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Note

The Effect of Cyclosporin A on the Growth of Human Glioma Cell Lines

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Cyclosporin A, an potent immunosuppressant, has been known to be one of the modulators of drug resistance as well as a cytostatic drug. Despite many attempts to basic or clinical application of cyclosporin A, there are few reports on the inhibition of brain tumor cells. In the present experiment, the possibility of cyclosporin A as synergic adjuvant was investigated by MTT assay, [^{3}H] thymidine uptake and through flowcytometric anaysis. Sole treatment of cyclosporin A on the CRT and CH235-MG glioma cell line revealed dose dependent cytotoxicity within a range of tested dose. Combined treatment of cyclosporin A with ACNU, BCNU and hydroxyurea on various glioma cancer cell line led to a significant synergistic cytotoxicity as well as inhibition of DNA synthesis with dose-dependency. In addition, cyclosporin A alone or combined treatment caused discernible changes of cell cycle in the tested cells. These data provide that cyclosporin A could potentiate the effect of nitrosourea compounds *in vitro* on human glioma cells.

Key words : Cyclosporin A, Glioma, Adjuvant, Nitrosourea

INTRODUCTION

Malignant glioma is one of the most lethal disease because within 6 months from its diagnosis, about 50% of the suffered were reported to die with poorer prognosis (Paoletti *et al.*, 1990). Furthermore, glioma is the most common primary brain tumor originated from neuroepthelial cells, accounting for about 15% of all primary brain tumors (Golden *et al.*, 1972). Based on statistics in domestic cancer surveys, the incidence of glioma approximately has been 1.9% through out the overall cancer with increasing trend (Ministry of Health and Social Affairs, 1989). As a mean to overcome intracranial astrocytoma and glioblastoma, cytoreductive surgery and radiation have been the mainstay of its conventional treatment (Waker *et al.*, 1980). This approach, although effective in prolonging survival, is rarely curative. Chemotherapy, in general, have been of some additional value in better prognosis (Chang *et al.*, 1983). Nevertheless, these therapeutic strategies little contribute to remarkable improvement of survival in glioma patient (Blasberg and and Groothuis, 1986; Kaye and Laidlaw, 1992). In clinical field, some nitrosourea-based alkylating agents such as hydroxyurea, ACNU(3-[4-amino-2-methylpyrimidine-5-yl) methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride) and BCNU(1,3-bis[2-chloroethyl]-1-nitrosourea) also have been introduced to treat various stage of glioma patients in a combined regimen (Rogers *et al.*, 1991; Shapiro and Shapiro, 1986). These drugs have been most frequently used for brain tumors, partly due to its ability to cross the blood-brain-barrier (Sakata *et al.*, 1994). Clinical problems in nitrosourea-based chemotherapy are related to a variety of factors, such as drug sensitivity or tumor resistance to drugs, as well as adverse effects on normal tissues (Linskey and Gilbert, 1995). Thus, lowering doses of the nitrosourea-based drug without losing its antitumoral efficacy should be benificial (Soma *et al.*, 1992). Therefore, to solve this limitation, the development of effective adjuvant may be considered. Recently, cyclosporin A, has been reported to had a potent cytostatic activity (Berguli *et al.*, 1994; Botling *et al.*, 1994; Gordon *et al.*, 1993; Kronke *et al.*, 1984; Piontek and Gilbert, 1994; Thornton *et al.*, 1995). Despite many attempts to basic or clinical application of cyclosporin A, there are few document on the inhibition of brain tumor cells. Therefore, in first, it is important to determine whether cyclosporin A, on the cellular level *in vitro,* inhibit the cellular proliferation of glioma alone or in cytotoxic

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agent-combined form. In the present experiment, the effects of cyclosporin A as a synergic adjuvant were investigated by MTT (methyltetrazolium bromide) assay, [^{3}H] thymidine uptake and through flowcytometric analysis.

MATERIALS AND METHODS

Chemicals

Cyclosporin A, ACNU, BCNU and hydroxyurea were obtained from Sigma Co., Ltd. (St. Louis, MO USA).

Cancer cell lines

The cancer cell lines for cytotoxicity test were as follows: CRT (astrocytoma, human), CH235-MG (glioblastoma multiforme, human). Both cell lines were kindly supplied from Dr Joo-Young Park, Institute of Basic Medical Sciences, Wonju, Korea. They were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated in a humidified 5% CO_2 chamber at 37°C.

