Promoter activation and following induction of the p21/WAF1 gene by flavone is involved in G_1 phase arrest in A549 lung adenocarcinoma cells

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Abstract Flavonoids are present in many plants including edible fruits and vegetables. Recently, many of the biological activities of flavonoids have been elucidated. Flavone is a well known flavonoid, and many of its derivatives have been shown to have anti-proliferative effects on several cancer cells. We report here that flavone can effectively inhibit the cell growth of human lung adenocarcinoma A549 cells in a dose-dependent manner, and 100 μM flavone causes cell cycle arrest at the G_1 phase. As a mechanism underlying the cell cycle arrest, flavone markedly increases the mRNA and protein levels of a universal inhibitor of cyclin-dependent kinase, p21/WAF1, and inhibits phosphorylation of retinoblastoma (RB) protein. Although A549 cells possess wild-type p53, flavone does not induce the p53 protein, suggesting that p21/WAF1 induction is p53-independent. In addition, 100 μM flavone significantly increases the promoter activity of the p21/WAF1 gene by 5-fold. These results suggest that the G_1 phase arrest by flavone is due to p53-independent transcriptional induction of the p21/WAF1 gene and the subsequent dephosphorylation of RB protein.

Key words: Flavone; A549 cell; Growth inhibition; Cell cycle; p21/WAF1; Promoter

1. Introduction

Flavonoids, ubiquitously occurring and widely consumed metabolites of plants, constitute some of the active components in vegetables and fruits. People consume about 1 g of flavonoids per day in the common diet [1]. Many types of flavonoids prevent or inhibit cancer development in vivo [2] and inhibit cell growth in vitro [3–6]. Flavone is a well known flavonoid and is present in many cereal grains and wheat [7]. Flavone has a basic structure, and many of its derivatives have been reported to have strong anti-cancer effects in human breast cancer cells [8] and in transplanted colon adenocarcinoma [9]. Because of these effects, it is expected that flavone may be used as an anti-cancer drug.

p21/WAF1 protein inhibits the activities of various cyclin-dependent kinases [10–12], and inhibits the phosphorylation of retinoblastoma (RB) protein, thereby inhibiting the G_1-S phase transition [10,13]. It is also well known that the p21/WAF1 gene is transcriptionally activated by wild-type p53 protein [14], suggesting that p21/WAF1 could play a key role as a downstream mediator of the p53-induced cell growth arrest.

In our previous study, we found that flavone inhibits cell growth in vitro and causes G_1 phase arrest in a human gastric cancer cell line, HGC-27 [5]. However, the mechanism of G_1 arrest by flavone is still unclear. To clarify the mechanism of the anti-proliferative effect of flavone, we determined whether flavone can induce the expression of the p21/WAF1 gene, the product of which is involved in G_1 phase arrest, and stimulate the activity of the p21/WAF1 gene promoter in a human lung cancer cell line A549.

2. Materials and methods

2.1. Reagent

Flavone was purchased from Nacalai Tesque, dissolved in dimethyl sulfoxide (DMSO) and diluted to the respective final concentrations in each culture dish.

2.2. Cell culture and cell growth study

A549 (a human lung adenocarcinoma cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. A549 cells were seeded at a density of 2×10^4 cells/2 ml of medium in 35-mm diameter dishes. Various concentrations of flavone were added to cells 24 h after cell seeding. Twenty-four hours, 48 and 72 h after the addition of flavone or DMSO, the number of cells was counted by the Trypan-blue dye exclusion test. This cell growth study was carried out in triplicate and repeated at least three times.

2.3. Analysis of cell cycle progression

Cells were plated at a density of 1×10^5 cells/10 ml of medium in 100-mm diameter dishes. Flavone was added to cells 24 h after cell seeding. Twenty-four hours after the addition of flavone, cells were removed from the culture dishes by trypsinization and centrifugation. After washing with PBS, cells were suspended in PBS containing 0.1% Triton X-100 to prepare nuclei. After the suspension was filtered through a 50-mm nylon mesh, 0.1% RNase and 50 mg/ml of propidium iodide were added. DNA contents in stained nuclei were analyzed with FACScan (Becton Dickinson). A suspension of 1×10^4 cells was analyzed for each DNA histogram. The number of stained nuclei in each phase was measured using the S-fit program in the FACScan [15].

