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A mechanism of apigenin-induced apoptosis is potentially related to anti-angiogenesis and anti-migration in human hepatocellular carcinoma cells

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ABSTRACT

Apigenin (APG) has been shown to have a strong anti-cancer effect on various cancer models via a programmed cell death, apoptosis. However, the fundamental mechanisms of these effects are still unclear. In the present study, we examined the question of whether or not APG can inhibit proliferation of hepatocellular carcinoma (HCC), huh-7 cells, resulting in apoptosis. In APG-treated cells, we observed typical features of apoptosis. To identify the proteins related to APG-induced apoptosis, we performed two-dimensional electrophoresis analysis and identified differentially expressed proteins. Among these proteins, we focused on vimentin, which plays a physiological role, such as cell migration and adhesion. We validated expression of vimentin in both mRNA and protein levels, verifying its decrease. In addition, we observed that APG down-regulated the expression levels of type I collagen, which collaborated with vimentin in cell migration and decreased the releasing amounts of VEGF and MMP-8, which are closely relevant to angiogenic activity. Finally, we confirmed the decreased capacity of cell migration due to down-regulation of vimentin, type I collagen, VEGF, and MMP-8 induced by APG. Based on the overall results, we suggested that vimentin was potentially associated with APG-induced apoptosis, as a key regulator in angiogenesis and migration.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant cancer (Parkin et al., 2005). Estimated attack rate per year of HCC is nearly 560,000 and the mortality is about 1 million annually worldwide (Bosch et al., 2004). Unlike other solid tumors, most HCC patients have chronic background liver diseases, including hepatic fibrosis, cirrhosis, hepatitis B virus, and hepatitis C virus, which give rise to considerable trouble in treatment (Azam and Koulaouzidis, 2008; Burroughs et al., 2004). Although chemotherapy, hepatectomy, and liver transplantation are representative treatments for HCC, these treatments have a limited range of applications (Cahill and Braccia, 2004). In particular, in patients for whom cancer could not be detected early, chemotherapy shows poor tolerance and low efficacy; also, liver resection and transplantation have a poor subsequent survival rate with a high recurrence rate, despite being potentially curative types of therapy used in HCC treatment (Burroughs et al., 2004). Therefore, alternative medicines with good efficacy are urgently needed.

There are two distinct types of cell death in cell-biology mechanisms; necrosis and apoptosis, which differ in their process and features. For example, DNA fragmentation, a phenomenon that involves regular DNA cleavage into an unit length of 180–200 bp, is a typical feature of apoptotic cells induced only in apoptotic stimulus. While necrosis is caused by mechanical force or trauma, apoptosis is induced by cellular signals and programmed in the cells for control of human homeostasis, such as removal of damaged or unnecessary cells. Because certain steps of apoptotic processes, including DNA fragmentation, are impaired in many cancer cells, cancer cells proliferate endlessly and threaten multiplication of other normal cells (Kiechle and Zhang, 2002). For these reasons, many researchers have been looking for apoptotic inducers in cancer cells.

All types of cancer cells require angiogenesis, the growth of new capillary vessels for energy supply, due to multiple cell divisions. Angiogenesis is induced by certain angiogeneic signals, like vascular endothelial growth factor (VEGF), which is up-regulated during proliferation and invasion into other tissues of cancer cells. When

Abbreviations: APG, apigenin; HCC, hepatocellular carcinoma; VEGF, like vascular endothelial growth factor; MMPs, matrix metalloproteinases; IF, intermediate filament; 2-DE, two-dimensional electrophoresis; MALDI-TOF/MS, matrix-associated laser desorption/ionization-time of flight mass spectrometery; RT-CES, real-time cell electronic sensing; ELISA, enzyme-linked immunosorbent assay; IC50, half-maximal inhibitory concentration; TdT, terminal deoxynucleotidyl transferase; VIM, vimentin; PRDX2, peroxiredoxin-2; KRT17, keratin type I cytoskeletal 17; CDA, cytidine deaminase; C19orf10, UPF0556 protein C19orf10; PRDX1, peroxiredoxin-1; RPLP2, 60S acidic ribosomal protein P2; DUT, deoxyuridine 5′-triphosphate nucleotidohydrolase, mitochondrial.

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angiogenesis begins, matrix metalloproteinases (MMPs) are required for breakdown of surrounding tissues in order to grow new vessels in downstream signals (Jiang et al., 2000; Kim, 2003). Angiogenesis is closely associated with several factors, including vimentin, which is a type of intermediate filament (IF), as structural proteins. Vimentin has shown correlation with type I collagens and other proteins in poor prognosis and has received the attention of researchers because it plays a key role in angiogenesis, cell migration, attachment, wound healing, and cell signaling. Moreover, over-expression of vimentin was reported in a metastastic hepatocellular carcinoma, which suggests that vimentin is a key factor in angiogenic processes (Ivaska et al., 2007).

