Pretreatment with Paclitaxel Enhances Apo-2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis of Prostate Cancer Cells by Inducing Death Receptors 4 and 5 Protein Levels

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ABSTRACT

We have demonstrated that Apo-2 ligand (Apo-2L)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis of human prostate cancer PC-3, DU145, and LNCaP cells in a dose-dependent manner, with PC-3 cells displaying the greatest sensitivity to Apo-2L/TRAIL. Susceptibility of the prostate cancer cell types to Apo-2L/TRAIL-induced apoptosis did not appear to correlate with the levels of the Apo-2L/TRAIL receptors death receptor (DR) 4 (TRAIL receptor 1) or DR5 (TRAIL receptor 2), decoy receptor (Dcr) 1 and Dcr2, Flame-1, or the inhibitors of apoptosis proteins family of proteins. Apo-2L/TRAIL-induced apoptosis of PC-3 cells was associated with the processing of caspase-8, caspase-10, and the proapoptotic Bid protein, resulting in the cytosolic accumulation of cytochrome c as well as the processing of procaspase-9 and procaspase-3. Cotreatment with the caspase-8 inhibitor z-IETD-fmk or DR4:Fc significantly inhibited Apo-2L/TRAIL-induced apoptosis. Treatment with paclitaxel or taxotere increased DR4 and/or DR5 protein levels (up to 8-fold) without affecting the protein levels of Dcr1 and Dcr2, Apo-2L/TRAIL, Fas, or Fas ligand. Up-regulation of DR4 and DR5 was not preceded by the induction of their mRNA levels but was inhibited by cotreatment with cycloheximide. Importantly, sequential treatment of PC-3, DU145, and LNCaP cells with paclitaxel followed by Apo-2L/TRAIL induced significantly more apoptosis than Apo-2L/TRAIL treatment alone (P < 0.01). This was also associated with greater processing of procaspase-8 and Bid, as well as greater cytosolic accumulation of cytochrome c and the processing of caspase-3. These findings indicate that up-regulation of DR4 and DR5 protein levels by treatment with paclitaxel enhances subsequent Apo-2L/TRAIL-induced apoptosis of human prostate cancer cells.

