HPCI/RNASEL Mediates Apoptosis of Prostate Cancer Cells Treated with 2',5'-Oligoadenylates, Topoisomerase I Inhibitors, and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

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ABSTRACT

The hereditary prostate cancer 1 (HPCI) allele maps to the RNASEL gene encoding a protein (RNase L) implicated in the antiviral activity of interferons. To investigate the possible role of RNase L in apoptosis of prostate cancer cells, we decreased levels of RNase L by severalfold in the DU145 human prostate cancer cell line through the stable expression of a small interfering RNA (siRNA). Control cells expressed siRNA with three mismatched nucleotides to the RNase L sequence. Cells deficient in RNase L, but not the control cells, were highly resistant to apoptosis by the RNase L activator, 2',5'-oligoadenylate (2-5A). Surprisingly, the RNase L-deficient cells were also highly resistant to apoptosis by combination treatments with a topoisomerase (Topo) I inhibitor (camptothecin, topotecan, or SN-38) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Apol2L). In contrast, cells expressing siRNA to the RNase L inhibitor RLI (HP68) showed enhanced apoptosis in response to Topo I inhibitor alone or in combination with TRAIL. An inhibitor of c-Jun NH2-terminal kinases reduced apoptosis induced by treatment with either 2-5A or the combination of camptothecin and TRAIL, thus implicating c-Jun NH2-terminal kinase in the apoptotic signaling pathway. Furthermore, prostate cancer cells were sensitive to apoptosis from the combination of 2-5A with either TRAIL or Topo I inhibitor, whereas normal prostate epithelial cells were partially resistant to apoptosis. These findings indicate that RNase L integrates and amplifies apoptotic signals generated during treatment of prostate cancer cells with 2-5A, Topo I inhibitor, and TRAIL.

INTRODUCTION

Genetic changes that lead to the discrete stages of malignant transformation provide insight into the molecular pathobiology of cancer (1). Prostate cancer, a common cancer of men in most developed countries, has a complex genetic basis that is influenced by environmental risk factors (2). Hereditary prostate cancer genes are predicted to account for about 43% of early-onset disease (age ≤55 years) and 9% of all cases of prostate cancer (3). These inherited prostate cancer susceptibility genes may function at a relatively early stage in the molecular pathogenesis of prostate cancer, during the progression of normal prostate epithelium to proliferative inflammatory atrophy (4). HPCI, the prototype prostate cancer susceptibility locus, was linked to chromosome 1q24–25 in 1996 (5) and to the RNASEL gene at 1q25 in 2002 (6). Involvement of RNASEL/HPCI in hereditary prostate cancer is supported by identification and association of different mutations (M1I, E265X, 471AAAG, and R462Q) with disease onset and/or frequency (6–10). Functional or epidemiologic data for a role of RNASEL in hereditary prostate cancer have been observed in most, but not all, studies (11, 12).

RNASEL encodes a regulated nuclease (RNase L) that functions in the interferon (IFN) antiviral response (13, 14). IFN treatment of cells induces a family of 2-5A synthetases that are stimulated by double-stranded RNA to convert ATP to PPi, and a series of short 2'-5'-linked oligoadenylates, collectively referred to as 2-5A (15). The only well-established function of 2-5A is activation of RNase L, leading to inhibition of the replication of certain viruses (16). On binding 2-5A, RNase L converts from inactive monomers to active dimers (17). RNase L activity can also be suppressed by RLI (HP68), a cytoplasmic member of the ATP-binding cassette transporter family implicated in assembly of HIV-1 Gag polyepitides into immature capsids (18, 19). The mechanism of inhibition of RNase L by RLI is not well defined; however, the ribosomal localization of RLI suggests that it could perhaps protect rRNA from cleavage by RNase L (20). Sustained activation of RNase L by 2-5A binding leads to cleavage of 28S and 18S rRNA at unique sites that are characteristic of RNase L action. Damage to rRNA by different agents, including α-sarcin, ricin, and RNase L, leads to c-Jun NH2-terminal kinase (JNK) activation (21–23). Apoptosis induced by 2-5A is defective in JNK1−/− JNK2−/− mouse embryonic fibroblasts, thus implicating JNK in the apoptotic signaling pathway initiated by RNase L activity (23). RNase L-mediated apoptosis is accompanied by cytochrome c release from mitochondria and requires caspase-3 activity (24). Previously, we demonstrated (25) that activation of RNase L by 2-5A leads to apoptosis of late-stage human prostate cancer cell lines, whereas naturally occurring mutations in RNASEL allow cell survival. However, because inactivating mutations in RNASEL are relatively rare, especially in sporadic cases (26), stimulation of RNase L activity toward apoptosis could potentially provide a unique platform for the treatment of the majority of prostate cancer cases.

