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Pretreatment of Docetaxel Enhances TRAIL-Mediated Apoptosis in Prostate Cancer Cells

Jinsang Yoo, Sung-Soo Park, and Yong J. Lee*

Department of Surgery and Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

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 treatment of docetaxel (DTX) can enhance apoptotic cell death by TRAIL against androgen-independent prostate cancer (AIPC). The cell death effect of combinations of TRAIL and docetaxel on prostate cancer cell lines (androgen-dependent LNCaP and its derived androgen-independent, metastatic C4-2B) was evaluated by synergisms of apoptosis. Western blot assay and DNA fragmentation assay were used to study the underlying mechanisms of cell death and search for any mechanisms of enhancement of TRAIL induced apoptosis in the presence of docetaxel. In addition, we investigated the in vitro anti-tumor effects of combined docetaxel and TRAIL using MAP kinase inhibitors. Docetaxel itself could not induce apoptotic cell death in 24 h even in high concentration. Apoptotic cell death, however, was drastically enhanced by pretreatment of docetaxel 20 h before TRAIL treatment. Docetaxel enhanced the PARP-1 cleavage and caspases activation by TRAIL especially in androgen-independent, metastatic C4-2B cell line, mainly by phosphorylation of Bcl-2 by JNK activation. It appears that apoptotic cell death was protected by the JNK inhibitor SP600125. The results of our study show that pretreatment of docetaxel is able to enhance the apoptosis produced by TRAIL in prostate cancer cells, especially in hormone-refractory prostate cancer (HRPC). J. Cell. Biochem. 104: 1636–1646, 2008.

Key words: hormone-refractory prostate cancer; docetaxel; Bcl-2; TRAIL; apoptosis; caspase

In the United States, prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related mortality in men [Jemal et al., 2007]. Age is a contributing factor, as 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 [Jemal et al., 2007]. Androgen deprivation has been used as an effective first-line therapy for metastatic prostate cancer. Although this therapeutic approach often leads to significant cancer control lasting for 2–3 years, in as many as 10–50% of men with prostate cancer the disease will ultimately progress to an androgen independent hormone-refractory prostate cancer (HRPC) status and spread to the pelvic lymph nodes and bone resulting in significant morbidity and death [Isaacs et al., 1994; Goktas, 1999; Prostate Cancer Trialists' Collaborative Group, 2000].

To treat prostate cancer, novel agents have been proposed such as nucleotide-based targeted therapies, small-molecule inhibitors, anti-angiogenic agents, novel cytotoxic therapeutics, and calcitriol [Hadaschik et al., 2007] and there are also a substantial number of protocols including hormonal therapy as well as conventional chemo- and immunotherapy.

Abbreviations used: AIPC, androgen-independent prostate cancer; HRPC, hormone-refractory prostate cancer; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; JNK, (c-Jun N-terminal kinase).

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*Correspondence to: Yong J. Lee, Department of Surgery, University of Pittsburgh, Hillman Cancer Center, 5117 Centre Ave. Room 1.46C, Pittsburgh, PA 15213.
E-mail: leeyj@upmc.edu

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However, currently, 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 [Petrylak, 1999; Pisters, 1999; Richie, 1999]. Death is the result of metastatic hormone-refractory disease in virtually the majority of patients. Hence, the successful development of a novel and effective therapeutic strategy to effectively inhibit HRPC is urgently needed.

Paclitaxel and docetaxel are taxanes, which are produced by the genus *Taxus* (yews), and are currently used as chemotherapy for the treatment of ovarian, breast and non-small cell lung cancers. Docetaxel, a semi synthetic analogue of paclitaxel, entered clinical trials in 1990 [Bissett and Kaye, 1993]. It is well known that phosphorylation of BCL-2 by taxane inactivates the function of BCL-2. Taxane treatment induces G2/M arrest, microtubule polymerization, and JNK (c-Jun N-terminal kinase) activation that phosphorylates at serine 70 amino acid residue of BCL-2 and consequently inactivates BCL-2. Moreover, taxane treated cells are more susceptible to Fas death signal, and overexpression of mutant type (Ser70Ala) BCL-2 restores resistance [Yamamoto et al., 1999].

Docetaxel chemotherapy is now the standard treatment for patients with metastatic HRPC. In two randomized trials on HRPC, docetaxel chemotherapy reduced serum PSA levels and palliated pain in 35% of the patients [Petrylak et al., 2004; Tannock et al., 2004]. However, the survival benefits were limited, suggesting that a novel therapeutic approach is required [Clarke, 2006; Calabro and Sternberg, 2007].

Tumor necrosis factor-related apoptosis-inducing ligand (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 (TNF) family, induces apoptosis in a broad range of cancer cells but spares normal cells and tissues [Ashkenazi and Dixit, 1999; Ashkenazi et al., 1999]. Preclinical studies clearly demonstrate that TRAIL has excellent anti-tumoral activity [Ashkenazi et al., 1999; de Jong et al., 2001; Chawla-Sarkar et al., 2003]. However, many tumor cells have been shown to be resistant to TRAIL [Bouralexis et al., 2003; Tillman 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], and matrix metalloprotease inhibitors [Nyormoi et al., 2003] that are able to render TRAIL-resistant tumor cells sensitive to TRAIL.

