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Fisetin, a dietary flavonoid, induces apoptosis of cancer cells by inhibiting HSF1 activity through blocking its binding to the hsp70 promoter

Joo Ae Kim¹,², Somyoung Lee¹,², Da-Eun Kim¹,², Moonil Kim²,³, Byoung-Mog Kwon¹,² and Dong Cho Han¹,²,*

¹Genomics Structure Research Center, Korea Research Institute of Bioscience and Biotechnology and ²Department of BioMolecular Science, University of Science and Technology in Korea and ³Bio-Nano Research Center, Korea Research Institute of Bioscience and Biotechnology, 111 Gwahangno, Yuseong-gu, Daejeon 305–806, Korea

*To whom correspondence should be addressed. Tel: +82 42 860 4568; Fax: +82 42 861 2675; E-mail: dchan@kribb.re.kr
Correspondence may also be addressed to Byoung-Mog Kwon. Tel: +82-42-860-4557; Fax: +82 42 861 2675; E-mail: kwonbm@kribb.re.kr

Abstract

Heat shock factor 1 (HSF1) is a transcription factor for heat shock proteins (HSPs) expression that enhances the survival of cancer cells exposed to various stresses. HSF1 knockout suppresses carcinogen-induced cancer induction in mice. Therefore, HSF1 is a promising therapeutic and chemopreventive target. We performed cell-based screening with a natural compound collection and identified fisetin, a dietary flavonoid, as a HSF1 inhibitor. Fisetin abolished heat shock-induced luciferase activity with an IC₅₀ of 14 μM in HCT-116 cancer cells. The treatment of HCT-116 with fisetin inhibited proliferation with a GI₅₀ of 23 μM. When the cells were exposed to heat shock in the presence of fisetin, the induction of HSF1 target proteins, such as HSP70, HSP27 and BAG3 (Bcl-2-associated athanogene domain 3), were inhibited. HSP70/BAG3 complexes protect cancer cells from apoptosis by stabilizing anti-apoptotic Bcl-2 family proteins. The downregulation of HSP70/BAG3 by fisetin significantly reduced the amounts of Bcl-2, Bcl-xL and Mcl-1 proteins, subsequently inducing apoptotic cell death. Chromatin immunoprecipitation assays showed that fisetin inhibited HSF1 activity by blocking the binding of HSF1 to the hsp70 promoter. Intraperitoneal treatment of nude mice with fisetin at 30 mg/kg resulted in a 35.7% (P < 0.001) inhibition of tumor growth.

Introduction

Cancer cells have multiple oncogenes and a high degree of signal redundancy, which causes a single anticancer drug to have relatively weak therapeutic effects. Therefore, combination therapy or multi-targeted therapy is a way to solve these obstacles. Most natural compounds have multiple target molecules and thus are useful for treating cancers. A nutraceutical is any substance considered to be a food or part of a food that provides medical benefits (1,2). Intake of dietary foods, such as fruits or spices, has been reported to reduce the risk of cancer genesis.

Fisetin (3,7,3′,4′-tetrahydroxyflavone) is a flavonoid found in many edible fruits and vegetables, such as apples, grapes, kiwis, persimmons, strawberries, cucumbers and onions that has multiple properties, such as antioxidant (3) and anti-inflammatory (4) activity. The highest levels of fisetin (160 μg/g wet food) are found in strawberries (5). Fisetin induced apoptosis in LNCaP human prostate cancer cells (6), and mice receiving fisetin at 45 mg/kg inhibited tumor growth by 74.8% through androgen receptor inhibition (7). Similarly, fisetin also inhibited melanoma cell growth by disrupting Wnt/β-catenin signaling, and mice receiving fisetin at 45 mg/kg suppressed tumor growth by 66.6% (8). Furthermore, fisetin has been reported as an inhibitor of Aurora B kinase (9), NF-κB (10), mammalian target of rapamycin...
Bcl-2, Bcl-x, from apoptosis by stabilizing the Bcl-2 family proteins, such as HSF1 is a highly potent target for cancer therapy.

Fisetin decreased the levels of not only HSP70/BAG-3 but also p-HSF1, PARP, HSP27 had an increased tumorigenicity when inoculated into nude mice, HSF1 knockout suppresses chemically induced skin cancer, and HSF1 activity occurred by blocking HSF1 binding to target gene promoters. This study is the first report that the anticancer effect of fisetin was mediated by blocking HSF1 binding to target gene promoters, suggesting that HSF1 is a highly potent target for cancer therapy.

In this study, we identified fisetin as an HSF1 inhibitor. Fisetin decreased the levels of not only HSP70/BAG-3 but also p-HSF1, PARP, HSP27 had an increased tumorigenicity when inoculated into nude mice, HSF1 knockout suppresses chemically induced skin cancer, and HSF1 activity occurred by blocking HSF1 binding to target gene promoters. This study is the first report that the anticancer activity of fisetin involves HSF1 inhibition.

