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Aspirin Enhances Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Mediated Apoptosis in Hormone-Refractory Prostate Cancer Cells through Survivin Down-Regulation

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic agent because of its tumor selectivity. TRAIL is known to induce apoptosis in cancer cells but spare most normal cells. In this study, we examined whether acetylsalicylic acid (ASA), so-called aspirin, enhances TRAIL-induced apoptosis in androgen-dependent LNCaP and androgen-independent LNCaP-derived prostate cancer cells. To evaluate the cell death effects of TRAIL in combination with ASA on tumor cells, we performed DNA fragmentation assay and immunoblot analysis for poly(ADP-ribose) polymerase-1, caspases, and anti-apoptotic proteins. We observed that ASA promoted TRAIL-induced apoptotic death in both LNCaP and its derived cells (C4, C4-2, and C4-2B). These enhancements of TRAIL's effect were related to the decrease in survivin protein expression by pretreatment with ASA. We also confirmed that knockdown in survivin expression by transfecting survivin small interfering RNA increased TRAIL-induced apoptosis. To study the mechanism of survivin down-regulation, we determined the levels of mRNA and the activities of survivin promoter in the ASA-treated and untreated cells. Reduction of the intracellular levels of survivin protein was due to a decrease in transcriptional activity. Data from electrophoretic mobility shift assay and chromatin immunoprecipitation analyses revealed that ASA inhibited the transcription factor E2F-1 binding activity to the survivin promoter region, which is known to regulate survivin gene transcription. Taken together, our studies suggested that ASA-promoted TRAIL cytotoxicity is mediated by down-regulating survivin, and the down-regulation of survivin is due to inhibition of E2F-1 binding activity to the survivin promoter region.

It is believed that, as in other neoplastic diseases, the accumulation of genetic alterations causes and promotes prostate cancer even though the exact molecular mechanisms underlying the onset and progression of prostate cancer are unknown. In the United States, prostate cancer is the leading cancer diagnosis and the second leading cause of cancer-related deaths in men (Jemal et al., 2007). The incidence of prostate cancer for men under the age of 40 years is 1 in 10,000 compared with 1 in 7 for those aged over 60 years (Jemal et al., 2007). In as many as 10 to 50% of men with prostate cancer, the disease will progress from androgen-dependent to androgen-independent growth (Isaacs et al., 1994) and spread to the pelvic lymph nodes and bone. This development of an androgen-independent phenotype leads to the incurable hormone-refractory state of the disease, suggesting the need for better treatment strategies. Novel agents such as nucleotide-based targeted therapies, small-molecule inhibitors, antiangiogenic agents, novel cytotoxic therapeutics, and calcitriol have been proposed (Hadaschik et al., 2007). Although there are a substantial number of novel protocols, including hormonal therapy and conventional chemo- and immunotherapy, only limited treatment options are available for prostate cancer because chemotherapy and radiotherapy have been found to be largely ineffective, and metastatic disease frequently develops even after surgery (Pisters, 1999). Death is the result of metastatic hormone-refractory disease in virtually the majority of patients. Hence, the development of a novel and effective therapeutic strategy to effectively inhibit hormone refractory prostate cancer is urgently needed.

Tumor necrosis factor-related apoptosis-inducing ligand

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ABBREVIATIONS: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ASA, acetylsalicylic acid; IAP, inhibitor of apoptosis protein; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; Sp1, specific protein 1; NF-κB, nuclear factor-κB; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(TRAIL) is possibly one of the best candidates for a new form of cytokine therapy. TRAIL, a type II integral membrane protein belonging to the tumor necrosis factor family, induces apoptosis in a broad range of cancer cells types but spares normal cells and tissues (Ashkenazi et al., 1999). Preclinical studies clearly demonstrate that TRAIL has excellent antitumoral activity (Ashkenazi et al., 1999). However, many tumor cells have been shown to be resistant to TRAIL (Bouralexis et al., 2003). Several researchers have reported that TRAIL resistance can be overcome by various sensitizing agents such as chemotherapeutic drugs (Lee et al., 2001; Fulda et al., 2004), cytokines (Park et al., 2002), aspirin (Oh et al., 2003; Kim et al., 2005), and matrix metalloprotease inhibitors (Nyormoi et al., 2003) that are able to render TRAIL-resistant tumor cells sensitive to TRAIL. Several researchers have demonstrated that down-regulation of expression of antiapoptotic genes such as *SURVIVIN, BCL-2,* and *XIAP* is responsible for the augmentation of TRAIL-induced apoptotic death (Kim et al., 2005a,b,c; Lu et al., 2005; Retzer-Lidl et al., 2007). In this study, we examined whether aspirin-promoted TRAIL cytotoxicity is mediated through this pathway.

