Research Article

Molecular Cancer Therapeutics

Butein Sensitizes Human Hepatoma Cells to TRAIL-Induced Apoptosis via Extracellular Signal-Regulated Kinase/Sp1–Dependent DR5 Upregulation and NF-κB Inactivation

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces cell death in various types of cancer cells but has little or no effect on normal cells. Human hepatoma cells are resistant to TRAIL-induced apoptosis. Although butein is known to mediate anticancer, anti-inflammatory, and antioxidant activities, little is known about the mechanism of butein in terms of TRAIL-induced apoptosis of human hepatoma cells. In this study, we determined that butein enhances TRAIL-induced apoptosis in hepatoma cells through upregulation of DR5. Luciferase analysis showed that a 5′-flanking region containing four Sp1-binding sites within the DR5 promoter was enhanced by butein (−305/−300). Electrophoretic mobility shift assays and chromatin immunoprecipitation studies were used to analyze the elevation of Sp1 binding to DR5 promoter sites by butein. Point mutations of the Sp1-binding site also attenuated promoter activity. Furthermore, pretreatment of the blocking chimeric antibody and small interfering RNA for DR5 significantly suppressed TRAIL-mediated apoptosis by butein in Hep3B cells. Butein also stimulated extracellular signal-regulated kinase (ERK) activation, and the ERK inhibitor PD98059 blocked butein-induced DR5 expression and suppressed binding of Sp1 to the DR5 promoter. Additionally, generation of reactive oxygen species had no effect on cell viability, although pretreatment with *N*-acetyl-L-cysteine or glutathione inhibited combined treatment-induced reactive oxygen species. Indeed, butein repressed the TRAIL-mediated activation of NF-κB and decreased its transcriptional activity. Our results suggest that butein could sensitize certain human hepatoma cells to TRAIL-induced apoptosis through stimulating its death signaling and by repressing the survival function in these cells. *Mol Cancer Ther; 9(6); 1583–95. ©2010 AACR.*

Introduction

Tumor necrosis factor (TNF) family members have limited use in anticancer therapy because they cause severe cytotoxicity to normal cells (1). However, for a couple of decades, TNF-related apoptosis-inducing ligand (TRAIL) has been thought to be a new candidate for anticancer therapy because TRAIL selectively suppresses tumor growth *in vivo* and *in vitro* but has little or no effect on normal cells (2, 3). TRAIL can bind to two death receptors (DR), DR4 and DR5, which contain a cytoplasmic functional death domain. After TRAIL binds to DRs, TRAIL triggers cell death through at least two fundamental apoptotic pathways, referred to as the extrinsic pathway and the intrinsic pathway, depending on the cell type (4). TRAIL-induced apoptosis initiated by the extrinsic pathway involves DR engagement, death-inducing signaling complex formation, and proteolytic activation of caspase-8 (5). Proteolytic caspase-8 further activates Bid, which, in turn, translocates to the mitochondria and activates the intrinsic pathway (6). Activated caspase-8 is released into the cytoplasm and induces a protease cascade that activates effector caspases such as caspase-3 and caspase-7 (7). DR4 and DR5 not only give the apoptosis signal through the Fas-associated death domain and caspase-8 but also activate NF-κB, which regulates the expression of survival factors such as members of the inhibitor of apoptosis (IAP) family (IAP1, IAP2, and XIAP) and Bcl-xL.

Many studies have attempted to find new anticancer agents, which could augment apoptosis induced by TRAIL, because some cancer cells are resistant to the apoptotic effects of TRAIL (8–10). The mechanism underlying the augmentation of TRAIL-induced apoptosis is largely related to their ability to upregulate the expression level of TRAIL receptors (i.e., DR4 and

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DR5). Therefore, agents that upregulate the expression of DR4 and/or DR5 may have potential for clinical management of cancer. Because DR4 and DR5 can be regulated by either a p53-dependent or a p53-independent mechanism (11–13), it is possible that augmentation of TRAIL-induced apoptosis by anticancer agents may be either p53 dependent or p53 independent.

Natural products have played a highly significant role over the years in the discovery of new drugs. This is particularly evident in the treatment of cancers and infectious diseases in which more than 60% and 75% of drugs, respectively, are of natural origin (14). Therefore, a natural product with strong synergistic activity with TRAIL but minimal toxicity could be a new tool for cancer therapy. Butein is one such agent that has been identified from numerous plants, including the stem bark of *Semecarpus anacardium*, the heartwood of *Dalbergia odorifera*, and the traditional Chinese and Tibetan medicinal herbs *Caragana jubata* and *Rhus verniciflua* Stokes. Previous studies suggested that butein sensitizes apoptosis in human promyelocytic leukemia cells and B16 melanoma cells (15, 16). In the *in vitro* experiment, butein also suppressed the proliferation of many human cancers, including breast carcinoma, colon carcinoma, osteosarcoma, and hepatic stellate cells (17–20). But the anticancer mechanisms of butein in TRAIL-mediated apoptosis are little understood.

In this study, we showed DR5 expression and NF-κB–mediated mechanism underlying butein-sensitized apoptosis induced by TRAIL in human hepatoma cancer cells.

Materials and Methods

Reagents

Antibodies against Sp1, nucleolin, p50, p65, Bid, caspase-3, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP), DR4, DR5, IAP-1, IAP-2, XIAP, Bcl-2, Bcl-xL, Bax, and Bad were purchased from Santa Cruz Biotechnology. Antibodies against c-Jun NH_2-terminal kinase (JNK), phospho-JNK, extracellular signal-regulated kinase (ERK), phospho-ERK, p38, and phospho-p38 were purchased from Cell Signaling. The antibody against β-actin was purchased from Sigma. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin, recombinant human TRAIL/Apo2 ligand (the nontagged 19-kDa protein, amino acids 114–281), and TNF-α were purchased from KOMA Biotechnology. Antibodies for Fas and PP2A were purchased from Upstate Biotechnology. The blocking antibody against DR5 was purchased from R&D Systems. 6-Carboxy-2′,7′-dichlorofluorescein diacetate (H_2DCFDA) and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Molecular Probes. Glutathione, *N*-acetyl-L-cysteine (NAC), pyrrolidine dithiocarbamate (PDTC), MG132, and PS341 were purchased from Sigma. Butein was also purchased from Sigma and dissolved in DMSO (vehicle). PD98059, SP600125, SB239063, z-VAD-fmk, and z-IETD-fmk were purchased from Calbiochem.

Cell culture

Human hepatoma cell lines Hep3B and HepG2 were cultured in DMEM (Life Technologies). PC3, HCT116, and U937 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum and antibiotics (Sigma). MTT assays were used to determine cell viability.

Flow cytometric analysis

Cells were fixed with 1 unit/mL of RNase A (DNase free) and 10 μg/mL of propidium iodide (Sigma) overnight at room temperature in the dark. A FACSCalibur flow cytometer (Becton Dickinson) was used to analyze the level of apoptotic cells containing sub-G_1 DNA content. For Annexin V and $DiOC_6$ staining, live cells were incubated with Annexin V (R&D Systems) and $DiOC_6$.

DNA fragmentation assay

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 × *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.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology). The preparation of cytoplasmic and nuclear extracts was conducted using NE-PER nuclear and cytosolic extraction reagents (Pierce). Total cell extracts were separated on polyacrylamide gels, and then standard procedures were used to transfer them to the nitrocellulose membranes. The membranes were developed using an enhanced chemiluminescence reagent (Amersham).

