Pretreatment of Acetylsalicylic Acid Promotes Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis by Down-regulating BCL-2 Gene Expression

Received for publication, April 5, 2005, and in revised form, September 19, 2005 Published, JBC Papers in Press, September 30, 2005, DOI 10.1074/jbc.M503713.200

Ki M. Kim1, Jae J. Song1, Jee Young An2, Yong Tae Kwon3, and Yong J. Lee1

From the 1Department of Surgery and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213 and 2Center for Pharmacogenetics and Department of Pharmaceutical Sciences, University of Pittsburgh School of Pharmacy, Pittsburgh, Pennsylvania 15261

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues. However, not all cancers are sensitive to TRAIL-mediated apoptosis. Thus, TRAIL-resistant cancer cells must be sensitized first to become responsive to TRAIL. In this study, we observed that pretreatment by acetylsalicylic acid (ASA) augmented TRAIL-induced apoptotic death in human prostate adenocarcinoma LNCaP and human colorectal carcinoma CX-1 cells. Western blot analysis showed that pretreatment of ASA followed by TRAIL treatment activated caspases (8, 9, and 3) and cleaved poly(ADP-ribose) polymerase, the hallmark feature of apoptosis. Most interestingly, at least 12 h of pretreatment with ASA was prerequisite for promoting TRAIL-induced apoptosis and was related to down-regulation of BCL-2. Biochemical analysis revealed that ASA inhibited NF-κB activity, which is known to regulate BCL-2 gene expression, by dephosphorylating IκB-α and inhibiting IKKβ activity but not by affecting the HER-2/neu phosphatidylinositol 3-kinase- Akt signal pathway. Overexpression of BCL-2 suppressed the promotive effect of ASA on TRAIL-induced apoptosis and changes in mitochondrial membrane potential. Taken together, our studies suggested that ASA-promoted TRAIL cytotoxicity is mediated through down-regulating BCL-2 and by decreasing mitochondrial membrane potential.

Recent studies have also revealed that TRAIL, which act as decoy receptors by inhibiting TRAIL signaling (4–7). Tumor necrosis factor-related- apoptosing ligand (TRAIL) is a cytotoxic molecule that has been shown to exert, selectively, anti-tumor cytotoxic effects both in vitro and in vivo with minimal toxicity to normal tissues (9, 10).

TRAIL has been considered a new therapeutic agent, and preclinical studies demonstrate its antitumor activity alone or in combination with drugs (10–13). However, many tumor cells have been shown to be resistant to TRAIL (14, 15). Several researchers have reported that TRAIL resistance can be overcome by various sensitizing agents like chemotherapeutic drugs (16, 17), cytokines (18), and matrix metalloprotease inhibitors (19) that are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis.

In recent studies, nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (aspirin; ASA), have been used as chemopreventive agents of cancers to induce apoptosis or to reduce the incidence of tumor formations in a variety of organs, i.e. colon (20), lung (21), stomach (22), and colorectum (23). ASA is known to act by directly suppressing the cyclooxygenase enzyme (COX-1 and COX-2), the rate-limiting enzyme catalyzing the biosynthesis of prostaglandins, thereby blocking the production of proinflammatory prostaglandins. ASA was also shown to be effective in the inhibition of ultraviolet radiation and carcinogen-induced tumor formations in animal models (24, 25).

In this study, we examined whether ASA in combination with TRAIL increases TRAIL-induced apoptotic death in TRAIL-resistant human cancer cells. We hypothesized that pretreatment with ASA enhances TRAIL-induced apoptosis by promoting the mitochondria-dependent apoptotic pathway. Our studies demonstrate that ASA augments TRAIL-induced apoptosis by down-regulating BCL-2 gene expression and by decreasing mitochondrial membrane potential, which subsequently leads to an increase in caspase activation.

EXPERIMENTAL PROCEDURES

Reagents—ASA, NS-398, indomethacin, sulindac sulfide, and carbonyl cyanide p-trifluoromethoxyphenylhydrazone were obtained from Sigma. Tetramethylrhodamine methyl ester (TMRM) was purchased from Molecular Probes (Eugene, OR). Anti-Bcl-XL, anti-phospho(Tyr...
Aspirin and TRAIL-induced Apoptosis

**FIGURE 1.** Effect of pretreatment of acetylsalicylic acid on TRAIL-induced cytotoxicity in human prostate adenocarcinoma LNCaP cells. A, cells were pretreated with various concentrations of ASA (0.01–1 mM) for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent the means ± S.E. from three separate experiments. B, cells were pretreated with 1 mM ASA for 20 h and treated with/without various concentrations of TRAIL (1–200 ng/ml) for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent the means ± S.E. from three separate experiments. C, cells were pretreated with 1 mM ASA for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. After treatment, apoptosis was detected by the TUNEL assay. Apoptotic cells are indicated by arrows. a, untreated control; b, ASA only; c, TRAIL only; d, ASA → TRAIL.

