行政院國家科學委員會專題研究成報告

戊乙醯去氫栀子甘誘導癌細胞凋謝死亡及機制之探討 (一)
Induction of tumor cells apoptosis and mechanisms of action of pentaacetyl gesniposide (一)

計劃類別：個別型計劃

計劃編號：NSC89-2320-B040-002

執行期間：88 年 8 月 1 日至 89 年 7 月 31 日

計劃主持人：王朝鐘

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執行單位：中山醫學院生物化學研究所

中 華 民 國 八十九 年 十 月 二十 日

行政院國家科學委員會專題研究計畫成果報告

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一、 中文摘要

戊乙醯去氫梔子甘, penta-acetyl geniposide, $(Ac)_5$-GP, 分離自梔子果實中去氫梔子甘 (geniposide), 再經乙醯化而來。在過去我們的研究中發現它能抑制培養及老鼠體中增生 C6 glioma 細胞,本研究發現處理$(Ac)_5$-GP 於 C6 glioma 細胞,造成細胞產生凋謝死亡 (apoptosis), 其現象包括染色質聚集,DNA 斷裂成 180-200 bp 之倍數,進一步也發現$(Ac)_5$-GP 造成細胞週期停止於 G_0/G_1。顯示$(Ac)_5$-GP 之抗癌作用 C6 為抑制細胞週期及促進凋謝死亡。

進一步研究發現處理$(Ac)_5$-GP, 增加 p53, c-Myc 及 Bax 蛋白表現,且抑制 Bcl-2 蛋白表現, 顯示$(Ac)_5$-GP 抑制細胞週期及促進凋謝死亡 p53,c-Myc 與及 Bcl-2 family 蛋白表現有關,進一步詳細機轉正進行中。

關鍵詞: 戊乙醯去氫梔子甘; 細胞凋謝死亡; 細胞週期停止; p53 蛋白; c-Myc 蛋白; Bcl-2 蛋白; Bax 蛋白。

Abstract

Penta-acaetyl geniposide, $(Ac)_5$-GP, was produced by acetylation of a glycoside, isolated from an extract of *Gardenia fructus*. Previously, we have reported that C6 glioma cells could be inhibited in culturing as well as in bearing rats by treating with $(Ac)_5$-GP. In this study, the effect of $(Ac)_5$-GP on inducing internucleosomal DNA fragmentation wsa examined in rat C6 glioma cells. Treatment of C6 glioma cells with the $(Ac)_5$-GP caused cell death, chromatin condensation, and internuclesomal DNA ladder. Also, cell cycle arrest at G_0/G_1 phase revealed that $(Ac)_5$-GP induced cell death appears to be mediated by apoptosis. In addition, the results also showed that p53 and c-Myc increased due to treatment of $(Ac)_5$-GP in a dose-response and time-dependent manner. Concomitant with the expression of p53 and c-Myc, decreased level of Bcl-2 and increased level of Bax protein were observed. These results suggest that cell death caused by $(Ac)_5$-GP through apoptosis and cell cycle arrest at G_0/G_1 may be associated with the induction of p53, c-Mye and may be mediated with apoptosis-related Bcl-2 family proteins.

KEY WORDS : Penta-acetyl geniposide; C6 glioma cell apoptosis; cell cycle arrest; p53; c-Myc; Bcl-2; Bax.

二、緣由與目的

Gardenia Fructus (San-jee-chee in Chinese), the fruit of *Gardenia jasminoides* Ellis (Rubiaceae), has been used for many years in Chinese medicine for the treatment of various inflammatory diseases and hepatic disorders. However,

the pharmacological basis of this medication is completely unknown. Numerous previous studies [1-7] have suggested the inhibitory effects of its compounds on tumorgenesis.Penta-acetyl geniposide, $(Ac)_5$-GP, 1-(β – D - 2', 3', 4', 6' – tetraacetylglucopyranosyloxyl) - 1,4a , 5, 7a - tetarhydro-7-(acetomythyl)-cyclopentapyran–4 carboxylic acid methyl ester (Fig. 1), was produced by acetylation of a iridoid glycoside isolated from an extract of *Gardenia Fructus* [8]. Previously, we reported that C6 glioma cells can be inhibited in culturing [8], as well as in bearing rats [9] through the treatment with $(Ac)_5$-GP. $(Ac)_5$-GP has also been shown to inhibit aflatoxin B_1- induced genotoxicity in rats [10].