Materials and methods

Reagents

The natural chemical library has 716 compounds, some of them isolated from natural resources in our lab. Chemicals used in this study, including fisetin, quercetin, naringenin, kaempferol, baicalein, dimethyl sulfoxide (DMSO) and monochloroacetic acid from Sigma (St Louis, MO). Antibodies against HSF1 and HSP70 were purchased from Enzo Life Sciences (Farmington, NY). Antibodies against HSP27, PARP, Bcl-2, Caspase-3 (#9662) and Caspase-7 (#9492) were purchased from Cell Signaling Technology (Danvers, MA). Anti-BAG3 antibody was purchased from Abcam (Cambridge, UK). Antibodies against α-Tubulin, Histone H1 (FL-219), c-Myc (#9010), Bcl-xl (H-5) and Mcl-1 (S-19) were obtained from Santa Cruz Biotechnology (Dallas, TX). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phospho-Ser303 HSF1 were obtained from Ab Frontier (Seoul, Korea).

The heat shock response protects cells from a wide range of stresses, including heat shock, oxidative stress, heavy metals, fever or protein misfolding (reviewed in (14 and 15)). The heat shock response is mediated by heat shock transcription factor 1 (HSF1) that controls the transcription of heat shock proteins (HSPs), as such as HSP70, HSP47, HSP27 and BAG3 (Bcl-2-associated anathogene domain 3). Cells overexpressing HSP70 or HSP27 has an increased tumorigenicity when inoculated into mice (16,17). The expression of hsp70 is induced by several onco-genes, such as H-ras (18), c-myc (19), c-myb, SV40 large T antigen and adenovirus E1a (20).

HSF1 knockout suppresses chemically induced skin cancer (21) and hepatocellular carcinoma induced by diethylnitrosamine (22) in mice. In addition, HSF1 knockdown has a minimal effect on normal primary human cells but significantly impairs the proliferation of several malignant cell lines (21). Similarly, the downregulation of HSP70 or HSP27 was found to inhibit cell proliferation and induce apoptosis (23,24), suggesting that HSF1 is a highly potent target for cancer therapy.

BAG3 is a HSF1-inducible gene and protects cancer cells from apoptosis by stabilizing the Bcl-2 family proteins, such as Bcl-2, Bcl-x, and Mcl-1 (25). The BAG1 family was first reported as a Bcl-2 interacting protein (26). Six BAG family members were reported to regulate HSF70 function either positively or negatively. Whereas BAG-1 interacts with the proteasome and increases the degradation of HSF70 client proteins (27), BAG3 inhibits their proteasomal degradation (28).

In this study, we identified fisetin as an HSF1 inhibitor. Fisetin decreased the levels of not only HSP70/BAG-3 but also Bcl-2 family proteins; these changes induced apoptosis of cancer cells. Furthermore, we demonstrated that the inhibition of HSF1 activity occurred by blocking HSF1 binding to target gene promoters. This study is the first report that the anticancer activity of fisetin involves HSF1 inhibition.

Cell culture and cell line authentication

HCT-116 (colon carcinoma, ATCC® CCL-247™) in the year 2008, SW620 (colon carcinoma, ATCC® CCL-227™) in the year 2008, Mia PaCa-2 (pancreatic carcinoma ATCC® CRL-14259™) in the year 2008, MDA-MB-231 (breast adenocarcinoma ATCC® CR-1435™) in the year 2011, DU-145 (prostate carcinoma, ATCC® HTB-81™) in the year 2011 and LNCap (prostate carcinoma, ATCC® CRL-1740™) in the year 2011 were purchased from the American Type Culture Collection (Manassas, VA). Because most of the results in this study were obtained using HCT-116 colon cancer cells, KCTC (Korean Collection for Type Cultures) authenticates HCT-116 cancer cells using short tandem repeat analysis. HCT-116 (human colon cancer) was maintained in McCoy's 5A medium (GIBCO; Carlsbad, CA). SW620 (human colorectal cancer cell), Mia PaCa-2 (human pancreatic cancer cells), PC-3 (human prostate cancer), DU-145 (human prostate cancer), LNCaP (human prostate cancer), MDA-MB-231 (human breast cancer) and HFF (human foreskin fibroblast) were cultured in RPMI 1640 media (GIBCO). All culture media were supplemented with 10% heat-inactivated fetal bovine serum (GIBCO). Cell cultures were maintained at 37°C under 5% CO₂ in an incubator.

Luciferase reporter construct and dual-luciferase™ reporter assay

pHSE-TA-Luc plasmid was constructed as reported previously (29). The activity of the reporter was measured using a Dual-Luciferase™ reporter system (Promega; Madison, WI). HCT-116 cancer cells were seeded at a density of 2.5 × 10⁴ cells in 100 × 20 mm culture dishes. Cells were co-transfected with 9 µg of p[HSE]-TA-Luc vector and 1 µg of pRL-TK vector containing the Renilla luciferase gene as an internal control. The transfection was performed using TransFection (Roche; Basel, Switzerland) according to the manufacturer's protocol. Five hours after transfection, cells were detached by trypsin with ethylenediaminetetraacetic acid (EDTA) and seeded onto steroid, black-bottom 96-well plates at a density of 2 × 10⁴ cells per well. After incubation for 24h, cells were treated with chemicals for 30min, exposed to heat shock at 44°C for 15min and then incubated further at 37°C for 5h. Firefly and Renilla luciferase activities were measured using a dual-light reporter gene assay kit (Promega; Madison, WI).