2.4. Northern blot analysis

Cells were plated at a density of 4×10^5 cells/10 ml of medium in 100-mm diameter dishes. Flavone was added to cells 24 h after cell seeding. Twenty-four hours after the addition of flavone, cells were washed twice in PBS and total RNA was isolated from cells using the TRIzol RNA isolation kit (Gibco-BRL), and 10 μg of total RNA per lane was subjected to Northern blot analysis. The p21/WAF1 cDNA as the probe was isolated from the pCEP-WAF1 plasmid (a kind gift from Dr. B. Vogelstein), by digesting with *Not*I. Northern blot analysis was performed using standard methods. To confirm the amount and quality of RNAs loaded in each lane, RNAs were stained with

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Abbreviations: PBS, phosphate buffered saline; CDK, cyclin dependent kinase; DMEM, Dulbecco's modified Eagle's medium

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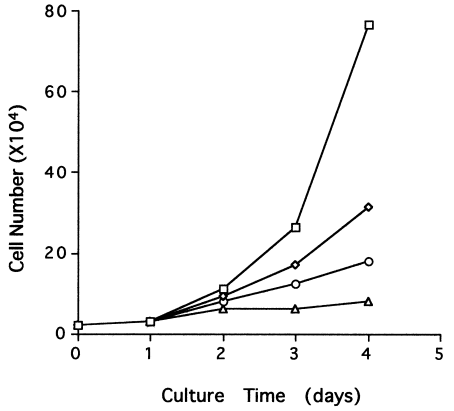

Fig. 1. Effect of flavone on the proliferation of A549 cells. Cells were seeded at a density of 2×10^4 cells/2 ml of DMEM per dish on day 0. DMSO or various concentrations of flavone were added to the cultures on day 1 (□, DMSO; ◇, 30 μM flavone; ○, 60 μM flavone; △, 100 μM flavone). The numbers of viable cells were counted on days 2, 3 and 4 by the Trypan-blue dye exclusion method. Representative data from three independent experiments are shown.

ethidium bromide (EtBr). The mRNA level was determined using the bio-imaging analyzer BAS 2000 (Fujix).

2.5. Western blot analysis

Cells were plated at a density of 4×10^5 cells/10 ml of medium in 100-mm diameter dishes. Flavone was added to cells 24 h after cell seeding. Twenty-four hours after the addition of flavone, cells were washed twice in PBS, resuspended in sample buffer (10% glycerol, 1% SDS, 5% mercaptoethanol, 50 mM Tris-HCl (pH 6.8) and 0.025% BPB), and the samples were boiled for 5 min. Equivalent amounts of total protein from each sample were run on 12% SDS-polyacrylamide gels and blotted onto a nitrocellulose filter. The filter was pretreated with PBS containing 0.2% Tween 20 and 4% dry milk, and incubated overnight at 4 with anti-human p21/WAF1 antibody in the same buffer. The filter was washed in washing buffer containing PBS and 0.2% Tween 20, soaked for 1 h with horseradish peroxidase-linked anti-mouse Ig, and then washed in washing buffer and finally in PBS only. Detection was achieved using an enhanced chemiluminescence kit (Amersham Life Science) with a 2-min exposure of the autoradiograph.

2.6. Transient transfection and luciferase assay

A549 cells were transfected using the calcium phosphate coprecipitation technique. A549 cells were inoculated at a density of 4×10^5 cells/5 ml of DMEM in 100-mm diameter dishes. After 24 h, 4 μg of wild-type p21/WAF1-luciferase reporter plasmid, WWP-Luc, in a calcium phosphate precipitate was used for transfection, and added to the medium. Eight hours after the transfection, the medium was changed. Twenty-four hours after the transfection, flavone was added, and 24 h after the addition of flavone, cell lysates were collected for a luciferase assay. The luciferase activities of the cell lysates were measured as described previously [16]. Luciferase activities were normalized by the amount of protein in the cell lysates. All the luciferase assays were carried out at least in triplicate, and the experiments were repeated at least three times.

3. Results

3.1. Flavone inhibits the proliferation of A549 cells

We first investigated the effect of flavone on the proliferation of human lung adenocarcinoma A549 cells using three concentrations (30, 60 and 100 μM) of flavone. Equivalent volumes of DMSO were used as controls. Twenty-four, 48 and 72 h after the addition of flavone, the growth inhibitory effect of flavone was observed in a dose-dependent manner and 100 μM flavone had a cytostatic effect on A549 cells.

