Transformation-Dependent Silencing of Tumor-Selective Apoptosis-Inducing TRAIL by DNA Hypermethylation Is Antagonized by Decitabine

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Abstract

TNF-related apoptosis-inducing ligand (TRAIL) kills tumor cells selectively. We asked how emerging tumor cells escape elimination by TRAIL and how tumor-specific killing by TRAIL could then be restored. We found that TRAIL expression is consistently downregulated in HRASG12V-transformed cells in stepwise tumorigenesis models derived from four different tissues due to DNA hypermethylation of CpG clusters within the TRAIL promoter. Decitabine de-silenced TRAIL, which remained inducible by interferon, while induction of TRAIL by blocking the HRASG12V-activated mitogen-activated protein kinase pathway was subordinated to epigenetic silencing. Decitabine induced apoptosis through upregulation of endogenous TRAIL in cooperation with favorable regulation of key players acting in TRAIL-mediated apoptosis. Apoptosis induction by exogenously added TRAIL was largely increased by decitabine. In vivo treatment of xenografted human HRASG12V-transformed human epithelial kidney or syngenic mice tumors by decitabine blocked tumor growth induced TRAIL expression and apoptosis. Our results emphasize the potential of decitabine to enhance TRAIL-induced apoptosis in tumors and thus provide a rationale for combination therapies with decitabine to increase tumor-selective apoptosis. Mol Cancer Ther; 10(9); 1–13. ©2011 AACC.

Introduction

TNF-related apoptosis-inducing ligand (TRAIL) is a unique member of the TNF family, as it is the only known factor that induces apoptosis selectively in a large variety of tumor cells without affecting normal cells. Indeed, recombinant TRAIL and TRAIL-agonistic antibodies are currently evaluated in clinical trials and early results are promising (1, 2). Unlike other members of this family, human TRAIL interacts with a complex system of receptors, comprising the 2 death-inducing receptors DR4 and DR5, 2 potentially antiapoptotic decoy receptors DcR1 and DcR2, and a soluble receptor osteoprotegerin. TRAIL suppressed spontaneous tumor formation in mice expressing in several types of immune cells and is crucial for tumor surveillance (3, 4). TRAIL−/− mice exhibited increased sensitivity to chemical carcinogenesis, decreased suppression of tumor growth in syngenic models, and developed lymphoma at later age (5–7). TRAIL suppressed spontaneous tumor formation in mice that possess only one functional p53 allele (7). TRAIL action is not limited to natural killer or natural killer T cells, as it acts ubiquitously in a cell autonomous and paracrine mode (8).

The notion that the TRAIL signaling pathway induces apoptosis selectively in tumor cells is supported by a plethora of in vitro and in vivo experiments with xenografted human tumor cells (1, 2). The molecular basis accounting for the cancer selectivity of TRAIL was investigated on a wide spectrum of tumor cells. The divergent expression of certain factors in normal and tumor cells has previously been correlated with the tumor-selective action of TRAIL; examples are the TRAIL decoy receptors DcR1 and DcR2, the death receptors DR4 and DR5, MYC, FLIP or O-glycosyltransferases. However, no general mechanism accounting for this selectivity has emerged from these studies (1, 2).
The tumor-selective action of TRAIL may contribute to a number of therapeutic paradigms that exhibit some degree of tumor selectivity. For example, it has been reported that TRAIL can be induced by retinoids or histone deacetylase inhibitors (9–11). A great number of cancer therapeutics synergize with TRAIL and have been tested in combinatorial settings (2). The TRAIL signaling pathway seems to be a central weapon that can be activated by a variety of drugs and exerts its function in the tumor cell through neighboring cells and through the innate immune system.

To date, the information on the relationship between tumorigenesis and TRAIL levels in solid cancers is limited (12, 13). Considering the tumor-killing activity of TRAIL and its implication in cancer surveillance, it is reasonable to expect that tumor cells are under selective pressure to silence the TRAIL pathway. In this study, we investigated whether tumorigenesis affects the levels of endogenous TRAIL in a cell-autonomous manner by using stepwise tumorigenesis models in vitro and in vivo.

