

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

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Abstract

Sulforaphane is a chemopreventive agent present in various cruciferous vegetables, including broccoli. Here, we show that treatment with tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) in combination with subtoxic doses of sulforaphane significantly induces rapid apoptosis in TRAIL-resistant hepatoma cells. Neither TNF- α - nor Fas-mediated apoptosis was sensitized in hepatoma cells by cotreatment with sulforaphane, suggesting that sulforaphane can selectively sensitize cells to TRAIL-induced apoptosis but not to apoptosis mediated by other death receptors. We found that sulforaphane treatment significantly up-regulated mRNA and protein levels of DR5, a death receptor of TRAIL. This was accompanied by an increase in the generation of reactive oxygen species (ROS). Pretreatment with *N*-acetyl-L-cysteine and overexpression of catalase inhibited sulforaphane-induced up-regulation of DR5 and almost completely blocked the cotreatment-induced apoptosis. Furthermore, the sulforaphane-mediated sensitization to TRAIL was efficiently reduced by administration of a blocking antibody or small interfering RNAs for DR5. These results collectively indicate that sulforaphane-induced generation of ROS and the subsequent up-regulation of DR5 are critical for triggering and amplifying TRAIL-induced apoptotic signaling. We also found that sulforaphane can sensitize both Bcl-xL- and Bcl-2-overexpressing hepatoma cells to TRAIL-induced apoptosis, indicating that treatment with a combination of TRAIL and sulforaphane may be a safe strategy for treating resistant hepatomas. (Cancer Res 2006; 66(3): 1740-50)

Introduction

The 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 (1). TRAIL induces apoptosis in tumor cells via the death receptor pathway using a mechanism similar to that of TNF (2). TRAIL cross-links with the death receptors DR4 or DR5,

leading to aggregation of the receptors, recruitment of the adaptor molecule FADD, and activation of initiator caspase-8 (3). The activated caspase-8 is released into the cytoplasm and initiates a protease cascade that activates “effector” caspases, such as caspase-3 and caspase-7 (4).

Hepatocellular carcinoma is the most common type of liver cancer and is the fourth leading cause of cancer deaths worldwide (5). Surgical resection has been considered the optimal treatment approach, but only a small proportion of patients qualify for surgery, and there is a high rate of recurrence. Approaches to prevent recurrence have included chemoembolization before and neoadjuvant therapy after surgery, neither of which has been proven to be beneficial (6). Therefore, new therapeutic options are needed for more effective treatment of this malignancy. Although TRAIL has garnered considerable attention as a novel anticancer agent, recent studies have shown that many cancer cells, including hepatoma cells, are resistant to the apoptotic effects of TRAIL (7, 8). A better understanding of the molecular mechanisms underlying TRAIL resistance and identification of the sensitizing agents capable of overcoming this resistance may facilitate the establishment of TRAIL-based combination regimens for the improved treatment of hepatocellular carcinoma.

Epidemiologic studies have shown that increased dietary consumption of cruciferous vegetables may protect against tumorigenesis (9), suggesting the potential use in the chemoprevention of cancer. Sulforaphane [1-isothiocyanato-4-(methylsulfanyl)-butane], a naturally occurring member of the isothiocyanate family, has received particular attention because of its anticancer effects (10). Sulforaphane seems to modulate the carcinogenic metabolism by inhibiting cytochrome *P*450–dependent monooxygenases, which are involved in the activation of carcinogenic chemicals (11), and/or by inducing phase II detoxification enzymes (12). Furthermore, accumulating evidence indicates that sulforaphane can inhibit growth of human cancer cells by causing cell cycle arrest and apoptosis (13, 14), suggesting its potential therapeutic value as an anticancer agent or an adjunct to current cancer therapies.

We show herein for the first time that sulforaphane is a potent sensitizer for TRAIL-induced apoptosis not only in a variety of TRAIL-resistant human hepatocellular carcinoma cells but also in hepatoma cells overexpressing Bcl-xL or Bcl-2. Moreover, we present the first evidence that subtoxic doses of sulforaphane up-regulate expression of DR5 via generation of reactive oxygen species (ROS), leading to rapid induction of TRAIL-mediated signaling and cell death in these hepatoma cells.

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Materials and Methods

Chemicals and antibodies. Recombinant human TRAIL/Apo2 ligand (the nontagged 19-kDa protein, amino acids 114-281) and TNF- α were from KOMA Biotech, Inc. (Seoul, South Korea). Anti-Fas antibody was from Upstate Biotechnology (Lake Placid, NY). Calcein-AM, EthD-1, and 6-carboxy-2',7'-dichlorofluorescein diacetate (H₂DCFDA) were from Molecular Probes (Eugene, OR). Sulforaphane and *N*-acetyl-L-cysteine (NAC) were from Sigma (St. Louis, MO). The following antibodies were used: anti-caspase-8, caspase-3, caspase-7, survivin, and X-linked inhibitor of apoptosis (XIAP; Stressgen, British Columbia, Canada); anti-caspase-9, caspase-2, FAK, Cdk2, DR4, c-IAP1, c-IAP2, Bcl-2, and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA); poly(ADP-ribose) polymerase (PARP; Upstate Biotechnology); Bid (Cell Signaling, Beverly, MA); anti-Flag M2 and FITC-conjugated anti-goat IgG (Sigma); anti-DR5 for Western blotting (Calbiochem, San Diego, CA); anti-DR5 antibody for fluorescence-activated cell sorting (FACS) analysis and DR5-specific blocking chimera antibody (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 and rat hepatocytes. The human hepatoma cell lines Hep3B, Huh-7, and HepG2 were cultured in DMEM (Life Technologies, Grand Island, NY). SNU-398, SNU-423, and SNU-449 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (Life Technologies). Pregnant BD rats were used in this study. Hepatocytes from 5-week-old fetal rats were isolated by collagenase disruption as described previously (15). The cells were incubated in 7.5% CO₂ at 37°C to facilitate attachment and the medium was changed after 4 hours. The hepatocytes used were at least 90% to 95% viable immediately after isolation.

DNA fragmentation assay. After treatments, cells were lysed in a buffer containing 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, and 0.5% Triton X-100 for 30 minutes on ice. Lysates were vortexed and cleared by centrifugation at 10,000 \times *g* for 20 minutes. Fragmented DNA in the supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 1.8% agarose gels containing 0.1 μ g/mL ethidium bromide.

Plasmids, transfections, and luciferase assays. The pDR5/*SacI* plasmid [containing DR5 promoter sequence (-2,500/+3)] and pDR5/-605 [containing DR5 promoter sequence (-605/+3)] were gifts from Dr. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). To localize the promoter regions responsible for sulforaphane-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 (16). In brief, Hep3B cells were plated onto 60-mm dishes at a density of 5 \times 10⁵ per plate and grown overnight. Cells were cotransfected with 1 μ g of various plasmid constructs and 0.2 μ g of the pCMV- β -galactosidase plasmid for 3 hours using LipofectAMINE Plus reagent (Life Technologies) following the manufacturer's instructions. After incubation for 24 hours, transfected cells were further treated or untreated with 10 μ mol/L sulforaphane. Luciferase and β -galactosidase activities were assayed according to the manufacturer's protocol (Promega, Madison, WI). Luciferase activity was normalized for β -galactosidase activity in cell lysates and expressed as an average of three independent experiments.

Semiquantitative reverse transcription-PCR analysis. Total RNA was extracted from Hep3B cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Following the manufacturer's protocol [RNA PCR kit (avian myeloblastosis virus); TaKaRa Shuzo Co., Ltd. Japan], reverse transcription-PCR (RT-PCR) was done. Conditions for final analysis were chosen when amplification of mRNA was in the middle of the exponential amplification phase for 10 μ mol/L sulforaphane. Human DR5 mRNA was amplified using the sense primer 5'-GTCTGCTCTGATCACCCAAC-3' and the antisense primer 5'-CTGCAACTGTGACTCCTATG-3' (corresponding to a 424-bp region of DR5). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sense primer 5'-CGTCTTCACCATGGAGA-3' and the antisense primer 5'-CGGCCATCACGCCACAGTTT-3' was used (corresponding to a 310-bp region of GAPDH).

