

行政院國家科學委員會專題研究計畫 成果報告

Wogonin 與 fisetin 抑制肝腫瘤細胞增生之體外與體內活性 與機轉研究:p53 與 p21 在天然物抗腫瘤之角色探討

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Wogonin and Fisetin Induction of Apoptosis Through Activation of Caspase 3 Cascade and Alternative Expression of p21 Protein in Hepatocellular Carcinoma Cells SK-HEP-1

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Abstract

Wogonin and fisetin are flavonoids and widely distributed in plants. Our recent study demonstrated that wogonin and fisetin showed the most potent apoptosis inducing activities in human promyeloleukemic cells HL-60 among seven structurally related flavonoids. In the present study, we performed molecular studies to assess the apoptotic effects of wogonin and fisetin on hepatocellular carcinoma cells SK-HEP-1. Both wogonin and fisetin show the dose dependent cytotoxic effects on SK-HEP-1 cells, accompanied by the occurrence of DNA fragmentation. Microscopic observation showed that wogonin and fisetin, at the dose of 80 μ M, induced cellular swelling and appearance of apoptotic bodies by Giemsa staining, characteristics of apoptosis, in SK-HEP-1 cells. And, flow cytometry analysis showed an increase of hypodiploid cells in wogonin- and fisetin-treated SK-HEP-1 cells. These data demonstrated that wogonin and fisetin were effective apoptotic inducers on SK-HEP-1 cells. Treatment with an apoptosis-inducing concentration of wogonin or fisetin causes induction of caspase 3/CPP32 activity, but not caspase 1 activity. And, a caspase 3 inhibitor Ac-DEVD-CHO, but not caspase 1 inhibitor Ac-YVAD-CHO, reverses the cytotoxic effects of wogonin and fisetin on SK-HEP-1 cells. Further, cleavage of caspase 3 substrates including poly(ADP-ribose) polymerase (PARP) and D4-GDI, and decrease of pro-caspase 3 protein were detected in wogonin- and fisetin-treated SK-HEP-1 cells. Increase of p53 protein was associated with wogonin- and fisetin-induced apoptosis, however a p53 controlled gene p21^{Waf/Cip-1} was only

induced in wogonin (not fisetin)-treated SK-HEP-1 cells. Serum starvation elevated p21^{Waf/Cip-1} protein expression, and enhanced the apoptotic induction activity of wogonin (not fiseitn) in SK-HEP-1 cells. Our study provided molecular evidences to demonstrate that wogonin and fisetin showed the effective cytotoxic effects through apoptosis induction in hepatocellular carcinoma cells SK-HEP-1; activation of caspase 3 cascade, induction of p53 protein and alternative expression of p21^{Waf/Cip-1} protein were involved.

Introduction

Hepatocellular carcinoma is the most invasive and frequently diagnosed malignancy and one of the leading causes of cancer death in the world. Therefore, development of new effective agents is necessary in treatment of hepatocellular carcinoma. Apoptosis and necrosis are two distinct modes of cell death, and morphological and biochemical studies demonstrated the differences between these two forms of cell death (Ashwell et al., 1994; Fisher et al., 1994; Laster et al., 1999). Apoptosis is characterized by cell shrinkage, blebbing of the plasma membrane, and chromatin condensation that are associated with DNA cleavage into ladders (Chen et al., 1999; Yu et al., 1999). Decreased ability to undergo apoptosis occurred in the human malignant tumor cells in response to some treatments (Barry et al., 1990; Hoffman and Liebermann, 1994). Therefore, developing effective agents that can induce or enhance the extent of apoptosis might be a promising strategy in the treatment of cancer.

