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Induction of Differentiation in Rat C6 Glioma Cells with Saikosaponins

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The effects of saikosaponins (a, b_1, b_2, c, d), isolated from *Bupleurum* Radix, on the induction of differentiation in rat C6 glioma cells were studied. Saikosaponins a and d were shown to inhibit cell proliferation and alter cell morphology. In addition to cytostasis, the enzymatic activities of glutamine synthetase (GS) and 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) were also noticeably increased after treatment with saikosaponin a. Nevertheless, saikosaponin d only showed an increase of GS activity, no significant changes in CNP activity were found. These results suggest that saikosaponin a can induce the differentiation of C6 glioma cells into astrocytes and/or oligodendrocytes, but saikosaponin d can only induce the differentiation of C6 glioma cells into astrocytes.

Keywords: saikosaponins; differentiation induction; C6 glioma cells.

INTRODUCTION

Despite aggressive treatments that include surgical resection, radiotherapy and cytotoxic chemotherapy, the survival rate of patients with malignant glioma, the most common primary tumour of the brain, is still low (Brandes *et al.*, 1991). Human malignant gliomas remain an important, unresolved oncological challenge. Thus, the novel approach of differentiation induction of tumour cells has become especially highlighted.

C6 glioma cells, derived from rat brain treated with N-nitroso-methylurea (Benda *et al.*, 1968), may differentiate into either oligodendrocytes and/or type 2 astrocytes (Parker *et al.*, 1980; Kumar *et al.*, 1986; Mangoura *et al.*, 1989). In the CNS, glutamine synthetase (GS) is localized primarily in astrocytes and has long been considered as a specific enzyme marker for astrocytes (Norenberg, 1979). It synthesizes glutamine from glutamate to protect the central nervous system from injury. On the other hand, 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) is considered to be a specific enzyme marker for oligodendrocytes and myelin membranes (Poduslo and Norton, 1972).

Saikosaponins, as the biologically active components, are obtained by extraction from the roots of *Bupleurum falcatum* L., which has been widely used in Chinese herbal medicine (Kubota and Hinoh, 1968; Shimaoka *et al.*, 1975). In this present study, the effects of saikosaponins (a, b_1, b_2, c, d) on the induction of differentiation in cultured rat C6 glioma cells have been investigated.

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MATERIALS AND METHODS

Materials. Rat C6 glioma cells were purchased from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were procured from Gibco (Gaithersburg, MD). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were obtained from Sigma (St Louis, MO). N,N-Dimethylfluoramide (DMF) was purchased from Merck (Germany). Saikosaponins (a, b_1, b_2, c, d) were a gift from Dr H. Abe (University of Kinki, Japan) and were dissolved completely in 70% ethanol. These agents were then sterilized by millipore filtration, dried and stored at −20 °C.

Cell culture. C6 cells were maintained as a monolayer culture in DMEM medium with 10% FCS in the presence of penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Subculture was made by using 0.05% trypsin with 0.02% EDTA every 3 to 4 days.

Cell growth and morphology. 135 μL of cell suspension (2.5×10^4 cell/mL) was inoculated into 96-well microplates. Four hours later, 15 μL of saikosaponins in a concentration ranging from 0.1 to 100 μg/mL was added. After 3 days incubation, cultures were fixed for 10 min by 10% formaldehyde and stained with Mayer's haematoxylin for 5–10 min, washed and dyed again with 1% eosin B for 3–5 min. Cultures were observed with a phase contrast microscope.

MTT colorimetric assay. MTT was dissolved at a concentration of 5 mg/mL in phosphate-buffered saline (PBSA), sterilized by filtration and stored at 4 °C (Mosmann, 1983; Hansen *et al.*, 1989). After treatment

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with saikosaponins for 4 days, the medium was discarded, and the cultures were rinsed with PBS solution, and replaced with 100 μL of fresh medium. Then 25 μL of MTT stock solution was added, and the cultures were incubated for an additional 2 h. Then 100 μL of lysis buffer (20% SDS–50% DMF) was added and mixed thoroughly for overnight incubation at 37 °C. The absorbance of each well at 570 nm was measured with an ELISA multiscanner (Bio-Rad model 450).

