Genistein sensitizes human hepatocellular carcinoma cells to TRAIL-mediated apoptosis by enhancing Bid cleavage

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily, and it has been shown that many human cancer cell lines are refractory to TRAIL-induced cell death. However, the molecular mechanisms underlying resistance are unclear. In this study, we show that TRAIL resistance is reversed in human hepatoma cells by genistein, an isoflavone found in soy products. Synergistic induction of apoptosis in cells treated with genistein plus TRAIL was associated with cleavage of Bid, a proapoptotic BH3-only protein. Silencing of Bid expression reduced decreases in mitochondrial membrane potential and reduced apoptosis in cells treated with genistein and TRAIL, confirming that Bid cleavage is required for the response. Pretreatment with caspase-3 and caspase-8 inhibitors reduced cotreatment-induced apoptosis. However, treatment with TRAIL alone induced caspase-8 activity that was not different than TRAIL plus genistein; both effectively induced Bid cleavage. These data suggest that genistein abolishes resistance to the Bid cleavage of TRAIL, and that genistein does not interfere with signals upstream of Bid in hepatoma cells. *Anti-Cancer Drugs* 20:713–722 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the fourth leading cause of cancer deaths worldwide [1]. The incidence of HCC is increasing worldwide, and surgical resection has been considered the optimal treatment approach; but only a small proportion of patients qualify for surgery. In addition, there is a high rate of cancer recurrence after surgery. Approaches used to prevent recurrence have included chemoembolization before surgery and neoadjuvant therapy after surgery, neither of which has been proven to be beneficial [2]. Therefore, new therapeutic options are needed for more effective treatment of this malignancy. Other unmet needs include resection/local ablation in the adjuvant setting and combination therapies.

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is considered as a promising anticancer agent because of its ability to induce apoptosis in a variety of tumor cell types [3]. Cellular sensitivity to TRAIL depends on the expression of cell membrane TRAIL receptors and caspase-8 [4,5]. Caspase-8 is activated in response to TRAIL, and is released into the cytoplasm, where it initiates a protease cascade that activates effector caspases, such as caspase-3 and caspase-7 [6,7]. Recent studies have shown that many tumor cells, including hepatoma cell lines, acquire resistance to the apoptotic effects of TRAIL [8–10]. Mutations of the proapoptotic protein Bax and increased expression of inhibitor of apoptosis protein (IAP) family members, such as XIAP and cIAP, increase resistance to TRAIL-mediated apoptosis [11,12]. Recently, it was found that hepatitis C virus core protein modulates TRAIL-mediated apoptosis by enhancing Bid cleavage and activation of a mitochondrial apoptosis signaling pathway [13]. Shigeno *et al.* [14] suggested that interferon-α could sensitize certain human hepatoma cells to TRAIL-induced apoptosis by stimulating death signaling in and repressing the survival function of these cells. Treating hepatoma cells with a combination of TRAIL and sulforaphane, an isothiocyanate found in many cruciferous vegetables including broccoli and cauliflower, enhanced TRAIL-mediated apoptosis, which was associated with elevated levels of death receptor 5 (DR5) [15]. These findings suggest that anticancer agents can be used in combination with TRAIL to sensitize resistant cells to TRAIL-mediated apoptosis. A better understanding of the molecular mechanisms underlying TRAIL resistance and identification of

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sensitizing agents that can overcome this resistance may facilitate the development of TRAIL-based combination regimens for improved treatment of HCC.

Epidemiological studies have shown that increased intake of dietary isoflavonoids may protect against tumorigenesis, which suggests their potential use in the chemoprevention of cancer [16,17]. Genistein (4′,5,7-trihydroxy isoflavone), a natural isoflavone compound found in soy products, inhibits activation of protein tyrosine kinases and exhibits numerous effects on cell functions. For example, this compound inhibited tumor cell proliferation and metastasis, induced tumor cell differentiation, triggered cell cycle arrest at G_2/M phase and apoptosis in some cell types, and blocked apoptosis under other circumstances [18–26]. Other evidence indicated that isoflavone consumption might be associated with an increased risk of HCC in women. Women with hepatitis virus infection may be advised to abstain from isoflavone consumption [27]. In addition, in-vivo and in-vitro studies have clearly shown that genistein is a promising agent for cancer chemoprevention and further suggest that it could be an adjunct to cancer therapy by virtue of its effects on reversing radioresistance and chemoresistance [28–32].

A recent study showed that the combination of genistein and TRAIL inhibited the growth of pancreatic carcinomas [33], and enhanced TRAIL-induced apoptosis in lung cancer cells by regulating the phosphoinositide-3 kinase / Akt pathway [34]. Earlier, our own study showed that genistein sensitizes TRAIL-resistant human gastric carcinoma AGS cells through the activation of caspase-3 [35]. This indicates that treatment with a combination of TRAIL and genistein may be a safe and effective strategy for the treatment of TRAIL-resistant cells, and suggests the potential therapeutic value of this combination as an anticancer agent or an adjunct to current cancer therapies.

Here, we show that genistein is a potent sensitizer for TRAIL-induced apoptosis in TRAIL-resistant human hepatoma cells. Moreover, we present the first evidence that subtoxic doses of genistein abolishes resistance to the Bid cleavage of TRAIL, which leads to significant induction of TRAIL-mediated signaling and cell death in human hepatoma cells.

