Role of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand in Interferon-Induced Apoptosis in Human Bladder Cancer Cells

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ABSTRACT

Immunomodulators such as Bacillus Calmette-Guerin and interferon are clinically active in transitional cell carcinoma of the bladder, but their mechanisms of action remain unclear. Here we investigated the effects of IFNα on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and apoptosis in a panel of 20 human bladder cancer cell lines. Six (30%) displayed significant DNA fragmentation in response to increasing concentrations of IFNα (10–100,000 units/mL). In these lines IFNα induced early activation of caspase-8, and DNA fragmentation was blocked by a caspase-8–selective inhibitor (IETDfmk), consistent with the involvement of death receptor(s) in cell death. IFNα stimulated marked increases in TRAIL mRNA and protein in the majority of IFNα-sensitive and IFNα-resistant cell lines. A blocking anti-TRAIL antibody significantly inhibited IFNα-induced DNA fragmentation in four of six IFNα-sensitive cell lines, confirming that TRAIL played a direct role in cell death. Bortezomib (PS-341, Velcade), a potent TRAIL-sensitizing agent, increased sensitivity to IFNα in two of the IFNα-resistant cell lines that produced large amounts of TRAIL. In response to IFN treatment. Our data show that IFNα-induced apoptosis in bladder cancer cells frequently involves autocrine TRAIL TRAIL production. Combination therapy strategies aimed at overcoming TRAIL resistance may be very effective in restoring IFNα sensitivity in a subset of human bladder tumors.

INTRODUCTION

The immunomodulator Bacillus Calmette-Guerin (BCG) is the current frontline therapy for superficial transitional cell carcinoma (TCC) of the bladder and produces response rates 50 to 89% in previously untreated patients with locally invasive disease (1). BCG seems to induce tumor regression by stimulating host cells to produce inflammatory cytokines, including tumor necrosis factor-α (2, 3), IFNs (4, 5), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a death receptor ligand that triggers tumor cell apoptosis after binding its surface receptors (DR4, DR5; ref. 6). Recent work indicates that IFNs also display promising activity in TCC (7) and that they can augment the effects of local BCG (8). Importantly, unlike BCG, IFNα can be delivered systemically, allowing it to be used in patients with disseminated cancer. Preclinical studies have shown that IFNs prevent the growth of orthotopic human bladder tumors in nude mice by inhibiting angiogenesis (9–11). However, the possibility that IFNs can also directly induce apoptosis in human bladder cancer cells has not been systematically addressed. We therefore undertook the present study to characterize the effects of IFNα on apoptosis within a panel of common TCC cell lines and identify the molecular mechanisms underlying cell death.

MATERIALS AND METHODS

Cell Lines and Reagents. RT4, 253J-P, and T24 were purchased from American Type Culture Collection (Manassas, VA). The 253J B-V metastatic variant was isolated from the 253J-P cells by orthotopic “recycling” as described previously (12). Cell lines in the UM-UC series were provided by Dr. Barton Grossman (Department of Urology, University of Texas M. D. Anderson Cancer Center). KU7 cells were provided by Dr. William Benedict. All cell lines are human bladder TCC with the exception of UC4, which is an adenocarcinoma, and UC5 and UC15, which are squamous cell carcinomas. Cells were grown in MEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum and 1% each of MEM vitamin solution (Life Technologies, Inc., sodium pyruvate (BioWhitaker, Walkersville, MD), l-glutamine (BioWhitaker), L-glutamine, penicillin/streptomycin solution, and nonessential amino acids (Life Technologies, Inc.) in a 5% CO2 incubator. Interferon-α-2A (Roferon, Roche Applied Science, Indianapolis, IN) was purchased from the University of Texas M. D. Anderson Cancer Center Pharmacy. Recombinant TRAIL, the antihuman TRAIL neutralizing monoclonal antibody, and the TRAIL ELISA kit were purchased from R&D Systems (Minneapolis, MN). Other antibodies were obtained from the following commercial sources: IFN regulatory factor-1 (IRF-1, Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated STAT1 (Cell Signaling, Beverly, MA), caspase-8 (BD Pharmigen, San Diego, CA), caspase-8–selective inhibitor (IETDfmk) and synthetic substrate (IETD-AFC) were obtained from Enzyme Systems Products, Inc. (Dublin, CA).