In this study, we examined whether pretreatment of docetaxel in combination with TRAIL increases TRAIL-induced apoptotic death in androgen-independent prostate cancer (AIPC). We employed human prostate adenocarcinoma LNCaP cell line and its metastatic derivative, C4-2B cells [Thalmann et al., 1994], which is an excellent model system to improve our understanding of the mechanisms of androgen-independence and osseous metastasis, and tumor-host determinants of PSA expression [Thalmann et al., 1994, 2000; Wu et al., 1994]. Although docetaxel is currently the standard of care for metastatic HRPC, this hard-to-cure cancer often progresses because of clonal selection of therapy-resistant cells or the development of cells with a drug-resistant phenotype. In this study, we demonstrate that docetaxel can be used to augment apoptotic cell death induced by TRAIL through inactivating (phosphorylating) BCL-2.

MATERIALS AND METHODS

Cell Culture and Survival Assay

Human prostate adenocarcinomas LNCaP and PC-3, and human prostate carcinoma DU 145 cells were purchased from American Tissue Type Culture Collection (Manassas, VA) and LNCaP's derivative C4-2B was a gift kindly given by Dr. Leland WK Chung (Emory University). 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 dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. One day prior to the drug treatment, 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 solution (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

Docetaxel (DTX) was purchased from Sigma Chemical Co. (St. Louis, MO). The MAP kinase inhibitors PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor) were purchased from Calbiochem (San Diego, California), and SB203580 (p38 inhibitor) was purchased from TOCRIS (Ellisville, MO). These MAP kinase inhibitors were prepared and dissolved in dimethylsulfoxide (DMSO) and applied to the cells at 20 μg/ml. Treatments of drugs were accomplished by aspirating the medium and replacing it with medium containing these drugs.

Antibodies

Anti-BCL-2, anti-caspase-3, anti-cyclin B1 and anti-JNK (for JNK 1,2,3) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8 and anti-phosphorylated BCL-2 (Ser70) were purchased from Cell Signaling (Beverly, MA) and anti-p53 antibody from Calbiochem. Anti-caspase-9 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-PARP antibody was purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Anti-actin antibody was purchased from MP Biomedicals (Solon, OH). For the secondary antibodies, anti-mouse-IgG-HRP and anti-rabbit-IgG-HRP were purchased from Santa Cruz Biotechnology.

Immunoblot Analysis

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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, Arlington Heights, IL).

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 His-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 prior to drug treatment. Twenty hours after docetaxel treatment and 200 ng/ml TRAIL treatment, cells were washed with 1X PBS and harvested. Cells were then 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. And 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 TE (10 mM Tris-1 mM EDTA, pH 8.0) buffer. Five micrograms of DNA were loaded on each lane of 1.5% agarose gel.

Cytochrome *c* Release

To measure the release of cytochrome *c* from mitochondria, non-mitochondrial cytosolic fractions were prepared by the following procedure. Subconfluent LNCaP and C4-2B cells growing in 60 mm dishes were treated as indicated in Figure 3. After treatment, cells were scraped, washed in PBS, washed in buffer H (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin) and resuspended in 200 μl buffer H. After 1 h incubation on ice, cells were lysed by forcing them through a 27-gauge needle 10 times. The lysate was centrifuged at 20,000*g* for 15 min and the supernatant was collected. Cytochrome *c* levels in the resulting supernatant were analyzed by immunoblotting.

Treatment of λ-Protein Phosphatase

λ-protein phosphatase was purchased from New England BioLabs (Beverly, MA). LNCaP and C4-2B cells were treated with DTX for 20 h, harvested and then lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.1 mM EDTA). After three times of freezing and thawing using dry ice, cell lysates

were harvested by centrifugation 10 min, 8,000 rpm at 4°C. To make a reaction, 1X λ-protein phosphatase reaction buffer, 2 mM $MnCl_2$, and 200 U of λ-protein phosphatase were applied to each 100 μg (in 100 μl) of protein lysate, and incubated at 30°C for 1 h. Lysates were boiled with 1X sample loading buffer loaded to the gel to resolve proteins.

