Expression of RASSF1A, an Epigenetically Silenced Tumor Suppressor, Overcomes Resistance to Apoptosis Induction by Interferons

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Abstract

Resistance of human renal cell carcinoma (RCC) and melanoma to the apoptosis-inducing effects of IFNs was postulated to result from epigenetic silencing of genes by DNA methylation, a common feature of human cancers. To reverse silencing, 5-AZA-deoxycytidine (5-AZA-dC) or selective depletion of DNA methyltransferase 1 (DNMT1) by phosphorothioate oligonucleotide antisense (DNMT1 AS) were employed in cells resistant (<5% terminal deoxynucleotidyl transferase–mediated nick-end labeling positive) to apoptosis induction by IFN-α2 and IFN-β (ACHN, SK-RC-45, and A375). 5-AZA-dC and DNMT1 AS similarly depleted available DNMT1 protein and, at doses that did not cause apoptosis alone, resulted in apoptotic response to IFNs. The proapoptotic tumor suppressor RASSF1A was reactivated by DNMT1 inhibitors in all three cell lines. This was associated with demethylation of its promoter region. IFNs augmented RASSF1A protein expression after reactivation by DNMT1 inhibition. In IFN-sensitive WM9 melanoma cells, expression of RASSF1A was constitutive but also augmented by IFNs. RASSF1A small interfering RNA reduced IFN-induced apoptosis in WM9 cells and in DNMT1-depleted ACHN cells. Conversely, lentiviral expression of RASSF1A but not transduction with empty virus enabled IFN-induced apoptosis. IFN-induced tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and TRAIL-neutralizing antibody inhibited apoptotic response to IFN in RASSF1A-expressing ACHN cells. Accordingly, RASSF1A markedly sensitized to recombinant TRAIL. Normal kidney epithelial cells, although expressing RASSF1A, did not undergo apoptosis in response to IFN or TRAIL but had >400-fold higher TRAIL decay receptor 1 expression than transduced ACHN cells (real-time reverse transcription-PCR). Results identified RASSF1A as regulated expression than transduced ACHN cells (real-time reverse transcription-PCR). Results identified RASSF1A as regulated

Introduction

As a result of direct effects on cell function, IFNs play a critical role in host response to virus infection and cancer through influences on viral replication and on tumor cell survival, differentiation, and motility (1–3). Activation of a signal cascade by IFNs culminates in induction of several hundred IFN-stimulated genes (ISG) that produce the cellular effects (4, 5). Resistance of cells to IFNs has been related not only to defects in specific components of the signaling cascade but also to homeostatic mechanisms that modulate receptor activation and transcriptional response (1–3). Epigenetic silencing of genes critical for effects of IFNs is a mechanism that also could influence cellular response and resistance. Although direct consequences for cell function have been little evaluated, gene profiling and expression studies indeed have identified genes involved in IFN pathways that are epigenetically silenced by hypermethylation of their 5′ regulatory regions (6–9).

Maintenance of DNA methylation in the promoter region of genes can result in heritable silencing of expression just as do mutational deletions (10). The degree of enzymatic redundancy in this process with the maintenance DNA methyltransferase 1 (DNMT1) and de novo DNMTs, such as DNMT3b (11), is still an unresolved issue. However, at least in a colon (12), a breast, and a lung cancer (13) cell line, inhibition of DNMT1 by oligonucleotide antisense or small interfering RNA (siRNA) was sufficient for reexpression of silenced genes. Epigenetic inactivation of genes that control DNA stability, cell proliferation, and apoptosis is integral to the neoplastic process (10). IFNs have increased expression of tumor suppressor genes, such as p53 (14), that are frequently affected by mutational loss of function in cancer, and other ISGs that can be silenced by DNA methylation, such as XAF1, may have tumor suppressor gene function (5, 6). Nephrectomy specimens and resections of primary renal cell carcinomas revealed a high frequency of silencing by promoter hypermethylation of the tumor suppressor gene RASSF1A in papillary (up to 100% of patients; ref. 15) and clear cell renal cell carcinoma (RCC; up to 90%; refs. 15, 16) and in melanomas (55%; ref. 17). RASSF1A plays a role in mitosis control; it can furthermore interact with the proapoptotic kinase MST1 and the scaffolding protein CNK1 to induce apoptosis and has facilitated death receptor–induced Bax conformational change and apoptosis by relieving inhibition of MAP-1 (18–24). Apoptosis has been increasingly recognized as one of the mechanisms that may play a role in antitumor effects of IFNs (25). We postulated that epigenetic silencing of RASSF1A might confer resistance to apoptosis induction by IFNs in RCC and melanoma. Thus, the effects of the well-established potent DNA demethylating agent 5-AZA-deoxycytidine (5-AZA-dC) were assessed in RCC and melanoma cell lines that were resistant to apoptosis induction by IFNs and did not express RASSF1A. The specific role of DNMT1 for gene silencing and IFN resistance was evaluated using a selective phosphorothioate antisense oligonucleotide inhibitor (DNMT1 AS) in the more readily transfectable RCC cells.