Many types of cancer therapeutic agents currently in use induce apoptosis of cancer cells and thus regression of malignancies. DNA topoisomerase (Topo) I inhibitors, such as camptothecin and its derivatives, SN-38, CPT-11, and topotecan, have been widely used as therapeutic agents for a variety of solid tumors (27). These compounds bind Topo I-DNA complexes in a manner that prevents DNA religation, resulting in breaks during the replication process. Camptothecin also activates JNK and sensitizes androgen-independent prostate cancer cell lines to Fas-induced apoptosis (28, 29). Despite the efficacy of Topo I inhibitors as anticancer drugs, resistance has been a major factor in the outcome of treatments. Tumor necrosis factor-related apoptosis-inducing ligand [TRAIL (Apol2L)], a member of the tumor necrosis factor family, is an inducer of apoptosis in various cancer cell lines, whereas most normal cells are resistant to TRAIL-induced apoptosis (30, 31). TRAIL induces receptor-mediated apoptosis through a caspase-dependent signaling cascade (32, 33). Recruitment of Fas-associated death domain and caspase-8 to the receptor activates effector caspases-3 and -7. These caspases cleave cellular substrates, including poly(ADP-ribose) polymerase (PARP), Rb, p53, and lamins, which ultimately leads to cell death. TRAIL can also activate JNK in some cell lines; however, the activation is insufficient for induction of apoptosis (34). Prostate cancer cell lines PC3, DU145, and LNCaP have been shown to be resistant to TRAIL-
induced apoptosis, and resistance does not correlate with the TRAIL receptor levels (35). Several studies have demonstrated that TRAIL-resistant cell lines can be sensitized by chemotherapy agents, inhibitors of RNA and protein synthesis, and radiation therapy (36–40). In particular, combined treatment with TRAIL and Topo I inhibitor (CPT-11 or topotecan) has been shown to induce apoptosis in androgen-independent prostate cancer cell lines C4-2 and DU145 (37, 38). In this study, we investigated the role of HPCI/RNASEL in inducing apoptosis of prostate cancer cells. Furthermore, we have explored the potential of a RNase L activator-based approach for inducing apoptosis of prostate cancer cells by combining 2-5A with Topo I inhibitors or TRAIL.

