行政院國家科學委員會補助專題研究計畫成果報告
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Justicidin A induces apoptosis through mitochondria dependent pathway in human colon carcinoma cell lines.

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一、中文摘要

*J. procumbens* 的天然抗癌萃取物 Justicidin-A(JA)，可以藉由誘導細胞凋亡而抑制人類大腸直腸癌細胞株 HT-29 及 HCT 116 的生長。隨著藥劑量的增加，以及作用的時間延長，抑制效果更佳。在 HT-29 細胞中，與 JA 一起培養 6 天後，其 50% 細胞凋亡濃度（IC₅₀）為 0.11 μM，而在 HCT 116 細胞則為 0.4 μM，在正常人類周邊單核球細胞中，其 IC₅₀ 高達 23.0 μM。此兩種細胞株在 JA 處理後，發現磷脂酰絲氨酸 (phosphatidylserine) 由細胞膜內面外翻至外面，sub-G₁ DNA 含量增加，細胞去核糖核酸 (DNA) 有呈現梯度的斷裂的現象，顯示 JA 毒殺癌細胞是經由細胞凋亡的途徑。另外也發現 JA 使細胞粒線體膜電位喪失，粒線體間區蛋白質如細胞色素 c (cytochrome c) 及 Smac/DIABLO 釋出到細胞質，XIAP 細胞內表現量降低，這些結果相繼促成 caspase-2, caspase-9 及 caspase-3 的活化。除此之外，我們也發現了抑制細胞凋亡的蛋白質 Bcl-2 與 Bcl-Xₐ 的量下降。更進一步，caspase-3 的活化會切割 poly (ADP-ribose) polymerase (PARP)，最終導致 DNA 的斷裂。本研究明確指出 *J. procumbens* 的萃取物 JA 可以經由誘導細胞凋亡途徑毒殺並抑制癌細胞的生長，或許 JA 與其衍生物可以成為有效而且安全的藥物，提供未來在大腸直腸癌患者治療上的幫助。

關鍵詞：胃癌 A，人類大腸直腸癌，細胞凋亡，cytochrome c, caspases, Smac/DIABLO, 嚴重複合免疫缺陷老鼠 e

Abstract

A natural product justicidin A (JA), isolated from *Justicia Procumbens* (Chinese herb medicine), effectively suppressed the growth of human colon cancer HT-29 and HCT 116 cells through apoptosis. The 50% inhibition concentration (IC₅₀) of JA on HT-29 and HCT 116 cells were 0.11 μM and 0.4 μM, respectively as compared to the normal human peripheral blood mononuclear cells (23 μM) at 6 day post-treatment. Susceptibility of the cells to JA is caused by apoptosis, which is demonstrated by phosphatidylserine exposure, increase of Sub-G₁ content, and DNA fragmentation. The molecular basis of JA triggered apoptosis seems to associate with the activation of caspase-2 and up expression of mitochondrial pro-apoptotic Bad, and down expression of cellular anti-apoptotic Bcl-2 and Bcl-Xₐ. Concomitantly, including loss of mitochondrial membrane potential, release of Smac/DIABLO and cytochrome c from the mitochondria into cytosol, decrease of the XIAP protein contents and activation of caspase-9 and -3, which in turn cleaves poly (ADP-ribose) polymerase (PARP) and DFF45, and causes DNA fragmentation. Our understanding the mechanism of JA action at molecular level will shed light on novel chemotherapeutic agent design against
human colon cancer progression.

Keywords: Justicidin A, human colon cancer, apoptosis, cytochrom c, caspases, Smac/DIABLO, SCID mice