Materials and Methods

Cell lines and reagents

The cell lines used in this study were provided by William C. Hahn (Dana Faber Cancer Institute, Boston, Massachusetts). The isogenic background for each pair of immortalized and transformed cell lines was confirmed by analysis of autosomal short-tandem repeats and on a 250K-SNP array (Affymetrix). Cell lines were immortalized with SV40TAg and hTERT (HA1E, BJEL, HMLE, and SALE) and subsequently transformed by introduction of HRASG12V (HA1ER, BJELR, HMLE/PR, and SALE-HR; refs. 14–16). They are derived from normal human epithelial kidney (HEK), foreskin fibroblast (BJ), mammary epithelial (HMEC), and small airway lung epithelial (SALE) cells, respectively. Expression of SV40-ER in immortalized and expression of SV40-ER and HRASG12V in the transformed cells were regularly verified by Western blot. The cell lines were cultivated on 100 mm dishes. Immediately before use, stock solutions of decitabine (Sigma), DR4 (Chemicon), BCL2, IRF1 (Santa Cruz), goat anti-rabbit IgG (Amersham), rabbit polyclonal antibodies directed against caspase 9, cleaved caspase 3, Bax, FasL, cleaved PARP, STAT1 (all from Cell Signaling), DR5 (BD Biosciences-Pharmingen), and 38B4 were used. Antibodies used were nonspecific rabbit IgG (Santa Cruz) or the MEK1/2 inhibitor U0126 (10 mmol/L in dimethyl sulfoxide; Promega) or TRAIL, cells were trypsinized from a 105 cells were seeded. One day before treatment with decitabine or the MEK1/2 inhibitor U0126 or TRAIL, 3 × 104 cells were seeded. One day before treatment with decitabine, 1 × 105 cells were seeded. Decitabine and medium were replenished every 24 hours. For cotreatment with decitabine and U0126 or IFNγ or TRAIL, cells were treated with decitabine for 72 hours and, after renewing medium and decitabine, further incubated for 24 hours with decitabine and the respective agent. Decitabine and DR5-Fc-chimeras were coincubated for 96 hours.

RT-qPCR

Real-time quantitative PCR (RT-qPCR) was used to quantify mRNA levels. Total RNA was extracted using the GenElute Kit (Sigma). Two μg RNA were reverse transcribed with AMV Reverse Transcriptase (Roche). RT-qPCR was conducted with a 1:10 dilution of the cDNA using SYBR Green (Qiagen) on a Light Cycler 480 (Roche) with 15 minutes of denaturation (95°C) and 50 cycles of amplification (20 seconds, 95°C; 20 seconds annealing, 62°C; and 25 seconds 72°C). For calculation standard, curves were run in parallel. mRNA levels were normalized relative to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or mouse 36B4 mRNA. Primer sequences are listed in Supplementary Table S1.

ELISA

TRAIL was measured in cell lysates using the TRAIL human ELISA Kit (Biomol) according to the manufacturer’s instruction. For normalization of TRAIL protein relative to cell number, cells were trypsinized from a 105 cells were seeded in parallel for identical treatment and the cell number calculated using a hemacytometer.

Western blot

Cells were scraped and lysed in RIPA-buffer supplemented with protease inhibitor cocktail (Roche), 25 mmol/L sodium fluoride and 1 mmol/L sodium orthovanadate (Sigma). Forty μg of total protein from cell lysates were separated on 8% to 12% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 1 hour with 5% nonfat dry milk or 5% bovine serum albumin (as indicated by the manufacturer) in TBS containing 0.05% Tween 20, incubated with primary antibody overnight, washed, incubated with horse-radish peroxidase-conjugated secondary antibody (Cell Signaling) and visualized by chemiluminescence (Western blotting reagent; GE Healthcare). Rabbit polyclonal antibodies directed against caspase 9, cleaved caspase 3, cleaved PARP, STAT1 (all from Cell Signaling), DR5 (Sigma), DR4 (Chemicon), BCL2, IRF1 (Santa Cruz), goat polyclonal antibodies against TRAIL (R&D Systems) and β-actin (Santa Cruz), and mouse monoclonal antibodies against FLIP (Alexis) were used. β-Actin was used as a loading control.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was conducted according to the protocol of Upstate Biotechnology with minor modifications. The 2.5 × 106 cells were seeded per 140 mm dish. The cross-linked chromatin was sheared by sonication using a Vibra cell sonicator (Biorad Scientific Technology) set to 30% of maximum power for 20 bursts of 15 seconds with 45 seconds on ice. Extracts from 2 × 106 cells were incubated with antibody according to the manufacturer’s suggestions. Antibodies used were nonspecific rabbit IgG (Santa Cruz) as negative control, RNA-Polymerase II (Covance), IRF1.
STAT1 (both from Santa Cruz), SP1, SP3, H3K27me1, H3K27me3 (all from Upstate) and H3K4me1, H3K4me2, H3K4me3, H3K9me1, and H3 (all from Abcam). ChiP- and input-DNA were analyzed by RT-qPCR. A standard PCR protocol was conducted with an annealing temperature of 62°C for 50 cycles.

