行政院國家科學委員會專題研究計畫 成果報告

PKC, AKT 及 NF-κB 參與 Justicidin A 誘發人類大腸癌細胞之凋亡

計畫類別： 個別型計畫
計畫編號： NSC92-2314-B-006-102-
執行期間： 92 年 08 月 01 日至 93 年 07 月 31 日
執行單位： 國立成功大學醫學系外科

計畫主持人： 李政昌
共同主持人： 翁舷誌

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中華民國 93 年 12 月 23 日
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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本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

執行單位：國立成功大學醫學院外科部

中華民國 93 年 12 月 20 日
The Anti-tumor Mechanism of Formosanin C in Human Colorectal Cancer Cells

Our previous study showed that justicidin A, isolated from the Chinese herb Justicia Procumbens, inhibits the growth of human colorectal cancer cells HT-29. Further study reveals that the justicidin A treated colorectal cancer cells died of apoptosis, and detailed mechanism exploration showed that Ku70 was initially decreased and followed by increased Bax and decreased Bcl-XL expression in the mitochondria. Concomitantly, Δψm was lost, which lead to the release of cytochrome c and Smac into the cytosol. Smac then decreased XIAP expression which further activates caspase-9 and is followed with caspase-3 activation. Caspase-3 activation increased the amounts of nuclear 89-kDa PARP and DFF-40, as well as DNA fragmentation. In present study, translocation of NF-κB from cytoplasm to nucleus and its DNA binding activity in justicidin A-treated HT-29 cells exhibited justicidin A time- and dose-dependent manner. Degradation of phosphorylation of IκB, but not IκB in justicidin A-treated HT-29 cells was observed. Concomitantly, the p-Akt, PKC-ε and membrane-PKC-ε in justicidin A-treated Ht29 cells was decrease. Taken together, justicidin A suppress colorectal cancer cell growth by inhibiting PKC-ε, Akt and NF-κB related apoptosis.

Keywords: Justicidin A, human colon cancer, apoptosis, PKCs, NF-κB, NIK, IKK, IκB, Akt/PI3K

二、緣由與目的

Colon cancer is one of the most common malignancies and causes major public health problems in Western countries and in Taiwan. Development of colon
cancer is a multiple process and activation of oncogene K-ras is an early event. The 5-fluorouracil is the most widely used drug in chemotherapy of human colon cancer. The therapeutic response is limited at 15% in advanced human colon cancer. Therefore, new chemotherapeutic agents have to develop of treatment. An important aim of cancer therapeutic agents is to lower the side effects. Thus, this led us to search natural products from Chinese traditional herbs for potential anticancer agents. Herbaceous plants, traditional Chinese herbal medicines, 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. The Chinese herbal medicine is verified by the National Center Institute (NCI) of USA to use as anticancer or chemopreventive agents. The whole plant water extracts of *J. procumbens* is used for treatments of pain and fever in Taiwan, and are also very common folklore in the treatment of human colon cancer in median area of Taiwan. Previous report have shown that the methanolic extract of the whole plant of *J. procumbens* inhibited P-388 lymphocytic leukemia growth in BDF1 mice and also exhibited cytotoxicity in 9-KB cell. Recently, five 2,3-naphtalide lignans including justicidin A, justicidin E, neojusticin A, B and diphyllin have been isolated from methanolic extract of the whole plant of *J. procumbens* Linn. An earlier report also indicated that JA significantly inhibited the growth of KB cells in vitro. In our recent works, JA induced 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. However, the mechanism of apoptosis caused by JA in human colon cancer cells is not thoroughly understood yet.