Quantification of Apoptosis by Propidium Iodide Staining and Fluorescence-Activated Cell Sorter Analysis. DNA fragmentation was measured by propidium iodide staining and fluorescence-activated cell sorter (PI/FACS) as described previously (13, 14). Cells were stored for at least 1 at 4°C in PI solution before analysis by flow cytometry. Cells that contain a subdiploid DNA content are considered apoptotic (14).

Measurement of STAT-1 Phosphorylation and IRF-1 Protein Accumulation. Cells were incubated with 10,000 units/mL IFNα for the times indicated and lysed for 4 hours at 4°C in lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 25 mmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride and a protease inhibitor mixture (Complete Mini tablet, Roche Applied Science)]. Postnuclear extracts were obtained by centrifuging the lysates for 15 minutes at 14,000 rpm (4°C). Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA.). Total lysates (20 μg of protein) were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membranes as described previously (14). Blots were probed for 16 hours at 4°C with relevant primary antibodies diluted 1:1,000 in blocking buffer and developed with species-specific secondary antibodies (sheep antimouse horseradish peroxidase, donkey antirabbit horseradish peroxidase, 1:2,000, diluted in blocking buffer, obtained from Amersham, Arlington Heights, IL) for 2 hours at 4°C. Blots were developed by enhanced chemiluminescence (Renassance, NEN, Boston, MA).

Electrophoretic Mobility Shift Assays. Cells were incubated with 10,000 units/mL IFNα in MEM containing 1% serum, harvested by trypsinization, resuspended in 0.5 mL buffer A [10 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 1.0 mmol/L EDTA and 3% glycerol], lysed by addition of 0.5 mL buffer B (buffer A containing 10% NP40) and gently layered onto a cushion of 3 mL buffer C (containing 10 mmol/L Tris (pH 7.4), 1.5 mmol/L MgCl2, 25% glycerol). Nuclei were collected by centrifugation for 5 minutes at 3,000 rpm. The pellets were washed with 1 mL cold PBS, and nuclear protein was extracted by resuspending the nuclei and rotating them in a buffer containing 20 mmol/L HEPES (pH 7.9), 400 mmol/L NaCl, 1.0 mmol/L EDTA, 1 mmol/L DTT and 1 mmol/L phenylmethylsulfonyl fluoride.
at 4°C for 30 minutes. Insoluble material was collected by centrifugation at 12,000 × g for 15 minutes at 4°C, and supernatants were snap-frozen and stored at −80°C. Protein concentrations were determined by the Bradford method.

The cis-inducible element oligonucleotide (GTGCATTGCGCCAATCT- TGTCTACA) containing a consensus binding site for Stat1 (15) was obtained from Santa Cruz Biotechnology. An aliquot of nuclear extract (10 µg of protein) in 19 µL of binding reaction mixture was incubated at room temperature for 20 minutes with 5X binding buffer (5 mmol/L MgCl₂, 2.5 mmol/L EDTA, 250 mmol/L KCl, 50 mmol/L Tris (pH 7.5), and 20% glycerol) and 1 µg of poly(dI-dC). The [γ-³²P]ATP-labeled cis-inducible element oligonucleotide (50,000 cpm) was incubated with the above binding reaction for 20 minutes at room temperature. For competition experiments, a 150-fold excess of specific unlabeled double-stranded oligonucleotide was added to the binding reaction. The identity of shifted complexes was confirmed by including an anti-Stat-1 E-23X (Santa Cruz Biotechnology) antibody (2 µg) in the reaction mixture (“supershift” analysis). Protein-DNA complexes were resolved by electrophoresis for 3.5 hours at 150V on 5% polyacrylamide gels, and protein-DNA complexes were detected by autoradiography.