Nonsteroidal anti-inflammatory drugs, 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, such as the colon (Qiao et al., 1998), lung (Hosomi et al., 2000), and stomach (Wong et al., 1999). ASA also potentiates apoptotic cell death by promoting the onset of the mitochondrial permeability transition and augments the cell killing by TRAIL (Oh et al., 2003; Kim et al., 2005c). ASA is known to act by directly suppressing the cyclooxygenase enzyme (COX-1 and COX-2), which is 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 formation in animal models (Bair et al., 2002). In this study, we examined whether ASA in combination with TRAIL increases TRAIL-induced apoptotic death in androgen independent prostate cancer, which is also referred to as hormone-refractory prostate cancer. For this study, we used human prostate adenocarcinoma LNCaP cell line and its derivatives (C4, C4-2, and C4-2B) (Thalmann et al., 1994). This is an excellent model system that will help improve our understanding of the mechanisms of androgen-independence and osseous metastasis and tumor-host determinants of prostate-specific antigen expression (Thalmann et al., 1994; Wu et al., 1994). Our studies demonstrate that ASA augments TRAIL-induced apoptosis by down-regulating *SURVIVIN* gene expression and by decreasing binding affinity of the transcription factor E2F-1 to survivin promoter, which results in a decrease in the intracellular level of survivin.

Materials and Methods

Cell Culture and Survival Assay. Human prostate adenocarcinoma LNCaP from American Tissue Type Culture Collection (Manassas, VA) and its derivatives, C4, C4-2, and C4-2B, were a gift kindly given by Dr. Leland W. K. Chung (Emory University, Atlanta, GA). LNCaP and its derivative cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM L-glutamine, and 26 mM sodium bicarbonate for monolayer cell culture. The media were calibrated by adding sodium bicarbonate and HEPES, pH 7.4. The dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. One day before the experiment, cells were plated in 60-mm dishes. For trypan blue exclusion assay (Burow et al., 1998), trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined under a light microscope. At least 300 cells were counted for each survival determination.

Drug Treatment. ASA was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution was prepared in dimethyl sulfoxide. Medium with drug was prepared, and pH was adjusted with HEPES buffer. Drug treatments were accomplished by aspirating the medium and replacing it with medium containing drug.

Antibodies. Anti-survivin and anti-caspase-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8, anti-E2F-1, and antihistone H3 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-caspase-9 antibody was purchased from Upstate Biotechnology (Charlottesville, VA). Anti-PARP antibody was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Anti-actin antibody was purchased from Valeant Pharmaceuticals (Costa Mesa, CA).

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; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Production of Recombinant TRAIL. A human TRAIL cDNA fragment (amino acids 114–281) obtained by RT-PCR was cloned into a pET-23d (Novagen, Madison, WI) plasmid, and histidine-tagged TRAIL protein was purified using the QIAGEN express protein purification system (QIAGEN, Valencia, CA).

DNA Fragmentation Assay. For detection of apoptosis by the DNA fragmentation assay, 5×10^5 cells were plated to 60-mm dishes 1 day before drug treatment. After treatment, cells were trypsinized, washed with 1× PBS, and harvested. Cells were resuspended in 0.5 ml of lysis buffer (20 mM EDTA, 10 mM Tris, pH 8.0, 200 mM NaCl, 0.2% Triton X-100, and 100 μg/ml Proteinase K) and incubated for 1.5 h in a 37°C incubator. Then, the samples were centrifuged (12,000 rpm) at room temperature for 5 min. The supernatant was transferred to a new Eppendorf tube, and equal volumes of isopropanol and 25 μl of 4 M NaCl (100 mM final concentration) were added followed by overnight incubation of the samples at −20°C. DNA was acquired by centrifugation of the samples, washed, dried, and dissolved in 30 μl of buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0. DNA (5 μg) was loaded on each lane of 1.5% agarose gel.

Luciferase Assay. To achieve transgene expression, plasmid containing minimal promoter, plasmid containing Luciferase coding sequence (pLuc), or plasmid containing −1430 base of survivin promoter sequence followed by Luciferase coding sequence (p1430Luc) (Health Research Inc., Buffalo, NY) was transfected to LNCaP and C4-2B cells using Lipofectamine Plus (Invitrogen). Transfected cells were grown for 30 h and assayed for luciferase activities with the luminometer TD20/20 (Turner Designs Instruments, San Jose, CA).

