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ORIGINAL CONTRIBUTION

Proteomic study of granulocytic differentiation induced by apigenin 7-glucoside in human promyelocytic leukemia HL-60 cells

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Abstract
Background Nutritional factors is one of the most important regulators in the progression of cancer. Some dietary elements promote the growth of cancer but others, such as plant-derived compounds, may reverse this process.
Purpose We tried to investigate yet another approach of cancer prevention through cancer cell differentiation, using a common non-mutagenic flavonoid apigenin 7-glucoside.
Methods HL-60 cells were treated with or without apigenin 7-glucoside. Cell proliferation was measured by MTT assay, and the cell cycle distribution was estimated by propidium iodide staining of DNA. To determine cellular differentiation, cell surface differentiation markers CD11b and CD14 were used. Two-dimensional gel electrophoresis was then performed to identify proteins that may be important in HL-60 cell differentiation following apigenin 7-glucoside treatment.
Results Apigenin 7-glucoside inhibited HL-60 cell growth, dose- and time-dependently, but did not cause apoptosis. The distribution of cells at different stages in the cell cycle indicated an accumulation of treated cells in G_2/M phase. Moreover, apigenin 7-glucoside induced granulocytic differentiation of HL-60 cells. Ten proteins that might play essential role in granulocytic differentiation were identified by proteomics.
Conclusions A complete understanding of the preventive effects of plant-based diet on cancer depends on the mechanisms of action of different plant components on processes. We hope these findings may contribute to the understandings of the different approaches for chemoprevention of cancer.

Keywords Differentiation · Proteomics · Apigenin 7-glucoside · HL-60 cells · Granulocyte

Introduction

The development of diet-based chemopreventive approaches is very important in many incurable diseases such as cancer. Even though epidemiological studies provide us valuable suggestions, the molecular events on cellular and tissue levels present the solid confirmation of beneficial effects. Currently available research shows that plant-based diets do not only reduce the incidence of cancer but in some cases inhibit its progression [1–3].

Cancer generally develops over long period of time with the initiation occurring by the change of some genes in normal cells by chemicals or other agents. The body generally repairs most such damage but if the cell reproduces itself before it is repaired, the daughter cell retains this genetic damage becoming the cancer cell. These cells continue to replicate themselves and grow into cell masses, thus promoting cancer. Nutritional factors might be the most important regulators in this step: some dietary elements may promote the growth of cancer but others such as plant-derived compounds may reverse this promotion process [4]. Recent cancer cell research mainly focused on

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the death of malignant cells through apoptosis. However, inducing cancer cell apoptosis is usually achieved when cells cannot resist the high enough cytotoxic concentration of chemicals [5]. With respect to dietary factors, the question is always aroused: can we achieve such a high concentration through the diet? There is another mild approach when we can induce cancer cells to differentiate and eliminate them from the body in natural way [6]. With regular consumption of plant-based diet, phytochemicals may reverse cancer promotion process by inducing cancer cell differentiation [7].

Flavonoids are considered as one of the main bioactive constituents of plants. Many of them have shown to possess anticarcinogenic effects by interfering with the initiation, development, and progression of tumors. In this study, we focused on apigenin which is abundantly present flavone and has shown remarkable promise as a potent chemopreventive agent. It received much attention in recent years for its low toxicity in normal cells, whereas numerous studies with different human cancer cell lines have shown that apigenin inhibits cancer cell growth via the promotion of cell cycle arrest and apoptosis [8–10]. We used the natural glycosylated form of apigenin to elucidate its cancer cell differentiation capacity. Apigenin 7-glucoside is more stable and has better solubility to compare with aglycone [8].

Leukemia cell lines are widely used as in vitro model to investigate the effect of different chemicals on cancer cells differentiation. HL-60 cells are human promyelocytic leukemia cell line that has a potential to differentiate into granulocytes or monocytes. Many compounds have been reported to induce differentiation of these cells; all-*trans* retinoic acid (ATRA) and dimethylsulfoxide (DMSO) are well-known inducers for granulocytes while 1α,25-dihydroxy vitamin D_3 [1,25$(OH)_2D_3$] and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induce HL-60 cells to monocytes [5]. In particular, ATRA which was initially a dietary factor have been used clinically for treatment for acute leukemia [11].

We could observe the differentiation-inducing effect of apigenin 7-glucoside on HL-60 cells, and proteomics approach was utilized to explain this phenomenon.

Materials and methods

Cell culture

Human promyelocytic leukemia cell line HL-60 was obtained from the Riken Cell Bank (Tsukuba, Ibaraki, Japan) and grown in RPMI 1640 medium (Gibco Invitrogen, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, BioWest, France) and 1% penicillin (5000 IU/mL) streptomycin (5000 IU/mL) solution (ICN Biomedicals) at 37 °C in a 5% CO_2 atmosphere. Cells were subcultured every 3 days.

Cell proliferation assay

At approximately 80% confluence, HL-60 cells were harvested and seeded in 96-well plates at 2.0×10^3 cells per well in medium. After overnight incubation, apigenin 7-glucoside (Sigma-Aldrich, St. Louis, MO, USA) in dilutions with medium was added to obtain final concentrations of 5, 10, 25, and 50 μM. The cells were cultured for 24, 48, and 72 h, followed by the addition of 10 μL of 5 mg/mL of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Dojindo, Japan). After 24 h of incubation, 100μL of 10% sodium dodecyl sulfate (SDS) was added and the cells were incubated for another 24 h to completely dissolve the formazan produced by the cells. The absorbance was spectrophotometrically determined at 570 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, NJ, USA). Blanks were prepared at the same time to correct for the absorbance caused by sample color and by the inherent ability of the samples to reduce MTT in the absence of cells.