Quantification of Caspase-8-Like Protease Activity. Cells were then treated with 10,000 units/mL IFNα or with or without 50 µmol/L IETDfmk for 48 hours. In control experiments, cells were treated with 50 ng/mL recombinant human TRAIL. (R&D Systems) with or without 50 µmol/L IETDfmk for 3 hours. Caspase-8 activity was measured in cytosolic extracts as described for caspase-3 previously (16). Liberated APC fluorescence was determined at 400 nm excitation and 505 nm emission on a Shimadzu 1500 spectrofluorometer (Shimadzu, Kyoto, Japan).

RNase Protection Assays. Cells were preincubated overnight in MEM medium containing 1% serum and then exposed to 10,000 units/mL IFNα for 8 hours. We isolated total RNA from cultured cells using an RNeasy kit (Qiagen, Valencia, CA), and RNase protection assay was done using a RibonQuant Multi-Probe kit and the Apo-3D probe set (BD Biosciences, San Diego, CA) according to the manufacturers’ instructions.

Quantification TRAIL Protein Expression. Cells were incubated with 10,000 units/mL IFNα for 48 hours. The Centricron filtration system (10,000 kDa cutoff, Amicon, Bedford, MA) was used to concentrate conditioned media, and cell pellets were lysed for 30 minutes in a buffer supplied by the manufacturer. The concentrated conditioned media and cellular extracts were assayed for TRAIL content by ELISA according to the manufacturer’s instructions. TRAIL standard curves were generated for each experiment and were used to calculate sample TRAIL content by linear regression analysis. Surface TRAIL expression was measured by immunofluorescence staining and flow cytometry as described previously (17).

RESULTS

Concentration-Dependent Effects of IFNα on Apoptosis. We exposed a panel of 20 human bladder cancer cell lines to increasing concentrations of recombinant IFNα (Roferon) and measured apoptosis-associated DNA fragmentation 48 hours later by PI staining and FACS analysis. Six of the cell lines displayed statistically significant (P < 0.05) increases in apoptosis (Fig. 1; Table 1). Further analyses with representative IFN-sensitive and IFN-resistant cell lines revealed that resistance was not caused by major defects in IFN receptor-
mediated signal transduction. Specifically, although the levels of IFNα-induced STAT-1 phosphorylation (Fig. 2A) and IRF-1 protein accumulation (Fig. 2B) seemed to be somewhat lower in IFN-resistant cells, IFNα stimulated comparable increases in STAT-1 DNA binding activity in all of the lines examined (Fig. 2C).

**IFN-Induced Apoptosis Is Associated with Caspase-8-Like Protease Activation.** Recent studies suggest that death receptors are involved in IFN-mediated apoptosis in other model systems (18–29). Therefore, we investigated the effects of IFNα caspase-8 activation, which is an early event associated with death receptor ligation. Consistent with the hypothesis, IFNα induced time-dependent (data not shown) caspase-8-like protease activation in both of the IFN-sensitive cell lines examined (Fig. 3A). The levels of caspase-8 activation were similar to those observed in cells treated with recombinant human TRAIL, and caspase-8 activation was blocked by the peptide caspase-8 inhibitor, IETDfmk (ref. 30; Fig. 3A and data not shown).

Immunoblotting confirmed that IFNα promoted the conversion of procaspase-8 into the processed/active form of the protease (Fig. 3B). IETDfmk also reduced the levels of IFN-induced DNA fragmentation to background in all of the cell lines tested (Fig. 3C).