Bisulphite sequencing
DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) and bisulphite modification conducted with the EpiTect Bisulphite Kit (Qiagen). Eighty ng of the bisulphite-modified DNA was used as a template in each PCR reaction conducted with Taq-Polymerase (Roche). A standard PCR protocol was used with primer-specific annealing for 40 cycles. Primers for amplification of bisulphite-modified DNA were designed with the assistance of the Oligo3 Software and MethPrimer (17). PCR products were resolved by PAGE (12%), the specific band excised, and the PCR product purified. PCR products were cloned using the pGem-T Vector System I (Promega). Clones were sequenced on an ABIPrism3100-seequencer. Primer sequences for Bisulphite sequencing are listed in the Supplementary Table S1.

Results

Transformation by the oncogene HRASG12V induces downregulation of TRAIL
The presently available information suggested that TRAIL expression varies in normal tissue, while it is low or undetectable in tumors. This could be due to (i) selection resulting in escape from immune surveillance or (ii) a phenomenon that is linked to molecular events during the transformation process. The establishment of stepwise tumorigenesis models which recapitulate critical steps of human tumorigenesis (20) and the observation that TRAIL sensitivity was acquired in 2 of these models (21) provided an ideal experimental system to address the question if tumorigenic transformation itself may, directly or indirectly, alter TRAIL expression.

To gain evidence for such a phenomenon, stepwise tumorigenesis models derived from BJ, HEK, HMEC, and SALE (14–16) were analyzed. TRAIL mRNA (Fig. 1A) and protein (Fig. 1B and C) were significantly downregulated when immortalized and HRAS G12V-transformed cells were compared, irrespective of cell type and tissue origin. As no difference in the rate of TRAIL mRNA decrease was observed between HA1E and HA1ER (Fig. 1D), we excluded that altered mRNA stability in HA1ER accounts for downregulation of TRAIL. We concluded that HRASG12V-mediated transformation is associated with downregulation of TRAIL expression in a cell-autonomous manner.

Epigenetic modifications, not Interferon-signaling, are implicated in downregulation of TRAIL in transformed cells
The silencing of TRAIL could be due to interference with an upstream TRAIL activating pathway(s) or due to epigenetic deregulation. As the interferon pathway is known to activate TRAIL expression (22), we tested whether TRAIL is still inducible in HRAS G12V-transformed cells. Indeed, IFNγ induced TRAIL mRNA similarly in immortalized and transformed cells. Notably, the absolute levels of TRAIL mRNA induced by IFNγ in the
transformed cells remained below those of the IFNγ-treated immortalized cells (Fig. 2A). It is well established that STAT1 and IRF1 mediate the action of interferon at the TRAIL promoter and Western blot analysis revealed indeed that IFNγ induced IRF1, STAT1, and TRAIL similarly in both HA1E and HA1ER (Fig. 2B).

The putative TRAIL promoter (−1 kb to +1 kb relative to the TSS) harbors 2 interferon response elements (IRF-E, ISRE), 2 sites for SP1, and 1 AP1 site (Fig. 2C). We tested by ChIP whether the downregulation of TRAIL mRNA in the transformed cells was due to differential recruitment of transcription factors involved in interferon signaling. STAT1 binding was not detectable at the TRAIL promoter, neither without nor with IFNγ stimulation, while it was detectable on the IRF1 promoter (Supplementary Fig. S1A and B). Consistently, downregulation of STAT1 by short interfering RNA did not affect TRAIL expression in either cell line (data not shown). IRF1 was recruited to the TRAIL promoter in HA1E after treatment with IFNγ, but the same induction of IRF1 binding was observed for HA1ER (Fig. 2D). Differential RNA polymerase II recruitment to the TRAIL promoter paralleled TRAIL expression and corresponded to differences seen for the untreated cells (Fig. 2D). These results indicate that neither STAT1 nor IRF1 are causally implicated in downregulation of TRAIL mRNA in the tumorigenesis models. SP1/3 recruitment to the TRAIL promoter was reported to be crucial for histone deacetylase-mediated TRAIL induction in breast cancer cell lines (23) but not detected in HA1E or HA1ER (Supplementary Fig. S1C and D). Thus differential recruitment of SP1 and/or SP3 did not account for silencing of TRAIL upon transformation.

