Formosanin-C 抗人類大腸直腸癌之作用機轉

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Formosanin C 抗人類大腸直腸癌之作用機轉
The Anti-tumor Mechanism of Formosanin C in Human Colorectal Cancer Cells

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一、中文摘要

Formosanin C (PF-C) 是從 Paris formosana Hayata 純化出之天然物，可誘使大腸直腸癌細胞株 HT-29 引起細胞凋亡 (apoptosis)。以 PF-C 濃度 HT-29 細胞 6 天，其 50%之抑制濃度 (IC{sub}50) 為 1.04 μM，而以 PF-C 處理人類頭頸部腫瘤細胞 (PBMC) 與人類臍靜脈內皮細胞 (HUVEC) 其 IC{sub}50 分別為 24 μM 與 12.6 μM。以 PF-C 處理 HT-29 細胞後，細胞膜內面之磷脂酰胺醇 (phosphatidylerine) 翻轉至細胞外表面，且細胞週期 sub-G1之 DNA 含量增加，並觀察到 DNA 片段化 (DNA fragmentation) 之現象，結果顯示 PF-C 為透過細胞凋亡的過程抑制 HT-29 細胞生長。PF-C 可造成 caspase-2之活化、粒線體之 Bax 和 Bak 表現量增加，以及粒線體之 Bcl-X{sub}_L 表現量減少。另外，以 PF-C 濃度 HT-29 細胞後，會造成粒線體膜電位 (ΔΨ{sub}m) 之喪失，cytochrome c 與 Smac/DIABLO 由粒線體膜間區釋放至細胞質，XIAP 蛋白質表現量減少並造成 caspase-9 與 caspase-3之活化，進而活化 DFF45，最後誘發 DNA 斷裂。由流式細胞儀與共聚焦顯微鏡分析，發現 PF-C 引發細胞凋亡之作用與造成粒線體ΔΨ{sub}m喪失之情形可被 caspase-2 之抑制劑 Z-VDVAD-fmk、caspase-9 之抑制劑 Z-LEHD-fmk 和 cycloporin A 所回復。以上實驗結果顯示，PF-C 處理大腸直腸癌細胞 HT-29 造成，caspase-2之活化、粒線體ΔΨ{sub}m之喪失、cytochrome c 與 Smac/DIABLO 由粒線體釋放至細胞質，XIAP 蛋白質表現量減少，進而活化下游分子，最終造成細胞凋亡。此研究針對 PF-C 進行分子機制之探討，有助於新藥之設計，以減緩大腸直腸癌疾病之進程並

關鍵詞：Formosanin C、大腸直腸癌、細胞凋亡、caspases、粒線體、Bcl-2、cytochrom c、Smac/DIABLO

Abstract

Formosanin C (PF-C), isolated from Paris formosana Hayata, suppresses the growth of human HT-29 cells via apoptosis. The 50% inhibition concentration (IC{sub}50) of PF-C in HT29 cells is 1.04 μM, compared to 24 μM for normal human peripheral blood mononuclear cells and 12.6 μM for HUVEC at day 6 post-treatment. Cells that perished to PF-C undergo apoptosis, which is demonstrated by phosphatidylerine exposure, an increase in sub-G1 content, DNA fragmentation and nuclear morphological changes. The molecular epidos of PF-C-triggered apoptosis includes activation of caspase-2, increased mitochondrial Bax and Bak expression, and decreased the expression of anti-apoptotic mitochondrial Bcl-X{sub}_L. Furthermore, we detected loss of loss of mitochondrial membrane potential (ΔΨ{sub}m), releases of cytochrome c and Smac/DIABLO from mitochondria to cytosol, a decrease in XIAP protein and activations of caspase-9 and -3, and in turn to cleave PARP and DFF45, and then induces DNA fragmentation. The PF-C-induced loss of ΔΨ{sub}m and apoptosis in HT-29 cells can be reversed by caspase-2 inhibitor Z-VDVAD-fmk, cycloporin A, caspase-9 inhibitor Z-LEHD-fmk indicating PF-C activates caspase-9 via caspase-2 activation and mitochondrial dysfunction. These results suggest that the molecular
mechanism of PF-C may shed light on novel drugs design against human colorectal cancer.