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Materials and Methods

Cell lines/IFN/tumor necrosis factor–related apoptosis-inducing ligand reagents. ACHN, A375, HeLa (American Type Culture Collection, Manassas, VA), SK-RC-45 (Memorial Sloan Kettering, NY), WM9 (Wistar Institute, PA), and normal kidney epithelial cells (isolated after review by a staff pathologist from abundant tissue nephrectomies done at the Cleveland Clinic Foundation (CCF)), OH, and used in 2nd or 3rd passage, kindly provided by Dr. J. Finke, CCF] were cultured at 37 °C in 5 % CO2 using MEM (or DMEM; Life Technologies/Invitrogen, Carlsbad, CA) with 0.1 mM/L nonessential amino acids, 1.0 mM/L pyruvate, 10% fetal bovine serum, penicillin G (50 units/mL), and streptomycin (50 µg/mL) and were regularly confirmed Mycoplasma free.

RASSF1A antisense oligonucleotide. RASSF1A siRNA sequence was as published (26). Transfections were done like DNMT1 AS transfections but only once and every 2 days into complete media not containing 5-AZA-dC. siRNA solution and diluted into media. Cells were replated 4 hours after 5-AZA-dC over 4 hours. Before and after transfections, cells were washed with PBS; transfections were performed in 35-mm dishes with 2 × 106 cells/mL using Lipofectamine (Invitrogen): denaturation at 94 °C for 4 minutes (95 °C), followed by final extension at 68 °C for 8 minutes.

Bisulfite modification and methylation-specific PCR. Genomic DNA (1 µg), harvested with a blood DNA mini kit (Qiagen, Valencia, CA), for bisulfite modification with the CpGenome kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions; 4 µl of bisulfite modified DNA was used per 25 µl methylation-specific PCR (MSP) reaction. Primers for RASSF1A MSP were as published (26). For PCR, methylated (M) primer pairs were denatured at 95 °C for 5 minutes followed by 35 cycles with a 1-minute denaturation step, 30 seconds of annealing at 60 °C, and 30 seconds of extension at 72 °C. Final extension after 35 cycles was at 72 °C for 4 minutes. For sequences specific for unmethylated (U) DNA, annealing was at 55 °C.

RASSF1A reactivation and sequencing. RT-PCR was with primers that amplified RASSF1A variants regulated by a promoter hypermethylated in cancer (19). Primers were 5’-AGGCTGCAAGGCGTGCAT-3’ (sense) and 5’-CAGGCTGCACCGTGCAT-3’ (antisense). Settings were 95 °C for 4 minutes, 55 °C for 45 seconds, 72 °C for 50 seconds for 15 to 25 cycles) and 72 °C for 4 minutes. GAPDH primers were 5’-CAGCTACCTAAGGGATCTC-3’ (sense) and 5’-GAGCCAGACCGTCTGG-3’ (antisense). For sequencing of full-length RASSF1A cDNA in DTN1 AS-treated ACHN cells, RT-PCR with primers 5’-CGGAACTTGTGGATC-3’ (sense) and 5’-CTCAATGCTTCCATTTTCT-3’ (antisense) was done using proofreading platinum Pfx polymerase (Invitrogen): denaturation at 95 °C for 4 minutes followed by 30 cycles at 95 °C for 45 seconds, annealing at 58 °C for 30 seconds, and extension at 68 °C for 3 minutes followed by final extension at 68 °C for 8 minutes. Products were cloned into Zero Blunt cloning vector and then into pcDNA4 for expansion. Sequencing of four independent bacterial clones all identified RASSF1A (NM_007182) with a single nucleotide polymorphism at nucleotide 528 (T instead of G), leading to a conservative change at amino acid position 133 (serine for alanine).