Cell morphology

The cells were examined by light microscopy (Leica Microsystems) to observe cell morphology. HL-60 cells were seeded in 100-mm dish at a density of 2.0×10^5 cells per dish. After overnight incubation, cells were treated with 25 μM of apigenin 7-glucoside for 24, 48, and 72 h and the pictures were taken accordingly.

Cell viability assay

Cell viability was assessed using flow cytometry according to the manufacturer's instructions. HL-60 cells were seeded in 100-mm dish at 2.0×10^5 cells per dish in 10 mL of medium. After overnight incubation, cells were treated with 25 and 50 μM of apigenin 7-glucoside for 24, 48, and 72 h. After treatment, cells were suspended in Guava ViaCount reagent (Guava technologies, CA, USA) and incubated for 30 min in darkness at room temperature. The cell number and viability were measured by Guava PCA flow cytometry (Guava technologies, CA, USA).

Cell cycle analysis

To determine cell cycle phase distribution, flow cytometric analysis of cellular DNA content was performed. HL-60 cells were seeded in 100-mm dish at 2.0×10^5 cells per dish in 10 mL of medium. After overnight incubation, cells

were treated with 25 μM of apigenin 7-glucoside for 24 and 48 h. After treatment, cells were collected, washed with phosphate-buffered saline (PBS) twice, and fixed in 70% of ice-cold ethanol at 4 °C for more than 12 h. The fixed cells were centrifuged at 500×*g* for 5 min, the supernatant was removed, and the pellets were washed with PBS twice. The cells were suspended in Cell cycle reagent (Guava technologies, CA, USA), incubated for 30 min in darkness at room temperature, and measured by a Guava PCA flow cytometry (Guava technologies, CA, USA).

Cell differentiation assay

HL-60 cell differentiation was determined by the expression of cell surface markers CD11b and CD14 measured by flow cytometry. 2.0×10^5 cells per dish in 10 mL of medium were seeded in 100-mm dish. After overnight incubation, cells were treated with 25 μM of apigenin 7-glucoside for 48 h. 100 nM ATRA- (Sigma-Aldrich, St. Louis, MO, USA) and 100 nM $1,25(OH)_2D_3$ (Sigma-Aldrich, St. Louis, MO, USA)-treated cells were used as positive controls. After washing with cold PBS twice, 1.0×10^5 cells were labeled with R-phycoerythrin-cyanine 5 (PC5) conjugated anti-CD11b antibody and phycoerythrin (PE) conjugated anti-CD14 antibody (Beckman Coulter CA, USA) for 30 min on ice in darkness. The cells were washed with cold PBS twice and finally resuspended in 500 μL of PBS for measurement.

Two-dimensional gel electrophoresis and image analysis

HL-60 cells were seeded in 100-mm dish at a density of 2.0×10^5 cells per dish. After overnight incubation, cells were treated with or without 25 μM of apigenin 7-glucoside followed by incubation for 48 h. The medium was then removed, and the cells were washed twice with PBS before the total protein was extracted using lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 40 mM dithiothreitol [DTT], 1 mM EDTA, 25 mM Spermine base) containing protease inhibitor according to the manufacturer's instructions. Samples were stayed for 1 h at room temperature and centrifuged at 17,000×*g* for 100 min at 15 °C, and then, supernatants were carefully collected. The protein concentration was determined by 2D Quant Kit (GE Healthcare, UK). The protein samples (350 μg) were rehydrated with rehydration buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 15 mM DTT) and isoelectrically focused in IPG Buffer (0.5%) using Immobiline IPG DryStrips (240 mm, pH 3–10, GE Healthcare, UK) for 12 h at 45 kVh on Ettan IPGphor system (GE Healthcare, UK). Focused strips were equilibrated, and SDS–PAGE was performed using 12.5% polyacrylamide gels in Ettan DALT *six* Large Electrophoresis System (GE Healthcare, UK). Following electrophoresis, gels were fixed and stained with 0.1% Coomassie Brilliant Blue G-350 in 30% methanol and 10% acetic acid. Gel image analysis was performed using software ImageMaster 2D Platinum ver. 5.0 (GE Healthcare, UK). Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel. Protein spots with normalized volume of over twofold differences were excised.

In gel digestion

Excised spots were destained with 25 mM ammonium bicarbonate for 30 min. The spot pieces were washed with 100% acetonitrile (ACN) and dried completely followed by reduction and alkylation with 10 mM DTT and 25 mM iodoacetamide, respectively. After washing with ACN and drying in a speedVac (Genevac Inc, NY, USA), gels were kept at 4 °C for 30 min in trypsin solution (10 μg/mL modified sequence-grade trypsin, 50 mM ammonium bicarbonate), then 50 mM ammonium bicarbonate was added to prevent gel pieces from drying during the digestion at 37 °C overnight. After digestion, the resulted peptides were extracted twice with 5% formic acid in 50% ACN.