508)-PI3K, anti-caspase-3, anti-caspase-9, anti-histone H1, anti-IKKα, and anti-IKKβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HER-2/neu antibody was purchased from DakoCytomation (Carpinteria, CA). Anti-DR5 and anti-DcR2 bodies were purchased from R&D Systems (Minneapolis, MN). Anti-FLIP (amino acids 114–281) obtained by RT-PCR was cloned into pET-3d (Novagen, Madison, WI) plasmid, and His-tagged TRAIL protein was purified using the QIAexpress protein purification system (Qiagen, Valencia, CA).

**TUNEL Assay**—For detection of apoptosis by the TUNEL method, cells were plated in slide chambers. After treatment, cells were fixed with 70% ethanol in PBS. Cells were washed once, permeabilized by incubating with 100 μl of 0.1% Triton X-100, 0.1% sodium citrate, and then washed twice in PBS. The TUNEL reaction was carried out at 37 °C for 1 h with 0.3 nmol of fluorescein isothiocyanate-12-dUTP, 3 nmol of dATP, 2 μl of CoCl2, 25 units of terminal deoxynucleotidyltransferase, and TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate) in a total reaction volume of 50 μl. The reaction was stopped with 2 μl of 0.5 M EDTA. Cells were observed under a fluorescence microscope.

**RNA Interference by siRNA of COX-2**—To down-regulate the COX-2, COX-2 siRNA (Santa Cruz Biotechnology) was used. COX-2 siRNA was transfected into LNCaP cells and incubated for 36 h. The interference of COX-2 protein expression was confirmed by immunoblot using anti-COX-2 antibody (Cayman Chemical).

**Transfection**—In order to generate Bcl-2 overexpressing LNCaP cells and CX-1 cells, cells were transfected with pcDNA3-Bcl-2 or pcDNA3-neo using Lipofectamine Plus (Invitrogen). Transfected cells were selected for 3 weeks in growth medium containing 0.5 mg of G-418 (geneticin; Invitrogen) per ml. The clone expressing the highest level of Bcl-2 was used for this study. The expression level was determined by immunoblot analysis.
Protein Extracts and PAGE—Cells were lysed with 1× Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce). The samples were diluted with 1× lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli (27) using a Hoefer gel apparatus.

Immunoblot Analysis—Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS/Tween 20 (0.1%, v/v) at 4 °C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer’s instructions) for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham Biosciences).

In Vitro Kinase Assay—For immunocomplex kinase assay, cells were lysed with 500 μl of buffer A (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% deoxycholate, 5 mM EGTA, 150 mM NaCl, 10 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protein inhibitor mixture solution (Sigma)). Cell lysates were immunoprecipitated with anti-IKKα or anti-IKKβ antibody. Immune complexes were washed twice with buffer B (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5 mM DTT, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride) at 4 °C and then incubated with 2 μg of purified GST-1xIκBα protein (Upstate Biotechnology) in the presence or absence of 1 mM ASA in a volume of 50 μl of a kinase buffer (100 μM ATP, 20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 0.1 mM EDTA) for 30 min at 30 °C. Thereafter, the sample was subjected to SDS-PAGE, and the phosphorylation of GST-1xIκBα was analyzed by anti-phospho-IκBα antibody (Cell Signaling).

Measurement of Mitochondrial Membrane Depolarization—The mitochondria-specific dye TMRM (Molecular Probe, Eugene, OR) was used to measure the mitochondrial potential. CX-1 cells were grown in 6-well plates and were pretreated with 1 mM aspirin in the presence or absence of TRAIL (200 ng/ml). After treatment, the cells were collected, washed in PBS, and resuspended in 500 μl of FASC buffer. Cells were incubated for 20 min with 200 nM TMRM (Molecular Probe) at 4 °C in the dark, washed in cold PBS twice, and then resuspended in 500 μl of PBS buffer. The cells were visualized by flow cytometry. Positive samples were stained with carbonyl cyanide p-trifluoromethoxyphenylhydrazone (mitochondrial membrane-depolarized inducer), and the surface markers were analyzed by an EPICS XL-MCL flow cytometer with a single argon laser at 488 nm (Beckman Coulter, Inc., Hialeah, FL).