Materials and methods

Reagents and antibodies

Propidium iodide (PI) and 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St Louis, Missouri, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine Iodide (JC-1), caspase-3 inhibitor, z-DEVD-fmk and caspase-8 inhibitor, z-IETD-fmk were obtained from Calbiochem (San Diego, California, USA). Caspase-8 activity assay kits and enhanced chemiluminescence kits were obtained from R&D Systems (Minneapolis, Minnesota, USA) and Amersham (Arlington Heights, Illinois, USA), respectively. RPMI 1640 medium and fetal bovine serum were purchased from Invitrogen Corporation (Carlsbad, California, USA) and GIBCO-BRL (Gaithersburg, Maryland, USA), respectively. All other chemicals were purchased from Sigma. Antibodies against $FLIP_{S/L}$, cIAP-1, cIAP-2, XIAP, actin, Bcl-2, Bcl-X_L, Bim, Bax, Bad, Bid, cytochrome *c*, and caspase-3 and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Cox IV was purchased from cell signaling (Beverly, Massachusetts, USA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham.

Cell culture and cell viability

Human hepatoma HepG2, Hep3B, SNU-423, and SNU-449 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were cultured at 37°C in a 5% CO_2 humidified incubator, and were maintained in the RPMI 1640 culture medium containing 10% heat-inactivated fetal bovine serum. For the cell viability study, cells were grown to 70% confluence and treated with TRAIL (Koma Biotech Inc., Seoul, Korea) and genistein (Sigma). TRAIL and genistein were dissolved in PBS and dimethyl sulfoxide (DMSO), respectively. Control cells were supplemented with complete media containing 0.05% DMSO (control) for various lengths of time. After treatment, cell viability was determined by MTT assays [36].

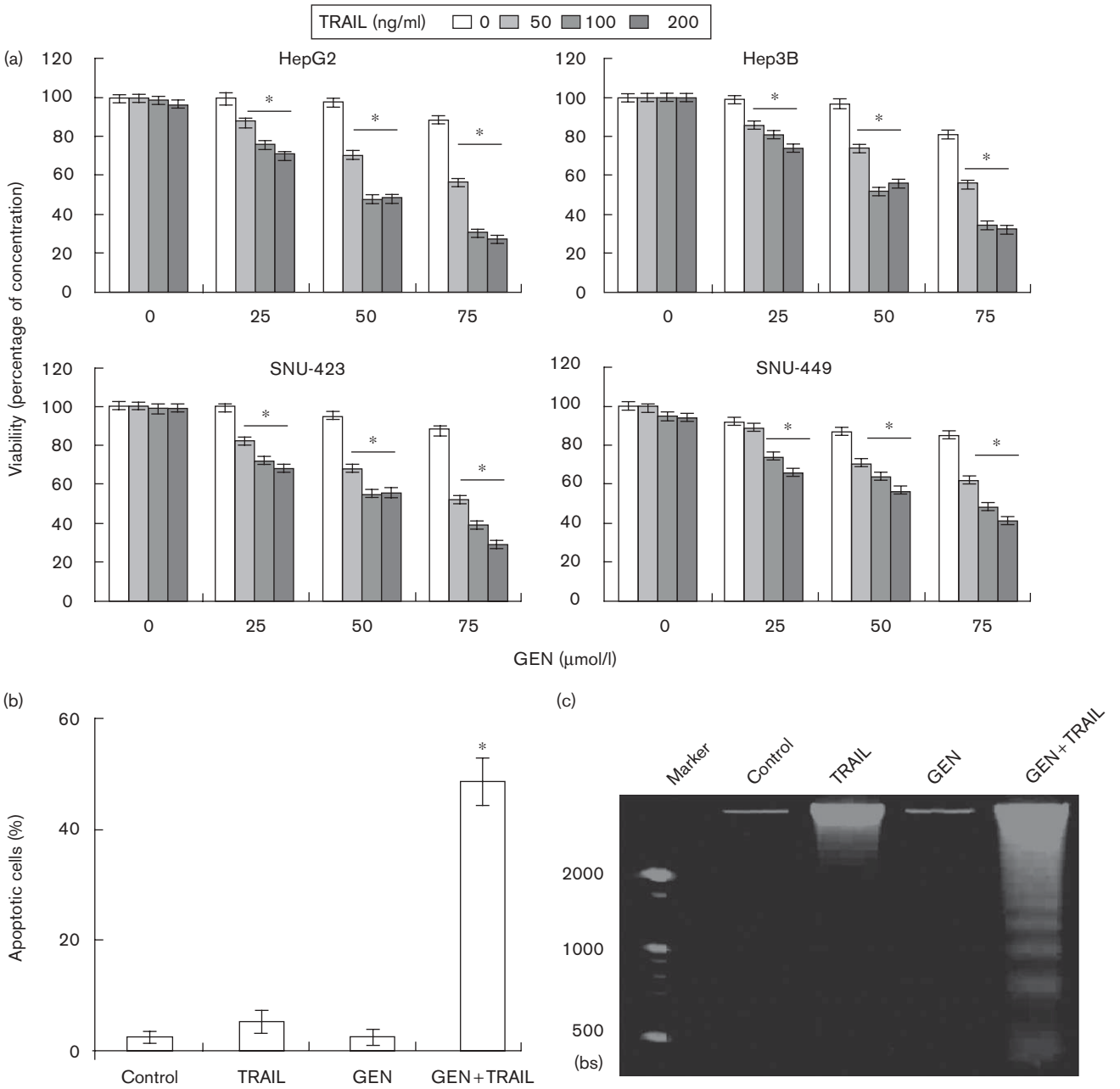
Analysis of apoptosis

Cells had been treated with genistein, TRAIL, and genistein plus TRAIL, and were double labeled with annexin-V–FITC and PI using an apoptosis detection kit (BioVision Research Products, Mountain View, California, USA) according to the manufacturer's protocol. The distribution of apoptotic cells was identified by a flow cytometer (Becton Dickinson, San Jose, California, USA).