RNA Interference by siRNA of Survivin and E2F-1. To knock down survivin or E2F-1 gene expression, survivin siRNA, E2F-1 siRNA, or control siRNA (Santa Cruz Biotechnology) was used. Survivin siRNA, E2F-1 siRNA, or control siRNA was transfected into LNCaP and C4-2B cells using Lipofectamine (Invitrogen) and then incubated for 48 h. The interference of survivin or E2F-1 expression

was confirmed by immunoblotting using anti-survivin or anti-E2F-1 antibody, respectively.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared by modifying the procedure reported by Dignam et al. (1983). After treatment with or without ASA for 20 h, LNCaP cells were washed three times with PBS and incubated on ice for 15 min in hypotonic buffer (10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l DTT, 0.5 mmol/l 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 (20 mmol/l HEPES, pH 7.9, 25% glycerol, 0.4 mol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, and 0.5 mmol/l 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 Laboratories, Hercules, CA). These nuclear extracts were used for electrophoretic mobility shift assay. 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, and 0.2% Tween 20) and 1 μg of poly(dI-dC) for 10 min on ice. Biotin-labeled probe survivin promoter-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% Tris borate-EDTA for 50 min at 120 V. The samples were then transferred in 0.5% Tris borate-EDTA 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, Indianapolis, IN) at room temperature for 30 min. The biotin-labeled probe was then detected with streptavidin-horseradish peroxidase diluted 1:20,000 (Pierce, Rockford, IL). 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).

RT-PCR Analysis of Survivin 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, Madison, WI). The following sense and antisense primers were used at 0.5 μM for each: survivin primer: sense, 5-GGCATGGGTGCCCCGACGTTG-3, and antisense, 5-CAGAGGCCTCAATCCATGGCA-3. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control: GAPDH primer: sense, 5-CGTCTTCACCATGGAGA-3, and antisense, 5-CGGCCATCACGCCCACAGTTT-3. The reaction was reverse-transcribed at 45°C for 1 h followed by 26 cycles of PCR. The initial PCR activation was at 94°C for 2 min, denatured at 94°C for 1 min, annealed at 53°C for 30 s, and final extension at 72°C for 5 min. After amplification, the products were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

Chromatin Immunoprecipitation Assay. LNCaP and C4–2B cells were pretreated with or without 5 mM ASA for 20 h. A total of 5×10^6 cells was used per immunoprecipitation reaction. Cells were cross-linked with 1% formaldehyde for 15 min at room temperature. Then, cells were harvested and sonicated, and chromatin fragments containing cell lysates were prepared. The lysates were immunoprecipitated with monoclonal E2F-1 antibody (Cell Signaling Technology). The sequences of the PCR primers used are as follows: E2F-1 (region −131 to −46), forward primer: 5-CGCCTCTACTCCCAGAAG-3; E2F-1 (region −131 to −46), reverse primer: 5-TGTAGAGATGCGGTGGTC-3. The binding of E2F-1 was analyzed by PCR.

Results

ASA Promoted TRAIL-Induced Apoptotic Cell Death. To examine the effect of ASA on TRAIL-induced apoptotic death in human prostatic adenocarcinoma LNCaP cells and their derivative C4, C4-2, and C4-2B cells, cells were pretreated with ASA and treated with TRAIL in the presence of ASA. Figure 1, A and B, shows that little or no cytotoxicity was observed with 5 mM ASA alone or 200 ng/ml