MATERIALS AND METHODS

Reagents and Antibodies. Antibodies to phospho-stress-activated protein kinase (SAPK/JNK (Thr183/Tyr185), total JNK, cleaved caspase-8, and PARP were from Cell Signaling Technology (Beverly, CA). Polyclonal antibody to human cleaved caspase-3 was from Chemicon (Temecula, CA). Monoclonal antibody to human RNase L was reported previously by us (17). Polyclonal antibody to RLI/HP68 was a gift from Jaisri Lingappa (University of Washington, Seattle, WA; ref. 19). Antibody to β-actin was from Sigma (St. Louis, MO). The JNK inhibitor SP600125 was from Calbiochem (San Diego, CA). TRAIL (killer/TRAIL, soluble human, recombinant) was from Alexis Bio-MO). The peak areas of 28S and 18S rRNA were used for experiments. The cell-based RNase L assay using RNA chips (Agilent, Palo Alto, CA) was performed as described previously (25). The peak areas of 28S and 18S rRNA were used for experiments. Two independent clones were analyzed with Western blots, and two clones reduced expression, were used for additional experiments. Twelve clones each of vector and siRNLm3 cells were analyzed with Western blots, and two clones were from Cell Signaling Technology (Beverley, CA). Polyclonal antibody to phospho-stress-activated protein kinase (SAPK/JNK (Thr183/Tyr185), total JNK, cleaved caspase-8, and PARP were from Cell Signaling Technology (Beverley, CA). Polyclonal antibody to human cleaved caspase-3 was from Chemicon (Temecula, CA). Monoclonal antibody to human RNase L was reported previously by us (17). Polyclonal antibody to RLI/HP68 was a gift from Jaisri Lingappa (University of Washington, Seattle, WA; ref. 19). Antibody to β-actin was from Sigma (St. Louis, MO). The JNK inhibitor SP600125 was from Calbiochem (San Diego, CA). TRAIL (killer/TRAIL, soluble human, recombinant) was from Alexis Bio-Mo). The 2-5A is a mixture of ppp(A2p)pppA, where n = 1 to 3, was prepared enzymatically from ATP and recombinant 2-5A synthetase (a generous gift from Rune Hartmann, University of Aarhus, Aarhus, Denmark; ref. 25). The 2-5A is a mixture of ppp(A2p)pppA, in the following proportions: n = 1, 42%; n = 2, 41%; n = 3, 10%; and n = 4, 2%. Individual 2-5A oligomers were purified using a fast protein liquid chromatography monQ column. Camptothecin and all other chemicals were purchased from Sigma.

Cell Culture and Transfections. DU145 and PC3 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), 2 mmol/L glutamine, and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). Normal prostate epithelial cells (PrECs) were obtained from Clonetics (San Diego, CA) and maintained in PrEGM medium supplemented with a mixture of various growth factors (SingleQuots; Clonetics) and 10% (v/v) fetal bovine serum. DU145 small interfering RNA (siRNA) clones were grown in the presence of 1 μg/ml puromycin (Sigma). Transfection of 2-5A or plasmids was done using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol.

Generation of DU145 Small Interfering RNA Stable Cell Lines. Vector pSUPER (41) containing inserts for short hairpin RNA homologous to RNase L mRNA (5'-CGAAAGATTTGACCGTTGC-3', 109 nucleotides from the mRNA start site), a 3-bp mismatch control for RNase L (5'-CGAAGATTTGACCGTGTGC-3', the lower case letters indicate mismatch at positions 5, 9, and 14), and RL/HF68 (5'-AGAAGTTGACCTGATTGTC-3', 84 nucleotides from the mRNA start site) was cotransfected into DU145 cells using LipofectAMINE 2000 with pBabe-puro (ratio, 10:1) containing a puromycin resistance gene as a selectable marker. After puromycin (1 g per mL) selection, 42% of the clones were resistant to puromycin, and 2% of the clones were selected by growth in selective medium. Antibodies to phospho-stress-activated protein kinase (SAPK/JNK (Thr183/Tyr185), total JNK, cleaved caspase-8, and PARP were from Cell Signaling Technology (Beverley, CA). Polyclonal antibody to human cleaved caspase-3 was from Chemicon (Temecula, CA). Monoclonal antibody to human RNase L was reported previously by us (17). Polyclonal antibody to RLI/HP68 was a gift from Jaisri Lingappa (University of Washington, Seattle, WA; ref. 19). Antibody to β-actin was from Sigma (St. Louis, MO). The JNK inhibitor SP600125 was from Calbiochem (San Diego, CA). TRAIL (killer/TRAIL, soluble human, recombinant) was from Alexis Bio-MO). The peak areas of 28S and 18S rRNA were used for experiments. Two independent clones were analyzed with Western blots, and two clones reduced expression, were used for additional experiments. Twelve clones each of vector and siRNLm3 cells were analyzed with Western blots, and two clones were used for experiments.

Monitoring RNase L-Mediated Ribosomal RNA Cleavages in Intact Cells. The cell-based RNase L assay using RNA chips (Agilent, Palo Alto, CA) was performed as described previously (25). The peak areas of 28S and 18S rRNA and their main cleavage products were measured using Agilent BioSizing program Version A.02.01 S1232. Two independent clones were analyzed, and the experiments were repeated three times.