DNA fragmentation assay

Cells were treated with genistein and/or TRAIL and lysed in a buffer containing 10 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 5 mmol/l EDTA, and 0.5% Triton X-100 for 30 min on ice. After centrifugation for 5 min at 12 000*g*, supernatants were treated with RNase A and proteinase K. Subsequently, 20 µl of 5 mol/l NaCl and 120 µl isopropanol were added to the supernatants and kept at −20°C for 6 h. After centrifugation for 15 min at 12 000*g*, the pellets were dissolved in 20 µl of TE buffer (10 mmol/l Tris–HCl and 1 mmol/l EDTA) as loading samples. To assay the DNA fragmentation pattern, samples were loaded onto 1.5% agarose gel, and electrophoresis was carried out.

Fig. 1



Effect of combined treatment with genistein plus TRAIL on hepatoma cell viability. (a) Hepatoma cells were treated with genistein with or without TRAIL for 24 h at the indicated concentrations. Cellular viability was assessed by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assays. (b) HepG2 cells treated with 50 μmol/l genistein alone, 100 ng/ml TRAIL alone, or genistein plus TRAIL for 24 h. Apoptosis was quantitated by staining cells with annexin-V–FITC and propidium iodide, which detects phosphatidylserine externalization, followed by flow cytometric analysis. Each point represents the mean ± SD of three independent experiments. Significance was determined using Student's *t*-test (**P* < 0.05 vs. treated control). (c) DNA fragmentation by combined treatment with genistein and/or TRAIL. After treatment of Hep3B cells as indicated for 24 h, fragmented DNAs were extracted from the treated cells and analyzed on 1.5% agarose gel. GEN, genistein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Cell cycle analysis

Cells were harvested and washed once with cold PBS, fixed in ice-cold 70% ethanol and stored at 4°C. Before analysis, cells were washed once again with PBS, suspended in 1 ml of a cold PI solution containing 100 μg/ml RNase A, 50 μg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer, and CellQuest software (Becton Dickinson, San Jose, USA) was used

to determine the relative DNA content based on the presence of a red fluorescence signal. The sub-G_1 population was calculated to estimate the apoptotic cell population.

Mitochondrial membrane potential assay

To measure the mitochondrial membrane potential (MMP, $\Delta\Psi_m$), JC-1, a dual-emission fluorescent dye, was used. JC-1 is internalized and concentrated by respiring mitochondria and can reflect changes in MMP in living cells. There are two excitation wavelengths, 527 nm (green) for the monomer form and 590 nm (red) for the JC-1 aggregate form. Briefly, the cells were collected and incubated with 10 μmol/l JC-1 for 30 min at 37°C. Cells were then washed once with cold PBS and analyzed using a DNA flow cytometer.

Protein extraction and western blotting

Cells were treated with genistein, TRAIL, or genistein plus TRAIL and harvested with ice-cold PBS. The mitochondrial and cytosolic fractions were then isolated using a mitochondrial fractionation kit (Activemotif, Carlsbad, California, USA). For the isolation of total cellular protein, cells were gently lysed for 20 min in ice-cold lysis buffer (20 mmol/l sucrose, 1 mmol/l EDTA, 20 μmol/l Tris–HCl, pH 7.2, 1 mmol/l DTT, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, California, USA). Cell lysates (30 μg protein per lane) were fractionated in SDS–polyacrylamide gels and then transferred to nitrocellulose membranes for immunoblot analysis using the indicated antibodies and an enhanced chemiluminescence detection system according to the manufacturer's recommendations.

Determination of caspase-8 activity

Caspase-8 activity was determined by colorimetric assays using caspase-8 activation kits according to the manufacturer's protocols. The kit uses synthetic tetrapeptides [Ile-Glu-Thr-Asp (IETD)] labeled with *p*-nitroanilide. Briefly, cells were lysed in the supplied lysis buffer. The supernatants were then collected and incubated at 37°C with the supplied reaction buffer, which contained dithiothreitol and substrates. Caspase activity was then determined by measuring changes in absorbance at 405 nm using a microplate reader.

Treatment with small interference RNA

Cells were transfected with Bid small interference RNA (siRNA), or an equal amount of nonspecific control RNA as a control (Dharmacon, Lafayette Co., Chicago, Illinois, USA). Transfection of synthetic RNA was then performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. After transfection with siRNA, cells were incubated for 24 h followed by incubation under the indicated conditions.

Statistical analysis

All data are presented as mean ± SD. Significant differences between groups were determined using an unpaired Student's *t*-test. A *P* value of less than 0.05 was considered to indicate statistical significance. The results shown in each of the figures in this study are representative of at least three independent experiments.

