Abstract. Kaempferol belongs to the flavonoid family and has been used in traditional folk medicine. Here, we investigated the antitumor effects of kaempferol on cell cycle arrest and autophagic cell death in SK-HEP-1 human hepatic cancer cells. Kaempferol decreased cell viability as determined by MTT assays and induced a G2/M phase cell cycle arrest in a concentration-dependent manner. Kaempferol did not induce DNA fragmentation, apoptotic bodies or caspase-3 activity in SK-HEP-1 cells as determined by DNA gel electrophoresis, DAPI staining and caspase-3 activity assays, respectively. In contrast, kaempferol is involved in the autophagic process. Double-membrane vacuoles, lysosomal compartments, acidic vesicular organelles and cleavage of microtubule-associated protein 1 light chain 3 (LC3) were observed by transmission electron microscopy, LysoTracker red staining, GFP-fluorescent LC3 assays and acridine orange staining, respectively. In SK-HEP-1 cells, kaempferol increased the protein levels of p-AMPK, LC3-II, Atg 5, Atg 7, Atg 12 and beclin 1 as well as inhibited the protein levels of CDK1, cyclin B, p-AKT and p-mTOR. Taken together, CDK1/cyclin B expression and the AMPK and AKT signaling pathways contributed to kaempferol-induced G2/M cell cycle arrest and autophagic cell death in SK-HEP-1 human hepatic cancer cells. These results suggest that kaempferol may be useful for long-term cancer prevention.

Introduction

Kaempferol (3,5,7,4-tetrahydroxyflavone) is a phytoestrogen found in a variety of vegetables and fruits, such as tomatoes, hops, red grapes and strawberries. Kaempferol has been used in traditional medicine and is believed to have various biological functions, including inhibition of inflammation (1). Kaempferol is a member of the flavonoid compounds, which are known for defensive properties against micro-organisms or pests and are a part of the oxidative stress protection pathway (2). It has been shown that kaempferol suppresses oxidative stress in animal cells by inhibiting the induction of nitric oxide synthase (iNOS) mRNA expression (3,4) and prostaglandin E2 production. In addition, kaempferol has been reported in treatment of various types of tumors, including breast cancer (5,6), lung cancer (7), colon cancer (8,9) and leukemia (10). Kaempferol has been shown to have a protective effect in CCl4-induced liver damage. These studies suggest that kaempferol possesses beneficial activities as an inherent antioxidant and may be an attractive anticancer agent, but the underlying mechanisms remain to be elucidated.

Autophagy is a conserved catabolic process used by all eukaryotic cells to degrade long-lived proteins and damaged organelles. The disintegration of vesicles is a hallmark morphological characteristic of autophagy (11). Double-membrane vesicles transport contents, such as cytoplasmic proteins, to lysosomes and then form autophagosomes (12), where degradative enzymes break the engulfed materials; the resulting
macromolecules are recycled (12-14). In addition, autophagy allows cells to adapt and survive under adverse conditions, such as nutrient deficiency. Moreover, autophagy has been implicated in development and pathophysiology (15). Potential links between autophagy and a number of human diseases have been proposed. For instance, cancer is associated with increased autophagic activity (16). Autophagy can protect from cell death in times of stress (12). Therefore, the impact of autophagy induction on the efficacy of chemotherapeutic drugs can be highly variable and depends on both the cell type and treatment. However, the precise role that autophagy plays in cancer development is very controversial (17,18).

Although kaempferol has been reported to have antifungal, antitumor, anti-inflammatory, anti-oxidative, anti-carcinogenic and anti-mutagenic abilities (2,19-21), the underlying mechanisms of kaempferol-mediated autophagy remain to be elucidated. In this study, we aimed to elucidate the role of kaempferol-mediated autophagy. Furthermore, the study of kaempferol-mediated autophagy may shed light on long-term cancer prevention.

Materials and methods

Chemicals and reagents. Acridine orange (AO), anti-actin primary antibody, kaempferol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Triton X-100 were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, LysoTracker Red, penicillin/streptomycin, propidium iodide (PI), Premo™ Autophagy Sensor LC3B-GFP Kit and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). The caspase-3 activity assay kit was purchased from R&D Systems Incorporated (Minneapolis, MN, USA). The anti-Atg 5, Atg 7, Atg 12, AKT, p-AKT (Ser473), AMPKα, p-AMPKα (Thr172), beclin, LC3, mTOR and p-mTOR primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-CDK1, cyclin B and peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) detection kit was obtained from Pierce Chemical (Rockford, IL, USA).