Isolation of Nuclear Proteins—Nuclear extracts were prepared by the modified procedure of Dignam et al. (28). Following treatment with ASA for 20 h, LNCaP cells were washed three times with PBS and incubated on ice for 15 min in hypotonic buffer A (10 mmol/liter HEPES, pH 7.9, 10 mmol/liter KCl, 0.1 mmol/liter EDTA, 0.1 mmol/liter EGTA, 1 mmol/liter DTT, 0.5 mmol/liter phenylmethylsulfonyl fluoride, and 0.6% Nonidet P-40). Cells were vortexed gently for lysis, and the nuclei were separated from the cytosol by centrifugation at 12,000 × g for 1 min. Nuclei were resuspended in buffer C (20 mmol/liter HEPES, pH 7.9, 25% glycerol, 0.4 mol/liter NaCl, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 1 mmol/liter DTT, 0.5 mmol/liter phenylmethylsulfonyl fluoride, and 0.6% Nonidet P-40). Nuclei were resuspended in buffer C (20 mmol/liter HEPES, pH 7.9, 25% glycerol, 0.4 mol/liter NaCl, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 1 mmol/liter DTT, 0.5 mmol/liter phenylmethylsulfonyl fluoride) and shaken for 30 min at 4 °C. Nuclear extracts were obtained by centrifugation at 12,000 × g, and protein concentration was measured by Bradford assay (Bio-Rad). NF-κB in nuclear extracts was detected by Western blotting as described above.

FIGURE 2. Effect of pretreatment of acetylsalicylic acid on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in LNCaP (A), DU-145 (B), or YPEN (C) cells. Cells were pretreated with various concentrations of ASA (1–10 μM) for 20 h, treated with/without TRAIL (200 ng/ml in LNCaP and YPEN, 50 ng/ml in DU-145) for 4 h, and then harvested. Cell lysates were subjected to immunoblotting for PARP, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects inactive form (55 kDa) and cleaved intermediates (41 and 43 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa) and cleaved active form (17 kDa). Immunoblots of PARP show the 116-kDa PARP and the 85-kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane. Con, control; Casp, caspase.
Electrophoretic Mobility Shift Assay—LNCaP cells were treated with various concentrations of ASA (0.01–1 mM) or 200 ng/ml TRAIL for 20 h, and nuclear extract was prepared as described above. The nuclear extract (10 μg of protein) was incubated with binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM ammonium sulfate, 1 mM DTT, 30 mM KCl, 0.2% Tween 20) and 1 μg of poly(dI-dC) for 10 min on ice. Biotin-labeled probe NF-kB-specific oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') was used. The reaction was incubated at room temperature for 30 min. The negative control consisted of free probe only. A competition control was set up by adding non-biotin-labeled cold probe to the reaction. The samples separated on a 6% native polyacrylamide gel in 0.5% TBE for 50 min at 120 V. The samples were then transferred in 0.5% TBE onto a nylon membrane at 300 mA for 40 min. After transfer, the sample was fixed on the membrane by UV cross-linking. The membrane was first blocked with 1% blocking reagent (Roche Applied Science) at room temperature for 30 min. The biotin-labeled probe was then detected with streptavidin-horseradish peroxidase diluted 1:20,000 (Pierce). After washing three times and equilibrating in buffer, the membrane was overlaid with lumino/enhancer and substrate for 5 min. The image was acquired using a Kodak X-Omat 2000A (Eastman Kodak, Rochester, NY).

Flow Cytometry—Cells were treated with ASA for the indicated time points with or without TRAIL. After washing, cells were blocked for 30 min with 1% bovine serum albumin in PBS. Cells were then incubated with 1 μg of primary antibodies to DR4 or DR5 (Alexis) in 1% bovine serum albumin for 30 min followed by washing with PBS. Finally, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probe) for 30 min. After washing, the cells were analyzed on a FACScan flow cytometer. Matched isotype using control IgG antibodies was included.