Before cloning into lentivirus, all Cpf sites 5’ of the translation start site were excluded by amplification with primers 5’-GGATCCACATGGATC-3’ (sense) and 5’-CTCTCGAGCTGATCTTTACAG-3’ (antisense) and using proofreading platinum Pfx polymerase (Invitrogen): denaturation at 94 °C for 4 minutes followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 63 °C for 30 seconds and extension at 68 °C for 2 minutes followed by final extension at 68 °C for 8 minutes).

Construction of RASSF1A lentivirus and transduction. RASSF1A cDNA from DTN1 AS–treated cells was used for overexpression. The BrunH and EcoRV fragment of the RASSF1A open reading frame was subcloned into the corresponding sites of a modified self-inactivating lentiviral vector LRV (6.9 kb), with a blastcidin resistance marker (confirmed by sequencing) and was then transfected into 293T cells along with the packaging plasmids possessing the gag-pol, rev, and vesicular stomatitis virus glycoprotein G for production and propagation of transducible Lenti-RASSF1A virus. Transductions into ACHN cells were two to three times at 20 to 30 multiplicities of infection of Lenti-RASSF1A virus (10 µg/mL polybrene); 3 days later, blastcidin (10 µg/mL) was added. Colonies were pooled, expanded, and RASSF1A verified by Western blot using RASSF1A mAb (eBioscience). Subsequently, the RASSF1A-expressing cells were selected in medium containing blastcidin, and sequence confirmation was repeated.

Results

Effects of DNMT1 inhibition on resistance of RCC and melanoma cells to IFN-induced apoptosis. ACHN and SK-RC-45 RCC and A375 melanoma cells, treated with IFN-α2 or IFN-β alone, were resistant to induction of apoptosis at doses up to 500 units/mL (~5% apoptotic cells on TUNEL; Fig. 1A-C). To determine
whether a potent DNMT1 inhibitor would influence apoptosis induction by IFNs, cells were treated with 5-AZA-dC for 2 to 6 days. After incorporation into DNA, 5-AZA-dC covalently binds DNMT1, leading to reduction of available DNMT1 protein in cells and whole-cell lysates (10, 12). Although 5-AZA-dC alone resulted in some apoptosis, apoptosis was markedly increased after the addition of IFN-α2 and even more so after IFN-β (Fig. 1A-C).

In contrast, no augmentation of apoptosis by IFN-β was observed in normal human neonatal melanocytes (NHEM) or normal kidney epithelial cells (NKE) after pretreatment with 5-AZA-dC (D). In nonmalignant cells 5-AZA-dC toxicity correlated with doubling times, which were ~4 days for NHEM, 3 days for NKE 01, and 2 days for NKE 02 (data not shown). To confirm reduction in DNMT1, cell lysates were subjected to Western blot analysis. 5-AZA-dC markedly decreased DNMT1 protein (immunoblots in Fig. 1A-D).

To determine whether DNMT1 depletion was sufficient for overcoming resistance to IFN-induced apoptosis, ACHN cells were transfected daily with 40 nmol/L DNMT1 AS. Nearly complete suppression of DNMT1 protein was achieved by day 4 in ACHN cells (Fig. 2A). In contrast, DNMT1 AS did not influence expression of STAT1, STAT2, or STAT3 (Fig. 2A). This latter result was further confirmed by RT-PCR for STAT1 (data not shown) and cRNA array (data not shown). In the absence of DNMT1, cell divisions should yield daughter cells with demethylated genes; therefore, transfections were continued for an additional 2 to 4 days before apoptosis assessment. The duration of DNMT1 depletion correlated with sensitization of ACHN cells to even low doses (50 units/mL) of IFN-α2 or IFN-β (Fig. 2A). Mismatch control oligonucleotide did not sensitize to IFN-induced programmed cell death (Fig. 2A). Apoptosis was confirmed by immunoblotting for detection of cleaved (activated) caspase-3 (Fig. 2A) and by caspase-3 activity assays (data not shown).

