ARTICLE
Journal of Cellular Biochemistry 105:1386–1398 (2008)

Journal of Cellular Biochemistry

Quercetin Sensitizes Human Hepatoma Cells to TRAIL-Induced Apoptosis Via Sp1-Mediated DR5 Up-Regulation and Proteasome-Mediated c-FLIP$_S$ Down-Regulation

Jin Yeop Kim,[1] Eun Hee Kim,[1] Seok Soon Park,[1] Jun Hee Lim,[2] Taeg Kyu Kwon,[2] and Kyeong Sook Choi[1*]

[1]*Department of Molecular Science & Technology, Institute for Medical Sciences, Ajou University School of Medicine, Suwon, Korea*

[2]*Department of Immunology, Keimyung University School of Medicine, Taegu, Korea*

ABSTRACT

This study demonstrates that combined treatment with subtoxic doses of quercetin (3′,3′,4′,5,7-pentahydroxyflavone), a flavonoid found in many fruits and vegetables, plus tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces rapid apoptosis in TRAIL-resistant hepatocellular carcinoma (HCC) cells. Effective induction of apoptosis by the combined treatment with quercetin and TRAIL was not blocked by overexpression of Bcl-xL, which is known to confer resistance to various chemotherapeutic agents. These results suggest that this combined treatment may provide an attractive strategy for treating resistant HCCs. While the proteolytic processing of procaspase-3 by TRAIL was partially blocked in various HCC cells treated with TRAIL alone, co-treatment with quercetin efficiently recovered TRAIL-induced caspase activation. We found that quercetin treatment of HCC cells significantly up-regulated the mRNA and protein levels of DR5, a death receptor of TRAIL, in a transcription factor Sp1-dependent manner. Furthermore, treatment with quercetin significantly decreased the protein levels of c-FLIP, an inhibitor of caspase-8, through proteasome-mediated degradation. Finally, administration of small interfering RNA against DR5 or overexpression of c-FLIP$_S$, but not c-FLIP$_L$, significantly attenuated quercetin-stimulated TRAIL-induced apoptosis. Collectively, these findings show that quercetin recovers TRAIL sensitivity in various HCC cells via up-regulation of DR5 and down-regulation of c-FLIP$_S$. J. Cell. Biochem. 105: 1386–1398, 2008.

KEY WORDS: QUERCETIN; TRAIL; HEPATOMA; DR5; c-FLIP$_S$

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm worldwide (>500,000 death/year) [Bruix et al., 2004]. Most hepatocellular carcinoma patients are diagnosed at advanced stages that are unsuitable for the current curative therapies of resection and transplantation [Avila et al., 2006]. The current chemotherapeutic options yield poor responses and low patient survival, making them ineffective for treatment of advanced HCC [Avila et al., 2006]. Therefore, new therapeutic alternatives are needed for more effective treatment of this malignancy.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is considered a promising anticancer agent due to its ability to induce apoptosis in a variety of tumor cell types while having only negligible effects on normal cells [Ashkenazi et al., 1999]. Binding of TRAIL to either DR4 or DR5, two death receptors of TRAIL, leads to oligomerization and clustering of their intracellular death domains. The subsequent interaction of DR4 or DR5 with the adaptor molecule, FADD (Fas-associated death domain), via their respective death domains leads to recruitment and activation of caspase-8 [Thomas et al., 2004]. Finally, caspase-8 activates the executioner caspases (e.g., caspase-3 and caspase-7), leading to apoptotic cell death [Srivastava, 2001]. Although TRAIL has garnered considerable attention as a novel anticancer agent, recent studies have shown that many cancer cells, including HCC cells, are resistant to the apoptotic effects of TRAIL, possibly due to

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Korea Science & Engineering Foundation; Grant number: BAERI-M20708630003-07B0863-00310; Grant sponsor: Korea Research Foundation; Grant number: KRF-2005-042-E00020.

*Correspondence to: Dr. Kyeong Sook Choi, Department of Molecular Science & Technology, Institute for Medical Sciences, Ajou University School of Medicine, Suwon, Korea. E-mail: kschoi@ajou.ac.kr

Received 15 May 2008; Accepted 4 September 2008 • DOI 10.1002/jcb.21958 • 2008 Wiley-Liss, Inc.

Published online 3 November 2008 in Wiley InterScience (www.interscience.wiley.com).

intrinsic or acquired TRAIL resistance [Yamanaka et al., 2000; Shankar and Srivastava, 2004]. Therefore, studies aimed at improving our understanding of the molecular mechanisms underlying TRAIL-resistance and identifying sensitizing agents capable of overcoming this resistance may facilitate the establishment of TRAIL-based combined regimens for the improved treatment of HCC.

Recent epidemiological and dietary intervention studies in animals and humans have suggested that diet-derived phenolic compounds, particularly the flavonoids, may play a beneficial role in inhibiting, reversing or retarding tumorigenesis, suggesting that these agents could have a potential use in the chemoprevention of cancer [Brit et al., 2001; Surh, 2003]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid that serves as the backbone for many other flavonoids, and many medicinal plants owe much of their activity to their high quercetin content [Havsteen, 1983]. Several studies have shown that quercetin exerts antiproliferative effects [Csokay et al., 1997] and/or apoptosis-inducing activity selectively in cancer cells but not in normal cells [Aghdassi et al., 2007], suggesting that it may have potential therapeutic value as an anticancer agent or an adjunct to current cancer therapies. We herein show that quercetin is a potent sensitizer for TRAIL-induced apoptosis in a variety of TRAIL-resistant HCC cells. Moreover, we present clear evidence showing that subtoxic doses of quercetin commonly and effectively overcome TRAIL-resistance in various hepatoma cells via Sp1-mediated DR5 up-regulation and proteasome-mediated c-FLIP$_S$ down-regulation, resulting in amplification of TRAIL-mediated apoptotic signaling.

MATERIALS AND METHODS

CHEMICALS AND ANTIBODIES

Recombinant human TRAIL/Apo2 ligand (the nontagged 19-kDa protein, amino acids 114–281) was from KOMA Biotech, Inc. (Seoul, South Korea). Quercetin was purchased from Sigma Chemical Corp. (St. Louis, MO). Calcein acetoxymethyl ester (calcein-AM) and ethidium homodimer-1 (EthD-1) were from Molecular Probes (Eugene, OR). The following antibodies were used: anti-caspase-8, caspase-3, caspase-6, and caspase-7 (Stressgen, British Columbia, Canada); anti-caspase-9, caspase-2, FAK, Cdk2, DR4, and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA); poly (ADP-ribose) polymerase (PARP; Upstate Biotechnology); anti-Bid (Cell Signaling, Beverly, MA); anti-c-FLIP (NF6) (Alexis, San Diego, CA); FITC-conjugated anti-goat IgG (Sigma); -tubulin, anti-DR5 for Western blotting (KOMA); anti-DR5 antibody for fluorescence-activated cell sorting (FACS) analysis (R&D Systems, Minneapolis, MN); and anti-rabbit IgG horseradish peroxidase, mouse IgG, and goat IgG (Zymed Laboratories, Inc., South San Francisco, CA).

CULTURE OF HEPATOMA CELLS

The human hepatoma cell lines HepG2 and SK-Hep1 were cultured in DMEM (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (Gibco-BRL). SNU-387, SNU-423, SNU-449 and SNU-475 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics.

MEASUREMENT OF CELLULAR VIABILITY

Cell viability was assessed by double labeling of cells with 2 M calcein-AM and 4 M EthD-1.The calcein-positive live cells and EthD-1-positive dead cells were visualized using a fluorescence microscope (Axiovert 200M, Zeiss, Oberkohen, Germany).

DNA FRAGMENTATION ASSAY

After treatments, cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10,000*g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of phenol/chloroform/ isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 g/ml ethidium bromide.

REVERSE TRANSCRIPTION-PCR ANALYSIS

Total RNA was extracted from SK-Hep1 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription-PCR (RT-PCR) was done following the manufacturer's protocol (RNA PCR kit (TaKaRa Co., Ltd. Japan)). Conditions for final analysis were chosen when amplification of mRNA was in the middle of the exponential amplification phase for 200 M quercetin. Human DR5 mRNA was amplified using the sense primer 5′-GTCTGCTCTGATCACCCAAC-3′ and the antisense primer 5′-CTGCAAACTG TGACTCCTATG-3′ (424-bp) For c-FLIP$_L$, the sense primer 5′-CGGACTATAGAGTGCTGATGG-3′ and the antisense primer 5′-GATTATCAGGCAGATTCCTAG-3′ (655-bp); c-FLIP$_S$, the sense primer 5′-CGGACTATAGAGTGCT-GATGG-3′ and the antisense primer 5′-AGATCAGGACAATGGGC-ATAG-3′ (561-bp). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sense primer 5′-CGTCTTCACCATGGAGA-3′ and the antisense primer 5′-CGGCCATCACGCCCACAGTTT-3′ was used (310-bp). The PCR cycling conditions (30 cycles) were chosen were as follows: (a) 30 s at 94°C, (b) 1 min at 70°C for DR5 and 30 s at 60°C for GAPDH, and (c) 1 min at 72°C, with a subsequent 10-min extension at 72°C. Reaction products were analyzed on 1.3% agarose gels. The bands were visualized by ethidium bromide.