Cell proliferation assay

Cells were seeded onto 96-well plates at a density of 5 × 10³ cells per well in McCoy's 5A medium with 10% fetal bovine serum. After 24h, the medium was replenished with fresh complete medium containing fasetin, quercetin or 0.1% DMSO. After incubation for 48h, the cell proliferation reagent WST-1 (Dojindo; Kaminashi gun, Kunitomo, Japan) was added to each well. The amount of WST-1 formazan produced was measured at 450nm using an ELISA reader (Bio-Rad).

Chromatin immunoprecipitation (ChIP) assay

Cells were treated with either no heat or heat in the presence of different concentrations (25 and 50 µM) of fasetin. Cells were then fixed by adding formaldehyde (Sigma; St Louis, MO) to the medium to a final concentration of 1.5% for 15min, after which glycine was added to a final concentration of 125mM. The cells were then scraped and centrifuged for 5min at 2400g at room temperature. Pelleted cells were washed with ice-cold phosphate-buffered saline containing protease inhibitors (1mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin and 1 µg/ml pepstatin A). After centrifugation, cells were resuspended in sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1 and protease inhibitors) and incubated for 10min on ice. After incubation, chromatin was sheared by sonication. After the removal of nuclear debris by centrifugation at 13000g for 10min at 4°C, the lysates were diluted 10-fold with ChIP Dilution Buffer (0.01% SDS, 1% Triton-X100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA and 150mM NaCl). Chromatin was then sonicated (4 × 30s) with a Branson sonicator. DNA fragments were precipitated with a 10-fold molar excess of Protein A beads over DNA amount. After precipitation, chromatin was eluted with 1% SDS and 0.1% SDS at 65°C for 4h. Eluates were analyzed by 2% agarose gel electrophoresis.
1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, 167 mM NaCl) and then precleared for 30 min using 30 μl of Salmon Sperm DNA/Protein A Agarose (Millipore; Darmstadt, Germany). Immunoprecipitation was carried out at 4°C overnight, and the immune complexes were collected with Salmon Sperm DNA/Protein A Agarose (Millipore). The antibodies used included anti-HSF1 (StressGen; Pennsylvania, Collegeville, SPA-901), or pre-immune rabbit serum as a control for non-specific interactions. After washing three times with Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 500 mM NaCl), LiCl Immune Complex Wash Buffer (0.25M LiCl, 1% NP-40, 1% Sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8.1) and twice with TE Buffer (10 mM Tris–HCl pH 8.1, 1 mM EDTA pH 8.0), immunocomplexes were eluted with Elution Buffer (1% SDS, 0.1 M NaHCO3). Protein-DNA cross-links were reversed by incubating at 65°C for 4 h. After proteinase K digestion, DNA was extracted with a PCR-Purification Kit (Bioneer; Daejeon-gu, Daejeon, Korea). Real-time PCR analysis was performed with the IQ5 thermocycler (Bio-RAD; Hercules, California), using FastStart SYBR Green Master (Roche; Basel, Switzerland) to prepare the reaction mixes. The primers used for real-time PCR of human hsp70 genes were the following for the HSF1 ChIP assay: HSP70A forward primer, 5′-CAGCTGCGTTCCTGGAG-3′; HSP70A reverse primer, 5′-TCCCTTCTGACGCCATCG-3′. The relative quantities of hsp70 were normalized to the input DNA. Data were expressed as the mean ± SD of triplicate samples.

Quantification of mRNA using quantitative reverse transcription–polymerase chain reaction

RNA was isolated using an RNAsesy kit (Qiagen; Valencia, CA). Two micrograms of isolated RNA for each sample was reverse-transcribed with TOPscript™ RT DryMix(DT18) kit (Enzynomics©; Yuseng-qu, Daejeon, Korea) according to the manufacturer’s instructions. Real-time PCR was performed using IQ™ SYBR Green supermix (Bio-Rad) according to the manufacturer’s instructions using an IQ5 real-time PCR detection system. The following primers were used for RT–PCR: HSP70 forward primer, 5′-ACCAAGGAGGAGCAGAG-3′; HSP70 reverse primer, 5′-GGCACTGGTTCTCAAGCCA-3′; GAPDH forward primer, 5′-GCGAGATTTTCGTCAGAC-3′; GAPDH reverse primer, 5′-CATGAA GCCACAGTTGTC-3′; HSP27 forward primer, 5′-GGATTCTTGTTGATG-3′; HSP27 reverse primer, 5′-AGGAGGCACTCGACATAGGTC-3′; BAG3 forward primer, 5′-ATGCCCATCTTCCACTTG-3′; BAG3 reverse primer, 5′-AGTAGTACGACACCAGG-3′; GAPDH forward primer, 5′-GGAAGGAAAAGGCTCATATC-3′; GAPDH reverse primer, 5′-CCATGCCAGTGGGTTCCGTCT-3′. The relative quantities of hsp70 mRNA were normalized against the GAPDH mRNA.