In A375 melanoma cells, only minimal reduction of DNMT1 protein resulted from nontoxic concentrations of DNMT1 AS (data not shown), whereas treatment with 200 nmol/L 5-AZA-dC over 4 days reduced available DNMT1 protein and increased frequency of apoptotic cells from 1.5 ± 1.1% in controls to 22.9 ± 3%

Figure 1. 5-AZA-dC effects on resistance of ACHN (A), SK-RC-45 (B), A375 (C), and normal melanocytes and kidney epithelial cells (D) to IFN-induced apoptosis. TUNEL assay: FITC-positive DNA was used to assess apoptosis of cells that had been treated with IFNs over 4 to 5 days 16 hours after plating. All three malignant cell lines were resistant to apoptosis induction by up to 500 units/mL of IFN-α2 or IFN-β (A-C). In renal carcinoma (A and B) and melanoma (C) cells, pretreatment with 200 nmol/L 5-AZA-dC (AZA), daily over 2 to 6 days before IFN treatment overcame resistance to apoptosis induction by 50 to 100 units/mL IFN-α2 or IFN-β, while causing little to moderate apoptosis alone (5-20% TUNEL+ cells). NHEM and NKE cells did not become sensitive to apoptosis induction by IFN-β (100 units/mL) after pretreatment with 200 nmol/L 5-AZA-dC (AZA) daily over 4 days, which alone caused little apoptosis (up to 10% TUNEL+ cells); D). Reduction in DNMT1 protein was confirmed in whole-cell lysates isolated after 4 days of 5-AZA-dC at 200 nmol/L and subjected to SDS-PAGE and Western blot analysis (A-D). 5-AZA-dC markedly decreased free DNMT1 protein in ACHN, SK-RC-45, A375, NHEM, and NKE cells (A-D). Columns, means of independent experiments; bars, SD.
mean F SD) after 100 units/mL IFN-α2, while alone causing little apoptosis (4.7 ± 1.85% apoptotic cells; Fig. 1C).

Because the lowest nontoxic schedule of 5-AZA-dC resulted in less IFN-induced apoptosis than the DNMT1 AS in the RCC cell lines, DNMT1 AS was mostly used for subsequent experiments with these cells, and subsequent experiments in A375 used 5-AZA-dC.

Effects of DNMT1 depletion on expression and promoter region of RASSF1A. To determine whether RASSF1A might be involved in induction of apoptosis by IFNs after DNMT1 inhibition, its expression was assessed in the IFN-resistant cell lines after both DNMT1 AS and 5-AZA-dC. Duration of DNMT1 depletion correlated with reexpression of RASSF1A mRNA, as shown in ACHN cells (Fig. 3A). DNMT1 AS (40 nmol/L over 6 days) or 5-AZA-dC (200 nmol/L over 4 days) also reactivated RASSF1A expression in SK-RC-45 cells (Fig. 3A). Although it was not possible to deplete DNMT1 by AS at doses allowing for continuing cell divisions in A375 cells 5-AZA-dC led to reactivation of RASSF1A expression (Fig. 3A). To confirm that RASSF1A expression was related to methylation status of its promoter region, MSP was undertaken. Reactivation of RASSF1A was associated with demethylation of a promoter CpG island in ACHN cells (Fig. 3B).

Figure 2. Depletion of DNMT1 by oligonucleotide antisense overcomes resistance to IFN-induced apoptosis. ACHN (A) and SK-RC-45 (B) cells were transfected daily with 40 nmol/L DNMT1 AS or mismatch oligonucleotide (MM) or Lipofectin transfection reagent only (L or Lipo). Every other day, 4 hours after the preceding transfection, cells were replated at 15,000/cm² (ACHN) or 5,000/cm² (SK-RC-45) to keep confluencies optimal for transfection efficiency. A, near-complete depletion of DNMT1 was achieved by day 4 of AS treatment and maintained to day 8 allowing for cell divisions in the absence of the maintenance DNA methyltransferase. Expression of STAT1, STAT2, and STAT3 was not affected. After 6 to 8 days, cells were plated at 5,000/cm² for IFN treatment 16 hours later. Apoptosis was assessed by immunoblotting for cleaved caspase-3 after 48 hours of IFN (following 8 days of DNMT1 AS) and by TUNEL assay to detect cells containing fragmented DNA after 5 days of IFN (following 6 and 8 days of DNMT1 AS). Duration of DNMT1 depletion correlated with apoptosis induction by 50 units/mL IFN-α2 or IFN-β as determined by TUNEL assay; while mismatch did not sensitize. B, DNMT1 AS (AS) at 40 nmol/L was sufficient for near-complete depletion of DNMT1 protein by day 4 in SK-RC-45 cells. Resistance to apoptosis induction by 500 units/mL of IFN-α2 or IFN-β over 4 days was overcome by pretreatment with DNMT1 AS over 6 days.

