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Pretreatment of indole-3-carbinol augments TRAIL-induced apoptosis in a prostate cancer cell line, LNCaP

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Abstract **Prostate cancer is one of the most common cancers in men and is the second leading cause of cancer-related deaths in the USA. Many anti-tumor agents against prostate cancer cells have been developed, but their unacceptable systemic toxicity to normal tissues frequently limits their usage in clinics. Several previous studies have demonstrated that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce cell death in a variety of transformed cells including prostate cancer cells, but not normal cells. Indole-3-carbinol (I3C), a phytochemical that is produced in fruits and vegetables, may play an important role in the prevention of many types of cancer, including hormone-related ones such as breast and prostate cancer. In this study, we examined the potential sensitizing effects of I3C on TRAIL-mediated apoptosis in a prostate cancer cell line, LNCaP. When LNCaP cells were incubated with I3C (either 30 or 90 μM) for 24 h and then treated with TRAIL (100 ng/ml), enhanced TRAIL-mediated apoptosis was observed. The enhanced apoptosis measured by poly(ADP-ribose) polymerase and caspase 3 cleavage. We also observed that loss of cell viability after treatment with I3C/TRAIL is greater compared with I3C and TRAIL alone. To determine the molecular mechanisms involved in the enhanced apoptosis, we examined the expression of two TRAIL death receptors (DR4 and DR5) and two TRAIL decoy receptors (DcR1 and DcR2). We found that treatment with I3C induced DR4 and DR5 expression at both transcriptional and translational levels. These findings suggest that I3C may be an effective sensitizer of TRAIL treatment against TRAIL-resistant prostate cancer cell lines such as LNCaP.**

Key words: Death receptor 4; Death receptor 5; Cell death; Chemosensitivity

1. Introduction

Prostate cancer is one of the most common cancers in men and remains the second leading cause of cancer-related deaths in the USA. It is estimated that in 2002, 189 000 new cases will be diagnosed and 30 200 men will die from prostate cancer in the USA alone [1]. Androgen can regulate programmed cell death of normal and malignant prostatic cells and its ablation is still a major therapy for the treatment of androgen-dependent prostatic cancer cells [2–4].

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family and can induce apoptotic cell death in a variety of cell types including prostate cancer cells [5–14]. The apoptotic signal induced by TRAIL is transduced by its binding to the death receptors TRAIL-R1 (designated death receptor 4, DR4) and TRAIL-R2 (designated death receptor 5, DR5) [15–17]. Both DR4 and DR5 contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. TRAIL also binds to antagonistic decoy receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which inhibit TRAIL signaling [18–22]. Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif [23]. The relative resistance of normal cells to TRAIL has been explained by the presence of large numbers of decoy receptors on normal cells [18,19]. Recently, this hypothesis was challenged based on results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell lines [24]. This discrepancy indicates that other factors such as death inhibitors (FLIP, FAP-1, or IAP) are also involved in the differential sensitivity to TRAIL. Several studies also suggest that constitutively active AKT is an important regulator of TRAIL sensitivity in prostate cancer cells [25–27].

Many anti-tumor drugs or cytokines have been developed for prostate cancer patients, but their intolerable systemic toxicity often limits their clinical usage. TRAIL has been a focus for anti-tumor studies because of its potential low cytotoxicity. However, recent studies have revealed that a polyhistidine-tagged TRAIL induces apoptosis in normal human hepatocytes in culture [28]. This is probably due to an aberrant conformation and subunit structure of TRAIL in the presence of low zinc concentrations [29]. In contrast, native-sequence, non-tagged recombinant TRAIL, when produced

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Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; I3C, indole-3-carbinol; PARP, poly(ADP-ribose) polymerase; DR4, death receptor 4; DR5, death receptor 5; DcR1, decoy receptor 1; DcR2, decoy receptor 2; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

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under optimized zinc concentrations, is markedly more active against tumor cells than the polyhistidine-tagged ligand, but has minimal toxicity toward human hepatocytes in vitro [29]. Moreover, preclinical studies in mice and primates have shown that administration of TRAIL can induce apoptosis in human tumors, but no cytotoxicity to normal organs or tissue [30]. In addition, unlike TNF and FasL, TRAIL mRNA is expressed constitutively in many tissues [6,31].

Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in all members of the cruciferous vegetable family, which includes cabbage, broccoli, Brussels sprouts, cauliflower and kale. Recently, it has become clear that I3C has the potential to prevent and even treat a number of common cancers, especially those that are estrogen-related [32–35].

In this study, we examine the potential sensitizing effects of I3C to TRAIL-mediated apoptosis in human prostate cancer cells. Thus, we investigated whether I3C can be involved in the enhancement of apoptosis mediated by TRAIL. The molecular analysis of I3C's effect on TRAIL may provide information on the molecular mechanisms by which I3C elicits its biological effects on prostate cancer (LNCaP) cells. Our data show that I3C is an effective sensitizer to TRAIL-mediated apoptosis in LNCaP cells. The sensitizing effect of I3C on TRAIL was associated with the up-regulation of DR4 and DR5 at both mRNA and protein levels.

2. Materials and methods

2.1. Cell lines and culture

Human prostate cancer cells (DU-145 and LNCaP) were originally obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle's medium for DU-145 and RPMI 1640 for LNCaP. Culture media were supplemented with either 5% (DU-145) or 10% (LNCaP) fetal bovine serum, L-glutamine (5 mM), non-essential amino acids (5 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all obtained from Bio Whittaker, Walkersville, MD, USA).

2.2. TRAIL preparation

A human soluble TRAIL cDNA fragment (amino acids 114–281) obtained by reverse transcription polymerase chain reaction (RT-PCR) was cloned into a prokaryotic expression vector pET-23d (Novagen, Madison, WI, USA) to tag the C-terminus with hexahistidine. Expressed TRAIL protein was purified using Ni-NTA His-Bind Resin Superflow according to the manufacturer's instructions (Novagen).

2.3. MTT assays

Growth inhibition of TRAIL was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [36]. Briefly, subconfluent cells plated on 96-well tissue culture plates were treated or not with TRAIL for 16 h. MTT (2 μg/ml) was added into each well for 4 h. After the medium containing MTT was aspirated, the formazan crystals were dissolved in 120 μl of dimethyl sulfoxide (DMSO). The absorbance was recorded using a Dynatech 96-well spectrophotometer at 570 nm, with 630 nm as the reference wavelength. The wells that contained only medium and 10 μl of MTT were used as blanks for the plate reader. Cell viability was expressed as the amount of dye reduction in treated cells relative to that of untreated control cells. Four sets of experiments were performed in 10 wells for each treatment. The data are presented as the mean ± S.D. of triplicate samples from at least three separate representative experiments and were analyzed by Student's *t*-test. A level of $P < 0.05$ was accepted as statistically significant.

2.4. I3C treatment

I3C was purchased from Sigma (St. Louis, MO, USA), dissolved in 95% EtOH and stored at −20°C before use. In this study we used 30–90 μM/l for each dose of I3C as previously described [37]. The cells were seeded at a density of 9×10^5 cells in a six-well culture dish. After 24 h, cells were treated with 30, 60, or 90 μM of I3C and the control cells with 95% DMSO.

2.5. Western blotting

Whole cell lysates were prepared and Western blotting was performed as described earlier [38]. Equal aliquots of total cell protein (50 μg per lane) were electrophoresed, transferred, and blotted using the following primary antibodies: DR4, DR5, DcR1 and DcR2 (rabbit polyclonal IgG, Alexis Biochemicals, 1:500 dilution), anti-poly-(ADP-ribose) polymerase (PARP) (H-250, rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500 dilution), anti-caspase 3 (H-277, rabbit polyclonal IgG, Santa Cruz Biotechnology, 1:1000). β-Tubulin (D-10, mouse monoclonal IgG, Santa Cruz Biotechnology, 1:500) and β-actin (I-19, rabbit polyclonal IgG, Santa Cruz Biotechnology, 1:1000) were used to assure equal loadings. Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz.

2.6. Isolation of RNA

After cell treatments with I3C (90 μM), the total cellular RNA was extracted using TRIzol reagents (Life Technologies), according to the manufacturer's instructions. Isolated RNA was electrophoresed through 1.0% agarose-formaldehyde gels to verify the quality of the RNA.