Measurement of reactive oxygen species

Cells were plated at a density of 5×10^4, allowed to attach for 24 hours, and exposed to 5 mmol/L NAC alone, 5 mmol/L glutathione alone, 10 μmol/L butein alone, or NAC or glutathione plus butein for 1 hour. The cells were stained with 10 μmol/L H_2DCFDA for 10 minutes at 37°C, and flow cytometry was used to determine the fluorescence intensity.

In vitro caspase activity assay

A caspase activation kit was used according to the manufacturer's protocol (R&D Systems) to measure the activity of caspase-like protease.

Reverse transcription-PCR analysis

Total RNA was extracted using the Trizol reagent (Invitrogen), and reverse transcription-PCR (RT-PCR) was conducted. The sense primer 5′-GTCTGCTCTGATCACCCAAC-3′ and the antisense primer 5′-CTGCAACTGTGACTCCTATG-3′ were used to amplify human DR5 mRNA. For glyceraldehyde-3-phosphate dehydrogenase, the

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sense primer 5′-CGTCTTCACCATGGAGA-3′ and the antisense primer 5′-CGGCCATCACGCCCACAGTTT-3′ were used.

Analysis of surface DR5 expression

Indirect staining with primary rabbit anti-human DR4 or DR5 followed by FITC-conjugated IgG was used to analyze cells for the surface expression of DR4 and DR5. Flow cytometry was used to analyze the expressions of these DRs.

Luciferase assays

The pDR5/*Sac*I plasmid [containing DR5 promoter sequence (−2,500/+30)] and pDR5/−605 [containing DR5 promoter sequence (−605/+3)] were gifts from Dr. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). To analyze the promoter regions responsible for butein, 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 the DR5 promoter were used. The detailed procedure to generate these mutants has been previously described (21).

Small interfering RNA

The 25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Santa Cruz Biotechnology. The cells were transfected with siRNA oligonucleotides using FuGENE 6 Transfection Reagent according to the manufacturer's recommendations.

Electrophoretic mobility shift assay

DNA-protein binding assays were carried out with a nuclear extract. Synthetic complementary Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′) and NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) binding oligonucleotides (Santa Cruz Biotechnology) were 3′-biotinylated using the biotin 3′-end DNA labeling kit (Pierce) according to the manufacturer's instructions.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was done using the EZ-Chip assay kit according to the manufacturer's protocol (Upstate Biotechnology). The primers used for the amplification of the Sp1-binding site of DR5 promoter region were 5′-GCCAGGGCGAAGGTTA-3′ (sense) and 5′-GGGCATCGTCGGTGTAT-3′ (antisense; 276-bp DNA product; ref. 22).

Statistical analysis

All data were derived from at least three independent experiments. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Photoshop. Scion Imaging software (23) was used to quantify. Statistical analyses were conducted using SigmaPlot software (version 6.0). Values were presented as mean ± SD. Significant differences between the groups were determined using the unpaired Student's t test. Statistical significance was regarded at $P < 0.05$.

Results

Butein sensitizes TRAIL-induced apoptosis regardless of cell type specificity through activation of caspase

Antiproliferative activity of TRAIL was first analyzed in four human cancer cell lines: Hep3B, HepG2, HCT116, and U937. Treatment with 100 ng/mL TRAIL induced limited inhibition of cell proliferation (<20%) at 24 hours, suggesting that these cells are resistant to the apoptotic effects of TRAIL (Fig. 1A). Next, we examined the antiproliferative effects of butein alone or in combination with TRAIL in these cells. Butein alone did not significantly induce any morphologic signs of cell death up to 5 μmol/L, although the cellular activity to reduce MTT formazan was slightly decreased at this concentration. However, cell proliferation was significantly reduced by treatment with a combination of butein and TRAIL when the concentration of TRAIL (100 ng/mL) was fixed. These results show that treatment with a combination of butein and TRAIL effectively inhibits cell proliferation in TRAIL-resistant hepatoma cells in a cell type–nonspecific manner. Next, we investigated whether the combination treatment is dependent on apoptosis. As shown in Fig. 1B, the treatment of Hep3B cells with a combination of 5 μmol/L butein and 100 ng/mL TRAIL for 24 hours significantly increased the accumulation of sub-G_1 phase cells (top) and Annexin V staining (middle), whereas treatment with butein or TRAIL alone slightly increased. Pretreatment with a pan-caspase inhibitor z-VAD-fmk significantly blocked the accumulation of sub-G_1 phase cell populations and Annexin V induced by treatment with butein and TRAIL. When phase-contrast microscopy was used to examine the change of cell morphology, the cells treated with a combination of 5 μmol/L butein and 100 ng/mL TRAIL for 24 hours displayed decreased cell numbers with some apoptotic shrinkage (bottom) compared with the untreated control cells (Fig. 1B). Furthermore, DNA fragmentation analysis also showed a typical ladder pattern of internucleosomal DNA fragmentation in hepatoma cells treated with a combination of butein and TRAIL but not in cells treated with butein or TRAIL alone (Fig. 1C). These apoptosis phenomena were also completely blocked by the pretreatment with z-VAD-fmk. Taken together, these results suggest that butein stimulates TRAIL-induced apoptosis through caspase activation but is not cell specific.

Treatment with a combination of butein and TRAIL activates extrinsic and intrinsic pathways

Hepatoma cells had been shown to be resistant to TRAIL because of insufficient activation of the extrinsic pathway and, more importantly, because of the blocking of death signaling at the mitochondrial level (4). Therefore, Western blot analyses of proapoptotic and

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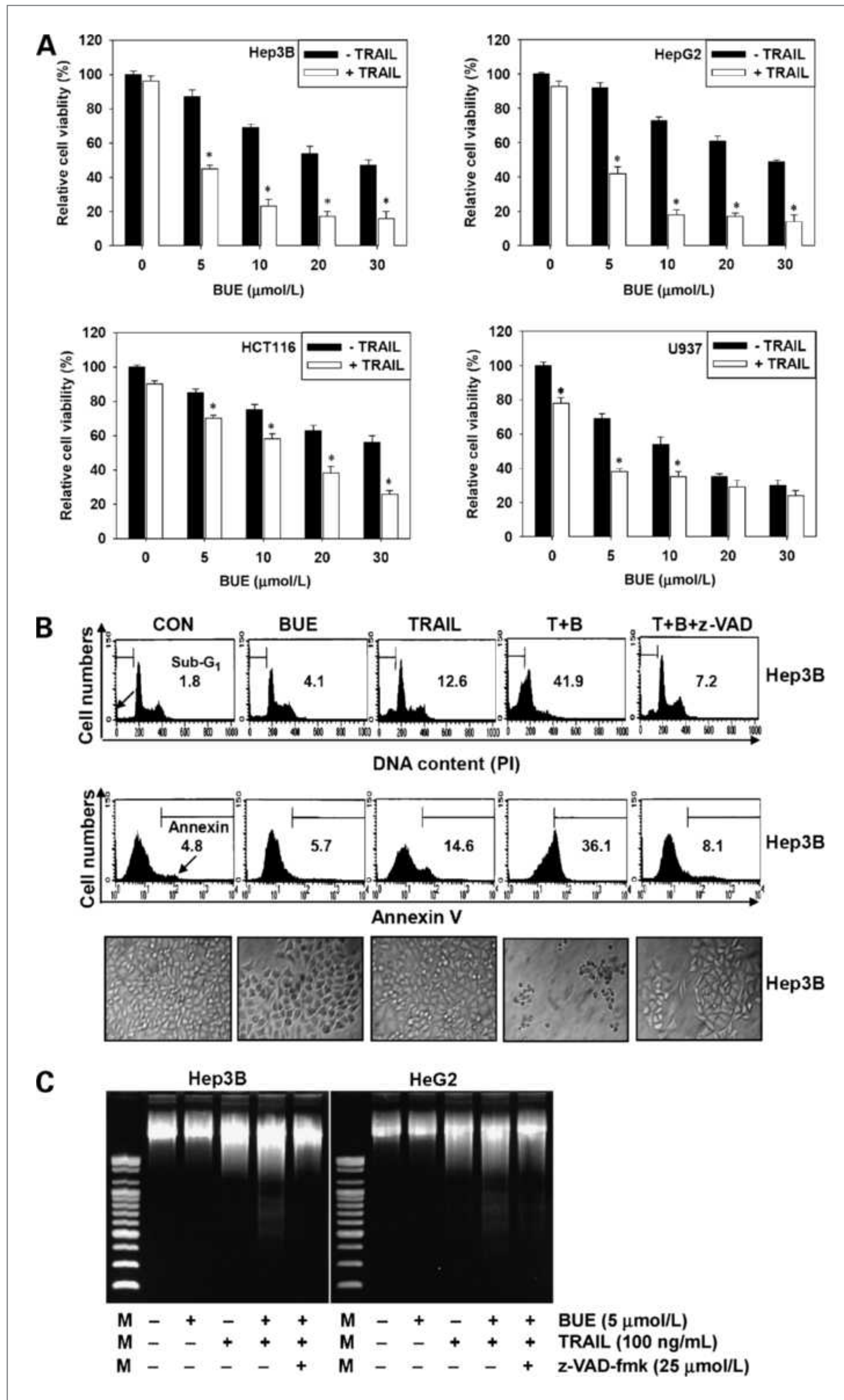