Columns, means of independent experiments; bars, SD.

(mean ± SD) after 100 units/mL IFN-β, while alone causing little apoptosis (4.7 ± 1.85% apoptotic cells; Fig. 1C).

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Effect of DNMT1 depletion and IFNs on RASSF1A protein expression. To determine whether reactivation of RASSF1A transcription was followed by translation into protein and whether this was influenced by IFN treatment, immunobLOTS were done. Transfection with DNMT1 AS reactivated RASSF1A protein expression, an effect that was further augmented by IFNs (Fig. 3C). Similar results occurred in A375 cells treated with 5-AZA-dC; after pretreatment with 5-AZA-dC, IFN-β induced RASSF1A protein (Fig. 3C).
Hypothesizing that RASSF1A might be involved in IFN-induced apoptosis, its expression was determined in a melanoma cell line (WM9) known to undergo programmed cell death upon treatment with IFN-β (27). In contrast to ACHN cells, RASSF1A mRNA and protein were constitutively expressed in WM9 cells (Fig. 3A and C). Addition of IFN-β to WM-9 cells further augmented RASSF1A protein expression (Fig. 3C). Thus, unless RASSF1A was silenced, IFNs could increase RASSF1A expression.

To further assess interactions between IFNs and RASSF1A, activation of MST1, an apoptotic partner molecule of RASSF1A, was also assessed. MST1 was cleaved (activated; ref. 28) after IFNs in DNMT1 AS but not control oligonucleotide (mismatch)–pretreated ACHN cells (Fig. 3C). Thus, not only did IFNs augment RASSF1A expression, but the increased protein was associated with enhanced proapoptotic protein activation.

Effect of suppression of RASSF1A by siRNA on IFN-induced apoptosis. To determine whether RASSF1A played a role in apoptosis induction by IFNs, ACHN cells depleted of DNMT1 by AS were transfected with RASSF1A siRNA followed by IFN treatment. Inhibition of RASSF1A by siRNA decreased IFN-induced apoptosis in DNMT1 AS–pretreated cells from 63.9 ± 9.19% in control siRNA treated cells to 35 ± 4.1% (mean ± SD), as determined by frequency of TUNEL-positive cells (Fig. 4A). Parallel assessment of RASSF1A protein expression by densitometry of Western blots identified 67% reduction in DNMT1 AS–treated cells and 61% reduction in DNMT1 AS– and IFN-β–treated cells by specific compared with control siRNA (Fig. 4A). Absence of IFN induction by the control siRNA was assessed by real-time RT-PCR for the ISGs XAF1, IRE1, and USP18. None of these genes were up-regulated compared with treatment with Lipofectin transfection reagent alone (0.6-, 1.1-, and 1.0-fold induction, respectively, for each gene).

To further confirm the importance of RASSF1A for IFN-induced apoptosis, WM9 cells, sensitive to apoptosis induction by IFNs alone, were treated with IFN after depletion of RASSF1A by siRNA. Similar reduction of IFN-induced apoptosis by RASSF1A siRNA occurred in WM9 melanoma cells (from 60.45 ± 3.18% in control

**Figure 3.** Effects of DNMT1 inhibition on expression of RASSF1A and its promoter region and influence of IFNs on RASSF1A protein expression. A, to reactivate RASSF1A mRNA expression, indicated cell lines were treated daily with 40 nmol/L DNMT1 AS (AS) over 4 to 8 days (ACHN) or 6 days (SK-RC-45). Mismatch control oligonucleotide (MM) or Lipofectin transfection reagent alone (L) were used as control treatments. Alternatively, RASSF1A mRNA expression was reactivated by daily 5-AZA-dC treatments at 200 nmol/L over 2 to 6 days (ACHN) or 4 days (SK-RC-45, A375). For RASSF1A RT-PCR, 500 ng RNA transcribed into cDNA was amplified over 35 cycles with RASSF1A primers; for GAPDH detection, 250 ng RNA transcribed into cDNA was amplified over 20 cycles. HeLa cells served as positive controls (19, 21, 23, 24) and WM9 cells as well as NHEM and NKE cells were found to express RASSF1A mRNA at baseline. B, ~100 ng of bisulfite modified DNA from ACHN cells was used for methylation specific PCR. Bisulfite modified DNA from HeLa cells was unmethylated control. Forty nanomoles per liter of DNMT1 AS over 8 days led to partial demethylation of RASSF1A promoter CpG island in ACHN cells. C, ACHN cells were transfected daily with 40 nmol/L DNMT1 AS or mismatch control oligonucleotide (MM) over 8 days before treatment with IFNs over 48 hours. Floating and adherent cells were harvested for RASSF1A mRNA expression, which was further augmented by IFNs. Cleavage (activation) of MST1 by IFNs occurred only in RASSF1A-expressing, DNMT1-pretreated ACHN cells. Augmentation of RASSF1A protein expression by IFN was also observed in A375 cells pretreated with 5-AZA-dC (AZA) at 200 nmol/L daily over 4 days, and without pretreatment in WM9 cells, known to be sensitive to apoptosis induction by IFNs (27). Similar results were obtained in independent experiments.
siRNA-treated cells to 39.2 ± 3.11%; Fig. 4B). RASSF1A protein was reduced by 65% at baseline and 57% in IFN-treated cells by specific control siRNA, as determined by densitometry (Fig. 4B). Thus, a limited suppression of RASSF1A partially inhibited apoptosis induced by IFN-β.