Cell Viability Assays. The viability of cells was determined using the colorimetric CellTiter 96 Aqueous Cell Proliferation Assay (3-[4,5-
Caspase-3 and -7 Assays. Caspase activity was determined using an ApoONE homogenous caspase-3 and -7 assay kit (Promega). The assay used rhodamine-labeled caspase-3 and -7 substrate, Z-DEVD-R110, which, on cleavage and excitation at 485 nm, emits light at 521 nm. Cells (2 \times 10^4 cells per well) were seeded in a 96-well plate and treated with chemotherapy agents, TRAIL, or 2-5A, either alone or in combination as indicated in the figure legends. At 20 hours after treatment, 100 \mu L of the substrate (diluted 1:100 in buffer provided) were added and incubated at room temperature for 30 minutes. Fluorescence was measured with a microtiter plate reader (model Spectra Max 340; Molecular Devices). Substrate alone was used to calculate background values, and caspase-3 activity was normalized to untreated cells. Experiments were performed in triplicate, and SDs were calculated.

RESULTS

Impact of RNase L on Prostate Cancer Cell Survival. To probe the role of RNase L in controlling apoptosis of human prostate cancer cells, we stably down-regulated either RNase L (siRNL) or its inhibitor, RLI, (siRLI), with siRNAs in DU145 cells. As a control, we generated a siRNA with 3-base mismatches and compensatory base changes in the opposite strand to maintain base pairing (siRNLm3). Two clones each with reduced RNase L and RLI expression were used for this study (Fig. 1A). RNase L and RLI levels were decreased 4- to 5-fold as estimated by normalization to \beta-actin levels (Fig. 1A). We did not observe a general IFN response in any of the siRNA-expressing cell lines by probing an IFN-stimulated gene microarray (data not shown; ref. 42).

The effect of the siRNAs on RNase L activity was determined by transfecting cells with 10 \mu mol/L 2-5A for 3 hours and measuring relative levels of specific rRNA cleavage products in RNA chips (Fig. 1B). Cells containing empty vector or expressing the control siRNA (siRNLm3) produced 18% to 19% rRNA cleavage during 2-5A transfections (Fig. 1B, Lanes 1–4). In contrast, rRNA remained largely intact (1% cleavage) in 2-5A-transfected cells expressing siRNL (Fig. 1B, Lanes 5–8). Remarkably, RNase L activity was greatly elevated (52% to 54% rRNA cleavage) in cells expressing siRLI (Fig. 1B, Lanes 9–12).

Fig. 1B. Involvement of RNase L in apoptosis caused by TRAIL and camptothecin. A. Cells were treated with 50 ng/mL TRAIL or 0.1 \mu g/mL (0.28 \mu mol/L) camptothecin or a combination of TRAIL and camptothecin at the same concentrations for 24 hours, and representative areas were photographed at \times 63 magnification with an inverted microscope. Cytotoxicity (MTS assay; B) and caspase-3/-7 activity (C) on cells treated as described in A for 3 hours; media were then replaced, and cells were incubated for an additional 20 hours. Analyses were performed in triplicate, and SDs were calculated. D. Immunoblots (100 \mu g of cell protein) were probed with antibodies for cleaved caspase-8, cleaved caspase-3, PARP, and \beta-actin. Lanes 1, 5, 9, and 13 were untreated. Lanes 2, 6, 10, and 14 were treated with 0.1 \mu g/mL (0.28 \mu mol/L) camptothecin. Lanes 3, 7, 11, and 15 were treated with 50 ng/mL TRAIL. Lanes 4, 8, 12, and 16 were treated with a combination of TRAIL and camptothecin [50 ng/mL and 0.1 \mu g/mL (0.28 \mu mol/L)]. The percentage of apoptosis was determined by DNA content analysis using propidium iodine staining and fluorescence-activated cell sorting. P values were determined using two-tailed tests.