Preparation of GS and CNP extracts. 2×10^5 cells/well were inoculated to 6-well plates. 4 h later, the cultures were treated with a different concentration of saikosaponin a or d for 3 days at 37 °C, in a 5% CO_2 incubator. Then the cells were harvested and lysed by a repeated freezing and thawing technique in 100 μL of deionized water. Cell pellets were centrifuged at 40 000 $\times$ **g** for 1 h at 4 °C. The supernatants were prepared for GS assay. The precipitates were solubilized with 100 μL of extraction solution (0.15 M NaCl, 0.5% Triton X-100 and 10 mM Tris-HCl, pH.7.5) and sonicated twice for 15 s at 0 °C and centrifuged. The supernatants were collected for CNP assay.

Measurement of GS activity. GS activity was determined according to the method described by Iqbal and Ottaway (1970). 40 μL of the GS extracts prepared as above were assayed with 60 μL of reaction mixture containing 0.2 M $MgCl_2$, 0.5 M glutamate, 1 M hydroxylamine, 0.25 M 2-mercaptoethanol, 19 mM ATP, 0.1 M 2-phosphoenol pyruvate and 1 mg/mL pyruvate kinase (pH 7.2). The reaction mixture was incubated at 37 °C for 15 min, then 150 μL of 0.37 M ferric chloride (0.67 M HCl-5% TCA) was added to terminate the reaction. After centrifugation at 3000 rpm for 5 min, the absorbance of the supernatant at 535 nm was detected using synthetic r-glutamylhydroxamate as a standard.

Measurement of CNP activity. The assay for CNP activity was carried out essentially as described by Prohaska *et al.* (1973). 5 to 25 μg of protein of CNP extracts was added to 7.5 mM 2′,3′-c-AMP, 50 mM Tris-maleate buffer (pH 6.2) to make a final volume of 200 μL. The initial reaction was carried out at 30 °C for 10 min, and terminated by 90 °C for 1 min. 100 μL of 21 mM $MgCl_2$ in 0.3 M Tris-HCl buffer (pH 9.0) was added with 60 μg of 0.72 U alkaline phosphatase (pH 8.5) at 30 °C for 20 min. Then 0.7 mL of 10% ascorbic acid: 0.42% ammonium molybdate in 1 N H_2SO_4 (1:6) mixture was added at 45 °C for 20 min. After centrifugation at 3000 rpm for 5 min, the absorbance of supernatants at 820 nm was detected using 2′-AMP as a standard (Ames, 1966).

RESULTS

Effect of saikosaponins on cell growth

After treatment with saikosaponins in concentrations of 0.1–100 μg/mL for 4 days, the proliferation of C6 glioma cells was studied by the MTT colorimetric method (Fig. 1). At lower concentrations of 0.1 or 1.0 μg/mL, none of the saikosaponins showed an effect on the growth of C6 glioma cells. But at a concentration of 10 μg/mL, saikosaponins a and d caused not only a cytostatic effect, but also an alteration in cell morphology (Fig. 2). At a higher concentration of 100 μg/mL, saikosaponins a, b_1 and d showed unexpected cytotoxic effects and caused cell death.

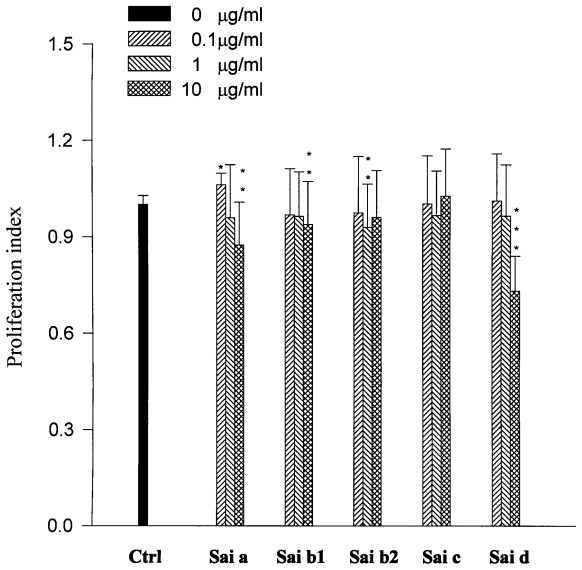

Figure 1. Effects of saikosaponins on the proliferation of C6 glioma cells. C6 glioma cells were cultured with each extract for 4 days and detected by MTT assay (*** $p < 0.001$, ** $p < 0.02$, * $p < 0.01$).