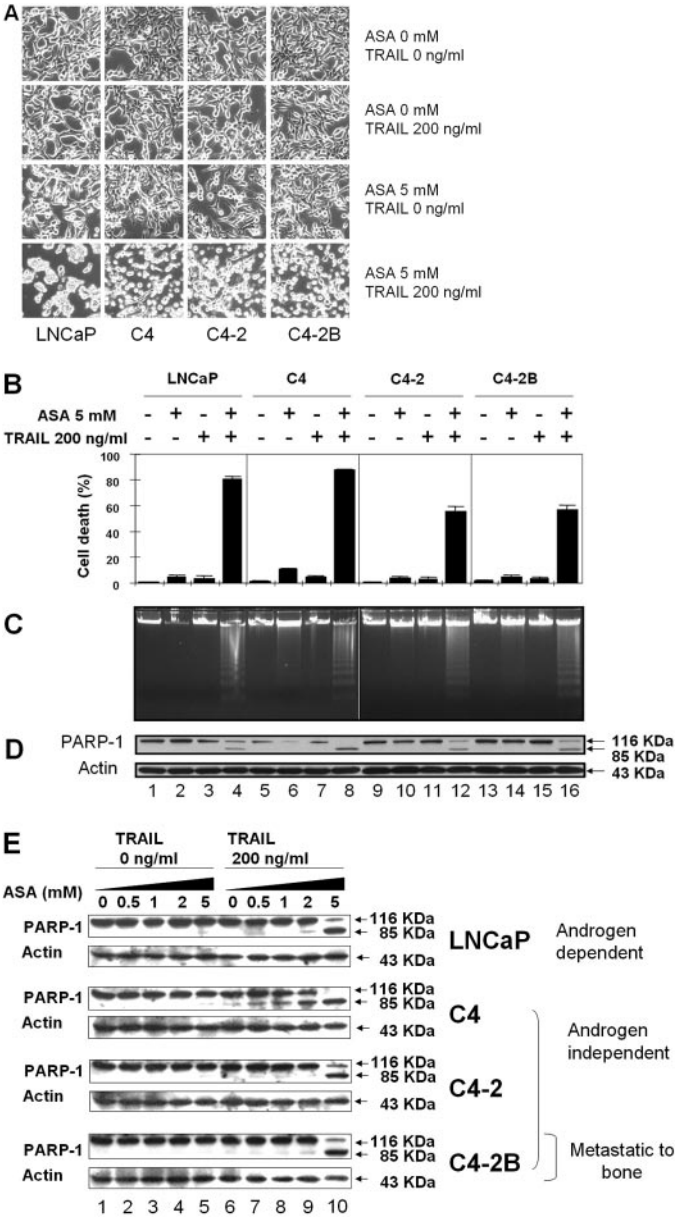

Fig. 1. Effect of pretreatment of ASA on TRAIL-induced apoptosis in androgen-dependent LNCaP and its androgen-independent derivative cell lines. LNCaP, C4, C4-2, and C4-2B cells were pretreated with or without various concentrations of ASA (0.5–5 mM) for 20 h and treated with or without 200 ng/ml TRAIL for 4 h (A–D). A, the morphological features were analyzed with a phase-contrast microscope (200×). B, cell death was determined by the trypan blue exclusion assay. Error bars represent S.E.M. from three separate experiments. C, after treatment, DNA was isolated from cell lysates and analyzed with agarose gel. D and E, cell lysates were subjected to immunoblotting for PARP-1. Immunoblots of PARP-1 show the 116-kDa PARP-1 and the 85-kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

TRAIL alone. Similar results were observed with DNA fragmentation assay (Fig. 1C). Additional studies also show that pretreatment with ASA followed by treatment with TRAIL caused PARP cleavage, the hallmark feature of apoptosis, in LNCaP and its derivatives (Fig. 1D). With the pretreatment of ASA (0.5–5 mM), TRAIL-induced apoptosis was promoted in an ASA concentration-dependent manner in LNCaP and its derivatives (Fig. 1E). These data demonstrate that TRAIL-induced apoptotic cell death was promoted by pretreatment with ASA regardless of androgen dependence, because LNCaP cells are androgen-dependent, whereas C4, C4-2, and C4-2B cells are androgen-independent.

Caspase Activation Was Responsible for ASA-Pretreated TRAIL-Induced Apoptosis. Additional experiments were conducted to investigate whether pretreatment with ASA followed by treatment with TRAIL activates caspases. 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 androgen-dependent LNCaP and androgen-independent and metastatic LNCaP subline C4-2B cells. The combined treatment of TRAIL and ASA also resulted in an increase in caspase-9 and caspase-3 activation in a dose-dependent manner in both LNCaP and C4-2B cells (Fig. 2). The precursor form of caspases 9 and 3 was cleaved to the active form of 37 and 17 kDa, respectively. As expected from Fig. 1, ASA alone did not activate caspases. Figure 2 shows that PARP (116 kDa) was cleaved, yielding a characteristic 85-kDa fragment in the presence of 200 ng/ml TRAIL and ASA (2–5 mM) in both LNCaP and C4-2B cells. The cleavage of PARP was not observed by treatment with ASA alone.

ASA Down-Regulated the Intracellular Level of Survivin. We reported previously that ASA does not significantly alter the total cellular levels of the TRAIL receptors (DR4, DR5, and DcR2) and antiapoptotic proteins (FLIPL, FLIPS, IAP-1, IAP-2, and Bcl-xL) (Kim et al., 2005c). In this study, we observed that ASA treatment resulted in a significant decrease in the level of survivin among IAP family proteins in LNCaP, C4, C4–2, and C4–2B cells (Fig. 3A). The reduction of survivin during treatment with ASA (5 mM) in LNCaP and its derivative prostate cancer cells was dependent on exposure time (Fig. 3B). The role of survivin in TRAIL sensitivity was examined by knock-downing survivin gene expression. Data from Fig. 4 show that the expression of survivin was effectively inhibited by survivin siRNA transfection in androgen-dependent LNCaP and androgen-independent and bone metastatic C4–2B cells. TRAIL-induced PARP-1 cleavage was also observed in both siRNA-transfected cell lines (Fig. 4A). Similar results were observed with DNA fragmentation assay (Fig. 4B). These results suggest that down-regulation of survivin leads to an increase in sensitivity to TRAIL regardless of androgen dependence.