Apoptosis, programmed cell death, is a regulated process involving activation of molecular episode and induction of cell death characterized by morphological changes, DNA fragmentation, phosphatidylserine externalization, and generation of apoptotic bodies. The apoptotic body is engulfed by phagocyte that is reported to lead little or no inflammation. The induction of apoptosis is a potentially promising approach for cancer therapy. Apoptosis can be induced by stimuli including radiation, hyperthermia, and chemotherapeutic agents including etoposide, Adriamycin, mitoxantrone, cisplatin, and 5-fluorouracil (5-FU). Mitochondria play a pivotal role in apoptotic process including tumor cell death induced by chemotherapeutic agents. Upon stimulation, apoptotogenic factors, such as cytochrome c, Smac/DIABLO (Second mitochondria-derived activator of caspase/direct IAP binding protein with low pl), endonuclease G, and release of AIF from mitochondria into the cytoplasm. In the cytosol, cytochrome c binds to Apaf-1 and form Apaf-1/cytochrome c complex in the presence of ATP and activates downstream effector caspases including caspase-7, -6, and -3. XIAP, X-linked inhibitor of apoptosis, widely expressed in various of human cancer, thought to be a potent modulator of programmed cell death and specific inhibitor of caspase-9, -7, and -3. Anti-apoptotic members of the Bcl-2 family proteins also bind to mitochondria and inhibit the release of cytochrome c and Smac/DIABLO. Protein kinas C (PKC) is a family of enzymes which contain 11 different isotypes, such as alpha, beta 1 and 2, gamma, delta, epsilon, eta, lambda ( iota), mu, theta and zeta. Based on their structure and activators, these enzymes can be divide into...
three groups, which are conventional PKC (cPKCs: α, β, γ), novel PKC (nPKCs: δ, ε, θ, η) and atypical PKC (aPKCs: ζ, λ) 59. cPKCs are calcium-dependent, and activated by diacylglycerol (DAG) and 12-O-tetradecanoylphorbol-13-acetate (TPA). nPKCs are calcium-independent and activated by DAG and TPA. PKCs are calcium-independent and can not be activated by DAG and TPA 60. The expression patterns of PKC isoforms vary in cell and tissues 61. The changes of PKC protein expression pattern induce apoptosis 62, modulate cell proliferation 63 and differentiation 64. Notably, previous experiments have shown that signals mediated via PKC isoforms play a role in the process of tumor promotion 65,66 67. Moreover, overexpression of PKCs prevents NO-induced apoptotic cell death in RAW 264.7 macrophage 68. Koriyama et al. have reported that proteolytic activation of PKCδ and ε in U937 cells during chemotherapeutic agent-induced apoptosis 69. Current report indicated that decrease in the expression of PKCδ and ε may play a pivotal role in alo-emodin- and emodin-induced apoptosis in human lung non-small carcinoma cells 70. Therefore, inhibition or overexpression of certain types of PKC isoforms has been suggested to be useful for therapeutic purpose in cancer 58,71.

Nuclear factor-κB (NF-κB), a mammalian transcription factor, is pivotal in regulation of many genes involved in immune system, inflammatory responses, tumour development, as well as in control of cell proliferation and apoptosis 72-74 75 76-78. An inactive NF-κB resides in the cytoplasm bound to an inhibitory protein known as IkB 72. Activation of NF-κB is trigged by extracellular stimuli, and then IkBα is phosphorylated and proteolytically processed by proteasomes and other proteases 79. This proteolytic process allows NF-κB translocate from cytosol to the nucleus, and therefore bind to the promoter region of the target gene 80. Recent work provides direct evidence that NF-κB mediates a critical antiapoptotic signal and leads to rescue cells from apoptosis, and therefore contribute to oncogenesis 74,81. Other lately evidence showed that the activation of NF-κB is through Ras and phosphatidylinositol-3-kinase (PI3-K) involving activation of Akt and IKK. Phosphorylation and degragation of IkBα, and activation of NF-κB result in the translocation of NF-κB from cytosol to the nucleus. These processes induce NF-κB DNA-binding activity 82,83. Moreover, current reports indicate clearly that NF-κB-dependent transcriptional activation of cIAP1 and cIAP2 (inhibitor of apoptosis protein) play a pivotal role in regulating apoptosis, and directly inhibit the activity of caspase 3, 7 and 9 42,84,85. There are several evidences indicating that PKC and Akt kinase are crucial factors for cell death or survival in vivo 86,87 and in vitro 88-90, and these two kinases are able to phosphorylate Bad protein 91,92. The phosho-Bad can interact with 14-3-3 protein and restrict in inhibition of mitochondrially located Bcl-2 family and inhibits its function 46,53,94.

Multiple mechanisms of action involved in the regulation of cell death demonstrated for almost all anticancer agents. To obtain more insight in the molecular mechanisms of justicidin A-induced apoptosis, we will extend our knowledge about PKC, Akt/PI3K and NF-κB pathway that could underlie the cytotoxic effects of justicidin A in human colon cancer HT-29 cells.