FLOW CYTOMETRY OF DEATH RECEPTORS

Cells were analyzed for the surface expression of DR4 and DR5 by indirect staining with primary goat anti-human DR4 and DR5 (R&D Systems) followed by FITC-conjugated rabbit anti-goat IgG. Briefly, 4×10^5 cells were stained with 200 l PBS containing saturating amounts of anti-DR4 or anti-DR5 antibody on ice for 30 min. After incubation, cells were washed twice and reacted with FITC-conjugated rabbit anti-goat IgG on ice for 30 min. After washing with PBS, the expressions of these death receptors were analyzed by a FACS sorter (Becton Dickinson and Co., Franklin Lakes, NY).

SMALL INTERFERING RNA

The small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and have the following sequences: DR5, AUCAGCAUCGUGUACAAGGUGUCCC; FLIPS, AACAUGGAACU-GCCUCUACUU; FLIPL, AAGGAACAGCUUGGCGCUCAA; FLIP$_{S/L}$, AAGCAGUCUGUUCAAGGAGCA. Cells were transfected with siRNA oligonucleotides using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations.

PLASMIDS

Sp1 reporter constructs were purchased from Clontech (Palo Alto, CA). The pDR5/*Sac*I plasmid [containing DR5 promoter sequence −2500/+3] and pDR5/−605 [containing DR5 promoter sequence (−605/+3)] were gift from Dr. Sakai (Kyoto Prefectural University). To localize the promoter regions responsible for quercetin-induced DR5 up-regulation, deletion mutants of the DR5 promoter, including pDR5/−1036 [containing DR5 promoter sequence −1036/+3] and pDR5/−717 [containing DR5 promoter sequence −717/+3], were constructed. To identify the specific Sp1 binding sites responsible for quercetin-induced DR5 up-regulation, the reporter constructs containing single (mSp1-1 and mSp1-2), double (mSp1-3 and mSp1-4), or triple (mSp1-5 and mSp1-6) point mutations at putative Sp1-binding sites of DR5 promoter were used. The detailed procedure to generate these mutants was previously described [Kim et al., 2004b].

TRANSFECTIONS AND LUCIFERASE ASSAYS

Cells were seeded in 60 mm dishes at a density of 2.5×10^5 cells/dish and grown for 24 h before transfection. Cells were co-transfected with 2 g of various plasmid constructs and 1 g of the pCMV--galactosidase plasmid for 5 h by the Lipofectamine method. After transfection, cells were cultured in 10% FBS medium with vehicle or quercetin for 24 h. Luciferase and -galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for -galactosidase activity in cell lysate and expressed as an average of three independent experiments.

CONSTRUCTION OF THE EXPRESSION VECTORS FOR c-FLIP$_L$ AND c-FLIP$_S$

The cDNAs encoding human c-FLIP$_L$ and c-FLIP$_S$ were PCR amplified from plasmids (pCA-FLAG-hFLIP$_L$ and pCA-FLAG-hFLIP$_S$; kindly provided by Dr. Park SI (Korea Centers for Disease Control and Prevention, Seoul, Korea)) to contain these sequences with the specific primers. c-FLIP$_L$ and c-FLIP$_S$ cDNA fragment were digested with *Kpn*I and *Xho*I and subcloned into the pcDNA 3.1(+) vector (Invitrogen, Carlsbad, CA), and the resulting constructs were confirmed by nucleotide sequencing.

ESTABLISHMENT OF THE CELL LINES STABLY OVEREXPRESSING Bcl-xL, FLIP$_L$ OR FLIP$_S$

Mammalian expression vectors encoding Flag-tagged Bcl-xL was kindly provided by Prof. A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Australia). SK-Hep1 cells were transfected with the expression vectors encoding Flag-tagged Bcl-xL, FLIP$_L$ or FLIP$_S$. Stable SK-Hep1 cell lines overexpressing Bcl-xL was selected with changes of fresh medium containing puromycin (4 g/ml). Overexpression of Bcl-xL in the stable cell lines was analyzed by Western blotting using anti-Flag antibody (Sigma). Stable SK-Hep1 cell lines overexpressing FLIP$_L$ or FLIP$_S$ was selected with changes of fresh medium containing G418 (500 g/ml). Overexpression of FLIP$_L$ or FLIP$_S$ in the stable cell lines was examined by Western blotting using anti-FLIP antibody.

STATISTICAL ANALYSIS

All data are presented as means ± SE of at least three independent experiments. The statistical significance of differences was assessed using ANOVA (GraphPad software; GraphPad, Santiago, CA), followed by Student–Newman–Keuls multiple comparison tests. $P < 0.05$ was considered significant.

RESULTS

SUBTOXIC DOSES OF QUERCETIN SIGNIFICANTLY SENSITIZE TRAIL-RESISTANT HEPATOMA CELLS TO TRAIL-INDUCED APOPTOSIS

First, the cytotoxic activity of human recombinant soluble TRAIL (amino acids 114–281) was tested in six hepatoma cell lines; HepG2, SK-Hep1, SNU-449, SNU-387, SNU-423, and SNU-475. Treatment of various hepatoma cells with 100 ng/ml TRAIL for 24 h induced a limited cell death (<10%), as assessed using calcein-AM and EthD-1 (Fig. 1A). These results suggest that these hepatoma cells are resistant to the cytotoxic effect of TRAIL. Next, we examined whether quercetin could sensitize these TRAIL-resistant hepatoma cells to TRAIL-mediated cell death. While treatment with quercetin alone up to 200 M did not induce any significant cell death, cell viability was significantly reduced by the combined treatment with 100 ng/ml TRAIL and quercetin in a dose-dependent manner. To exclude the possibility that the sensitizing effect of quercetin on TRAIL-mediated apoptosis may result from the non-specific effects by use of its high dose, we compared the effects of quercetin with those of other flavonol compounds, including quercitrin, myricetin, rutin, and morin [Habtemariam, 1997; Xiao et al., 2008], as well as dihydroquercetin, an inactive analog of quercetin [Budagova et al., 2003; Loke et al., 2008]. We found that neither these flavonol compounds nor dihydroquercetin at the concentration of 200 M did stimulate SK-Hep1 cells to TRAIL-mediated apoptosis, suggesting that quercetin specifically sensitizes hepatoma cells to TRAIL-mediated apoptosis (Supplementary Fig. 1). Elevated protein levels of Bcl-xL in HCC cells are known to contribute to resistance to various chemotherapeutic agents [Takehara et al., 2001]. Therefore, we next examined whether Bcl-xL overexpression in hepatoma cells affects the cell death induced by combined treatment with quercetin and TRAIL. Interestingly, quercetin-stimulated TRAIL-mediated cell death was not inhibited by Bcl-xL overexpression (Fig. 1B). These results suggest that combined treatment with quercetin and TRAIL may have a therapeutic effect on hepatoma cells overexpressing Bcl-xL, which are resistant to many other chemotherapeutic drugs.

INCOMPLETE TRAIL-INDUCED PROTEOLYTIC PROCESSING OF CAPASE-3 IN HEPATOMA CELLS IS COMPLETED BY QUERCETIN CO-TREATMENT

Since typical apoptotic morphologies, including cellular shrinkage, membrane blebbing, formation of apoptotic bodies, and DNA fragmentation, were observed in SK-Hep1 cells co-treated with quercetin and TRAIL (Fig. 2A,B), we further examined whether the sensitizing effect of quercetin on TRAIL-mediated cell death is associated with caspase activation. Treatment of HepG2 or SK-Hep1 cells with 200 M quercetin alone did not induce any proteolytic