INTRODUCTION

TRAIL, also called Apo-2L, has been shown to induce apoptosis of a variety of tumor cell types (1–3). Apo-2L/TRAIL suppresses human mammary adenocarcinoma growth in mice without any of the significant toxic effects seen with the in vivo use of tumor necrosis factor and FasL (CD95L) (Ref. 4). Apo-2L/TRAIL can bind to five members of the tumor necrosis factor receptor family, i.e., DR4, DR5, DcR1, DcR2, and osteoprotegerin (1, 2). DR4 and DR5 contain a cytoplasmic region consisting of a stretch of 80 amino acids designated as the death domain responsible for transducing the death signal (1, 2). Binding of Apo-2L/TRAIL to DR4 and DR5 leads to the cleavage and activation of caspase-8 and caspase-10 (1, 2). These, in turn, activate downstream effector caspsases such as caspase-3, caspase-6, and caspase-7 (5). Processed and activated caspase-8 can also cleave and activate the BH3 domain containing the proapoptotic Bid protein, which then translocates to the mitochondria, triggering the cytosolic release of cyt c (6). In the cytosol, cyt c and dATP bind to Apaf-1 and cause its oligomerization (7). Apaf-1, in turn, binds one-to-one, oligomerizes, and processes procaspase-9 into an active caspase, which recruits, cleaves, and activates the executioner caspase-3 (7, 8). This can proteolytically cleave a number of cellular proteins, e.g., poly(ADP-ribose) polymerase, DNA fragmentation factor 45 [inhibitor of caspase-activated deoxyribonuclease (ICAD)], and so forth, resulting in the morphological features and DNA fragmentation of apoptosis (9). There are several known determinants of Apo-2L/TRAIL-induced apoptotic signaling. Treatment with DNA-damaging anticancer agents can induce p53 and/or nuclear factor κB, which, in turn, can up-regulate DR5 and/or DR4 expression, thereby enhancing Apo-2L/TRAIL-induced apoptotic signaling (10, 11). In contrast, Dcr1 and Dcr2 bind and titrate Apo-2L/TRAIL and can act as inhibitors of Apo-2L/TRAIL-induced apoptosis (1, 2). An intracellular protein, Flame-1 (also known as c-FLIP, CASH, CLARP, MRIT, and usurpin), which has an NH2 terminus Fas-associated death domain (FADD) homology and COOH terminus caspase homology domains without caspase activity, has a dominant negative effect against caspase-8 and caspase-10 and can potentially inhibit Apo-2L/TRAIL-induced death signaling (12). Finally, the levels of IAP family members, which include cIAP1, cIAP2, X-linked XIAP, and survivin, may also inhibit TRAIL-induced apoptosis by specifically binding to and inhibiting the activities of caspase-3, caspase-9, and caspase-7 (13). Taxanes, such as paclitaxel and taxotere, are highly active anticancer drugs that are currently being investigated for their activities against human prostate carcinoma (14). Taxane-induced apoptosis is triggered by mitochondrial ΔΨm (permeability transition), release of cyt c into the cytosol, and induction of Apaf-1-mediated caspase-9 and caspase-3 activities (15, 16). In the present studies, we determined the sensitivity and molecular steps of apoptosis triggered by Apo-2L/TRAIL and/or paclitaxel in human prostate cancer cells. We demonstrate that Apo-2L/TRAIL triggers the molecular events of both the extrinsic (DR) and intrinsic (mitochondrial) pathway of apoptosis. Importantly, we also show that pretreatment with paclitaxel or taxotere induces DR4 and DR5 protein levels and enhances Apo-2L/TRAIL-induced apoptosis of prostate cancer cells.

MATERIALS AND METHODS

Reagents. z-IETD-fluoromethyl ketone (fmk) and z-LEHD-fmk were purchased from Enzyme Systems Products (Livermore, CA). Anti-Apaf-1 and anti-Bid antisera (6, 7) were kindly provided by Dr. Xiaodong Wang (University of Texas Southwestern School of Medicine, Dallas, TX). The recombinant human homotrimeric Apo-2L/TRAIL (leucine zipper construct) was a gift from Immunix Corp. (Seattle, WA; Ref. 4). Fas receptor (CD95) and FasL monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Monoclonal Anti-XIAP antibody was purchased from Boehringer Mannheim (Indianapolis, IN), whereas anti-cIAP-1 and anti-Flame-1 (IFLICE)
antibodies were purchased from PharMingen (San Diego, CA). Polyclonal anti-DR4, anti-Drk1, and anti-Apo-2L/TRAIL antibodies were purchased from Alexis Corp. (San Diego, CA). Polyclonal anti-caspase-8 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-survivin antibody was purchased from R&D Systems (Minneapolis, MN).

**Cell Culture.** Prostate cancer cell lines PC-3, DU145, and LNCaP were obtained from American Type Culture Collection and maintained in a humidified 5% CO₂ environment in RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids, 1% essential amino acids, and 10% bovine calf serum (Life Technologies, Inc., Grand Island, NY).

**Western Analyses of Proteins.** Western analyses of DR4, DR5, DcR1, DcR2, Apo-2L/TRAIL, procaspase-9, caspase-10, caspase-3, Fas receptor, Fasl, survivin, Bid, poly(ADP-ribose) polymerase, and β-actin were performed using specific antisera or monoclonal antibodies according to protocols reported previously (15, 16). Horizontal scanning densitometry was performed on Western blots by using acquisition into Adobe Photo Shop (Apple, Inc., Cupertino, CA) and analysis by the NIH Image Program (NIH, Bethesda, MD). The expression of β-actin was used as a control.