The PCR cycling conditions (30 cycles) were chosen were as follows: (a) 30 seconds at 94°C, (b) 1 minute at 70°C for DR5 and 30 seconds at 60°C for GAPDH, and (c) 1 minute at 72°C with a subsequent 10-minute 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, 5 \times 10⁵ cells were stained with 200 μ L PBS containing saturating amounts of anti-DR4 or anti-DR5 antibody on ice for 30 minutes. After incubation, cells were washed twice and reacted with FITC-conjugated rabbit anti-goat IgG on ice for 30 minutes. 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 25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and had the following sequences: DR5 (F01), UUUAGCCACCUUUAUCUCAUUGUCC; DR5 (E11), ACAGCAUCGUGUACAAGGUGUCCC; DR5 (E09), UACAACACCGACCUUGACCAUCC; and green fluorescent protein (GFP), AAGACCCGCGCGAGGUGAAG. Cells were transfected with siRNA oligonucleotides using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations.

Measurement of ROS. Hep3B or HepG2 cells were plated at a density of 5 \times 10⁵ or 1 \times 10⁶, respectively, in 60-mm dishes, allowed to attach overnight, and exposed to 5 mmol/L NAC alone, 10 μ mol/L sulforaphane alone, or NAC plus sulforaphane for specified time intervals. The cells were stained with 10 μ mol/L H₂DCFDA for 10 minutes at 37°C and then observed under a fluorescence microscope (Axiovert 200M, Carl Zeiss). Alternatively, the fluorescence intensity of dichlorofluorescein in cells was determined using the flow cytometer (Becton Dickinson and Co.).

Construction of the expression vector encoding human catalase. Plasmid DNA encoding human catalase was kindly provided by Dr. M. Akashi (National Institute of Radiological Sciences, Chiba, Japan). The human catalase cDNA was amplified by PCR using primers designed to incorporate a 5' hemagglutinin (HA) epitope. The PCR product was subcloned into the pcDNA3.1(+) expression vector (Invitrogen). The fidelity of the PCR and cloning procedures was verified by nucleotide sequencing.

Establishment of the cell lines stably overexpressing Bcl-2, Bcl-xL, or catalase. Mammalian expression vectors encoding Flag-tagged Bcl-xL and Bcl-2 were kindly provided by Prof. A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia). Hep3B cells were transfected with the expression vectors encoding Flag-tagged Bcl-xL, Bcl-2, or HA-tagged catalase. Stable Hep3B cell lines overexpressing Bcl-xL or Bcl-2 were selected with changes of fresh medium containing puromycin (4 μ g/mL). Overexpression of Bcl-xL or Bcl-2 in the stable cell lines was analyzed by Western blotting using anti-Flag antibody (Sigma). Stable Hep3B cell lines overexpressing catalase were selected with changes of fresh medium containing G418 (500 μ g/mL). Overexpression of catalase in the stable cell lines was examined by Western blotting using anti-HA antibody (Covance, Princeton, NJ).

Results

Subtoxic doses of sulforaphane significantly sensitize TRAIL-resistant hepatoma cells to TRAIL-induced apoptosis.

The cytotoxic activity of human recombinant soluble TRAIL (amino acids 114-281) was tested in six hepatoma cell lines: Huh-7, Hep3B, HepG2, SNU-398, SNU-423, and SNU-449 (Fig. 1A). Treatment with 50 to 200 ng/mL TRAIL induced a limited cell death (<10%) over 24 hours, suggesting that these hepatoma cells are resistant to the apoptotic effects of TRAIL. Next, we examined the cytotoxic effects of sulforaphane alone or in combination with TRAIL in these cells. Sulforaphane alone did not induce any morphologic signs of cell death up to 10 μ mol/L, although the cellular activity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was slightly decreased at this concentration. However, cell

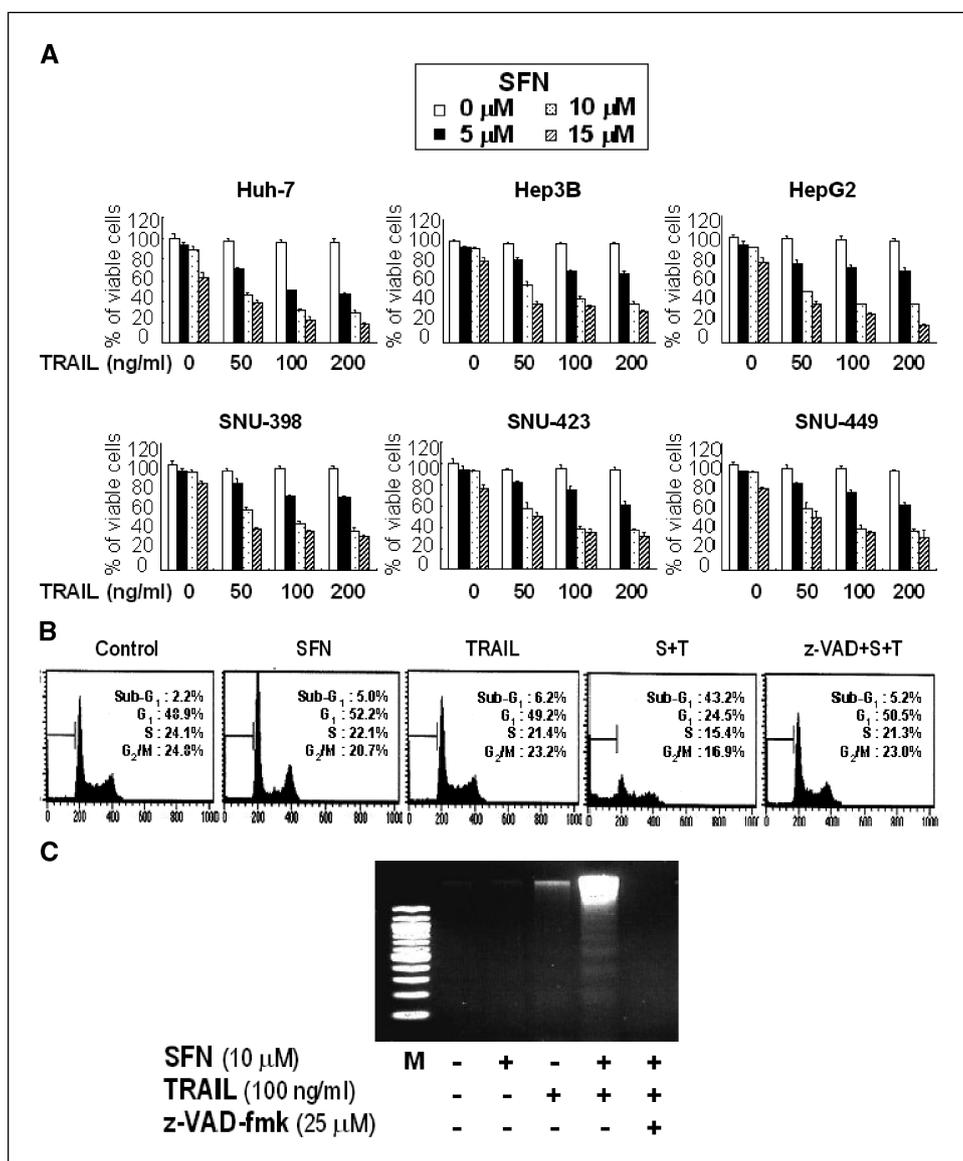


Figure 1. Effect of sulforaphane and/or TRAIL on hepatoma cell viability. **A**, hepatoma cells were treated with sulforaphane (SFN) for 30 minutes and further treated with or without TRAIL for 24 hours at the indicated concentrations. Cellular viability was assessed by MTT assay. *Columns*, average of three individual experiments; *bars*, SD. **B**, DNA contents of Huh-7 cells treated with 10 μmol/L sulforaphane alone, 100 ng/mL TRAIL alone, or sulforaphane + TRAIL (S+T) for 16 hours. To examine the effect of the inhibition of caspases, Huh-7 cells were pretreated with 25 μmol/L z-VAD-fmk (z-VAD) for 30 minutes and further treated with sulforaphane + TRAIL for 16 hours. DNA contents of the cells were analyzed by flow cytometry. **C**, DNA fragmentation by the combined treatment with sulforaphane and/or TRAIL. After treatment of Hep3B cells as indicated for 16 hours, fragmented DNAs were extracted from the treated cells and analyzed on 1.8% agarose gel.