Caspases cascade has been demonstrated to be involved in apoptosis signal transduction and execution (Salvesen and Dixit, 1997; Green, 1998). Human caspases-1 to -10 have been described, and activation of caspase cascade is involved in chemicals- and agents-induced apoptosis (Martin and Green, 1995). Apaf-1 binds to an initiator caspase 9, and causes caspase 9 activation through inducing its oligomerization (Goyal, 2001; Kanuka et al., 1999). In turn, activated caspase 9

cleaves and activates executioner caspase 3. Caspase 3 usually exists as an inactive pro-caspase 3 that becomes proteolytically activated by multiple cleavages of its precursor 32 kDa to generate the 20/11 or 17/11 kDa active forms in cells undergoing apoptosis (Busihardjo et al., 1999). Downstream of caspase 3 activation, some specific substrates are cleaved that are important for the occurrence of apoptosis such as PARP and D4-GDI proteins. PARP is required for the DNA repair, and activated caspase 3 cleaves PARP at Asp 216 to generate the 85 kDa and 31 kDa apoptotic fragments in coordination with DNA fragmentation during apoptosis. D4-GDI is a negative regulator of the ras-related Rho family of GTPases, and activation of Rho GTPases promoted cytoskeletal and membrane changes associated with apoptotic cells. Activated caspase 3 cleaved D4-GDI to 23 kDa and 5 kDa fragments, and release of activated Rho GTPases to produce the apoptotic morphological changes (Stadheim et al., 2001). However, the defined role of D4-GDI in apoptosis is still unclear.

A lot of oncogenes and tumor suppressor genes are involved in mediating apoptosis. Tumor suppressor gene p53 is one of the critical genes that regulates the onset of DNA replication around G1/S boundary. p53 transcription factor also is an important regulator of apoptosis and, induction of apoptosis by p53-mediated tumor suppression appears to be critical for therapeutic potential in treatment of tumor (Ryan et al., 2001). Previous studies demonstrated that a p53-dependent and a p53-independent apoptosis were detected in tumor cells in response to apoptosis inducers (Chao et al., 2000; Vogelstein et al., 2000). And, numerous p53-dependent target genes have been identified that play a role as downstream effectors of p53 function. For example, the cyclin dependent kinase inhibitor p21 Waf/Cip-1 is a direct p53 target and deletion of this gene reduces the cell cycle arrest response to p53 (Niculescu et al., 1998).

However, the importance of p21 played in p53-dependent or –independent apoptosis is still undefined clearly.

Flavonoids are one type of polyphenols including flavones, isoflavones, and flavanones, and possess anti-lipoperoxidant, anti-tumoral, anti-platelet and anti-inflammatory activities (Chen et al., 2000; Chen et al., 2001a; Naasani et al., 1998). In contrast to the beneficial effects, mutagenic effects of flavonoids have also been found through their prooxidant rather than the antioxidant action (Hanasaki et al., 1994). Wogonin and fisetin are popular flavonoids in plants, and anti-inflammatory activity of wogonin have been identified (Chen et al., 2001b). Our recent study demonstrated that wogonin and fisetin exhibit significant apoptosis inducing activities in HL-60 cells among seven structurally related flavonoids (Lee et al., 2002). In the present study, we provided molecular evidences to demonstrate that wogonin and fisetin are effective apoptotic inducers on hepatocellular carcinoma cells SK-HEP-1, interestingly, activation of caspase 3 cascade and alternative induction of p21 protein were found.