**Effect of lentiviral expression of RASSF1A on apoptosis induction by IFNs.** To further confirm the role of RASSF1A in overcoming resistance to IFN-induced apoptosis, independent of any other genes reactivated by DNMT1 depletion, forced expression of RASSF1A using a lentiviral construct (Fig. 5A) was undertaken. After transduction, the population of transduced cells was kept in selective antibiotic for 14 days before RASSF1A protein expression and sensitivity to IFN-induced apoptosis were assessed. After transduction, the population of transduced cells was more than 90% at baseline (Fig. 5A). Real-time RT-PCR showed >400-fold higher H19 siRNA expression in NKE cells expressing RASSF1A (Fig. 3B) but even after 5-AZA-dC treatment remained resistant to IFN (Fig. 3A) and Apo2L/TRAIL-induced apoptosis (Fig. 4B and 4D). Real-time RT-PCR identified >400-fold higher TRAIL decoy receptor 1 (TRAIL DcR1) expression in NKE cells expressing RASSF1A (Fig. 4B) and Fig. 5D). This suggested that RASSF1A sensitized to IFN-induced apoptosis at least in part by sensitization to Apo2L/TRAIL and that strong expression of TRAIL receptors but modestly increased them all (Table 1). This suggested that RASSF1A sensitized to IFN-induced apoptosis at least in part by sensitization to Apo2L/TRAIL and that strong expression of TRAIL receptors but modestly increased them all (Table 1). This suggested that RASSF1A sensitized to IFN-induced apoptosis at least in part by sensitization to Apo2L/TRAIL and that strong expression of TRAIL receptors.

**Discussion**

Maintenance of DNA methylation in the promoter region of genes can lead to heritable epigenetic silencing of expression with
consequences similar to mutational deletions (10). Antitumor activity of IFNs, commonly used in the treatment of metastatic RCC and melanoma, depends on induction of gene expression in cancer cells, immune cells, and cells regulating angiogenesis (1).

Hypothesizing that unsatisfactory response rates of melanoma and RCC to IFN (about 15%) are in part due to epigenetic silencing of genes, the role of a tumor suppressor gene that is frequently silenced in both malignancies (15–17) was examined.

Figure 5. Effect of lentivirus expression of RASSF1A on IFN-induced and Apo2L/TRAIL–induced apoptosis in ACHN cells. A, map of lentiviral RASSF1A construct. B, ACHN cells were transduced with RASSF1A carrying lentivirus or empty virus. Seventy-two hours later, selective antibiotic was added, and the population of clones was grown in selection media for 14 days before assessing apoptotic response to IFN-β (50 units/mL over 5 days) by TUNEL assay. After confirmation of stable expression of RASSF1A by immunoblotting, clonal selection was done to identify clones that expressed similar amounts of RASSF1A protein as achieved by DNMT1 AS (40 nmol/L) over 8 days. Treatment with DNMT1 mismatch control oligonucleotide (MM) and empty lentivirus–transduced cells served as negative controls. In clones expressing RASSF1A at levels comparable with DNA-demethylating treatment, IFN-β (50 units/mL over 5 days) resulted in 20% to 40% apoptotic cells, whereas stable transduction with empty lentivirus (kept in selective antibiotic for equal amounts of time) did not. C, concurrent treatment of ACHN RASSF1A clone 1.3 with 2 μg/mL TRAIL neutralizing antibody (TRAIL-N AB) and IFN-β (50 U/ml) over 5 days inhibited IFN-induced apoptosis compared with cotreatment with 2 μg/mL control rabbit immunoglobulin (CTRL AB). D, RASSF1A expression markedly increased sensitivity to apoptosis induction by Apo2L/TRAIL in ACHN cells. NKE cells, even after pretreatment with 5-AZA-dC (200 nmol/L) over 4 days, which alone resulted in moderate apoptosis induction, remained resistant to the apoptosis-inducing effects of Apo2L/ TRAIL. Columns, means of independent experiments; bars, SD.
Table 1. TRAIL receptor and decoy receptor expression compared with ACHN cells transduced with empty lentivirus by real-time RT-PCR