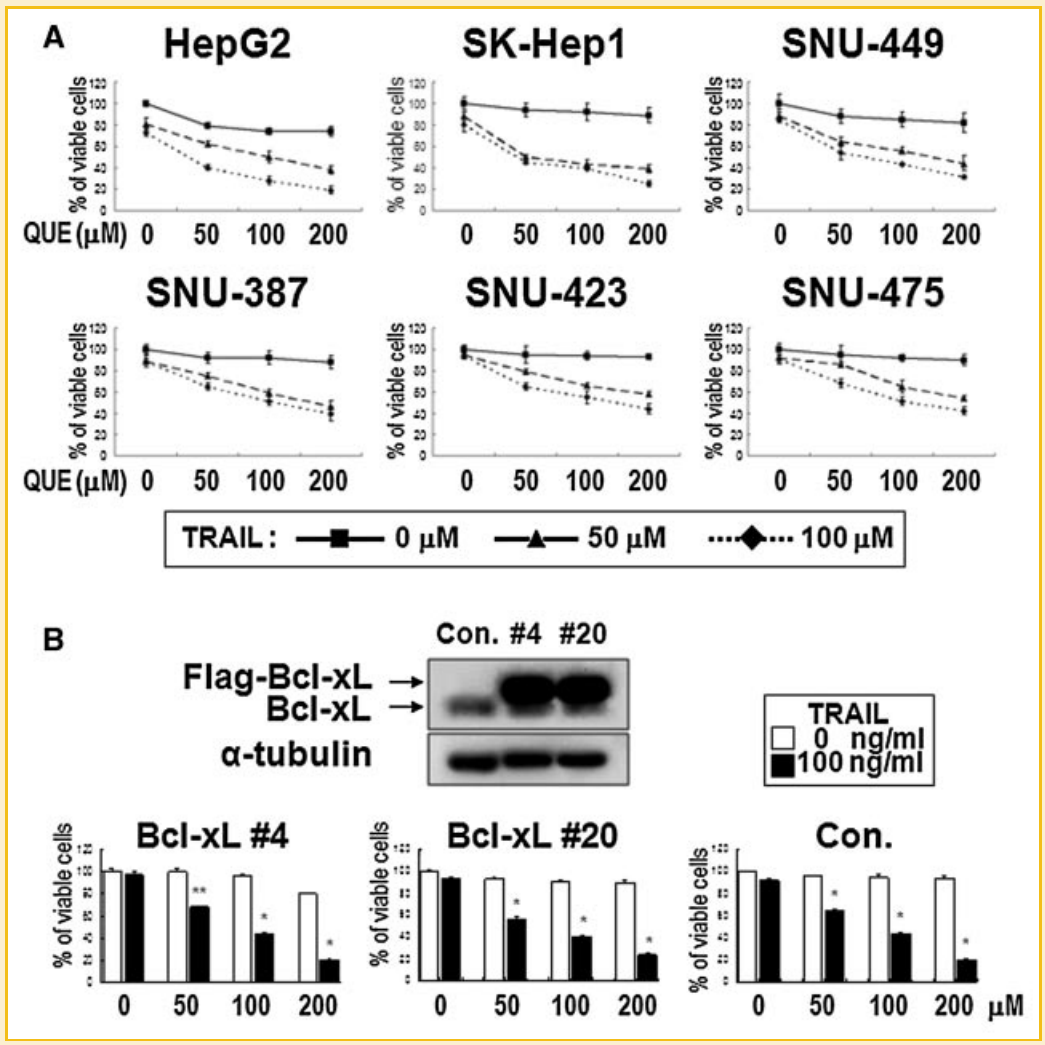

Fig. 1. Subtoxic doses of quercetin sensitize human hepatoma cells to TRAIL-induced apoptosis via activation of caspases. A: Viabilities of hepatoma cells treated with quercetin and/or TRAIL. Various hepatoma cells were treated with 100 ng/ml TRAIL alone, quercetin alone at the indicated concentrations, or 100 ng/ml TRAIL plus quercetin at the indicated concentrations for 24 h. Cellular viabilities of cells were assessed using calcein-AM and EthD-1. Results shown are the means of three independent experiments; bars, SE. B: Cell death induced by the combined treatment with quercetin and TRAIL is not blocked by Bcl-xL overexpression. Protein levels of Flag-tagged Bcl-xL in SK-Hep1 stable cell lines overexpressing Bcl-XL were analyzed by Western blotting using anti-total Bcl-xL antibody (top). SK-Hep1 cells stably transfected with pcDNA3 (Control cells) was used as a control. Control cells or Bcl-xL overexpressing SK-Hep1 cells were treated with 100 ng/ml TRAIL alone or 100 ng/ml TRAIL plus quercetin at the indicated concentrations for 24 h. Cellular viability were measured using calcein-AM and EthD-1 (bottom). Columns, average of three independent experiments; bars, SE. $^{*}P < 0.001$ compared with untreated cells; $^{**}P < 0.01$ compared with untreated cells.

processing of caspases (Fig. 2C). In contrast, the 32-kDa procaspase-3 was partially cleaved to a 20-kDa intermediate form in these cells treated with 100 ng/ml TRAIL alone for 12 h. Further cleavage into the active p17 subunit was not detected nor was other caspase-processing events in these cells treated with TRAIL alone. However, combined treatment with quercetin and TRAIL induced the complete processing of caspase-3 into p17/p12. The precursor protein levels of caspase-8, -9, -2, -6 or -7 were also significantly decreased in cells exposed to combined treatment with quercetin and TRAIL. Similar activation patterns of caspases in SK-Hep1 cells were observed in response to quercetin plus TRAIL. In parallel with the proteolytic processing of caspases, several key death substrates that indicate the activation of caspases, including FAK (caspase-3 and -7) and Bid (caspase-8), were also progressively degraded from 4 to 8 h after the combined treatment, whereas they were not degraded following treatment with quercetin alone or TRAIL alone (Fig. 2C). Flow cytometric analysis demonstrates that co-treatment of SK-Hep1 cells with 200 M quercetin and 100 ng/ml TRAIL for 8 h significantly increased the accumulation of sub-G1 phase cells with hypodiploid having slightly fewer than the diploid number of chromosomes, whereas treatment with quercetin or TRAIL alone did not (Fig. 2D). Pretreatment with a pan-caspase inhibitor z-VAD-fmk significantly blocked the accumulation of sub-G1-phase cell populations induced by quercetin plus TRAIL. Collectively, these results demonstrate that quercetin enhances TRAIL-mediated apoptosis via activation of caspases.

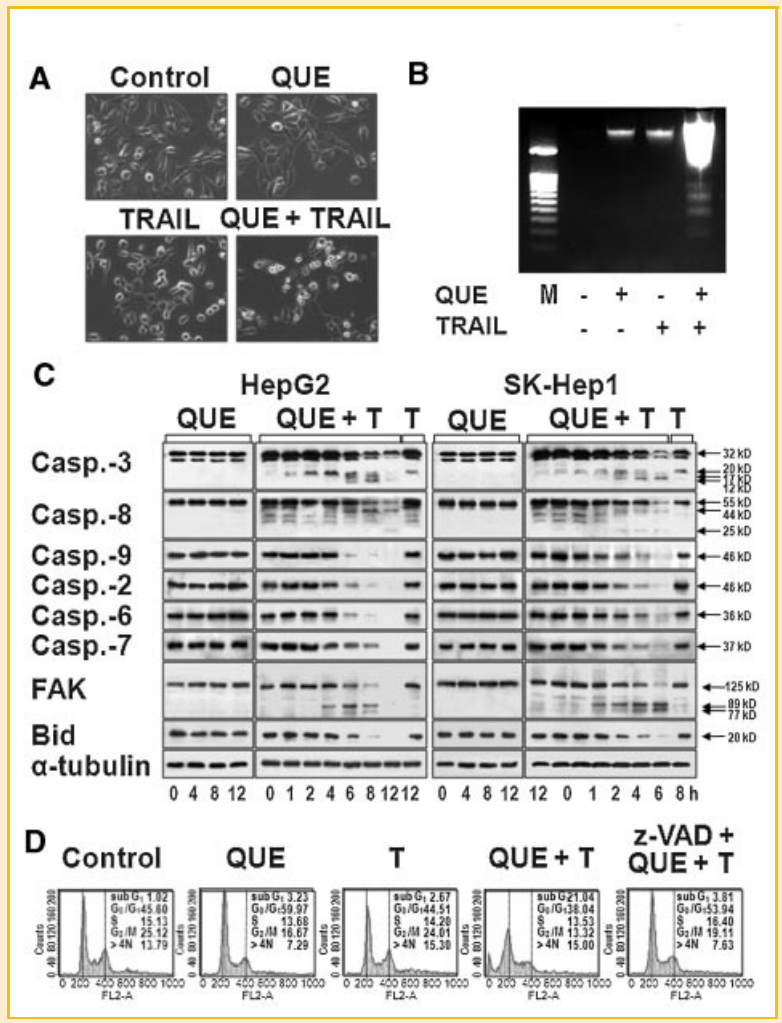

Fig. 2. Apoptosis induced by the combined treatment with quercetin and TRAIL is dependent on caspases. A: Morphologies of SK-Hep1 cells treated with quercetin and/or TRAIL. SK-Hep1 cells were untreated or treated with 200 M quercetin and/or 100 ng/ml TRAIL for 12 h. Representative phase contrast images of cells are shown. B: DNA fragmentation induced by the combined treatment with quercetin and TRAIL. SK-Hep1 cells were treated with 200 M quercetin alone, 100 ng/ml TRAIL alone, or 200 M quercetin plus 100 ng/ml for 12 h. Only fragmented DNA was isolated from the treated cells and subjected to agarose gel electrophoresis. C: Activation of caspases during quercetin-facilitated TRAIL-induced apoptosis. HepG2 or SK-Hep1 cells were treated with 200 M quercetin alone, 100 ng/ml TRAIL alone, 200 M quercetin plus 100 ng/ml TRAIL for the indicated time points and cell extracts were prepared to detect the changes in the expression of caspases by Western blotting. To confirm the activation of caspases, Western blotting of FAK and Bid was performed. D: Inhibition of caspases blocks quercetin-stimulated TRAIL-induced apoptosis. SK-Hep1 cells were treated with 200 M quercetin alone, 100 ng/ml TRAIL alone, or 200 M quercetin plus 100 ng/ml TRAIL for 8 h. To examine the effect of caspase inhibition, SK-Hep1 cells were pretreated with 50 M z-VAD-fmk for 30 min and further treated with 200 M quercetin plus 100 ng/ml TRAIL for 8 h. Cells were stained with propidium iodide and FACS analysis was performed.