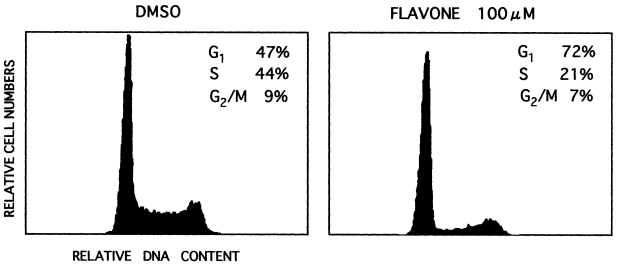

Fig. 2. DNA histograms of the cell cycle in A549 cells. A549 cells were seeded at a density of 1×10^5 cells/10 ml of DMEM per dish. DMSO or 100 μM flavone was added to the cells 24 h after cell seeding. The cell cycle stage was analyzed 24 h after the addition of the drug.

On day 4, the growth of cells was inhibited to 40.9, 23.6 and 10.6% of the control level by 30, 60 and 100 μM flavone, respectively (Fig. 1).

3.2. Flavone arrests A549 cells in the G_1 phase of the cell cycle

The effect of flavone on the cell cycle progression of A549 cells was determined by flow cytometric analysis 24 h after its addition. DNA histograms in Fig. 2 show that flavone increased the population of G_1 phase cells compared with the control. Using the cytostatic dose of flavone (100 μM), the percentage of cells in the G_1 phase increased from 47% to 72%, and that in the S phase decreased from 44% to 21%. These data demonstrate that flavone arrests the cell cycle of A549 cells at the G_1 phase.

3.3. Flavone increases p21/WAF1 mRNA level

To investigate whether the cyclin-dependent kinase inhibitor p21/WAF1 is involved in the flavone-induced growth ar-

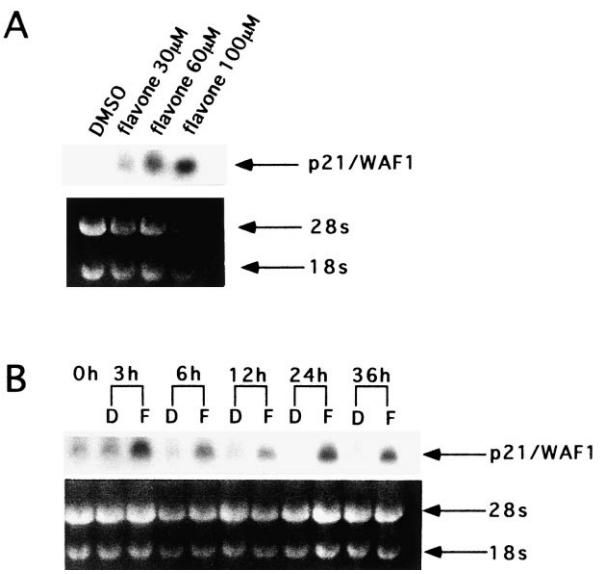

Fig. 3. Induction of p21/WAF1 mRNA by flavone in A549 cells. A549 cells were seeded at a density of 4×10^5 cells/10 ml of DMEM per dish. A: Various concentrations of flavone or equivalent volume of DMSO was added to the cells 24 h after cell seeding. Total RNA was collected 24 h after the drug addition and Northern blot analysis was carried out. B: Sixty μM flavone or an equivalent volume of DMSO was added to the cells 24 h after cell seeding. Total RNA was collected at the indicated times after the drug addition and Northern blot analysis was carried out.

rest of A549 cells, three concentrations of flavone (30, 60 and 100 μM) and equivalent volume of DMSO were added to A549 cells, and p21/WAF1 mRNA expression was assayed by Northern blot analysis. Marked induction of p21/WAF1 mRNA was observed in a dose-dependent manner following a 24-h exposure to flavone (Fig. 3A). This is consistent with the result that flavone inhibited the growth of A549 cells dose-dependently. In the time course study, the p21/WAF1 mRNA level of A549 cells started to increase 3 h after treatment with 100 μM flavone, and it continued until 36 h after exposure (Fig. 3B).