<table>
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<th>NKE 02</th>
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<td>16.09 (7.94)</td>
<td>35.67 (27.3)</td>
<td>1.03 (0.04)</td>
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NOTE: Values are the mean (SD). RNA was harvested from cells in exponential growth phase (NKE 02, ACHN RASSF1A 1.3) or after 4 days of treatment with 5-AZA-dC (200 nmol/L; NKE 02 AZA) and transcribed into cDNA followed by determination of relative expression compared to empty virus-transduced ACHN cells using real-time RT-PCR. GAPDH was used for normalization. Results show the mean and SD of three independent measurements.

*Not expressed in ACHN cells, displayed value derived from assuming a Cv value of 40 for ACHN empty lentivirus cells.

in vitro. Treatment of three cell lines (two renal and one melanoma) that were resistant to apoptosis induction by high doses of IFN-α2 and IFN-β (500 units/mL) with the DNA-demethylating nucleoside analogue 5-AZA-dC overcame resistance to IFN-induced apoptosis (Fig. 1A–C) and reactivated expression of RASSF1A (Fig. 3).

5-AZA-dC is a nucleoside analogue that after incorporation into DNA inhibits DNMT1 by covalent binding (10). DNMT1 is thus trapped and not available at the DNA replication fork to copy methylation patterns from mother to daughter strand, resulting in demethylation upon cell division. The covalent binding of the ~190-kDa DNMT1 protein to DNA, however, also results in DNA damage; thus, 5-AZA-dC may have effects in cells, independent of its DNA-demethylating activity (29, 30). Sensitization to IFN-induced apoptosis was observed at 5-AZA-dC doses that did not result in apoptosis alone (Fig. 1) associated with reactivation of RASSF1A mRNA expression (Fig. 3A). More importantly, specific inhibition of DNMT1 AS similarly reactivated RASSF1A and overcame resistance to apoptosis induction by IFNs (Figs. 2–3). Compared with mismatch control oligonucleotide, transfection reagent alone, and media alone, DNMT1 AS did not induce ISGs (Fig. 2A; data not shown), suggesting that its effect on IFN resistance was due to DNMT1 depletion (Fig. 2A) and associated DNA demethylation (Fig. 3B). These results further support that at least in RCC cells, which were more amenable to downregulation of DNMT1 by AS than studied melanoma cells, DNMT1 was critical for silencing of genes.

Containing a diacylglycerol and a rasGTP binding domain but no catalytic activity, RASSF1A has influenced function of binding partners, including the E1A-regulated transcription factor p120 (E4F; ref. 31), the proapoptotic kinase MST1 (20, 22, 32), the scaffold protein CNK1 (22), and cdc20, an activator of anaphase promoting complex (24). Interaction of RASSF1A with cdc20 regulated mitotic progression (21, 24) and apoptosis resulted when RASSF1A was coexpressed with the scaffold protein CNK1 (22). RASSF1A directed MST1 to the cell membrane, where MST1 can be activated influencing apoptosis (20, 22). Because it was activated by IFNs after RASSF1A reexpression (Fig. 3C), MST1 may be contributory to the apoptotic effects of IFNs. Interestingly, IFNs also increased protein expression of RASSF1A after DNMT1 depletion and in cells with baseline expression (Fig. 3C). The exact mechanism of RASSF1A protein regulation by IFNs will need to be studied in future experiments, but real-time RT-PCR results suggest post-transcriptional events (data not shown).