Liquid chromatography–tandem mass spectrometry

Liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis was performed for protein identification using 3200 QTRAP MS/MS system (Applied Biosystems) coupled with Ultimate 3000 LC (Dionex). After sample injection by autosampler, the column was washed for 5 min with 98% mobile phase A (0.1% formic acid in water) at a flow rate of 0.3 μL/min. Peptides were eluted from the column using a linear gradient of 2% mobile phase B (0.1% formic acid in ACN) to 40% mobile phase B in 10 min at a flow rate of 0.3 μL/min, then to 90% mobile phase B for an additional 5 min. The column effluent was directed into the electrospray source. The electrospray voltage was set at 2.3 kV. Full MS scan range was 400–1,200 m/z, spectra were acquired automatically in Information Dependent Acquisition (IDA) analysis. Protein identification was performed using Mascot software (www.matrixscience.com) and searched against swiss prot database. Search criteria were defined as follows: enzyme as trypsin, taxonomy as *Homo sapiens*, missed cleavage of 1, fixed modifications as carbamidomethylation of cysteine and variable modifications as oxidation of methionine. MASCOT protein score >34 was considered to be significantly different ($p < 0.05$).

Statistical analysis

Results are expressed as mean ± SD of triplicate experiments. Statistical analysis was performed using Student's *t* test. The $p < 0.05$ was considered statistically significant.

Results

Apigenin 7-glucoside inhibits cell proliferation of HL-60 cells

First, in order to elucidate whether apigenin 7-glucoside has biochemical effects in a physiologically relevant dose, we investigated the cell proliferation with apigenin 7-glucoside using a MTT assay. HL-60 cells were treated with different doses from 0 to 50 μM of apigenin 7-glucoside up to 72 h. In this experimental condition, apigenin 7-glucoside inhibited HL-60 cell proliferation in a time- and dose-dependant manner (Fig. 1a). After 48 h treatment with 25 μM of apigenin 7-glucoside, the cell proliferation was inhibited to 35% compared with the control.

The morphological changes observed in apigenin 7-glucoside-treated HL-60 cells were compared to control cells (Fig. 1b). The control cells had the same morphology with round shape and smooth cell surface throughout treatment times (24–72 h), while at 24 h of treatment, treated cells became bigger with rough surface and were attached weakly to the bottom of the dish.

Apigenin 7-glucoside induces growth inhibition of HL-60 cells

Since MTT assay is based on mitochondrial activity of cells, we cannot conclude whether the anti-proliferative effect was derived from apoptosis or growth inhibition only. To precisely define the cell growth inhibition effect by apigenin 7-glucoside, we performed more specific cytotoxicity assay by flow cytometric analysis. This assay is based on a proprietary mixture of two DNA-binding dyes. The first, a membrane-permeant dye, stains all nucleated cells, helping to eliminate cellular debris. The second, a membrane-impermeant dye, stains only damaged cells and thus indicates compromised cell health in cells that it stains. HL-60 cells treated with apigenin 7-glucoside exhibited slow cell growth compared to control cells in a dose-dependent manner (Fig. 2a); at concentration of 25 μM, the cell number had little increase. The viability of both control and treated cells at 5 μM was nearly 97% during all incubation periods (24–72 h), while at 25 μM treatment, it was slightly decreased time-dependently (Fig. 2b). Nevertheless, the cell viability was over 90% throughout the experimental conditions, suggesting apigenin 7-glucoside did not cause

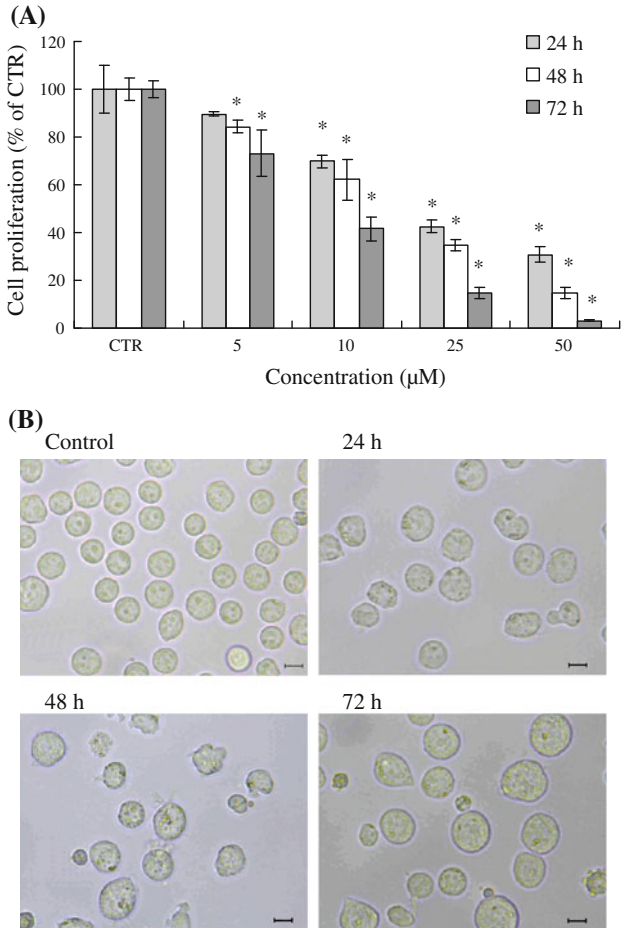

Fig. 1 Effect of apigenin 7-glucoside on the proliferation of HL-60 cells. **a** The cells were treated with various concentrations (5–50 μM) of apigenin 7-glucoside for 24, 48, and 72 h. The cell proliferation was measured by MTT assay. CTR represents control cells treated with 1.0% ethanol in medium at the final concentration. Data are presented as a percentage of the control and as the mean ± SD of three independent experiments. *$p < 0.05$ significantly different from the control. **b** The cells were treated for 24, 48, and 72 h with control or 25 μM of apigenin 7-glucoside. The cells were observed by light microscopy (magnification ×400). *Scale bar* represents 10 μm. The photos are the representative of three independent experiments