2.7. Semiquantitative RT-PCR

The first strand cDNA was generated from 1 μg of total RNA in a final volume of 20 μl using SuperScript II (Life Technologies) and oligo(dT) primers. One μl of the diluted cDNA was used for each PCR reaction. PCR amplification was performed using a Perkin-Elmer DNA thermal cycler. The PCR primer sets used in this study were as follows: DR4 F, 5′-TTG TGT CCA CCA GGA TCT CA-3′; DR4 R, 5′-GTC ACT CCA GGG CGT ACA AT-3′; DR5 F, 5′-AGA GGG ATT GTG TCC ACC TG-3′; DR5 F, 5′-AAT CAC CGA CCT TGA CCA TC-3′; β-actin F, 5′-TAG CGG GGT TCA CCC ACA CTG TGC CCC ATC TA-3′; β-actin R, 5′-CTA GAA GCA TTT GCG GTG GAC CGA TGG AGG G-3′.

To determine the expression level of DR4 and DR5, 1 μl (out of 20 μl) of synthesized cDNA was amplified in a total volume of 50 μl containing 200 μM each of all four dNTPs, 2 μM each of death receptor-specific primer sets, and 1 U of Taq DNA polymerase (Perkin-Elmer). PCR conditions were as follows: 1 cycle, 5 min/95°C; 29 cycles (for DR4 and DR5) or 22 cycles (for β-actin), 1 min/95°C, 1 min/53°C and 1 min/72°C. β-Actin was used as a loading control. PCR products were analyzed by electrophoresis through 1.0% agarose gels containing 0.1 mg/ml of ethidium bromide. The gels were photographed under ultraviolet illumination.

3. Results and discussion

3.1. TRAIL induces apoptosis of human prostate cancer cells

TRAIL has been reported to induce the apoptosis of a variety of tumor cell types [5–13]. However, the molecular determinants of TRAIL-induced apoptosis have not been well characterized in human prostate cancer cells. We used the human prostate cancer cell lines DU-145 and LNCaP for this study. When DU-145 cells were treated with 0–400 ng/ml TRAIL for 16 h, the cellular viability decreased in a dose-dependent manner as determined by a MTT assay (Fig. 1A). Relative viabilities of DU-145 cells treated with TRAIL (100 ng/ml) for various intervals were also analyzed (Fig. 1B). Consistent with the results from the MTT assay in Fig. 1A, approximately 50% of cells were dead by treatment with TRAIL (100 ng/ml) for 16 h. A time-dependent reduction in cell survival was observed in TRAIL-treated cells. The mechanism of cell death by TRAIL is apoptosis (data not shown). In contrast to DU-145, LNCaP was relatively resistant to treatment with TRAIL (Fig. 1A,B). These results are consistent with a previous study showing that LNCaP is TRAIL-

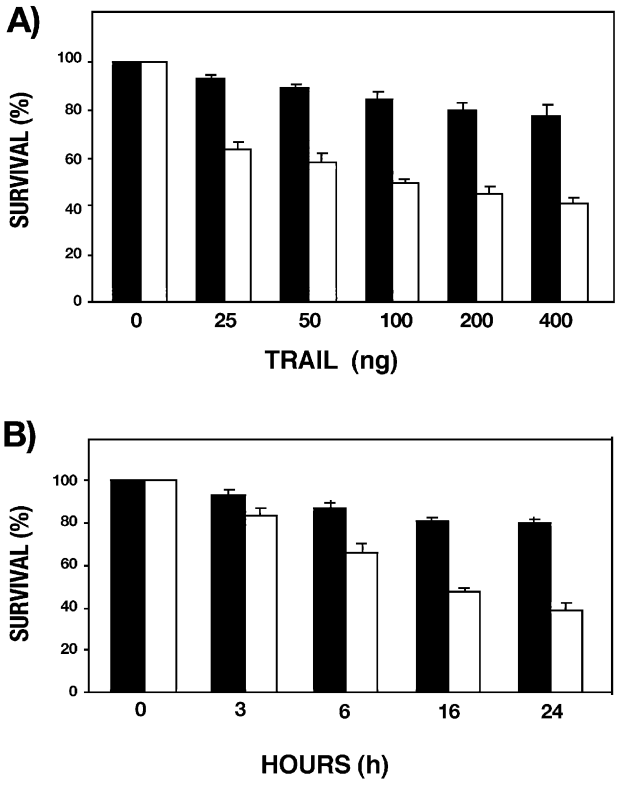

Fig. 1. TRAIL-induced cytotoxicity in DU-145 and LNCaP cells. Cells were treated with TRAIL and survival (%) was analyzed by the tetrazolium conversion assay as described in Section 2. A: Relative viabilities of DU-145 and LNCaP cells treated with increasing doses of TRAIL (0–400 ng/ml) for 16 h. B: Relative viabilities of DU-145 and LNCaP cells treated with TRAIL (100 ng/ml) for various intervals. Black bar represents LNCaP and white bar represents DU-145 cells.

resistant, while DU-145 is TRAIL-sensitive compared with LNCaP cells [39].