HPC1/RNASEL IN APOPTOSIS OF PROSTATE CANCER CELLS

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Lanes 9–12). The RNase L activity did not vary significantly in two independent clones each of siRNL and siRLI cells (Fig. 1B).

To measure the effect of RNase L activity on cell viability, the DU145 cell lines were treated with 3 μmol/L 2-5A on 3 consecutive days (Fig. 1C). Treatment with 2-5A of cells containing vector or siRNLm3 reduced viability to 58% and 59% of the control level on day 1 compared with 27% viability of identically treated siRLI cells. In striking contrast, 87% of the cells with siRNL were viable on day 1. Viability of cells with vector and siRNLm3 was reduced further by day 3 to only 4%, compared with 2% in the siRLI cells and 44% in the siRNL cells, presumably because of residual low levels of RNase L.

Cells Deficient in RNase L Are Resistant to Apoptosis Induced by Camptothecin and TRAIL. Prior studies have demonstrated the sensitivity of prostate cancer cell lines to apoptosis induced by combined treatments of Topo I inhibitors and TRAIL (37, 38). To determine whether RNase L participates in the apoptotic pathway, the DU145 siRNA clones were treated with TRAIL and camptothecin. Membrane blebbing and other morphologic changes associated with apoptosis and cytotoxicity were observed when vector, siRNLm3, or siRLI cells were treated with camptothecin in combination with TRAIL (Fig. 2A and B). The single treatments were not cytotoxic, with the exception of siRLI cells treated with camptothecin alone. Remarkably, cells expressing siRNL were resistant to camptothecin plus TRAIL. These results demonstrate the involvement of RNase L in the apoptotic effects of combined TRAIL and camptothecin treatments.

To probe the apoptotic signaling pathway, we measured activation of caspase-8, -3, and -7 at 20 hours (Fig. 2C and D). There was a similar level of caspase-8 activation in response to TRAIL in all of the cell lines. However, caspase-8 activation was specifically deficient in the siRNL cells treated with both TRAIL and camptothecin (Fig. 2D, Lane 8). In the vector and siRNLm3 cells, there was a 6-fold increase in caspase-3 and -7 activity during the combined treatment as compared with single treatments (Fig. 2C). Consistent with the cell viability assays, siRLI cells showed a 5-fold increase in caspase-3 and -7 activity with camptothecin alone, which further increased to about 8-fold in the combined treatments. In contrast, cells expressing siRNL were resistant to apoptosis, showing <2-fold activation of caspase-3.

Fig. 3. Role of JNK in apoptosis induced by 2-5A, TRAIL, and camptothecin. A. Cells were treated as described in Fig. 2B and C, and cell lysates (100 μg protein) were analyzed in immunoblots for total JNK (T-JNK1/2), phospho-JNK (P-JNK1/2), and β-actin. B. Cells were pretreated with or without SP600125 at 25 μmol/L for 30 minutes and then transfected with or without 2-5A for 3 hours (as indicated). Cell viability was determined at 20 hours with the MTS reagent. Analyses were performed in triplicate, and SDs were calculated. C. Immunoblots were performed using lysates (100 μg of protein) of cells treated as described in B and probed with antibodies for PARP, total JNK (T-JNK1/2), phospho-JNK (P-JNK1/2), and β-actin. Columns (B) and lanes (C) are aligned to indicate treatment groups. D and E. Cells were pretreated with JNK inhibitor SP600125 at 25 μmol/L for 30 minutes and then treated with TRAIL and/or camptothecin as described in Fig. 2B and C. Viability (MTS assay) (D) and caspase-3/-7 activity (E) were measured at 20 hours. Analyses were performed in triplicate, and SDs were calculated.
and -7. We confirmed the activation of caspase-3 by appearance of its cleaved p17 subunit (Fig. 2D). PARP was cleaved after combined treatments in the vector, siRNLm3, and siRLI cells, but not in the siRLI cells (Fig. 2D, Lane 8). A high level of PARP cleavage was also observed in siRLI cells treated with camptothecin alone (Fig. 2D, Lane 14). By propidium iodine labeling of DNA by fluorescence-activated cell-sorting analysis for DNA content, we confirmed that reductions in cell viability were due to apoptosis (Fig. 2D). These data suggest synergistic induction of apoptosis by TRAIL and camptothecin in a pathway that is significantly influenced by RNase L.