二、缘由与目的

Chemotherapeutic agents, such as etoposide, adriamycin, mitoxanthrone, cisplatin, and 5-fluorouracil (5-FU), has been shown to induce apoptosis in several leukemias and solid tumors (1) (2-6). The activation of caspase-2 induced by cytotoxic stress is an early process in apoptosis, which is required for the permeabilization of mitochondria (7-12). Mitochondria play a pivotal role in apoptotic process including tumor cell death induced by chemotherapeutic agents (13-15). Upon stimulation, the apoptotic factors, such as cytochrome c [Liu, 1996 #1678], Smac/DIABLO (Second mitochondria-derived activator of caspase/direct IAP binding protein with low pl) (16-18), endonuclease G (19), and AIF (20) release from mitochondria into the cytoplasm. In the cytosol, cytochrome c binds to Apaf-1 and forms Apaf-1/cytochrome c complex in the presence of ATP and activates downstream effector caspases including caspase-9, -7, and -3 (16, 21, 22). In addition, Smac/DIABLO is also released from mitochondria to the cytosol during apoptosis and promote caspase activation by binding to XIAP (23)[Shi, 2001 #2034] (24) (22, 25). Once activation of effector caspase can cleave various of death substrates, which in turn lead to cause apoptosis in cancer cells (26) (27).

Colorectal cancer is one of the most common malignancies and causes major public health problems in Western countries (28-30). The 5-fluorouracil is the most widely used drug in chemotherapy of colorectal cancer (31, 32). The therapeutic response is limited at 15% in advanced human colon cancer, therefore, new chemotherapeutic agents have to develop. Thus, led us to search natural products from Chinese traditional herbs for potential anticancer agents. Herbaceous plants have been used as food and as medicine for thousands of years in China. They play significant roles in treating and maintaining Chinese health and in improving the characteristic of Chinese life for many centuries (33). Recently, The herbal medicine was verified by National Center Institute (NCI) of USA using as anticancer agent to show cancer preventive properties (34-36). The whole plant water extracts of *J. procumbens* is used for treatments of pain and fever in Taiwan (37-39), and are also very common folklore in the treatment of human colon cancer in median area of Taiwan. Previous reports have shown that the methanolic extract of the whole plant of *J. procumbens* inhibited P-388 lymphocytic leukemia growth in BDF1 mice in vivo and showed cytotoxicity in the KB cell in vitro (40). Recently, five 2,3-naphtalide lignans including justicidin A (JA), justicidin E, neojusticin A, B and diphyllyn have been isolated from methanolic extract of the whole plant of *J. procumbens* Linn (41, 42). An earlier report also indicated that JA significantly inhibited the growth of KB cells in vitro (43). However, the mechanism of JA induces colon cancer cell death is not thoroughly understood yet.

Here, we first explore the induction of
apoptosis signaling pathway by JA in human colon cancer cell lines, HT-29 and HCT116. We clearly demonstrate that JA induces cell death through an apoptotic pathway involving phosphatidylserine exposure, increase of sub-G1 content and DNA fragmentation related to programmed cell death. Among molecular events that accompany these cytotoxic effects of JA, activation of caspase-2 and induction of Bax protein expression are early events resulting in mitochondrial dysfunction and precedes downstream apoptotic cascades and cell death. Notably, we also demonstrate that JA-induced apoptosis requires the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol to antagonize the expression of XIAP protein leading to induction of downstream effector caspases cleavage of various death substances. Therefore, our findings suggest that the natural JA induces apoptosis through caspases and mitochondria mechanism, which may contribute to chemoprevention or chemotherapy in human colon cancer.

三・ Results and Discussion

**Growth Inhibition by JA in human colon cancer cells.** The cytotoxic activity of justiciedin A (JA) on HT-29 and HCT116 human colon cancer cells was studied by MTT assay. Cells were treated with JA for 6 day, JA reduced the viable cells in a dose-and time-dependent manner (Fig. 1 and 2). The IC₅₀ (50% cell inhibitory concentration) value on HT-29 and HCT-116 cells was 0.11 μM and 0.4 μM, respectively (Table 1). The inhibiting efficacy of HT-29 and HCT-116 cell colony formations in soft agar assay was also evaluated. At 15 days post-treatment of JA, the numbers of colonies in the plate treated with 0.03 μM for HT29 and 0.1 μM for HCT116 are approximately half compared with those in the control plates, indicating that JA is more effective in inhibiting colony formation in soft agar than that in MTT assay (Table 1, Fig. 3). Strikingly, Human PBMC from normal volunteers was also tested for their susceptibility to JA. As shown in Table 1, the IC₅₀ of JA in these PBMC is 25 μM, indicating increased sensitivity to JA than normal PBMC.