Keywords: Formosanin-C, human colorectal cancer, apoptosis, caspases, mitochondria, Bcl-XL, cytochrome c, Smac/DIABLO

二、縁由與目的

Herbaceous plants, traditional Chinese herbal medicines, have been used as food and as medicine for thousands of years. These traditional Chinese medicines have significant roles in treating and maintaining Chinese health, and improving the qualities of Chinese life for many centuries. Many herbal medicines have been verified by the National Center Institute (NCI) in USA as of anticancer and cancer-preventive properties. The whole plant water extracts of Paris formosana Hayata (P. formosana) is used for treatments of snake bite, inflammation and cancer in Taiwan, and P. formosana is also a very common folklore in the treatment of human colorectal cancer in Taiwanese aborigines. Previous report had shown that the water extract of the leaves of P. formosana inhibited tumor growth in mice in vivo and showed cytotoxicity in human cancer cells in vitro. Recently, formosanin-C (PF-C) (Fig. 1) had been isolated from extract of the leaves of P. formosana. An earlier report also indicated that PF-C showed significantly antitumor effect on SK-OV-3 and HT-29 in nude mice. However, the mechanisms of PF-C induced colorectal cancer cell death are poorly understood.

Apoptosis (programmed cell death) represents a general and a delicately autonomous cellular suicide pathway that helps to govern cell numbers and can occur throughout normal development. Cell death by apoptosis is characterized by reduced cell volume, blebbing, condensed chromatin in the nucleus, DNA fragmentation, and phosphatidyserine externalization, as well as generation of apoptotic bodies. The apoptotic bodies are engulfed by phagocytes and lead to little or no inflammation. Apoptosis has been observed in vitro and can be induced by stimuli including radiation, hyperthermia, and chemotherapeutic agents. These results suggest that different type of cellular damage may lead to the production of common signal pathways that initiate the death of cells.

The current aim of cancer research is to ascertain therapeutic agents with fewer side effects than the presently used cytostatic or cytotoxic drugs. This has led to emphasis of the studies on effectiveness of various natural products from Chinese traditional herbs and apoptosis have become a therapeutic aim to evaluate the induction of tumor remission by detecting the production of apoptotic mediators in tumors to accomplish the effectiveness of cancer therapies. Two major pathways related to caspase for the induction of apoptosis have been verified extrinsic and intrinsic. The extrinsic pathway is depicted by tumor necrosis factor family receptors that utilize protein interaction to modulate death domains and death effector domains and to activate certain caspase family such as caspase 8, 9 or 10. The intrinsic pathway comprises mitochondria which releases of cytochrome c, resulting in activations of caspase 9, 7 and 3, and this activation is through the effects of Apaf-1. Colorectal cancer is one of the most common malignancies and causes major public health problem in Western countries and Taiwan. The 5-fluorouracil is the most widely used drug in chemotherapy of colorectal cancer. The therapeutic response is limited at 15% in advanced colorectal cancer. Therefore, treatment concentrates on chemoprevention in at-risk and chemotherapy for the inhibition of human colorectal cancer is important.

三、Results and Discussion

The cytotoxic activity of PF-C on HT-29 human colorectal cancer cells was studied. Cells were treated with the PF-C for up to 6 days. PF-C inhibited the growth of HT-29 cells in a dose- and time-dependent manner (Fig. 2). The IC₅₀ (50% cell inhibitory
concentration) value on HT-29, Hep 3B, PBMC, and HUVEC cells was 1.04, 0.78, 23.8 and 12.64 μM, respectively (Table 1). The inhibiting efficacy of HT-29 cell colony formations in soft agar assay was evaluated. At 14 days post-treatment, the numbers of colonies in the plates treated with 0.9 μM of PF-C for HT-29 cells (Table 1, Fig. 3).