Western blotting and immunoprecipitation

Twenty micrograms of lysate was resolved by 7.5 or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% skim milk in TBS-T (50 mM Tris–HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20). Proteins were detected with the indicated primary antibodies. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit, anti-mouse and anti-rat IgG from Jackson ImmunoResearch. The antibodies were used at dilutions recommended by the manufacturers. The membrane was incubated with the primary antibody for 2 h at room temperature, washed five times with TBS-T and visualized with LuminataTM Forte Western HRP Substrate (Millipore). For immuno-precipitation, 800 μg of the lysates were incubated with the primary antibody overnight at 4°C with rotation, and then 50 μl of protein G magnetic beads (Millipore) were added. After 1 h, the lysates were removed and beads were washed three times with RIPA buffer. Bead-bound proteins were resolved by SDS–PAGE and detected using specific antibodies.

Fluorescence-activated cell sorting analysis

HCT-116 cancer cells were treated with fisetin at various concentrations for 48 h. Cells were then harvested, fixed with 70% chilled ethanol and preserved at −20°C before fluorescence-activated cell sorting (FACS) analysis. Fixed cells were washed three times with phosphate-buffered saline solution before being suspended in 500 μl phosphate-buffered saline and treated with 100 μg/ml RNase A at 37°C for 30 min. Propidium iodide was added to a final concentration of 50 μg/ml for DNA staining, and 20000 fixed cells were analysed on a FACScalibur system (BD Biosciences; Franklin Lakes, NJ). The cell cycle distribution was analysed using the ModFit program (BD Biosciences).

Nuclear and cytoplastic extraction

Cells were seeded onto 100 × 20 mm plates at a density of 2 × 10⁵ cells in McCoy’s 5A medium with 10% fetal bovine serum. After 24 h, cytoplastic and nuclear protein extracts were isolated using the NE-PER kit (Thermo Scientific; Waltham, MA) according to the manufacturer’s instructions. A volume of samples corresponding to 20 μg was subjected to SDS–PAGE and transferred to a PVDF membrane (Millipore). Proteins were detected with the indicated primary antibodies. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit, anti-mouse and anti-rat IgG (Jackson ImmunoResearch). The blots were developed with LuminataTM Forte Western HRP Substrate (Millipore).

Nude mouse xenograft assay

Xenograft experiment with HCT-116 cells was carried out in accordance with the guidelines and under the approval of the Institutional Review Committee for Animal Care and Use, Korea Research Institute of Bioscience and Biotechnology. Seven-week-old female inbred specific-pathogen-free Balb/c nude mice were housed under sterile conditions with 12 h light/dark cycles, and fed food and water ad libitum. For the evaluation of the in vivo anti-tumor activity of fisetin, HCT-116 cells (0.3 ml of 4 × 10⁶ cells/ml) were implanted subcutaneously into the right flank of the mice on day 0. Fisetin was dissolved in 10% DMAC, 10% Tween80, and 80% [20% 2-hydroxypropyl-β-cyclodextrin (in distilled water)]. Vehicle control and fisetin (30 mg/kg, intraperitoneal injection) were administered 3 times per week for 25 days. Tumor volumes were estimated using the formula length (mm) × width (mm) × height (mm)/2. To determine the toxicity of the compound, the body weight of tumor-bearing animals was recorded. On day 25, the mice were killed and the tumors were weighed.

Statistical analysis

The data are expressed as the means ± standard deviations (SD), and the degree of significance was analysed using Student’s t-test. P-values below 0.05 were considered to indicate statistical significance.

Results

Screening for inhibitors of HSF1 activity

A heat shock–dependent luciferase reporter plasmid, p(HSE)–TA–Luc, was used for screening of HSF1 inhibitors as described previously (29). Seven hundred and sixteen structurally diverse natural compounds were screened to identify HSF1-inhibitory chemicals. For this, HCT-116 cancer cells were cotransfected with a p(HSE)–TA-Luc reporter and an internal control vector constitutively expressing pRL-TK to normalize for transfection efficiency. There was 75-fold induction of the luciferase activity in HCT-116 cancer cells by heat shock stress (Fig. 1A). Then, cells were treated with compounds at 10 μM for 30 min and then exposed to heat shock at 44°C for 15 min. After 5 h of recovery at 37°C, luciferase activity was measured. From these experiments, we identified fisetin as a HSF1 inhibitor.