Effect of saikosaponins on cell morphology

After saikosaponin treatment for 3 days, an alteration of cell morphology was observed. The results showed that no significant differences were observed between the control and 0.1 or 1.0 μg/mL treatment (data not shown). Saikosaponins a and d at 10 μg/mL (Fig. 2B, F), showed not only growth inhibition but also alteration in cell morphology. Figure 2B, F shows a decrease of cell density and an extension of long cytoplasmic processes. These results indicate that saikosaponins (especially a, d) may have the ability to induce cultured C6 glioma cells to differentiate into phenotypes.

Changes in the enzymatic activity of GS and CNP

After treatment of C6 cells with 0, 2.5, 5.0, 10 μg/mL of saikosaponins a or d for 3 days, the activities of GS and CNP were measured. As seen in Fig. 3, a significant increase in the specific GS activity was observed at 10 μg/mL of saikosaponin a. In addition, saikosaponin a can induce an increase of CNP enzyme activity (Fig. 4). A maximal effect was found at 5 μg/mL concentration. Saikosaponin d at 10 μg/mL can also significantly increase the activity of GS enzyme (Fig. 3). Nevertheless, by comparison with the control group, there was no obviously change in CNP activity in saikosaponin d treated cells (Fig. 4). Therefore, it seems that saikosaponin a at 5–10 μg/mL may induce C6 glioma cells to differentiate into astrocytes and/or oligodendrocytes, but saikosaponin d only can induce C6 glioma cells to differentiate into astrocytes.