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Results

Induction of Apoptosis by Wogonin and Fisetin in SK-HEP-1 cells

Flavonoids are diphenylpropanes that commonly occur in plants, and more than 4000 flavonoids have been found and are frequently components of the human diet. However, several biological activities of flavonoids are still undefined. The chemical structures of the wogonin and fisetin used in the present study are shown in Fig. 1, and the effects of both compounds on the viability of SK-HEP-1 cells were investigated by MTT assay (Fig. 2A). When cells were treated with various concentrations of wogonin or fisetin (20, 40, and 80 μ M) for 24 h, significant concentration-dependent inhibition on the viability of SK-HEP cells was detected. DMSO, even at the highest dose of 0.5%, showed no effect on cellular viability of SK-HEP-1 cells. To characterize cell death induced by wogonin and fisetin, the integrity of genomic DNA, morphological changes, and the hypodiploid cells in SK-HEP-1 cells were examined. On analysis of DNA integrity by agarose electrophoresis, the results show that wogonin and fisetin caused the digestion of genomic DNA into ladders in dose- and time-dependent manners, associated with a decrease in intact DNA (Fig. 2B). Morphological alternations in wogonin- and fisetin-treated SK-HEP-1 cells were detected under microscopic observation. Under wogonin or fisetin treatment for 24 hr, SK-HEP-1 cells showed the swelling and rounded morphology and appearance of significant apoptotic bodies around the rounded cells was detected in wogonin- and fisetin-treated SK-HEP-1 cells by Giemsa staining (Fig. 3A). Results of flow cytometry analysis showed that an increase of hypodiploid cells was detected in wogonin- or fisetin-treated SK-HEP-1 cells. However, no obvious DNA ladders, apoptotic bodies, or hypodiploid cell were detected in DMSO-treated SK-HEP-1 cells (data not shown). These data demonstrated that wogonin and fisetin

are effective apoptosis inducers in hepatocellular carcinoma cells SK-HEP-1.

Activation of Caspase 3-like Activities, not Caspase 1-like Activities, in Wogonin and Fisetin-induced Apoptosis

Caspases cascade has been demonstrated to be involved in the apoptosis induction. During apoptosis progression, caspases were activated and cleaved the specific sites such as DXXD↓X for caspase 3 and YXXD↓X for caspase 1. To detect if enzymatic activities of caspases were involved in wogonin- and fisetin-induced apoptosis in SK-HEP-1 cells, two colorimetric substrates, Ac-DEVA-pNA for caspase 3-related activities and Ac-YVAD-pNA for caspase 1 related activities, were used in this study. As illustrated in Figure 4, wogonin and fisetin induced a dramatic increase in DEVD-specific caspase activity in treated SK-HEP-1 cells, and Ac-DEVD-CHO, an inhibitory peptide of caspase 3 activity, inhibited the increase of caspase 3 like activities induced by wogonin or fisetin. However, no significant induction of YVAD-specific activity was observed in wogonin- and fisetin-treated SK-HEP-1 cells. In order to further identify if caspase 3 involved in wogonin- or fisetin-induced cell death, caspase 3 peptidyl inhibitor Ac-DEVD-CHO and caspase 1 peptidyl inhibitor Ac-YVAD-CHO were used to block intracellular caspase 3 and caspase 1 activity, respectively, and wogonin- or fisetin-induced cell death was measured by MTT assay. Results of Figure 4 showed that caspase 3 inhibitor Ac-DEVD-CHO, but not caspase 1 inhibitor Ac-YVAD-CHO, attenuated wogonin- and fisetin-induced cell death, represented here as the recovery of absorbance intensity in MTT assay. These data indicated that activation of caspase 3-like activity was participated in wogonin- and fisetin- induced apoptosis in SK-HEP-1 cells.

Cleavage of PARP and D4-GDI proteins in Wogonin- and Fisetin-treated SK-HEP-1 cells

Activation of caspase 3 leads to the cleavage of a number of proteins such as PARP and D4-GDI, and caspase 3 cleaves the 28 kDa mature form of D4-GDI to give a 23 kDa and a 5 kDa fragments, and cleaves the 115 kDa PARP to a 86 and a 31 kDa fragments during apoptosis. Figure 5 shows that exposure of SK-HEP-1 cells to wogonin and fisetin caused the degradation of 116-kDa PARP into 85-kDa fragments, and an increase of a 23 kDa fragment of D4-GDI in a concentration- and time-dependent manners, associated with the activation of caspase 3 brought about by its cleavage, represented here as a decline in its pro-level on the western blot (Figure 5).