Based on the cell growth inhibition results, we next investigated whether PF-C treatment can induce cell death, and if so, which cell death mechanism(s) may be triggered by PF-C. PF-C treated- or untreated-tumor cells were stained with Annexin V or PI for various time intervals and analyzed by flow cytometry. DNA fragmentation was determined by gel electrophoresis. Figure 4A and 4B show the quantified percentages of apoptotic cells following staining with Annexin V. Exposure to PF-C (0.87 μM) resulted in a significant increase in binding of Annexin V-fluorescein to the cytoplasmic side of the cell membrane (46.89%) at 24 h compared to cells exposed to vehicle alone. In addition, the highest numbers of Annexin V-positive cells (83.02%) was observed in HT-29 after 72 h treatment with PF-C (Fig. 4A). Figure 4B shows that PF-C treatment in HT-29 cells for 24 h significantly increased the amount of Annexin V-positive cells in HT-29 in a PF-C dose-dependent manner. Apoptotic activity was confirmed by staining cells with PI, and the accumulation of cells at sub-G1 phase in HT-29 cells was measured by flow cytometry. As shown in Fig. 4C and 4D, PF-C treatment of HT-29 (0.87 μM) for 48 h, resulted in a time-dependent increase in the accumulation of sub-G1 cells. Furthermore, treatment with vehicle or PF-C (0.22-1.74 μM) for 48 h, a PF-C dose-related increase in the accumulation of sub-G1 cells was observed (Fig. 4D). In addition, the distribution of cells in G0/G1, S, and G2/M phases decreased in a time- and PF-C dose-related manner in HT-29 cells (Fig. 4C and 4D). To confirm these results, cells were treated with vehicle or PF-C (0.22-1.74 μM) in HT-29 cells for 48 or 72 h, DNA laddering was observed by gel electrophoresis (Fig. 4E). Moreover, we next confirm the nuclei of HT-29 cells by fluorescence microscopy after Hoechst 33258 staining. Figure 4F and 4G show that many PF-C-treated HT-29 cells had condensed and fragmented nuclei in a time and PF-C related manner after treatment of PF-C. Taken together, these data demonstrate that PF-C treatment of HT-29 cells results in an altered phosphatidylserine distribution, accumulation of sub-G1 cells, degradation of DNA and condensed nuclei, indicating that PF-C-induced death of HT-29 human colorectal cancer displays typical apoptotic characteristics. These results were reproducibly repeated at least four times (data not shown).

Apoptosis involves a cascade of proteolytic activity, much of which is performed by caspases 32. To investigate whether caspases are involved in PF-C-induced apoptosis in colorectal cancer cells, the expression of activated forms of caspase-2, caspase-9, and caspase-3 following PF-C treatment in HT-29 was assessed by immunoblotting. As shown in Fig. 5A, the expression level of 45-kDa pro-caspase-2 slightly decreased at 72 h after PF-C treatment in HT-29 cell lines, whereas the 35-kDa active form of caspase-2 reached a peak at 12 h and declined from 24 to 96 h in HT-29 cells. Further, the 14-kDa active form of caspase-2 appeared within 12 h following PF-C treatment, reaching a peak at 72 h in HT-29 cells. As shown in Fig. 5B, PF-C treatment for 24 h in HT-29 significantly increased the 35- and 14-kDa active form of caspase-2 in HT-29 cells in a PF-C dose-dependent manner. Additionally, both the 37- and 35-kDa activated forms of caspase-9 also appeared in HT-29 cells in a time- and PFC dose-related manner following PF-C treatment (Fig. 5C and 5D). Moreover, treatment with either the caspase-2 inhibitor Z-VDVAD-fmk or the caspase-9 inhibitor Z-LEHD-fmk almost completely blocked PF-C-induced apoptosis in tumor cells (Fig. 4E and 5F). Finally, both the 17- and 12-kDa activated forms of
caspase-3 appeared within 24 h of PF-C treatment, reaching peak levels at 72 h in HT-29 cells (Fig. 5G). In addition, treatment of HT-29 cells with various concentrations of PF-C (0.22–1.7 μM) resulted in a dose-dependent increase in 17- and 12-kDa activated forms of caspase-2 protein levels (Fig. 5H). No active forms of any of the caspases were observed in untreated cells (Fig. 5A, 5B, 5C, 5D, 5G and 5H). Caspase-8 was not involved in PF-C-induced apoptosis in HT-29 cells (data not shown). These results indicate that PF-C-induced apoptosis occurs via activation of caspase-2 and -9 in colorectal HT-29 cell lines.