QUERCETIN UP-REGULATES DR5 IN VARIOUS HEPATOMA CELLS

Since TRAIL is known to trigger apoptotic signals via two types of death receptors, DR4 and DR5 [Pan et al., 1997; Sheridan et al., 1997], we examined whether the modulation of DR4 and/or DR5 protein levels by quercetin might be involved in its sensitizing effect on TRAIL-induced apoptosis in various hepatoma cells. We found that quercetin treatment of HepG2 cells induced a time-dependent increase in the protein levels of DR5 but did not affect the protein levels of DR4 (Fig. 3A). Furthermore, treatment with quercetin dose-dependently increased DR5 protein levels in HepG2, SK-Hep1, and SNU-387 cells, whereas DR5 up-regulation was prominent in SNU-449, -423, and -475 cells treated with 50 M quercetin (Fig. 3B). These results indicate that up-regulation of the TRAIL death receptor, DR5, is a common response of various hepatoma cells to quercetin exposure. FACS analysis showed that the quercetin-induced surface expression of DR5, but not DR4, was also significantly increased in SK-Hep1 cells (Fig. 3C). To clarify the functional role of DR5 in the quercetin-mediated stimulation of TRAIL-induced apoptosis, we employed siRNA-mediated knockdown of DR5. Suppression of DR5 expression by transfection with its siRNA effectively inhibited quercetin-stimulated TRAIL-induced cell death of SK-Hep1 cells (Fig. 3D). Collectively, these results demonstrate that quercetin-induced DR5 up-regulation is critical for the enhancement of TRAIL sensitivity in hepatoma cells.

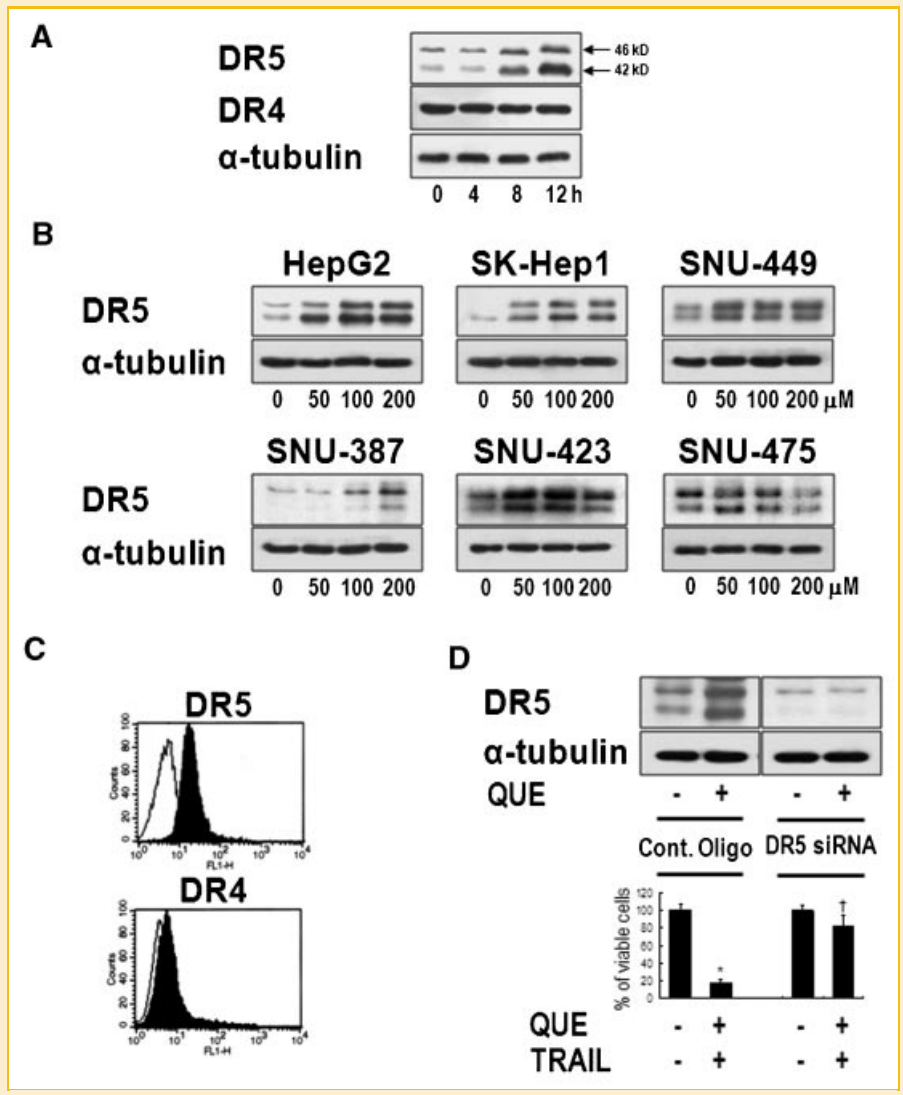

Fig. 3. Quercetin-induced DR5 up-regulation is important for quercetin-stimulated TRAIL-induced apoptosis. A: Quercetin up-regulates the protein levels of DR5 but not DR4. HepG2 cells were treated with quercetin at the indicated concentrations and Western blotting of DR5, DR4, and -tubulin was performed. B: Quercetin-induced DR5 up-regulation is a common response in human hepatoma cells. Various hepatoma cells were treated with quercetin for the indicated time periods and Western blotting of DR5 was performed. C: Quercetin up-regulates the surface expression of DR5 but not DR4. SK-Hep1 cells were treated with 200 M quercetin for 12 h and the surface protein levels of DR5 and DR4 were measured by FACS analysis. D: Suppression of DR5 expression by its siRNA transfection blocks quercetin-stimulated TRAIL-induced apoptosis. SK-Hep1 cells were transfected with the control fluorescent oligonucleotide or siRNA against DR5. Transfected cells were untreated or treated with 200 M quercetin plus 100 ng/ml TRAIL for 12 h. Suppression of DR5 protein levels were confirmed by Western blotting (top). Cellular viability was measured using calcein-AM and EthD-1 (bottom). Columns, average of three independent experiments; bars, SE. $^{*}P < 0.001$ compared with untreated cells; $^{\dagger}P < 0.001$ compared with cells transfected with control oligonucleotide and further treated with quercetin plus TRAIL.

Sp1 MEDIATES QUERCETIN-INDUCED DR5 UP-REGULATION

Next, we investigated the underlying mechanism of quercetin-induced DR5 up-regulation. RT-PCR analysis demonstrated that DR5 mRNA levels increased dose-dependently with respect to quercetin (Fig. 4A), demonstrating that quercetin modulates DR5 expression at the transcriptional level. Since p53 [Wu et al., 1999], NF-B [Ravi et al., 2001], CHOP [Son et al., 2007], and Sp1 [Kim et al., 2004b] have been implicated in regulating DR5 expression, we examined their possible involvement in quercetin-induced DR5 up-regulation. DR5 was significantly up-regulated in not only hepatoma cells with wild-type p53 (e.g., HepG2) and but also those with mutant p53 (e.g., SK-Hep1, SNU-387, SNU-423, SNU-449, and SNU-475), suggesting that p53 is not critical for quercetin-induced DR5 up-regulation in hepatoma cells. Furthermore, the activity of NF-B, which was assessed by Western blotting using an anti-phospho-p65 antibody, was not increased by quercetin (data not shown), suggesting that quercetin-induced DR5 up-regulation is independent of NF-B. When we further examined the promoter activity of DR5 using a luciferase gene expression system, we found that treatment of SK-Hep1 cells with 100 M quercetin triggered