apoptosis in HL-60 cells at selected concentrations (Fig. 2b, c). The results of cell number correlate well with the results of MTT assay (Fig. 1a).

Apigenin 7-glucoside modulates cell cycle progression in HL-60 cells

As apigenin 7-glucoside induced significant growth inhibition of HL-60 cells, we further analyzed its effect on the cell cycle distribution by measuring DNA content after staining with Propidium iodide (PI). Cells treated with apigenin 7-glucoside showed the accumulation of the cells in G_2/M phase compared with the control (Fig. 3a, b). The

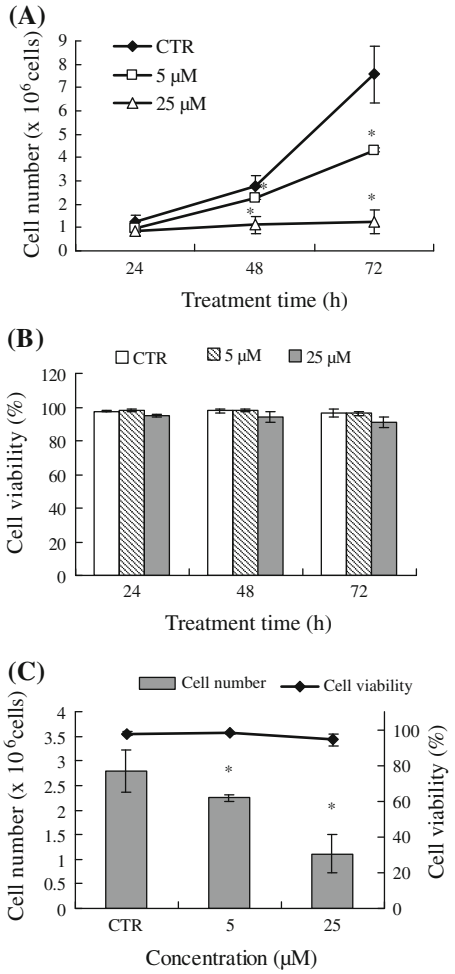

Fig. 2 Effect of apigenin 7-glucoside on the cell number and viability of HL-60 cells. The cells were treated with 5 and 25 μM of apigenin 7-glucoside for 24, 48, and 72 h. The time-dependant effect of apigenin 7-glucoside on cell number (**a**) and viability (**b**) was measured by flow cytometry. **c** The dose-dependant effect of apigenin 7-glucoside (5, 25 μM) on cell number and viability after 48 h incubation. CTR represents control cells treated with 0.5% ethanol in medium. Data are presented as the mean ± SD of three independent experiments. $*p < 0.05$ significantly different from the control