ASA-Suppressed SURVIVIN Gene Transcription Was Mediated by Inhibiting DNA Binding Activity of E2F-1. We further examined whether the down-regulation of *SURVIVIN* gene expression by treatment with ASA is due to inhibition of transcriptional activity. Data from RT-PCR in Fig. 5A show that the level of *SURVIVIN* mRNA was decreased during treatment with 5 mM ASA. These results suggest that the reduction of survivin levels during treatment with ASA was related to the suppression of *SURVIVIN* gene transcription. To confirm our observations, pLuc and pLuc-1430 (the 1430 base of *SURVIVIN* promoter sequence followed by pLuc) plasmids were transfected to LNCaP cells, and luciferase activities were determined with or without 5 mM ASA. Figure 5B clearly shows that luciferase activity from cell lysate of pLuc-1430-transfected cells was dramatically decreased in the presence of ASA. These results suggest that down-regulation of *SURVIVIN* gene expression by treatment with ASA is mediated through inhibition of transcriptional activity. It is well known that the *SURVIVIN* promoter

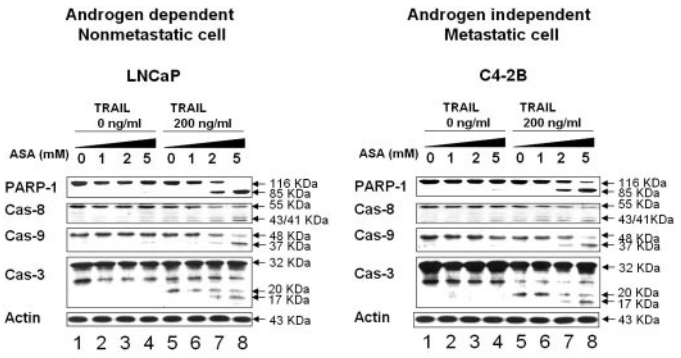

Fig. 2. Effect of pretreatment of ASA on TRAIL-induced proteolytic cleavage of PARP-1 and activation of caspases in LNCaP and C4–2B cells. Cells were pretreated with various concentrations of ASA (1–5 mM) for 20 h and treated with or without TRAIL (200 ng/m) for 4 h and then harvested. Equal amounts of protein (20 μg) from cell lysates were separated by SDS-PAGE and subjected to immunoblotting for PARP-1, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects the 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-1 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.

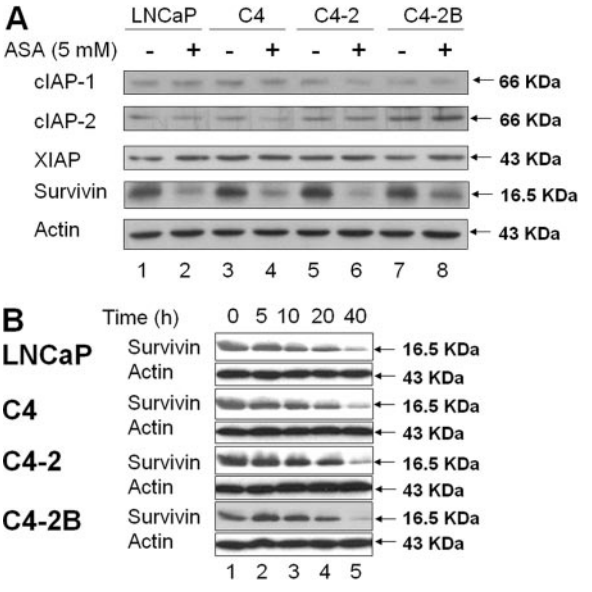

Fig. 3. ASA-mediated down-regulation of survivin expression in LNCaP, C4, C4-2, and C4-2B cells. A, cells were treated with or without 5 mM ASA. Cells were harvested 20 h after treatment and subjected to Western blot. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-cIAP-1, anti-cIAP-2, anti-XIAP, anti-survivin, or anti-actin antibody. Actin was shown as an internal standard. B, time-course expression of survivin. Cells were treated with 5 mM ASA for various times (5–40 h) and then harvested. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-survivin or anti-actin antibody.

region contains binding sites of several putative transcription factors such as specific protein 1 (Sp1), Stat3, E2F-1, and p53 (Fig. 6A). We hypothesized that ASA affects binding affinity of these transcription factors in the *SURVIVIN* promoter region and subsequently inhibits transcription of the *SURVIVIN* gene. To test the hypothesis, we chose two potential candidates, Sp1 and E2F-1, because there are eight putative binding sites for these transcription factors in the *SURVIVIN* promoter region. Data from electrophoretic mobility shift assay show that Sp1 from the nuclear extracts of LNCaP and C4-2B bound to the Sp1-specific oligomer. This binding activity was not affected by treatment with ASA.