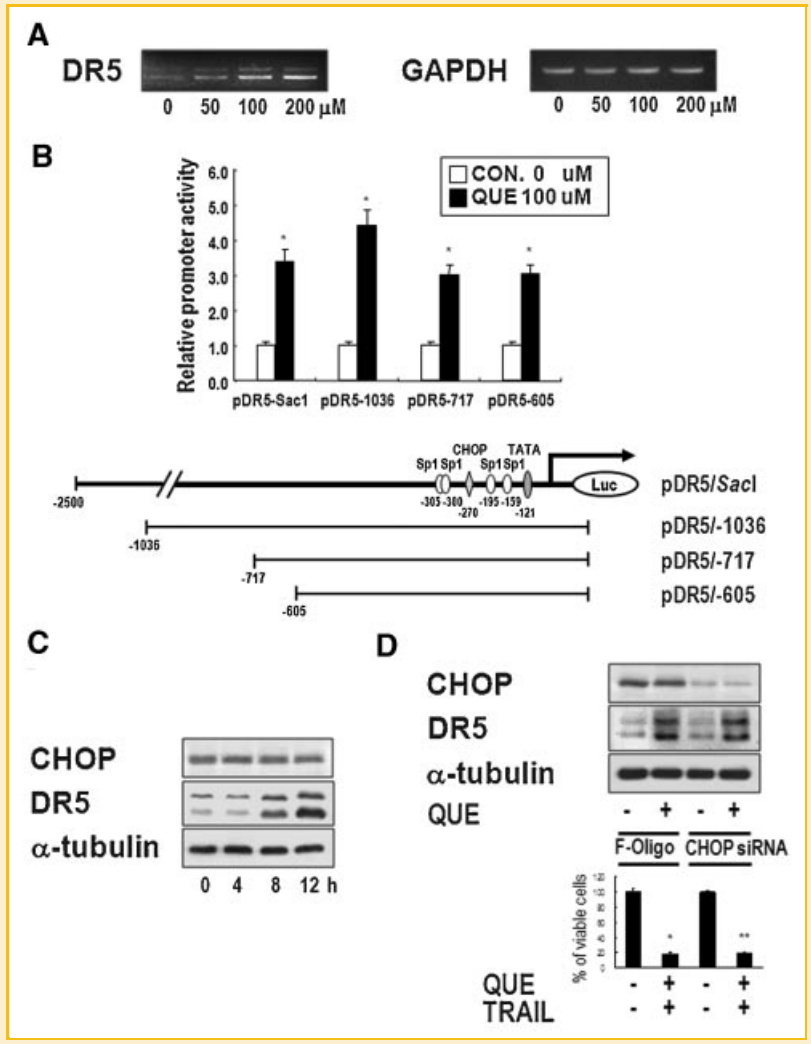

Fig. 4. CHOP is not involved in quercetin-induced enhancement of DR5 up-regulation. A: Changes in the mRNA levels of DR5. SK-Hep1 cells were treated with quercetin at the indicated concentrations. Total RNA was prepared for RT-PCR of DR5. B: Effect of quercetin on DR5 promoter activity. pDR5/SacI, pDR5/−1036, pDR5/−717, or pDR5/−605 promoter construct was transfected into SK-Hep1 cells, which were then treated with varying concentrations of quercetin, lysed, and assayed for luciferase activity. Columns, average of three independent experiments; bars, SE. $^{*}P < 0.001$ compared with untreated cells (top). Schematic structures of the DR5 promoter constructs used for the luciferase activity assays (bottom). C: Effect of quercetin on CHOP protein levels. HepG2 cells were treated with 200 M quercetin for the indicated time points and Western blotting of CHOP and DR5 was performed. Western blotting of -tubulin was used for the loading control. D: Suppression of CHOP expression by its siRNA transfection does not block quercetin-induced DR5 up-regulation and the cell death induced by the combined treatment with quercetin and TRAIL. Effect of CHOP suppression on quercetin-induced DR5 up-regulation was examined by Western blotting (top). Cellular viability was measured using calcein-AM and EthD-1 (bottom). Columns, average of three independent experiments; bars, SE. $^{*}P < 0.001$ compared with untreated cells; $^{**}P < 0.05$ compared with cells transfected with control oligonucleotide and further treated with quercetin plus TRAIL.

similar enhancements of the transcriptional activity from reporter constructs containing 2,500- 1,036-, 717-, and 605 bp fragments of the DR5 gene promoter region (pDR5/*Sac*I, pDR/−1036, pDR5/−717 and pDR5/−605) (Fig. 4B). This suggests that one or more quercetin-responsive elements may be localized within the 605 bp fragment. Recently, Yoshida et al. [2001] showed that a CHOP binding site is localized at −270 bp of the DR5 promoter, and the region of the DR5 promoter spanning nucleotides −605 to +3 contains four putative Sp1 binding sites and a TATA-like box site (Fig. 4B). Therefore, we tested the possible involvement of CHOP or Sp1 in quercetin-mediated DR5 up-regulation. However, CHOP protein levels in HepG2 cells were not up-regulated by quercetin (Fig. 4C) and neither quercetin-induced DR5 up-regulation nor quercetin-stimulated TRAIL-induced apoptosis was blocked by siRNA-mediated knock-down of CHOP expression (Fig. 4D). These results suggest that CHOP is not involved in quercetin-induced DR5 up-regulation in these hepatoma cells. We next examined whether Sp1 is responsible for quercetin-mediated DR5 up-regulation. We first tested whether Sp1 activity is positively controlled by quercetin using a Sp1 reporter construct containing three Sp1 binding sites. Treatment of SK-Hep1 cells with quercetin increased the Sp1-mediated transcriptional activity in a dose-dependent manner (Fig. 5A). Furthermore,

pretreatment with a specific inhibitor of Sp1, mithramycin A, dose-dependently attenuated the quercetin-mediated up-regulation of both DR5 promoter activity and DR5 protein levels (Fig. 5B,C). We further examined which Sp1 site(s) in the DR5 promoter might be important for quercetin-induced DR5 up-regulation. While mSp1-3 (mutated at the −305 and −309 Sp1 sites; Kim et al., 2004b) showed slight decrease in the promoter activity, the promoter activities of Sp1-1 (mutated at the −195 Sp1 sites), mSp1-2 (mutated at the −159 Sp1 sites), mSP1-4 (mutated at the −195 and −159 sites), mSp1-5 (mutated at the −305, −300, and −195 Sp1 sites), and mSP1-6 (mutated at the −305, −300, and −159 sites) were significantly decreased compared with that from the wild-type construct (pDR5/−605) (Fig. 5D). These results suggest that the two putative Sp1-binding sites present at −195 and −159 bp may play important roles in quercetin-induced enhancement of DR5 promoter activity.

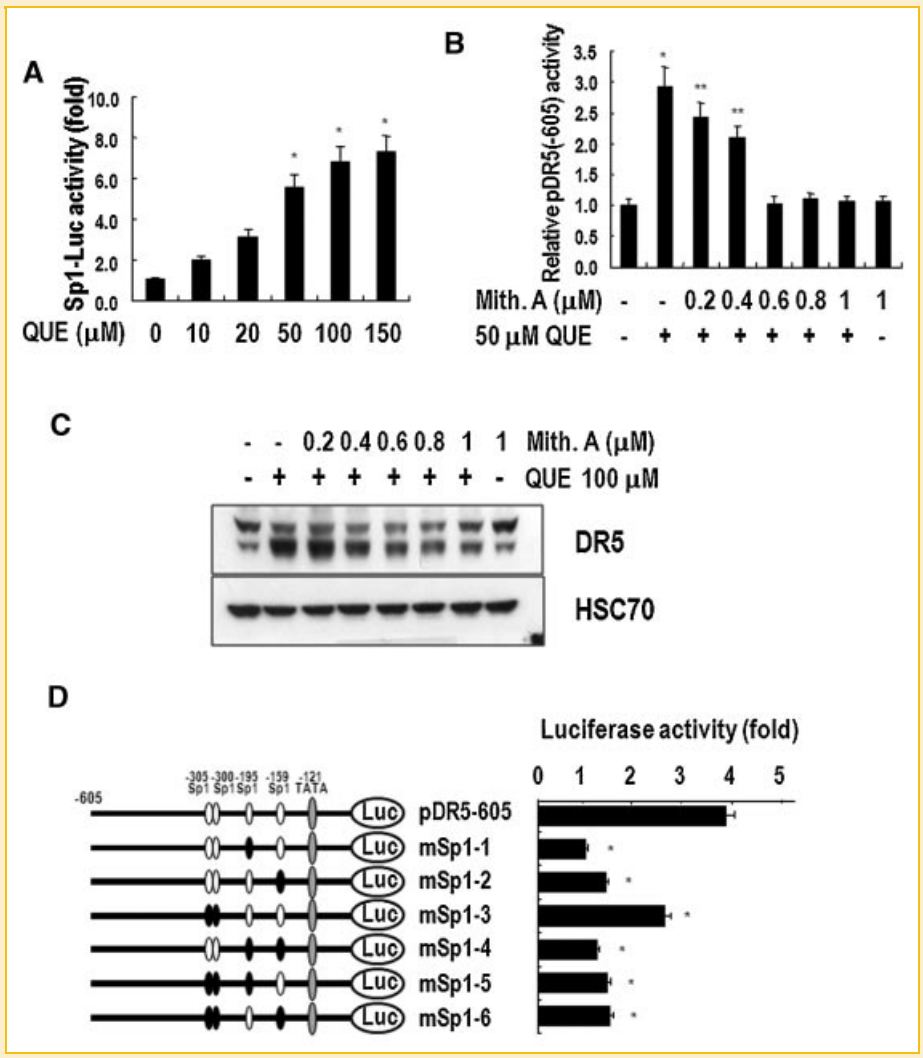

Fig. 5. Sp1 mediates quercetin-induced DR5 up-regulation. A: Effect of quercetin on the transcriptional activity of Sp1. A reporter vector (pSp1-Luc) that has three Sp1-binding sites was transfected into SK-Hep1 cells. Transfected cells were treated with quercetin at the indicated concentrations for 24 h, and lysed and luciferase activity was measured. Columns, average of three independent experiments; bars, SE. $^{*}P < 0.005$ compared with untreated cells. B: Inhibition of Sp1 by treatment with mithramycin A abolishes quercetin-mediated increase in DR5 promoter activity. SK-Hep1 cells were transfected with pDR5-605 promoter construct and further treated with 50 M quercetin, together with or without mithramycin A at the indicated concentrations for 24 h. Cells were lysed and luciferase activity was measured. Columns, average of three independent experiments; bars, SE. $^{*}P < 0.001$ compared with untreated cells; $^{**}P < 0.05$ compared with cells treated with quercetin alone. C: Inhibition of Sp1 by treatment with mithramycin A abolishes quercetin-mediated increase in DR5 protein levels. SK-Hep1 cells were treated with 100 M quercetin, together with or without mithramycin A at the indicated concentrations for 24 h. Cell extracts were prepared for Western blotting of DR5. Western blotting of HSC70 was performed to confirm the equal loading of protein samples. D: Mutational analysis of transcriptional regulatory elements in the DR5 promoter region. Schematic structures of the DR5 promoter constructs used to measure luciferase activity (left). Mutations were introduced into the Sp1 consensus sites of DR5 promoter, SK-Hep1 cells were transfected with the reporter constructs, and lysates from cells treated with or without quercetin were assayed for luciferase activity (right). Columns, average of three independent experiments; bars, SE. $^{*}P < 0.005$ compared with cells transfected with pDR5/−605.