Cell culture. The SK-HEP-1 human hepatic cancer cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were grown in Eagle's Minimum Essential medium fortified with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin and incubated at 37°C under a humidified 5% CO₂ atmosphere (11).

Cell viability and morphology. Cell viability was assessed by MTT assays. Briefly, SK-HEP-1 cells were cultured in a 96-well plate at a density of 2.5x10³ cells/well and incubated with 0, 25, 50, 75 or 100 µM kaempferol for 24 h. At the end of the kaempferol treatment, culture medium containing 0.5 mg/ml MTT was added to each well. The cells were then incubated at 37°C for 4 h, and the blue formazan crystal products were dissolved with isopropanol, 0.04 N HCl. The absorbance of each well was measured at 570 nm with an ELISA reader using a reference wavelength of 620 nm. The cell viability after each treatment was defined as the percentage of the control. Kaempferol-treated cells were morphologically examined for autophagic vacuoles under a phase-contrast microscope (11).

Cell cycle analysis. The cell cycle distribution was analyzed by flow cytometric analysis using DNA staining with PI. SK-HEP-1 cells were cultured in a 24-well plate at a density of 2.5x10³ cells/well and incubated with 0, 25, 50, 75 or 100 µM kaempferol for 24 h. After treatment, the cells were collected, washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at -20°C. The cells were collected by centrifugation and re-suspended in PBS containing 50 mg/ml PI at room temperature. Stained cells were analyzed by flow cytometry on a FACSCalibur™ flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) as previously described. The percentage of cells in the different phases of the cell cycle was determined using the CellQuest software program (22).

DNA fragmentation assay. SK-HEP-1 cells were seeded into 75-T flasks (1x10⁶ cells/flask). Kaempferol (0, 50, 75 or 100 µM) was added, and the cells were incubated for 24 h. At the end of the incubation, the cells were harvested and washed with ice-cold PBS. The cells were re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.2% Triton X-100) containing 0.1 mg/ml proteinase K and 50 µg/ml RNase A, and the samples were incubated at 37°C overnight. The DNA was subjected to electrophoresis using a 1.5% agarose gel (Sigma-Aldrich). The agarose gel was stained with 1 µg/ml ethidium bromide (Invitrogen Life Technologies) in 0.5X TBE buffer to visualize the DNA fragmentation, and the gel was examined using photographs taken under UV light as previously described (23,24).

DAPI stain. DNA condensation was detected using the DAPI staining method as previously described. SK-HEP-1 cells were seeded in 24-well plates at 2.5x10³ per well and followed by treatment with 0 or 100 nM kaempferol. The cells were fixed gently by the addition of 70% ethanol, stained with DAPI and then photographed using a fluorescence microscope (25,26).

Caspase-3 activity assays. The caspase-3 activity was determined using the caspase-3 colorimetric assay kit as described by the manufacturer (Caspase Colorimetric Kit; R&D Systems Inc.). Briefly, after 24 h of incubation with 0, 25, 50, 75 or 100 µM kaempferol, the SK-HEP-1 cells were harvested. The collected cells were then lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT. The cell lysates were used to determine caspase-3 activity by adding the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). Cleavage of the peptide by the caspase releases pNA, which has a high absorbance at 405 nm. The concentration of pNA released from the substrate was calculated using a calibration curve prepared from defined pNA solutions. The experiments were performed in triplicate (27).

Transmission electron microscopy (TEM). SK-HEP-1 cells were seeded in 6-well plates at 1x10⁶ per well and treated with kaempferol (100 µM) for 24 h. The cells were washed with PBS and then fixed in 2% paraformaldehyde and 2.5% glutaralde-
LysoTracker red staining. SK-HEP-1 cells were seeded in 6-well plates at 1x10^6 per well and treated with kaempferol (100 µM) for 24 h. After treatment, the cells were stained with LysoTracker Red (50 nM) at 37°C for 15 min. After washing three times with PBS, the cells were immediately visualized by fluorescence microscopy (Nikon, Melville, NY, USA) for the detection of acidic lysosome compartments (11).