**RNase Protection Assay.** A RiboQuant Multi-Probe RNase Protection Assay System was used according to the manufacturer’s instructions (PharMingen). A hAPO3c probe set including the DNA templates for caspase-8, Fasl, Fas, Drk1, Drk2, receptor-interacting protein (RIP), L32, gyceraldehyde-3-phosphate dehydrogenase, and so forth was used for T7 RNA polymerase-directed synthesis of [32P]UTP-labeled antisense RNA probes. The probes were hybridized with 20 μg of RNA isolated from prostate cancer cells (PC-3) after treatment with Taxol at different time points using RNaizol (Cupertino, CA) and analysis by the NIH Image Program (NIH, Bethesda, MD).

**Preparation of the S-100 Fraction for the Analysis of Cytosolic Accumulation of Cyt c.** Untreated and drug-treated cells were harvested by centrifugation, and the cell homogenates were centrifuged at 100,000 × g for 30 min at 4°C to obtain the S-100 fraction, as described previously (15, 16). The supernatants were collected, and the protein concentrations of S-100 were determined by the Bradford method (Bio-Rad, Hercules, CA). Samples were then analyzed for the release of cyt c from the mitochondria into the cytosol by Western blot, as described previously (15, 16).

**Flow Cytometric Analysis of Sub-G₁ (Hypodiploid) Apoptotic Cells.** The flow cytometric evaluation of PI-stained apoptotic cells was performed according to a method described previously (17). The percentage of sub-G₁ (hypodiploid) apoptotic cells was calculated using Multicycle software (Phoenix Flow Systems, San Diego, CA).

**Apoptosis Assessment by Annexin V Staining.** After drug treatment, cells were resuspended in 100 μl of staining solution (containing annexin V fluorescein and PI in a HEPES buffer; Annexin-V-FLUOS Staining Kit; Boehringer Mannheim). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin V and PI; Ref. 17).

**Statistical Analysis.** Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions...
RESULTS AND DISCUSSION

Apo-2L/TRAIL Induces Apoptosis of Human Prostate Cancer Cells. Apo-2L/TRAIL has been reported to induce apoptosis of a variety of tumor cell types (3, 4). However, the molecular determinants of Apo-2L/TRAIL-induced apoptosis had not been comprehensively examined in human prostate cancer cells. Fig. 1A demonstrates that exposure of human prostate cancer PC-3 cells to 100–250 ng/ml Apo-2L/TRAIL for 24 h caused a dose-dependent increase in the percentage of apoptotic cells, as determined by annexin V staining followed by flow cytometry. This was confirmed by light microscopic morphological examination of Wright-stained, cytospun, Apo-2L/TRAIL-treated PC-3 cells (data not shown). Apo-2L/TRAIL was less active against DU145 or LNCaP cells. For example, exposure to 100 ng/ml Apo-2L/TRAIL for 24 h induced apoptosis of 11.6 ± 2.5% of DU145 and 7.6 ± 1.2% of LNCaP cells, as compared with 39.8 ± 1.2% of PC-3 cells (mean ± SE of three experiments).

Previous reports have suggested that the sensitivity to Apo-2L/TRAIL-induced apoptosis can be correlated to the relative expressions of DR4 and DR5 versus DcR1 and DcR2 or the intracellular levels of Flame-1 (2, 3). Fig. 1D demonstrates that, as compared with LNCaP cells, which have the lowest sensitivity to Apo-2L/TRAIL-induced apoptosis, highly sensitive PC-3 cells displayed similar or lower protein levels of DR4 and DR5 and higher levels of DcR2. DcR1 expression was barely detectable in all cell types. Counterintuitively, Flame-1 and XIAP levels were also higher in PC-3 cells than in LNCaP cells. Fig. 1D also shows that PC-3 cells display a lower Bax:Bcl-2 expression ratio as compared with the relatively resistant LNCaP cells. Although the data are not shown, survivin levels were also not significantly different between PC-3 and LNCaP cells (13). The expression levels of these regulators of apoptosis also did not offer a clear explanation for the intermediate sensitivity to Apo-2L/TRAIL-induced apoptosis of DU145 cells. The lack of correlation between the expression of DRs (DR4 and DR5) and DcRs (DcR1 and DcR2) or Flame-1 with the sensitivity to Apo-2L/TRAIL-induced apoptosis shown by our findings differs from some but not all previously reported findings (3, 11, 18–20). This may be because our data are derived from prostate cancer cells, unlike other reports in which other cell types were studied.