RESULTS

DTX Alone Does Not Induce Apoptotic Cell Death

Prior to investigating the effect of DTX on TRAIL-induced cytotoxicity, we studied whether DTX induces apoptotic death in human prostate cancer LNCaP, C4-2B, PC-3, and DU 145 cells. Cells were treated with various concentrations (1–25 μM) of DTX for 4 or 24 h. Figure 1A shows that no cleavage of poly(ADP-ribose) polymerase (PARP), which is the hallmark feature of apoptosis, occurred during treatment with the drug even in 25 μM. These results suggest that treatment with DTX for 24 h does not induce apoptosis in these prostate cancer cells. To examine whether DTX promotes TRAIL-induced apoptosis, hormone-sensitive LNCaP cells and its derivative hormone-insensitive, metastatic, C4-2B cells were pretreated with 10 nM DTX or various concentrations (0.1–20 nM) of DTX for 20 h and then treated with various concentrations (20–200 ng/ml) of TRAIL or 200 ng/ml TRAIL, respectively, for 4 h in the presence of the drug (Fig. 1B,C). TRAIL in combination with DTX induced apoptosis in a dose dependent manner in TRAIL resistant LNCaP and C4-2B cells (Fig. 1B,C). Figure 1B shows that the minimal amount of 100 ng/ml TRAIL was required for PARP-1 cleavage after pretreatment with 10 nM DTX. Figure 1C shows that the minimal amount of 1–2 nM DTX was required for PARP-1 cleavage in the presence of 200 ng/ml TRAIL. TRAIL-induced apoptosis was also promoted in a DTX concentration dependent manner in DU 145 and PC-3 cells (data not shown) and in a time dependent manner in LNCaP and C4-2B cells (Fig. 1D). Figure 1D shows that at least 20 h of pretreatment with DTX was required for PARP-1 cleavage in the presence of TRAIL. Interestingly, DTX more effectively enhanced TRAIL-induced apoptosis in C4-2B cells compared with LNCaP cells.

DTX Pretreatment Promotes TRAIL-Induced Apoptotic Cell Death

To confirm whether DTX in combination with TRAIL indeed induces apoptotic death in LNCaP cells and C4-2B cells, cells were pretreated with 10 nM DTX for 20 h and/or treated with 200 ng/ml TRAIL in the presence of DTX. Figure 2A shows that little or no cytotoxicity was observed with 200 ng/ml TRAIL alone. Similar results were observed with PARP-1 cleavage, cell death assay, and DNA fragmentation assay (Fig. 2B–D). When treated with DTX alone, LNCaP and C4-2B cells formed a round shape and detached from the plates; however, apoptotic DNA fragmentation and PARP-1 cleavage were not detected (Fig. 2A–D). DTX in combination with TRAIL induced apoptotic death, which is associated with typical morphological features like cell shrinkage and cytoplasmic membrane blebbing, in both cell lines (Fig. 2A). In addition, PARP-1 cleavage, cell death, and DNA fragmentation were observed in both cell lines (Fig. 2B–D). These data demonstrate that TRAIL-induced apoptotic cell death was promoted by pretreatment with DTX regardless of androgen dependency, since LNCaP cells are androgen-dependent while C4-2B cells are androgen-independent.

Caspase Activation Is Responsible for DTX-Pretreated TRAIL-Induced Apoptosis

Additional experiments were conducted to investigate whether pretreatment with DTX 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 DTX and treatment with TRAIL in androgen-dependent LNCaP and androgen-independent and metastatic LNCaP subline C4-2B cells. The combined treatment of TRAIL and DTX also resulted in an increase in caspase-9 activation as well as caspase-3 activation in both LNCaP and C4-2B cells (Fig. 3). The precursor form of caspase-9 and -3 was cleaved to the active form of 37 and 17 kDa, respectively. As expected from Figure 1A, DTX alone did not activate caspases. Figure 3 shows that PARP-1 (116 kDa) was cleaved yielding a characteristic 85-kDa fragment in the presence of 200 ng/ml TRAIL and DTX (10 nM) in both LNCaP and C4-2B cells. The cleavage of PARP was not observed by treatment with DTX or TRAIL