Surprisingly, fisetin (3,7,3′,4′-tetrahydroxyflavone) but not quercetin (3,3′,4′,5,6-pentahydroxyflavone) was identified in our screening even though the latter was previously reported as a HSF1 inhibitor (30–32). We measured the inhibitory activity of fisetin and quercetin on the HSF1 reporter in several cancer cell lines (Figure 1A–D). Fisetin inhibited HSF1 reporter activity in a concentration-dependent manner, with 50% inhibition at 14 μM for HCT-116 cancer cells. In addition, fisetin inhibited HSF1 reporter activity in all cancer...
cell lines and had stronger activity compared with quercetin. We tested four structural analogs of fisetin, quercetin, naringenin, kaempferol and baicalein (Figure 1E). Quercetin and kaempferol showed 54 and 66% inhibitory activity at 100 μM, respectively. Naringenin and baicalein did not inhibit reporter activity, even at 100 μM.
Inhibition of heat shock-induced HSP mRNAs and proteins by fisetin

Because fisetin decreased HSF1-dependent reporter activity, the inhibitory effect of fisetin on the endogenous hsp70, hsp47 and hsp27 gene expression was investigated. HCT-116 cells were exposed to heat shock at 43°C for 1 h in the presence or absence of fisetin. Then, the cells were incubated at 37°C for 30 min to allow for recovery, and the total RNAs were isolated. The expression of hsp70 mRNA was evaluated by quantitative real-time reverse transcription-PCR. As shown in Figure 2A, heat shock treatment caused an 11-fold increase in hsp70 mRNA expression relative to the non-heat shock condition. Pretreatment of HCT-116 cells with fisetin blocked heat shock-induced hsp70 mRNA expression in a concentration-dependent manner, with 50% inhibition at 17 μM. Similarly, fisetin inhibited hsp47, hsp27 and BAG3 mRNA expression in a concentration-dependent manner (Figure 2D). The IC₅₀ for each of the HSP's mRNA expression is very similar to the IC₅₀ for the HSF1 reporter assay (Figures 1 and 2).

Next, we analysed effect of fisetin of the expressions of HSP proteins. For this, HCT-116 cells were subjected to heat shock at 43°C for 1 h in the presence or absence of fisetin and incubated at 37°C for 6 h to allow recovery. Consistent with its effect on HSPs mRNA expression, fisetin also significantly downregulated HSF1 downstream target proteins, such as HSP70, HSP27 and BAG3, in a concentration-dependent manner (Figure 2E). Interestingly, amounts of HSF1 were decreased by treating HCT-116 cells with heat shock and further decreased when the cells were treated with heat shock and fisetin (Figure 2E). However, when HCT-116 cells were subjected to heat shock at 43°C for 1 h in the absence or presence of fisetin without recovery at 37°C, amounts of HSF1 was not decreased by fisetin (Figure 5D), suggesting that HSF1 was degraded during the recovery time at 37°C. In addition, HSF1 migrated slowly on SDS–PAGE by hyperphosphorylation of HSF1 (Figure 2E).

Fisetin induces apoptosis through the downregulation of HSP70/BAG3/Bcl-2

BAG3 was reported to be induced by HSF1 and enhance cancer cell survival by stabilizing the anti-apoptotic Bcl-2 family proteins (25). As shown in Figure 2D, heat shock treatment increased BAG3 mRNA expression more than 4-fold relative to the non-heat shock condition. The pretreatment of HCT-116 cancer cells with fisetin inhibited heat shock-induced BAG3 expression in a concentration dependent manner (Figure 2D). Fisetin also decreased heat shock-induced BAG3 protein expression (Figure 2E).

Because HSP70 and BAG3 are molecular chaperones that stabilize of anti-apoptotic Bcl-2 family proteins, we analysed the effects of fisetin on the levels of Bcl-2, Bcl-Δ and Mcl-1 proteins. HCT-116 cells were treated with fisetin at various concentrations (0–50 μM) for 48 h. As shown in Figure 3A, fisetin caused a decrease in the expression of HSF70, BAG3, Bcl-2, Bcl-Δ and Mcl-1 in a concentration-dependent manner. As the amounts of Bcl-2, Bcl-Δ and Mcl-1 were decreased by treating cells with fisetin, PARP cleavage appeared, indicating apoptotic cell death brought on by the fisetin treatment.

To analyse the temporal changes in protein expression caused by fisetin, HCT-116 cancer cells were treated with 50 μM
Fisetin for different amounts of time. As shown in Figure 3B, treating HCT-116 cancer cells with fisetin for 12 h decreased the expression of HSP70 and BAG3 followed by the downregulation of Bcl-2 after 24 h of treatment. Significant downregulation of HSP70/BAG3/Bcl-2 is coincident with PARP cleavage, indicating apoptosis of the HCT-116 cancer cells. Unexpectedly, the downregulation of Mcl-1 and Bcl-xL was detected 3 h after treating cells with fisetin. Because Mcl-1 and Bcl-xL were downregulated earlier than HSP70/BAG3 by fisetin, their expression might be mediated by another target molecule and not by HSF1 inhibition. However, the downregulation of Mcl-1 and Bcl-xL could not induce PARP cleavage (Figure 3B). These results suggested that HSP70/BAG3/Bcl-2 downregulation is important to induce HCT-116 apoptosis. Because, PARP cleavage was strongly induced by treating HCT-116 cancer cells with fisetin for 24 h (Figure 3B), we analysed whether fisetin activated executive caspases. As shown in Figure 3C, active caspase-3 and caspase-7 was induced by fisetin in a concentration-dependent manner and this caspases activation is coincident with PARP cleavage.