**Role of TRAIL in IFN-Induced Apoptosis.** We next investigated the effects of IFNα on the expression of death receptor pathway components using multiprobe RNase protection assays. IFN increased TRAIL mRNA levels in all of the IFN-sensitive lines (Fig. 4A) and in most of the IFN-resistant lines (Fig. 4B). The TRAIL receptor, DR4, was also up-regulated by IFN in most of the cell lines (Fig. 4). Some of the cell lines also displayed increases in Fas expression (Fig. 4A and B), but these changes were less dramatic and were not observed in the majority of IFN-sensitive cells (Fig. 4A). A small subset of IFN-resistant cells (n = 3) failed to display any increase in TRAIL mRNA levels in response to IFN treatment (Fig. 4C). IFN also increased TRAIL protein production in most of the cell lines (15 of

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**Fig. 2.** IFN-induced signal transduction in representative IFN-sensitive and IFN-resistant cell lines. A, IFN-induced STAT1 phosphorylation. Phosphorylated STAT1 levels were determined by immunoblotting as described in “Materials and Methods.” Results are from one experiment that was characteristic of three. B, IFN-induced accumulation of IRF-1. IRF-1 protein levels were determined by immunoblotting as described in “Materials and Methods.” Results of one experiment typical of 3. C, IFN-induced STAT1 DNA binding. STAT1 DNA binding activity was measured by electrophoretic mobility shift assay as described in “Materials and Methods.” Lanes c, cold competition; Lanes ss, supershift with anti-STAT1 antibody. Results are representative of those obtained in three separate experiments.
20) as measured by ELISA (Table 1). Surface staining and FACS analysis confirmed that IFN increased surface TRAIL expression in IFN-sensitive (RT-4) as well as IFN-resistant (UM-UC5, UM-UC7, UM-UC11) bladder cancer cells (Fig. 4D).

We used a neutralizing anti-TRAIL antibody (25, 26, 29) to determine whether or not IFN-induced apoptosis was dependent on TRAIL production. The antibody significantly inhibited IFN-induced DNA fragmentation in four of six of the cell lines (Fig. 5 and data not shown). The antibody also consistently reduced levels of IFN-induced proteolytic processing of caspase-8, which was measured by immunoblotting as described in “Materials and Methods.” Results shown were typical of those obtained in three separate experiments. C, effects of a peptide caspase-8 inhibitor. Cells were treated with 10,000 units/mL IFN for 48 hours in the presence or absence of 50 μM IETDfmk, and DNA fragmentation was quantified by propidium iodide staining and FACS analysis as described in “Materials and Methods.” Mean ± SD, n = 3.

Effects of Exogenous TRAIL on Apoptosis. Because most of the IFN-resistant cell lines produced TRAIL in response to IFNα, we wondered whether TRAIL resistance could account for IFN resistance. To address this possibility, we incubated the 20 bladder cancer cell lines in our panel for 24 hours in the absence or presence of 50 ng/mL recombinant human TRAIL and quantified the levels of DNA fragmentation by PI/FACS. Most of the cell lines (16 of 20) displayed significant increases in DNA fragmentation, but the magnitudes of the responses were heterogeneous (Table 1).

Effects of Bortezomib on IFN-Induced Apoptosis. Some of the most impressive IFN-induced increases in TRAIL production were observed in cell lines that were resistant to TRAIL-induced apoptosis (i.e., UM-UC-7, Table 1). We therefore wondered whether an agent that is capable of enhancing TRAIL sensitivity would also sensitize cells to IFNα. The proteasome inhibitor, bortezomib, functions as an extremely potent TRAIL sensitizing agent in TRAIL-resistant bladder cancer cells (Fig. 6A). Furthermore, bortezomib synergized with IFN to promote apoptosis in cells that were completely resistant to IFN alone (Fig. 6A). The blocking anti-TRAIL antibody partially inhibited these effects and also reduced the levels of DNA fragmentation observed in the UM-UC5 cells treated with bortezomib alone (Fig. 6B), presumably because direct cytotoxic effects of bortezomib involved the TRAIL that was produced by the cells at baseline (Fig. 6D). These results strongly suggest that modulation of TRAIL sensitivity can enhance IFN-induced apoptosis.