Measurement of cytotoxicity

To evaluate cytotoxicity, modified MTT method was performed essentially as described previously (Carmichael *et al.*, 1987; Kim *et al.*, 1996). Briefly, monocellular suspension was seeded at 1×10^4 cells per well in 96 well plates with 100 μl of medium per well. Cytotoxic agents alone (ACNU, BCNU and hydroxyurea) or cytotoxic agents plus cyclosporin A were added at varying concentrations and cultures were incubated for 72 hours in an incubator maintaining a highly humidified atmosphere, 5% CO_2 and 95% air. Fifty μl of the medium containing MTT (5 mg/ml) was added to each well. After 4 hours of exposure, the medium was partly decanted and the wells were washed with PBS, and then 150 μl of DMSO was added to each well to solubilize the precipitates. The plates were transferred to an ELISA reader to measure absorbance at 570 nm with a reference wave length, 630 nm. IC_{50} value, 50% inhibition of cell growth, was calculated by nonlinear regression analysis (plotting the viability versus the concentration of the test compound) using Graphpad Prism 2.0 (GraphPad Software, Inc.). All experiments were done at least 3 times, with 3 wells for each concentrations of test agents. The *in vitro* data were analyzed for significance by the Student's t test.

Measurement of DNA synthesis

To determine the effect on DNA synthesis by tested drugs, [^{3}H] thymidine incorporation was done. Following 48 hr treatment with various doses of ACNU, BCNU and hydroxyurea to CH235-MG cells, cells were pulsed with 1 μCi of [^{3}H] thymidine (20 Ci/mmol; 740 GBq/mmol, NEN, Wilmington, DE, U.S.A) for 1 hour. Cells were harvested with a cell harvestor (Skatron Inc., Stering, VA, U.S.A) collected on filter paper and dried. The uptake of radioisotopes was measured with liquid scintillation, and the results were expressed as the mean of three counts per minute (cpm).

Cell cycle analysis

About 1×10^6 CH235-MG cells were treated with 25 μg/ml of ACNU alone or with 2, 5 and 10 μg/ml of cyclosporin A in combination for 18hr and followed by a subsequent washing with PBS. Single cell suspensions were stained with a solution containing 0.05 mg propidium iodide/ml, 0.1% sodium citrate, and 0.1% Triton X-100, and vortexed vigorously. Ten μl DNase-free RNase (Calbiochem, La Jolla, CA) at 0.2mg/ml in PBS were added to each sample and incubated for 30 min at room temperature (Cho *et al.*, 1988). Cells were analyzed on a FACScan flowcytometer (Becton Dickinson, Mountain View, CA). Cell cycle distribution was determinated using RFit method.

RESULTS AND DISCUSSION

To explore the synergistic cytotoxicity of cyclosporin A on glioma cells, we first examined the effect of cyclosporin A on tested cell lines *in vitro*. Sole treatment of cyclosporin A on the CRT and CH235-MG cell line revealed dose dependent cytotoxicity within a range

Table 1. 50% inhibitory concentration[a] following sole or combined treatment of cyclosporin A, ACNU, BCNU and hydroxyurea in CRT and CH235-MG cell line

Compound	IC_{50} (μg/ml)[a] ± S.D[b]	
	CRT	CH235-MG
CSA	11.4±2.32	13.68±3.15
ACNU(Control 1)	49.07±11.3	7.97±3.24
ACNU+CSA(2)[c]	5.18±2.23**	3.82±1.16*
ACNU+CSA(5)[d]	2.69±1.22**	0.21±0.04**
ACNU+CSA(10)[e]	0.1±0.06**	0.13±0.08**
BCNU(Control 2)	205.1±67.2	314.5±34.6
BCNU+CSA(2)	125.1±23.8**	304.4±21.1
BCNU+CSA(5)	159.5±34.5**	192.2±20.7**
BCNU+CSA(10)	23.54±8.25**	N.D
HYU(Control 3)	18.84±5.41	30.3±9.23
HYU+CSA(2)	2.11±1.56**	4.29±1.43**
HYU+CSA(5)	2.16±0.75**	6.70±3.42**
HYU+CSA(10)	1.2±0.07**	2.50±1.15**

[a]Measured by MTT assay, IC_{50} value which is defined as the concentration that caused 50% inhibition of cell growth.
[b]Standard deviation.
[c,d,e]CSA (2 μg/ml), CSA (5 μg/ml) and CSA (10 μg/ml) which was added to ACNU or BCNU, hydroxyurea.
Note: hydroxyurea, HYU; cyclosporin A, CSA.
N.D: Not done.
*P<0.05, **P<0.01.