Results

Subtoxic doses of genistein sensitized TRAIL-induced apoptosis

The cytotoxic effects of genistein and TRAIL were tested against four hepatoma cell lines: HepG2, Hep3B, SNU-423, and SNU-449 (Fig. 1a). Treatment with 50–200 ng/ml TRAIL induced a limited amount of cell death over 24 h, suggesting that many of these hepatoma cells were resistant to the cytotoxic activity of TRAIL. Then, we examined the cytotoxic activity of genistein alone or in combination with TRAIL. Genistein, up to 50 μmol/l, alone did not induce any signs of cell death, although cell viability was slightly decreased at this concentration. However, cell viability was markedly reduced by the combined treatment, both when holding the concentration of TRAIL fixed and varying the concentration of genistein and when holding the concentration of genistein fixed and varying TRAIL. These results indicate that combined treatment with genistein plus TRAIL effectively induces cell death in TRAIL-resistant hepatoma cell lines. We then examined whether apoptotic cell death is induced by cotreatment of genistein and TRAIL using flow-cytometry analysis with annexin-V and PI staining. Combined treatment of HepG2 cells with 50 μmol/l genistein plus 100 ng/ml TRAIL for 24 h significantly increased apoptosis, whereas treatment with genistein or TRAIL alone did not (Fig. 1b). Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed a typical ladder pattern of internucleosomal DNA fragmentation in Hep3B cells cotreated with 50 μmol/l genistein and 100 ng/ml TRAIL but not in cells treated with genistein or TRAIL alone (Fig. 1c). These results suggest that genistein sensitizes TRAIL-resistant hepatoma cell lines to TRAIL-induced apoptosis.

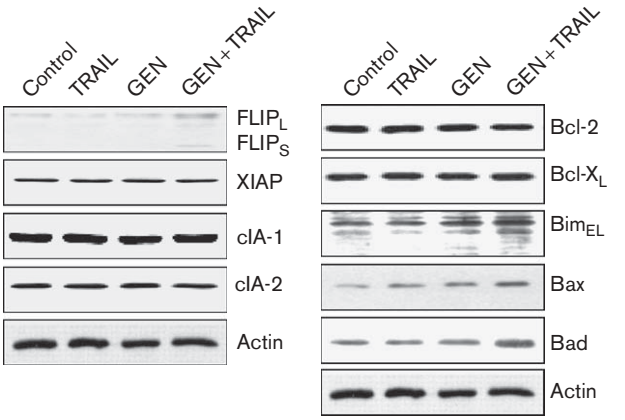
Combined treatment with genistein plus TRAIL upregulates the expression of proapoptotic proteins and downregulates MMP levels

The proapoptotic and antiapoptotic members of the Bcl-2 and IAP families play pivotal roles in regulating cell viability. These proteins induce a wide array of diverse upstream survival and distress signals to decide the fate of the cells. Therefore, we investigated whether combined treatment with genistein plus TRAIL induces apoptosis by modulating the expression of Bcl-2 and IAP family members. Treatment of cells with 50 μmol/l genistein and 100 ng/ml TRAIL alone or in combination did not affect the expression levels of antiapoptotic

proteins, such as XIAP, cIAP-1, cIAP-2, and Bcl-2, Bcl-X_L or FLIP in HepG2 cells (Fig. 2a). In contrast, expression of proapoptotic Bax, Bad, and Bim_{EL} in HepG2 cells were significantly upregulated by cotreatment with 50 μmol/l genistein and 100 ng/ml TRAIL, but not in cells treated with genistein or TRAIL alone (Fig. 2b). Similar results were also found in other cell lines such as Hep3B and SNU-423 (data not shown).

The role of mitochondria in genistein and TRAIL-induced apoptosis of cells was further investigated by examining the effect of genistein and TRAIL on MMP levels, as well as on the levels of cytosolic and mitochondrial Bax and cytochrome *c*. Treatment of cells with 50 μmol/l genistein for 3–24 h had no effect on MMP and little effect was observed by 100 ng/ml TRAIL alone. Unlike genistein and TRAIL alone, a significant and time-dependent loss of MMP by cotreatment with genistein and TRAIL in HepG2 cells was observed (Fig. 3a). Similar results were found in other cell lines such as Hep3B, SNU-423 and SNU-449 (Fig. 3c). Furthermore, cotreatment with genistein and TRAIL led to a significant increase in the level of cytosolic cytochrome *c* and a decrease in cytosolic Bax; this did not occur after treatment with genistein or TRAIL alone (Fig. 3b). Cells cotreated with 50 μmol/l genistein and 100 ng/ml TRAIL showed a decrease in mitochondrial cytochrome *c* and a significant increase in mitochondrial Bax. These results suggest a direct role of the mitochondria in apoptosis in TRAIL-resistant hepatoma cells by a combined treatment with genistein plus TRAIL.

Fig. 2



Effect of combined treatment of genistein plus TRAIL on the expression of various intracellular regulators of apoptosis. HepG2 cells were treated with 100 ng/ml TRAIL alone, 50 μmol/l genistein alone, or a combination of the two for 24 h. Cell extracts were prepared for western blotting. Actin was used as an internal control. Each protein was assayed at least twice using independently prepared lysates, and the results were similar. GEN, genistein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