**Activation of JNK in Response to Camptothecin and TRAIL Requires RNase L.** Previous results suggest that JNK is an essential factor in the apoptotic signaling pathway mediated by RNase L (22, 23). In addition, activation of JNK by camptothecin has been shown to sensitize DU145 cells to Fas-induced apoptosis (29). Therefore, we compared JNK activation in the DU145 siRNA cell lines treated with camptothecin and TRAIL, in combination or alone. Treatment with camptothecin, but not TRAIL, activated JNK as determined by phosphorylation of p46 and p55 JNK isoforms (Fig. 3A, compare Lanes 2 and 3). Combined treatment showed JNK phosphorylation, except in the siRLN cells. The different treatments did not affect endogenous levels of JNK (Fig. 3A, T-JNK1/2).

To determine the contribution of RNase L to JNK activation and apoptosis, cells were transfected with 2-5A for 3 hours. 2-5A caused JNK phosphorylation and cleavage of PARP in vector control, siRNLm3, and siRLI cells and reduced viability at 20 hours (Fig. 3B and C, Lanes 2, 10, and 14). SiRNL cells showed neither enhanced JNK phosphorylation nor cleavage of PARP, correlating with viability (Fig. 3B and C, Lane 6). To determine whether there was a causal relationship between JNK activation and 2-5A–induced apoptosis, cells were pretreated with the JNK inhibitor SP600125 (43). The inhibitor blocked JNK phosphorylation and suppressed PARP cleavage after 2-5A treatment, correlating with increased cell survival (Fig. 3B and C). The endogenous levels of JNK (Fig. 3C, T-JNK1/2) remained unchanged. These experiments demonstrated that JNK participates in prostate cancer cell death initiated by RNase L activation.  

**Role of JNK in Apoptosis Mediated by Camptothecin and TRAIL.** Cytotoxicity induced by camptothecin and TRAIL was suppressed by the JNK inhibitor, and in the siRNL cell line, cytotoxicity from camptothecin alone was similarly suppressed (Fig. 3D). However, cells are not fully protected by SP600125, perhaps due to JNK-independent cytotoxic mechanisms. Caspase-3 and -7 activity measured during identical treatments demonstrated that cell death was due to apoptosis and partially suppressed by the JNK inhibitor (Fig. 3E). As expected, no apoptosis was observed in the siRLN cells.

**RNase L Is Required for Apoptosis by TRAIL in Combination with Different Topo I Inhibitors.** Camptothecin and its derivatives, SN-38 and topotecan, specifically target Topo I. Cells were treated for 20 hours with varying concentrations of camptothecin, SN-38, and topotecan, each combined with 50 ng/mL TRAIL. In vector and siRNLm3 cells, all three of the Topo I inhibitors produced dose-dependent decreases in cell survival and increased caspase-3 and -7 activation (Fig. 4). SiRLI cells were robust in activation of caspase-3 and -7, and cell viability dropped steeply in response to each of the three Topo I inhibitors in combination with TRAIL. SiRNL cells were resistant to the cytotoxic effects of TRAIL when combined with the different Topo I inhibitors. These observations suggest a role for RNase L in the cell death pathway induced by Topo I inhibitors plus TRAIL.