PROTEASOME-MEDIATED DEGRADATION OF c-FLIP$_S$ CONTRIBUTES TO QUERCETIN-SENSITIZED TRAIL-INDUCED APOPTOSIS

Recently, c-FLIP protein levels were shown to correlate with TRAIL resistance in some tumor types [Zhang et al., 2004; Dolcet et al., 2005], and c-FLIP down-regulation has been implicated in chemotherapy-sensitized TRAIL-induced apoptosis [Kelly et al., 2002; Palacios et al., 2006]. Although c-FLIP is expressed as multiple splice variants at the transcript level, two main forms are expressed at the protein level: c-FLIP short form (c-FLIP$_S$), which is 28 kDa in size and contains two death effector domains: and c-FLIP long form (c-FLIP$_L$), which is 55 kDa in size and has two death effector domains and an inactive caspase-like domain [Irmler et al., 1997]. We examined the possibility that c-FLIP might be involved in quercetin-sensitized TRAIL-induced apoptosis in hepatoma cells. Both c-FLIP$_S$ and c-FLIP$_L$ protein levels were significantly reduced by quercetin treatment in all tested hepatoma cells (Fig. 6A), showing that c-FLIP down-regulation is a common response of various hepatoma cells to quercetin. Interestingly, lower doses of quercetin were required for down-regulation of c-FLIP$_S$ protein levels than c-FLIP$_L$. Since RT-PCR analysis showed that the mRNA levels of c-FLIP$_L$ and c-FLIP$_S$ were unchanged following quercetin treatment (Fig. 6B), we examined the possible involvement of the proteasomal pathway in the quercetin-mediated down-regulation of c-FLIP. Pretreatment of cells with the proteasome inhibitor, MG132, considerably recovered quercetin-induced down-regulation of c-FLIP$_L$ and c-FLIP$_S$ (Fig. 6C), suggesting that proteasomal degradation may mediate their quercetin-induced c-down-regulation of c-FLIP$_L$ and c-FLIP$_S$. We next examined the significance of c-FLIP$_L$ and c-FLIP$_S$ down-regulation in quercetin-stimulated TRAIL-induced apoptosis. When we treated SK-Hep1 cell lines stably over-expressing c-FLIP$_L$ or c-FLIP$_S$ with quercetin and TRAIL, the cell death induced by the combined treatment was significantly attenuated by over-expression of c-FLIP$_S$ but not c-FLIP$_L$ (Fig. 6D). Furthermore, TRAIL-mediated apoptosis was dramatically stimulated by siRNA-mediated suppression of c-FLIP$_S$ alone or suppression of both c-FLIP$_S$ and c-FLIP$_L$, compared with suppression of c-FLIP$_L$ alone (Fig. 6E). Therefore, these results demonstrate that down-regulation of c-FLIP$_S$ may be more critical than c-FLIP$_L$ down-regulation for quercetin-sensitized TRAIL-mediated apoptosis in hepatoma cells. Taken together, quercetin recovers TRAIL-sensitivity in various hepatoma cells via Sp1-mediated DR5 up-regulation and proteasome-mediated downregulation of c-FLIP.

DISCUSSION

The cytokine, TRAIL, is considered a promising substance in the field of innovative cancer therapy because it is selectively cytotoxic to tumor cells while sparing normal cells [Walczak et al., 1999; Bruix et al., 2004]. However, various tumor cells, including HCC cells, are resistant to the apoptotic effects of TRAIL [Yamanaka et al., 2000; Pei et al., 2004; Kim et al., 2006]. This resistance could be caused by a variety of factors. For example, TRAIL is known to trigger apoptosis through binding to DR4 and DR5 [Sheridan et al., 1997; Avila et al., 2006; Pan et al., 1997], suggesting that the expression levels of DR4 and/or DR5 may play a role in determining the intensity and/or duration of TRAIL-induced death receptor-mediated apoptotic signaling. In addition, endogenous expression levels of c-FLIP, a protein that has a high level of homology to caspase-8 and -10 but no protease activity, has been correlated with TRAIL resistance in some tumor types [Zhang et al., 2004; Dolcet et al., 2005]. Finally, overexpression of various anti-apoptotic proteins, including IAPs, Bcl-2, Bcl-xL, NF-B, and Akt, may also confer cancer cells resistance to TRAIL [Chawla-Sarkar et al., 2004; Shankar and Srivastava, 2004; Kim et al., 2004a; Bai et al., 2005]. Thus, identification of sensitizers capable of modulating the expression and/or activity levels of these apoptotic regulators may be an effective strategy for overcoming TRAIL resistance in cancer cells.

Quercetin, one of the major dietary flavonoid enriched in various fruits and vegetables, has been reported to exert a broad range of pharmacological effects, including antioxidant and anti-inflammatory activities [Lamson and Brignall, 2000]. For example, chronic quercetin treatment was shown to lower blood pressure or prevent the development of hypertension in various experimental models [Duarte et al., 2001; Perez-Vizcaino et al., 2006]. Consumption of quercetin, enriched in onions and apples, was found to be inversely associated with lung cancer risk [Le Marchand et al., 2000]. Furthermore, quercetin was shown to inhibit cancer growth and induce apoptosis in animal models [Mouria et al., 2002]. Moreover, quercetin treatment has been associated with selective antiproliferative effects [Nair et al., 2004] and induction of cell death, predominantly through an apoptotic mechanism, in cancer cell lines but not in normal cells [Chowdhury et al., 2005]. Although the underlying mechanisms governing these effects are not yet fully understood, the available evidence collectively indicates that quercetin may be of therapeutic benefit in clinical settings, suggesting its potential use as an anticancer agent or an adjunct to current cancer therapies. We demonstrate here that combined treatment with subtoxic doses of quercetin and TRAIL effectively induces apoptotic cell death in various TRAIL-resistant hepatoma cells.

Bcl-xL is a significant prognostic factor for the progression of human hepatocellular carcinoma, and its expression is known to confer resistance to various pro-apoptotic therapeutics [Takehara et al., 2001]. Furthermore, mutations in the *p53* gene have been found in many human cancers, including up to 50% of HCCs [Hsu et al., 1991], and many cancer cells with mutations in p53 resist chemotherapy-induced cell death [Velculescu and El-Deiry, 1996]. Interestingly, the cell death induced by quercetin plus TRAIL was not blocked by Bcl-xL overexpression, and occurred both in human hepatoma cells expressing wild-type p53 (HepG2 cells; Hsu et al., 1993) and hepatoma cells expressing mutant p53 (SK-Hep1, SNU-387, SNU-423, SNU-449, and SNU-474; Hsu et al., 1993; Kang et al., 1996). Therefore, these results suggest that combination treatment with quercetin and TRAIL may provide an effective therapeutic strategy for the treatment of HCC, which are often resistant to various anti-cancer therapies due to Bcl-xL overexpression and/or defects in p53 function. Combined treatment with quercetin and TRAIL as a potential new strategy for treating TRAIL-resistant hepatomas seems to warrant additional study including in vivo experiments with animal models.