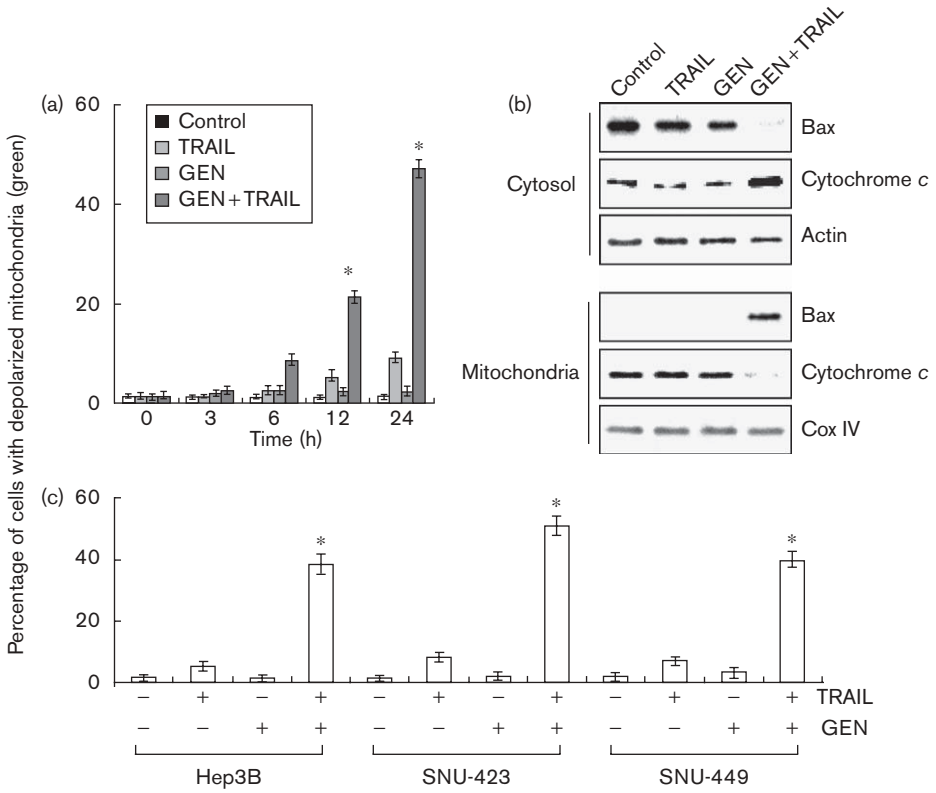
Caspase-dependent induction of apoptosis by combined treatment with genistein plus TRAIL

Caspases are known to act as important mediators of apoptosis, and are also known to contribute to overall apoptotic morphology through cleavage of various cellular substrates. Therefore, we investigated caspase activation in hepatoma cells treated with genistein and TRAIL alone or together. Western blots showed that treatment with 100 ng/ml TRAIL alone slightly decreased pro-caspase-9 levels and increased cleavage of caspase-3, but this did not occur after treatment with 50 μmol/l genistein alone in HepG2 cells. However, combined treatment with genistein plus TRAIL for 6–24 h significantly decreased pro-caspase-9 levels, induced cleavage of caspase-3, and reduced full-length Bid expression in a time-dependent manner (Fig. 4a). Then, we assessed the effect of genistein and TRAIL on cell cycle distribution in the presence or absence of caspase-8 or caspase-3 inhibitors using a flow cytometer. Treatment of cells with 50 μmol/l genistein for 24 h resulted in a markedly increased accumulation of G_2/M phase cells as compared with genistein or TRAIL alone; however, combined treatment with 50 μmol/l genistein plus 100 ng/ml TRAIL resulted in a significant accumulation of cells with sub-G_1 DNA content, which was reduced by pretreatment of the caspase-8 or caspase-3 inhibitors, z-IETD-fmk or z-DEVD-fmk, respectively (Fig. 4b). Consistent with these results, pretreatment of z-IETD-fmk or z-DEVD-fmk markedly reduced apoptosis induced by genistein plus TRAIL (Fig. 4c) indicating that cotreatment induces apoptosis through activation of caspase-3 and caspase-8. Then, cell lysates containing equal amounts of total protein from cells treated with genistein plus TRAIL were assayed to assess in-vitro caspase-8 activity. Treatment with 25–50 μmol/l genistein alone did not induce caspase-8 activation, but TRAIL alone (100–200 ng/ml) or in combination with genistein (100 ng/ml TRAIL plus 25–50 μmol/l genistein) increased caspase-8 activity. This was significantly inhibited in the presence of z-IETD-fmk. TRAIL alone induced caspase-8 activity but this was not different than the effect of TRAIL plus genistein in HepG2 cells (Fig. 4d). These data indicate that genistein does not interfere with signals upstream of Bid in TRAIL-resistant HepG2 cells.

Genistein sensitizes cells to TRAIL-induced apoptosis through Bid cleavage

Then, we examined in detail the effects of genistein and TRAIL on Bid expression in hepatoma cells. Bid cleavage was assessed as a reduction in full-length Bid protein – the antibody used in this study–recognized only the full-length Bid molecule and not the cleavage product (Fig. 5a). TRAIL alone (100 ng/ml) resulted in a slight reduction in wild-type Bid (indicating slight Bid cleavage); treatment with 50 μmol/l genistein alone did not induce any cleavage of Bid. However, the reduction in Bid was markedly increased by combined treatment with