Induction of p53 protein but a distinct increase of p21 protein in wogonin- and fisetin-treated SK-HEP-1 cells

SK-HEP-1 cell is a hepatocellular carcinoma cell line with wild type p53 alleles. In this study, an increase of p53 protein was detected in wogonin- or fisetin-treated SK-HEP-1 cells. At the dose of 80 μ M, both wogonin and fisetin induced the accumulation of p53 protein in SK-HEP-1 cells about 2-5 fold. Upon analysis of p21 protein expression by western blotting, induction of p21 protein expression associated with an increase of p53 protein was detected in wogonin-treated cells. However, there is no significant p21 protein induction in fisetin-treated cells although an increase of p53 protein was found. These data indicated increasing p53 protein was involved in both wogonin- and fisetin-induced apoptosis, whereas induction of p21 was only detected in wogonin-treated cells (Figure 5).

An increase of p21 protein by a prior serum starvation enhances the apoptosis inducing activity of wogonin

In order to identify if p21 is an important regulator for wogonin (not fisetin)-induced apoptosis, SK-HEP-1 cells were serum-starved for 24 hr followed by wogonin or fisetin (80 μ M) treatment a further 24 hr in the presence or absence of serum. Wogonin performed more effective apoptosis inducing activity in SK-HEP-1 cells under serum free condition (SF) than those in 10 % FBS containing condition (FBS) or a prior 24 hr serum starvation followed by wognin or fiseitn treatment in the presence of serum (SF→FBS) by MTT assay and DNA fragmentation assay (Figure 6). In contrast, there is no significant difference appeared in fisetin-induced apoptosis activity in the condition with or without of serum in SK-HEP-1 cells. Western blotting analysis of PARP cleavage, D4-GDI cleavage, p53, p21 and caspase 3 proteins in SK-HEP-1 cells under different treatment conditions, the results appeared that serum starvation (SF) significantly stimulated p21 protein expression in SK-HEP-1 cells, but no change on p53 protein expression was found. Wogonin showed the more potent apoptotic effects such as increasing PARP cleavage and D4-GDI cleavage, and enhancement of p53 induction in serum free condition (SF) than that in FBS or SF→FBS condition. However, no significant alternation on PAPR cleavage, D4-GDI cleavage, and p53 protein induction in fisetin-treated SK-HEP-1 cells in SF, FBS or SF→FBS conditions (Figure 7).

Discussion

Wogonin and fisetin are functional natural compounds and exist widely in plants. This study provided firstly evidences to demonstrate that wogonin and fisetin showed the significant cytotoxic activities on human hepatocellular carcinoma cells SK-HEP-1. Activation of caspase 3 cascade including induction of PARP and D4-GDI cleavage, and an increase of p53 protein were associated with wogonin- and fisetin-induced apoptosis. Interestingly, induction of p53 regulated gene p21^{Waf/Cip-1} was found in wogonin-, but not fisetin-, treated cells, and an enhancement of p21^{Waf/Cip-1} protein by a prior serum starvation treatment potentiated the apoptosis inducing activity of wogonin. Our results demonstrated that both of them are apoptosis inducers and distinct apoptosis pathways were participated in wogonin- and fisetin-induced apoptosis.

Recent studies demonstrated that the proteolytic degradation of specific substrates is responsible for many of the morphologic and biochemical features of apoptosis (Cardone et al., 1998; Cecconi, 1999). Caspase 3 is a member of the caspase family and requires proteolytic cleavage for its activation. Activated caspase 3 cleaves down-stream substrates such as PARP, D4-GDI or α -actin, and induces the progression of apoptosis (Stadheim et al., 2001; Goyal, 2001). Our in vitro results in SK-HEP-1 cells indicate the concurrent occurrence of apoptotic DNA fragmentation, caspase 3 activation, PARP cleavage and D4-GDI cleavage in wogonin- and fisetin-treated SK-HEP-1 cells. Elevation of caspase 3 activity, but not caspase 1 activity, was detected in wogonin- or fisetin-treated SK-HEP-1 cells. And, wogonin- and fisetin-induced apoptotic responses and DNA ladders are attenuated by a prior treatment of caspase 3 inhibitor, Ac-DEVD-CHO, but not by the caspase 1 inhibitor, Ac-YVAD-CHO. Therefore, activation of caspase 3 but not caspase 1 plays an