3.4. Flavone induces p21/WAF1 protein in A549 cells

We clarified whether the p21/WAF1 protein could also be induced by treatment of A549 cells with flavone. Thirty-six hours after the addition of flavone (30, 60 and 100 μM), p21/WAF1 protein was induced in a dose-dependent manner (Fig. 4A). In the time course study, treatment with 100 μM flavone induced p21/WAF1 protein 12 h after exposure, and a strong induction of p21/WAF1 protein was observed from 24 h to 48 h after exposure (Fig. 4B). We investigated the induction in A549 cells of another CDK inhibitor, p27, but only slight induction was observed upon treatment with flavone (data not shown). A549 cells lack the CDK inhibitor, p16. Therefore, p21/WAF1 gene induction could be the major cause of G_1 phase arrest in A549 cells.

3.5. Flavone induces p21/WAF1 in a p53-independent manner

The p21/WAF1 gene was first characterized as a p53-dependent gene [14]. As A549 is a p53 positive cell line [17], we investigated the induction of p53 protein in A549 cells to clarify whether p21/WAF1 induction by flavone is p53-dependent. Surprisingly, treatment with 100 μM flavone did not change or slightly decreased the p53 level (Fig. 5A,B), suggesting that p21/WAF1 induction by flavone in A549 cells is p53-independent.

3.6. Flavone inhibits phosphorylation of RB protein

p21/WAF1 is known to inhibit cyclin-dependent kinase activity and the subsequent dephosphorylation of RB protein arrests the cell cycle at the G_1 phase. We clarified whether flavone can change the phosphorylation status of the RB protein using Western blotting. When A549 cells were exposed to 100 μM flavone for 12–48 h, the majority of the RB protein

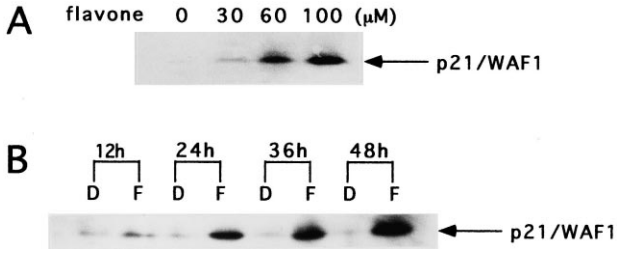

Fig. 4. Induction of p21/WAF1 protein by flavone in A549 cells. A549 cells were seeded at a density of 4×10^5 cells/10 ml of DMEM per dish. A: Various concentrations of flavone or an equivalent volume of DMSO was added to the cells 24 h after cell seeding. Total protein was collected 36 h after the drug addition and Western blot analysis was carried out. B: One hundred μM flavone or an equivalent volume of DMSO was added to the cells 24 h after cell seeding. Total protein was collected at the indicated times after the drug addition and Western blot analysis was carried out.

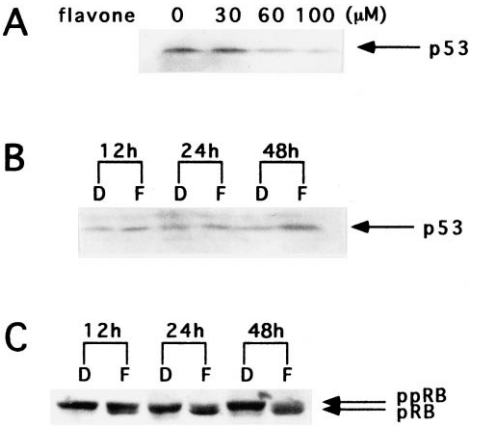

Fig. 5. Induction of p53 protein by flavone in A549 cells. A: Various doses of flavone (30 μM, 60 μM, and 100 μM) or DMSO was added to dishes. Twenty-four hours after the drug addition, total protein was collected and Western blot analysis was carried out. B: One hundred μM flavone or an equivalent volume of DMSO was added to the cells 24 h after cell seeding. Total protein was collected at the indicated times after the drug addition and Western blot analysis was carried out. C: Dephosphorylation of RB protein by flavone in A549 cells. One hundred μM flavone or an equivalent volume of DMSO was added to the cells. Total protein was collected at the indicated times after the drug addition and Western blot analysis was carried out.

molecules was converted to the hypophosphorylated form (Fig. 5C). These results suggest that the G_1 phase arrest caused by flavone is due to dephosphorylation of the RB protein.