of tested dose. IC_{50} of cyclosporin A on tested cell lines is 11.4 μg/ml in CRT cell and 13.68 μg/ml in CH 235-MG cell, respectively (Table 1). In higher dose (over 20 μg/ml), it was noted that cyclosporin A alone could be cytotoxic to tested cell lines. At least, less than 10 μg/ml of cyclosporin A had little influence on the value of cytotoxicities. Thus we adopted 2 to 10 μg/ml of cyclosporin A for MTT test in synergism. According to our results, IC_{50} of ACNU on CRT cell line could be decreased to 0.1-5.18 μg/ml than IC_{50} (49.07 μg/ml) without cyclosporin A if 2 to 10 μg/ml of cyclosporin A is added (Table 1). This phenomenon was also observed at cotreatment of cyclosporin A plus nitrosourea drugs (ACNU,BCNU and hydroxyurea) on CRT cell lines. Combined effect of ACNU plus cyclosporin A was stronger than that of BCNU or hydroxyurea plus cyclosporin A on both cell line (Table 1). Among tested cytotoxic drugs, hydroxyurea showed most excellent cytotoxicity on CRT cell lines whereas ACNU was highly cytotoxic to CH235-MG cells. In view of synergism, the enhrncement of cytotoxicity by cyclosporin A was occurred in both cell lines at varying degree (1-490 fold). Interestingly, ACNU-based combination gave the strongest inhibition of cellular proliferation in tested cell lines. These results would be related with the report that cyclosporin A had an antiproliferative effect on astrocytoma cells through inhibition of substance P receptor *in vitro* (Gitter *et al.*, 1995). On [^{3}H]thymidine incorporation assay to confirm the potentiating effect of cyclosporin

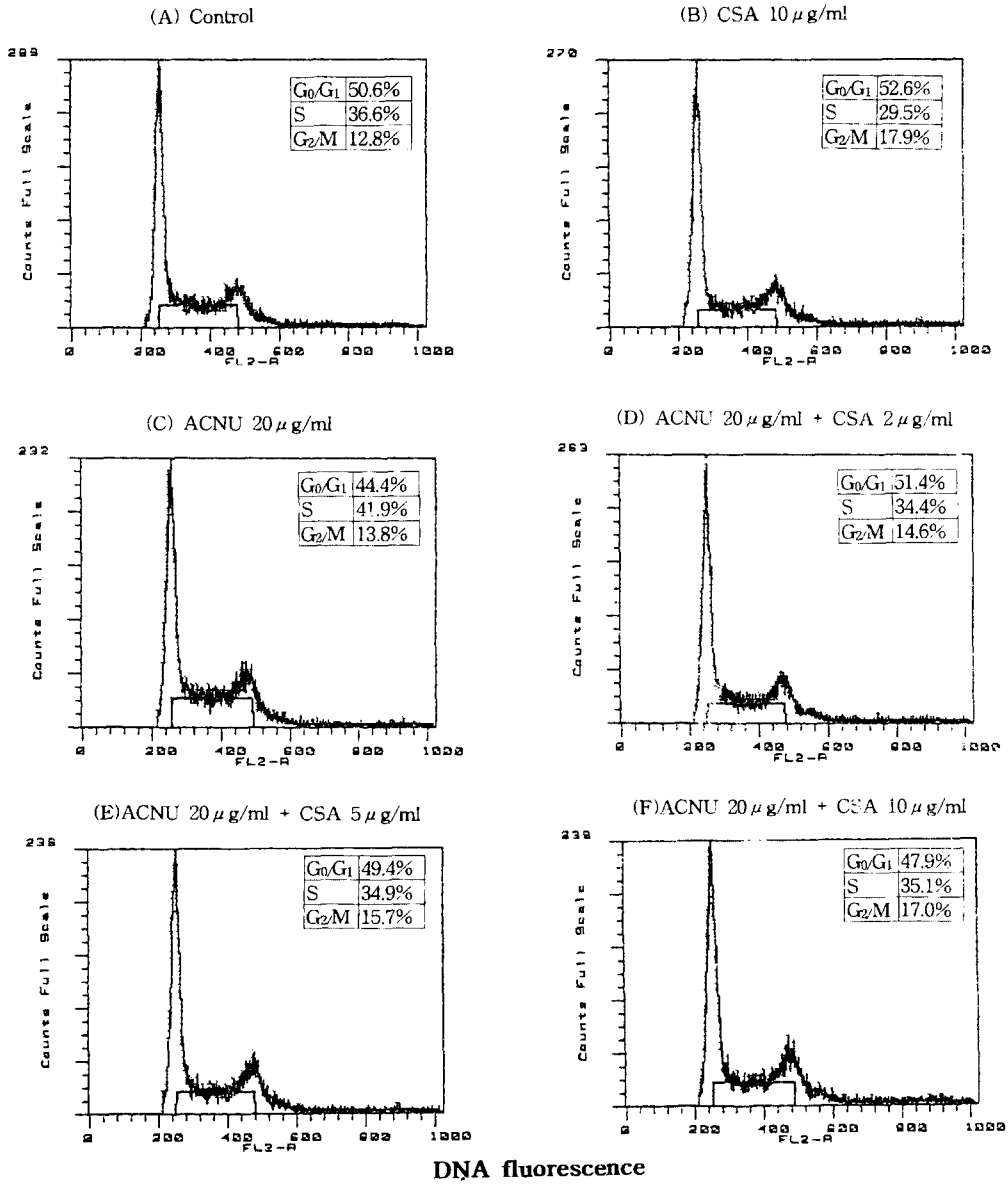

Fig. 1. Effect of cyclosporin A plus ACNU on DNA ploidy pattern of asynchronous CH235-MG cells.