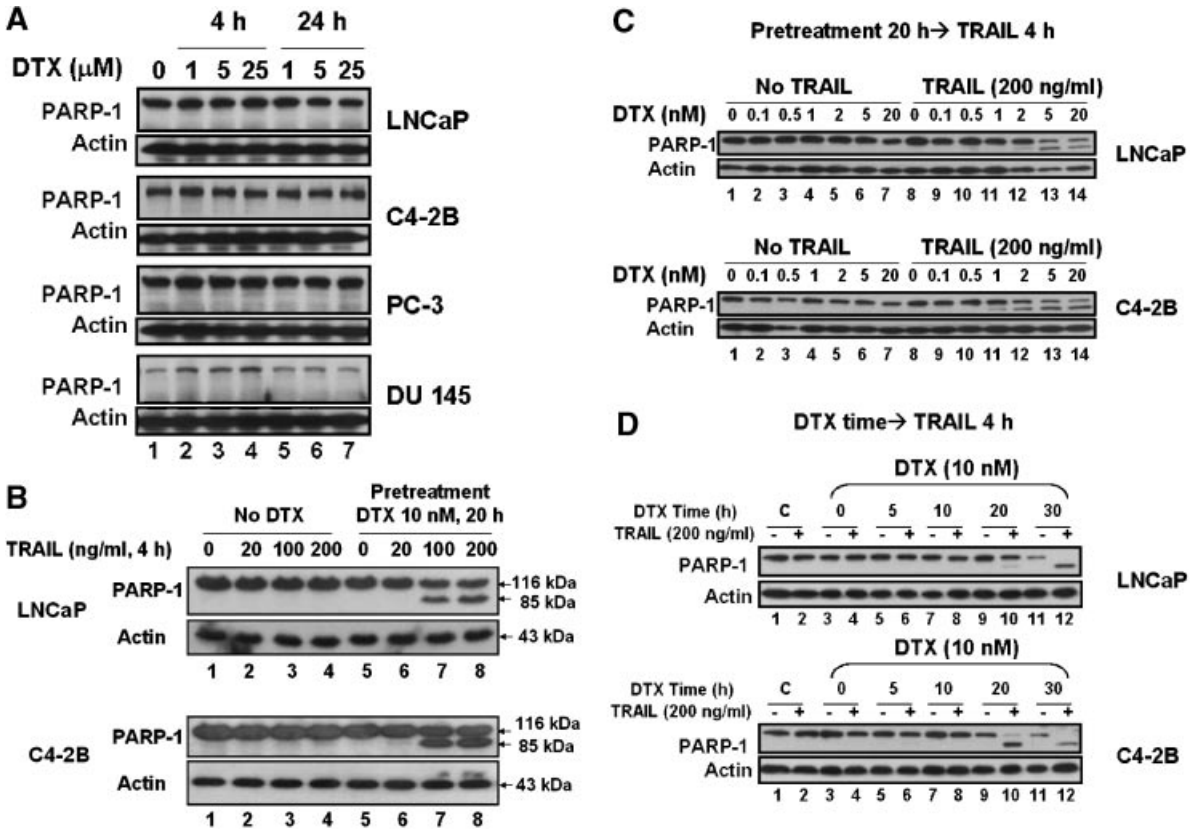

Fig. 1. Effect of pretreatment of docetaxel (DTX) on TRAIL-induced apoptosis in prostate cancer cells. **A**: LNCaP, C4-2B, PC-3, and DU 145 cells were treated with various concentrations of DTX (1–25 μM) for 4 or 24 h. **B**: LNCaP and C4-2B cells were pretreated with/without 10 nM DTX for 20 h and then treated with various concentrations of TRAIL (20–200 ng/ml) for 4 h. **C**: LNCaP and C4-2B cells were pretreated with various concentrations of DTX (0.1–20 nM) for 20 h and treated with/without TRAIL (200 ng/ml) for 4 h. **D**: LNCaP and C4-2B cells were pretreated with 10 nM DTX for various times (0–30 h) and then treated with/without TRAIL (200 ng/ml) for 4 h. A–D: Cells were harvested and equal amounts of protein (20 μg) from cell lysates were separated by SDS–PAGE and then subjected to immunoblotting for PARP-1. Actin was used to confirm the amount of proteins loaded in each lane.

alone. Previous studies have demonstrated that apoptotic signaling via a mitochondria-dependent pathway plays an important role in the TRAIL-induced activation of caspases [Kim et al., 2005]. It is also well known that the cytochrome *c* release from mitochondria activates various caspases which are responsible for apoptotic death. These findings are supported by Figure 3, which shows that DTX in combination with TRAIL induced cytochrome *c* release.

DTX Upregulates the Intracellular Level of p53 in Prostate Cancer

It has been well known that p53, the most commonly mutated gene in human cancers, can mediate the apoptosis response to chemotherapeutic agents [Vassilev, 2005]. Also, it has been reported that LNCaP cells overexpressing cyclin B1 by chemotherapeutic agents are more sensitive to apoptosis [Gomez et al., 2007]. We hypothesized that DFX deregulates the activation of cyclin B1 kinase, which leads to the induction of overexpression of p53 and apoptotic cell death when combined with TRAIL therapy. In our study, we observed that DTX treatment resulted in a significant increase in the levels of cyclin B1 and p53 in C4-2B cells in time dependent manner (Fig. 4A). Similar results were observed in LNCaP and DU 145 cells (Fig. 4B). Nonetheless, DTX still promoted TRAIL-induced apoptosis in prostate cancer cells which are devoid of p53 (PC-3 cells) or have an inactive mutant form of p53 (DU 145 cells; Fig. 4B). These results suggest that upregulation of p53 is not required for promotion of TRAIL-induced apoptosis by DTX.