The biochemical actions of various cytotoxic agents have been extensively investigated, although little is known about the precise mechanisms by which they kill normal and malignant cells. Recent studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin, cytarabine, camptothecin, amsacrine, etoposide and teniposide [11-17]. There is an accumulating evidence that the efficacy of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [11-14 , 18-20]. Apoptosis, a physiological mode of cell death, is characterized by reduced cell volume, condensed chromatin in the nucleus, formation of internucleosomal DNA fragmentation and loss of membrane integrity, and generation of apoptic bodies [11, 21, 22].

Previous evidence suggested that two cellular responses to p53 activation are well described – growth arrest and apoptosis [18, 23]. Many studies also indicate that apoptosis involves in the regulation of oncogenes. One of the first oncogenes demonstrated to have proapoptotic activity was c-Myc. Ectopic expression of c-Myc is sufficient to drive many cells into the cycle and also promotes apoptosis [24-26]. In a previous study [8,9] we have reported that $(Ac)_5$-GP inhibits the growth of C6 glioma cells in culturing and in bearing rats. The exact mechanisms responsible for the antitumor effect of $(Ac)_5$-GP, however, are not yet throughly understood. In this paper we further investigate whether $(Ac)_5$-GP exerts cytotoxic activity against tumor cells by inducing apoptosis and cell cycle arrest, also examining the roles of p53, and c-Myc in the phenomenon. The Bax stimulation effect on apoptosis and cell cycle arrest induced by $(Ac)_5$-GP and Bcl-2 family mediated apoptosis pathway in $(Ac)_5$-GP induced apoptosis of C6 glioma cells.

三、結果與討論

Fig. 2 illustrates the results of MTT assays performed with various concentrations of $(Ac)_5$-GP administered to logarithmically growing C6 glioma cells for 24 hr. The resulting survival curve shows that the $(Ac)_5$-GP had a dose-dependent effect on the cytotoxicity of cells.

The effect of treatment with $(Ac)_5$-GP (0.15 to 0.60 mM) for 6 hr on internucleosomal DNA fragmentation in C6 glioma cells is shown in Fig. 3. Exposure to $(Ac)_5$-GP produced the characteristic ladder of oligonucleosomal DNA fragments that were 180 bp integer multiples in size Fig. 3A. Fig. 3B shows quantitative analyzes of the fragmented DNA in the supernatant of the lysed C6 cells, expressed as the mean percent of the total starting DNA.Fig. 3B shows that a marked increase in the DNA fragmentation was observed when C6 glioma cells were treated with $(Ac)_5$-GP. The resulting DNA fragmentation shows

that the $(Ac)_5$-GP had a dose-dependent effect on the C6 cells. The DNA histogram of the PI-stained cells in Fig. 4 shows that various concentrations of $(Ac)_5$-GP-treated cells (24 hr) had hypodiploid DNA, indicative of apoptosis.

The cells were treated with 0.075 mM $(Ac)_5$-GP for various time periods (0, 12, 24 and 48 hr) and analyzed by flow cytometry. $(Ac)_5$-GP caused a dramatic increase from 59 to 80% in the proportion of G_0/G_1-phase cells (Fig. 5), whereas the S phase decreased compared with the control.

As shown in Fig. 6A, the expression of p53 was gradually increased resulting in a peak at 4 hr after $(Ac)_5$-GP treatment, and then decreased to basal. Concommitantly, the expression of C-Myc and Bax proteins were gradually increased during the period of treatment of $(Ac)_5$-GP (Figs. 7A and 8B). However, the expression of Bcl-2 was gradually decreased at the same period of $(Ac)_5$-GP treatment. The expression of all these proteins showed dose-dependence in $(Ac)_5$-GP-treated C6 glioma cells. These results indicated that $(Ac)_5$-GP-induced apoptosis and cell cycle arrest at G_0/G_1 was associated with the induction of p53, C-Myc and Bax and inhibition of Bcl-2.