Flavonoids plentifully contained in fruits and vegetables are a class of plant secondary metabolites with an ubiquitous phenolic structure. Apigenin (APG), a type of flavonoid found mainly in orange, tea, chamomile, onion, and wheat sprouts, has a potential for biological activities, including antioxidant and anti-inflammatory. It also functions as an immune system modulator and carbohydrate metabolism promoter. APG has recently received much attention, due to its strong anticancer effect in various cancer cells, including breast cancer, colon cancer, lung cancer, neuroblastoma, liver cancer, prostate cancer, pancreas cancer, and oral cancer cells (Cai et al., 2011 ; Khan and Sultana, 2006; Vargo et al., 2006).

Although many researchers have studied the effect of APG, a biomolecular mechanism of APG is still ambiguous. The primary aim of our study was to find a possible molecular mechanism of APG-induced apoptosis in HCC cell line, huh-7, using proteomic analysis. Two-dimensional electrophoresis (2-DE) has been used widely to investigate intracellular protein expression in proteomics study (Bae et al., 2005). In this study, several protein candidates were selected using 2-DE followed by matrix-associated laser desorption/ionization-time of flight mass spectrometery (MALDI-TOF/MS). Then, the candidates were confirmed using RT-PCR in mRNA level. Among these candidates, we focused our research on the decreased expression of vimentin in APG-induced apoptosis. To widely understand the change of vimentin in HCC, the correlation of vimentin and other angiogenic proteins, such as type I collagen, were confirmed using immunoblot. We detected the released amount of VEGF and MMP-8 in APG-treated huh-7 cells. Finally, we performed a migration assay for investigation of the overall effects of vimentin and other angiogenic proteins in cell migration. The results suggested that APG induced apoptosis in hepatocellular carcinoma cells, huh-7, through dysfunction of cell migration and angiogenic factors.

2. Materials and methods

2.1. Cell line and cell culture

Huh-7 is a human hepatocellular carcinoma (HCC) cell line. Huh-7 cells were purchased from the Korean Cell Line Bank and cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum, 100 μm/ml antibiotics at 37 °C, and 5% CO_2 in a humidified incubator. Apigenin (APG), purchased from Sigma (Deisenhofen, Germany), was dissolved in DMSO and stocked at −20 °C. Huh-7 cells were seeded and cultured in 100 mm culture dishes until the cells were grown to 70% confluency; the medium was then replaced with a new medium containing 40, 80, or 120 μM of APG. Control cells were cultured in a culture medium.

2.2. Cell viability assay (RT-CES)

The real-time cell electronic sensing (RT-CES) system was used for analysis of the viability of APG treated cells in vitro. This cell-based screening assay is a real-time measurement of electronic impedance which represents cell viability (Xing et al., 2006). Cells were seeded into E-plates at a density of 2×10^5 cells/well and the E-plates were inserted into a 16× E-plate station that was placed in an incubator. After 24 h incubation, various concentrations of APG were added to each well. The 16× E-plate station was linked with an analyzer and computer in order to monitor the status of the cells. The scanning interval was set up to 10 min.

2.3. DNA fragmentation assay

Cells treated at various concentrations of APG for 24 h were collected and centrifuged for 5 min. The DNA of the cells was extracted using the TACS® Apoptotic DNA laddering kit (Trevigen, Gaithersburg, MD). The assay was performed according to the manufacturer's protocol. Obtained DNA was stained by 1 μM of ethidium bromide loaded on each well of a 1.5% agarose gel. The gel was visualized under an UV light and photography was performed with a transilluminator (VILBER LOURMAT, Marne-la-Vallee, France).

2.4. Tunel assay

The presence of early apoptosis-related DNA fragmentation was determined by use of the tunel assay. Cells were cultured on a slide for a day and treated at various concentrations of APG. After 24 h of treatment, the cells were fixed with 4% methanol-free formaldehyde solution in PBS (pH 7.4) at RT for 10 min and dipped in PBS for 5 min twice. Tunel staining was performed using the DeadEnd™ Flourometric tunel System kit (Promega, Madison, WI) according to the manufacturer's protocol. Tunel-positive (green staining) and negative (red staining) cells were observed through a fluorescence microscope. Observed microscopic fields were randomly selected.