Aspirin and TRAIL-induced Apoptosis

Flow Cytometry—Cells were treated with ASA for the indicated time points with or without TRAIL. After washing, cells were blocked for 30 min with 1% bovine serum albumin in PBS. Cells were then incubated with 1 μg of primary antibodies to DR4 or DR5 (Alexis) in 1% bovine serum albumin for 30 min followed by washing with PBS. Finally, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probe) for 30 min. After washing, the cells were analyzed on a FACScan flow cytometer. Matched isotype using control IgG antibodies was included.
RT-PCR Analysis of Bcl-2 mRNA Levels—Total cellular RNA was extracted using the Trizol method (Invitrogen) according to the manufacturer’s instructions. For each RT-PCR, 1 µg of total RNA was used with Novagen One-step RT-PCR kit (EMD Bioscience). The following sense and antisense primers were used at 0.5 µM for each: Bcl-2 primer, sense, 5'-CGACGACTTCTCCCGCCGCTACCGC-3', and antisense,
Aspirin and TRAIL-induced Apoptosis

5′-CCGCAATGCTGGGCGGTCACAGTCC-3′; glyceraldehyde-3-phosphate dehydrogenase primer, sense, 5′-TCCACCACCTGGTTGCTGTA-3′, and antisense, 5′-ACCACAGTCCATGCCCACAC-3′. The reaction conditions were 40 cycles at reverse transcription at 60 °C for 30 min, initial PCR activation at 94 °C for 2 min, denature at 94 °C for 1 min, anneal at 60 °C for 90 s, and final extension at 60 °C for 7 min. After amplification, the products were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

Northern Blot Analysis—LNCaP cells were treated with either 200 ng/ml TRAIL or concentrations of aspirin (0.01, 0.1, 0.5, and 1 mM) for 20 h. For Northern blot hybridization, total RNAs (10 μg) isolated from the above-treated cells were fractionated by electrophoresis in formaldehyde-1.2% agarose gels, blotted onto Nytran Plus (Schleicher & Schuell), and hybridized with the 32P-labeled BCL-2 cDNA probe. The 448-bp BCL-2 cDNA probe was amplified by using PCR with primers 5′-CACCTGACCCTGGCCGTACAGTTCC-3′ (forward) and 5′-TGTTGACTTTCACTTGGCCGACAG-3′ (reverse).

RESULTS

ASA Promotes TRAIL-induced Cytotoxicity—To investigate the effect of ASA on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma LNCaP cells were pretreated with ASA and treated with TRAIL in the presence of ASA. Fig. 1, A and B, shows that little or no cytotoxicity was observed with 1 mM ASA alone or 200 ng/ml TRAIL alone. However, pretreatment of ASA promoted TRAIL-induced cytotoxicity that was dependent upon concentrations of ASA (Fig. 1A) and TRAIL (Fig. 1B). Similar results were observed with TUNEL staining (Fig. 1C). Data from TUNEL assays show that apoptotic cell death occurred when LNCaP cells were pretreated with ASA followed by TRAIL.

Effect of ASA on TRAIL-induced Apoptosis—Additional studies were designed to examine whether pretreatment with ASA followed by treatment with TRAIL causes PARP cleavage, the hallmark feature of apoptosis, in prostate cancer LNCaP and DU-145 cells and normal prostate YPEN cells. Fig. 2 shows that PARP (116 kDa) was cleaved yielding a characteristic 85-kDa fragment in the presence of TRAIL (50–200 ng/ml) and ASA (1–10 μM) in prostate cancer cells, but not in normal prostate cells. The cleavage of PARP was not observed by treatment with ASA alone. These results were similar to the observations of cytotoxicity (Fig. 1, A and B). Western blot analysis shows that procaspase-8 (55 kDa) was cleaved to the intermediates (41 and 43 kDa) by pretreatment with ASA and treatment with TRAIL in LNCaP and DU-145 cells. The combined treatment of TRAIL and ASA also resulted in an increase in caspase-9 activation as well as caspase-3 activation in LNCaP and DU-145 cells (Fig. 2, A and B). The precursor form of caspase-9 and -3 was cleaved to the active form of 37 and 17 kDa, respectively. ASA alone did not activate caspases. We extended our studies to investigate a time course and dose response on PARP cleavage. Fig. 3A shows that at least 12 h of pretreatment with ASA was required for PARP cleavage in the presence of TRAIL. Fig. 3, B and C, shows that a minimal amount of 10 ng/ml TRAIL or 0.01 mM ASA was required for PARP cleavage in the presence of 1 mM ASA or 200 ng/ml TRAIL, respectively, in LNCaP cells. We further investigated whether treatment with ASA is a prerequisite. Fig. 4 shows that combined treatment with TRAIL and ASA did not activate caspases. Taken together, these results suggest that pretreatment...
with ASA for 12 h is essential for inducing apoptotic death in the presence of TRAIL.