Figure 1. Butein (BUE) sensitizes TRAIL-induced cell death regardless of cell type specificity. A, effect of treatment with a combination of butein and TRAIL on cell viability. Human hepatoma Hep3B and HepG2, human colon cancer HCT116, and human leukemia U937 cells were treated with butein for 30 min at the indicated concentrations and further treated with or without 100 ng/mL TRAIL for 24 h. MTT assay assessed cellular viability. B, effect of treatment with a combination of butein and TRAIL on cell death and apoptosis. Hep3B cells treated with 5 μmol/L butein alone, 100 ng/mL TRAIL alone, or TRAIL + butein (T+B) for 24 h. To examine the effect of the inhibition of pan-caspase, Hep3B cells were pretreated with 25 μmol/L z-VAD-fmk (z-VAD) for 30 min and further treated with butein + TRAIL for 24 h (T+B+z-VAD). Flow cytometry analyzed the DNA content (top) and Annexin V^+ (middle) of the cells. Morphology of cells (bottom) was examined under light microscopy. Magnification, ×400. C, effect of treatment with a combination of butein and TRAIL on DNA fragmentation. After treatment of Hep3B and HepG2 cells as indicated for 24 h, fragmented DNAs were extracted from the treated cells and analyzed on 1.5% agarose gel. Data are expressed as overall mean ± SD from three independent experiments. Statistical significance was determined by Student's *t* test. *, $P < 0.05$ versus vehicle control.

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antiapoptotic factors were done; it was presumed that the inhibitory factors in mitochondria were suppressed by treatment with a combination of butein and TRAIL. As shown in Fig. 2A, time-dependent cleavage of caspase-8 and Bid was observed by treatment with the combination of butein and TRAIL. In contrast, little or no change was observed in cells treated with a single agent. Similarly, caspase-9, caspase-3, and PARP were

also activated after the combined treatment. Next, apoptotic events in the mitochondria were evaluated by measuring the mitochondrial membrane potential using $DiOC_6$. Marked reduction in the mitochondrial membrane potential had occurred in cells treated with butein plus TRAIL (Fig. 2B). In addition, this process was accompanied by the release of cytochrome *c* from the mitochondria into the cytosol (Fig. 2C). However, pretreatment with caspase-8 inhibitor z-IETD-fmk normalized mitochondrial membrane potential and completely blocked the release of cytochrome *c* into cytosol (Fig. 2C). Furthermore, these caspases activity was significantly increased by the combined treatment (Fig. 2D). The combined treatment also degraded the expression of Bcl-2, XIAP, IAP-1, and IAP-2. In contrast, the level of Bad expression was upregulated by the combined treatment (Fig. 2E). These results indicated that treatment with a combination of butein and TRAIL reduces the expression of multiple proteins associated with cell survival through the extrinsic and intrinsic apoptotic signal pathway.

DR5 upregulation is important for butein-stimulated TRAIL-induced apoptosis

As the TNF superfamily members share similar protein structures and DR-mediated apoptotic signaling pathways (24), we next examined whether butein sensitizes TNF-α–mediated apoptosis, possibly targeting the

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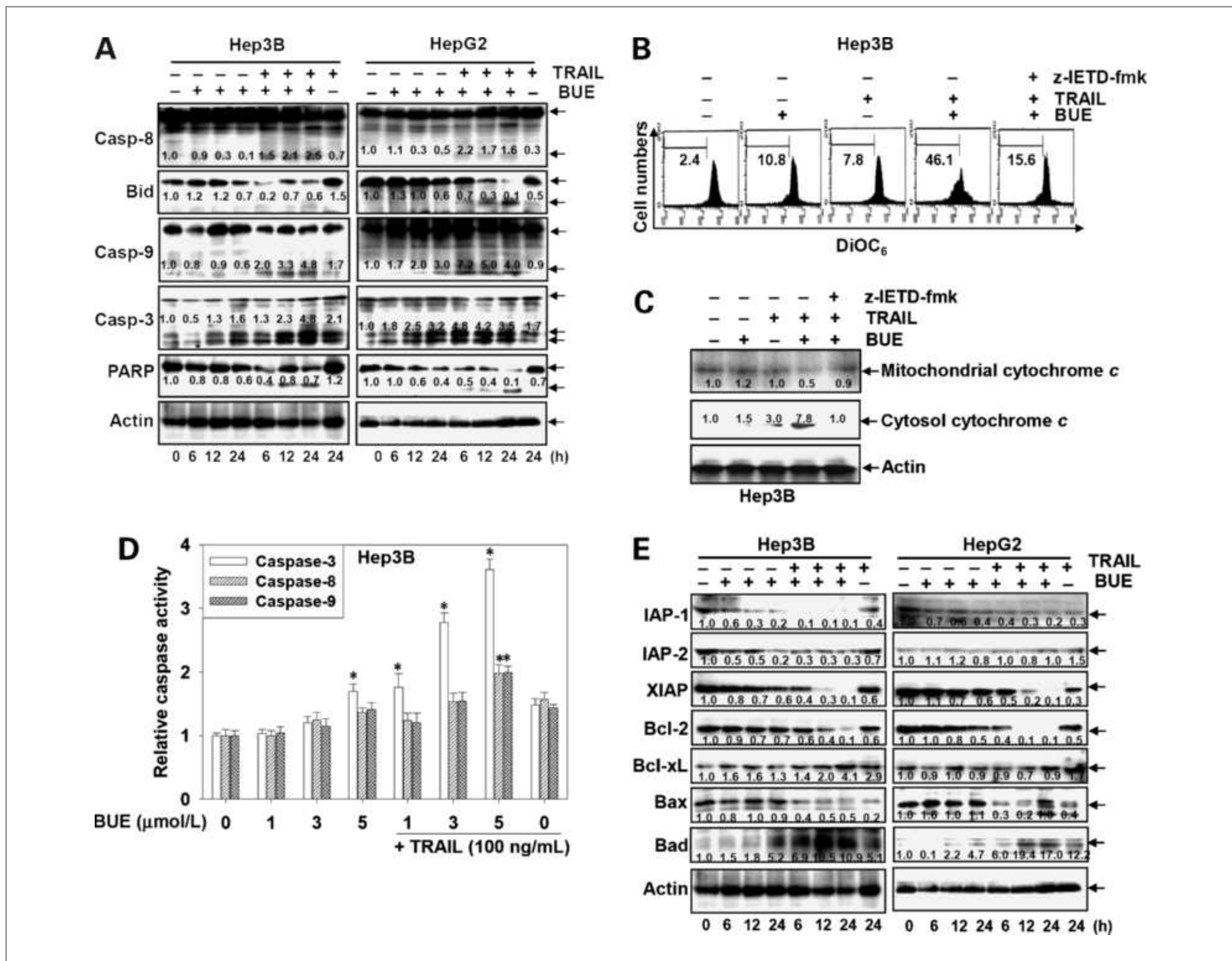