**Induction of Apoptosis by JA in human colon cancer cells.** Based on the results of cell growth inhibition, the experiments were carried out to investigate whether the cell death induced by JA was due to the process of apoptosis, JA treated or untreated tumor cells were performed an annexinV (a lipophilic dye and high affinity for phosphatidylserine, PS) and analyzed by flow cytometry. Redistribution of the cytoplasmic membrane annexin V-binding PS to the outer leaflet of cytoplasmic membrane is a hallmark of apoptosis. Both HT-29 and HCT116 cells treated with JA (0.75 μM for HT-29 and 5 μM for HCT116) from 12 to 72 hours, respectively. After incubation, cells were harvested and stained with annexin V for quantifying the percentage of apoptotic cells. As shown in Fig. 4, exposure of JA resulted in a significant increase in the binding of annexin V-fluorescein to the cytoplasmic membrane for 23.8% in HT29 and 20.12 % for HCT116 at 24 h compared with those of cells exposure to vehicle alone (5.76 for HT-29 and 9.5% for HCT116) (Fig.4). In addition, JA induced the PS of cytoplasmic membrane translocation from the inner layer to the outer leaflet of the cell.
surface is in a time-dependent manner (Fig. 4). To confirm apoptotic process, the presence of apoptosis was examined by PI staining. Flow cytometry was performed to measure the accumulation of cells with sub-G1 DNA content at 6-96 h after JA (0.75 μM for HT-29 and 5 μM for HCT116) treatment. As shown in Fig. 5 and 6, treatment of HT29 cells with JA resulted in about 37-fold at 72 h for HT-29 and 18-fold at 92 h for HCT116 increase of sub-G1 peak, respectively. In addition, at the earlier time points (24 h) the cells only stained with annexin V. Whereas, at later time points (from 48 h to 92 h) staining sub-G1 with PI was found. Moreover, JA-induced sub-G1 peak increased significantly dose-dependent manner in both cells. To further confirm these results, agarose gel electrophoresis were performed by purifying DNA from cells treated with vehicle or JA (0.25-1.0 μM for HT29 and 1.25-5 μM for HCT116) at 48 h for HT-29 or 72 h for HCT116 cells, respectively. As shown in Fig. 7, a dose-dependent DNA ladder-like pattern fragment was observed. To determine whether JA-induced DNA fragmentation depended on protein synthesis and/or gene expression (44, 45), both cells were incubated with cycloheximide (0.5 μg/ml) prior to addition of JA. JA-induced DNA fragmentation was markedly inhibited in the presence of cycloheximide, indicating that intact protein biosynthesis was required for JA-triggered apoptosis (Fig. 8). Taken together, the present data suggest that JA induces cell death in HT29 and HCT116 cells by changing the distribution of PS, amount of DNA content, and degradation of DNA, indicating JA-induced human colon carcinoma HT29 and HCT116 cell death is a typical apoptosis processes.

Activation of Caspases by JA in human colon cancer cells. To investigate whether caspases were involved in JA-induced apoptosis, activated form of cleavage caspase-2, caspase-9 and caspase-3 were examined by immunoblotting. As shown in Fig. 9, 10 and 11, the cleavage of procaspase-2, procaspase-9 and procaspase-3 were detected in a dose-dependent manner in HT-29 and HCT 116 cells at 48 h and 72 h exposure of various concentrations of JA (0.25-1.0 μM), respectively. Furthermore, treatment of both tumor cells with Z-VAD-fmk (a pan caspase inhibitor) almost completely blocked DNA fragmentation induced by JA (Fig. 12). These results indicated that induction of apoptosis by JA in HT-29 and HCT 116 cells was related to the activations of caspase-2, caspase-9 and -3.