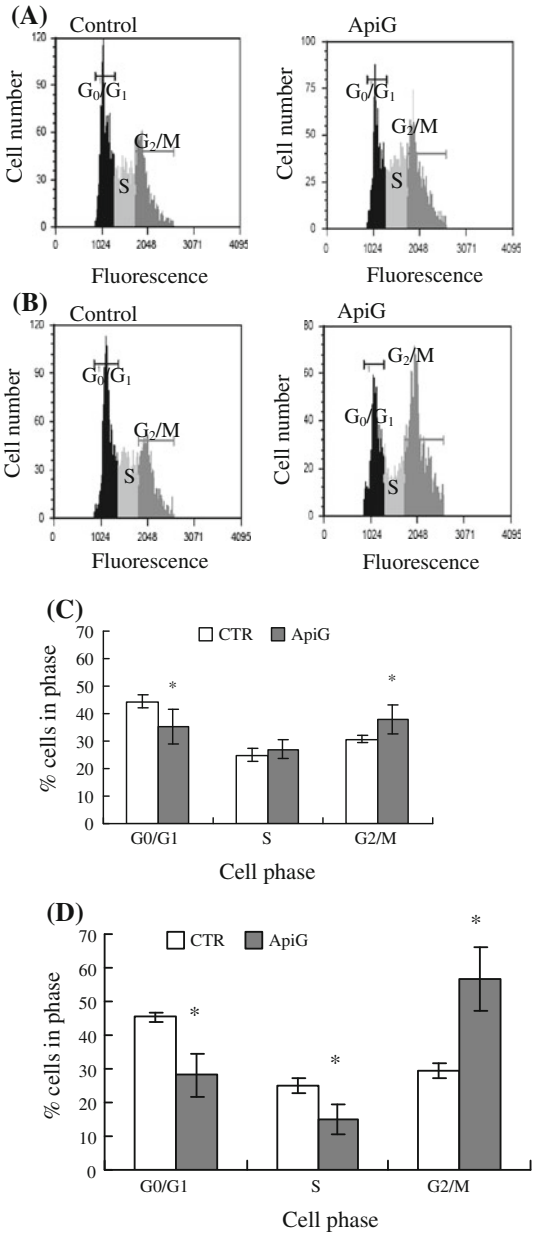

Fig. 3 Effect of apigenin 7-glucoside on the cell cycle distribution of HL-60 cells. The cells were treated with 25 μM of apigenin 7-glucoside for 24 h (**c**) and 48 h (**d**). The cell cycle distribution according to their DNA content revealed by propidium iodide-derived fluorescence was determined by flow cytometry. An example of the obtained flow cytometric profiles is shown as **a** treated for 24 h and **b** treated for 48 h. CTR represents control cells treated with 0.5% ethanol in medium and ApiG represents apigenin 7-glucoside-treated cells. The left peak indicates that the cells in G_0/G_1 phase while the right peak indicates the cells in G_2/M phase, and the region between two peaks defines the cells at S phase. Data are presented as the mean ± SD of three independent experiments. $*p < 0.05$ significantly different from the control

number of cells in G_2/M phase with apigenin 7-glucoside treatment was increased to 37.8 and 56.7% after 24 and 48 h of treatment, respectively, while it was 30.7 and 29.7% in control cells, respectively (Fig. 3c, d). The accumulation of cells in G_2/M phase was time-dependent. This result suggests that the growth inhibition driven by apigenin 7-glucoside was due to a block of cell cycle at G_2/M phase.

Apigenin 7-glucoside induced granulocytic differentiation in HL-60 cells

In order to determine whether growth inhibition via cell cycle arrest by apigenin 7-glucoside is associated with differentiation, we detected the expression of cell surface makers in HL-60 cells by flow cytometry. CD11b and CD14 are both expressed in monocytes while the

expression of CD11b only indicates granulocytic differentiation of the cells. As shown in Fig. 4a, the control cells did not express both markers of CD11b and CD14 antigens. ATRA-treated cells which is the positive control of granulocytic differentiation showed increase in the number of CD11b-positive cells only, while $1,25(OH)_2D_3$-treated cells used for the positive control of monocytic differentiation showed increase in the percentage of both CD11b- and CD14-positive cells. After treatment with apigenin 7-glucoside, HL-60 cells expressed only CD11b marker similar with ATRA treatment and the population of CD11b-positive cells was 39.1% while the control was 2.9% (Fig. 4b). These results indicate that apigenin 7-glucoside induced differentiation of HL-60 cells to granulocytes.

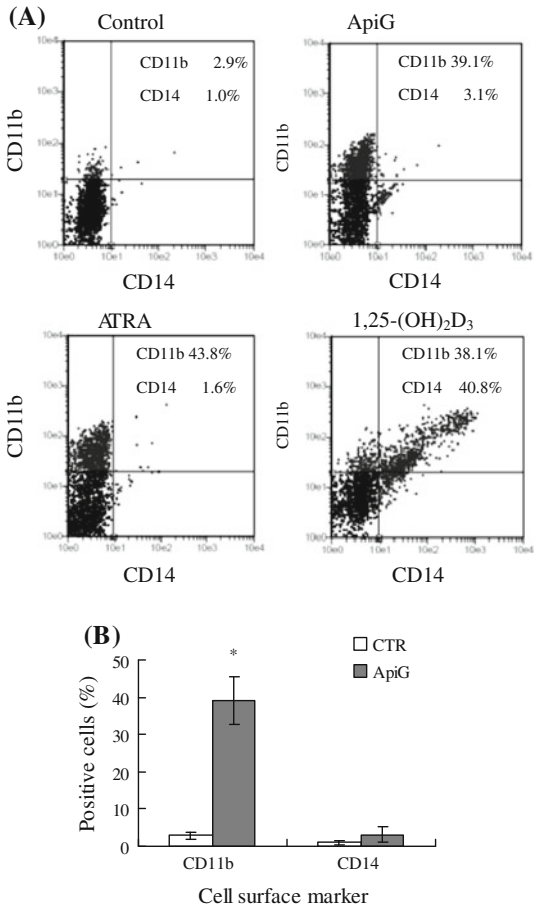

Fig. 4 Effect of apigenin 7-glucoside on induction of differentiation of HL-60 cells. The cells were treated for 48 h with 0.5% ethanol (CTR), 25 μM of apigenin 7-glucoside (ApiG), 100 nM of ATRA, and 100 nM of $1,25\text{-}(OH)_2D_3$. The differentiation analysis according to the expression of cell surface markers CD11b (granulocyte and monocyte specific) and CD14 (only monocyte specific) was determined by flow cytometry. Representative flow cytometric profiles are shown (**a**). The lines delimit the region of positive cells. The total percentage of positive cells (CD11b—two upper quadrants; CD14—two right-side quadrants) is showed within each panel. **b** The differentiation data are expressed as a percentage of differentiated cells with the mean ± SD of three independent experiments. $^*p < 0.05$ significantly different from the control

Identification of differential expression of proteins in HL-60 cells with or without apigenin 7-glucoside

To further characterize the mechanism by which apigenin 7-glucoside induced granulocytic differentiation of HL-60 cells, we analyzed the protein expression changes in treated HL-60 cells compared to control cells using proteomics. We detected 330 spots in each gel after CBB staining, and more than 80% of protein spots were matched (Fig. 5). From the gels, we selected 10 spots, which were found to be differentially expressed and showed >2-fold change (Fig. 6). These spots were excised and digested in trypsin, and the proteins were identified by LC/MS/MS. In Table 1, identified protein names, their mascot score, pI/MW, and value of fold change compared to control are listed. All identified proteins were up-regulated by apigenin 7-glucoside and associated with the process of differentiation. The up-regulation of proteins was confirmed by the expression of mRNAs of selected proteins by RT–PCR experiments (Online resource, Fig. S1).