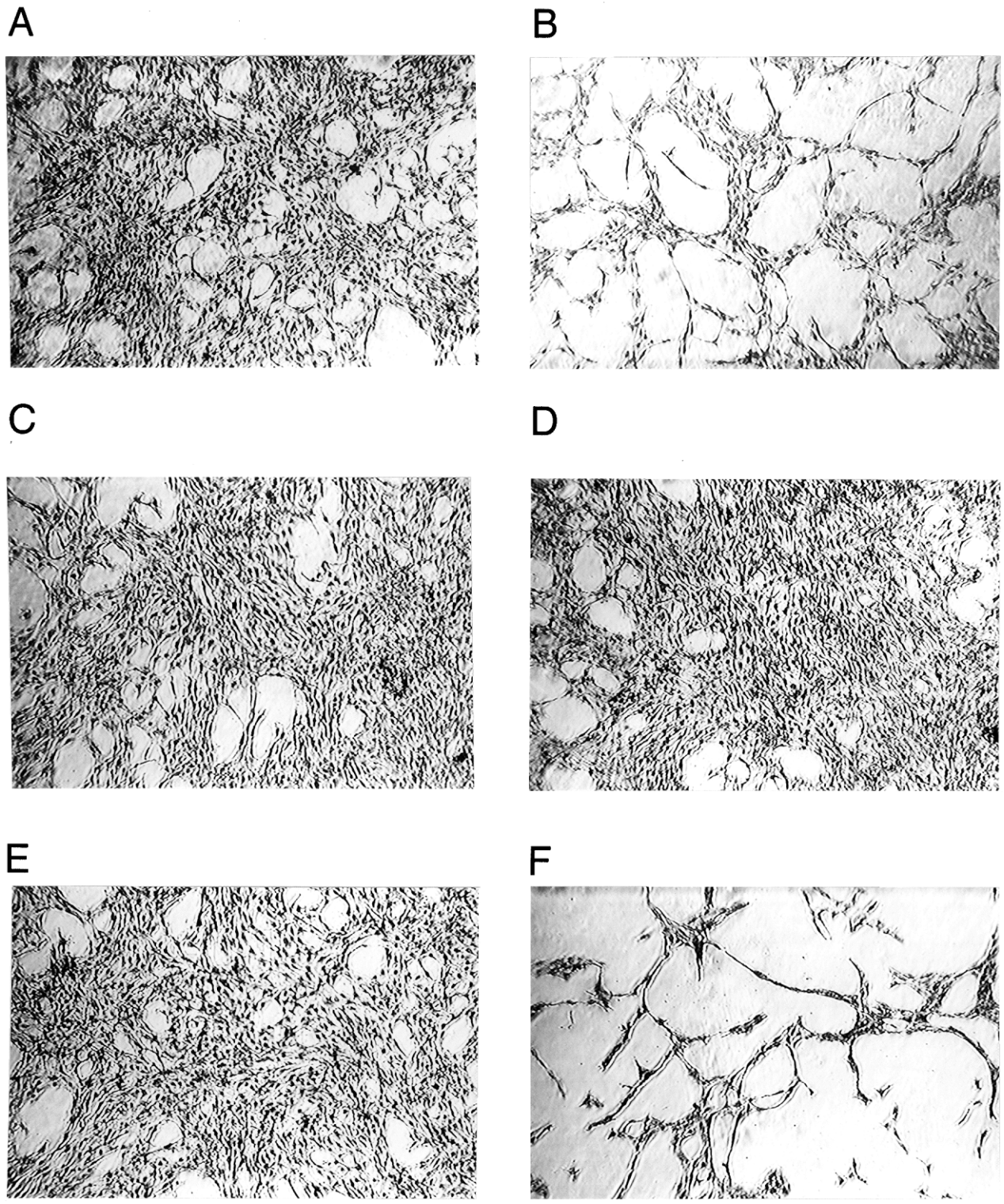

Figure 2. Effects of saikosaponins on the morphologic changes of C6 glioma cells. C6 glioma cells (2×10^4 cells/mL) were cultured with (A) control, (B) saikosaponin a (10 μg/mL), (C) saikosaponin b1 (10 μg/mL), (D) saikosaponin b2 (10 μg/mL), (E) saikosaponin c (10 μg/mL), (F) saikosaponin d (10 μg/mL). After 3 days, the morphology was observed by phase-contrast microscopy.

DISCUSSION

Because the number of glial cells is much greater than neural cells in the central nervous system, glioma is the most common malignant tumour in the human brain. Its prognosis is very poor because of the blood–brain barrier to drugs, the low sensitivity of the immune response, and the heterogeneity of glioma. Most clinically used cytotoxic antitumour drugs are unsatisfactory for glioma treatment since they would cause unnecessary harm to healthy brain cells.

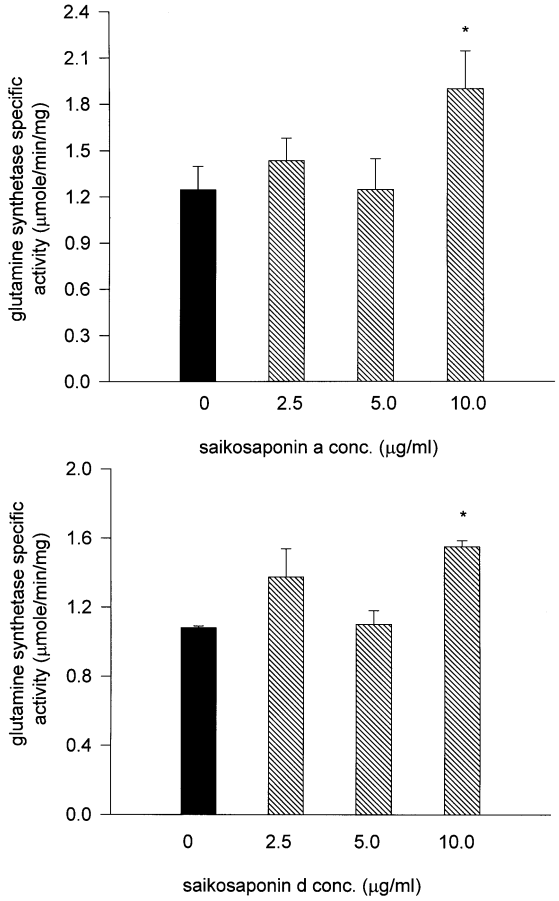

Figure 3. Effect of saikosaponin a and saikosaponin d on the GS activity. C6 glioma cells were cultured with saikosaponin a and saikosaponin d for 3 days (* $p < 0.01$). Values are the mean ± SEM ($n = 3$–6).