Paclitaxel-induced Apoptosis of Prostate Cancer Cells Is Associated with Up-Regulation of DR4 and DR5 Protein Levels. Paclitaxel and taxotere are active anticancer agents and are being investigated in combination regimens in the treatment of prostate cancer (14). With the aim of preclinically investigating the anti-prostate cancer activity of the combination of paclitaxel and Apo-2L/TRAIL,
we first determined the sensitivity of PC-3 cells to paclitaxel-induced apoptosis. Fig. 1B demonstrates that exposure to 20–500 nM paclitaxel for 24 h induced apoptosis of PC-3 cells in a dose-dependent manner (P < 0.01). We have previously reported that subsequent to causing microtubule bundling and cell cycle G2-M-phase arrest, paclitaxel induces apoptosis by engaging the intrinsic mitochondrial pathway of apoptosis (15, 16). It was shown to trigger the mitochondrial Dr5, release of cyt c into the cytosol, and the generation of Apaf-1-mediated caspase-9 and caspase-3 activities, resulting in apoptosis (15, 16). In the present studies, we determined the role of the extrinsic pathway in Apo-2L/TRAIL- and paclitaxel-induced apoptosis. Fig. 1C demonstrates that cotreatment with either caspase-8-specific inhibitor z-IETD-fmk (50 μM), Apo-2L/TRAIL R1:Fc (10 ng/ml), or TRAIL receptor 2:Fc (data not shown) did not affect paclitaxel-induced apoptosis but inhibited Apo-2L/TRAIL-induced apoptosis of PC-3 cells. Taken together with the observation that exposure to paclitaxel did not induce FasL or Apo-2L/TRAIL protein levels in PC-3 cells (vide infra), these findings (Fig. 1C) indicate that paclitaxel-induced apoptosis of PC-3 cells is not mediated through the induction of FasL or Apo-2L/TRAIL and the engagement of the extrinsic pathway of apoptosis. These findings also suggest that sensitivity to Apo-2L/TRAIL, but not to paclitaxel, would be mitigated by cotreatment with the extracellular domains of DR4 and/or DR5.

Recent reports have demonstrated that DNA-damaging agents such as etoposide, doxorubicin, CPT-11, and ionizing radiation can augment Apo-2L/TRAIL-induced apoptosis of breast cancer cells, perhaps by inducing p53 and/or nuclear factor κB activity and DR5 and/or DR4 expression (10, 11, 18–22). In the present studies, we determined the effect of antimicrotubule taxanes on DR4 and DR5 expression and Apo-2L/TRAIL-induced apoptosis of prostate cancer cells. Fig. 2A demonstrates that treatment with clinically relevant concentrations of paclitaxel (500 nM) or taxotere (100 nM) increases DR4 and DR5 protein levels in a time-dependent manner; maximal enhancement was observed within 12 h of exposure to the drugs (Fig. 2A). Exposure to paclitaxel (Fig. 2B) and taxotere (data not shown) also increased DR4 and DR5 levels in a dose-dependent manner. It should be noted that PC-3 cells are known to have a mutant p53 and display an absence of p53 nuclear immunostaining (23). Although wild-type p53 can induce DR5, a previous report has also suggested that DR4 or DR5 expression and its induction are not affected by a compromised p53 function (20). Fig. 2C shows the results of a representative RiboQuant Multi-Probe RNase Protection Assay demonstrating that treatment of PC-3 cells with paclitaxel did not induce the mRNA level of Apo-2L/TRAIL, DR4, DR5, Fas, or caspase-8. This suggested that the induction of DR4 and DR5 levels by paclitaxel may be posttranscriptional. Whereas exposure to paclitaxel also in-
increased DR4 and DR5 levels, in DU145 and LNCaP cells, this effect was decreased when compared with that seen in PC-3 cells (Fig. 2D). Findings shown in Fig. 2E demonstrate that whereas cotreatment with actinomycin D (500 ng/ml) had no effect, cotreatment with cycloheximide (100 μg/ml) reduced paclitaxel-mediated DR4 and, to a lesser extent, DR5 induction in PC-3 cells. However, our studies did not address the mechanism of posttranscriptional induction of DR4 or DR5 by paclitaxel.