important role in executing apoptosis in wogonin- and fisetin-treated SK-HEP-1 cells. P53 acts as guardian of the genome, and is one of the major factors controlling cell proliferation, suppressing growth and transformation of cells. The p53 tumor suppressor is mutated in over 50 % of human cancers, and the oncogenic activity of p53 mutants is their ability to interfere with p53-dependent apoptosis by a dominant negative mechanism (Sigal and Rotter, 2000). Activation of p53 is often detected by natural chemotherapeutic agents, and p53-negative tumors are generally less sensitive or even insensitive to these agents. In human, p53 may even protect rather than sensitize tumor cells to stimuli (Mahyar-Roemer and Roemer, 2001). The p21^{Waf/Cip-1}, GADD45 and 14-3-3 δ and other products are downstream effectors of p53-induced growth arrest and apoptosis. p21^{Waf/Cip-1} has been suggested to be involved p53-induced growth arrest triggered by DNA damage by halting the cell cycle in G1 phase. In contrast, apoptosis in response to genomic damage appears to require p53 but not p21^{Waf/Cip-1} (Klas et al., 1999). Therefore, the relationship between p53 and p21 in the response of human tumors to stimuli are controversial. In the present study, both wogonin and fisetin showed the similar apoptosis induction effects associated with increasing p53 protein expression in SK-HEP-1 cells. An increase of p21 protein was only detected in wogonin-treated cells, and induction of p21 protein by a prior serum starvation treatment enhances the apoptosis induction activity of wogonin. However, no significant change on p21 protein expression in fisetin-treated SK-HEP-1 cells, and a prior serum starvation showed no obvious effect on apoptosis inducing activity of fisetin. Our previous study demonstrated wogonin and fisetin were able to induce apoptosis in HL-60 (p53^{-/-}) cells (Lee et al., 2002). Based on our recent studies, we demonstrated that p53-dependent and -independent mechanisms were involved in wogonin- and fisetin-induced apoptosis, and p21 protein is an

important factor in wogonin induction apoptosis. The in vivo role of p21 played in anti-tumor activity of wogonin is still unclear, and deserved to be pursued further.

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LEGENDS

Figure 1. Structure of the wogonin and fisetin

Figure 2. Cytotoxic effects of wogonin and fisetin associated with time- and dose-dependent DNA ladders in hepatocellular carcinoma cells SK-HEP-1. (A) SK-HEP-1 cells were plated into 24 well plates for 24 h, and then treated with different concentration of wogonin or fisetin (20, 40 and 80 μ M) for a further 24 h. MTT was added into medium at the end of reaction for an additional 4 h. The viability of cells was detected by measuring the absorbance at a wavelength of 600 nm. (B) (left panel) SK-HEP-1 cells were treated with different concentrations (20, 40, 80 μ M) of wogonin or fisetin for 24 h, and integrity of DNA was analyzed through a 1.8% agarose gel and visualized by staining with ethidium bromide. (right panel) SK-HEP-1 cells were treated with wogonin or fisetin (80 μ M) for different time points (6, 12, 24 hr), and the integrity of DNA was analyzed through a 1.8 % agarose gel, and visualized by staining with ethidium bromide. C: control group.

Figure 3. Appearance of apoptotic bodies by Giemsa stain under microscopic observation and an increase of hypodiploid cell in wogonin- and fisetin- treated SK-HEP-1 cells by flow cytometry analysis. (A) SK-HEP-1 cells were treated with or without wogonin or fisetin (80 μ M) for 24 hr, and morphological changes (B, D, F) and apoptotic bodies appearance by Giemsa staining (A, C, E) were detected under a light microscopic observation. A, B: control; C, D: wogonin treatment for 24 hr; E, F: fisetin treatment for 24 hr: (B) SK-HEP-1 cells were treated with or without wogonin or fisetin (80 μ M) for 24 h, and ratio of hypodiploid cells was measured by flow cytometry analysis by PI staining. Each value is presented as the mean $\pm$ SE of three independent experiments.