Enhanced apoptosis is responsible for many of the adverse effects of chemotherapy and for tumor regression. $(Ac)_5$-GP, a potential anticancer compound developed in our laboratory, has demonstrated [8,9] excellent antitiumor activity in culturing and bearing C6 glioma cells. In view of these encouraging results, it will be worthwhile to investigate the $(Ac)_5$-GP-induced cell death by apoptosis through induction of p53 and c-Myc and modulation of Bcl-2 family proteins. Previously, we found that $(Ac)_5$-GP is no toxic in treated animals (55). All of the above results reflect the therapeutic potential of this new compound.

In conclusion, the data presented in this paper indicate that $(Ac)_5$-GP, a potential chemotherapy agent, inhibits C6 glioma cells proliferation by inducing apoptotic death and cell cycle arrest at G_0/G_1, which is accompainied , at least partily, by the induction of expression of p53 and c-Myc and mediated Bcl-2 family proteins.

四、計畫成果自評

1. 本計畫結果發現$(Ac)_5$-GP具有抗癌作用，其抑制癌細胞之能力係經由促進細胞凋謝死亡及停止細胞週期在G_0/G_1.
2. 其作用機轉，初步研究顯示增加癌細胞 p53 及 c-Myc 表現，並影響 Bcl-2 family 蛋白.
3. 詳細的抗癌分子機轉正進行中.

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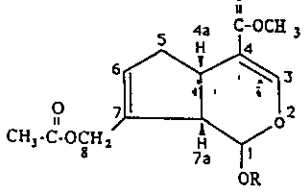

R=β-D-2, 3, 4, 6-tetra-acetyl-glucose

Fig. 1 The structure of penta-acetyl geniposide, $(Ac)_5$-GP.

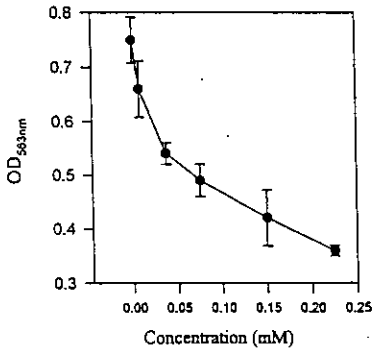

Fig. 2 Dose-response of the viability of C6 glioma cells after treatment with $(Ac)_5$-GP. After overnight attachment of cells, the culture was exposed to indicated concentration of $(Ac)_5$-GP for 24 hr. Then the medium was removed and isopropanol was added to dissolve the formazan crystal for MTT assay. The viable cell number is directly proportional to the production of formazan. Results are shown as mean±SD from three independent experiments.

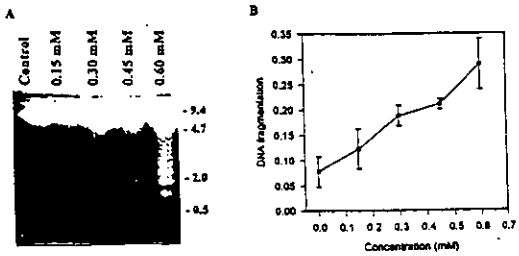

Fig. 3 Agarose gel electrophoretic (A) and quantation (B) analysis of the fragmented DNA from $(Ac)_5$-GP -treated glioma cells. Cells were incubated for 6 hr in the presence of indicated concentration of $(Ac)_5$-GP. DNA fragmentation is measured by anti-Brdu and is directly proportional to the absorbance at 450 nm.

