blotting

Protein samples were obtained from B65 neuroblastoma cells that had been exposed to various concentrations of yokukansan or individual medicinal herbs for 24, 48 and 72 h. For the preparation of protein extract from B65 neuroblastoma cells, the cells were washed with ice-cold homogenizing buffer (pH7.4; 250 mM sucrose, 2 mM EDTA 2Na, 10 mM EGTA and 20 mM Tris-HCl), and centrifuged to obtain a cell pellet. The cell pellet was treated with lysis buffer (homogenizing buffer containing 1% Triton X-100 and a protease inhibitor cocktail) and sonicated on ice. After the sample was centrifuged, the supernatant was collected as a total protein extract. For western blotting, protein extracts were placed on SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA, U.S.A) and immunoblotted with anti-p44/42 mitogen-activated protein kinase (MAPK) polyclonal antibody (Cell Signaling Technology, Danvers, MA, U.S.A., 1:1000), anti-phospho-p44/42 MAPK polyclonal antibody (Cell Signaling Technology, Danvers, MA, U.S.A., 1:1000) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Millipore, Billerica, MA, U.S.A., 1:5000). The blots were developed with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A., 1:20000) and visualized by chemiluminescence using an ECL prime Western Blotting Detection System (Amersham, Piscataway, NJ, USA) and Western BLoT Quant HRP Substrate (TaKaRa, Ootsu, Japan).

2.5. [^{3}H]Serotonin uptake assay

Approximately 4×10^4 B65 neuroblastoma cells were divided equally into 24-well plates. [^{3}H]Serotonin (5-HT) uptake assays