PARP protein is a pivotal substrate for active caspases, and cleavage of PARP is indicative of apoptosis. PARP (116 kDa) is cleaved into a 85-kDa product during apoptosis. To monitor the cleavage of PARP, HT-29 cells were treated with PF-C (0.87 μM). The 85-kDa cleaved form of PARP slightly appeared within 24 h and expression peaked at 96 h (Fig. 5I) and concomitantly increased the full-size 116-kDa PARP (Fig. 5I). Figure 5I also shows that PF-C treatment for 48 h in HT-29 cells significantly increased the amount of cleaved 85 kDa protein of PARP in HT-29 in a PF-CA dose-dependent manner. Similarly, no PARP cleavage was detected in untreated cells.

Caspase-3 can cleave its substrate DFF45 prior to the initiation of apoptotic DNA fragmentation. Therefore, reduced expression of DFF45 was used as an indicator of caspase-3 activation. As shown in Fig. 5K, treatment of HT-29 cells with 0.87 μM PF-C for the indicated time intervals resulted in slight decrease in DFF45 levels at 48 h, with the lowest levels occurring at 96 h. In addition, the levels of 30-kDa product of DFF45 were also increased from 48 to 96 h HT-29 cells following treatment with PF-C at various time intervals (Fig. 5K). Treatment of HT-29 cells with various concentrations of JA (0.22–1.7 μM) for 48 h resulted in a dose-dependent decrease in 35- and 45-kDa DFF45 and increased in 30-kDa protein levels (Fig. 5L). These data demonstrate that caspase activation induced by PARP cleavage and DFF45 degradation is involved in PF-C-induced apoptosis in HT-29 tumor cell lines.

Because the mitochondrial release of both cytochrome c and Smac/DIABLO are major events in mitochondria-mediated apoptosis that are required for the activation of caspasess and DNA fragmentation, we assessed these two events by immunoblot analysis. As shown in Fig. 6A and 6C, cytosolic levels of cytochrome c and Smac/DIABLO increased significantly in HT-29 cells within 24 h and reached peak levels at 48–72 h following treatment with PF-C (0.87 μM). Conversely, level of cytochrome c and Smac/DIABLO decreased in the mitochondria-enriched fractions of HT-29 cells within 24 h and became undetectable 72 h after PF-C treatment. The levels of cytochrome c and Smac/DIABLO were significantly increased in the cytosol and decreased in the mitochondria-enriched fraction in a PF-C dose-related manner after 48 h of PF-C treatment (0.22–1.74 μM) (Fig. 6B and 6D). In contrast, in the absence of PF-C treatment, cytosolic levels of cytochrome c and Smac/DIABLO remained low (Fig. 6A, 6B, 6C, and 6D). Our data suggest that the release of cytochrome c and Smac/DIABLO from mitochondria into the cytosol plays a role in PF-C-induced apoptosis in human colorectal cancer HT-29 cells.

Smac/DIABLO has been reported to antagonize the antiapoptotic function of XIAP, an inhibitor of apoptosis that acts through caspasess. We therefore investigated whether Smac/DIABLO and XIAP are involved in PF-C-induced apoptosis. The cell lysates of vehicle- or PF-C-treated HT-29 cells were analyzed by Western blotting. As shown in Fig. 6E, XIAP levels dramatically decreased within 24 h and reached a minimum 96 h after PF-C treatment (0.87 μM) in HT-29 cells. In addition, XIAP protein levels were significantly reduced in a PF-C dose-dependent fashion (0.22–1.74 μM) (Fig. 6F). In contrast, XIAP levels were not
affected in vehicle-treated cells. These results confirm that the activation of caspase-9 and caspase-3 by PF-C in HT-29 may correlate with an increase of cytochrome c and Smac/DIABLO in the cytosol and a decrease in XIAP protein levels.