viability was significantly reduced by the combined treatment both when holding the concentration of TRAIL fixed and varying the concentration of sulforaphane and when holding the concentration of sulforaphane fixed and varying TRAIL. These results show that combined treatment with sulforaphane and TRAIL effectively induces cell death in the tested TRAIL-resistant hepatoma cell lines. We then investigated whether apoptotic cell death is induced by a combination of sulforaphane and TRAIL using flow cytometric analysis, which detects the increase in hypodiploid cell populations. Cotreatment of Huh-7 cells with 10 μmol/L sulforaphane and 100 ng/mL TRAIL for 16 hours significantly increased the accumulation of sub-G₁ phase cells, whereas treatment with sulforaphane or TRAIL alone did not (Fig. 1B). Pretreatment with a pan-caspase inhibitor z-VAD-fmk significantly blocked the accumulation of sub-G₁-phase cell populations induced by sulforaphane plus TRAIL. Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed a typical ladder pattern of internucleosomal DNA fragmentation in Hep3B cells cotreated with 10 μmol/L sulforaphane and 100 ng/mL TRAIL but not in cells treated with sulforaphane or TRAIL alone (Fig. 1C). DNA fragmentation induced

by sulforaphane plus TRAIL was completely blocked by the pretreatment with z-VAD-fmk. Collectively, these results suggest that sulforaphane stimulates TRAIL-induced, caspase-dependent apoptosis.

We next examined whether caspases were actually activated during sulforaphane-facilitated, TRAIL-induced cell death of hepatoma cells (Fig. 2A). Huh-7 cells were treated with 10 μmol/L sulforaphane alone for 16 hours or 100 ng/mL TRAIL alone or pretreated with sulforaphane (30 minutes) followed by TRAIL for the indicated times. Treatment with 10 μmol/L sulforaphane alone for 16 hours did not induce any proteolytic processing of caspases. In response to TRAIL, the 32-kDa procaspase-3 was partially cleaved to a 20-kDa intermediate form after 4 hours, but further cleavage into the active p17 subunit was not detected nor was other caspase-processing events. However, treatment with sulforaphane plus TRAIL induced the cleavage of caspase-3 into the p20 intermediate form and its subsequent cleavage into the active p17 subunit after 4 hours. Caspase-2, caspase-8, caspase-9, and caspase-7 were also progressively processed after 4 to 8 hours of the combined treatment. We further assessed the cleavage of several

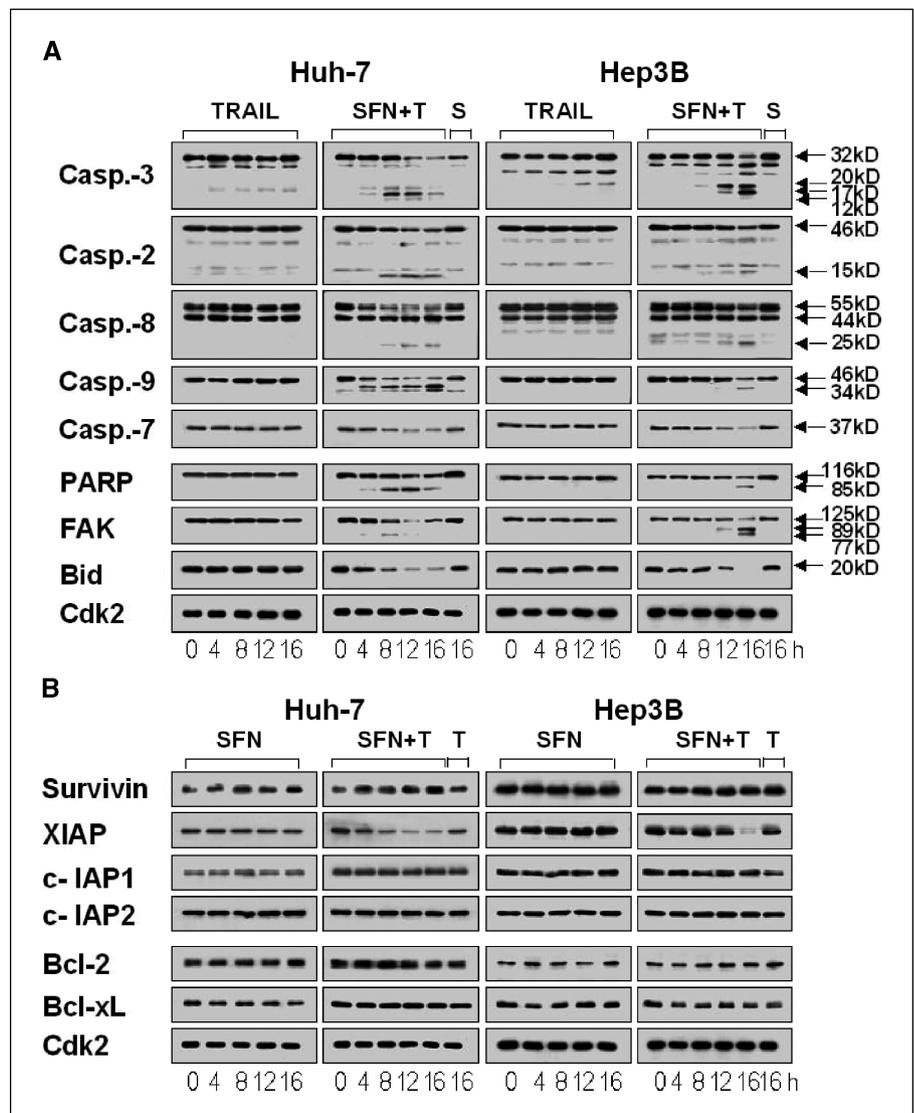
key death substrates that indicate the activation of caspases, including PARP (substrate for caspase-3 and caspase-7), FAK (caspase-3), and Bid (caspase-8). In parallel with the proteolytic processing of caspases, these substrate proteins were progressively degraded from 4 to 8 hours after the combined treatment, whereas they were not degraded following treatment with TRAIL or sulforaphane alone. Similar but slower activation patterns of caspases in Hep3B cells were observed in response to sulforaphane plus TRAIL, consistent with the slower progression of cell death in this cell line compared with that in Huh-7 cells.

Sulforaphane does not affect the expression levels of IAPs or antiapoptotic Bcl-2 family proteins. Recent reports have shown that several intracellular proteins, including survivin, XIAP, Bcl-2, and Bcl-xL, are capable of inhibiting death receptor-mediated apoptosis when present at sufficient levels in cancer cells (17–19). To explore the underlying mechanisms by which sulforaphane enhances TRAIL-induced apoptosis in TRAIL-resistant hepatoma cells, we first examined the possibility that sulforaphane might down-regulate the expression levels of these antiapoptotic proteins. We did not observe any significant differences in the protein levels of the tested IAP protein (survivin, XIAP, c-IAP1, and c-IAP2) or the

tested antiapoptotic Bcl-2 family proteins (Bcl-2 and Bcl-xL) following treatment with 10 $\mu\text{mol/L}$ sulforaphane alone or 100 ng/mL TRAIL alone (Fig. 2B). Cotreatment with sulforaphane and TRAIL induced down-regulation of XIAP proteins but did not alter the expressions of the other proteins in Huh-7 and Hep3B cells. Because XIAP has been reported previously to be a substrate of caspase-3, caspase-7, and caspase-9 during apoptosis (20) and pretreatment with z-VAD, a pan-caspase inhibitor, blocked down-regulation of XIAP following treatment with sulforaphane and TRAIL (data not shown), this reduction of XIAP protein levels might be the result of caspase activation in response to the combined treatment rather than the cause of sulforaphane-stimulated TRAIL-induced apoptosis.