Degradation of PARP and DFF45 by JA in human colon cancer cells. Poly (ADP-ribose)polymerase (PARP) protein is pivotal substrate for active caspases and cleavage of PARP is a hallmark of apoptosis [Lazebnik, 1994 #1709]. The PARP (116 kDa) is cleaved into 89 kDa fragment during apoptosis (46, 47). To investigate the cleavage fragment of PARP in JA-induce apoptosis, treatment of tumor cells with various concentrations of JA (ranging from 0.25 to 1.0 μM) for 48 h caused a dose-dependent of cleavage of PARP (89 kDa) (Fig. 13). In kinetic studies, the appearance of cleavage PARP (89 kDa) was observed within 12 h and reach its peak at 72 h after treatment of JA (0.75 μM) (Fig. 13). Similar results were obtained in JA-treated HCT116 cells (Fig. 13). The present data
indicated that PARP cleavage is involved in JA-induced apoptosis in HT29, which may relate to the appearance of caspase activity.

Caspase-3 must cleave its substrate DFF45 before apoptotic DNA fragmentation can be proceeded (48, 49). The possibility of activation in caspase-3 was tested if it may also induce DFF45 cleavage. As shown in Fig. 14, treated tumor cells with various concentration of JA (0.25 to 1 μM) for 48 h results in decreasing the protein level of DFF45. In addition, treatment of tumor cells with 0.75 μM of JA for indicating time interval, illustrated in Fig. 14 shown that DFF45 is decreased within 24 h in the JA treatment of JA. The cleavage of caspase-3 at same amount of JA was observed 12 h latter, indicating time course is paralleled with decrease in DFF45 decreased and fragmentation of DNA.

Release of Cytochrome c from mitochondria by JA in human colon carcinoma cells. Cytochrome c releases from mitochondria into the cytosol is required for the activation of caspases and fragmentation of DNA (50)(Cell 86: 147, 1996). Therefore, the release of mitochondrial cytochrome c into the cytosol was examined by immunoblot analysis. As shown in Fig. 15, cytochrome c was significantly increasing in the cytosol in a dose dependent (ranging from 0.25 to 1.0 μm) manner after 48 h of treatment with JA HT-29 cells. In contrast, without treatment, most of the cytochrome c was undetectable in cytosol. The effect of JA on cytochrome c in the mitochondria was also examined. The amount of cytochrome c in mitochondria showed a significant corresponding decrease and became undetectable after 48 h treatment with JA (0.75 μM) (Fig. 16). The increasing of cytochrome c in cytosol and decreasing in mitochondria after treatment of JA showed in a time-dependent manner (Fig. 16). These results indicated that cytochrome c release from mitochondria to cytosol is involved in HT29 apoptosis induced by JA. A similar effect was also observed in JA-treated HCT116 cells (Fig 15 and 16). These findings indicated that the cytochrome c release from mitochondria to cytosol is involved in JA-induced apoptosis in HT29 and HCT116 cells.

Release of Smac/DIABLO from mitochondria by JA in human colon carcinoma cells. Smac/DIABLO was reported to antagonize the antiapoptotic function of XIAP (23, 51). To further examine the relation between Smac/DIABLO and XIAP in JA-induced apoptosis, cytosolic and mitochondrial lysates of vehicle- or JA-treated HT29 or HCT116 cells were analyzed by Western blotting. As shown in Fig. 17, the Smac/DIABLO were significantly increasing in the cytosol in a dose-dependent fashion at 48 h after treatment of JA (0.25-1.0 μM). The protein level of Smac/DIABLO in the mitochondria showed a significant decrease and became unable to detect (Fig. 17). Treatment of HT29 cells with JA (0.75 μM) resulted in a time-dependent increasing in the release of Smac/DIABLO from mitochondria into the cytosol, and decreasing at the level of Smac/DIABLO in mitochondria (Fig. 18). Similar results were also found in JA-treated HCT116 cells (Fig. 17 and 18). These data indicated that the Smac/DIABLO release from mitochondria to cytosol is also involved
in JA-induced apoptosis in HT29 and HCT116 cells.