The ΔΨm is an early event of apoptosis, and a decrease of ΔΨm is associated with mitochondrial dysfunction. Changes of ΔΨm in apoptotic cells were analyzed using the mitochondrial dye rhodamine 123. As shown in Fig. 7A, a significant time-dependent decrease in rhodamine 123 intensity in the mitochondria in PF-C-treated HT-29 (0.87 μM PF-C) cells was observed. The loss of ΔΨm was observed as early as 12 h and reached a minimum 72 h after PF-C treatment in this cell lines (Fig. 7A). Furthermore, treatment of HT-29 cells with various concentrations of PF-C (0.22–1.74 μM) for 24 h resulted in a dose-dependent loss of ΔΨm (Fig. 7B). This time-dependent loss was confirmed with confocal microscopy using rhodamine 123 staining, which showed a decrease within 12 h and reached its lowest levels 72 h after PF-C treatment in tumor cell lines (Fig. 7C). In addition, a PF-C dose-dependent (0.22 μM –1.74 μM) loss of ΔΨm was also confirmed with confocal microscopy (Fig. 7C). In contrast, there was no change of ΔΨm in vehicle-treated cells as measured by rhodamine 123 flow cytometry or confocal microscopy staining (Fig. 7A, 7B, and 7C). Moreover, treatment of tumor cell lines with 20 μM Z-VDVAD-fmk or 10 μM cyclosporin A (cyclophilin D inhibitor) prior to the addition of PF-C blocked mitochondrial dysfunction as imaged by confocal microscopy and PF-C-induced DNA fragmentation measured by gel electrophoresis (Fig. 7D and 7E), indicating that mitochondrial dysfunction in PF-C-induced apoptosis requires the upstream action of caspase-2 as well as changes in mitochondrial membrane permeability. Taken together, our results confirm that a loss of ΔΨm is essential for the PF-C-induced apoptotic release of cytochrome c and Smac/DIABLO from the mitochondria into cytosol.

To determine whether the levels of death-related proteins in human colorectal cancer cells are influenced by PF-C-induced apoptosis, we measured the expression levels of Bcl-2, Bcl-XL, Bax, and Bak following PF-C treatment by Western blotting. Tumor cell lines were treated with varying concentrations of PF-C at the indicated time intervals. As shown in Fig. 8A and 8B, treatment of HT-29 cells with various concentrations of PF-C (0.22–1.74 μM) for the indicated time intervals resulted in a time- and dose-related decrease in Bcl-XL expression was observed in mitochondria-enriched fractions, but no significant effect on whole-cell lysates, of PF-C-treated HT-29 cells. We also measured any PF-C-induced changes in Bax and Bak expression levels in the cytosol- and mitochondria-enriched fractions in tumor cell lines. As shown in Fig. 8C, the mitochondria-enriched fraction of Bax was slightly increased within 12 h and peaked from 48 to 96 h in PF-C-treated HT-29 cells. In addition, mitochondrial Bak was markedly increased at 48 h and peaked from 72 to 96 h in tumor cells after PF-C treatment (Fig. 8E). In contrast, the amount of cytosolic Bax and Bak in tumor cell lines significantly decreased as the PF-C treatment time increased (Fig. 8B and 8E). Moreover, expression of both Bax and Bak dramatically increased in a dose-dependent manner in the mitochondria-enriched fraction and a significantly decreased in cytosolic fraction of tumor cell lines (Fig. 8D and 8F). These results indicate that Bcl-XL, Bax, and Bak play a role in PF-C-induced apoptosis in human colorectal tumor cell lines.

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