Sequential Exposure to Paclitaxel followed by Apo-2L/TRAIL

Increases Apoptosis of Prostate Cancer Cells. To determine the functional significance of paclitaxel- and taxotere-mediated up-regulation of DR4 and DR5 levels, we compared the apoptotic effects of sequential treatment with paclitaxel followed by Apo-2L/TRAIL with the effects of the drugs administered in the reverse sequence. Exposure to 500 nM paclitaxel for 24 h followed by 100 ng/ml Apo-2L/TRAIL for 24 h induced significantly more apoptosis of PC-3 cells than the reverse sequence of exposure to the drugs or exposure to either drug alone (Fig. 3A; P < 0.01). This was also observed with the sequential exposure of PC-3 to lower concentrations of paclitaxel (100 nM) for 24 h followed by 100 ng/ml Apo-2L/TRAIL for 24 h (data not shown). As shown in Fig. 3B, exposure of DU145 and LNCaP cells to paclitaxel (100 nM) followed by Apo-2L/TRAIL (100 ng/ml) also resulted in more apoptosis than the reverse sequence of exposure to the drugs or exposure to either drug alone. Concurrent treatment of all cell types with paclitaxel plus Apo-2L/TRAIL for 24 h also induced significantly more apoptosis than the sequential exposure to Apo-2L/TRAIL followed by paclitaxel or exposure to either drug alone (Fig. 3, A and B; P < 0.01). Fig. 4 again shows that exposure to paclitaxel alone or sequential treatment with paclitaxel followed by Apo-2L/TRAIL up-regulates DR4 and DR5 expression. However, as compared with treatment with each agent alone, the sequential treatment with paclitaxel followed by Apo-2L/TRAIL resulted in greater processing of caspase-8, Bid, procaspase-9, and caspase-3 as well as greater cytosolic accumulation of cyt c. These findings suggest that pretreatment with paclitaxel enhances Apo-2L/TRAIL-induced death-inducing signaling complex, which causes greater processing of caspase-8 and Bid, resulting in the engagement of the mitochondrial pathway to apoptosis. Treatment with paclitaxel followed by Apo-2L/TRAIL was also associated with down-regulation of XIAP, cIAP1, and survivin levels, which were not significantly different from those observed with treatment with Apo-2L/TRAIL alone. Although the precise mechanism underlying this observation is not clear, a previous report had demonstrated that XIAP is processed into two fragments during Fas-mediated apoptotic signaling (24). Because survivin expression has been related to cell cycle status and shown to increase during mitosis (25), it is possible that the observed decline in the survivin levels after treatment with Apo-2L/TRAIL may be due to a concomitant decrease in the mitotically arrested cells. These results indicate that the processing and down-regulation of IAP family of proteins may be one mechanism by which Apo-2L/TRAIL overcomes barriers to apoptosis of prostate cancer cells.

The present findings, taken together with those that previously demonstrated that Apo-2L/TRAIL exerts a relatively selective in vivo anticancer activity, suggest that Apo-2L/TRAIL may be a potentially promising, novel anti-prostate cancer therapeutic agent (3, 4, 23). Apo-2L/TRAIL is also likely to be used with conventional chemotherapeutic drugs, e.g., taxanes. By demonstrating that DR4 and DR5 protein levels are up-regulated by taxanes, the present findings also highlight the optimum schedule of Apo-2L/TRAIL administration with taxanes to achieve the maximal apoptotic effect against prostate cancer cells.

REFERENCES

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