Sulforaphane up-regulates DR5 in various hepatoma cells. As the TNF superfamily members reportedly share similar protein structures and death receptor-mediated apoptotic signaling pathways (21), we next tested whether sulforaphane could also sensitize TNF- α - and/or Fas-mediated apoptosis, possibly targeting the common component(s) of these death receptor-mediated apoptotic pathways. Consistent with our TRAIL results, treatment of Hep3B cells with TNF- α or anti-Fas antibody alone did not induce

Figure 2. Effect of TRAIL and/or sulforaphane on the expressions of caspases and various intracellular regulators of apoptosis. **A**, activation of caspases during apoptosis induced by the combined treatment with sulforaphane and TRAIL. Huh-7 and Hep3B cells were treated with 100 ng/mL TRAIL alone, 10 $\mu\text{mol/L}$ sulforaphane alone, or a combination of both for the indicated time points. Cell extracts were prepared for Western blotting for caspases. To confirm the activation of caspases, Western blotting of the substrate proteins of caspases was also done. **B**, Western blotting of IAPs, Bcl-2, and Bcl-xL. The immunoblotting for each protein was done at least twice using independently prepared lysates, and the results were similar.



significant cell death (Fig. 3A). Interestingly, cotreatment of these agents plus sulforaphane had no effect on cell death in Hep3B cells, which were very sensitive to sulforaphane/TRAIL cotreatment. These results indicate that sulforaphane selectively facilitates cell death induced by TRAIL but not the other tested death ligand(s). Because TRAIL is known to trigger apoptotic signals via two types of death receptors, DR4 and DR5 (22, 23), we next examined whether the modulation of DR4 and/or DR5 protein levels by sulforaphane might be involved in its sensitizing effect on TRAIL-induced apoptosis. We found that treatment of Hep3B cells with sulforaphane induced a dose-dependent increase in the protein levels of DR5 but did not affect the levels of DR4 (Fig. 3B). FACS analysis showed that the sulforaphane-induced surface expression of DR5 but not DR4 was also significantly increased in Hep3B cells (Fig. 3C). Consistent with this, treatment with 10 $\mu\text{mol/L}$ sulforaphane significantly increased DR5 protein levels in other human hepatoma cell lines, such as Huh-7, SNU-398, SNU-423, and HepG2 (Fig. 3D), showing that up-regulation of the DR5 TRAIL death receptor is a common response of hepatoma cells to sulforaphane treatment. To clarify the functional role of DR5 in stimulation of TRAIL-induced apoptosis by sulforaphane, we examined the effect of DR5-specific blocking chimera antibody on sulforaphane/TRAIL-induced apoptosis. Addition of DR5-specific blocking antibody dose-dependently inhibited sulforaphane/TRAIL-induced apoptosis of Hep3B cells (Fig. 3E). Furthermore, suppression of DR5 expression by transfection of Hep3B cells with three kinds of siRNAs also effectively inhibited sulforaphane-stimulated TRAIL-induced cell death (Fig. 3F), supporting the idea that sulforaphane-induced up-regulation of DR5 is critical for the enhancement of TRAIL sensitivity in Hep3B cells.

Sulforaphane activates transcription from the DR5 promoter. To examine whether sulforaphane-induced DR5 up-regulation is controlled at the transcriptional level, we did RT-PCR analysis of DR5 in sulforaphane-treated Hep3B cells. We found that treatment with 10 $\mu\text{mol/L}$ sulforaphane was followed by a gradual increase in DR5 mRNA levels by 12 hours (Fig. 4A). We further explored the underlying mechanisms involved in sulforaphane-induced transcriptional control of DR5 using the luciferase gene expression system. We first examined the effects of sulforaphane on the promoter activities of reporter constructs containing 2.5- and 0.6-kb fragments of the DR5 gene promoter region (pDR5/*SacI* and pDR5/-605, respectively; ref. 24). Hep3B cells were transfected with these constructs and the luciferase activities were assayed 24 hours after sulforaphane treatment at different doses. We found that sulforaphane significantly increased the promoter activities of both pDR5/*SacI* and pDR5/-605 in a dose-dependent manner (Fig. 4B), suggesting that sulforaphane-responsive elements are localized within the smaller fragment (605 bp). Previously, Yoshida et al. (24) showed that the region of the DR5 promoter spanning nucleotides -605 to +3 contains typical transcription factor binding sites, including four Sp1 sites and a TATA-like box site (Fig. 4B). To examine which Sp1 site(s) in the DR5 promoter is important for sulforaphane-induced DR5 up-regulation, we did luciferase assays using reporter constructs with mutations at the different Sp1-binding sites (Fig. 4C). Transfection with mSp1-3 (mutated at the -305 and -300 Sp1 sites), mSp1-5 (mutated at the -305, -300, and -195 Sp1 sites), and mSp1-6 (mutated at the -305, -300, and -159 sites; ref. 16) showed significantly decreased sulforaphane-induced DR5 promoter activities compared with the wild-type construct (pDR5/-605). Although introduction of any Sp1 site mutation decreased the DR5 promoter activity to some

extent, these significant decreases suggest that the two putative Sp1-binding sites present at -305/-300 may play an important role in sulforaphane-induced enhancement of DR5 promoter activity.

Sulforaphane-induced ROS generation plays a critical role in the up-regulation of DR5 and the induction of cell death by cotreatment with sulforaphane and TRAIL. As Singh et al. (25) reported recently that ROS initiate sulforaphane-induced cell death in prostate cancer cells, we next examined whether ROS were also generated in hepatoma cells treated with subtoxic doses of sulforaphane. H₂DCFDA-based FACS detection revealed that intracellular ROS levels increased in Hep3B cells following treatment with 10 $\mu\text{mol/L}$ sulforaphane (Fig. 5A). Increased dichlorofluorescein fluorescence was detected as early as 30 minutes after treatment with 10 $\mu\text{mol/L}$ sulforaphane (data not shown), peaked at 4 hours (Fig. 5A), and diminished afterward. NAC is a widely used thiol-containing antioxidant that is a precursor of reduced glutathione (GSH). GSH scavenges ROS in cells by interacting with OH \cdot and H₂O₂, thus affecting ROS-mediated signaling pathways. The sulforaphane-induced increases in ROS levels were completely blocked by pretreatment with NAC (Fig. 5A). Similar results were obtained from fluorescence microscopic observation after staining sulforaphane-treated Hep3B cells or HepG2 cells with H₂DCFDA (data not shown). Next, we examined the role of sulforaphane-induced ROS generation in the up-regulation of DR5. Western blotting showed that pretreatment with NAC caused a significant dose-dependent inhibition of sulforaphane-induced up-regulation of DR5 protein (Fig. 5B). Moreover, RT-PCR analysis showed that pretreatment with NAC significantly inhibited sulforaphane-induced increases in DR5 mRNA (Fig. 5C). We further investigated whether overexpression of catalase could inhibit sulforaphane-induced DR5 up-regulation. Sulforaphane-induced up-regulation of DR5 was significantly attenuated in stable cell lines overexpressing catalase compared with control cells (Fig. 5D). We also tested whether treatment of hepatoma cells with H₂O₂ could elevate DR5 protein levels. We found that H₂O₂ time- and dose-dependently increased the protein levels of DR5 in HepG2 cells (Fig. 5E). Collectively, these results clearly show that sulforaphane-induced up-regulation of DR5 requires the generation of ROS. We next tested whether scavenging of ROS could attenuate the cell death induced by cotreatment with sulforaphane and TRAIL. Pretreatment of Hep3B with NAC dose-dependently blocked cell death induced by the combination of sulforaphane and TRAIL (Fig. 5F). Moreover, the cell death induced by the combination of sulforaphane and TRAIL was significantly attenuated in Hep3B cells overexpressing catalase, suggesting that ROS generation is required for the induction of cell death. Taken together, these results indicate that sulforaphane-induced ROS generation plays a key role in its ability to up-regulate the DR5 TRAIL receptor and therefore contributes to its dramatic enhancement of TRAIL-induced apoptosis.