**Normal PrECs Are Relatively Resistant to 2-5A–Induced Cell Death Yet Have Comparable RNase L Activity.** To investigate RNase L as a potential specific target in prostate cancer treatments, the effects of 2-5A were determined in normal PrECs and in prostate cancer cell lines DU145 and PC3 (both of which contain functional RNase L; ref. 25). Cells were treated with 2-5A at 3 μmol/L on 3 consecutive days, and cell viability was determined by MTS assays. 2-5A reduced the viability of PrEC, DU145, and PC3 cells to 39%, 8%, and 9% (Fig. 5A). To determine whether this difference was due to RNase L or RLI, we measured the levels of these proteins in cell lysates; however, no differences were detected (Fig. 5B). Nevertheless, to determine whether RNase L activity was equivalent in the different cell types, cleavage of rRNAs was determined after 2-5A

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**Fig. 4.** RNase L enhances (A) caspase-3 and -7 activation and (B) cytotoxicity (MTS assay) induced by Topo I inhibitors and TRAIL. Cells were treated with Topo I inhibitors (camptothecin, SN-38, or topotecan) in combination with TRAIL (50 ng/mL) for 3 hours before replacing media. Caspase-3 and -7 and MTS assays were measured at 20 hours. Analyses were performed in triplicate, and SDs were calculated.
Augmentation of 2-5A–Induced Cell Death by TRAIL or Topo I Inhibitor. Additional analyses focused on using 2-5A in combination with TRAIL, camptothecin, SN-38, or topotecan. We used sub-cytotoxic levels of 2-5A (1 μmol/L) combined with standardized doses of Topo I inhibitors or TRAIL. In DU145 and PC3 cells treated with 2-5A and TRAIL, a 2.5- to 3-fold increase in caspase-3 activation was observed compared with treatment with TRAIL alone, which was reflected in decreases in cell viability (Fig. 6A). 2-5A reduced the viability of PC3 and DU145 cells to about 65%, and this cell viability was further reduced to 48% and 46% in the presence of TRAIL plus 2-5A (Fig. 6B). Certain Topo I inhibitors in combination with 2-5A reduced the viability of DU145 and PC3 cells to a greater than extent than treatment with 2-5A alone. SN-38 was more potent in PC3 cells, and topotecan was more effective in DU145 cells. In contrast, PrECs were highly resistant to combined treatment of 2-5A and Topo I inhibitors or TRAIL, and only low levels of caspase-3 activation and marginal effects on cytotoxicity and cell viability were seen, as determined by trypan blue dye exclusion assay (Fig. 6; data not shown).

DISCUSSION

Interdependent Roles of RNase L and JNK in Mediating Apoptosis of Prostate Cancer Cells. Our findings show that RNase L-deficient prostate cancer cells are remarkably resistant to apoptosis induced by Topo I inhibitors and TRAIL. The possible involvement of RNase L in apoptosis by TRAIL and a Topo I inhibitor was investigated because of the common involvement of the mitogen-activated protein kinase JNK in both pathways. We showed previously that 2-5A treatment leads to JNK phosphorylation and that RNase L-mediated apoptosis is greatly diminished when JNK is inhibited or ablated in either mouse embryonic fibroblasts or human ovarian carcinoma cells (22, 23). On the other hand, camptothecin was previously shown to activate JNK, thus sensitizing DU145 cells to Fas-induced apoptosis (28, 29). Although the sub-cytotoxic levels of camptothecin used in this study activate JNK, camptothecin does not induce apoptosis on its own (except in cells lacking RLI), indicating that an additional signal(s) or stimulus is required. JNKs participate in apoptotic signaling through activation of preexisting Bc/II family member proteins, Bim and Bax, which mediate mitochondrial release of cytochrome c and subsequent caspase activation (44). In the present study, Topo I inhibitor plus TRAIL caused JNK activation, caspase-8 and -3 activity, PARP cleavage, and, finally, cell death. Down-regulation of RNase L with siRNA or chemical inhibition of JNK suppressed these events, whereas down-regulation of RLI with siRNA had the opposite effect, sensitizing cells to apoptosis. Indeed, decreasing levels of RLI sensitized DU145 cells to apoptosis by camptothecin alone, suggesting a possible therapeutic strategy targeting RLI. These studies further suggest that RNase L and JNK are interdependent regulators of apoptosis in prostate cancer cells treated with Topo I inhibitors and TRAIL.