LC3B protein aggregation. Autophagy was detected by measuring the aggregation of LC3B protein coupled to green fluorescence protein (GFP) using Premo Autophagy Sensor kits (Invitrogen Life Technologies). Briefly, SK-HEP-1 cells were seeded in 12-well plates at 5x10^5 per well. Then, the cells were transduced with non-replicating baculoviral vectors expressing LC3B-GFP (component A) or LC3BΔ(G120A)-GFP (component B), which encodes a point mutation in the LC3B gene which prevents cleavage. Twenty-four hours after transduction, the SK-HEP-1 cells were treated with kaempferol (100 µM) for 24 h. The appearance of LC3B-GFP aggregates was observed and photographed using a fluorescence microscope (11).

Acridine orange (AO) stain. SK-HEP-1 cells were seeded in 6-well plates at 1x10^6 per well and treated with kaempferol (100 µM) for 24 h. After treatment, the cells were stained with acridine orange (AO) at 37°C for 15 min. After washing three times with PBS, the cells were immediately visualized by fluorescence microscopy (Nikon) for the detection of acidic vesicular organelles (AVO) (11).

Western blot analysis. SK-HEP-1 cells were seeded in 75-T flasks at 1x10^7 per flask and treated with 0, 50, 75 or 100 µM kaempferol for 6 or 24 h. Then, the cells were harvested and lysed; the total proteins were collected. Thirty micrograms of protein from each treatment were separated using 10 to 12% SDS-polyacrylamide gel electrophoresis and/or cyclin B at 4˚C overnight. The cell cycle distributions of kaempferol-treated SK-HEP-1 cells. The cell cycle distributions of kaempferol-treated SK-HEP-1 cells. The cell cycle distributions of SK-HEP-1 cells decreased in a dose-dependent manner. Morphological changes of SK-HEP-1 cells were visualized by optic microscopy after exposure to 75 µM kaempferol for 24 h. Arrows indicate the autophagic vacuoles.

Statistical analysis. All the statistical results are presented as the mean ± SEM for the indicated numbers of separate experiments. Statistical analyses of data were performed using a one-way ANOVA followed by Student’s t-test, and P<0.001 was considered significant (11).

Results

The effects of kaempferol on cell viability in SK-HEP-1 cells. In this study, we assessed the cytotoxic effects of kaempferol on SK-HEP-1 cells. Various doses of kaempferol (25, 50, 75 and 100 µM) were applied for 24 h and the cell viability was evaluated using MTT assays as described in the Materials and methods section. All results are expressed as the mean ± SEM of three independent experiments. P<0.05 and P<0.01 compared with control. (A) SK-HEP-1 cells were treated without or with kaempferol (25, 50, 75 or 100 µM) for 24 h. Cell viability was evaluated using MTT assays as shown in Fig. 1A. The cell viability of SK-HEP-1 cells decreased in a dose-dependent manner. Morphological changes of SK-HEP-1 cells were visualized by optic microscopy after exposure to 75 µM kaempferol for 24 h. Arrows indicate the autophagic vacuoles.

The cell cycle distributions of kaempferol-treated SK-HEP-1 cells. To characterize whether kaempferol caused SK-HEP-1 cells to arrest in a specific cell cycle phase, SK-HEP-1 cells were treated with different doses of kaempferol (25, 50, 75 or 100 µM) for 24 h. As shown in Fig. 2A and B, the sub-G1, G1, and G2 phases observed by PI staining and flow cytometry were significantly different between the control and kaemp-
ferol-treated groups. Kaempferol treatment resulted in G\(_2\)/M cell cycle arrest. As shown in Fig. 2C, the expression of CDK1 and cyclin B decreased in kaempferol-treated SK-HEP-1 cells. The data were consistent with the finding in Fig. 2A. These results indicated that kaempferol treatment resulted in G\(_2\)/M cell cycle arrest.

**Kaempferol had no effect on apoptosis in SK-HEP-1 cells.** Apoptosis is characterized by DNA fragmentation, chromatin condensation and caspase activation. We next examined whether kaempferol induced apoptotic cell death in SK-HEP-1 cells. SK-HEP-1 cells were treated with various concentrations of kaempferol (25, 50, 75 or 100 µM) for 24 h. The characteristic apoptotic laddering pattern of DNA fragments with multiple bands indicated the induction of apoptosis. As shown in Fig. 3A, not all of the samples showed the DNA fragment band. Next, SK-HEP-1 nuclei were stained by DAPI and observed by fluorescence microscope. There was no significant difference between the control and kaempferol-treated cells (Fig. 3B). Furthermore, caspase-3 activity remained unchanged in both the control and kaempferol-treated cells (Fig. 3C). These results indicated that kaempferol treatment did not induce a typical apoptotic response in SK-HEP-1 cells.