Inhibition of XIAP by JA in human colon carcinoma cells. XIAP is an inhibitor of apoptosis protein that can inhibit cell death by interaction with caspases (52). We next asked whether XIAP protein would be involved in the action of JA, cell lysates of vehicle- or JA-treated HT 29 and HCT 116 cells were analyzed by Western blotting. As shown in Fig. 19, the protein level of XIAP was significantly decreasing in a dose-dependent manner at 48 h after treatment of JA (ranging from 0.25 to 1.5 μM) (Fig. 19 A). The amount of XIAP became undetectable after treatment with 1.0 μM of JA. In addition, the decreasing of XIAP in JA (0.75 μM)-treated HT29 cells was significantly showed in a time-dependent fashion (Fig. 19). The decreasing of XIAP was observed beginning at 24 h and reached its peak at 48-72 h (Fig. 19). In contrast, the amount of XIAP was not affected by JA treatment for 6 and 12 h. In addition, similar results were obtained in JA-treated HCT116 cells. These data confirmed that JA treatment promoted to activate caspase-9 and caspase-3 activity in HT29 cells due to increase the Smac/DIABLO from mitochondria to cytosol and that leading to decrease in protein level of XIAP.

Mitochondria involvement in JA-induced apoptosis of human colon cancer cells. Mitochondrial membrane potential (Δψm) is an event of apoptosis (9). Decreasing Δψm is associated with mitochondrial dysfunction (53). To study this key events, both cells were treated with JA and apoptotic cells were analyzed by changes in mitochondrial Δψm using the mitochondria dyes DiO6 (54), and by staining with PI exclusion assay. As shown in Fig. 20, the significantly decreased in the accumulation the intensity of DiO6(3) on mitochondria in JA-treated HT-29 and HCT116 cells were observed in a time-dependent manner. The loss of Δψm was observed as early as 24 hours after 0.75 and 5 μM of JA treatment in HT-29 and HCT116 cells, respectively. As shown in Fig. 21, treatment of HT29 or HCT116 cells with various concentrations of JA (ranging from 0.25 to 10 μM) for 48 h resulting in loss of Δψm were also confirmed by confocal microscopy using rhodaime 123 stain of mitochondria in living cells (55)[Yang, 1997 #1828]. Moreover, the loss of Δψm was also observed at 24 h after 0.75 or 5 μM of JA in HT-29 or HCT 116 cells by confocal microscopy image, respectively (Fig. 22). In contrast, There is no change in Δψm in vehicle treated-cells measuring by DiO6(3) flow cytometry or confocal microscopy rhodamine staining image. Taken together, the results further indicate that the loss of Δψm is essential for the release of Smac/DIABLO and cytochrome c from mitochondria to cytosol, and thereby induces its apoptotic effect in human colon cancer cells by treatment of JA.

Involvement of death-related proteins in JA-induced apoptosis in human colon cancer cells. The Bcl-2 family proteins are the best characterized regulators of apoptosis such as Bcl-2 and Bcl-XL (suppress apoptosis), whereas others, such as Bax and Bak (promote apoptosis (56)[Thornberry, 1998 #1949](57). To examine whether the levels of these cell death-related proteins are involved in JA-induced apoptosis, we
determined the JA regulation of Bcl-2, Bcl-X<sub>L</sub>, and Bad protein levels during JA-induced apoptosis. Both HT-29 and HCT 116 cells were treated with different concentrations of JA at the indicated time intervals by Western blot analysis. As shown in Fig.23 and 24, HT-29 cells did not show any Bcl-2 protein level in this experiments, whereas HCT 116 cells were showed an decrease in the Bcl-2 protein 24 h after treatment of JA and remained a significant decreased after 72 h of exposure (Fig. 23). Moreover, treatment of HT-29 cells with various concentration of JA ranging from 0.25 to 1 μM for 48 h resulted in decreases Bcl-X<sub>L</sub> protein expression in whole cell lysates and mitochondrial fraction (Fig. 25). In this study, we also performed the distribution of Bax in total or mitochondrial fractions. As shown in Fig. 26, the mitochondrial Bax was increased in time-dependent fashion during apoptosis induced by JA in HT-29 cells, whereas Bax from JA-treated cells was lower than that from untreated cells. These results indicated that Bcl-X<sub>L</sub> and Bax protein of both cells are involved in the mechanism by which JA induces apoptosis.