Discussion

Cancer is one of the major public health burdens in many countries. Epidemiological findings strongly suggest that cancer occurrence is influenced by environmental factors including diet. Cancer prevention aims at blockage or reversal of the initiation phase of carcinogenesis or arrests it at progression stage. In this context, plant-derived, naturally occurring constituents may be largely involved, since increased consumption of fruits and vegetables correlates with lower incidence of cancer [7]. Here, we tried to investigate yet another approach of cancer prevention such as cancer cell differentiation using a common non-mutagenic flavonoid apigenin 7-glucoside.

Induction of differentiation is generally associated with a loss of proliferative capacity of cells. Several studies have been reported that the differentiation-inducing agents exhibit anti-proliferative effect [12–15]. Our results indicate that apigenin 7-glucoside inhibited the cell proliferation in time- and dose-dependant manner (Fig. 1a). However, the cell viability was not decreased by treatment with apigenin 7-glucoside (Fig. 2b), indicating apoptosis was not induced. Previous reports have presented anti-proliferative effect of apigenin in breast cancer, prostatic stromal, colon carcinoma as well as leukemia cells but the treatment concentrations were high enough for cytotoxic effect [10, 16–18]. Wang et al. [18] demonstrated that 60 μM apigenin induced apoptosis in HL-60 cells. In our

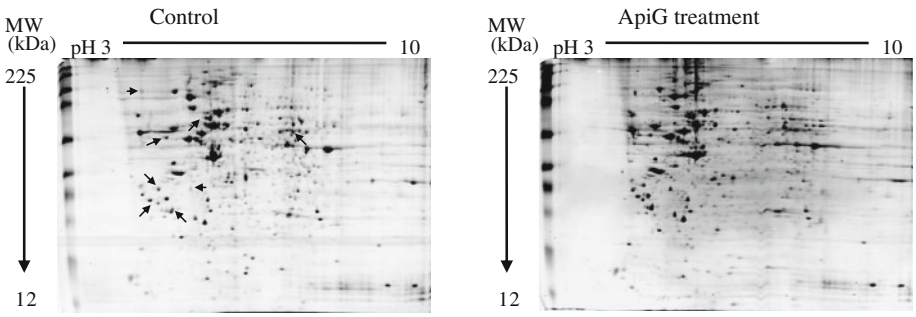

Fig. 5 An overview of representative 2D gel images obtained from control HL-60 cells and treated HL-60 cells with apigenin 7-glucoside (ApiG) for 48 h. 2DE gels were stained by CBB. Proteins differentially expressed were marked by *arrow*. Data presented are the representative of three independent experiments

experimental condition, the low dose of apigenin 7-glucoside (25 μM), contributes to the effect of anti-proliferation but not cytotoxicity on HL-60 cells.

It was demonstrated that apigenin arrested the cell cycle at G_2/M phase in various types of cancer cells [9, 16, 19]. Our results also show that apigenin 7-glucoside induced G_2/M-phase arrest on HL-60 cells, which is the possible explanation of growth inhibition by apigenin 7-glucoside. The cell cycle is mediated by many protein complexes such as CDKs, cyclins, and CKIs through the cell cycle checkpoints. Cdc2 (CDK1) and cyclinB complex is the master switch for the G2/M-phase transition [20]. Previous studies showed that apigenin inhibits cdc2 kinase activity through the exposure of a wide array of malignant cells [21]. Other potential regulatory effects of apigenin may increase the expression of p21, which is the negative cell cycle regulatory molecule [20]. Apigenin has also been reported to up-regulate the expression of p21 in prostate, cervical, and breast cancer cells [9, 16]. All these studies tried to explain the possible G2/M arrest mechanisms by apigenin treatment in different cell lines with connection to apoptosis. Normally, cells rely on the G_1 checkpoint to protect against DNA damage leading to cell cycle arrest at G_0/G_1 phase; however, cancer cells have defective G_1 checkpoint function [20]. Thus, the G_2 checkpoint could be a potential target for cancer therapy, and the compounds modulating G_2 checkpoint can be the attractive agents in cancer treatment. On the other hand, the cell cycle arrest in the common event observed during cell differentiation.