Silencing of TRAIL could occur at the chromatin level by histone modification. We therefore investigated whether activating or repressing histone marks were introduced during transformation. The levels of the activating methylations of histone H3 lysine 4 in the TRAIL

**Figure 1.** Oncogenic transformation by HRASG12V causes downregulation of TRAIL. A, RT-qPCR for TRAIL mRNA. B, ELISA measurement of TRAIL protein in immortalized (gray bars) and transformed (black bars) HEK, HMEC, BJ, and SALE cells, n = 3. C, Western blot for TRAIL in immortalized HA1E, HRASG12V-transformed HA1ER, and COS7 transfected with a pRK5-TRAIL expression vector. D, RT-qPCR for the kinetics of TRAIL expression in immortalized (gray bars) and transformed (black bars) HEK cells treated with actinomycin D, n = 2. In A, levels are normalized relative to the corresponding transformed cells (set to 1), and in D, relative to the untreated cells (set to 1). Data are means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
promoter (H3K4me3: regions 3, 4, and 5; H3K4me2: region 5) were reduced in the HRASG12V-transformed cells (Fig. 2D). IFNg increased H3K4me3 (regions 4 and 5) similarly in both cell lines. No changes were observed for H3K4me1, H3K9me1, H3K9me3, H3K27me1, and H3K27me3 (Supplementary Fig. S1E–J). We concluded that TRAIL silencing upon oncogenic transformation (i) is not a consequence of altered abundance/activity of IRF1 or STAT1, (ii) can be antagonized by exposure to IFNg, and (iii) occurs at the TRAIL promoter.

Figure 2. Interferon signaling is not implicated in the downregulation of TRAIL in HRASG12V-transformed cells. A, RT-qPCR for TRAIL mRNA induction after treatment with IFNg for 18 hours in immortalized (gray bars) and transformed (black bars) HEK, HMEC, BJ, and SALE cells, n = 3. TRAIL mRNA levels after treatment with IFNg are normalized relative to the corresponding untreated transformed cell line (set to 1). Data are means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, Western blot for STAT1, IRF1, and TRAIL in HA1E and HA1ER treated with IFNg. COS7 transfected with a pSG5-IRF1 expression vector served as positive control. C, illustration of the TRAIL proximal promoter region with the positions of transcription factor–binding sites relative to the TSS (+1) indicating the locations of the regions analyzed by qPCR after ChIP. D, ChIP assays on the TRAIL promoter conducted with antibodies against IRF1, RNA polymerase II, H3K4me3, and H3K4me2 for untreated HA1E and HA1ER cells (gray bars) and IFNg-treated (10 ng/mL; 18 hours) HA1E and HA1ER cells (black bars). The fold enrichment is the relative abundance of DNA fragments at the indicated regions over a transcriptional inactive control region (TSS of Myoglobin), n = 2.
DNA hypermethylation represses TRAIL expression in RAS-transformed cells

Silencing of tumor-suppressor genes by DNA methylation is frequently observed in tumors and implicated in tumor development (24, 25). The potential impact of DNA methylation on HRASG12V-dependent downregulation of TRAIL was analyzed by treating HEK, HMEC, BJ, and SALE cells with decitabine, an established DNA methylation inhibitor that is approved for treatment of myelodysplastic syndromes (26). TRAIL mRNA and protein induction was significantly stronger in tumor than in immortalized cells, resulting in TRAIL protein levels above those in the immortalized cells (Fig. 3A). TRAIL protein induction in the immortalized cells was low. Consistent with the kinetics expected for decitabine-mediated DNA demethylation TRAIL induction was not detected before 72 hours (Supplementary Fig. S2A; refs. 27, 28). Bisulfite sequencing of the TRAIL promoter and upstream region revealed hypermethylation within (i) 2 adjacent CpGs in intron 1 (region A) and (ii) the 5′ part of a CpG-island (region C) upstream of the TRAIL TSS in HAIER (Fig. 3B). Decitabine reduced DNA methylation in regions A and C (Supplementary Fig. S2B and C). No differences were observed in region B.

A common feature of RAS-transformed cells is the constitutive activation of the MAPK signaling pathway, which could contribute to TRAIL silencing. We observed repression of TRAIL in immortalized HAI1E when over-expressing the RAS effector loop mutant HRASG12V/S59 that specifically activates the MAPK pathway (Ref. 19; Fig. 3C). Although this observation suggests a link between MAPK activation and TRAIL repression, we did not observe induction of TRAIL after treatment with the MAPKK inhibitor U0126 in HAIER. The repression of TRAIL by DNA methylation inhibits derepression through blocking MAPK activity. Supporting this notion the combination of decitabine and U0126 in HAIER synergistically increased TRAIL mRNA (Fig. 3D).