To assess the sensitivity of HCT-116 cancer cells to fisetin, the cells were treated with fisetin or quercetin at different concentrations (0–100 μM) for 48 h (Figure 4A). Fisetin exhibited a dose-dependent inhibition of HCT-116 cell growth over a broad range of concentrations, with a GI50 of 23 μM, while quercetin was the inhibitor concentration at which a 50% inhibition of cell growth is observed. However, the GI50 of quercetin was more than 100 μM, suggesting a 4-fold weaker activity compared with fisetin. The relative inhibitory activity of fisetin and quercetin on the proliferation of HCT-116 cancer cells (Figure 4A) is very similar to the inhibitory activity results from the HSF1-dependent reporter assay (Figure 1). These results strongly suggest that HSF1 inhibition by fisetin is important for the anti-proliferative activity of the compound. The effect of fisetin on the proliferation of various other tumor cell lines was also analysed; these cell lines and the GI50 value for each are as follows: Mia-PaCa-2 (29 μM), SW620 (37 μM), MDA-MB-231 (38 μM), DU-145 (17 μM), PC3 (41 μM) and LNCaP (41 μM). When immortalized human foreskin fibroblast cells were used for proliferation, GI50 was 145 μM, indicating that cancer cells were more sensitive to fisetin compared with the immortalized normal cells.

Because fisetin inhibited cancer cell proliferation, the phase of the cell cycle affected by the compound was evaluated. HCT-116 cancer cells were treated with fisetin at different concentrations for 48 h and subjected to FACS analysis. Fisetin caused an increase in the proportion of the sub-G1 population in a concentration-dependent manner, indicating fisetin-induced apoptosis (Figure 4B).

**Fisetin did not block nuclear localization of HSF1 upon heat shock**

HSF1 is primarily localized in the cytosol under normal growth condition. Upon stress, it translocates into the nucleus where it binds to the promoters of HSPs. Because fisetin blocked HSF1-dependent HSPs expression, we examined the possibility that fisetin inhibited HSF1 translocation into the nucleus by performing a subcellular fractionation analysis. As shown in Figure 5A, under normal conditions, HSF1 was localized mainly in the cytoplasm and was translocated into the nucleus upon heat shock stress. In addition, nuclear translocation was not inhibited by fisetin treatment. This result suggested that fisetin did not block the translocation stage but inhibited the step(s) of HSF1 action in the nucleus, such as binding step to the promoters of HSPs.

**Fisetin inhibits heat shock-induced recruitment of HSF1 to the hsp70 promoter**

Upon heat shock, HSF1 is recruited to the promoters of heat shock genes. Because fisetin reduced transcription activation...
by HSF1, the effect of fisetin on the recruitment of HSF1 to the hsp70 promoter was analysed by ChIP analysis. For this, HCT-116 cells were subjected to heat shock at 43°C for 1 h in the presence or absence of fisetin without recovery time at 37°C. As shown in Figure 5B and C, HSF1 binding to the hsp70 promoter was increased by heat shock. However, treating cells with fisetin significantly inhibited heat shock-induced HSF1 association with the hsp70 promoter. Next, we tested whether decreased binding of HSF1 by fisetin was caused by HSF1 degradation. Treating cells with fisetin using the same conditions as for ChIP assays (pretreatment of fisetin at 25 or 50 μM for 30 min followed by heat shock at 43°C for 1 h without recovery time at 37°C) did not decrease the amount of HSF1 (Figure 5D), excluding the possibility that fisetin decreased HSF1 binding to the hsp70 promoter by downregulating HSF1 expression.

**Fisetin inhibits the growth of HCT-116 cells in BALB/c nude mice**

HCT-116 tumor xenografts in nude mice were used to investigate the inhibitory activity of fisetin on tumor growth in vivo. HCT-116 cells were implanted subcutaneously into the right flank of nude mice on day 0. Vehicle control and fisetin (30 mg/kg, intraperitoneal injection) were administered 3 times per week for 24 days. To determine the toxicity of the compound, the body weight of tumor-bearing mice was measured. On day 25, the mice were killed and the tumors were removed and weighed. Mice treated with fisetin showed a 35.7% (P < 0.05) decrease in tumor volume and a 34.3% (P < 0.001) decrease in tumor weight compared with control mice (Figure 6). These results suggest that fisetin could block growth of tumor in an animal model.