DISCUSSION

Previous studies in human bladder xenografts have shown that IFNα is a strong inhibitor of bladder cancer growth in vitro and in vivo. The antiangiogenic effects of IFN have been linked to its ability to downregulate tumor angiogenesis by inhibiting angiogenic factor production and stimulating the expression of antiangiogenic proteins (9–11, 31). Here we investigated effects of IFN on apoptosis within a diverse panel of 20 human bladder cancer cell lines. Thirty percent of the lines displayed statistically significant, IFN-induced increases in DNA fragmentation. Analysis of the molecular mechanisms involved revealed a central role for autocrine TRAIL production in the responses observed, consistent with observations made in other model systems (25, 26, 29, 32, 33). The failure of IFNα to stimulate apoptosis in the other 14 lines was not caused by global defect(s) in IFN signal transduction or TRAIL expression. Assuming that this panel of cell lines reflects the spectrum of tumors found in patients, our results suggest that IFN-induced apoptosis will contribute to tumor growth inhibition in a subset of cases and that specific strategies to reverse baseline IFN resistance will have a major impact on its clinical activity.

Although TRAIL expression was important for IFN-mediated apoptosis, other mechanisms also contributed to cell death. The most obvious example of this was found in the UM-UC-10 cells, which were among the most IFN-sensitive lines in the panel but did not express TRAIL in response to IFN treatment (Fig. 1, Table 1). Previous studies have implicated the transcription factor IRF-1 and the protein kinase regulated by RNA (PKR) in IFN-induced apoptosis (Fig. 6A). These results strongly suggest that modulation of TRAIL expression in the UM-UC5 cells treated with bortezomib alone (Fig. 6B), presumably because direct cytotoxic effects of bortezomib involved the TRAIL that was produced by the cells at baseline (Fig. 6D). These results strongly suggest that modulation of TRAIL sensitivity can enhance IFN-induced apoptosis.
Fig. 4. Effects of IFN on death receptor pathway mRNA and surface TRAIL expression. Death receptor pathway transcripts were measured by multiprobe RNase protection assay as described in “Materials and Methods.” Results shown are representative of three separate experiments. A, IFN-sensitive cell lines. TNFR, tumor necrosis factor receptor; RIP, receptor-interacting protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, IFN-resistant cell lines that expressed TRAIL. C, IFN-resistant cell lines that did not express TRAIL. D, surface TRAIL expression was measured by immunofluorescence staining and FACS analysis as described in “Materials and Methods.” Results are representative of those obtained in three independent experiments. Blue traces, staining controls; red traces, untreated cells; green traces, cells treated with 10,000 units/mL IFNα for 24 hours. The rightward shift in the green peak indicates increased surface TRAIL expression.

Fig. 5. Effects of a blocking anti-TRAIL antibody on IFNα-induced apoptosis. Cells were incubated with 10,000 units/mL IFNα for 48 hours in the absence or presence of a blocking anti-TRAIL antibody (10 μg/mL), and DNA fragmentation was measured by propidium iodide staining and FACS analysis as described in “Materials and Methods.” Mean ± SD, n = 3. Levels of inhibition were as follows: 253J-P, 59%; RT4, 56%; UM-UC-4, 37%; UM-UC-6, 23% (not statistically significant); UM-UC-10, 0%; UM-UC-12, 48%.
expression and apoptosis in biopsies obtained from patients enrolled in ongoing studies, we are using BCG for superficial TCC (6), and IFNs are among the cytokines which patients will benefit from them. A very recent study found that active bladder cancer therapies, but it has been impossible to predict early point in the course of therapy. To identify those patients who are benefiting from systemic IFN at an early point in the course of therapy, we will be able to monitor TRAIL expression and apoptosis, which is possible by monitoring TRAIL expression and apoptosis, we will be able to identify those patients who are benefiting from systemic IFN at an early point in the course of therapy.

REFERENCES


![IFN-induced Trail expression in TCC](image-url)
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