**Kaempferol induced autophagy in SK-HEP-1 cells.** Autophagic vacuoles appeared when SK-HEP-1 cells were treated with kaempferol (Fig. 4A). To determine whether kaempferol treatment induced autophagy, cells were stained with MDC to detect AVO formation. The AVO positive cells increased after kaempferol treatment in a concentration-dependent manner compared to the control (Fig. 4B). Based on these data, we concluded that kaempferol induced autophagy. Autophagy is a series of biochemical steps through which eukaryotic cells commit suicide by degrading their own cytoplasm and organelles. In this process, these components are engulfed and then digested in double membrane-bound
Figure 3. The effects of kaempferol on apoptosis in SK-HEP-1 cells. (A) Apoptosis was measured in kaempferol-treated cells using a DNA fragmentation analysis. DNA was isolated from the cells and analyzed using 1.5% agarose gel electrophoresis with 1X TBE buffer and ethidium bromide staining. Lane M, DNA ladder marker; lane 2, 24 h at 0 µM (negative control); lane 3, 24 h at 50 µM; lane 4, 24 h at 75 µM; lane 5, 24 h at 100 µM. (B) The nuclei of SK-HEP-1 cells were stained by DAPI and observed by fluorescence microscope. Control cells without kaempferol treatment are shown in the upper panel. Kaempferol-treated cells are shown in the lower panel. (C) SK-HEP-1 cells were treated with the indicated concentrations of kaempferol for 24 h. Extracts from untreated or kaempferol-treated cells were assayed for caspase activity using the Caspase-3 Colorimetric Assay kit. All results are expressed as the mean ± SEM of three independent experiments.

Figure 4. Autophagic vacuoles increase in SK-HEP-1 cells incubated with kaempferol for 24 h. (A) Representative images from SK-HEP-1 cells incubated with kaempferol (0, 50, 75 or 100 µM) for 24 h. Arrows indicate the autophagic vacuoles. (B) Quantification of autophagic vacuoles from SK-HEP-1 cells incubated with kaempferol (0, 50, 75 or 100 µM) for 24 h. The data represent the mean ± SEM from three separate experiments. Asterisks (*) indicate the values are significantly different from the control at ***P<0.001.
vacuoles called autophagosomes (15). To verify whether the kaempferol treatment induced autophagy, SK-HEP-1 cells were treated with kaempferol (0 or 100 µM) for 24 h and examined by fluorescence microscopy. Fig. 5A shows the formation of autolysosomes in SK-HEP-1 cells treated with 100 µM of kaempferol observed using electron microscopy. Moreover, an increase in autophagosomes was visualized and analyzed by LysoTracker Red staining, LC3B-GFP staining and acidic vesicular organelle formation in kaempferol-treated SK-HEP-1 cells compared to control SK-HEP-1 cells (Fig. 5B-D). Based on these data, we concluded that kaempferol could induce autophagic cell death in SK-HEP-1 cells.