**Discussion**

HSF1 is activated in a broad range of cancer specimens taken directly from cancer patients (33). HSF1 activation is an indicator of poor prognosis in breast, colon and lung cancers. HSF1-knockout mice have significantly reduced tumor incidence induced by chemical carcinogens on the skin or on the liver by the mutation of the p53 tumor suppressor (21,22). Therefore, HSF1 is a promising target for the chemopreventive purposes after surgery and for therapeutic treatment of cancer patients.

Mice that received fisetin at 45 mg/kg inhibited prostate tumor growth by 74.8% (7) and melanoma tumor growth by 66.6% (8). Mice treated with fisetin at 30 mg/kg showed a 35.7% (P < 0.05) decrease in colon tumor growth compared with control mice (Figure 6). These results suggested that fisetin can be effective in reducing tumor growth in an animal model.

Previously, we reported a novel compound KRB11 as an HSF1 inhibitor (29). Using affinity chromatography with biotinyl-KRB11, we found that KRB11 associated with HSF1 in vitro. ChIP analysis showed that KRB11 did not inhibit HSF1 binding to the hsp70 promoter. Instead, KRB11 decreased HSF1-dependent recruitment of p-TEFb to the hsp70 promoter. Unlike KRB11, fisetin inhibited HSF1 itself binding to the hsp70 promoter (Figure 5B C). Currently, we do not know whether fisetin directly associates with HSF1. It is important to identify the
Figure 5. Fisetin inhibits heat shock-induced HSF1 binding to the hsp70 promoter. (A) The analysis of the subcellular localization of HSF1. HCT-116 cancer cells were treated with either no heat or heat at 43°C for 1 h in the presence of fisetin (50 μM). After heat shock, nuclear and cytoplasmic protein extraction was performed as described in Materials and methods. α-Tubulin and histone H1 are markers for the purity of cytoplasmic and nuclear fractions, respectively. (B and C) ChIP analysis of HSF1 binding to the hsp70 promoter. HCT-116 cancer cells were incubated with the indicated concentrations of fisetin, heat shocked at 43°C for 1 h and analysed for the recruitment of HSF1 on the hsp70 promoter by the ChIP assay as described in Materials and methods. ChIP-enriched DNAs were prepared using preimmune IgG or anti-HSF1 antibodies. The quantification of the DNA fragment of the hsp70 gene (−216 to −24) was performed using quantitative real-time PCR (B) and PCR (C). The ChIP assay was performed three times, and similar results were obtained. Relative promoter occupancy is expressed as fold induction compared with the control prepared from samples that were not heat treated. Error bars indicate SD. Statistical significance (P value) was determined with an unpaired t test. *P < 0.01 versus the no heat control. **P < 0.01 versus the heat control. (D) HCT-116 cancer cells were incubated with the indicated concentrations of fisetin, heat-shocked at 43°C for 1 h with no recovery. HCT-116 lysates were analysed by western blotting using anti-HSF1 or GAPDH antibody as described in Materials and methods.

To estimate HSPs expressions at normal temperature compared with those at heat shock stress, it is better to use amounts of HSPs mRNA and protein in the HCT-116 cancer cells. As shown in Figure 2, depending on HSPs genes, there is 2.5- to 11-fold difference of mRNA between normal and heat shock stress cultures (Figure 2A–D). In addition, there is 1.4- to 2-fold difference of HSP proteins between normal and heat shock stress (Figure 2E). These results suggested that basal level of HSPs protein is at least 50% of heat shock-induced HSPs level in HCT-116 cancer cells. These basal expressions of HSPs proteins were efficiently downregulated by treating HCT-116 cancer cells with fisetin 50 μM for 24 h, inducing PARP cleavage (Fig. 3B).

Many HSF1 inhibitors (for review, see [36]) have been reported, including quercetin ([30,31]), QC12 ([37]), KNK437 ([38]), Stresgenin ([39], triptolide ([40]), Emunin ([41]), NZ28 ([41]), KIBB ([29]), phenyl isothiocyanate ([42]), roacaglates ([43]) and cantharidin ([44]). Strong efficacy and low toxicity are important factors to consider when developing a cancer therapeutic. Triptolide shows potent activity against HSF1, and it was used to show the antitumor effect of HSF1 inhibition in vivo ([45]). However, triptolide inhibits NF-xB and AP-1 as well as HSF1. Similarly, quercetin is a naturally occurring flavonoid that inhibits multiple target proteins, including HSF1, NF-xB, several kinases and CYP3A4.

Surprisingly, even though quercetin was included in our study, it was not initially identified as having inhibitory effects. Our reporter plasmid has four copies of HSF1 binding elements upstream from the luciferase promoter to induce luciferase expression only in response to HSF1 activation. In the case of quercetin, it inhibited HSF1 reporter activity at high
concentration (13.1% inhibition at 20 μM and 23.2% inhibition at 50 μM in HCT-116 cancer cells) (Figure 1). Because we screened compounds at 10 μM, quercetin was not selected as a hit. Recently, quercetin target proteins were identified by affinity chromatography with biotinylated quercetin (46). Biotinyl-quercetin binds directly to HSP70 and HSP90. Therefore, it is likely that quercetin inhibits HSP70 and HSP90 functions by directly binding to them.