2.5. Two-dimensional electrophoresis, in gel digestion of proteins and MALDI-TOF mass spectrometry

Cells treated at various concentrations of APG for a day were collected by centrifugation for 5 min and lysed in a lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, and 0.5% pharmalyte) containing protease inhibitor (Sigma–Aldrich). Proteins in rehydration buffer containing urea and thiourea were loaded on the Immobiline DryStrip (18 cm, pH 3–10, GE healthcare, Piscataway, JN) and the strips were rehydrated for 12 h. After rehydration, isoelectric focusing (IEF) was performed using a Multiphor II IEF system (GE healthcare) for the first dimensional separation. The strips were placed in equilibration buffer for 15 min twice and placed on 12.5% SDS–polyacrylamide gels for the second dimensional electrophoresis. The Ettan DALTsix vertical electrophoresis system (GE healthcare) was operated at 16 and 24 mA per gel. The SDS–polyacrylamide gel was stained with coomassie brilliant blue G250 dye (Bio-Rad, Hercules, CA) for analysis of differences in protein expression patterns between control and APG-treated cells. Proteins were extracted from 2-DE gels and identified by MALDI-TOF/MS, as described in a previous study (Yoo et al., 2009).

2.6. Reverse transcriptase-polymerase chain reaction

Following 24 h treatment with APG, $1–10 \times 10^6$, cells were harvested and lysed for extraction of RNA using the easy-spin™ total RNA extraction kit (Intron, Seoul, Korea). RNA concentration was determined using a Qubit™ Flourocytometer (Invitrogen, Carlsbad, CA). For synthesis of cDNA, the oligo (dT) Maxime RT premix kit (Intron) was used; cDNA was then amplified using the i-Star Taq Maxime PCR premix kit (Intron). An amplification step was repeated for 40 cycles. The primer sequences using in PCR are shown in Table 1.

2.7. Western blot analysis

To confirm the difference in protein expression between control and APG-treated cells, 5×10^6 cells were harvested and lysed in a lysis buffer (Cell signaling, Danvers, MA). The concentration of extracted proteins was determined by Qubit™ Flourocytometer (Invitrogen, Carlsbad, CA) by loading equal amounts of protein on each well of the NuPAGE Bis–Tris Gel (Invitrogen, Carlsbad, CA). Protein samples were separated using SDS–PAGE at 200 V for 30 min and transferred electrophoretically to a nitrocelluouse membrane at 200 mV for 2 h. Transferred membranes were blocked and the membranes were incubated with primary antibody (anti-vimentin monoclonal antibody from cell signaling, anti-caspases 3, 8, and 9 polyclonal antibody from Abcam, anti-collagen type I monoclonal antibody from Santa Cruz) at RT for 1–2 h. Membranes were washed again and detected using the western blot detection system (GE healthcare).

2.8. Cell migration assay

Huh-7 cells were cultured in 60 mm culture dishes with DEME until the cells were grown to 85% confluency, and wounded across the dishes using a scraper. To remove wounded cells and cell debris, cells were washed with DMEM twice and then treated with APG. After 48 h, photographs of the wound were taken using a Nikon eclipse TE2000 inverted microscope at a final magnification of 100×. The number of migrated cells was counted for analysis of cell migration.

Table 1
Sequences of RT-PCR primers used for validation of 2-DE result in mRNA levels.

Primer	Nucleotide sequence (5′–3′)	Amplicon size
FTH1 sense	TCCTACGTTTACCTGTCCATGT	268
FTH1 antisense	GTTTGTGCAGTTCCAGTAGTGA	
PRDX2 sense	TCCTCTTTTTCTACCCTCTGGAC	211
PRDX2 antisense	CTGGTCACGTCAGCAAGCA	
TRIM5 sense	AGACAAAGGAGAGAGTAGCTGC	71
TRIM5 antisense	GCCGATTAGGCCGTATGTTCT	
KRT17 sense	GCCGCATCCTCAACGAGAT	105
KRT17 antisense	CGCGGTTCAGTTCCTCTGTC	
CDA sense	CTGAACGGACCGCTATCCAG	169
CDA antisense	CCATCCGGCTTGGTCATGT	
C19orf10 sense	GCCACCGCCGACAAATATAC	64
C19orf10 antisense	ATTGCTCATTGGTCCCTCCTT	
PRDX1 sense	CGGAGATCATTGCTTTCAGTGA	113
PRDX1 antisense	AGGTGTATTGACCCATGCTAGAT	
RPLP2 sense	TTGGACAGCGTGGGTATCG	125
RPLP2 antisense	CCAGCAGGTACACTGGCAA	
DUT sense	CGCCATTTCACCCAGTAAGC	226
DUT antisense	AGCCACTCTTCCATAACACCC	
VIM sense	CGCCAGATGCGTGAAATGG	278
VIM antisense	ACCAGAGGGAGTGAATCCAGA	
β-Actin sense	AACACCCCAGCCATGTACG	254
β-Actin antisense	ATGTCACGCACGATTTCCC	

2.9. Determination of secreted VEGF and MMP-8 protein levels

To determine the levels of released VEGF and MMP-8 into media, enzyme-linked immunosorbent assay (ELISA) was performed. All reagents used in the ELISA assay were purchased from R&D systems (Minneapolis, MN). After 24 h APG treatment, media were collected and spun-down for removal of cells and unnecessary cell debris. Harvested-media were then transferred to antibody-precoated microplates. HRP-conjugated primary antibody was then added to each well for conjugation with antigen. The concentrations of each sample were measured using a spectrophotometer.