In DNMT1-depleted ACHN cells, selective suppression of RASSF1A by siRNA reduced apoptosis in response to IFN from 63.9 ± 9.19% to 35 ± 4.1% (mean ± SD; Fig. 4A). Rationalizing that cells sensitive to apoptosis induction by IFNs might express RASSF1A, WM9 cells (27) were studied. Without treatment, RASSF1A was expressed, IFN increased expression (Fig. 3A and C), and RASSF1A siRNA reduced IFN-β–induced apoptosis from 60.45 ± 3.18% to 39.2 ± 3.11%, accompanied by reduction of RASSF1A protein expression (Fig. 4B). Conversely, in ACHN cells, lentiviral expression of RASSF1A protein to levels comparable with the one achieved by DNA demethylation overcame resistance to IFN-induced apoptosis (Fig. 5).

Recent evidence has suggested requirement of RASSF1A for death receptor–induced Bax conformational change and apoptosis. RASSF1A enabled Bax apoptotic signaling by relieving an inactivating intramolecular conformation of a necessary partner molecule of Bax, BH3-like protein modulator of apoptosis-1 (MAP-1; ref. 18). Apo2L/TRAIL was induced by IFN-β (50 units/mL) in ACHN cells, 20- to 25-fold as determined by real-time RT-PCR (data not shown), and TRAIL-neutralizing antibody inhibited IFN-induced apoptosis of RASSF1A-expressing cells (Fig. 5C). Accordingly, RASSF1A markedly sensitized ACHN cells to Apo2L/TRAIL–induced cell death (Fig. 5D).

On the other hand, NKE cells did not undergo programmed cell death in response to IFN or TRAIL despite expression of RASSF1A and no synergism of 5-AZA-dC with either drug was observed (Fig. 1D, Fig. 3A, and Fig. 4D). Real-time RT-PCR before and after 4 days of 5-AZA-dC treatment revealed >400-fold higher TRAIL DcR1 expression in NKE compared with ACHN cells (Table 1). TRAIL decoy receptors bind Apo2L/TRAIL without transmission of apoptotic signals into the cell (33). Thus, RASSF1A overcame resistance to IFN-induced apoptosis at least in part by sensitization to Apo2L/TRAIL, and strong TRAIL decoy receptor expression might protect certain nonmalignant RASSF1A-expressing cells from cell death induction by IFN or Apo2L/TRAIL.

Promoter hypermethylation of ISGs, including DAPK (mainly lymphoid malignancies), XAF1 (gastric), and IRF7 (fibrosarcoma), as well as of genes essential for IFN apoptotic signaling, like TRAIL R1 and caspase-8 (lung), have been identified in cell lines and/or biopsy specimens (6–9). Although not described as frequently hypermethylated in renal cancer, reactivation of such genes could have contributed to sensitization of ACHN cells to IFN-induced apoptosis after DNMT1 depletion. However, except for one ISG of unknown function, IF27, no other ISGs or genes known to be essential for IFN apoptotic signaling (25) were increased in expression by DNMT1 AS treatment in ACHN cells, as determined by U133A Affymetrix cRNA array (data not shown). Although TRAIL R1 and TRAIL R2 were not represented on the array, quantitative RT-PCR did not identify evidence for their reactivation by DNMT1 AS (data not shown). However, among the 137 genes increased at least 2-fold in DNMT1 AS over mismatch treated cells, eight had roles in apoptosis and also may have contributed to overcoming resistance to IFN-induced programmed cell death by influencing apoptotic pathways (small GTPase ARHGDIB), inhibition of NF-κB (NFKBIA, IER3, and CSF1), or other mechanisms (PHLD1, NAC, STK17A, and ASC; data not shown).
IFN-α2 has increased survival of patients with metastatic RCC in randomized trials, albeit only for several weeks to months (1, 34). Prolongation of disease-free and possibly overall survival has resulted when IFN-α2 has been administered to melanoma patients for high-risk primary disease (1). Resistance of RCC and melanoma cells to the apoptosis-inducing effects of IFN-α2 and IFN-β was overcome by inhibition of DNMT1 (Figs. 1 and 2). This was at least in part due to reactivation of the tumor suppressor gene RASSF1A (Figs. 4 and 5) that is frequently silenced by DNA methylation in RCC and melanoma (15–17) and as shown herein up-regulated in expression by IFNs (Fig. 3C). By targeting DNMT1 in RCC and melanoma, clinical antitumor effects of IFNs may be augmented through reactivation of silenced RASSF1A and possibly by reactivation of other heterogeneously silenced genes in IFN pathways.

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References

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