Figure 2. Treatment with a combination of butein and/or TRAIL activates apoptotic signal via the extrinsic and intrinsic pathways. A and E, effect of treatment with a combination of butein and TRAIL on levels of antiapoptotic and proapoptotic protein. Hep3B and HepG2 cells were treated with 100 ng/mL TRAIL alone, 5 µmol/L butein alone, or a combination of both for the indicated time points. Cell extracts were prepared for Western blotting for caspase-8, Bid, caspase-9, caspase-3, PARP, IAPs, Bcl-2, Bcl-xL, Bax, and Bad. B, effect of treatment with a combination of butein and TRAIL on mitochondrial membrane potential. Hep3B cells were pretreated with caspase-8 inhibitor, z-IETD-fmk, and then the cells were treated with 100 ng/mL TRAIL alone, 5 µmol/L butein alone, or a combination of both for 24 h. Mitochondrial membrane potential was measured by flow cytometry using $DiOC_6$ dye. C, the translocation of cytochrome *c* was analyzed by Western blot analysis. D, effect of treatment with a combination of butein and TRAIL on caspase activity. Relative caspase activity was determined by the manufacturer's protocol. Columns, mean from three independent experiments; bars, SD. Statistical significance was determined by Student's *t* test. *, $P < 0.05$ versus vehicle control.

common component(s) of these DR-mediated apoptotic pathways. Consistent with the TRAIL results, TNF-α alone had no significant influence on cell proliferation in Hep3B cells (Fig. 3A). Interestingly, treatment with a combination of these agents plus butein had no effect on cell death in Hep3B cells, which were very sensitive to the butein/TRAIL-combined treatment. These results indicate that butein selectively facilitates the inhibition of cell growth induced by TRAIL but not the other tested death ligand. Because TRAIL is known to trigger apoptotic signals via two types of DRs, DR4 and DR5, we next examined whether the modulation of DR4 and/or DR5 protein levels by butein might be involved in its sensitizing effect on TRAIL-induced apoptosis. We found that treatment of Hep3B cells with butein induces a time-dependent (left) and dose-dependent (right) increase in the protein levels of DR5 but did not affect the levels of DR4 (Fig. 3B). Fluorescence-activated cell sorting (FACS) analysis also showed that the butein-induced surface expression of DR5 but not DR4 was also significantly increased in Hep3B cells (Fig. 3C). Consistent with this, treatment with butein significantly increased DR5 protein levels in other human cancer cell lines, HepG2, HCT116, PC3, and U937 (Fig. 3D and E). To confirm the functional role of DR5 in the sensitization of TRAIL-induced apoptosis by butein, we also examined the effect of DR5-specific blocking chimera antibody on butein/TRAIL-induced apoptosis. The addition of DR5-specific blocking antibody dose dependently reversed the inhibition of butein/TRAIL-induced cell proliferation in Hep3B cells (Fig. 3F). Similarly, treatment with anti-DR5 antibody in Hep3B cells decreased the DNA fragmentation as a marker of apoptosis (Fig. 3G). Furthermore, suppression of DR5 expression by transfection of Hep3B cells with DR5 siRNA also effectively inhibited butein-stimulated TRAIL-induced growth inhibition (Fig. 3H), supporting the idea that butein-induced upregulation of DR5 is critical for the enhancement of TRAIL sensitivity in Hep3B cells.

Butein activates DR5 transcription through the activation of Sp1 in the DR5 promoter regions

To examine whether butein-induced DR5 upregulation is controlled at the transcriptional level, we did RT-PCR analysis of *DR5*. After the cells were treated with 10 μmol/L butein, we observed a gradual increase in *DR5* mRNA levels of Hep3B (Fig. 4A). We further explored the effects of butein on the promoter activities of reporter constructs containing 2.5- and 0.6-kb fragments of the DR5 gene promoter region (pDR5/*Sac*I and pDR5/−605, respectively; ref. 25) in Hep3B cells. We found that butein significantly increases the promoter activities of both pDR5/*Sac*I (data not shown) and pDR5/−605 (Fig. 4B), suggesting that butein-responsive elements are localized within the smaller fragment (605 bp). Previously, Yoshida et al. (26) 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 are critical for butein-induced DR5 upregulation, we analyzed luciferase assays using reporter constructs with several Sp1 mutants of the promoter. As shown in Fig. 4B, transfection with double mutant mSp1-3 (mutated at the −305 and −300 Sp1 sites) construct significantly decreased butein-induced DR5 promoter activity compared with the wild-type (pDR5/−605) construct. Furthermore, the DR5 promoter activity derived from the triple mutant constructs mSp1-5 and mSp1-6 was significantly decreased by treatment with butein. However, single mutant constructs mSp1-1 and mSp1-2 and double mutant mSp1-4 had only a slight effect on DR5 promoter activity. These results suggest that the two putative Sp1-binding sites present at −305/−300 may play an important role in butein-induced enhancement of DR5 promoter activity. To determine whether butein has an effect on the DNA-binding activity of Sp1, we did electrophoretic mobility shift assay (EMSA) by incubating nuclear extracts from cells exposed to butein with a biotin-labeled Sp1 response element containing oligonucleotides. As shown in Fig. 4C, treatment with 10 μmol/L butein time dependently increased the specific DNA-binding activity of Sp1 in HepG2 and Hep3B cells. Furthermore, Sp1 DNA-binding complexes were undetected when a mutant Sp1 oligonucleotide was used in the binding reaction, and supershift assays using specific Sp1 antibody confirmed that the slowest migrating complex represents Sp1 (data not shown). Western blotting assay detected that treatment with 10 μmol/L butein increased Sp1 translocation to the nucleus (Fig. 4D). To further investigate the exact mechanism of butein on the regulation of DR5 expression, a ChIP assay was done to examine the binding of Sp1 on the DR5 promoter regions. As shown in Fig. 4E, butein increased Sp1 binding to the promoter regions of DR5. To additionally investigate whether butein-induced Sp1 DNA-binding activity was due to Sp1 phosphorylation, the nuclear extract was incubated with the serine-threonine phosphatase PP2A before the binding reaction. As observed in Fig. 4F, the increased Sp1 DNA-binding activity due to butein was blunted in the samples treated with PP2A. These data suggest that butein activates DR5 transcription by increasing Sp1 phosphorylation and consequent Sp1 DNA-binding activity.