2.10. Statistical analysis

Data are representative of three or more independent experiments. Student's t test was used for statistical analysis and $P < 0.05$ was considered significant.

3. Result

3.1. Decreased cell viability in APG-treated huh-7 cells

To investigate the effect of APG on human hepatocellular carcinoma cells, huh-7, were treated with 80, 160, and 240 μM of APG for 24 h and their viability was determined using an RT-CES assay (Fig. 1A). The obtained results showed that APG significantly decreased the viability of huh-7 cells in a dose-dependent manner. There was a significant decrease in cell viability, from 110.6 ± 5.7% in control cells to 58.6 ± 3.8%, 30.6 ± 3.0%, and 20.0 ± 1.6%, respectively, in 80, 160, and 240 μM of APG-treated cells for 24 h. Half-maximal inhibitory concentration (IC50) of a 24 h APG treatment on huh-7 cells was approximately 80 μM.

3.2. APG-induced apoptosis in huh-7 cells

DNA cleavage is a common feature of apoptosis, which was confirmed using a fluorescent tunel assay (Fig. 1B).

A damaged DNA strand has a number of 3′-OH ends and nicks, depending on the degree of DNA cleavage. Terminal deoxynucleotidyl transferase (TdT) enzymes used in the tunel assay catalyze incorporation of fluorescent stains with the nicks of DNA, 3′-OH ends. Therefore, broken DNA was stained brighter than undamaged DNA. As shown in Fig. 1B, the percentages of green fluorescence stained cells and the degree of stain brightness were increased, implying an increase of DNA fragmentation in a dose-dependent manner. Similarly, we confirmed corresponding results in a DNA laddering assay (Fig. 1C). Doses higher than 80 μM of APG for 24 h induced significant DNA laddering in huh-7 cells, whereas the DNA ladder of 40 μM of APG-treated cells was barely shown.

Western blot analysis was performed for detection of activated caspase members, a family of cysteine proteases, which are apoptosis-inducing factors (Sun et al., 1999). The expression levels of caspase 3, 8, and 9 proteins were gradually decreased in a dose-dependent manner, while the expression levels of cleaved-caspases 3, 8, and 9 proteins, activated forms, were dramatically increased (Fig. 1D). Based on the results, APG-induced apoptosis was activated via caspase cascade.

3.3. Two-dimensional electrophoresis and identification of differentially expressed proteins using MALDI-TOF/MS

Protein samples were visualized by 2-DE analysis to compare expression levels of proteins between control and APG-treated cells and to detect proteins related to APG-induced apoptosis (Fig. 2). More than 500 protein spots were differentially expressed. Among these proteins, we selected 10 protein spots whose degree of expression was evaluated using ImageMaster software. Ten spots were identified by MALDI-TOF/MS and MS-FIT database searches, as summarized in Table 2. Five proteins were up-regulated and five proteins were down-regulated in APC-treated huh-7 cells (Fig. 2B).

3.4. Validation of 2-DE results using RT-PCR and western blot analysis

The identified 10 proteins were confirmed at the post-transcriptional mRNA level using RT-PCR (Fig. 3A). Seven mRNA levels were changed in accordance with the 2-DE results; peroxiredoxin-2 (PRDX2), cytidine deaminase (CDA), UPF0556 protein C19orf10 (C19orf10), deoxyuridine 5′-triphosphate nucleotidohydrolase, mitochondrial (DUT), and vimentin (VIM) were down-expressed, while keratin type I cytoskeletal 17 (KRT17) and peroxiredoxin-1 (PRDX1) were up-expressed. The results of ferritin heavy chain (FTH1), tripartite motif-containing protein 5 (TRIM5), and 60S acidic ribosomal protein P2 (RPLP2) in RT-PCR, on the other hand, were away from 2-DE results. The intensity of protein spot for FTH1, TRIM5, and RPLP2 changed significantly, but FTH1 was barely expressed in mRNA levels. Also, the RT-PCR result of TRIM5 and RPLP2 has almost no change lower than 80 μM; TRIM5 was slightly increased, and RPLP2 was remarkably decreased in 120 μM treatment.

RT-PCR is a preliminary method for identification of the expression levels of certain proteins in the post-transcriptional step as one of the processes of protein expression; therefore, the results of RT-PCR do not always correspond to those of proteomic analysis, 2-DE. For this reason, we focused on one protein, vimentin, and detected vimentin and related proteins using western blot. As shown in Fig. 3B, protein expression of vimentin was dramatically down-regulated. Because type I collagen collaborates with vimentin in cell migration, detection of type I collagen was performed and the results showed a decrease of its expression (Fig. 3C).