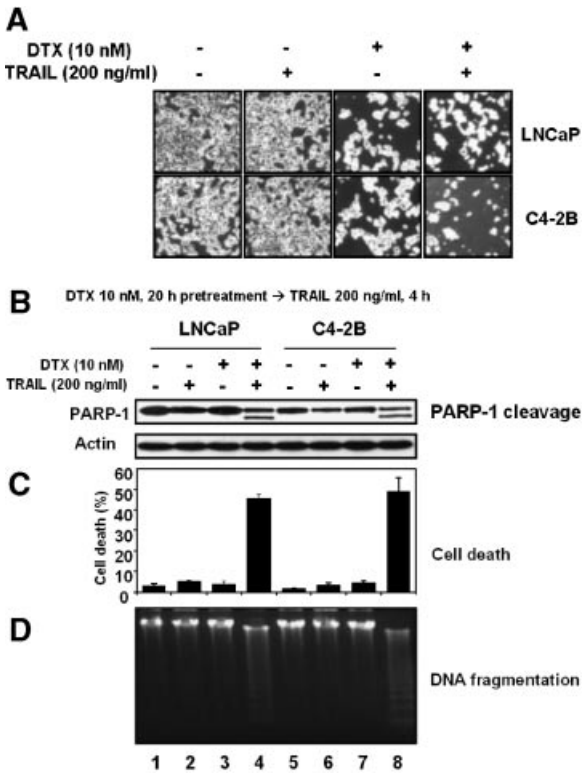

Fig. 2. Effect of pretreatment of DTX on TRAIL-induced apoptotic cell death in LNCaP and C4-2B cells. Cells were pretreated with 10 nM of DTX for 20 h and/or treated with or without TRAIL (200 ng/m) for 4 h. **A**: The morphological features were analyzed with a phase-contrast microscope (100 X). **B**: Equal amounts of protein (20 μg) from cell lysates were subjected to immunoblotting for PARP-1. Actin was used as an internal protein control to confirm the amount of proteins loaded in each lane. **C**: Cell death was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. **D**: DNA was isolated from cell lysates and DNA fragmentation was analyzed with agarose gel.

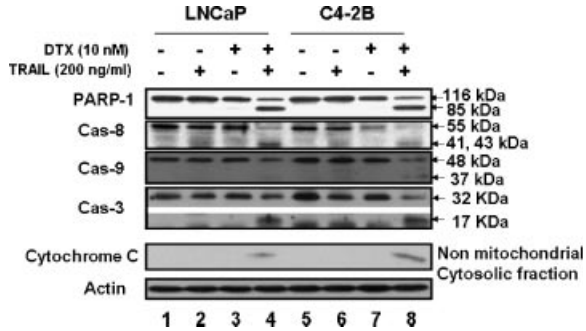

Fig. 3. Effect of pretreatment of DTX on TRAIL-induced proteolytic cleavage, activation of caspases and cytochrome *c* release from mitochondria in LNCaP and C4-2B cells. Cells were pretreated with 10 nM of DTX for 20 h and/or treated with or without TRAIL (200 ng/m) for 4 h. Cell lysates were separated by SDS–PAGE and subjected to immunoblotting for PARP-1, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects 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). Cytosolic fractions were prepared as described in the Materials and Methods Section and loaded for detecting cytosolic cytochrome *c*. Actin was used to confirm the amount of proteins loaded in each lane.

Phosphorylation of Anti-Apoptotic Protein BCL-2 by Treatment With DTX

Cancer cells which are resistant to chemotherapeutic drugs often overexpress anti-apoptotic proteins such as BCL-2 and BCL-XL. These proteins act on the mitochondrial membrane to prevent caspase activation by interfering with cytochrome *c* release [Cory et al., 2003]. It is well known that BCL-2 protein can be phosphorylated by treatment with taxol and that phosphorylated form of BCL-2 (pBCL-2) lose anti-apoptotic activity [Haldar et al., 1994, 1995]. We hypothesized that DTX's in inactivation of BCL-2 through phosphorylation of BCL-2 is responsible for promotion of TRAIL-induced cytotoxicity in hormone-sensitive LNCaP as well as hormone-insensitive C4-2B cells. Figure 5A,B shows that a mobility shifted form of BCL-2 was detected during treatment with DTX in LNCaP, C4-2B, PC-3, and DU 145 cells. This shifted form of BCL-2 might be pBCL-2 because the upper BCL-2 band disappeared by treatment with λ-phosphatase in both cell lines (Fig. 5A). Data from immunoblotting with anti-phospho-BCL-2 clearly confirmed that serine 70 amino acid residue of BCL-2 was phosphorylated during treatment with DTX (Fig. 5C). DTX phosphorylated BCL-2 in a time-dependent manner (Fig. 5D). Figure 5D shows that at least 8 h of pretreatment with DTX was required for phosphorylation of BCL-2.

JNK1 Is Involved in Phosphorylation of BCL-2 During Treatment With DTX

Our studies suggest that DTX-induced phosphorylation of BCL-2 is related to the promotion of TRAIL cytotoxicity. This leads to the question of how BCL-2 is phosphorylated during treatment with DTX. Several researchers have reported that JNK is associated with phosphorylation of BCL-2 during treatment with taxol [Brichese et al., 2004]. We hypothesized that JNK is also involved in the phosphorylation of BCL-2 during treatment with DTX. To test our hypothesis, the status of JNK1, JNK2, and JNK3 and their phosphorylated forms (pJNK)