Figure 4. Activation of caspase 3, but not caspase 1, activity in wogonin- and fisetin-induced cell death in SK-HEP-1 cells. SK-HEP-1 cells were treated with or without wogonin or fisetin (80 μ M) for 24 h. (A) Enzymatic activity of caspase 1- and caspase 3-like proteases were determined by incubation with specific colorigenic substrates, Ac-YVAD-pNA (caspase 1) or Ac-DEVD-pNA (caspase 3), as described in “Materials and Methods”. DEVE-CHO and YVAD-CHO indicate that the addition of 200 μ M of Ac-YVAD-CHO or Ac-DEVD-CHO with the reaction in wogonin or fisetin -treated HL-60 cell lysates. (B) Caspase 3 peptidyl inhibitor Ac-DEVD-CHO, not caspase 1 inhibitor Ac-YVAD-CHO, attenuated wogonin- or fisetin-induced cytotoxicity by MTT assay. Cells were treated with wogonin or fisetin (80 μ M) for 24 hr in the presence or absence of a 2 hr prior treatment of Ac-DEVD-CHO or Ac-YVAD-CHO (200 μ M). The cytotoxicity of wogonin and fisetin on SK-HEP-1 cells were measured by MTT assay as described in Figure 2.

Figure 5. Dose dependent and time dependent induction of PARP cleavage, D4-GDI cleavage, p53 protein, p21 protein and caspase 3 activation in wogonin- and fisetin-treated SK-HEP-1 cells. (A) SK-HEP-1 cells were treated with different concentrations (40 and 80 μ M) of wogonin or fisetin for 24 h, and p53, p21, pro-caspase 3, PARP cleavage and cleaved D4-GDI proteins were analyzed by western blotting using specific antibodies. (B) SK-HEP-1 cells were treated with wogonin or fiseitn (80 μ M) for different time points (6, 12 and 24 hr), and p53, p21, pro-caspase 3, PARP cleavage and cleaved D4-GDI proteins were analyzed by western blotting using specific antibodies.

Figure 6. Enhancement of apoptosis inducing activity of wogonin (not fisetin) under serum starvation condition. SK-HEP-1 cells were incubated in the 10 % FBS containing medium (FBS) or serum starvation for 24 hr followed by replacing with 10 % FBS containing medium (SF→FBS) or maintaining in serum free medium in the presence of wogonin or fisetin (80 μ M) for a further 24 hr. (A) The viability of cells under wogonin or fisetin (80 μ M) treatment for 24 hr was analyzed by MTT assay. Data represented here is the percentage of viability compared with indicated control. ** $p < 0.01$ significantly different from the control, as analyzed by Student's t test.

(B) Integrity of DNA was analyzed by electrophoresis in 1.8 % agarose.

Figure7. Expression of PARP cleavage, D4-GDI cleavage, p53, p21 and pro-caspase 3 was analyzed by western blotting analysis in SK-HEP-1 cells under different culture conditions (FBS: 10 % FBS containing medium; SF: serum starvation for 24 hr followed by wogonin or fisetin treatment for a further 24 hr in the condition without serum; SF→FBS: serum starvation for 24 hr followed by wogonin or fisetin treatment for a further 24 hr in the condition with 10 % FBS) were treated with wogonin or fisetin (80 μ M) for 24 hr. Expression of PARP, cleaved D4-GDI. P53, p21 and pro-caspase 3 proteins were analyzed by western blotting using specific antibodies. (B) Band intensities of p53 and p21 were quantified by densitometry (IS-1000 Digital Imaging System) from three independent experiments.