3.5. Anti-angiogenesis and anti-migration activity induced by APG in huh-7 cells

Many studies have suggested that VEGF and MMP-8 play an important role in angiogenesis (Jiang et al., 2000). Detection of released VEGF and MMP-8 in cultured media was performed using ELISA in order to determine whether anti-angiogenesis participates in mechanisms of APG-induced apoptosis (Fig. 3D and E). Released levels of VEGF and MMP-8 were decreased from 4836.4 ± 368.3 and 983.7 ± 62.2 upto 347.2 ± 47.5 and 517.9 ± 32.0 (pm/ml), respectively.

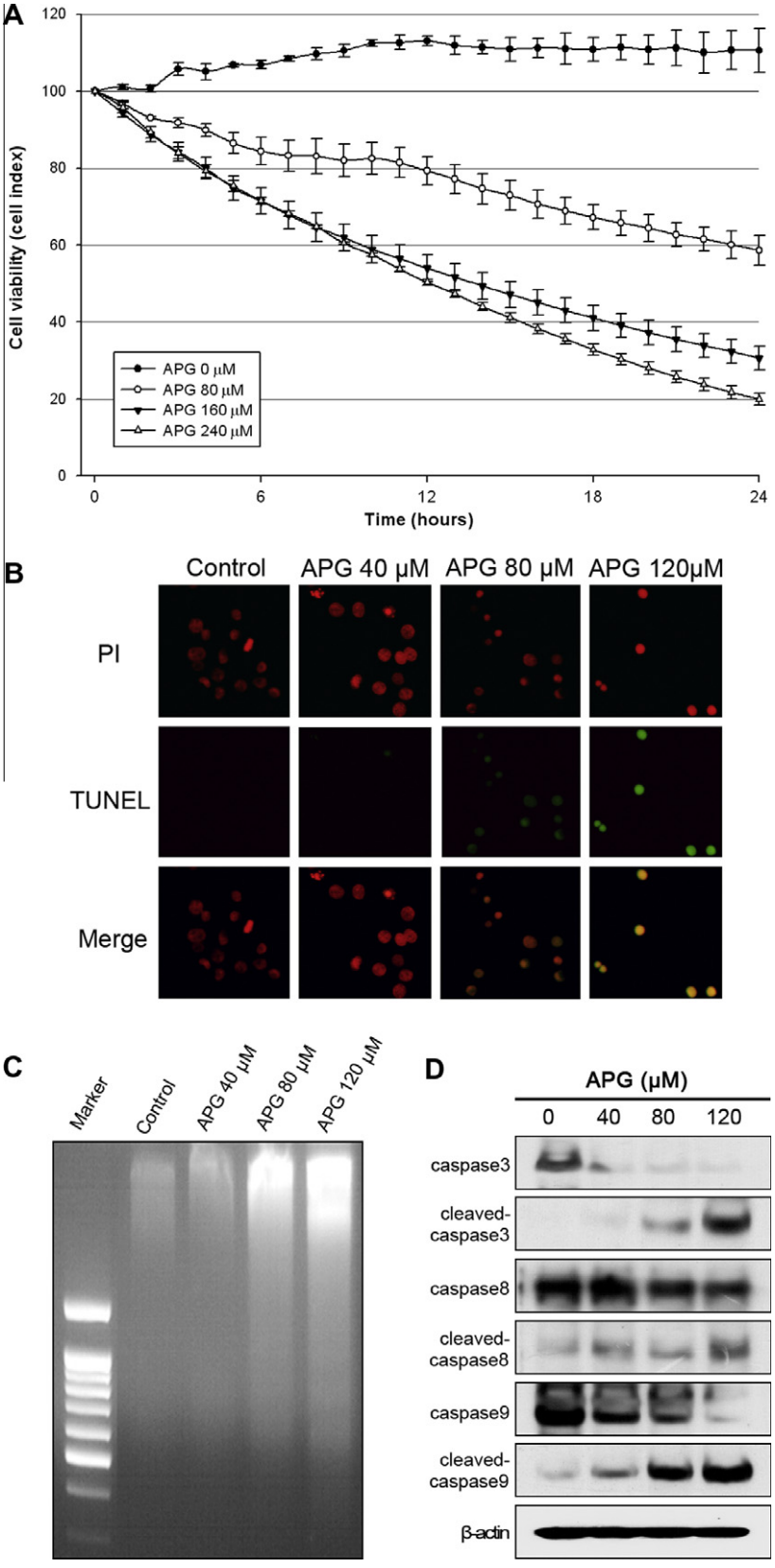

Fig. 1. Anti-proliferate effect of APG on huh-7 cells. (A) Huh-7 cells were treated at various concentrations of APG (0, 80, 160, and 240 μM) for 24 h. Viability of huh-7 cells was measured using RT-CES analysis. (B) A fluorescent tunel assay was performed for evaluation of apoptosis under 24 h treatment with APG (0, 40, 80, and 120 μM). Control and fragmented cells were assessed by PI (red) and tunel (green) staining, respectively. Images of the cells were observed by a fluorescence microscope. (C) APG-induced apoptosis was confirmed using a DNA fragmentation assay after cells were treated with various concentration of APG (0, 40, 80, and 120 μM) for 24 h. DNA was extracted and loaded on 1.5% agarose gel and visualized using EtBr staining. (D) Apoptotic proteins, caspase family members, were detected using western blot under 24 h treatment with APG (0, 40, 80, and 120 μM). Data are representative of at least three independent experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