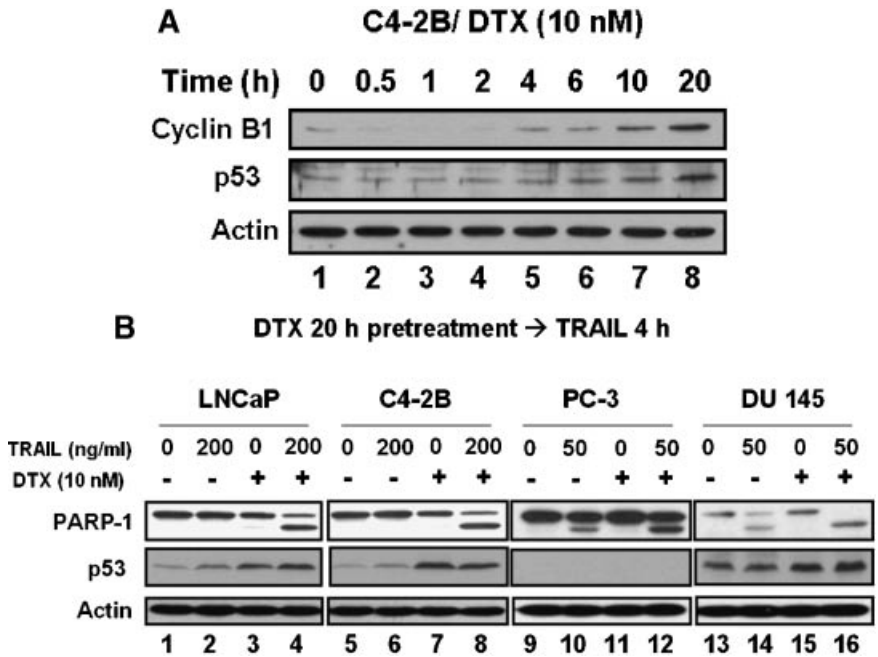

Fig. 4. DTX-mediated upregulation of cyclin B1 and p53 expression and involvement of p53 in enhancement of TRAIL-induced apoptosis in various human prostate cancer cells. **A**: C4-2B cells were treated for various times (0.5–20 h) with 10 nM DTX. **B**: LNCaP, C4-2B, PC-3, and DU 145 cells were pretreated with 10 nM of DTX for 20 h and/or treated with or without TRAIL (200 ng/m) for 4 h. A,B: Equal amounts of protein (20 μg) from cell lysates were subjected to immunoblotting for cyclin B1, p53, or PARP-1. Actin was used to confirm the amount of proteins loaded in each lane.

were examined during treatment with DTX in LNCaP and C4-2B cells. Figure 6A shows that mobility shifted form of JNK1 was detected during treatment with DTX in LNCaP and C4-2B cells. This shift is due to phosphorylation of JNK, because adding λ-phosphatase to the C4-2B cell lysate changed pJNK1 to non-phosphorylated JNK1 and inhibited shifting of JNK1 (Fig. 6B). These results indicate that there is a correlation between an increase in pJNK and inactivation of BCL-2 by phosphorylation.

TRAIL-Induced Apoptosis Is Protected by JNK Inhibitor But NOT by p38 or ERK Inhibitors

BCL-2 family members are well known to be important proapoptotic or anti-apoptotic regulators. However, the involvement of BCL-2 protein expression and/or modification and its relation to the MAPK pathways in DTX-induced apoptosis are not fully understood. Hence, we further examined which MAP kinases are responsible for the phosphorylation of BCL-2 and whether inhibition of the BCL-2 phosphorylating kinases can prevent TRAIL-induced apoptotic death. As shown in Figure 7, DTX-induced phosphorylation of BCL-2 and JNK1 was completely inhibited by JNK inhibitor SP600125, but not p38 inhibitor SB202190 or ERK inhibitor PD98059 (Fig. 7). These results suggest that DTX-induced phosphorylation of BCL-2 is mediated through activation of JNK1. Inhibition of JNK1 leads to suppression of apoptotic death by inhibiting BCL-2 phosphorylation during treatment with DTX.

DISCUSSION

Prostate cancer begins as an androgen-dependent tumor and undergoes clinical regression in response to pharmacological or surgical strategies that reduce testosterone concentration. While 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. 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]. Nonetheless, previous studies show that DTX is an effective anti-cancer agent