Transactivation of the DR5 promoter requires activation of mitogen-activated protein kinase by butein

The phosphorylation of Sp1 has been widely studied and the results showed that some kinases phosphorylate Sp1, which affects transactivation, but some other kinases phosphorylate Sp1, which affects its DNA binding affinity (27). Serine or threonine residues could be phosphorylated by different kinases, including DNA-dependent protein kinase, mitogen-activated protein kinase (MAPK), casein kinase II, and cyclin-dependent kinase 2. To investigate whether butein-mediated Sp1 DNA binding is MAPK

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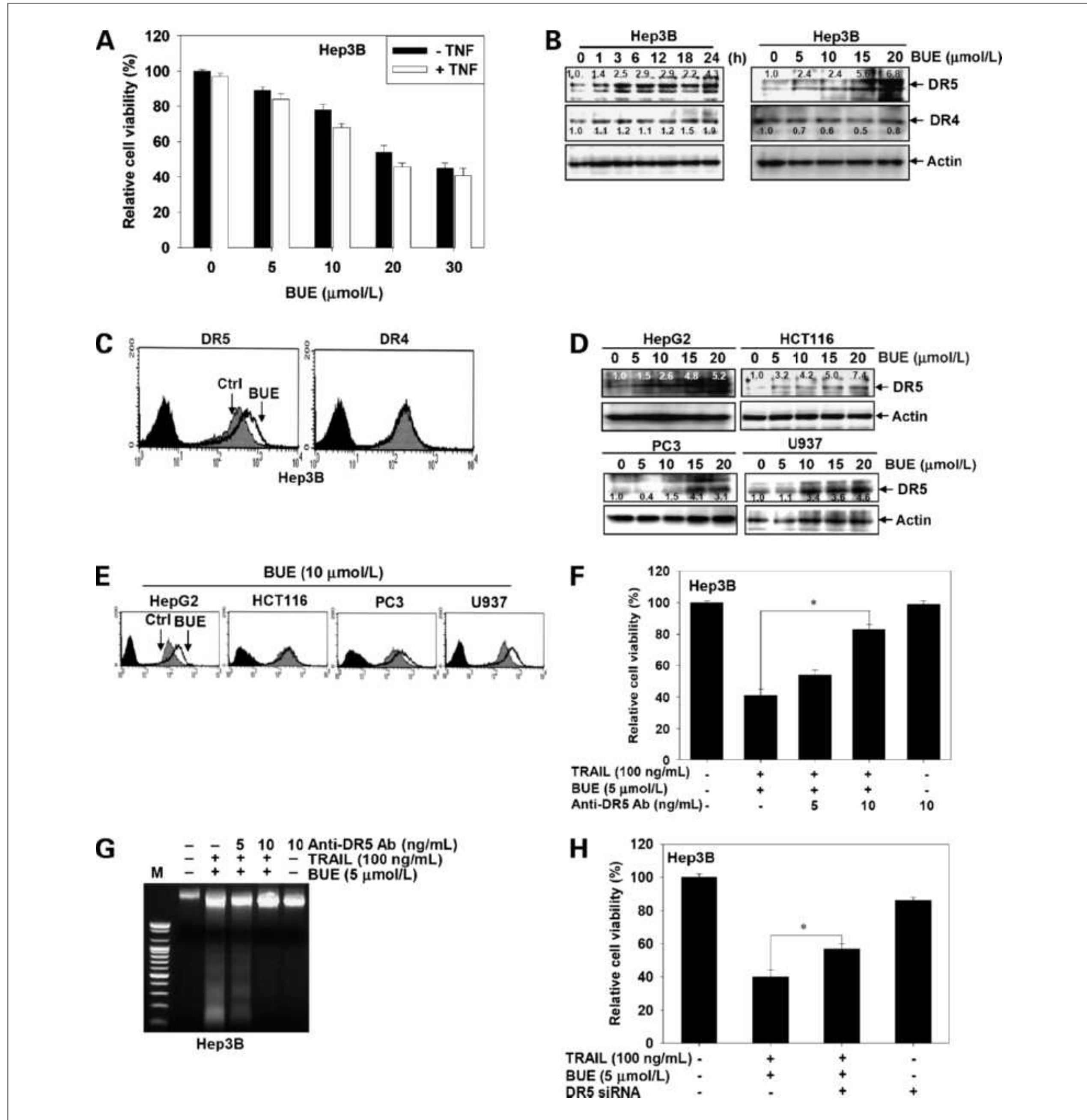

Figure 3. Butein increases DR5 but not DR4 levels in hepatoma cell lines. A, effect of butein on TNF-α–mediated cell death. Hep3B cells were pretreated with the indicated concentration of butein for 30 min and further treated with the indicated concentrations of TNF-α for 24 h. Cellular viability was analyzed by MTT. B, butein-induced DR5 upregulation in Hep3B cells. Hep3B cells were treated with 10 μmol/L butein for the indicated time and Western blot analysis of DR5, DR4, and β-actin as a loading control was conducted. C, effect of butein on the surface expression levels of DR5 and DR4. Hep3B cells were incubated with or without 10 μmol/L butein for 24 h, and flow cytometry was used to analyze the surface expression of DR5 and DR4. *X* axis, fluorescence intensity; *Y* axis, relative number of cells. Black histograms, isotype control; black line, treated cells with butein; gray histograms, untreated cells. D, butein-induced DR5 upregulation in other types of cancer cells. Cells were treated with up to 20 μmol/L butein for 24 h, and cell extracts were prepared for Western blot analysis of DR5. E, surface DR5 expression. Flow cytometry was used to analyze the surface expression of DR5. Black histograms, isotype control; black line, treated cells with butein; gray histograms, untreated cells. F, effect of DR5-specific blocking chimera antibody on butein/TRAIL-induced apoptosis. Hep3B cells were pretreated with or without 5 μmol/L butein for 30 min followed by treatment with or without 100 ng/mL TRAIL for 24 h in the presence of the indicated concentrations of DR5-specific blocking chimera antibody. G, fragmented DNA was extracted and analyzed on 1.5% agarose gel containing ethidium bromide. H, effect of siRNA DR5 on cell death. Hep3B cells were transfected with siRNA duplexes against DR5 mRNA. Twenty-four hours after the transfection, cells were treated with 5 μmol/L butein and 100 ng/mL TRAIL for 24 h. Cellular viability was determined by MTT assay. Columns, mean from three independent experiments; bars, SD. Statistical significance was determined by Student's *t* test. *, $P < 0.05$ versus vehicle control.