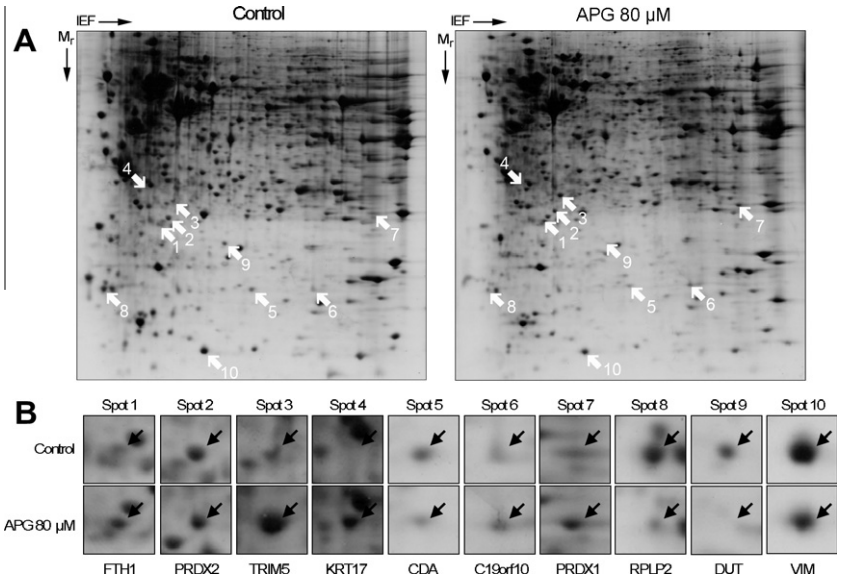

Fig. 2. Proteomic analysis of APG-treated huh-7 cells. The cells were treated with APG for 24 h in order to comprise protein expression between 80 μM of APG-treated cells and control. (A) Expression of 10 proteins was differentiated in 2-DE gel images of total proteins. (B) Amplified images of 10 protein spots showing a higher than 1.5-fold change in the protein expression levels.

Table 2
Identification of differentially expressed proteins between APG-treated cells and control.

No.[a]	Accession No.[b]	Protein name	Gene name	Sequence cov. (%)[c]	Theoretical MW (DA)/pI	Diff.[d]	Process	Function	Mowse score	Number of matched peptide
1	P02794	Ferritin heavy chain	FTH1	42.0	21,383/5.30	+	Cell proliferation	Oxidoreductase activity	113	7
2	P32119	Peroxiredoxin-2	PRDX2	48.0	22,014/6.84	–	Anti-apoptosis	Thioredoxin peroxidase activity	151	10
3	Q9C035	Tripartite motif-containing protein 5	TRIM5	30.0	31,861/5.00	+	Protein trimerization	Protein binding	69	7
4	Q04695	Keratin, type I cytoskeletal 17	KRT17	23.0	48,361/4.97	+	Epidermis development	Structural molecular activity	132	11
5	P32320	Cytidine deaminase	CDA	62.0	16,687/6.55	–	Cell growth regulation	Cytidine deaminase activity	109	7
6	Q969H8	UPF0556 protein C19orf10	C19orf10	56.0	11,383/7.04	+	–	–	118	7
7	Q06830	Peroxiredoxin-1	PRDX1	52.0	22,324/8.27	+	Cell proliferation	Antioxidant activity	129	8
8	P05387	60S acidic ribosomal protein P2	RPLP2	80.0	11,658/4.42	–	Translation	Structural constituent of ribosome	107	8
9	P33316	Deoxyuridine 5′-triphosphate nucleotidohydrolase, mitochondrial	DUT	48.0	26,975/9.65	–	DNA replication	dUTP diphosphatase activity	111	10
10	P08670	Vimentin	VIM	27.0	20,138/4.84	–	Apoptosis	Protein binding	78	6

[a] Spot number on 2-DE gel (Fig. 2).
[b] Swiss-Prot accession numbers.
[c] Sequence coverage.
[d] Difference.

For proliferation and metastasis, cancer cells need to migrate to other tissues. Numbers of migrated cells were counted, and the results showed that APG-treated cells appeared to be dramatically suppressed from 1679.6 ± 50.6 to 73.6 ± 30.0 in the ability of cell migration (Fig. 3F).