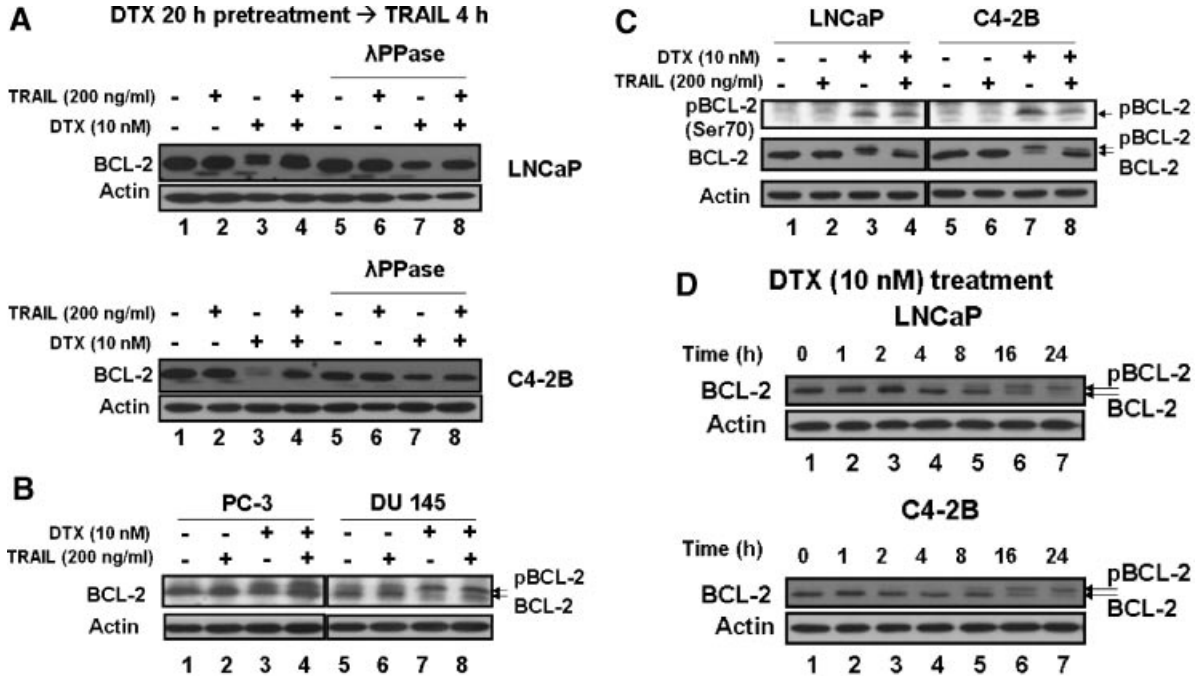

Fig. 5. Phosphorylation of BCL-2 by treatment with DTX in prostate cancer cells. **A**: LNCaP and C4-2B cells were pretreated with 10 nM DTX for 20 h and/or treated with or without TRAIL (200 ng/m) for 4 h. After treatment, NP-40 lysis buffer was applied to the samples and then lysates were incubated with or without λ-phosphatase as described in the ''Materials and Methods Section.'' **B**: PC-3 and DU 145 cells were pretreated with 10 nM DTX for 20 h and/or treated with or without TRAIL (200 ng/m) for 4 h. **C**: LNCaP and C4-2B cells were pretreated with 10 nM DTX for 20 h and/or treated with or without TRAIL (200 ng/m) for 4 h. **D**: LNCaP and C4-2B cells were treated for various times (0–24 h) with 10 nM DTX. A–D: Equal amounts of protein (20 μg) from cell lysates were separated by SDS–PAGE and immunoblotted with anti-BCL-2, anti-phospho-BCL (p-Ser70), or anti-actin antibody.

against HRPC [Chowdhury et al., 2007]. In this study, we observed that DTX in combination with TRAIL effectively induces apoptotic cell death to androgen-dependent LNCaP cells as well as its derivative androgen-independent C4-2B cells. Our results are consistent with previous observations which demonstrate that several chemotherapeutic agents including

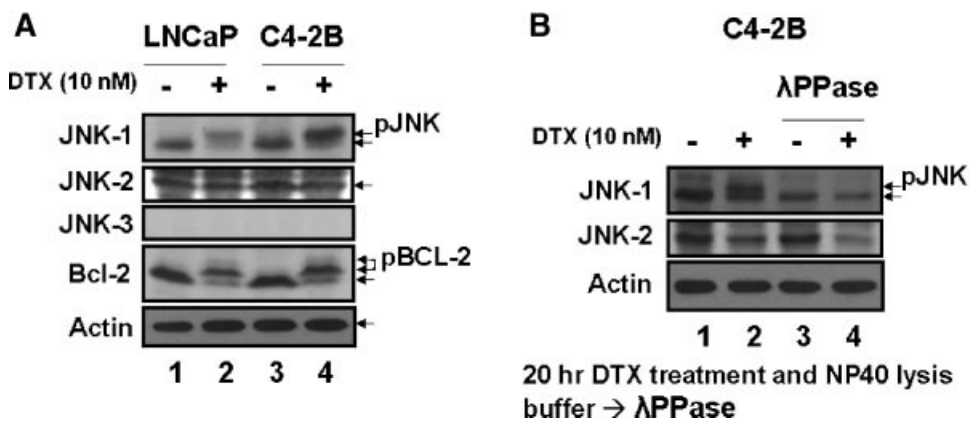

Fig. 6. Phosphorylation of JNK by treatment with DTX in LNCaP and C4-2B cells. **A**: LNCaP and C4-2B cells were treated with 10 nM DTX for 20 h and harvested. Equal amounts of protein (20 μg) from cell lysates were separated by SDS–PAGE and immunoblotted with anti-JNK-1, anti-JNK-2, anti-JNK-3, anti-BCL-2, or anti-actin antibody. **B**: C4-2B cells were treated with 10 nM DTX for 20 h and harvested. Cells were lysed with NP-40 lysis buffer and their lysates were incubated with or without λ-phosphatase. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-JNK1, anti-JNK2, or anti-actin antibody.