Combined treatment with sulforaphane and TRAIL enhances cell death in hepatoma cells overexpressing Bcl-xL or Bcl-2 but not in rat primary hepatocytes. Because increased expression of Bcl-xL in human hepatocellular carcinoma is important for the inhibition of apoptosis that is initiated by various cellular stresses (26), we next investigated whether overexpression of Bcl-xL or Bcl-2 could affect the sensitizing effect of sulforaphane on TRAIL-induced apoptosis. Stable Bcl-xL- and Bcl-2-overexpressing cell lines were established using Hep3B cells (Fig. 6A), and the viabilities of these cells following treatment with 10 $\mu\text{mol/L}$ sulforaphane and 100 ng/mL TRAIL for 24 hours were measured by staining with

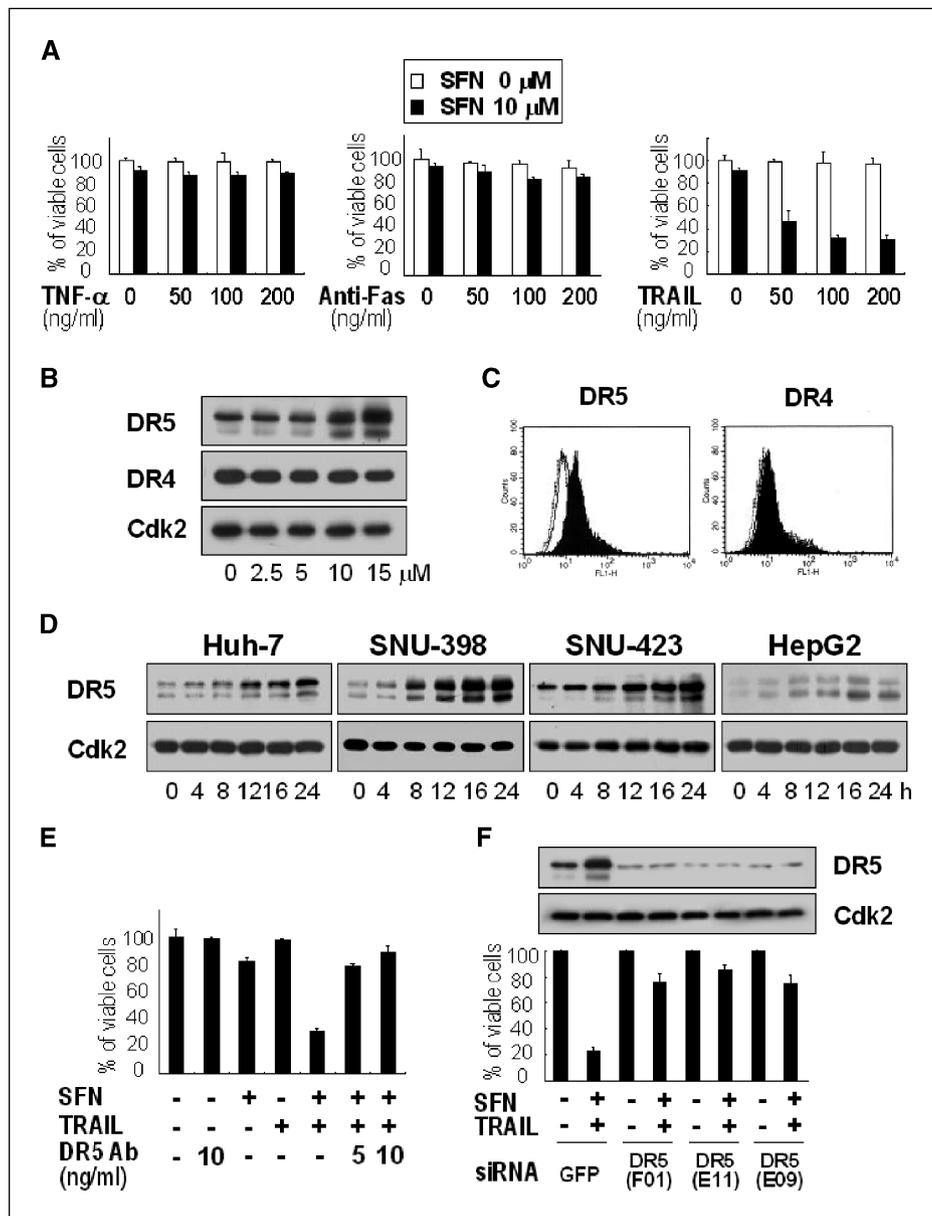


Figure 3. Sulforaphane increases DR5 but not DR4 protein levels in many hepatoma cell lines. **A**, effect of sulforaphane on TNF- α - or Fas-mediated cell death. Hep3B cells were pretreated with 10 μ mol/L sulforaphane for 30 minutes and further treated with the indicated concentrations of TNF- α , anti-Fas antibody, or TRAIL for 24 hours. Cellular viabilities were measured with calcein-AM and EthD-1 to detect live and dead cells, respectively. Similar results were obtained from three independent experiments. **B**, sulforaphane-induced DR5 up-regulation in Hep3B cells. Hep3B cells were treated with the indicated concentrations of sulforaphane for 16 hours, and Western blotting of DR5, DR4, and Cdk2 as a loading control was done. **C**, effect of sulforaphane on the surface expression levels of DR5 and DR4 proteins. Hep3B cells were incubated with or without 10 μ mol/L sulforaphane for 16 hours and the surface expression of DR5 and DR4 proteins was analyzed by flow cytometry. *X* axis, fluorescence intensity; *Y* axis, relative number of cells. *Black histograms*, treated cells with sulforaphane; *clear histograms*, untreated cells; *histograms with gray lines*, negative control (i.e., cells incubated in the absence of the respective primary antibody). Representative experiment from a total of three. **D**, sulforaphane-induced DR5 up-regulation in several human hepatoma cells. Cells were treated with 10 μ mol/L sulforaphane for the indicated times and cell extracts were prepared for Western blotting of DR5. Cdk2 was used for a loading control of Western blotting. Representative results of two independent experiments. **E**, effect of DR5-specific blocking chimera antibody on sulforaphane/TRAIL-induced apoptosis. Hep3B cells were pretreated with or without 10 μ mol/L sulforaphane for 30 minutes followed by treatment with or without 100 ng/mL TRAIL for 24 hours in the presence of indicated concentrations of DR5-specific blocking chimera antibody. Cellular viabilities were measured with calcein-AM and EthD-1 to detect live and dead cells, respectively. Similar results were obtained from three independent experiments. **F**, suppression of DR5 expression by siRNAs reduces sulforaphane-stimulated TRAIL-induced apoptosis in Hep3B cells. Hep3B cells were transfected with GFP siRNA or three kinds of siRNA duplexes against DR5 mRNA. Twenty-four hours after the transfection, cells were treated with 10 μ mol/L sulforaphane for 16 hours. First, Western blotting of DR5 was done to confirm the down-regulation of DR5 by siRNA transfection. Cdk2 levels were assessed to show equal gel loading (*top*). To examine the effect of DR5 down-regulation on sulforaphane/TRAIL-induced apoptosis, Hep3B cells were transfected with siRNAs, incubated for 24 hours, and further treated with or without 10 μ mol/L sulforaphane + 100 ng/mL TRAIL for 24 hours. Cellular viability was determined using calcein-AM and EthD-1 (*bottom*).

calcein-AM and EthD-1. Interestingly, overexpression of Bcl-xL or Bcl-2 in Hep3B cells had no effect on the sensitizing effect of sulforaphane on TRAIL-induced apoptosis (Fig. 6B). These results suggest that combined treatment with sulforaphane and TRAIL

may have a therapeutic effect on hepatoma cells overexpressing Bcl-xL, which are resistant to many other chemotherapeutic drugs.

Next, we examined whether sulforaphane plus TRAIL shows cytotoxicity in normal hepatocytes in addition to hepatoma cells.