Fig. 3



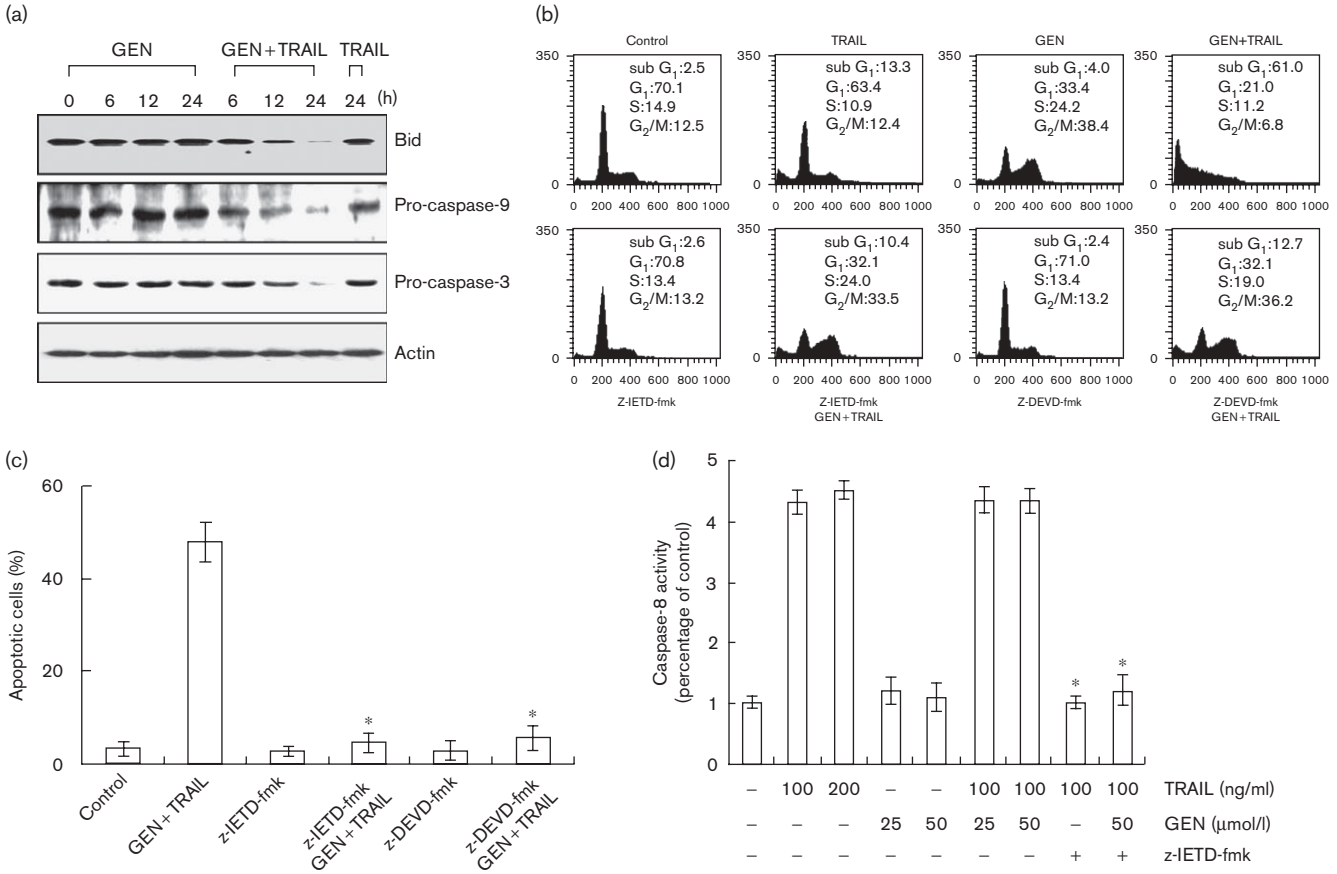
Effect of combined treatment with genistein plus TRAIL on loss of mitochondrial membrane potential (MMP), and expression of Bax and cytochrome *c* in hepatoma cells. (a) HepG2 cells were treated with 50 μmol/l genistein alone, 100 ng/ml TRAIL alone, or a combination, for the indicated time points. Loss of MMP was measured by JC-1 staining. (b) HepG2 cells were treated with 50 μmol/l genistein alone, 100 ng/ml TRAIL alone, or a combination of the two for 24 h. Mitochondrial or cytosolic levels of Bax and cytochrome *c* proteins were assessed by western blotting. Actin, a marker of cytoskeletal proteins, was included as a control for protein loading. A mitochondrial marker was detected by Cox IV. (c) Hepatoma cells were treated with 50 μmol/l genistein alone, 100 ng/ml TRAIL alone, or a combination of the two for 24 h and we measured decreases in MMP. Each point represents the mean ± SD of three independent experiments. Significance was determined using Student's *t*-test (*$P<0.05$ vs. untreated control). GEN, genistein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

genistein plus TRAIL (Fig. 5a). As the caspase-8 activity induced by TRAIL alone was not different after TRAIL plus genistein, and caspase-8 activity was not induced by genistein alone, the data suggest that genistein abolished resistance to Bid cleavage of TRAIL in hepatoma cells (Fig. 5b). To further confirm the role of Bid in cell death induced by genistein plus TRAIL, we examined whether ablated Bid expression (using siRNA) protects against cell death. As shown in Fig. 6, Bid knockdown significantly reduced apoptosis and loss of MMP induced by TRAIL alone or TRAIL plus genistein, showing that genistein lowers the threshold at which TRAIL-induced Bid cleavage triggers the mitochondrial apoptosis program.

Discussion

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 [37] and DR5 [37,38], which contain cytoplasmic death domains responsible for recruiting adapter molecules involved in caspase activation. TRAIL is a recently discovered molecule that induces apoptosis in some cell types. It has aroused great interest in medical circles because it can selectively induce cancer cells, transformed cells, and virus-infected cells to undergo apoptosis without toxicity to normal cells. Likewise, recombinant soluble human TRAIL has been shown to have a profound apoptotic effect on xenografted melanoma cells without toxicity to human hepatocytes *in vitro* and *in vivo* [39]. These results indicate that this form of TRAIL may be a safe and effective biological agent for use in cancer therapies in humans. However, recent studies have shown that a considerable number of cancer cells, including human hepatoma cells, are resistant to the proapoptotic effects of TRAIL [8–10]. Thus, scientists are currently attempting to identify TRAIL sensitizers that are able to overcome TRAIL resistance in cancer cells.