When HCT-116 cancer cells were treated with fisetin or quercetin at 20 μM, HSF1 luciferase reporter activity was inhibited by 74.1 and 13.1%, respectively (Figure 1A). Similarly fisetin and quercetin exhibited a dose-dependent inhibition of HCT-116 cell growth over a broad range of concentrations, with a GI50 of 23 μM and 100 μM, respectively (Figure 4A). These results indicate that fisetin has stronger HSF1 inhibitory and anti-proliferation activities than quercetin.

Fisetin was reported to inhibit AR (6,7). Therefore, we tested whether fisetin inhibited prostate cancer in an AR-dependent manner. AR-negative DU145 and PC3 prostate cancer cells and AR-positive LNCaP prostate cancer cells were treated with fisetin at various concentrations (0–300 μM) and the inhibition of proliferation was analysed. As described in the results, we obtained a GI50 of 17 μM, 41 μM and 41 μM for DU145, PC3 and LNCaP, respectively. This result was somewhat unexpected because if fisetin inhibited AR, it significantly inhibited growth of AR-positive LNCaP prostate cancer compared with AR-negative DU-145 and PC3 prostate cancer cells. However, fisetin inhibited the proliferation of both AR-negative and AR-positive prostate cancer cells, indicating that fisetin has other target molecule besides AR.

Cancer cells have multiple mutations and oncogenes. In addition, signaling has a high degree of pathway redundancy. Therefore, disruption of a single target function has frequently weak or little anticancer effect. Therefore, combination therapy with target-specific drugs is important approach to get therapeutic effect. However, multi-targeted therapy can be an alternative approach for the same purpose. In general, flavonoids have multiple target molecules in cells and fisetin might inhibit not only HSF1 but also other target proteins including NF-κB, β-catenin and AR. Therefore, antitumor effects of fisetin could be a sum of these multi-targets inhibitions. Even though we mentioned beneficial effects of multi-targeted natural compounds, they have detrimental properties, too. Because flavonoids have multiple targets, it can inhibit normal cell, producing off-target toxicity. Therefore, in the case of multi-targeted therapy, we have to carefully evaluate beneficial and detrimental effects for clinical use.

Bcl-2 family members are key regulators of cancer cell survival and consist of both anti- and pro-apoptotic proteins. Pro-survival Bcl-2 family proteins have been emerging as promising therapeutic targets, and small Bcl-2/Bcl-xL inhibitors, such as ABT-737 and ABT-236, are being developed (47,48). ABT-236 has
clinical activity in Bcl-2-dependent tumors. However, many tumors are not dependent on Bcl-2 but depend instead on Mcl-1. In addition, the overexpression of Mcl-1 causes resistance against ABT-737 in cancer cells (49–51). Furthermore, the amplification of the Mcl-1 locus was reported as one of the most frequent somatic genetic events in human cancer (52). These reports suggest that a triple inhibitor against both Bcl-2/ Bcl-x, and Mcl-1 is more effective than specific inhibitors that target one or the other. This concept was consistent with our result (Figure 3B). It is worth noting that even though Mcl-1 and Bcl-x, were decreased 3 h after fisetin treatment, there was no PARP cleavage, suggesting that the downregulation of Mcl-1 and Bcl-x, was insufficient to induce HCT-116 apoptosis (Figure 3B). HSF70 expression was decreased 12 h after treatment, and the significant downregulation of HSF70/BAG3 and Bcl-2 family proteins were observed 24 h after treatment. This temporal pattern correlated with PARP cleavage in HCT-116 cancer cells. Our HSF1 inhibitor fisetin acted as a triple inhibitor by simultaneously decreasing the expression of Bcl-2, Bcl-xL, and Mcl-1 through the downregulation of their chaperones, HSP70 and BAG3. Therefore, fisetin can be useful to overcome to the single agent-induced resistance problem.

Generally chemopreventive use of a drug requires long-term treatment; hence, it is necessary that the drug be less toxic than common therapeutic anticancer drugs. Interestingly, fisetin is an ingredient of food, such as apples, grapes, kiwis, persimmons, strawberries, cucumbers and onions. The amount of fisetin is 160 µg in 1 g of strawberries. In addition, fisetin was reported to have only a minimal effect on the growth of epithelial cells (6). Therefore, it is interesting to test whether fisetin can be a candidate compound to use as a chemopreventive agent for long-term administration after surgery.

The present study is the first report that fisetin, a dietary flavonoid, can inhibit HSF1 activity, interfere with cancer cell proliferation and induce apoptosis. In addition, we determined the inhibitory mechanism of fisetin; it blocked HSF1 binding to the promoters of the hsp70 gene (and presumably the bag3 gene), downregulating the expression of HSP70 and BAG-3. Low levels of HSP70/BAG3 might destabilize anti-apoptotic Bcl-2 family proteins, inducing apoptotic cancer cell death.

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