**Discussion**

This is the first study to demonstrate that JA can induce the death of human colon cancer HT 29 and HCT116 cells through apoptosis in a dose- and time-dependent manner. The mechanism of apoptosis includes activation of caspase-2, loss of the mitochondrial membrane potential (Δψm), release of Smac/DIABLO and cytochrome c to the cytosol, decrease of XIAP protein contents, activation of caspases, the cleavage of PARP and decrease of DFF-45. During apoptosis, certain characteristic events including phosphatidylserine (PS) externalization, DNA fragmentation, and generation of apoptotic bodies occur (4, 6, 58-62). In our additional study showed that the PS exposure on the cytoplasmic membrane, sub-G1 content and DNA fragmentation in JA-treated HT-29 and HCT 116 cells were observed. Based on these data, JA-induced human colon cancer HT29 and HCT116 cell death was proved to be a typical apoptosis processes.

Apoptosis can be triggered by many factors, including activation of cell surface death receptors of TNFR family, oxidative stress, γ-irradiation, chemotherapeutic and chemopreventive agents (63-66). Chemotherapeutic drug-induced apoptotic pathways are complicated, especially involving signal transductions in the cells (27). Caspases are selectively cleaved at an aspartate residue to produce the active enzyme (67, 68). Caspase-2 is activated in various cells in response to numerous stimuli such as TNF-α, Fas ligand and anticancer drugs (8, 9, 69, 70). When cells are induced to go through cell death, caspase-2 is cleaved into 32-35, 18, and 12 kDa active subunits (71). Notably, Lassus et al. reported that caspase-2 is required for chemotherapeutic agents-induced apoptosis of human cancer cell lines (8). In this study, JA treatment resulted in a dose- and time-dependent cleavage of caspase-2 in both cells. Moreover, we also showed that JA-treated cells induced activation of caspase-9 and caspase-3 in the later events.

Mitochondria play an important role in the regulation of apoptosis (72) and the mechanism involved in cytochrome c and
Smac/DIABLO-related apoptosis has well been defined (21, 23, 51, 73-75). In response to apoptotic stimuli, cytochrome c and Smac/DIABLO translocates from mitochondria to cytosol, and then binds to Apaf-1 and induces the aggregation and processing of caspase-9, which in turn activates downstream caspases (22, 24). Anti-cancer drugs induced apoptosis caused the loss of mitochondrial membrane potential ($\Delta\psi_m$) and the release of cytochrome c and Smac/DIABLO from the mitochondrial intermembrane space have been reported (76) (22, 77, 78). In JA-treated cells, we observed loss of $\Delta\psi_m$ followed by release of cytochrome c and Smac/DIABLO, indicating that the cytochrome c and Smac/DIABLO are released from mitochondria during mitochondrial dysfunction and then to activate downstream caspases. Moreover, we also demonstrated that JA treatment leads to the release Smac/DIABLO, which interacts with XIAP and subsequently leads XIAP dissociates from caspase-9 and together in the presence of cytochrome c to active caspase-9. The activated caspase-9 triggered the downstream caspase cascade to induces cell apoptosis.

PARP is a 116 kDa nuclear chromatin-associated enzyme that is cleaved during apoptosis by caspase-3 into an 89 kDa fragment catalytic domain, as a readout of apoptosis (79-81). In this study, this first time to our knowledge, the involvement of PARP cleavage and the activation of caspase-2, caspase-9, and caspase-3 during apoptosis execution induced by JA in human colon cancer cells. DNA fragmentation Factor (DFF), a heterodimer of 40 kDa (DFF40) and 45 kDa (DFF45) subunits, can induce DNA fragmentation (48, 49, 82) (83, 84). In nonapoptotic cells, DFF40 remains inactive because it is bound to its natural inhibitor DFF45/ICAD (49, 82, 85) (4-6). Caspase-3 functions to dissociate DFF40 from DFF45 leading DFF40 to enter the nucleus and degrade DNA (48, 49, 82, 85). JA treatment significantly decreased the level of DFF45 in a dose- and time- dependent manner (Fig.), suggesting that JA induced apoptosis in colon cancer cells is through cleavage of PARP, decreasing DFF45 and increasing caspase-activated deoxyribonuclease.