三、結果與討論

Our previous study showed that justicidin A, isolated from Chinese herb *Justicia Procumbens*, inhibited the growth of human colorectal cancer HT-29 cells. Further study revealed that justicidin A treated colorectal cancer cells died of apoptosis, and detailed mechanism exploration showed that Ku70 was initially decreased and followed by increased Bax and decreased Bcl-XL expression in the mitochondria. Concomitantly, Δψm was lost, which lead to the release of cyto c and Smac into the cytosol. Smac then decreased
XIAP expression which further activated caspase-9 and was followed with caspase-3 activation. Caspase-3 activation increased the amounts of nuclear 89-kDa PARP and DFF-40, as well as DNA fragmentation.

Justicidin A inhibited NF-κB activation in human colorectal cancer cell. To test whether translocation of NF-κB from cytoplasm to nucleus is involved in justicidin A-induced apoptosis, we performed western blotting and EMSA to determine the nuclear translocation and DNA-binding activity of NF-κB in justicidin A stimulated HT-29 cells. As Fig. 1 indicated, nuclear p65 protein expression was decreased as early as 6-12 h, and dramatically decreased at 24-48 h following justicidin A (1 μM) treatment of HT-29 cells. In addition, expression of nuclear p65 protein also decreased in a dose-dependent manner (Fig. 1). As shown in Fig. 2A and 2B, inhibition of DNA-binding activity treatment with justicidin A (1 μM) was detected at 48-72 h. The specificity of NF-κB band was completely eliminated in the present of 100-fold molar excess of unlabeled κB oligonucleotide (Fig. 2B, lane 10). In contrast, 100-fold molar excess of unlabeled mutant κB (Fig. 2A, lane 11), AP-1(Fig. 2A, lane 12) and SP1 (Fig. 2A, lane 13) oligonucleotide probe had no effect on the binding ability of NF-κB to DNA. In addition, coinubcation of justicidin A (1 μM)-treated HT-29 nuclear extract with the anti-p50 or anti-p60 polyclonal antibody resulted in a reduction of the intensity of NF-κB band (Fig. 2A lane 8 and 9). Supershift analysis also showed that the NF-κB was p50/p65 heterodimer (Fig. 2A, lane 8 and 9). Figure 2C and 2D also showed that justicidin A (1 μM) treatment for 12 h in HT-29 cells significantly decreased DNA-binding activity in a justicidin A dose-dependent manner. NF-κB in its inactive state resides in the cytoplasm bound to an inhibitory protein known as IkB. To examine whether IkB is involved in justicidin A-induced cell death, we performed Western blotting to determine the phosphorylation of IkB in justicidin A treated HT-29 cells. Figure 3 showed that dramatically decreased the phosphorylation of IkB-α, but not IkB-α, at 12-72 h following justicidin A (1 μM) treatment of HT-29 cells. Pommier et al. reported that Akt is activated in many cancers and Akt is a target for cancer chemotherapy. To examine whether the Akt is involved in justicidin A-induced apoptosis, we used Western blotting to determine the phosphorylation of IkB in justicidin A treated HT-29 cells.

Phosphorylation of Akt was reduced by justicidin A in a time- and dose-dependent manner (Fig. 4). The changes of PKC protein expression pattern induce apoptosis. To determine whether PKC proteins are involved in justicidin A-induced cell death, we performed Western blotting to determine the total or membrane PKC-ε expression in justicidin A treated HT-29 cells. Figure 5 indicates that total PKC-ε slightly decreased at 12 h and completely abolished at 24-72 h. Strikingly, the membrane fraction of PKC-ε dramatically decreased between 6 and 72 h in HT-29 cells after justicidin A treatment (Fig. 5). These results suggest that downregulation PKC-ε protein level triggers decreasing Akt protein and phospho-Akt expression to lead degradation of phospho-IkB-α, which in turn inhibited NF-κB nuclear translocation and its activity in justicidin A-induced apoptosis in colorectal tumor cell lines. We conclude, then that justicidin A may be considered as a potential agent for chemotherapy against human colorectal cancer.

References

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Fig. 1

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Fig. 2A

![Gel electrophoresis image showing NF-κB binding to DNA probes at different time points and with various treatments.](image)
Fig. 2B

NF-κB-DNA binding activity (fold of control) vs. Time (h)
Fig. 2C

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JA (µM) 0.50 1.00 1.50

→ p65Supershift
→ p50Supershift
Fig. 2D

NF-κB-DNA binding activity (fold of control)

JA (µM)
Fig. 3

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Fig. 4

**Table: JA (0.75μM)**

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**Table: JA (µM)**

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Fig. 5

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- **total PKC-\(\varepsilon\)**
- **membrane PKC-\(\varepsilon\)**

RACK 1