3.2. Treatment of LNCaP with I3C up-regulates DR4 and DR5

I3C is an active anti-cancer component and is being investigated as a treatment and for the prevention of a variety of cancers [32–35]. In order to investigate the potential effects of the combination of I3C and TRAIL on prostate cancer cell growth, we first determined the effect of I3C on growth of LNCaP cells. While lower doses (30 and 60 μM) of I3C slightly stimulated proliferation of LNCaP for the first 2 days, a higher dose of I3C (90 μM) slightly suppressed cell growth from the second day of treatment (Fig. 2). The precise mechanism of the differential growth regulation by various doses of I3C remains to be determined. Considering that a low dose of I3C (30 μM) inhibits the growth of PC-3 prostate cancer cells by the induction of G1 cell cycle arrest, which led to apoptosis [37], LNCaP is relatively resistant to low doses of I3C.

Recent studies have shown that glucose deprivation and doxorubicin enhanced TRAIL-induced cytotoxicity in prostate cancer cells [40,41]. Several DNA damaging agents such as VP-16, CPT-11, and ionizing radiation have been shown to enhance TRAIL-induced apoptosis in breast cancer cells [42–45]. In the case of VP-16 [43] and irradiation [42], increased levels of DR4 and/or DR5 were responsible for the augmentation of TRAIL-induced apoptosis. In our present study, we determined the effect of I3C on DR4 and DR5 expression and TRAIL-induced apoptosis of prostate cancer cells. Fig. 3A demonstrates that treatment of LNCaP cells with 90 μM of I3C for 24 h increased DR4 and DR5 protein. However, I3C did not change the expression of the two TRAIL decoy receptor DcR1 and DcR2. To determine whether the increases of DR4 and DR5 protein are due to transcriptional regulation, we performed semi-quantitative RT-PCR. Increased levels of DR4 and DR5 mRNA were found in I3C-treated cells compared to the control (Fig. 3B). Accordingly, I3C up-regulates DR4 and DR5 at the transcriptional level in LNCaP cells.

A recent study shows that treatment of I3C inhibits the phosphorylation and subsequent activation of Akt kinase in a human prostate cancer cell line, PC-3 [46]. Since constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer [25–27], we examined levels of Akt and its phosphorylation in LNCaP up to 2 days following I3C treatment and found no significant change (data not shown).

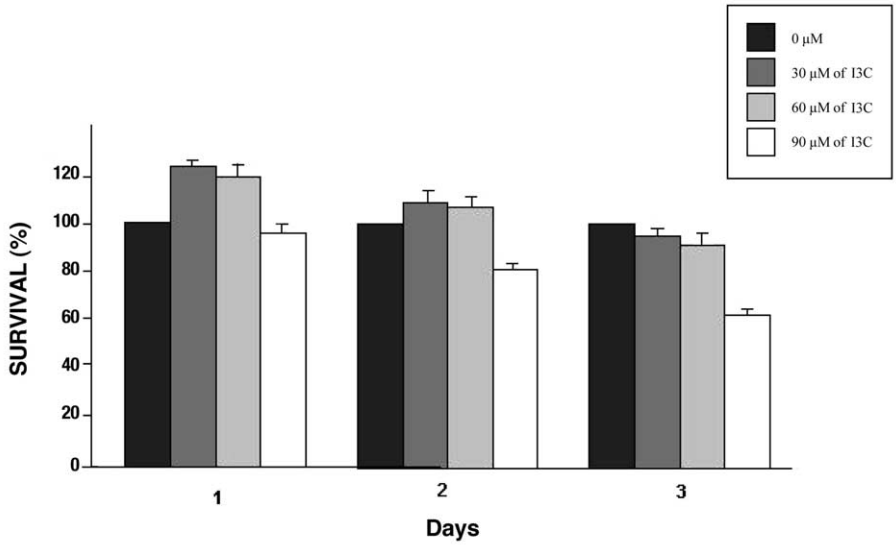

Fig. 2. Effects of I3C on cell survival in LNCaP cells. Cells treated with I3C (30, 60, or 90 μM) for 1–3 days were harvested and cell survival (%) was determined by the tetrazolium conversion assay as described in Section 2.