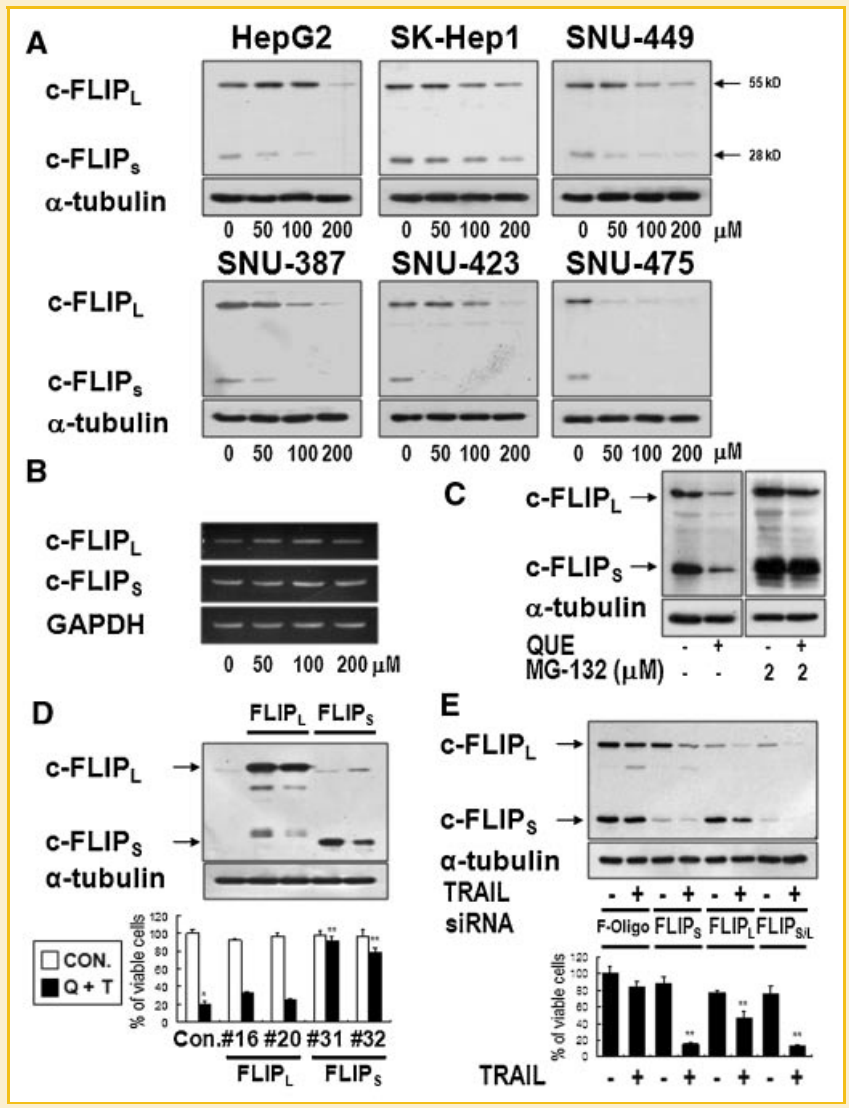

Fig. 6. Quercetin-induced c-FLIP$_S$ down-regulation contributes to quercetin-stimulated TRAIL-induced apoptosis. A: Quercetin-induced down-regulation of FLIP. Various hepatoma cells were treated with quercetin at the indicated concentrations for 12 h. Cell extracts were prepared for Western blotting of FLIP$_L$ and FLIP$_S$. Western blotting of -tubulin was performed to show equal loading of protein samples. B: mRNA levels of c-FLIP$_L$ or c-FLIP$_S$ are not altered by quercetin treatment. Total RNA was extracted from SK-Hep1 cells treated with 200 M quercetin for 24 h and RT-PCR of c-FLIP$_L$, c-FLIP$_S$, and GAPDH was performed. C: Proteasome-mediated degradation may be involved in quercetin-induced c-FLIP down-regulation. SK-Hep1 cells were pretreated with MG132 at the indicated concentration for 30 min and further treated with 200 M quercetin for 12 h. Protein levels of c-FLIP were analyzed by Western blotting. D: Quercetin-sensitized TRAIL-induced apoptosis is inhibited in the stable cell lines overexpressing c-FLIP$_S$. Protein levels of the c-FLIP$_S$ in the stable cell lines overexpressing c-FLIP$_L$ or c-FLIP$_S$ were examined by Western blotting (top). Control cells (SK-Hep1 cells stably transfected with pcDNA3) and the stable cell lines overexpressing c-FLIP$_S$ were untreated or treated with 200 M quercetin and 100 ng/ml TRAIL for 12 h and cell viability was measured using calcein-AM and EthD-1 (bottom). Columns, average of three independent experiments; bars, SE. $^{*}P < 0.001$ compared with untreated cells; $^{\dagger}P < 0.001$ compared with control cells treated with quercetin and TRAIL. E: Suppression of c-FLIP$_S$ expression, rather than c-FLIP$_L$, by its siRNA transfection blocks quercetin-stimulated TRAIL-induced apoptosis. SK-Hep1 cells were transfected with control fluorescent oligonucleotide, c-FLIP$_S$ siRNA alone, c-FLIP$_L$ siRNA alone, or siRNA against both c-FLIP$_S$ and c-FLIP$_L$. Transfected cells were untreated or treated with 200 M quercetin plus 100 ng/ml TRAIL for 12 h. Suppression of c-FLIP$_S$ protein levels were confirmed by Western blotting (top). Cellular viability was measured using calcein-AM and EthD-1 (bottom). Columns, average of three independent experiments; bars, SE. $^{*}P < 0.001$; $^{\dagger}P < 0.01$ compared with cells transfected with control oligonucleotide and further treated with TRAIL.

Recently, Kim and Lee [2007] reported that treatment with quercetin sensitizes prostate cancer cells to TRAIL-mediated apoptosis through Akt dephosphorylation. In addition, Chen et al. [2007] reported that treatment with quercetin sensitizes non-small cell lung cancer cells to TRAIL-mediated apoptosis via DR5 induction and survivin suppression. However, we found that Akt phosphorylation was somewhat enhanced in HepG2, SK-Hep1, SNU-449 and SNU-387 cells, whereas it was decreased in SNU-423 and SNU-475 cells (Supplementary Fig. 2A). Furthermore, survivin protein levels were considerably increased in SNU-449 and SNU-387 cells, while they were down-regulated in HepG2, SK-HEP1, SNU-423, and SNU-475 cells (Supplementary Fig. 2B). We cannot exclude the possibility that either down-regulation of Akt activity or survivin expression in the respective hepatoma cells may contribute

to the recovery of TRAIL sensitivity. However, quercetin-induced Akt inhibition or suppression of survivin may not be a common mechanism explaining the sensitizing effects of quercetin on TRAIL-induced apoptosis in hepatoma cells. In addition, quercetin is known to decrease HSP70 and many cancer cell lines show resistance to apoptosis because of high levels of HSP70 [Wei et al., 1994; Aghdassi et al., 2007]. Therefore, we examined the possible involvement of HSP70 in the quercetin-stimulated TRAIL-induced apoptosis. HSP70 protein levels were unaltered by treatment with 200 M quercetin for 24 h in various hepatoma cells, although they were slightly decreased in SK-Hep1 and SNU-387 cells treated with quercetin for 48 h (Supplementary Fig. 2C). Since about 60–80% of cells are killed by the combined treatment with quercetin and TRAIL within 24 h (Fig. 1A), these results suggest that HSP70 may not be associated with the sensitizing effect of quercetin on TRAIL-mediated apoptosis in hepatoma cells. In contrast, quercetin treatment induced significant increases in DR5 protein levels in all tested hepatoma cells. The critical role of DR5 up-regulation in quercetin-stimulated TRAIL-mediated apoptosis was confirmed by our finding that siRNA-mediated knockdown of DR5 expression inhibited the cell death induced by the combination of quercetin and TRAIL. Recently, we showed that the generation of reactive oxygen species (ROS) plays a critical role in the up-regulation of DR5 and the induction of TRAIL-mediated apoptosis [Kim et al., 2006]. However, pretreatment with the antioxidant, NAC, did not reduce the quercetin-induced DR5 up-regulation observed in our study (data not shown), suggesting that ROS are not involved in quercetin-induced DR5 up-regulation. Recent studies have shown that DR5 up-regulation is critical for endoplasmic reticulum stress-mediated apoptosis in human cancer cells, and that this process involves CHOP, a member of the C/EBP family of transcription factors [Yamaguchi and Wang, 2004; Yoshida et al., 2005]. However, quercetin treatment did not induce CHOP protein levels, and knockdown of CHOP expression by siRNA duplexes did not inhibit the cell death induced by combined treatment with quercetin and TRAIL, demonstrating that the sensitizing effect of quercetin is independent of CHOP. We did, however, develop a number of lines of evidence clearly demonstrating the involvement of the Sp1 transcription factor in quercetin-induced DR5 up-regulation, including the findings that: (a) quercetin treatment dose-dependently increased the Sp1-mediated transcriptional activity in hepatoma cells; (b) inhibition of Sp1 activity by mithramycin A abolished quercetin-induced DR5 up-regulation; and (c) quercetin-induced activation of DR5 promoter activity was abrogated by the mutations in the −195 bp and/or −159 bp Sp1 binding sites of the DR5 promoter.

Another mechanism potentially involved in the regulation of TRAIL sensitivity is expressional modulation of c-FLIP [Kelly et al., 2002; Zhang et al., 2004; Dolcet et al., 2005; Palacios et al., 2006; Son et al., 2007]. This caspase-8 inhibitor is predominantly expressed as two forms (c-$FLIP_L$ and c-$FLIP_S$; Irmler et al., 1997). c-$FLIP_S$ completely inhibits procaspase-8 activation at the death-inducing signaling complex, while c-$FLIP_L$ permits partial cleavage of procaspase-8 to an intermediate p41/p43 form but prevents further processing of procaspase-8 to its active p18/p10 subunits [Micheau et al., 2002]. In the present study, quercetin significantly down-regulated c-$FLIP_S$ protein levels versus c-$FLIP_L$ protein levels in all tested hepatoma cells. We found that quercetin-induced down-regulation of c-FLIP is not controlled at the transcriptional level, but rather is mediated via the proteasomal pathway, as shown by our observation that pretreatment with MG132 attenuated the quercetin-induced down-regulation of c-$FLIP_L$ and c-$FLIP_S$. Similar to our results, a recent study showed that $FLIP_S$ is more prone to ubiquitination and has a considerably shorter half-life than $FLIP_L$ [Poukkula et al., 2005]. Very interestingly, we found that overexpression of c-$FLIP_S$, but not c-$FLIP_L$, significantly reduced the apoptosis induced by quercetin/TRAIL treatment. Furthermore, siRNA-mediated suppression of c-$FLIP_S$ alone or c-$FLIP_S$ plus c-$FLIP_L$ (c-$FLIP_{S/L}$), but not suppression of c-$FLIP_L$ alone, led to significant enhancement of TRAIL-mediated apoptosis. These results highlight the functional significance of c-$FLIP_S$ down-regulation in quercetin-stimulated TRAIL-mediated apoptosis. Since both DR5 and c-FLIP are known to be key components controlling the initial steps of the death receptor-mediated apoptotic signaling pathway, our findings that quercetin up-regulates DR5 and down-regulates c-$FLIP_S$ provide new insights into the potential underlying mechanisms necessary to overcome TRAIL-resistance in hepatoma cells, via amplification of TRAIL-mediated apoptotic signaling pathway.

ACKNOWLEDGMENTS

This work was supported by the grants from KOSEF (BAERI-M20708630003-07B0863-00310) and Korea Research Foundation (KRF-2005-042-E00020).

REFERENCES

Aghdassi A, Phillips P, Dudeja V, Dhaulakhandi D, Sharif R, Dawra R, Lerch MM, Saluja A. 2007. Heat shock protein 7 increases tumorigenicity and inhibits apoptosis in pancreatic adenocarcinoma. Cancer Res 67:616–625.