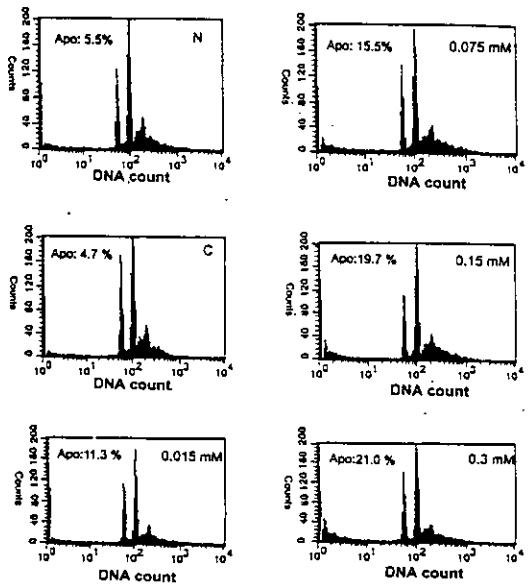

Fig. 4 Determination of pro-apoptotic bodies in $(Ac)_5$-GP-treated C6 glioma cell by flow cytometry. Cells were incubated with $(Ac)_5$-GP as indicated concentration of $(Ac)_5$-GP for 24 hr. Apo indicates the cells with hypodiploid

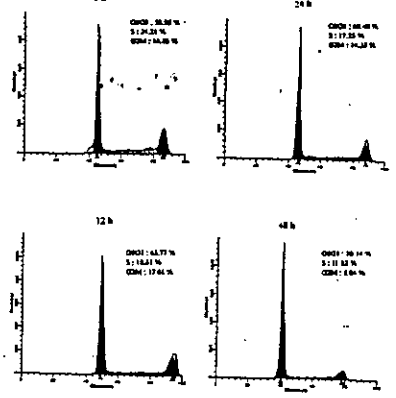

Fig. 5 Flow cytometric DNA fluorescence profiles of C6 glioma cells. PI-stained DNA hitograms of $(Ac)_5$-GP -treated cells are shown. Cells were treated with 0.3 mM for 0-48 hr.

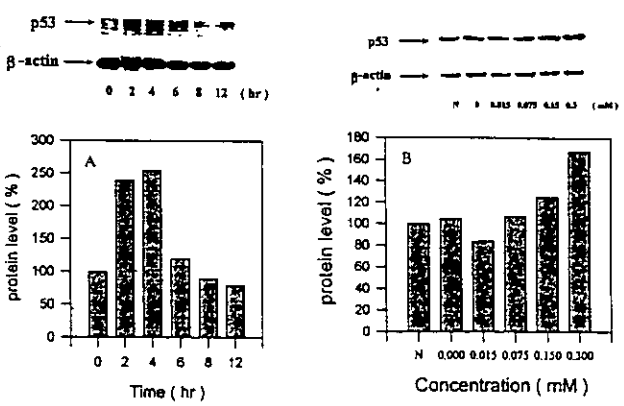

Fig. 6 Expression of p53 protein in the $(Ac)_5$-GP -treated C6 glioma cells. Cells were treated with 0.3 mM for indicated time (A) and treated with various concentration for 4 hr (B), then lysed and analyzed by Western blotting using anti-p53 Ab. Three independent experiments were conducted, a representative one in shown here.

Fig. 7 Expression of c-Myc protein in the $(Ac)_5$-GP -treated C6-glioma cells. Cells were treated with 0.3 mM for indicated time (A) and treated with various concentration for hr (B), then lysed and analyzed by Western blotting using anti-c-Myc Ab. Three independent experiments were conducted, a representative one in shown here.

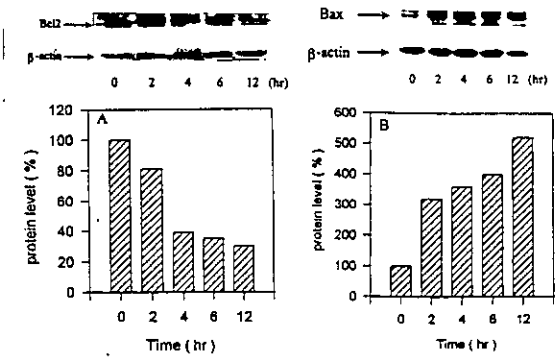

Fig. 8 Expression of Bcl-2 family (Bcl-2 and Bax) proteins in the $(Ac)_5$-GP-treated cells. Cells were treated with 0.3 mM for indicated time, then lysed and analyzed by Western blotting using anti-Bcl-2 Ab (A) and anti-Bax Ab (B). Three independent experiments were conducted, a representative one in shown