Role of COX in TRAIL-induced Apoptosis—It is well known that ASA inhibits only COX-1 at low concentrations (IC50 = 44 μM) but both COX-1 and COX-2 at higher concentrations (IC50 = 1100 μM) (29). To examine whether the promotive effect of ASA on TRAIL-induced apoptosis is mediated through inhibiting COX, LNCap cells were pretreated with various NSAIDs and then treated with TRAIL. Unlike ASA, Fig. 5, A–C, shows that no significant cleavage of PARP was observed by treatment with various concentrations of sulindac sulfide (IC50 = 1.02 μM for COX-1 and IC50 = 10.43 μM for COX-2), NS-398 (a selective COX-2 inhibitor; IC50 = 4.81 μM for COX-1 and IC50 = 0.47 μM for COX-2), or indomethacin (a nonselective COX inhibitor; IC50 = 0.16 μM for COX-1 and IC50 = 0.46 μM for COX-2). To confirm our observations, cells were transfected with COX-2 siRNA or mock siRNA. Fig. 5D shows that the expression of COX-2 was effectively inhibited by siCOX-2. However, knock-down of COX-2 expression did not promote TRAIL-induced apoptosis. Nonetheless, pretreatment with ASA promoted TRAIL-induced apoptosis regardless of the presence or the absence of COX-2. These results suggest that COX is not involved in ASA-promoted TRAIL cytotoxicity.

Effect of ASA on the Level of TRAIL Receptor Family and Anti-apoptotic Proteins—It is well known that TRAIL can interact with death receptors (DR4 and DR5), which trigger apoptotic signals (4). Such signals may be blocked by expression of the antagonistic decoy receptors (DcR1 and DcR2). Previous studies demonstrate that increased DR5 levels are induced by chemotherapeutic agents (30). Thus, we examined whether pretreatment with ASA affects the level of TRAIL receptors and anti-apoptotic proteins, and consequently promotes apoptosis by treatment with TRAIL. LN Cap cells were pretreated with ASA (1–10 mM) and treated with 200 ng/ml TRAIL in the presence of ASA. Data from Western blot analysis reveal that ASA treatment did not significantly alter the total cellular levels of the TRAIL receptors (DR4, DR5, and DcR2) and anti-apoptotic proteins (FLIPL, FLIPp, IAP-1, IAP-2, and Bcl-XL) (Fig. 6, A and C). Data from flow cytometric analysis show that TRAIL induced surface expression of DR5 but not DR4 (Fig. 6B). However, ASA treatment did not enhance the DR5 expression. Most interestingly, ASA treatment resulted in a decrease in the level of Bcl-2 (Fig. 6C). The reduction of Bcl-2 during treatment with 1 μM ASA was dependent upon exposure time (Fig. 6B). To confirm the effect of ASA on BCL-2 gene expression, LN Cap or DU-145 prostate cancer cells were treated with various concentrations of ASA, and expression of BCL-2 was examined. Fig. 7A shows that ASA reduced the level of Bcl-2 in both cell lines. Data from RT-PCR and Northern blot assay in Fig. 7, B and C, show that the level of BCL-2 mRNA was significantly decreased during treatment with ASA. The reduction of BCL-2 mRNA was dependent upon ASA concentration. These results suggest that the reduction of Bcl-2 levels during treatment with ASA was because of suppression of BCL-2 gene transcription.
Effect of ASA on the HER-2/neu-PI3K-Akt-NF-κB Signal Transduction Pathway—It is well known that BCL-2 expression is regulated by NF-κB, a dimeric transcription factor (31). We postulated that ASA inhibits NF-κB activity, which subsequently decreases transcription of BCL-2. To examine this possibility, the effect of ASA on upstream signal transduction of NF-κB was investigated. Fig. 8 shows that ASA treatment did not change the level of HER-2/neu, PI3K, and Akt or alter the phosphorylation of these proteins. In contrast, ASA treatment inhibited IKKβ activity, dephosphorylated IκB-α, increased the level of IκB-α, and prevented NF-κB nuclear translocation (Fig. 9). These results suggest that ASA down-regulates BCL-2 gene expression by inhibiting the IKKβ-IκB-α-NF-κB signal transduction pathway.