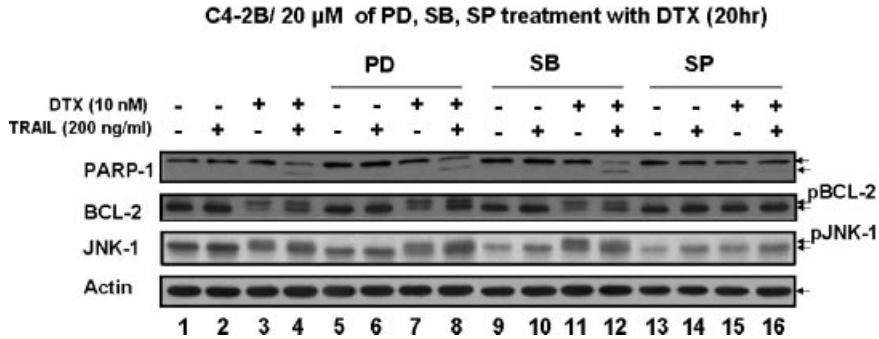

Fig. 7. Effect of MAP kinase inhibitors on DTX promoted TRAIL cytotoxicity, BCL-2 phosphorylation, and JNK phosphorylation in C4-2B cells. C4-2B cells were pretreated with 10 nM DTX for 20 h with or without 20 μM MAP kinase inhibitors (PD98059 (ERK), SB20219 (p38), and SP600125 (JNK)). After pretreatment, cells were treated with or without 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 μg) from cell lysates were separated by SDS–PAGE and subjected to immunoblotting for PARP-1, BCL-2, and JNK-1. Actin was used to confirm the amount of proteins loaded in each lane.

nitrosylcobalamin, doxorubicin, oxaliplatin, melphalan, cisplatin, and vincristine enhance TRAIL-induced apoptosis in a variety of human cancer cells [Baritaki et al., 2007; Bauer et al., 2007; Wu et al., 2007; Yoo and Lee, 2008]. In this study, we believe that administration of DTX in combination with TRAIL may offer an alternative protocol to treat HRPC patients.

Previous studies have shown that DTX arrests cells to G2/M and induces cyclin B1 accumulation which results in an increase in cyclin B1 kinase activity followed by induction of apoptotic cell death [Gomez et al., 2007]. Increased expression of cyclin B1 during treatment with DTX sensitizes prostate cancer cells to apoptosis induced by chemotherapeutic agents. Increased sensitivity to apoptosis by accumulation of cyclin B1 may be due to a decrease in the intracellular level of BCL-2 and an increase in the level of p53 [Estève et al., 2006; Gomez et al., 2007]. However, we observed no significant change in the intracellular level of BCL-2 (lanes 13–16 in Fig. 7) during treatment with DTX. Moreover, DTX still promotes TRAIL-induced apoptosis in p53-null PC-3 cells (Fig. 4B). These results suggest that modulation of intracellular levels of BCL-2 and p53 is not required for enhancing TRAIL-induced apoptosis by DTX. Nevertheless, our studies clearly demonstrated that DTX-induced phosphorylation of BCL-2 plays an important role in sensitization of prostate cancer cells to apoptosis induced by TRAIL.

Various kinases have been proposed to mediate the phosphorylation of BCL-2, following various stimuli [Abuharbeid et al., 2005; Sui et al., 2006; Chen et al., 2007; Mhaidat et al., 2007; Das et al., 2007, 2008]. Several researchers reported that taxol activates Raf-1 and JNK for phosphorylation and inactivation of anti-apoptotic Bcl-2 protein [Shiah et al., 2001; Nicolini et al., 2003; Brichese et al., 2004; Das et al., 2007, 2008]. In this study, we demonstrated that JNK, in particular JNK1, but not p38 and ERK, is involved in the phosphorylation of Bcl-2 during treatment with DTX. A fundamental question which remains unanswered is how DTX treatment activates JNK. At the present time, we can only speculate about the mechanism of activation of JNK during DTX treatment. Recently, Das et al. [2008] reported that taxol triggers production of reactive oxygen species (ROS) and subsequently induces phosphorylation of p38, JNK1, and Raf-1 kinase. It is possible that DTX elevates the intracellular level of ROS by either increasing production of ROS through the mitochondrial electron transport chain or decreasing elimination of ROS through blockage of the glutathione peroxidase/glutathione reductase system. Redox-regulatory proteins such as thioredoxin and glutaredoxin recognize DTX-induced oxidative stress and activate the apoptosis signal-regulating kinase 1 (ASK1)-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-JNK1-BCL-2 signal transduction pathway [Yamamoto et al., 1999]. We believe that many critical questions still remain to be answered to understand the mechanism of the phosphorylation of BCL-2 during treatment with DTX. However, this model will provide a framework for future studies.

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