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z, Schwall RH. 1999. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 104:155–162.

Avila MA, Berasain C, Sangro B, Prieto J. 2006. New therapies for hepatocellular carcinoma. Oncogene 25:3866–3884.

Bai J, Sui J, Demirjian A, Vollmer CM Jr, Marasco W, Callery MP. 2005. Predominant Bcl-XL knockdown disables antiapoptotic mechanisms: Tumor necrosis factor-related apoptosis-inducing ligand-based triple chemotherapy overcomes chemoresistance in pancreatic cancer cells in vitro. Cancer Res 65: 2344–2352.

Brit DF, Hendrich S, Wang W. 2001. Dietary agents in cancer prevention: Flavonoids and isoflavonoids. Pharmacol Ther 90:157–177.

Bruix J, Boix L, Sala M, Llovet JM. 2004. Focus on hepatocellular carcinoma. Cancer Cell 5:215–219.

Budagova KR, Zhmaeva SV, Grigor'ev AN, Goncharova AY, Kabakov AE. 2003. Flavonoid dihydroquercetin, unlike quercetin, fails to inhibit expression of heat shock proteins under conditions of cellular stress. Biochemistry (Mosc) 68:1055–1061.

Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ, Borden EC. 2004. Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. Cell Death Differ 11: 915–923.

Chen W, Wang X, Zhuang J, Zhang L, Lin Y. 2007. Induction of death receptor 5 and suppression of survivin contribute to sensitization of TRAIL-induced cytotoxicity by quercetin in non-small cell lung cancer cells. Carcinogenesis 28:2114–2121.

Chowdhury SA, Kishino K, Satoh R, Hashimoto K, Kikuchi H, Nishikawa H, Shirataki Y, Sakagami H. 2005. Tumor-specificity and apoptosis-inducing activity of stilbenes and flavonoids. Anticancer Res 25:2055–2063.

Csokay B, Prajda N, Weber G, Olah E. 1997. Molecular mechanisms in the antiproliferative action of quercetin. Life Sci 60:2157–2163.

Dolcet X, Llobet D, Pallares J, Rue M, Comella JX, Matias-Guiu X. 2005. FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. Lab Invest 85:885–894.

Duarte J, Pérez-Palencia R, Vargas F, Ocete MA, Pérez-Vizcaino F, Zarzuelo A, Tamargo J. 2001. Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. Br J Pharmacol 133:117–124.

Habtemariam S. 1997. Flavonoids as inhibitors or enhancers of the cytotoxicity of tumor necrosis factor-alpha in L-929 tumor cells. J Nat Prod 60:775–778.

Havsteen R. 1983. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 32:1141–1148.

Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. 1991. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 350:427–428.

Hsu IC, Tokiwa T, Bennett W, Metcalf RA, Welsh JA, Sun T, Harris CC. 1993. p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. Carcinogenesis 14:987–992.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schröter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. 1997. Inhibition of death receptor signals by cellular FLIP. Nature 388:190–195.

Kang MS, Lee HJ, Lee JH, Ku JL, Lee KP, Kelley MJ, Won YJ, Kim ST, Park JG. 1996. Mutation of p53 gene in hepatocellular carcinoma cell lines with HBX DNA. Int J Cancer 67:898–902.

Kelly MM, Hoel BD, Voelkel-Johnson C. 2002. Doxorubicin pretreatment sensitizes prostate cancer cell lines to TRAIL induced apoptosis which correlates with the loss of c-FLIP expression. Cancer Biol Ther 1:520–527.

Kim YH, Lee YJ. 2007. TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. J Cell Biochem 100:998–1009.

Kim EH, Kim SU, Shin DY, Choi KS. 2004a. Roscovitine sensitizes glioma cells to TRAIL-mediated apoptosis by downregulation of survivin and XIAP. Oncogene 23:446–456.

Kim YH, Park JW, Lee JY, Kwon TK. 2004b. Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells. Carcinogenesis 25:1813–1820.

Kim H, Kim EH, Eom YW, Kim WH, Kwon TK, Lee SJ, Choi KS. 2006. Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant hepatoma cells to TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of DR5. Cancer Res 66:1740–1750.

Lamson DW, Brignall MS. 2000. Antioxidants and cancer. Part 3. Quercetin. Altern Med Rev 5:196–208.

Le Marchand L, Murphy SP, Hankin JH, Wilkens LR, Kolonel LN. 2000. Intake of flavonoids and lung cancer. J Natl Cancer Inst 92:154–160.

Loke WM, Proudfoot JM, Stewart S, McKinley AJ, Needs PW, Kroon PA, Hodgson JM, Croft KD. 2008. Metabolic transformation has a profound effect on anti-inflammatory activity of flavonoids such as quercetin: Lack of association between antioxidant and lipoxygenase inhibitory activity. Biochem Pharmacol 75:1045–1053.

Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grütter MG. 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J Biol Chem 277:45162–45167.

Mouria M, Gukovskaya AS, Jung Y, Buechler P, Hines OJ, Reber HA, Pandol SJ. 2002. Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome c release and apoptosis. Int J Cancer 98:761–769.

Nair H, Rao K, Aalinkeel R, Mahajan S, Chawda R, Schwartz S. 2004. Inhibition of prostate cancer cell conlony formation by the flavonoid quercetin correlates with modulation of specific regulatory genes. Clin Diagnostic Lab Immunol 11:63–69.

Palacios C, Yerbes R, Lopez-Rivas A. 2006. Flavopiridol induces cellular FLICE-inhibitory protein degradation by the proteasome and promotes TRAIL-induced early signaling and apoptosis in breast tumor cells. Cancer Res 66:8858–8869.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM. 1997. The receptor for the cytotoxic ligand TRAIL. Science 276:111–113.

Pei Z, Chu L, Zou W, Zhang Z, Qiu S, Qi R, Gu J, Qian C, Liu X. 2004. An oncolytic adenoviral vector of Smac increases antitumor activity of TRAIL against HCC in human cells and in mice. Hepatology 39:1371–1381.

Perez-Vizcaino F, Duarte J, Andriantsitohaina R. 2006. Endothelial function and cardiovascular disease: Effects of quercetin and wine polyphenols. Free Radic Res 40:1054–1065.

Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamäki T, Johnson MS, Sistonen L, Eriksson JE. 2005. Rapid turnover of c-FLIP short is determined by its unique C-terminal tail. J Biol Chem 280: 27345–27355.

Ravi R, Bedi GC, Engstrom LW, Zeng Q, Mookerjee B, Gélinas C, Fuchs EJ, Bedi A. 2001. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. Nat Cell Biol 3:409–416.

Shankar S, Srivastava RK. 2004. Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: Mechanisms and clinical implications. Drug Resist Updat 7:139–156.

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P, Ashkenazi A. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277:818–821.

Son YG, Kim EH, Kim JY, Kim SU, Kwon TK, Yoon AR, Yun CO, Choi KS. 2007. Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. Cancer Res 67:8274–8284.

Srivastava RK. 2001. TRAIL/Apo-2L: Mechanisms and clinical applications in cancer. Neoplasia 3:535–546.

Surh YJ. 2003. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3:768–780.

Takehara T, Liu X, Fujimoto J, Friedman SL, Takahashi H. 2001. Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology 34:55–61.

Thomas LR, Henson A, Reed JC, Salsbury FR, Thorburn A. 2004. Direct binding of Fas-associated death domain (FADD) to the tumor necrosis factor-related apoptosis-inducing ligand receptor DR5 is regulated by the death effector domain of FADD. J Biol Chem 279:32780–32785.

Velculescu VE, El-Deiry WS. 1996. Biological and clinical importance of the p53 tumor suppressor gene. Clin Chem 42:858–868.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 5:157–163.

Wei YQ, Zhao X, Kariya Y, Fukata H, Teshigawara K, Uchida A. 1994. Induction of apoptosis by quercetin: Involvement of heat shock protein. Cancer Res 54:4952–4957.

Wu GS, Burns TF, McDonald ER III, Meng RD, Kao G, Muschel R, Yen T, el-Deiry WS. 1999. Induction of the TRAIL receptor Killer/DR5 in p53-dependent apoptosis but not growth arrest. Oncogene 18:6411–6418.

Xiao J, Suzuki M, Jiang X, Chen X, Yamamoto K, Ren F, Xu M. 2008. Influence of B-ring hydroxylation on interactions of flavonols with bovine serum albumin. J Agric Food Chem 56:2350–2356.

Yamaguchi H, Wang HG. 2004. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem 279:45495–45502.

Yamanaka T, Shiraki K, Sugimoto K, Ito T, Fujikawa K, Ito M, Takase K, Moriyama M, Nakano T, Suzuki A. 2000. Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. Hepatology 32:482–490.

Yoshida T, Maeda A, Tani N, Sakai T. 2001. Promoter structure and transcription initiation sites of the human death receptor 5/TRAIL-R2 gene. FEBS Lett 507:381–385.

Yoshida T, Shiraishi T, Nakata S, Horinaka M, Wakada M, Mizutani Y, Miki T, Sakai T. 2005. Proteasome inhibitor MG132 induces death receptor 5 through CCAAT/enhancer-binding protein homologous protein. Cancer Res 65:5662–5667.

Zhang X, Jin TG, Yang H, DeWolf WC, Khosravi-Far R, Olumi AF. 2004. Persistent c-FLIP$_L$ expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. Cancer Res 64:7086–7091.