Role of Bcl-2 in ASA-enhanced TRAIL Cytotoxicity—To determine whether ASA-mediated down-regulation of BCL-2 plays an important role in the augmentation of TRAIL-induced apoptotic death, LNCaP cells or human colorectal carcinoma CX-1 cells were stably transfected with either an empty control vector (pcDNA 3-neo) or vector containing BCL-2 (pcDNA3-Bcl-2). Figs. 10 and 11 show that pretreatment with ASA followed by treatment with TRAIL caused PARP cleavage, activation of caspases, as well as cytotoxicity in control vector transfected cells. However, overexpression of BCL-2 protected LNCaP and CX-1 cells from ASA-enhanced TRAIL cytotoxicity. These results suggest that ASA-promoted TRAIL cytotoxicity is mediated by down-regulating BCL-2.

Overexpression of BCL-2 Prevents Alteration of Mitochondrial Membrane Potential by Treatment with ASA and TRAIL—Bcl-2 is an anti-apoptotic protein that inhibits the release of cytochrome c from mitochondria into the cytoplasm, thereby down-regulation of BCL-2 may promote intrinsic mitochondria-mediated apoptosis (32, 33). To investigate whether ASA disrupts mitochondrial membrane potential and overexpression of BCL-2 protects cells from this disruption, CX-1/Bcl-2 or CX-1/neo cells were pretreated with ASA and treated with TRAIL. We used the mitochondria-specific dye TMRM to measure the mitochondrial membrane potential. Fig. 12 shows that overexpression of BCL-2 inhibited the loss of mitochondrial membrane potential during treatment with ASA alone or ASA in combination with TRAIL.

A Model for the Effect of ASA on the TRAIL-induced Apoptotic Pathway—Fig. 13 shows a schematic diagram of a model that is based on the literature and our data. ASA blocks the Akt-NF-κB survival signal pathway by inhibiting IKKβ. The inhibition of this pathway results in suppression of the expression of BCL-2, an anti-apoptotic molecule.

DISCUSSION

Aspirin (acetylsalicylic acid) is a nonsteroidal anti-inflammatory drug (NSAID) widely used for its anti-pyretic and analgesic properties. ASA is also known to induce gastrointestinal side effects, mainly in the form of gastric and duodenal ulcerations or erosions. However, epidemiological findings have revealed that ASA reduces the risk of colorectal cancer and adenoma (34, 35). In this study, we demonstrate that pretreatment with ASA promotes TRAIL-induced apoptotic death. The enhancement of apoptosis by treatment with ASA is probably because of down-regulation of BCL-2, activation of caspases, induction of conformational change, translocation of Bax, and cytochrome c release (Figs. 2, 6, and 7) (36–40). Our observations were similar to previous reports (41). Previous studies have shown that aspirin inhibits the transcription factor...
NF-κB (42, 43), which is critical for the expression of several anti-apoptotic genes including C-IAP1, C-IAP2, BCL-XL, FLIP, and BCL-2 (31, 44–46). The inhibition of NF-κB activity is mediated through preventing the phosphorylation and degradation of the inhibitory subunit IκB (Fig. 9) (47). Although the expression of these Bcl-2 family and IAP family proteins is known to be regulated by NF-κB, our data show that ASA inhibits preferentially BCL-2 gene expression (Fig. 6). Thus, a fundamental question that remains unanswered is how ASA inhibits selectively the expression of the BCL-2 gene among BCL-2 family and IAP family genes. It is well known that the NF-κB family of proteins, including NF-κB1, NF-κB2, RelA, RelB, and c-Rel, can form homo- and heterodimers in vitro, except for RelB. In mammals, the most widely distributed NF-κB is a heterodimer composed of p50 and p65 (also called RelA) subunits (48). NF-κB activity is regulated by the IκB family of proteins that interacts with and sequesters the transcription factor in the cytoplasm. IκB proteins become phosphorylated by the multisubunit IKK complex, which subsequently targets IκB for ubiquitination and degradation by the 26 S proteasome (49). At this time only speculation can be made concerning the role of NF-κB in the down-regulation of BCL-2 gene expression during treatment with ASA. One possibility is that differential activation of NF-κB may be responsible for selective inhibition of BCL-2 gene expression. As mentioned above, the inhibition of five members of the NF-κB family may differ during treatment with ASA, and this differential inhibition of the various members of NF-κB family causes a selective inhibition of BCL-2 gene expression (50). We believe that many critical questions still remain to be answered in order to understand the mechanisms of the regulation of BCL-2 gene expression by ASA. However, this model will also provide a framework for future studies.