Primary hepatocytes isolated from rats were treated for 24 hours with 10 $\mu\text{mol/L}$ sulforaphane alone, 100 ng/mL TRAIL alone, or sulforaphane plus TRAIL, and viabilities were assessed using calcein-AM and EthD-1 (Fig. 6C). We did not observe increased cell death in hepatocytes treated with TRAIL alone, sulforaphane alone, or sulforaphane plus TRAIL compared with untreated hepatocytes. When we further investigated the effect of sulforaphane on DR5 expression in primary hepatocytes, we found that DR5 protein levels were not altered in hepatocytes treated with either sulforaphane and/or TRAIL compared with those in Hep3B cells treated with the same agent(s) (Fig. 6D). These results suggest that differential regulation of sulforaphane-induced DR5 expression may be responsible for the selective toxicity of the combined treatment against hepatoma cells. In conclusion, our results collectively indicate that combined treatment with sulforaphane and TRAIL may provide an effective treatment strategy for TRAIL-resistant hepatomas.

Discussion

Members of the TNF receptor superfamily, including TNF receptor, Fas, and TRAIL receptor, share similar conserved structures (21). Although TNF- α and FasL can trigger apoptosis

in some solid tumors, their clinical usage has been limited by the risk of lethal systemic inflammation and hepatotoxicity, respectively (27, 28). In contrast, recombinant soluble human TRAIL (amino acids 114-281) has shown a profound apoptotic effect on the xenografted melanoma cells without toxicity to human hepatocytes *in vitro* and *in vivo* (29). These results indicate that this form of TRAIL may prove to be a safe and effective biological agent for cancer therapy in humans. However, recent studies have shown that considerable numbers of cancer cells, including human hepatocellular carcinoma cells, are resistant to the apoptotic effects of TRAIL (7, 8, 30, 31). Cellular sensitivity to TRAIL can be affected by the expression levels of the cell membrane TRAIL receptors, caspase-8, or c-FLIP (32). Further downstream in the TRAIL-induced apoptotic pathway, Bax mutations (33) or increased expression of IAP family members, such as XIAP and survivin, also cause resistance (17, 18). Thus, scientists are currently seeking to identify TRAIL sensitizers capable of overcoming TRAIL resistance in cancer cells.

In this study, we investigated the ability of sulforaphane, a naturally occurring member of the isothiocyanate family of chemopreventive agents, to enhance TRAIL-induced apoptosis in human hepatoma cells. We show for the first time that subtoxic

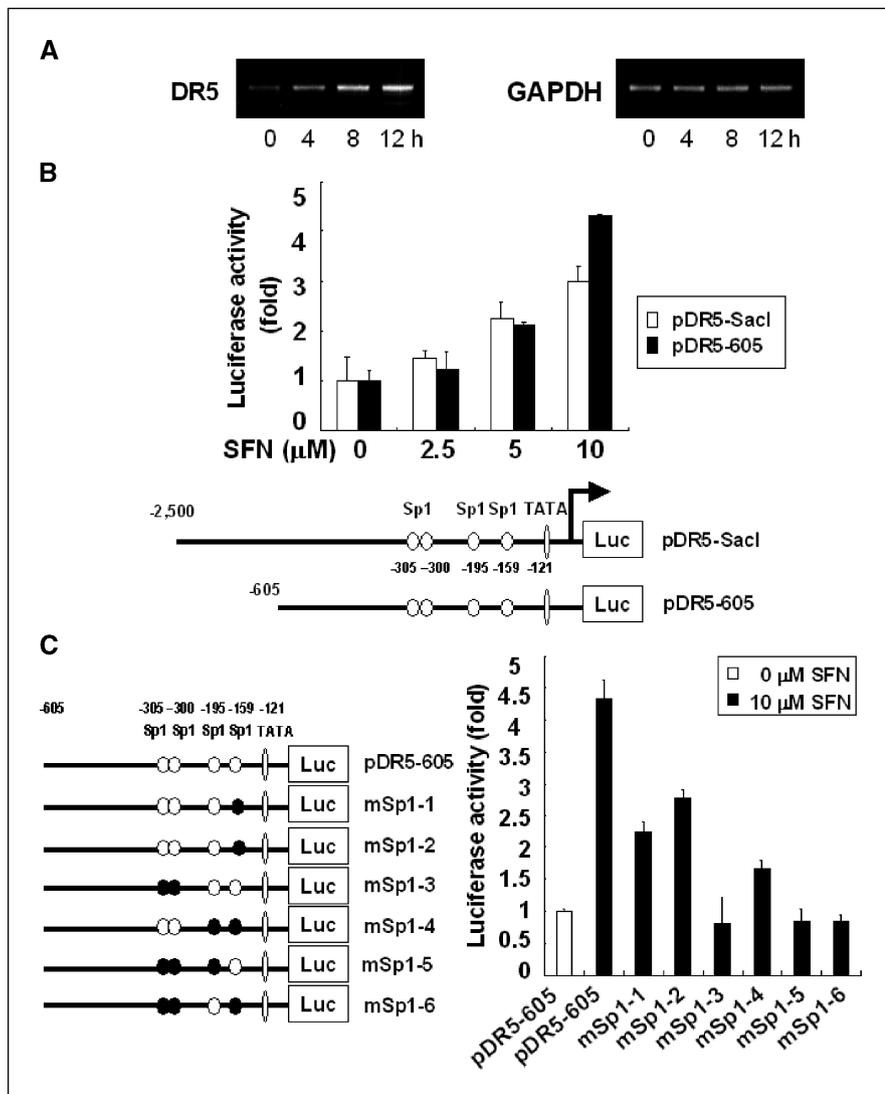
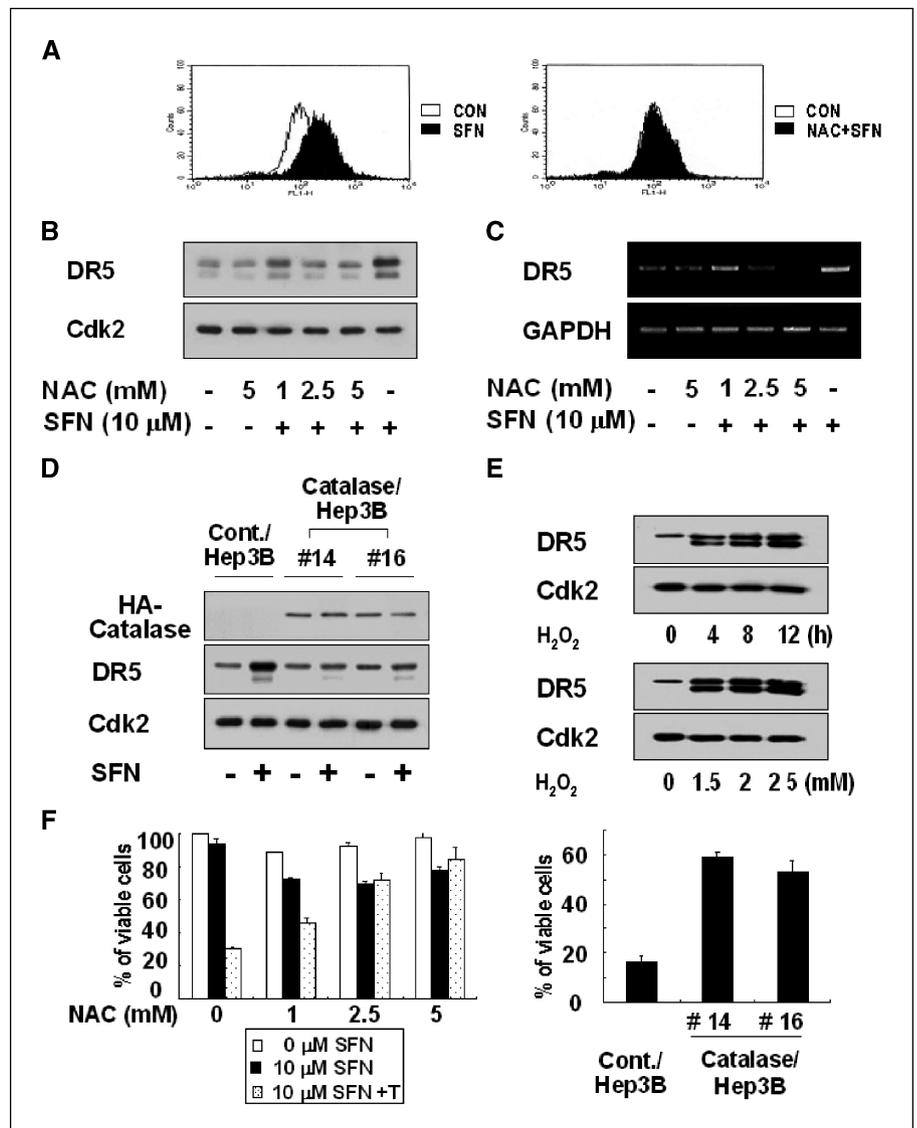