Fig. 4



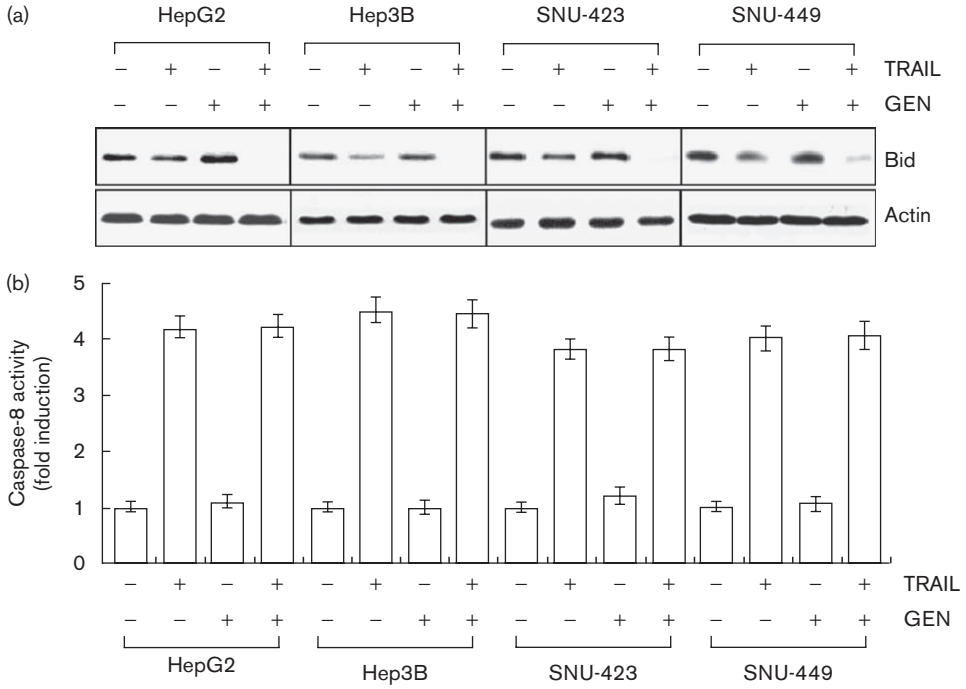
Effect of combined treatment with genistein plus TRAIL on caspase activation. (a) Activation of caspases during apoptosis induced by combined treatment with genistein plus TRAIL. HepG2 cells were treated with 50 μmol/l genistein alone, 100 ng/ml TRAIL alone, or a combination for the indicated time points. Cell extracts were prepared for western blotting for Bid and caspases. (b) For experiments using inhibitors of caspase-3 and caspase-8, HepG2 cells were preincubated with z-DEVD-fmk (50 μmol/l) or z-IETD-fmk (50 μmol/l) for 1 h before the addition of 50 μmol/l genistein and 100 ng/ml TRAIL for an additional 24 h. DNA content of the cells was analyzed by flow cytometry. Each point represents the mean of two independent experiments. (c) Apoptosis was quantitated by staining cells with annexin-V–FITC and propidium iodide, which detects phosphatidylserine externalization, followed by flow cytometric analysis. (d) Cells were preincubated with z-IETD-fmk (50 μmol/l) for 1 h before the addition of the indicated concentration of genistein and TRAIL for an additional 24 h. Caspase-8 activity was determined using caspase-8 assay kits. Data are expressed as the mean ± SD of three independent experiments. Significance was determined using Student's *t*-test (**P* < 0.05 vs. untreated control). GEN, genistein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Here, we show that combined treatment with genistein plus TRAIL causes a significant induction of apoptosis in human hepatoma cells that are resistant to either agent alone. Recently, researchers reported that genistein enhanced TRAIL-mediated pancreatic cancer growth through the activation of caspase-3 in mice with orthotopically transplanted AsPC1 cells [33], and combined treatment with genistein plus TRAIL potentiated apoptosis in A549 lung carcinoma cells [35] and HeLa cervix carcinoma cells [40]. Caspases belong to a family of cysteine proteases that are integral components of the apoptotic pathway. Many studies have determined that a variety of chemotherapeutic agents induce apoptosis through the activation of caspases [5–7]. Our findings indicate that caspases are critical protease mediators of apoptosis triggered by combined treatment with genistein plus TRAIL.

Caspase activation is regulated by various cellular proteins, including IAP, c-FLIP, and Bcl-2 family proteins [41,42]. Although other groups have reported that TRAIL alters the expression of antiapoptotic proteins, such as XIAP [43], we did not observe a significant downregulation of IAP and FLIP protein levels. We also found that combined treatment with genistein plus TRAIL increased the expression of the proapoptotic Bax, Bad, and Bim proteins, and caused truncation of the proapoptotic Bid protein. Combined treatment with genistein plus TRAIL did not downregulate protein levels of the antiapoptotic Bcl-2 or Bcl-xL. We found that the combination of genistein plus TRAIL increased loss of MMP in hepatoma cells.

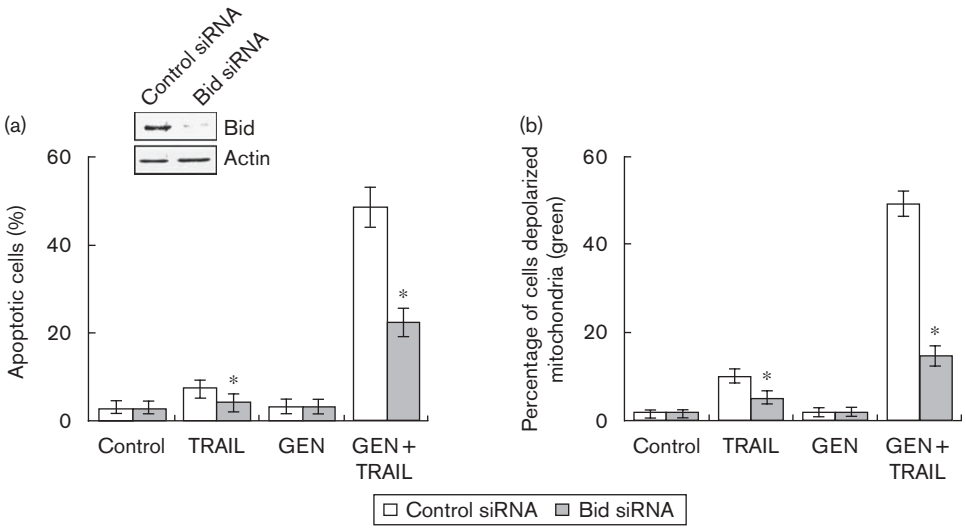
Earlier studies have implied that cleavage of Bid by caspase-8 mediates the mitochondrial damage that is