Previous studies have shown that ASA inhibits tumor necrosis factor-α-and interleukin-1-induced NF-κB activation and sensitizes HeLa cells to apoptosis (51). In this study, we observed that ASA augments TRAIL cytotoxicity in TRAIL-resistant LNCaP cells that contain high levels of HER-2/neu. It is well known that HER-2/neu has an intrinsic TRAIL cytotoxicity in TRAIL-resistant LNCaP cells that contain high levels of HER-2/neu. It is well known that HER-2/neu has an intrinsic

**REFERENCES**

1. Ng, C. P., and Bonavida, B. (2002) *Adv. Cancer Res.* 85, 145–174
2. Hersey, P., and Zhang, X. D. (2003) *Cancer Biol. Ther.* 2, 541–543
3. Takeda, K., Hayakawa, Y., Smyth, M. J., Kayagaki, N., Yamaguchi, N., Sakuta, S., Iwakura, Y., Yagita, H., and Okumura, K. (2001) *Nat. Med.* 7, 94–100
4. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. (1997) *Science* 277, 815–818
5. Sheridan, J. P., Marsters, S. A., Petti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, I. L., Goddard, A. D., Godowski, P., and Ashkenazi, A. (1997) *Science* 277, 818–821
6. Walczak, H., Degli-Espositi, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour, M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T. (1997) *EBMOL* 16, 5386–5397
7. Marsters, S. A., Sheridan, J. P., Petti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., and Ashkenazi, A. (1997) *Curr. Biol.* 7, 1003–1006
8. Pan, G., O’Rourke, K., Chinnaiyan, A. M., Gentz, R., Elben, R., Ni, J., and Dixit, V. M. (1997b) *Science* 276, 111–113
9. Ashkenazi, A., and Dixit, V. M. (1999) *Carr. Optic. Cell Biol.* 11, 255–260
10. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMcurtry, A. E., Hebert, A., DeForgo, L., Koumenis, I. I., Lewis, D., Harris, L., Russiere, J., Koeppe, H., Shaborzak, Y., and Schwall, R. H. (1999) *J. Clin. Invest* 104, 155–162
11. De, Jong, S., Timmer, T., Heienbrock, F. J., and de Vries, E. G. (2001) *Cancer Metastasis Rev.* 20, 51–56
12. Wajant, H., Pfizenmaier, K., and Scheurich, P. (2002) *Apoptosis* 7, 449–459
13. Chowdhury, S., Bauer, J. A., Luptica, J. A., Morrison, R. H., Tang, Z., Oates, R. K., Almasan, A., DiDonato, J. A., Borden, E. C., and Lindner, D. J. (2003) *J. Biol. Chem.* 278, 39461–39469
14. Bouralexis, S., Findlay, D. M., Atkins, G. J., Labrinidis, A., Hay, S., and Evdokias, A. (2003) *Br. J. Cancer* 7, 206–214
15. Tillman, D. M., Izardarjene, K., Szucs, K. S., Douglas, L., and Houghton, A. J. (2003) *Cancer Res.* 63, 5121–5125
16. Lee, Y. J., Lee, K. H., Kim, H. R., Jessup, J. M., Seol, D. W., Kim, T. H., Billar, T. R., and Song, Y. K. (2001) *Oncogene* 20, 1476–1485
17. Fulda, S., Jeremias, I., and Debatin, K. M. (2004) *Oncogene* 23, 7611–7620
18. Park, S. Y., Billar, T. R., and Seol, D. W. (2002) *Biochem. Biophys. Res. Commun.* 291, 233–236
19. Nyromi, O., Mills, L., and Bar-Eli, M. (2003) *Cell Death Differ.* 10, 558–569
20. Qin, L., Hanif, R., Spichacs, E., Shiff, S. J., and Rigas, B. (1998) *Biochem. Pharmacol.* 55, 53–54
21. Hosomi, Y., Yokose, T., Hirose, Y., Nakajima, R., Nagai, K., Nishiyaki, W., and Ochiai, A. (2000) *Lung Cancer* 30, 73–81
22. Wong, B. C., Zhu, G. H., and Lam, S. K. (1999) *Biomed. Pharmacother.* 53, 315–318
23. Williams, C. S., Smalley, W., and DufRois, R. N. (1997) *J. Clin. Invest.* 100, 1325–1329
24. Bair, W. B., III, Hart, N., Enspahr, J., Liu, G., Dong, Z., Alberts, D., and Bowden, G. T. (2002) *Cancer Epidemiol. Biomark. Prev.* 11, 1645–1652
25. Wargovich, M. J., Gjernes, G., Steele, V. E., Velasco, M., Woods, J., Price, R., Gray, K., and Kelloff, G. J. (2000) *Carcinogenesis* 21, 1149–1155
26. Burow, M. E., Weldon, C. B., Tang, Y., Navar, G. L., Krajewski, S., Reed, J. C., Hammond, T. G., Clejan, S., and Beckman, B. S. (1998) *Cancer Res.* 58, 4940–4946
27. Laemmli, U. K. (1970) *Nature* 227, 680–685
28. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acid Res.* 11, 1475–1489
29. Mitchell, J. A., Akarsereenont, P., Thieme, C., Flower, R. J., and Vane, J. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 90, 11693–11697
30. Nagane, M., Pan, G., Wieddle, J. J., Dixit, V. M., Cavenese, W. K., and Huang, H. J. (2000) *Cancer Res.* 60, 847–853
31. Chen, C., Edelstein, L. C., and Gelinas, C. (2000) *Mol. Cell. Biol.* 20, 2687–2695
32. Hengartner, M. O. (2000) *Nature* 407, 770–776
33. Costantini, P., Jacobot, E., Decaudin, D., and Kowarzew, G. (1997) *J. Natl. Cancer Inst.* 92, 1042–1053
34. Marnett, L. J. (1995) *Prev. Med.* 24, 103–106
35. Rosenberg, L., Louik, C., and Shapiro, S. (1998) *Cancer* 82, 2326–2333
36. Li, M., Lotan, R., Levin, B., Tahara, E., Lippman, S. M., and Xu, X. C. (2000) *Cancer* 92, 2326–2333
Aspirin and TRAIL-induced Apoptosis