Figure 4. Sulforaphane activates transcription from the DR5 promoter. *A*, effect of sulforaphane on DR5 mRNA levels. Hep3B cells were treated with 10 $\mu\text{mol/L}$ sulforaphane for the indicated time points, total RNA was isolated, and RT-PCR analysis of DR5 and GAPDH was done. *B*, effect of sulforaphane on DR5 promoter activity. pDR5/Sacl or pDR5/-605 was transfected into Hep3B cells, which were then treated with varying concentrations of sulforaphane, lysed, and assayed for luciferase activity. *Columns*, mean of at least three independent experiments; *bars*, SD (*top*). *Bottom*, schematic structures of the DR5 promoter constructs used for the luciferase activity assays. *C*, mutational analysis of transcriptional regulatory elements in the DR5 promoter region. *Left*, schematic structures of the DR5 promoter constructs used to measure luciferase activity. Mutations were introduced into the Sp1 consensus sites, Hep3B cells were transfected with the reporter constructs, and lysates from cells treated with or without sulforaphane were assayed for luciferase activity. Representative of at least three independent experiments.

Figure 5. ROS generation is critical for sulforaphane-induced DR5 up-regulation and sulforaphane/TRAIL-induced cell death. **A**, detection of sulforaphane-induced ROS accumulation by FACS analysis. Hep3B cells were pretreated with or without 5 mmol/L NAC and then treated with or without 10 μ mol/L sulforaphane for 4 hours followed by addition of 10 μ mol/L H₂DCFDA and subsequent FACS analysis for intracellular accumulation of ROS. *X axis*, fluorescence intensity; *Y axis*, relative number of cells. *Black histograms*, treated cells as indicated; *clear histograms*, untreated cells. Representative experiment from a total of three. **B**, NAC treatment blocks sulforaphane-induced increases in DR5 protein levels. Hep3B cells were pretreated with NAC at the indicated concentrations for 30 minutes and further treated with or without 10 μ mol/L sulforaphane for 12 hours. Cell extracts were prepared for Western blotting of DR5, with Cdk2 examined as a loading control. Representative of three independent experiments. **C**, NAC treatment blocks sulforaphane-induced increases in DR5 mRNA levels. Hep3B cells were treated as described in (B) and cell extracts were prepared for RT-PCR of DR5 and GAPDH. **D**, catalase overexpression blocks sulforaphane-induced DR5 up-regulation. Stable cell lines overexpressing HA-tagged catalase were established in Hep3B cells and their expressions were examined using anti-HA antibody. Control Hep3B or catalase-overexpressing Hep3B cells (*Catalase/Hep3B #14* and *#16*) were treated with or without 10 μ mol/L sulforaphane for 12 hours and cell extracts were prepared for Western blotting of DR5, with Cdk2 examined as a loading control. **E**, effect of H₂O₂ on DR5 protein levels. Cell extracts were prepared from HepG2 cells treated with 2 mmol/L H₂O₂ for the indicated time points or HepG2 cells treated with the indicated concentrations of H₂O₂ for 12 hours. Western blotting of DR5 was done. **F**, treatment with NAC or catalase overexpression blocks the cell death induced by sulforaphane + TRAIL. Hep3B cells were pretreated with NAC at the indicated concentrations for 30 minutes and further treated with 10 μ mol/L sulforaphane + 100 ng/mL TRAIL for 24 hours (*left*). Control Hep3B or catalase-overexpressing Hep3B cells were treated with 10 μ mol/L sulforaphane + 100 ng/mL TRAIL for 24 hours (*right*). Cellular viability was assessed using calcein-AM and EthD-1. Similar results were obtained from three independent experiments.



doses of sulforaphane sensitize many TRAIL-resistant hepatoma cells to TRAIL-induced apoptosis by up-regulating DR5. We showed that sulforaphane causes a significant dose- and time-dependent increase in the levels of mRNA and protein for DR5 but not DR4 in hepatoma cells. A DR5-specific blocking chimeric antibody and knockdown of DR5 expression by siRNA duplexes inhibited the cell death induced by the combination of sulforaphane and TRAIL in Hep3B cells, confirming that this up-regulation of DR5 is functionally significant.

The expression levels of death receptors may play a critical role in determining the intensity and/or duration of death receptor-mediated apoptotic signaling in response to death ligands. TRAIL is known to trigger apoptosis through binding to the death receptors, DR4 (22) and DR5 (22, 23), which contain cytoplasmic death domains responsible for recruiting adaptor molecules involved in caspase activation (3). At physiologic conditions (37°C), TRAIL is known to bind with a higher affinity to DR5 than to DR4 (34). Moreover, a recent study using phage display of death receptor-selective TRAIL variants showed that DR5 may play a more prominent role than DR4 in mediating apoptotic

signals emanating from TRAIL in cells expressing both death receptors (35). Recently, various agents, including DNA-damaging agents, such as ionizing irradiation and many anticancer drugs (36, 37), histone deacetylase inhibitors (16, 38), bile acids (39), IFN- α (40), triterpenoid methyl-2-cyano-3,12-dioxooleana-1 (41), and proteasome inhibitors (42), have been reported to up-regulate DR5 expression. This indicates that the combination of TRAIL and the agents that are capable of up-regulating DR5 may be a promising strategy for sensitizing tumors to TRAIL-induced apoptosis. Although c-Jun NH₂-terminal kinase (JNK) has been proposed to be involved in DR5 up-regulation by bile acid (39) or methyl-2-cyano-3,12-dioxooleana-1 (41), JNK was not significantly activated by sulforaphane, and pretreatment with SP600125, a specific inhibitor of JNK, could not reduce the sulforaphane-induced DR5 up-regulation in our study (data not shown), suggesting that novel mechanisms may be responsible for sulforaphane-induced DR5 up-regulation.

Sulforaphane has been reported to suppress proliferation or various cancer cells by causing cell cycle arrest, apoptosis, or both (14, 25, 43). For example, sulforaphane-induced cell cycle arrest

is associated with activation of Chk2 and the subsequent accumulation of Tyr¹⁵-phosphorylated (inactive) Cdk1 (43). Furthermore, up-regulation of Bax and down-regulation of Bcl-2 occur during sulforaphane-induced apoptosis in human T-cell leukemia (14). In our study, however, subtoxic doses of sulforaphane did not cause any significant changes in the protein levels for various cell cycle regulators (data not shown) or for Bcl-2 or Bcl-xL. Recently, Singh et al. reported that treatment with 40 μmol/L sulforaphane induces apoptosis in PC-3 cells and that the initial signal for sulforaphane-induced apoptosis is derived from ROS (25). They showed that pretreatment with NAC or overexpression of catalase significantly inhibits sulforaphane-induced apoptosis. Similarly, we found that exposure of Hep3B or HepG2 cells to a subtoxic dose (10 μmol/L) of sulforaphane induces ROS generation. Sulforaphane-induced ROS generation was evident as early as 30 minutes and as late as 6 hours after treatment with sulforaphane, whereas a significant increase in the mRNA and protein levels of DR5 was observed only after 8 hours. Pretreatment with NAC or overexpression of catalase blocked the sulforaphane-induced increase in DR5 expression. In addition, treatment with H₂O₂ significantly enhanced DR5 protein levels. Finally, pretreat-

ment of Hep3B cells with NAC or overexpression of catalase blocked the induction of cell death by cotreatment with sulforaphane and TRAIL. These results collectively show that ROS act as upstream signaling molecules for the initiation of sulforaphane-induced DR5 expression and are critical for the sensitization of the cells to TRAIL-induced apoptosis.