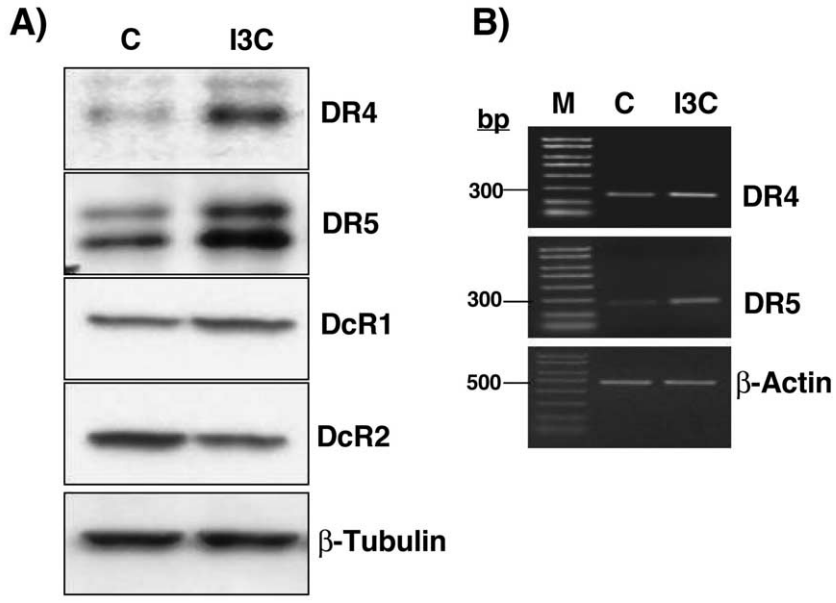

Fig. 3. Increased expression of DR4 and DR5 by I3C treatment. A: Cells treated with 90 μM I3C for 24 h were harvested and subjected to immunoblotting analysis. The levels of two TRAIL receptors, DR4 and DR5, were measured using each antibody described in Section 2. β-Tubulin was used for loading control. B: The change in DR4 and DR5 transcripts was determined by semi-quantitative RT-PCR. Information on primer sequences for DR4, DR5 and β-actin and RT-PCR conditions is given in Section 2.