3.7. p21/WAF1 promoter activation by flavone

To determine whether p21/WAF1 mRNA induction is mediated through p21/WAF1 gene promoter activation, we clarified whether flavone can stimulate the activity of the promoter of the p21/WAF1 gene using transient transfection. A 24-h exposure to 30, 60 and 100 μM flavone increased the p21/WAF1 promoter activity in a dose-dependent manner up to 5-fold compared with the untreated control (Fig. 6). Our Western blotting data suggests that the p21/WAF1 induction by flavone is p53-independent. Therefore, flavone might be able to respond to a specific region of other than the p53 binding sites.

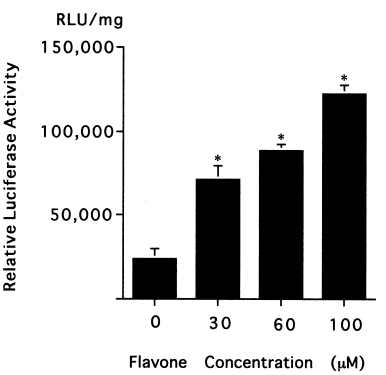

Fig. 6. Stimulation of p21/WAF1 promoter activity in A549 cells by treatment with flavone. A549 cells were transiently transfected with the wild-type p21/WAF1-luciferase reporter plasmid, WWP-Luc, and luciferase activity was measured 24 h after the addition of DMSO, or 30 μM, 60 μM and 100 μM flavone. Data are shown as means ± S.D. ($n=3$). *$P<0.05$.

4. Discussion

In this study, we showed that flavone arrests the cell cycle at the G_1 phase in A549 cells. In addition, we showed that flavone induces a cyclin-dependent kinase inhibitor, p21/WAF1, without increasing the p53 protein level which results in dephosphorylation of the RB protein which is involved in the G_1 phase arrest. Moreover, flavone stimulates p21/WAF1 gene promoter activity, suggesting that the p21/WAF1 gene induction is mediated through activation of its promoter in a p53-independent manner. These results suggest that stimulation of p21/WAF1 gene by flavone causes dephosphorylation of RB gene product resulting in G_1 phase arrest. However, it cannot be ruled out that flavone might dephosphorylate the RB protein by a p21/WAF1-independent pathway. In order to clarify this possibility, more study will be required at the next step.

Recently, several reports indicate p53-independent regulation of the p21/WAF1 gene. TGF-β [18], phorbol myristate acetate (PMA) [19], okadaic acid [19], butyrate [20] and trichostatin A [21] stimulate the p21/WAF1 gene promoter through Sp1 sites in the promoter. In addition, transcription factors such as signal transducers and activators of transcription (STAT) protein [22], vitamin D_3 receptor [23] and C/EBPβ [24] act via their specific binding sites in the p21/WAF1 promoter. We investigated the responsive region of flavone, and found that it was included in the 124-base pair region upstream from the transcription start site, in which two p53-binding sites are not located (data not shown). In this region there are six Sp1 sites and a TATA box [20] but no p53-binding site, which is consistent with our data that p21/WAF1 induction by flavone is p53-independent.

Recently, flavopiridol, a derivative of flavone, was reported to cause G_1 arrest in a p53-independent manner in a human breast carcinoma cell line MCF-7 [25]. Flavopiridol binds directly to cyclin-dependent kinases CDK2 and CDK4, thereby inhibiting their activities [26], and arrests the cell cycle in the G_1 phase. In our study, the induction of the CDK inhibitor p21/WAF1 by flavone was suggested to be the cause of G_1 phase arrest. However, the data for flavopiridol suggests the possibility that flavone might directly bind to CDKs.

In summary, our study demonstrates that flavone, which is a well known flavonoid, can arrest the cell cycle in the G_1 phase in A549 cells possibly through activation of the p21/WAF1 gene promoter and induction of p21/WAF1 protein in a p53-independent manner. Recently, we proposed a novel strategy for chemotherapy or chemoprevention which we termed 'gene-regulating chemotherapy or chemoprevention' [20,27]. This strategy is to activate growth inhibitory genes using several agents. Our present study suggests that flavone may be one candidate drug for use in gene-regulating chemotherapy or chemoprevention.

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