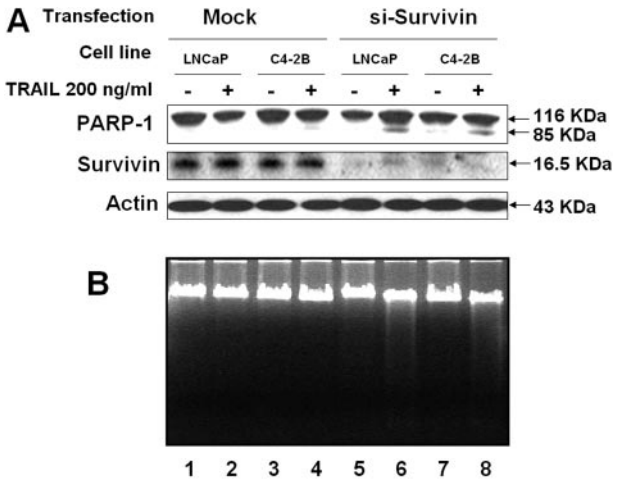

Fig. 4. Role of survivin in TRAIL-induced apoptosis in LNCaP and C4–2B cells. Cells were transfected with survivin siRNA or control siRNA (mock) and incubated for 36 h. Cells were then treated with or without 200 ng/ml TRAIL for 4 h. A, equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP-1, anti-survivin, or anti-actin antibody. B, DNA was isolated from cell lysates and analyzed with agarose gel.

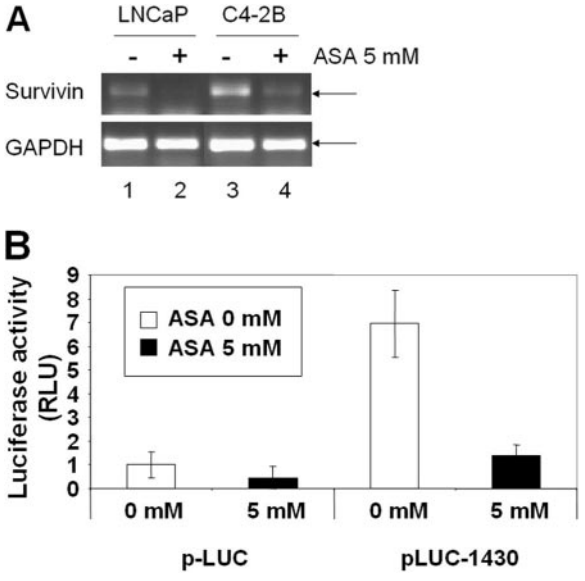

Fig. 5. Transcriptional down-regulation of SURVIVIN gene expression by treatment with ASA. A, RT-PCR analysis was performed for detecting *SURVIVIN* or *GAPDH* expression in LNCaP and C4–2B cells. *GAPDH* is shown as an internal standard. B, LNCaP or C4-2B cells were transfected with pLuc or p1430Luc plasmids and incubated for 30 h. After incubation, cells were treated with or without 5 mM ASA for 20 h. Cells were lysed, and luciferase activities were measured with a luminometer. Error bars represent S.E.M. from three separate experiments.

Unlike the DNA binding activity of Sp1, that of E2F-1 was changed in the presence of ASA (Fig. 6D). Even though the intracellular level of E2F-1 was not significantly altered in the presence of ASA (Fig. 6C), data from chromatin immunoprecipitation assay clearly demonstrate that ASA treatment markedly decreased recruitment of E2F-1 to the proximal site of *SURVIVIN* promoter (Fig. 6D) in both cell lines. To investigate whether ASA specifically alters E2F-1 binding activity to the *SURVIVIN* promoter region, total E2F-1 binding activity to sonicated chromatin was determined by immunoprecipitation with anti-E2F-1 antibody followed by immunoblotting with antihistone H3. Figure 6E shows that total E2F-1 binding activity was not significantly changed in the presence of ASA. Taken together, our data suggest that specific disruption of E2F-1-binding activity to survivin promoter occurs by treatment with ASA. The role of E2F-1 in the down-regulation of *SURVIVIN* gene expression was confirmed by knock-downing E2F-1. Figure 6F shows that transfection with E2F-1 siRNA effectively decreased the intracellular level of E2F-1 and significantly suppressed the level of survivin in LNCaP and C4-2B cells.

Discussion

Several conclusions can be drawn after considering the results presented. The ASA pretreatment significantly promotes TRAIL-induced apoptotic cell death by down-regulating *SURVIVIN* gene expression in androgen-dependent LNCaP cells and their androgen-independent derivative cells. Inhibition of E2F-1 binding activity in survivin promoter is responsible for ASA-mediated down-regulation of *SURVIVIN* gene expression. These results suggest that the combination of ASA and TRAIL may be a promising regimen for the treatment of both local and metastatic hormone-refractory prostate cancer.