4. Discussion

Although HCC is the sixth most common malignant cancer and has been recently reported as having the fifth rate of metastasis, present HCC treatments still have many side effects and are restricted in several cases of HCC patients (Bosch et al., 2004; Parkin et al., 2005). Because natural substances have a low possibility of side effects when compared with synthetic materials, researchers have focused on APG, a natural substance, as a potential chemotherapeutic agent; APG has been reported to have an anticancer effect in recent studies.

In our studies, we first investigated the effects of APG in HCC, huh-7 cells. The observed results showed that APG induced a decrease of viability in huh-7 cells and suggest that the effect of anti-proliferation induced by APG on HCC was related to a programmed cell death, apoptosis. According to the other results in our study, huh-7 cells went through apoptosis under treatment with APG and the mechanism of APG-induced apoptosis arose through a caspase cascade showing nuclear DNA fragmentation, which is a biochemical hallmark of apoptosis (Raffray and Cohen, 1997). Down-regulation of pro-form or up-regulation of the

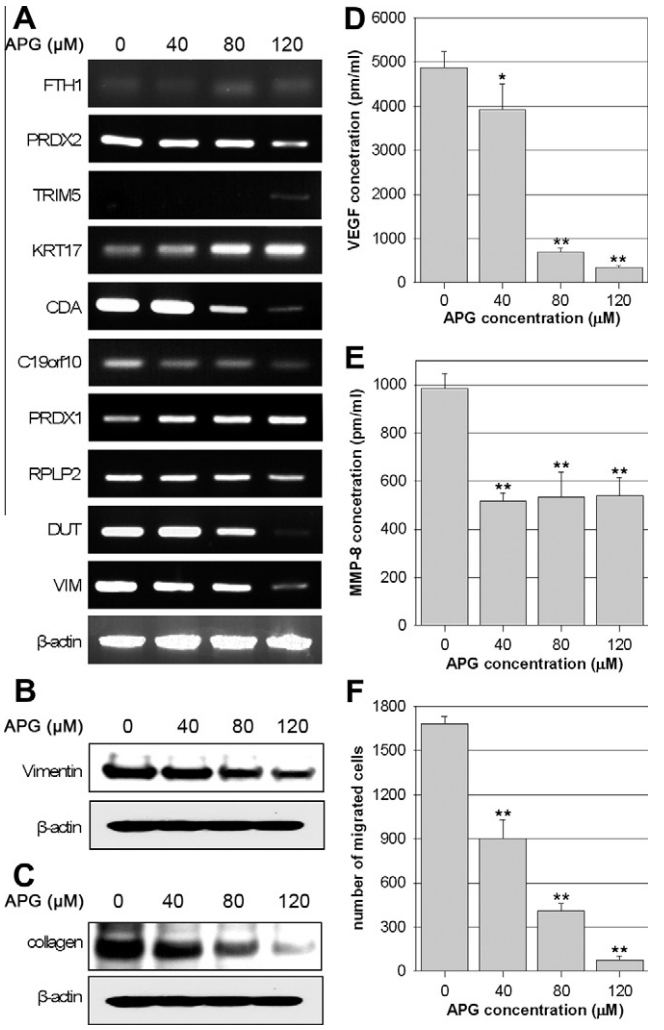

Fig. 3. Validation of the 2-DE result and detection of angiogenic proteins related to APG-induced apoptosis in huh-7 cells. (A) Synthesized cDNA from mRNA of 24 h APG-treated cells was amplified using RT-PCR to confirm the 2-DE result. (B) Expression of vimentin and (C) type I collagen were analyzed using western blot after cells were treated with APG (0, 40, 80, and 120 μM) for 24 h. (D) Released amounts of VEGF and (E) released amounts of MMP-8 were detected in control and APG-treated cells using ELISA (*$P < 0.05$, **$P < 0.001$). (F) Cells were treated with various concentration of APG for 48 h, and then the number of migrated cells was counted under an inverted microscope (**$P < 0.001$).