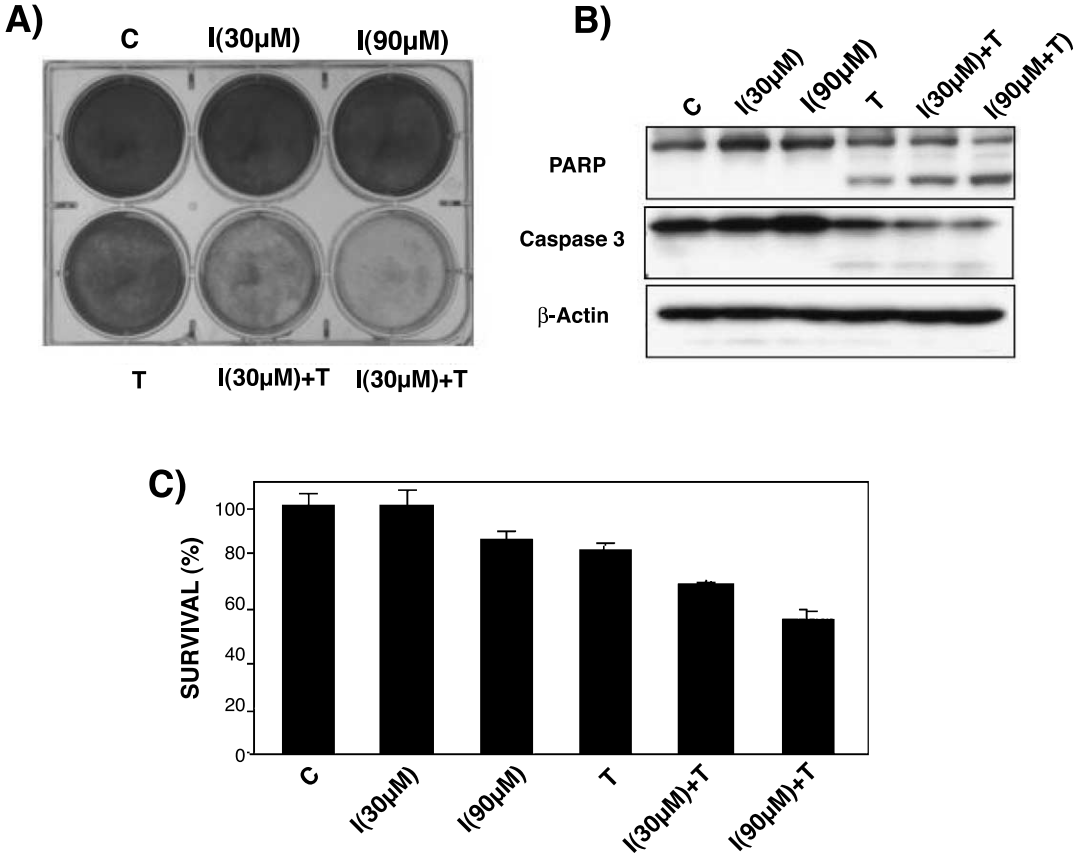

Fig. 4. Pretreatment with I3C sensitizes LNCaP cells to TRAIL-mediated apoptosis. A: Cells pretreated with either 30 or 90 μM I3C for 24 h were exposed to TRAIL (100 ng/ml) for 24 h and stained with Coomassie blue. B: Cells were lysed and subjected to immunoblotting for PARP and caspase 3. Immunoblots of PARP showed the 116-kDa PARP and the 85-kDa apoptosis-related cleavage fragment. Anti-caspase 3 antibody detected both inactive (32 kDa) and active forms (17 kDa and 12 kDa). β-Actin was used as an internal control protein. C: Survivability (%) of cells treated with I3C/TRAIL as in A was tested by the tetrazolium conversion assay as described in Section 2. C represents control cells; I represents I3C-treated cells; T represents TRAIL-treated cells.

3.3. Sequential exposure to I3C followed by TRAIL increases apoptosis of prostate cancer cells

To determine the functional significance of I3C-mediated up-regulation of DR4 and DR5 levels, we compared the apoptotic effects of sequential treatment with I3C followed by TRAIL with the effects of TRAIL alone. Exposure to I3C (30 or 90 μM) for 24 h followed by 100 ng/ml TRAIL for 24 h induced a higher level of apoptosis of LNCaP cells than exposure to TRAIL alone (Fig. 4A). To confirm that the reduced cell viability was due to apoptosis, we measured PARP cleavage. Compared to each agent alone, the sequential treatment of I3C followed by TRAIL resulted in increased processing of PARP and caspase 3 (Fig. 3B). Only one form of PARP (the full size, 116 kDa) protein was found in the control cells and cells treated with I3C (30 or 90 μM) for 24 h. This result implies that the reduced survivability observed following I3C treatment in Fig. 2 may not be due to apoptosis; rather it may be due to cell cycle arrest. The full-size PARP (116 kDa) protein was cleaved to yield an 85-kDa fragment after treatment with either TRAIL alone or a combination of I3C and TRAIL. The amount of full-size PARP declined during the combined treatment (I3C+TRAIL) at a greater rate than with TRAIL alone. We also found that loss of cell viability was greater than the sum of the viability losses observed in cultures treated with I3C and TRAIL alone (Fig. 4C). These results show that pretreatment with I3C (even at a low dose such as 30 μM) can sensitize DU-145 prostate cancer cells to TRAIL-mediated apoptosis. A recent study demonstrated that pretreatment of PC-3 with paclitaxel enhanced TRAIL-induced apoptosis of human prostate cancer cells by inducing DR4 and 5 protein levels [47].

The present findings, taken together with earlier ones demonstrating that TRAIL exerts itself relatively selectively in in vivo anti-cancer activity, suggest that TRAIL could potentially be a promising anti-prostate cancer therapeutic agent. TRAIL could also be used with chemopreventive agents such as I3C. By demonstrating that DR4 and DR5 are up-regulated by I3C, the present findings also highlight the potential feasibility of TRAIL administration with I3C to maximize the apoptotic effect against LNCaP cells.

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