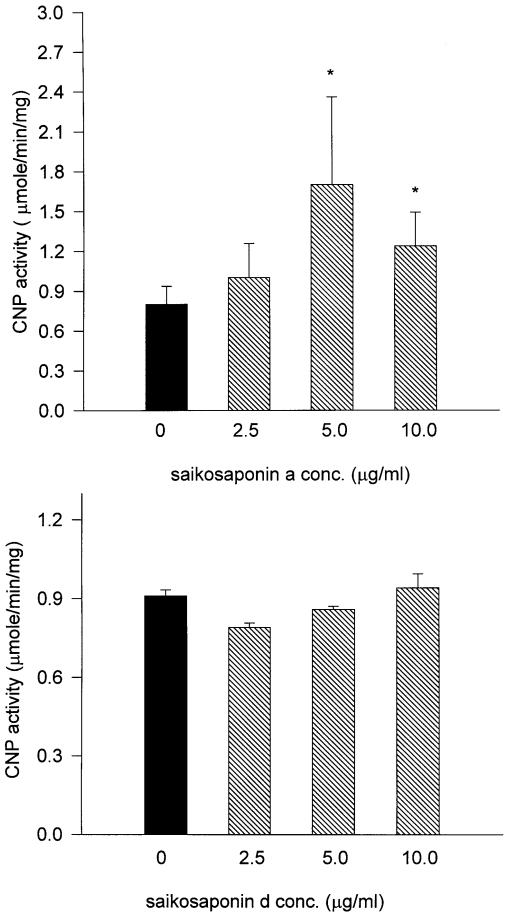

Figure 4. Effect of saikosaponin a and saikosaponin d on CNP activity. C6 glioma cells were treated with saikosaponin a and saikosaponin d for 3 days. Values are the mean ± SEM ($n = 3$, * $p < 0.01$).

Recent studies have suggested that cancer, including brain tumours, can be considered a disorder of cell differentiation. Some chemical agents, such as retinoic acid (Sidell, 1982; Thiele *et al.*, 1985), sodium butyrate (Hargreaves *et al.*, 1989), dibutyryl cyclic AMP (Pishak and Phillips, 1980; Jackson *et al.*, 1995), cytosine arabinoside (Ponzoni *et al.*, 1989), hexamethylene bisacetamide (Li *et al.*, 1996) and phenylacetate (Samid *et al.*, 1992;1994), have been found to induce certain kinds of tumour cells to differentiate with a loss of proliferative capacity and the expression of certain characteristics of mature cells.

Phenylacetate (PA), a naturally occurring plasma component, induces cytostasis and the reversal of malignant phenotypes in a variety of cultured human cancer cells, including malignant glioma (Stockhammer *et al.*, 1995). The *in vitro* and *in vivo* antitumour activity was noted at millimolar concentration levels. Since the effective doses of saikosaponins in our experiments are micromolar level (12.8 μM), saikosaponins may be valuable in the brain *in vivo*.

Many reports have indicated that saikosaponins have multiple biological activities including antihepatotoxicity (Abe *et al.*, 1980;1985), antiinflammatory action (Yamamoto *et al.*, 1975), immunomodulatory activity (Kato *et al.*, 1995) and antihepatoma activity (Qian *et al.*, 1995). It is interestering to note that saikosaponins a, b_1, b_2, c, and d have similar chemical structures in the aglycone framework (oleanene skeleton). The difference of chemical structure between saikosaponins a, d group and b group is the ether linkage between C13 and C28, and this linkage might cause the difference in biological activity between saikosaponins a, d, and saikosaponin b. Kohno (1995) showed a melanogenesis effect of saikosaponin b1 and b2 on cultured melanoma cells. It is likely to be due to the cell type specificity of these saikosaponins.

Parker *et al.* (1980) reported that C6 glioma cells have both astrocytic and oligodendrocytic glial properties with passage of the cells as shown by the presence of specific enzyme activities, GS and CNP, respectively. From our results, saikosaponin a can induce the differentiation of C6 glioma cells into astrocytes and/or oligodendrocytes, but saikosaponin d can only induce the differentiation of C6 glioma cells into astrocytes. Based on these results, we propose that saikosaponins may be applied in cancer therapy as a noncytotoxic differentiation inducer.

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