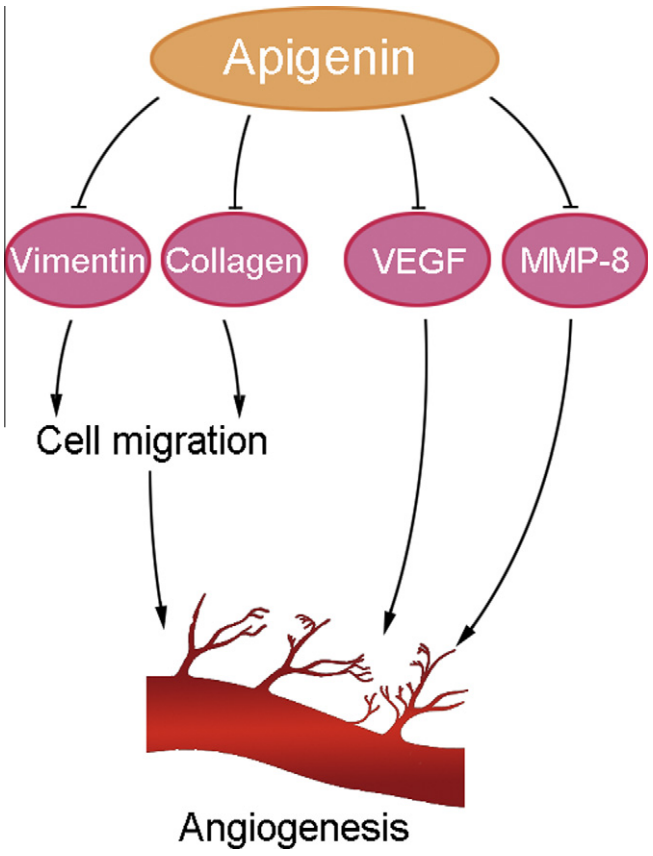

Fig. 4. A mechanism of APG-induced apoptosis in huh-7. Anti-angiogenesis and anti-migration were generated in APG-treated cells undergoing apoptosis.

activated form in caspase family members is considered a typical phenomenon in the apoptotic pathway due to their roles. Caspase-8 and -9 play a prerequisite role as an initiator which sequentially activates downstream effector caspases, such as caspases-3, -6, and -7. These types of effectors ultimately induced cleavage of critical cellular proteins resulting in apoptosis (Sun et al., 1999).

In a proteomic analysis for determination of biochemical mechanisms related to APG-induced apoptosis in huh-7, we identified differentially expressed proteins and selected 10 candidates. Among these candidates, we focused on one protein, vimentin, which has recently been reported to be related to anti-angiogenesis and anti-metastasis in several cancer cells. Of particular interest, vimentin maintains the structure of cells as a structural protein and also plays a critical role in several physiological mechanisms, including cell migration, adhesion, and signaling (McInroy and Maatta, 2007). For example, it was down-regulated in the mechanism of anti-angiogenesis and anti-metastasis in cancer cells and its expression was significantly increased in invasive cancer cells (Ebert et al., 2000). In addition, it was reported that the cells of mice were knocked down by vimentin, and, in fact, failed to maintain their adhesion and that caspase-induced degradation of vimentin implied apoptosis of cells (Muller et al., 2001). On the basis of these facts, it was not surprising that we focused on vimentin as a part of key regulators in APG-induced apoptosis. All results drawn from the total proteomic analysis showed that expression of vimentin in APG-treated cells was decreased in comparison with that of control. These results might indicate that vimentin is important in cell viability and might be a possible target in anti-angiogenesis activities.

To better understand a correlation between a decrease of vimentin expression and anti-angiogenesis, we performed a cell migration assay. Cell migration is closely associated with angiogenesis, because it is a critical processes for cell proliferation, formation of new tissue, and wound healing (Xiao and Singh, 2007). Identification of the capacity of cell migration is a representative method for verification of angiogenic activity. The results revealed that suppression of cell migration was significantly in accordance with down-regulation of vimentin and type I collagen, a cytoskeletal protein which is co-worked with vimentin in cell activities, such as cell migration, under 24 h AGP-treatment.

Angiogenesis, a sprouting of new capillaries from preformed blood vessels, is an essential process for cell proliferation, especially in cancer cells. Cancer cells show rapid and unlimited propagation because they have already been mutated in regulation of cell growth; to do that, they need to take in more nutrition through the blood vessels. In addition, tumors are known to be angiogenesis-dependent and many studies have reported that VEGF typified by an inducer in angiogenesis regulates cell proliferation (Jiang et al., 2000). Overall, these facts suggest the possibility that inhibition of angiogenesis becomes a main cause of anti-proliferation activities and apoptosis in cancer cells.

Because cell migration is an essential step in angiogenesis, decreased migration activities of cancer cells appear to have a hypo-function in development of new capillaries. Hence, the results of a decrease of metastatic activity under AGP treatment indicated that APG induced anti-angiogenesis in huh-7 cells. In order to confirm the anti-angiogenesis effect induced by APG, we investigated the released amounts of VEGF and MMP-8, which are closely relevant to angiogenesis in cultured media of APG-treated cells. According to our observation, the amounts of both proteins were decreased (Fig. 4). The results implied that APG has an anti-angiogenesis effect on huh-7 cells and this effect might potentially be a crucial cause of APG-induced apoptosis.

5. Conclusion

Based on the overall results, APG induced apoptosis in HCC and inhibited cell migration through a decrease of vimentin and type I collagen expression. On the other hand, it showed an anti-angiogenesis effect that was dependent on decreased activity of migration and down-regulation of VEGF and MMP-8 release. Therefore, vimentin might potentially be a key factor in the mechanism of APG-induced apoptosis in HCC, huh-7 cells, via anti-angiogenesis and anti-migration activities.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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