Prostate cancer begins as an androgen-dependent tumor and undergoes clinical regression in response to pharmacological or surgical strategies that reduce testosterone concentration. Although this treatment approach is effective initially in controlling the prostate cancer, these tumors ultimately fail to respond to androgen blockade. The failure of androgen ablation therapy leads to a hormone-refractory state of the disease. Androgen receptor mutations (6–10%), androgen receptor amplifications (20–30%), and HER-2/neu overexpression (67%) may explain relapse in some patients (Bartlett et al., 2005). Few anticancer reagents work on the hormone-refractory prostate cancer. It is well known that androgen-independent cancer cells are resistant to radiotherapy, chemotherapy, and Fas-mediated apoptosis (Suzuki et al., 2000; Curtin and Cotter, 2003). In this study, however, we observed that ASA in combination with TRAIL effectively induces cytotoxicity to androgen-dependent LNCaP and its derivative androgen-independent cells.

One of the mechanisms for enhancing tumor cell death is facilitation of apoptosis through the down-regulation of anti-apoptotic molecule expression. Previous studied have revealed that TRAIL-induced apoptosis is augmented by a variety of chemical compounds, such as CDK4 inhibitor 2-bromo-12,13-dihydro-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7(6*H*)-dione (Retzer-Lidl et al., 2007), rottlerin (Kim et al., 2005b), sodium butylate (Kim et al., 2005a), aspirin (Kim et al., 2005c), and thiazolidinedione (Lu et al., 2005), through