To explore the mechanisms underlying sulforaphane-induced ROS generation, we tested the possibility that depletion of GSH might be associated with sulforaphane-induced ROS generation in hepatoma cells. Consistent with the findings of Singh et al. (25), treatment of HepG2 cells with apoptosis-inducing doses of sulforaphane (>20 μmol/L) significantly decreased intracellular GSH levels (data not shown). However, treatment with 10 μmol/L sulforaphane only weakly decreased the intracellular GSH levels, suggesting that mechanisms other than reduced GSH levels may also contribute to the ability of subtoxic doses of sulforaphane to induce ROS generation in hepatoma cells. A recent study suggested that the increased expression of cytochrome P450 protein mediates sulforaphane-induced oxidative stress in the lungs of rats (44). Spontaneous hydrolysis of the -N=C=S moiety in isothiocyanates (45) and mitochondrial damage by isothiocyanates (46) may also

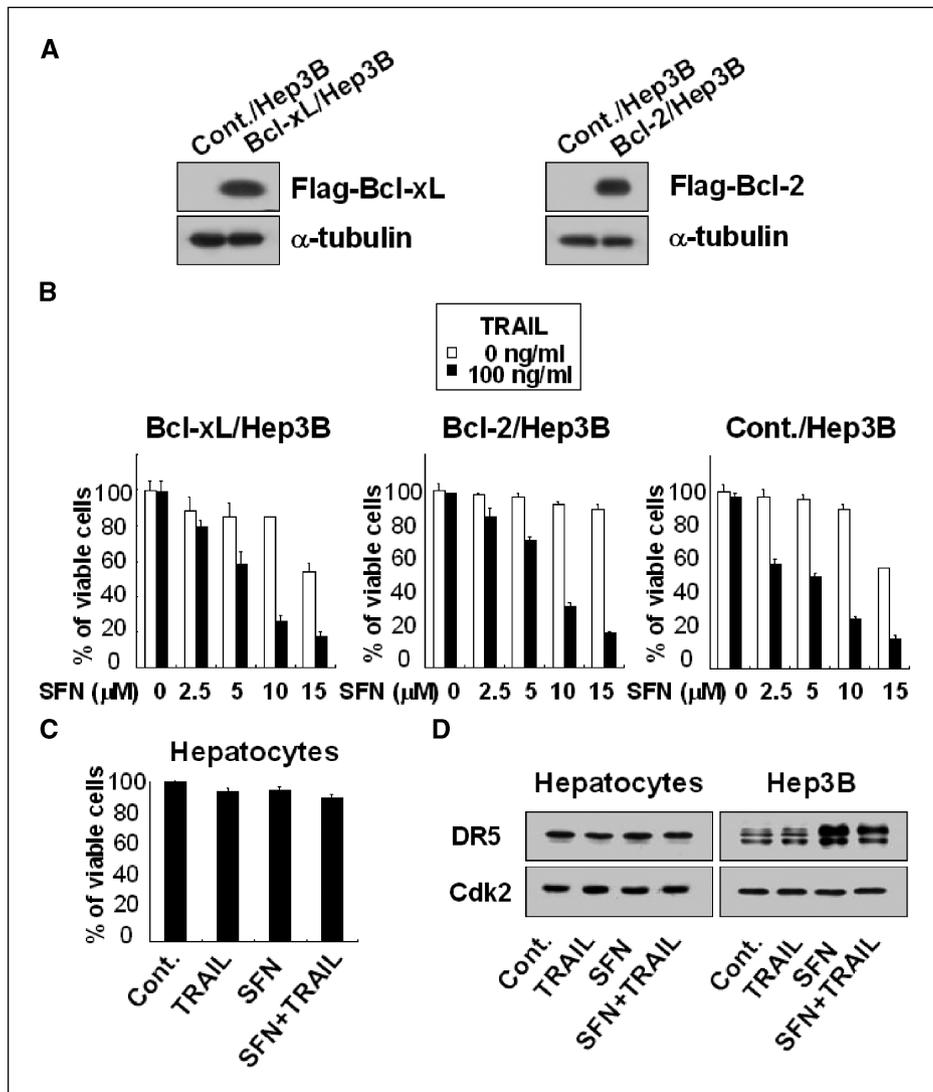


Figure 6. Effect of sulforaphane/TRAIL cotreatment on hepatoma cells overexpressing Bcl-xL or Bcl-2 and rat primary hepatocytes. **A**, analysis of Bcl-2 or Bcl-xL expression in the stably transfected cell lines. Western blotting using an anti-Flag antibody was done to detect Flag-tagged Bcl-xL or Flag-tagged Bcl-2 in the selected cell lines. **B**, effect of Bcl-xL or Bcl-2 overexpression on cell death induced by the combined treatment. Bcl-xL- and Bcl-2-overexpressing Hep3B cells or control Hep3B cells were treated with 100 ng/mL TRAIL alone or 100 ng/mL TRAIL + sulforaphane at the indicated concentrations for 24 hours. Cellular viability was measured using calcein-AM and EthD-1. Representative of three independent experiments. **C**, effect of sulforaphane and/or TRAIL on the viability of rat hepatocytes. Rat primary hepatocytes were treated with or without 10 μmol/L sulforaphane for 30 minutes and further treated with 100 ng/mL TRAIL for 24 hours. Viabilities of the treated cells were assessed using calcein-AM and EthD-1. The viabilities of the primary hepatocytes incubated for 24 hours without any treatment were used as a control (100%). **Columns**, average of three individual experiments; **bars**, SD. **D**, DR5 expression is not up-regulated by sulforaphane in primary hepatocytes. Rat primary hepatocytes or Hep3B cells were treated with 100 ng/mL TRAIL and/or 10 μmol/L sulforaphane for 16 hours and cell extracts were prepared for Western blotting of DR5. Cdk2 was used as a loading control of Western blotting. Representative of three independent experiments.

explain the generation of ROS in sulforaphane-treated cells. Clearly, further studies will be necessary to determine the mechanisms by which sulforaphane induces the generation of ROS in hepatoma cells.

Many cancer cells with mutations in p53 resist chemotherapy-induced apoptosis (47) and 30% to 60% of hepatocellular carcinoma patients have altered p53 expression (48). In our study, we showed that the combined treatment with sulforaphane and TRAIL effectively induced irreversible cell death not only in human hepatoma cells with wild-type p53 (HepG2 cells; ref. 49) but also in those with mutant p53 [Huh-7 cells, Hep3B (p53 deleted), SNU-398, and SNU-449; refs. 49, 50] commonly through DR5 up-regulation. These results suggest that this combined treatment with sulforaphane and TRAIL could be useful for hepatoma cells whose p53 function has been compromised by aflatoxin B–mediated mutations or by binding of the X protein of hepatitis B virus (51). Bcl-xL, which is overexpressed in human hepatoma cells, is a significant prognostic factor for the progression of human hepatocellular carcinoma (52). However, the cell death induced

by sulforaphane plus TRAIL was not blocked by overexpression of Bcl-xL or Bcl-2. In contrast, the combined treatment with sulforaphane and TRAIL was not cytotoxic to primary hepatocytes. In addition, treatment with sulforaphane selectively sensitized cells to TRAIL-induced apoptosis but not to apoptosis mediated by other death receptors that can cause lethal systemic inflammation or hepatotoxicity. In conclusion, the use of TRAIL in combination with subtoxic doses of sulforaphane may provide an effective therapeutic strategy for safely treating resistant hepatomas.

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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

Heesue Kim, Eun Hee Kim, Young Woo Eom, et al.

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