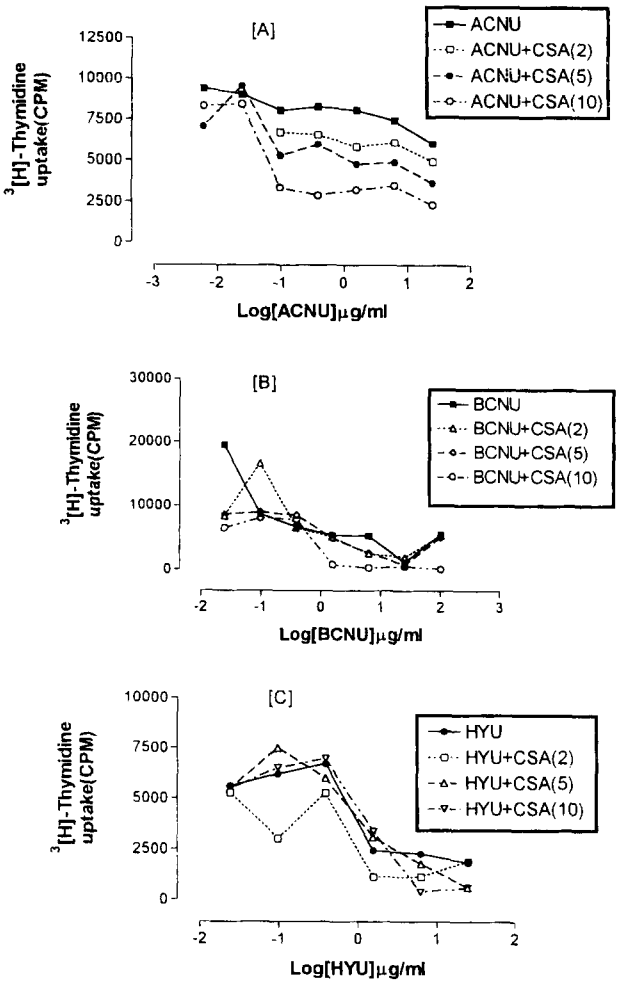

Fig. 2. Inhibition of DNA synthesis in CH235-MG cells treated with cyclosporin A plus ACNU, BCNU and hydroxyurea.

A, there was an inhibition of DNA synthesis in CH 235-MG cells treated with ACNU, BCNU and hydroxyurea plus cyclosporin A (Fig. 2). Notably, ACNU plus cyclosporin A displayed stronger inhibition of DNA synthesis in tested cells. These were closely compatible with MTT results in synergism obtained through ACNU-based treatment. The inhibition of DNA synthesis was disappeared following treatment with lower doses (below IC_{50} value) of cytotoxic drugs. Based on the observation so far achieved, it may be assumed that cyclosporin A has an action in accumulating cytotoxic drugs into cells with an easy access to DNA alkylation. Another explanation is that cyclosporin A can modulate P glycoprotein pump within tested cells. But, these hypotheses remain to be further testified. These results may be a first report that provides a theoretical clue on the applicability of cyclosporin A as adjuvant with nitrosourea-drugs in a field of neuro-oncology. On flowcytometric analysis, when asynchronous CH235-MG cells were treated with cyclosporin A for nearly doubling time (36 hr), the pattern of cell cycle distribution was dose-dependent. As seen in Fig. 1, treatment of CH235-MG cells with 20 μg/ml of ACNU induced decrease in G1 phase cells accompanying an increase in S phase cells. However, treatment with 10 μg/ml of cyclosporin A or ACNU plus 2, 5, 10 μg/ml of cyclosporin A increased the cell numbers in G_2+M phase (13.8%, 14.6 %, 15.7%, 17%) slightly high as those (12.8%) of control cells separately. There were similar DNA ploidy patterns among each cell groups treated with different doses of cyclosporin A (2, 5 and 10 μg/ml) and ACNU (20 μg/ml) (Fig. 1). However, in case of CRT cell line, unique pattern in cell cycle induced by cyclosporin A alone as well as discernible cell cycle shift on combined treatment could be better observed with a failure to quantitative analysis because of extreme aneuploid pattern (data not shown). To analyze cell cycle changes by cyclosporin A plus nitrosourea-based drug in detail, a variety of factors including drug concentration, cell line and incubation time should be considered.

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