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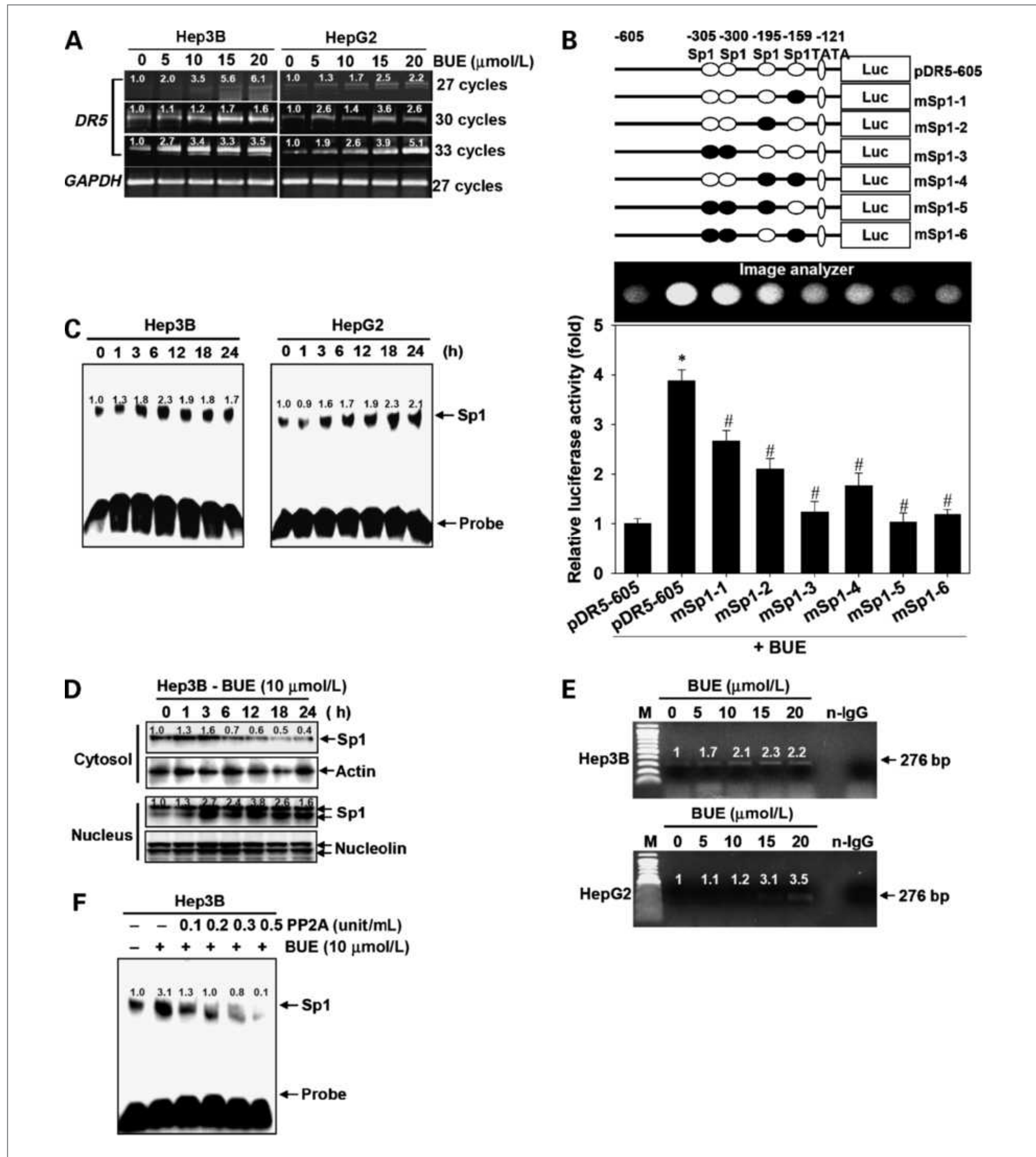

Figure 4. Butein activates transcription of DR5 through Sp1 activation at the −305/−300 region. A, effect of butein on *DR5* mRNA levels. Hep3B cells were treated with up to 10 μmol/L butein for 24 h, total RNA was isolated, and RT-PCR analysis of DR5 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was done. B, effect of butein on DR5 promoter activity. Schematic structures (top) 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 butein were assayed for luciferase activity. C, upregulation of Sp1 DNA binding activity at promoter regions of DR5 by butein. Cells were incubated with 10 μmol/L butein for the indicated time. Sp1 DNA binding activity was analyzed by LightShift chemiluminescent EMSA kit as described in Materials and Methods. D, butein activates Sp1 translocation to the nucleus. Cytosolic and nucleus proteins were prepared, and Western blotting analysis of Sp1 was conducted. E, *in vivo* binding of Sp1 on DR5 promoter regions by butein. ChIP assay was done using antibodies against Sp1 in Hep3B and HepG2 cells. Negative controls were done using antibody against rabbit IgG. F, Sp1 phosphorylation by butein. Nuclear extracts prepared from untreated Hep3B cells (lane 1), treated with 10 μmol/L butein (lanes 2–6), and treated with indicated concentration of PP2A (lanes 3–6) were incubated with a biotin end-labeled Sp1-binding sequence oligonucleotide.

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dependent, Western blotting analysis was used to test the change in phosphorylation of JNK, ERK, and p38. As shown in Fig. 5A, 10 μmol/L butein time dependently increased the phosphorylation of JNK, ERK, and p38. ERK, JNK1/2, and p38 inhibition abrogated butein-mediated Sp1-binding activity (Fig. 5B). Inhibition of ERK, JNK1/2, and p38 inhibited Sp1 translocation to the nucleus (Fig. 5C). However, only inhibition of ERK by PD98059 significantly blocked butein-induced DR5 upregulation and inhibited cleavage of caspase-3 (Fig. 5D) and induction of sub-G_1 population induced by treatment with butein plus TRAIL (Fig. 5E). FACS analysis also showed that PD98059 significantly suppresses the combined treatment-induced surface expression of DR5 in Hep3B cells (Fig. 5F). Moreover, pretreatment of 20 μmol/L PD98059 for 1 hour reduced DR5 promoter (pDR5/−605) activity induced by butein (Fig. 5G). Additionally, we investigated whether reactive oxygen species (ROS) are involved in butein-induced apoptosis in hepatoma cells treated with 5 μmol/L butein. H_2DCFDA-based FACS detection revealed that intracellular ROS levels slightly increased following treatment with butein (Fig. 5H). The butein-induced increases in ROS levels were completely blocked by pretreatment with antioxidants, NAC, and glutathione. We next tested whether scavenging of ROS attenuates the growth inhibition induced by the treatment of the combination of butein and TRAIL. Pretreatment with NAC and glutathione did not block the antiproliferative effects induced by the combination of butein and TRAIL (Fig. 5H). These results suggest that ERK may be involved in butein-induced DR5 expression via Sp1 activation.

Butein inhibits TRAIL-mediated NF-κB activation

Because TRAIL can trigger both death and survival signaling (e.g., NF-κB), thus regulating the expression of several factors involved in cell survival, we examined the effect of butein on TRAIL-mediated NF-κB by EMSA assay. As shown in Fig. 6A, 50 ng/mL TRAIL induced a strong increase in binding of NF-κB within 0.5 hour and was sustained for 3 hours. Pretreatment with butein inhibited TRAIL-induced NF-κB activation in a dose-dependent manner (Fig. 6B). Especially, at a dose of 10 μmol/L, butein significantly inhibited NF-κB activation induced by TRAIL as much as NF-κB inhibitors [PDTC, MG-132, and bortezomib (PS341)] in Hep3B cells (Fig. 6B). It is well known that NF-κB activation by TRAIL is mediated by phosphorylation and degradation of IκBα. As shown in Fig. 6C, butein blocked TRAIL-dependent IκBα degradation. Butein also repressed nuclear translocation of p65 and p50 induced by TRAIL. To further confirm the role of NF-κB in butein/TRAIL-induced apoptosis, cells were pretreated for 30 minutes with PDTC, MG132, and PS341. The cells were then treated with butein and TRAIL for an additional 24 hours, and flow cytometry was used to assess the sub-G_1 cell populations. As shown in Fig. 6D, cells incubated with TRAIL or PDTC, MG132, and PS341 alone resulted in a slight increase in the sub-G_1 percentage. However, treatment with a combination of the NF-κB inhibitors and TRAIL or TRAIL/butein markedly increased the accumulation of sub-G_1 phase cells. In addition, as observed by MTT assay (data not shown), butein-mediated repressed cell viability is enhanced by blocking of the NF-κB signal pathway. These results suggest that butein treatment is likely to inhibit the NF-κB pathway and that this pathway is likely involved in apoptosis.

Discussion

Although TRAIL is thought to be a highly promising candidate for cancer treatment, use of TRAIL has major limits in clinical applications because of resistance against TRAIL in a variety of cancer cells (2, 3). Nevertheless, many scientists have reported that conventional cytotoxic drugs such as antioxidants and novel molecular-targeted agents or irradiation markedly sensitize TRAIL-induced apoptosis in TRAIL-resistant cancer cells (8–10). Thus, efforts to identify agents that activate DRs or block antiapoptotic effectors may improve anticancer therapeutic design. Here, we provide evidence that butein is capable of triggering TRAIL-induced apoptosis at least in TRAIL-resistant hepatoma cells.