The effects of kaempferol on the expression of autophagy-related proteins in SK-HEP-1 cells. We next examined the protein level of LC3, which is a protein marker of autophagy. Fig. 6A shows that the LC-3 level increased with kaempferol treatment in a dose-dependent manner. In addition, the levels of p-AKT and p-mTOR (a negative regulator of autophagy and apoptosis) decreased in a dose-dependent manner after kaempferol treatment for 24 h. In contrast, p-AMPK, which is a positive regulator of autophagy, increased significantly. These results suggested the kaempferol induced autophagy in SK-HEP-1 cells through the downregulation of p-AKT or the upregulation of p-AMPK.
Kaempferol is a flavonoid that can be found in many edible plants, including broccoli, cabbage, strawberries and tomatoes (2,21). Kaempferol is also found in traditional Chinese medicines, such as Acacia nilotica, Aloe vera and Tilia spp. It was reported that kaempferol has a broad spectrum of pharmacological activities, including anti-inflammatory, anti-oxidant and cardio-protective (2,19-21). Many studies have demonstrated that kaempferol has anticancer activity in various human cancer cell lines, including osteosarcoma, breast cancer, lung cancer, colorectal cancer, leukemia, oral cancer and ovarian cancer (2,5-10,29-31). In contrast, kaempferol has low toxicity against normal cells. Berger et al demonstrated that kaempferol reduced the cell viability and proliferation rates of HepG2 and Hep3B hepatic cancer cell lines. Kaempferol also induced cell apoptosis and inhibited HIF-1 activity in Huh7 and H4IIE hepatic cancer cell lines. In this study, we investigated the anti-hepatic effects of kaempferol on SK-HEP-1 cells in vitro. In the present study, we focused on cell cycle arrest and autophagy induction by kaempferol in SK-HEP-1 cells. Our results showed that kaempferol exerted a significant antiproliferative effect in SK-HEP-1 cells (Fig. 1A). Kaempferol induced G2/M phase cell cycle arrest in a concentration-dependent manner (Fig. 2A and B). However, DNA fragmentation was observed in SK-HEP-1 cells treated with certain doses of kaempferol (Fig. 3A and B). The caspase-3 activity did not change in kaempferol-treated SK-HEP-1 cells (Fig. 3C). Our results suggested that kaempferol did not induce apoptosis after 24 h in SK-HEP-1 cells, and another mechanism might be involved in kaempferol-induced growth inhibition in SK-HEP-1 cells. Our results demonstrated that kaempferol could induce growth inhibitory effects through G2/M cell cycle arrest (Fig. 2A and B) and autophagy in SK-HEP-1 cells. In addition to the G2/M phase arrest, kaempferol treatment decreased the cyclin B and CDK1 protein levels in a concentration-dependent manner (Fig. 2C). Many studies have demonstrated that kaempferol induced G2/M arrest in HL-60 leukemia cells, mouse T lymphocytes, MDA-MB-453 breast cancer cells and OCM-1 melanoma cells (5,6). Our results agree with previous studies showing that kaempferol induces G2/M cell cycle arrest.
There are three types of morphological processes that lead to cell death: apoptosis, necrosis and autophagy (32). Autophagy, or cellular self-digestion, plays an important role in normal physiology in animals. Many pharmacologic studies have suggested that autophagy has an anticancer role. Kaempferol-induced autophagy in SK-HEP-1 cells was supported by several pieces of evidence, including the formation of autophagic vesicles (Fig. 4B) double-membrane vacuoles (Fig. 5A), acidic lysosomal compartments (Fig. 5B), and acidic vesicular organelles (Fig. 5D); the cleavage of microtubule-associated protein 1 light chain 3 (LC3) (Figs. 5C and 6A); and elevated levels of autophagic proteins, Atg complex (Atg 5, Atg 7 and Atg 12) and beclin-1 (Fig. 6A). Our results suggested that kaempferol-induced cell death may involve autophagy in SK-HEP-1 cells. This is the first study to present detailed evidence for kaempferol-induced autophagy in SK-HEP-1 cells. Our findings are in agreement with previous studies that demonstrated kaempferol-induced autophagy in HeLa cervical cancer cells (33).

Many protein kinases are involved in the cell survival response, and previous studies suggested that AMPK and AKT serine/threonine kinase [also called protein kinase B (PKB)] play key roles and are involved in the induction of cell autophagy (34,35). AKT activation was associated with anti-apoptotic responses, cell proliferation and cellular energy metabolism. Liu et al suggested that the AKT gene was overexpressed in hepatic cancer and AKT activation participates in the pathogenesis and progression of hepatic cancer (36). Therefore, regulation of the AKT pathway may be essential for developing therapeutic inhibitors for hepatic cancer. Park et al demonstrated that kaempferol was able to reduce LPS-induced inflammatory mediators via MAPK and AKT downregulation, suggesting that kaempferol has therapeutic potential for the treatment of neuro-inflammary diseases (34). Luo et al reported that kaempferol inhibited angiogenesis and VEGF expression through repression of AKT phosphorylation in human ovarian cancer cells (37). Nguyen et al also showed that kaempferol-induced apoptosis in A549 lung cancer cells is through AKT phosphorylation (7). In addition, Filomeni et al presented that kaempferol-induced autophagy in HeLa cells are mediated by the AMPK pathway (33). Our study demonstrated kaempferol-induced autophagy was accompanied with the upregulation of p-AMPKα (Thr172) and the downregulation of phospho-AKT (Ser473) and phospho-mTOR protein levels (Fig. 6B). Our results suggested that the AMPK and AKT/mTOR pathways are associated with the induction of autophagy in kaempferol-treated SK-HEP-1 cells. A schematic of the kaempferol-induced G/M arrest and autophagy pathways in SK-HEP-1 human hepatic cancer cells is presented in Fig. 7. Our findings imply that kaempferol may be used as a novel anticancer drug candidate for the treatment of human hepatic cancer.

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