We studied the cell morphological changes during apigenin 7-glucoside treatment. In the first 24 h, the surface of treated cells lost their smoothness and their size became bigger compared to control cells; later, these cells developed small protrusions and were attached weakly to the bottom of the dish (Fig. 1a). Granulocytes have functional characteristics including cell adhesion, migration, chemotaxis, and phagocytic activity. Besides, HL-60 cells which were differentiated by DMSO also exhibited adhesion [22]. On the other hand, apigenin 7-glucoside reduced cell

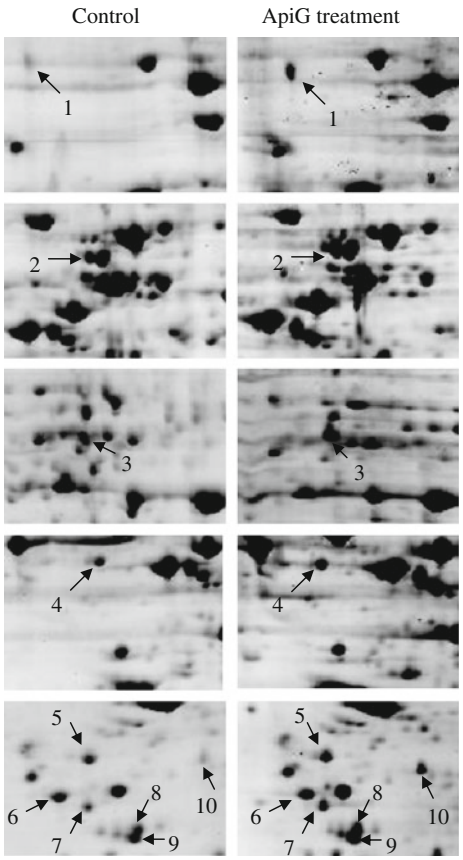

Fig. 6 Specific regions of 2D gel image indicating protein spots with altered expression compared to control. Information on each numbered spot is summarized in Table 1. ApiG represents apigenin 7-glucoside-treated cells

proliferation with no cytotoxic effect. Therefore, we hypothesized that apigenin 7-glucoside may have a potential of inducing differentiation of HL-60 cells accompanied with cell cycle arrest and performed further studies with differentiation marker analysis. The results support our hypothesis and reveals that apigenin 7-glucoside has a potential to be not only anticancer agent by induction of cell cycle arrest but also inducer of cellular

Table 1 List of proteins affected by apigenin 7-glucoside and identified by LC/MS/MS

Spot no.	Protein name	Score[a]	Theoretical[b]		Observed		Fold change[c]
			pI	MW	pI	MW	
1	Glucosidase 2 subunit beta	71	4.33	60.4	4.43	90.5	2.25
2	Plastin-2	221	5.20	70.8	5.80	67.0	2.35
3	Coronin-1A	83	6.25	51.7	7.63	58.0	2.25
4	Histone-binding protein RBBP4	45	4.74	47.9	4.97	53.0	2.12
5	Proliferating cell nuclear antigen	261	4.57	29.1	4.78	33.0	2.03
6	Elongation factor 1-beta	67	4.50	24.9	4.60	29.5	2.22
7	14-3-3 protein epsilon	122	4.63	29.3	4.77	28.5	2.29
8	14-3-3 protein beta/alpha	138	4.76	28.2	5.10	27.0	2.77
9	14-3-3 protein gamma	79	4.80	28.5	5.07	26.5	4.09
10	Annexin A5	132	4.94	36.0	5.63	30.5	2.74

[a] Mascot score of >34 was considered as significant ($p < 0.05$)

[b] Theoretical pI and MW were derived from the amino acid sequence in swiss prot

[c] Fold changes of spot volume (Apigenin 7-glucoside vs. control)

differentiation. Our previous study reported that apigenin 7-glucoside demonstrates the differentiation-inducing effect on human chronic leukemia K562 cells toward erythrocytes [23]. A number of agents have been studied over years for cancer cell differentiation, and some of them such as ATRA are used in clinical practice to treat leukemias. But none of them is ideal; they can be effective only in certain types of leukemia, and their long-term use possesses toxic effects. HL-60 and K562 cells are the models of acute and chronic myeloid leukemias, respectively. The development of these leukemias is thought to be different in terms of origin and stages of development. Our studies suggest that apigenin 7-glucoside is capable of triggering terminal differentiation of different types of leukemia and has the good potential for the differentiation inducer.

The cell cycle arrest is prerequisite for hematopoietic cell differentiation, generally at the G_0/G_1 phase. Differentiation initiates within the G_0/G_1 phase, and terminally differentiated cells are arrested at the G_0/G_1-phase cells because cells do not divide anymore and exit from the cell cycle. Although many studies attempt to clarify the correlation between cell cycle arrest and differentiation, the precise mechanisms are largely unidentified. The common view that the initiation of cell differentiation is most likely to occur at the G_0/G_1 phase was not followed in the case of apigenin 7-glucoside. Therefore, it might be interesting to investigate the differentiation pathway with apigenin 7-glucoside treatment and explore novel players in the complex process of cellular signaling. With this purpose, we performed proteomic analysis.

Proteomics is one of the most valuable tools to analyze the poorly unknown mechanisms. Traditional techniques of molecular biology were usually highly focused, targeting one or few molecules at a time, whereas proteomics provides possible overview of large-scale protein expression changes. We identified 10 proteins consistently modulated in response to apigenin 7-glucoside treatment by proteomic approach (Table 1). Apigenin has been reported to inhibit the proteasome activity in human leukemia cells, and it gives the possible explanation the up-regulation of all selected proteins [24].