DRs have a highly conserved extracellular region containing cysteine-rich repeats and a conserved intracellular region of 80 amino acids termed the death domain (28). The domain is essential for the rising death signal, resulting in the activation of caspase-8 (29). Therefore, it has been thought that regulation of DRs causes effective death of cancer cells through direct binding with TRAIL. Some scientists reported that a few chemicals or proteins sensitize TRAIL-induced apoptosis through overexpression of DR4 or DR5 (11–13). In this study, we also found that butein mainly increases the expression of DR5 at the transcriptional level but maintains the expression of DR4. Additionally, for the functional study of DR5, DR5-specific blocking chimeric antibody and transient knockdown of DR5 expression by siRNA duplexes significantly inhibited the growth-inhibitory effects induced by treatment with a combination of butein and TRAIL in Hep3B cells. Nevertheless, we cannot rule out the possibility that the upregulation of DR5 is not the only way for sensitizing to TRAIL because knockdown of DR5 caused only a modest decrease in TRAIL-induced apoptosis despite the almost complete loss of DR5 expression (8). Recently, it is well known that the tumor suppressor gene *p53* is a main regulator for the expression of genes engaged in apoptosis (30). Many scientists have reported that overexpression of p53 transactivates DR4 and DR5 gene-induced DNA damage–inducing agents, suggesting that DR4 and DR5 definitely are important regulators in p53-dependent apoptosis (11, 13). Nevertheless, there has been conflicting evidence about the involvement of p53 in regulating the expression of DR4 and DR5 until now (17). In this study, butein induced the expression of DR5, regardless of p53 status. These results may imply that

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Figure 5. Butein-induced Sp1 translocation is mediated by ERK. A, effects of butein on phosphorylation of MAPKs. Hep3B cells were treated with 10 μmol/L butein for the indicated times. Equal amounts of cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against phospho-JNK (p-JNK), JNK, phospho-ERK (p-ERK), ERK, phospho-p38 (p-p38), and p38. B and C, regulation of Sp1 activity by MAPK inhibitors. MAPKs are involved in butein-upregulated Sp1 DNA binding. Hep3B cells were stimulated with 10 μmol/L butein for 24 h after pretreatment with 20 μmol/L PD98059, 20 μmol/L SP600125, and 10 μmol/L SB203580 for 1 h. Then, Sp1 DNA binding activity and nuclear translocation were analyzed by EMSA (B) and Western blot analysis (C). D, effects of MAPKs on DR5 and caspsase-3 induced by butein and TRAIL. Hep3B cells were stimulated with 5 μmol/L butein and 100 ng/mL TRAIL for 24 h after pretreatment with 20 μmol/L PD98059, 20 μmol/L SP600125, and 10 μmol/L SB203580 for 1 h. Then, DR5 and caspase-3 were analyzed by Western blot analysis. E, flow cytometric analysis of sub-G_1 phase by combined treatment with butein (10 μmol/L) and TRAIL (100 ng/mL). After combined treatment for 24 h, cells were stained with propidium iodide (PI) and analyzed by flow cytometry. F, effect of PD98059 on butein-induced DR5 expression. Cells were incubated with or without 10 μmol/L butein for 24 h, and flow cytometry was used to analyze the surface expression of DR5. *X* axis, fluorescence intensity; *Y* axis, relative number of cells. Black histograms, isotype control; gray histograms, treated cells with butein. G, effect of PD98059 on DR5 promoter assay. PD98059 inhibits DR5 luciferase activity induced by butein. pDR5/−605 was transfected into Hep3B cells, which were then pretreated with 20 μmol/L PD98059 for 1 h and further treated with 10 μmol/L butein for 24 h, lysed, and assayed for luciferase activity. H, effect of ROS on butein/TRAIL-mediated apoptosis. Hep3B cells were pretreated with 5 μmol/L NAC and glutathione for 30 min and further treated with 5 μmol/L butein + 100 ng/mL TRAIL for 24 h. Intracellular H_2O_2 and the DNA contents of the cells were analyzed by flow cytometry. PD, PD98059; SP, SP600125; SB, SB203580; T, TNF-α; B, butein.

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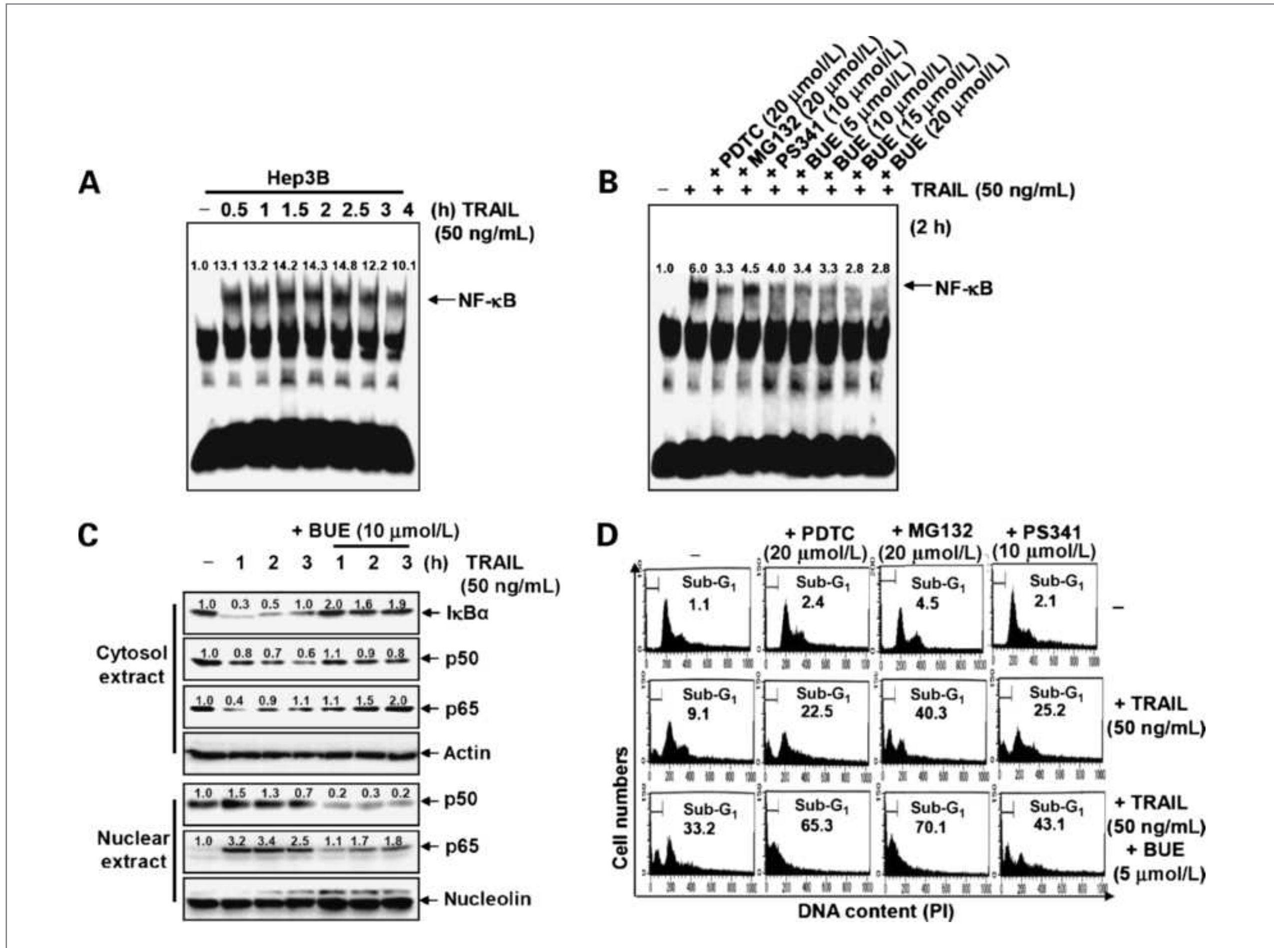