Epidermol. Biomark. Prev. 9, 545–549
37. Kim, K. Y., Seol, J. Y., Jeon, G. A., and Nam, M. J. (2003) Cancer Lett. 189, 157–166
38. Gu, Q., Wang, J. D., Xia, H. H., Lin, M. C., He, H., Zou, B., Tu, S. P., Yang, Y., Liu, X. G., Lam, S. K., Wong, W. M., Chan, A. O., Yuen, M. F., Kung, H. F., and Wong, B. C. (2004) Carcinogenesis 26, 541–546
39. Piquer, M., Barragan, M., Dalmau, M., Bellosillo, B., Pons, G., and Gil, J. (2000) FEBS Lett. 480, 191–196
40. Zimmermann, K. C., Waterhouse, N. J., Goldstein, J. C., Schuler, M., and Green, D. R. (2000) Neoplasia 2, 505–513
41. Li, X. A., Fang, D. C., Si, P. R., Zhang, R. G., and Yang, L. Q. (2003) Zhonghua. Gan. Zang. Bing. Za. Zhi. 11, 676–679
42. Kopp, E., and Ghosh, S. (1994) Science 265, 956–959
43. Yamamoto, Y., Yin, M. J., Lin, K. M., and Gaynor, R. B. (1999) J. Biol. Chem. 274, 27307–27314
44. Mischeau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. (2001) Mol. Cell. Biol. 21, 5299–5305
45. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) Science 281, 1680–1683
46. Bui, N. T., Livolsi, A., Peyron, J. F., and Prehn, J. H. M. (2001) J. Cell Biol. 152, 753–763
47. Yin, M. J., Yamamoto, Y., and Gaynor, R. B. (1998) Nature 396, 77–80
48. Baeuerle, P. A., and Baltimore, D. (1989) Gene Dev. 3, 1689–1698
49. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Science 282, 1318–1321
50. Cross, D. A., Alesi, D. R., Cohen, P., Andjelkovich, M., and Hennings, B. A. (1995) Nature 378, 785–789
51. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
52. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) Science 278, 687–689
53. Hetman, M., Cavanaugh, J. E., Kimeiman, D., and Xia, Z. (2000) J. Neurosci. 20, 2567–2574
54. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) Nature 401, 82–85