Fig. 5



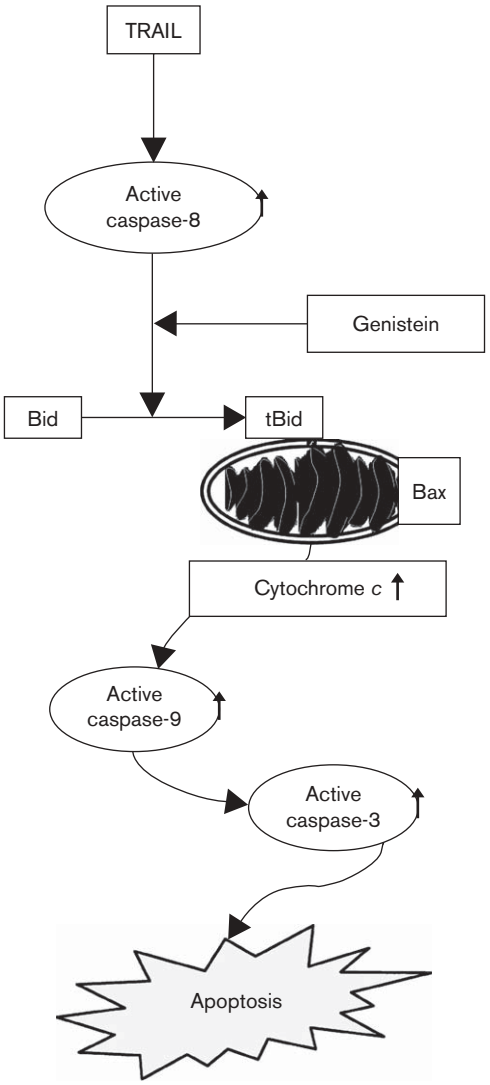
Combined treatment with genistein plus TRAIL upregulates Bid cleavage in various hepatoma cells. (a) Hepatoma cells were treated with 50 μmol/l genistein alone, 100 ng/ml TRAIL alone, or a combination of the two for 24 h. Cell extracts were prepared for western blotting. Each protein was assayed at least twice using independently prepared lysates, and the results were similar. (b) Caspase-8 activity was determined using caspase-8 assay kits. Data are expressed as the mean ± SD of three independent experiments. GEN, genistein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Fig. 6



Small interference RNA (siRNA) knockdown of Bid expression abrogates TRAIL-genistein apoptotic synergy. (a) HepG2 cells were transfected with either control or Bid-specific siRNAs. There was almost complete inhibition of Bid protein expression caused by Bid-specific siRNA 24 h after transfection (top panel). To examine the effect of Bid downregulation on genistein/TRAIL-induced apoptosis or decreases in mitochondrial membrane potential (MMP), HepG2 cells were transfected and further treated with or without 50 μmol/l genistein and 100 ng/ml TRAIL for 24 h. (a) Apoptosis and (b) loss of MMP were determined by flow cytometry, respectively. Data are expressed as the mean ± SD of three independent experiments. Significance was determined using Student's *t*-test (*$P < 0.05$ vs. untreated control). GEN, genistein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Fig. 7



Overview of pathways for genistein and TRAIL-induced apoptosis in human hepatocellular carcinoma cells. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

observed during FasL and TNF-induced apoptosis [44,45]. Moreover, Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis [46]. However, it was found that induction of caspase-8 activity by TRAIL alone was not different to that by TRAIL plus genistein. Bid cleavage is induced by genistein plus TRAIL but not in the genistein and TRAIL alone. In addition, pretreatment with caspase-8 inhibitor significantly decreased genistein plus TRAIL-induced apoptosis, and attenuated Bid cleavage (data not shown). The loss of MMP and apoptosis were markedly blocked by knockdown of Bid expression by siRNA. These data suggest that genistein abolishes resistance to Bid cleavage by TRAIL, and that genistein does not interfere with signals upstream of Bid in hepatoma cells. The elucidation of these intricate mechanisms of Bid points to the development of a possible therapeutic option that combines cytotoxic therapies to treat hepatoma cells, as shown in Fig. 7.

Resistance to apoptosis is a major obstacle to chemotherapeutic treatment of cancer. Its ability to induce apoptosis makes genistein a potentially effective preventive and therapeutic agent for combating malignancy. Although TRAIL represents a potentially important anticancer agent, recent studies have shown that many cancer cells are resistant to its apoptotic effects. Thus, the combined treatment of genistein plus TRAIL might be a good strategy for the treatment of cancers that are resistant to chemotherapy or to TRAIL alone. Taken together, the results of this study suggest that genistein sensitizes cells to TRAIL-mediated apoptosis by abolishing resistance to Bid cleavage of TRAIL. In conclusion, the use of TRAIL in combination with subtoxic doses of genistein might provide an effective therapeutic strategy for the safe treatment of some TRAIL-resistant HCC cancer cells.

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