Figure 6. Butein inhibits TRAIL-induced NF-κB activation. A, effect of TRAIL on NF-κB activity. TRAIL activates NF-κB in Hep3B cells. Hep3B cells were incubated with 50 ng/mL TRAIL for 4 h. Nuclear extracts were then prepared and assayed for NF-κB by EMSA. B, effect of butein on NF-κB activity. Butein suppressed NF-κB activity induced by TRAIL. After treatment with the indicated concentration of butein and 20 μmol/L PDTC, 20 μmol/L MG132, and 10 μmol/L PS341 for 2 h, the cells were treated with 50 ng/mL TRAIL for 2 h and assayed for NF-κB by EMSA. C, effect of butein on expression of IκBα, p65, and p50. Butein prevents degradation of IκBα and translocation of p65 and p50. Hep3B cells were treated with 10 μmol/L butein for 1 h followed by treatment with 50 ng/mL TRAIL for the indicated time intervals. Equal amounts of cytoplasmic proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies. β-Actin was used as a loading control. Nuclear extracts were prepared, resolved by 10% SDS-PAGE, and electrotransferred onto a nitrocellulose membrane. Western blot analysis was conducted with antibodies against p65 and p50. Nucleolin was used as a loading control. D, effect of NF-κB on cell death. Butein- and TRAIL-induced apoptosis is enhanced by blocking of the NF-κB signal pathway. Hep3B cells were pretreated with 20 μmol/L PDTC, 20 μmol/L MG132, and 10 μmol/L PS341 for 2 h before treatment with 50 ng/mL TRAIL alone or 50 ng/mL TRAIL plus 5 μmol/L butein for 24 h. The DNA contents of the cells were analyzed by flow cytometry.

upregulation of DR5 is functionally essential for butein-sensitized apoptosis at least in TRAIL-resistant hepatoma cells through a p53-independent mechanism.

Sp1 is well known to bind to G-rich elements such as GC-box (GGGGCGGGG) and GT-box (GGTGTGGGG; ref. 31). Recently, it has been well known that binding of Sp1 in the promoter regions tightly regulates DR5 transcription in a variety of cancer cells, and the required specific Sp1 site was mainly found at −195/−159 to the transcription start site (21, 22). In this study, we found that treatment with butein significantly induced expression of Sp1 in HepG2 and Hep3B cells that was correlated to the induction of DR5. Reporter gene analysis also showed two regions (−305 and −300) of the DR5 promoter that seemed to be important in butein regulation of DR5 promoter activity. Furthermore, the ChIP assay showed that Sp1 can directly bind to the DR5 promoter regions and regulated transcriptional expression. These results suggest that induction of Sp1 significantly sensitizes butein/TRAIL-dependent apoptosis through DR5 expression. Nevertheless, the regulation of DR5 expression is much less understood in the combined treatment. Currently, DR5 expression has been regulated by Sp1 transcription factor activity (21, 22). It was reported that ROS generation is a major target for triggering and amplifying TRAIL-dependent apoptosis through DR5

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(32, 33). JNK directly is activated by ROS and stimulates DR5 expression (25, 34). Therefore, we hypothesized that the combined treatment sensitizes the ROS-JNK-Sp1 signal cascades to activate DR5 expression; however, pretreatment of NAC or glutathione has no significant effect on cell growth inhibition and DR5 promoter activity. Nevertheless, our previous study reported that high doses of butein (<40 μmol/L) increase G_2-M phase arrest and cell growth inhibition through the ROS-JNK pathway (35). However, low doses of butein (<10 μmol/L) induced G_1 phase arrest without cell growth inhibition in a ROS-independent manner. Only the ERK inhibitor PD98059 significantly blocked butein-mediated Sp1 activation and cell death. The results indicate that Sp1 is a butein-regulated transcription factor, and ERK is an intermediary kinase in this relationship. Additionally, JNK could be correlated with DR5 expression via Sp1 activation because it is well known that pretreatment with SP600125 moderately decreased DR5 expression and cell death (8, 25, 34). We did further study to elucidate if JNK and p38 have influence on the DR5 promoter activity. SP600125 itself significantly increased promoter activity of DR5, but SP600125 evidently downregulated its activity in the presence of butein (data not shown). These data indicated that the JNK signal pathway may be also related to DR5 regulation in butein-induced apoptosis. Nevertheless, we cannot rule out the possibility of EKR inhibition by 20 μmol/L SP600125 because SP600125 quite selectively blocks JNK at nanomolar concentrations. Additionally, although SB203580 suppressed the expression of Sp1 and proapoptotic proteins, we cannot find any differences of DR5 promoter activity in the presence of SB203580. Therefore, more prudent experiments will be needed for the effects of p38 on TRAIL-induced apoptosis.

The NF-κB transcription factor family consists of several structurally related proteins such as c-Rel, Rel-A (p60), Rel-B, p50/p105, and p52/p100, which form homodimers or heterodimers with each other and regulate the expression of a number of genes (36). TRAIL induced additional binding activity of NF-κB composed of a Rel-A/p50 heterodimer in these cells. Ravi et al. (37) have recently reported that Rel-$A^{-/-}$ mouse fibroblasts are highly sensitive to TRAIL-induced apoptosis and that anti-CD40–mediated activation of NF-κB, including Rel-A, effectively blocked TRAIL-induced apoptosis. Therefore, NF-κB composed of Rel-A/p50 seems to play a key role in the resistance of Hep3B and HepG2 cells to TRAIL-induced apoptosis. Indeed, pretreatment with butein inhibited TRAIL-mediated activation of Rel-A/p50 NF-κB that was sensitized to TRAIL-induced apoptosis by butein. Similar observations have been reported that butein suppressed activation of Rel-A/p50 NF-κB induced by TNF-α (38). Taken together, these results indicate that butein could possibly sensitize to TRAIL-induced apoptosis by inhibiting the activation of NF-κB, including Rel-A. In contrast, recently important findings showed that the activation status of NF-κB is not sufficient to determine the fate of a cell with respect to TRAIL-induced apoptosis in hepatocellular carcinoma (10). Braeuer et al. (39) also reported that constitutively activated NF-κB, but not induced NF-κB, leads to TRAIL resistance by upregulation of XIAP in human cancer cells. Therefore, the effects of NF-κB will be investigated in TRAIL resistance. In addition, suppression of NF-κB activity by butein may also be involved in the stimulation of caspase-8 activity because the NF-κB–induced products, IAP-1, IAP-2, and XIAP, are known to cooperatively block caspase-8 activity (40).

In summary, we showed that butein sensitizes apoptosis in TRAIL-resistant cells via upregulation of DR5 expression. We also showed that DR5 expression is tightly regulated by Sp1 activity in the promoter regions of the gene. However, p53 is not necessary for butein-induced DR5 expression and triggers apoptosis in TRAIL-stimulated cells. We also found that the JNK inhibitor SP600125 partially suppresses Sp1 and DR5 expression and cell death; however, the ERK inhibitor PD98059 significantly abrogated the effect. Consequently, butein p53 independently upregulates DR5 expression through the ERK and Sp1 mechanism and sensitizes apoptosis in TRAIL-resistant cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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