First, the expression of glucosidase 2 subunit beta protein was increased in apigenin 7-glucoside-treated cells. This protein is regulatory subunit of β-glucosidases, which hydrolyses flavonoid glycosides. In human small intestine, two of the β-glucosidase, one of which bound in membrane and the other distributed in cytosol, hydrolyze flavonoid glucosides in distinct pathway [25]; for instance, quercetin glucoside has been reported to be absorbed via sugar transporters and to be hydrolyzed by β-glucosidase [26]. These previous reports provide a possible explanation for better bioavailability of glucosides. Expression of glucosidase 2 subunit beta suggests that apigenin 7-glucoside might be hydrolyzed by β-glucosidase and released aglycone could affect HL-60 cells.

When apigenin is taken orally, it reaches the gut and is extensively metabolized and converted by phase I and phase II enzymes. In vitro study has demonstrated that apigenin was converted to the three mono-hydroxylated derivatives via phase I metabolism [27]. Apigenin and luteolin, which are the major phase metabolite of these derivatives, were conjugated to three mono-glucuronated and one mono-sulfated compounds, and four mono-glucuronated, two sulfated, and one methylated compounds, respectively, via phase II metabolism in vitro [27]. These observations suggest that the bioavailability of apigenin is limited, but Gradolatto et al. [28] have shown that a single oral administration of radio-labeled apigenin in rats

resulted in 24.8% in the rest of the body within 10 days and also its radioactivity appeared in blood after 24 h but eliminated slowly. These results suggest slow metabolism of apigenin and give rise to the possibility of an accumulation of apigenin in the body and tissue for its effective chemopreventive properties. Furthermore, apigenin was shown to have no mutagenic activity and effects to normal cells such as liver and prostate cells [29, 30]; normal human peripheral blood lymphocytes were not affected by apigenin even at high concentrations: the viability of cells was over 80% with apigenin treatment at 200 μM [9]. These reports indicate that apigenin has low intrinsic toxicity even though flavonoids are present in systemic circulation at low micro-molar concentration and differential effects in normal versus cancer cells. However, further studies about the metabolism of apigenin and the usage of administration are needed to develop apigenin 7-glucoside as a food-based anti-leukemia compound.

Next, identified proteins were 3 isoforms of 14-3-3 proteins. 14-3-3 proteins are a family of highly conserved molecules that play important roles in a wide range of cellular processes within all eukaryotic cells, and seven isoforms are identified in mammals [31]. 14-3-3ε and 14-3-3γ were reported to make a complex with tyrosine phosphatase cdc25C and inhibit its entry into the nucleus [32]. In the cell cycle progression, the cytoplasmic localization of cdc25C does not activate the cyclinB/cdc2 complex and induce G_2/M arrest [33]. 14-3-3β expression is also related to G_2/M cell cycle arrest by indirect inhibition of cell division control protein cdc2 activity [34]. Overall, our observation supports the involvement of 14-3-3 proteins in G_2/M arrest particularly with apigenin 7-glucoside treatment for cancer cells.

Other regulatory proteins up-regulated during the HL-60 cells differentiation were proliferation cell nuclear antigen (PCNA) and elongation factor 1-β (EF-1β). Although all of the PCNA functions described to date reflect its crucial role in DNA synthesis and repair, recent studies of Witko-Sarsat et al. demonstrated its specific function in neutrophils exclusively [35, 36]. Unlike macrophages and other cells, differentiated neutrophils do not proliferate but highly expressed PCNA protein was shown to be associated with procaspases protecting neutrophils from apoptosis. Our observations with less apoptotic feature but cell differentiation with apigenin 7-glucoside support this evidence. Interestingly, the expression of PCNA protein increases steadily though the whole cell cycle term and remains high at G_2/M phase [37], further interacting with cdc25C at G_2/M transition [38] and with EF-1β [35]. EF-1β exchanges GDP for GTP to regenerate active EF-1α. This active EF-1α is then able to perform another round of elongation of polypeptide chain [39]. Increase in size of differentiated cells (Fig. 1b) may implicate the active protein synthesis, which could lead to the increase of EF-1β in protein level.

Histone acetylation is important chromatin modification reaction controlling gene transcription. In this process, histone deacetylase 1 (HDAC1) was reported to form a complex with PU.1 to act as transcriptional repression [40, 41], and PU.1 is known to be suppressed in human leukemic cells but in the process of granulocytic differentiation, its expression is increased [42]. The observed up-regulation of plastin-2, Annexin A5, and Coronin-1A with apigenin 7-glucoside treatment confirms our granulocytic differentiation in terms of granulocytes characteristic features. Plastin-2 and Annexin A5 are reported to be the components of NADPH oxidase system, which is the basis of NBT reduction assay to identify the differentiated granulocytes [43–49]. Coronin-1A is a member of actin-associated proteins that have been implicated in a variety of cellular processes dependant on actin rearrangements. In primary human neutrophils, the expression of coronins is required for actin-dependant changes in cell morphology that lead to migration and chemotaxis [50]. Our morphology observations of the differentiated cells with apigenin 7-glucoside also showed the protrusions and pseudopod developments in HL-60 cells with weak attachment to the dish. These 3 proteins support our results of apigenin 7-glucoside induced granulocytic differentiation in HL-60 cells.

In conclusion, we have demonstrated that plant flavone apigenin 7-glucoside not only induces apoptosis of different cancer cells but also has a potential to induce cell differentiation. The appropriate understanding of plant-based diet preventive effects from different diseases including cancer depends on the mechanisms of action of its different components at all levels of organism. We hope our findings may contribute to understand different approaches of chemopreventive properties of natural compounds against cancer disease.

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