The Bcl-2 family is a critical regulator of apoptosis that can either inhibit (Bcl-2 and Bcl-XL) or induce (Bax and Bad) apoptosis (13, 86, 87). The mitochondria-mediated apoptosis is initiated by the release of Smac/DIABLO and cytochrome c and this process is control by Bcl-2 family members (88 review ref). Our data showed that Bcl-XL protein levels decreased in both cell lines after treatment of JA. Bcl-2 was never detected before or after treatment with JA in HT-29, which is consistent with Gamet-Payrastre et al report (89). In contrast, Bcl-2 protein was substantially expressed in HCT 116 to protect HCT 116 cells against the apoptosis. Bax or Bak appears to be a pivotal gateway to mitochondrial dysfunction required for cell death in response to various apoptotic stimuli or stress (90, 91). The interaction between Bcl-2 family proteins in the mitochondrial membrane can regulate the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol (22, 90, 92, 93). Current previous studies demonstrated that anticancer agents-induced translocation of Bax to mitochondria from cytoplasm and for release of cytochrome c and Smac/DIABLO
to cytoplasm is required caspase-2 activation (8-10). In our present study, treatment of JA resulted in activation of caspase-2 and upregulation of Bax protein expression in both tumor cells. It appears that caspase-2 leads Bax translocation to mitochondria may play an essential role in response to JA in human colon cancer cells in which cytochrome c and Smac/DIABLO release from mitochondria and proceeds downstream apoptotic death cascades.

In summary, we clearly show that JA could induce apoptosis in two human colon cancer cell lines. JA initially induced activation of caspase-2 and inhibited the expression of the Bcl-X<sub>L</sub> and Bcl-2, and upregulated the expression of Bax, resulting in an altered ratio of Bax and Bax to Bcl-X<sub>L</sub> and Bcl-2, which then triggered mitochondria-mediated caspase cascade cell death. Taken together, our findings suggest that JA is a promising chemopreventive or chemotherapeutic agent at least for human colon cancers. Whether it is effective for other cancers remaining to be determined. It is also noteworthy using oral or i.p. application, and molecular pharmacology of JA-target cell interactions or its derivative analog substances which are currently under way.

References
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Growth inhibition of HT-29 treated with JA
MTT assay:

Fig. 1

Growth inhibition of HCT 116 by treated with JA
MTT assay:

Fig. 2

Effects of JA on by colony formation

Fig. 3

Table 1. Effects of JA response for 50% growth inhibition (IC50) and colony formation on human colon cancer cell lines HT-29, HCT 116 and human peripheral blood mononuclear cells (PBMC).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>growth inhibition (μM)</th>
<th>Colony forming inhibiton (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>0.11 ± 0.10</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>HCT 116</td>
<td>0.46 ± 0.12</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>PBMC</td>
<td>22.97</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4

Effects of JA on sub-G1 peaks in human colorectal carcinoma cells by flow cytometry

Fig. 5
Effects of JA on sub-G1 peaks in human colorectal carcinoma cells by flow cytometry

HCT 116 (72 h) vs. JA (5.0 μM)

Fig. 6

Effects of cycloheximide (Cyc) on DNA fragmentation in JA-treated HT-29 and HCT 116 cells

Cycloheximide: 0.2 μg/ml

Fig. 8

Inductions of DNA fragmentation in human colorectal carcinoma cells by JA

Fig. 7

Caspase-9 activation in HT-29 cells by JA

Fig. 10
Effects of Z-VAD-fmk on DNA fragmentation in JA-treated HT-29 cells.

Fig. 12

Decrease of DFF-45 in HT-29 cells by JA

Fig. 14

Poly(ADP-ribose) polymerase (PARP) in HT-29 cells by JA

Fig. 13

Fig. 15

Fig. 16

Fig. 17