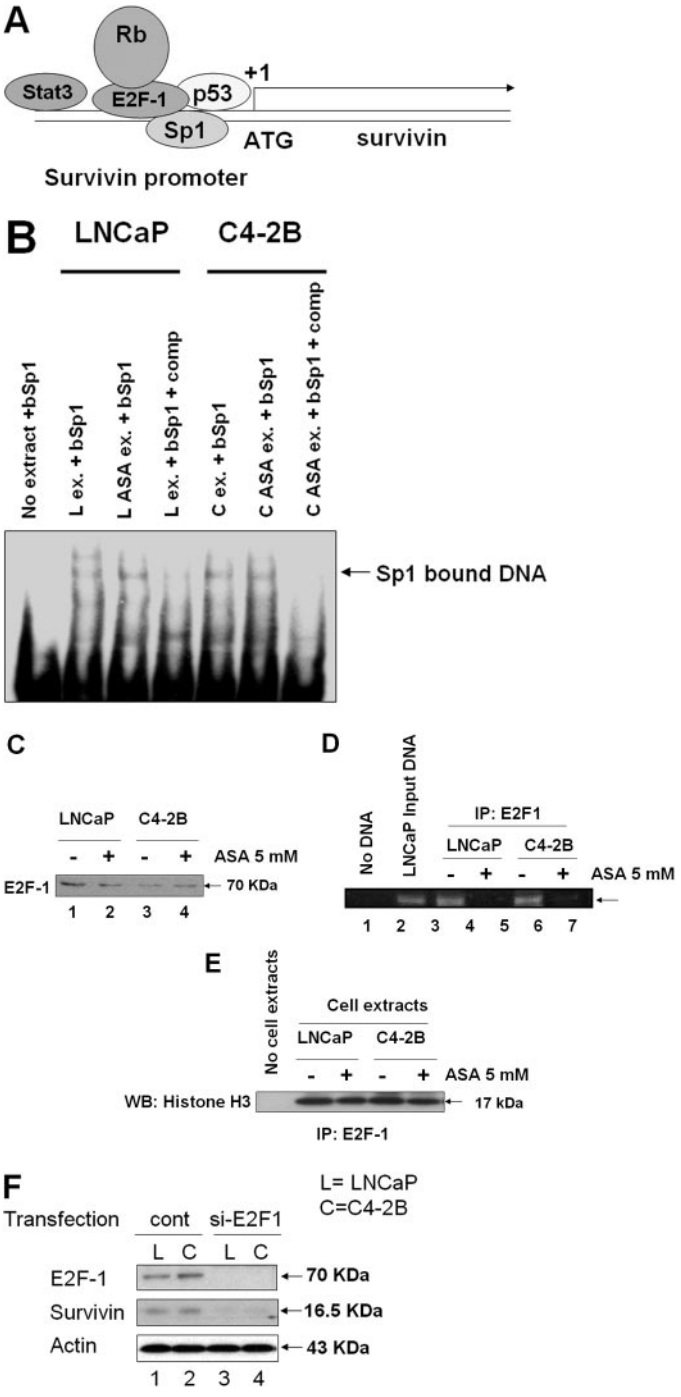

Fig. 6. Transcription factor binding activity in SURVIVIN promoter. A, diagram of putative transcription factors in the *SURVIVIN* promoter. *SURVIVIN* promoter contains stat-3 binding sites (−1231 to −1009, three putative binding sites), E2F-1 (−43 to −38), and Sp1 binding sites (−1409 to −24, eight putative binding sites). B to E, LNCaP and C4–2B cells were treated with or without 5 mM ASA for 20 h. B, their nuclear extracts were incubated with biotin-labeled *SURVIVIN* promoter oligonucleotide at room temperature for 30 min. Gel mobility shift assays for Sp1 binding activity were performed as described under *Materials and Methods.* L ex., nuclear extract from LNCaP cells; L ASA ex., nuclear extract from 5 mM ASA-treated LNCaP cells; C ex., nuclear extract from C4-2B cells; C ASA ex., nuclear extract from 5 mM ASA treated C4-2B cells; bSp1, biotin-labeled Sp1 probe; Comp, biotin unlabeled Sp1 oligomer. C, equal amounts of protein (20 μg) from cell lysates were separated by SDS-PAGE and immunoblotted with anti-E2F-1 antibody. D, cells were sonicated, and chromatin fragments were immunoprecipitated with anti-E2F-1. The binding of E2F-1 on *SURVIVIN* promoter was analyzed by PCR. E, chromatin fragments were immunoprecipitated with anti-E2F-1 antibody. Interaction between E2F-1 and histone H3 was performed with anti-histone H3 antibody. F, LNCaP and C4-2B cells were transfected with E2F-1 siRNA or control siRNA and incubated for 48 h. Equal amounts of protein (20 μg) from cell lysates were separated by SDS-PAGE and immunoblotted with anti-E2F-1, anti-survivin, or anti-actin antibody.

down-regulation of *SURVIVIN*, *XIAP*, and *BCL-2*. In the present study, we investigated the regulation of IAP family gene expression during treatment with ASA. IAP proteins were initially identified as antiapoptotic protein, which inhibits apoptosis and allows time for viral replication in baculoviruses (Crook et al., 1993). The IAP family of proteins is characterized by the presence of one to three domains known as baculoviral IAP repeat domains, rendering antiapoptotic activity by binding to caspases (Salvesen and Duckett, 2002). The effector caspases 3 and 7 and the initiator caspase-9 are among the caspases that are directly inhibited by human IAP family members (IAP-1, IAP-2, XIAP, and survivin). It is interesting that treatment of LNCaP, C4, C4-2, and C4-2B cells with 5 mM ASA down-regulates only the survivin level among the IAP family members in a time- and dose-dependent manner, suggesting that ASA-promoted TRAIL cytotoxicity is mediated through survivin down-regulation (Fig. 3). Survivin is a chromosomal passenger protein that is essential for mitosis but has also been implicated in protecting cells from apoptosis (Altieri, 2003). Previous reports show that Sp1 is overexpressed in cancer cells and tumors (Zannetti et al., 2000; Wang et al., 2003; Hosoi et al., 2004). *SURVIVIN* expression is dependent, in part, on Sp1 in some cancer cell lines (Li and Altieri, 1999; Wu et al., 2005; Li et al., 2006). Nevertheless, our data suggest that Sp1 is not involved in ASA-altered *SURVIVIN* expression (Fig. 6B).

Recent studies by ourselves and others have shown that putative E2F-1 binding sites are present on the *SURVIVIN* promoter (Jiang et al., 2004; Fig. 6D). Western blotting showed that E2F-1 siRNA selectively suppresses the levels of survivin and E2F-1 in these proteins in androgen-dependent LNCaP and its derivative androgen-independent C4-2B cells (Fig. 6F). Figure 6F shows that the transfection of E2F-1 siRNA effectively knocks down the *SURVIVIN* expression in LNCaP and C4-2B cells. It is likely that cellular/nuclear concentration of E2F-1 is not altered, although its interaction with *SURVIVIN* promoter in the nucleus is decreased (Fig. 6, D and E). A fundamental question that remains unanswered is how ASA inhibits E2F-1 binding activity. At the present time, we can only speculate about the role of E2F-1 in ASA-mediated down-regulation of *SURVIVIN* gene expression. Previous studies have shown that NF-κB, E2F-1, p130, and pRB proteins interact with target promoter and regulate gene expression (Jiang et al., 2004; Lin et al., 2006). Both pRB and p130 interact with the *SURVIVIN* promoter and repress *SURVIVIN* transcription. In contrast, E2F-1 bonds to the *SURVIVIN* promoter and induces *SURVIVIN* transcription (Jiang et al., 2004). ASA inhibits NF-κB activity by dephosphorylating IκB-α and inhibiting IκB kinase complex-β activity (Kopp and Ghosh, 1994; Yin et al., 1998; Kim et al., 2005c). Inhibition of NF-κB may prevent the replacement of pRB/p130 with E2F-1 and results in the suppression of *SURVIVIN* gene expression. Obviously, further studies at the molecular level are necessary to understand the mechanism of transcriptional regulation of the *SURVIVIN* gene and how ASA affects this process.

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