Synergistic Apoptotic Activity of TRAIL in Combination with Topo I Inhibitors. TRAIL induces apoptosis in many tumor cell lines; however, DU145 cells and other prostate cancer cell lines are TRAIL resistant (37, 38). Chemotherapeutic drugs have been shown to sensitize certain TRAIL-resistant tumor cells, including prostate cancer cells, to the cytotoxic effects of TRAIL (36–39). Increases in TRAIL receptor expression, particularly TRAIL receptor 1 (DR4) and TRAIL receptor 2 (DR5), and decreases in the levels of the antiapoptotic protein survivin play a critical role in apoptosis of DU145 cells treated with TRAIL and topotecan (38). In C4-2 prostate cancer cells, induction of apoptosis by combined treatment with TRAIL and CPT-11 (a water-soluble camptothecin derivative) was shown to involve increased expression and activation of Bax and activation of caspase-8 and -3 (36). We observed that when different Topo I inhibitors or TRAIL were used alone in DU145 cells, only a weak activation of caspase-3 was detected. However, when Topo I inhibitors were used in combination with TRAIL, greater-than-additive accumulation of cleaved caspase-3 and cleaved PARP and synergistic reductions in cell viability were observed. To determine whether RNase L activity was altered, we searched for rRNA cleavage products in DU145 cells treated with camptothecin and TRAIL. Although we were unable to consistently detect such rRNA breakdown products, these assays are relatively insensitive and would not reveal RNase L action on other RNA substrates, particularly mRNAs (data not shown). On the other hand, reductions in caspase-8 activation (an initiator caspase) by TRAIL plus camptothecin in siRNL cells suggest that RNase L does not result in indiscriminate induction of apoptosis.

Camptothecin and its derivatives, CPT-11, SN-38, and topotecan, are currently used in human clinical trials for cancer therapy (45). We determined that camptothecin, SN-38, and topotecan each synergize with TRAIL in enhancing apoptosis and that RNase L is required for this effect. The susceptibility to combined treatment with TRAIL and the different Topo I inhibitors was reduced when RNase L levels were reduced; however, no significant differences were observed. The cell survival signals that allow PrEC cells to resist apoptosis by 2-5A have yet to be identified (see Discussion).
decreased and enhanced in cells depleted of RLI. Indeed, RNase L knockdown cells (siRNL cells) were uniformly resistant to all treatment combinations performed during the course of these studies.

**Therapeutic Implications of RNase L in Prostate Cancer.** The mapping of the hereditary prostate cancer 1 (HPC1) allele to the RNase L gene strongly suggests that RNase L plays a protective role in suppressing prostate cancer in most men; however, in a subset of prostate cancer cases, mutations in RNASEL/HPC1 allow cancer to progress (6). In relatively small numbers of cases, there are mutations that ablate RNase L function (e.g., stop codons). In addition, however, a homozygous missense mutation that reduces RNase L activity may be responsible for up to 13% of unselected prostate cancer cases (9). Accordingly, the majority of prostate cancer cases (perhaps >80%) could potentially benefit from treatment with a RNase L activator drug or from therapies, including Topo I inhibitors and TRAIL, that depend on the presence of RNase L. Normal PrECs are relatively resistant to 2-5A, as well as to the combined treatments with Topo I inhibitors or TRAIL, despite having similar levels of RNase L protein and activity compared with DU145 and PC3 cells. Whereas the reasons for the resistance of normal cells are unknown, the intracellular levels of pro- and antiapoptotic proteins and the combination of death receptors (in the case of TRAIL) ultimately determine the fate of cells. Regardless, the sensitivity and resistance of prostate cancer and normal cells, respectively, to 2-5A, Topo I inhibitors, and TRAIL indicate a level of selectivity for cancer cells. Our data show that RNase L is a potent apoptotic factor in the late-stage prostate cancer cell lines DU145 and PC3. Therefore, these studies suggest that RNase L is a newly recognized “druggable” target in the search for prostate cancer therapeutics.

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**REFERENCES**


HPC1/RNASEL Mediates Apoptosis of Prostate Cancer Cells Treated with 2’,5’-Oligoadenylates, Topoisomerase I Inhibitors, and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Krishnamurthy Malathi, Jayashree M. Paranjape, Ram Ganapathi, et al.


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