We concluded that (i) DNA-hypermethylation silences TRAIL in RAS-transformed cells and (ii) that TRAIL induction by MAPKK inhibition requires prior reversal of the super-posed DNA methylation lock in RAS-transformed cells.

Synergistic apoptosis induction in tumor cells by TRAIL and decitabine

As decitabine and IFNγ induced high levels of TRAIL in HAIER, we reasoned that the combination of decitabine or IFNγ with sublethal doses of TRAIL may enhance apoptosis. Pretreatment with decitabine followed by coexposure to decitabine and TRAIL led to synergistic cell death (80%), whereas a 24-hour exposure to TRAIL alone had no effect and decitabine alone induced 40% cell death after 96 hours (Fig. 4A and Supplementary Fig. S3). The combination of TRAIL and decitabine induced the apoptosis cascade, including both initiator (CASP9) and executor (CASP3) caspase activation and resulted in enhanced PARP cleavage (Fig. 4B). Coincubation with TRAIL-neutralizing DR5-Fc–chimeras significantly reduced decitabine and decitabine plus TRAIL-induced apoptosis, revealing the major contribution of TRAIL (Fig. 4C). DR5-Fc–chimeras specifically abolished TRAIL-induced apoptosis but not other apoptosis pathways. Apoptosis induced by the DNA-damaging agent etoposide was not inhibited by blocking the TRAIL signaling pathway (Fig. 4D). We concluded that TRAIL induced by decitabine is involved in decitabine-induced apoptosis and that cotreatment with TRAIL and decitabine significantly increases apoptosis.

Decitabine induces a gene expression pattern favoring TRAIL-induced apoptosis

Treatment with IFNγ induced similar levels of TRAIL as treatment with decitabine, but in contrast to decitabine, IFNγ did not synergize with TRAIL for apoptosis induction. Thus decitabine is likely to exert further apoptosis-inducing activities which enhance the effect of its TRAIL induction. To identify activities induced by decitabine but not IFNγ, we compared their regulatory effects on key mediators of the interferon and apoptosis pathways in HAIER (Fig. 5A). Although IFNγ more significantly induced IRF1 and STAT1, decitabine specifically induced expression of several proapoptotic regulators, including DR4, DR5, BID, APAF1, and CASP8. Both compounds induced similarly strong expression of XAF1, which is likely to antagonize the action of the apoptosis inhibitors cIAP1, cIAP2, and XIAP that were induced by decitabine (29). DcR1 was induced by decitabine but did not interfere with TRAIL-induced apoptosis. Protein levels of BCL2 and FLIP, which antagonize TRAIL-induced apoptosis (30, 31), were dramatically reduced by decitabine, whereas those of TRAIL, DR4, and DR5 strongly increased (Fig. 5B). The transcriptional response pattern toward decitabine was conserved in the HRASG12V-transformed fibroblast (BJELR) and mammary epithelial (HMLE/PR) cells (Fig. 5C). The synergistic apoptogenic effect between decitabine and exogenous TRAIL was also observed in these cells (data not shown). Notably, silencing of BID abolished TRAIL-induced apoptosis via the intrinsic death pathway (Fig. 5D, right panel). However, tumor cell killing by decitabine alone is limited. Addition of exogenous TRAIL to decitabine largely increased apoptosis, thus indicating that TRAIL is a crucial limiting factor and that decitabine induces subthreshold TRAIL levels. Note that also lower concentrations of decitabine increase TRAIL and DR5 expression (Fig. 5D, left panel). We concluded that multiple actions of decitabine together with the induction of TRAIL and its cognate DR5 receptor enhance the sensitivity of tumor cells toward TRAIL and that exogenous TRAIL enhances apoptosis induction.

Anticancer action and TRAIL induction by decitabine in vivo

To assess whether decitabine induces TRAIL and exerts an anticancer activity in vivo, we established...
Figure 3. DNA hypermethylation silences TRAIL in transformed cells. A, left, RT-qPCR for induction of TRAIL mRNA by decitabine in immortalized (gray bars) and transformed (black bars) HEK, HMEC, BJ, and SALE cells, n = 3. TRAIL mRNA levels are normalized relative to the corresponding untreated transformed cell line (set to 1). Right, ELISA measurement for TRAIL protein after treatment with decitabine in immortalized (gray bars) and transformed (black bars) HEK cells, n = 2. Bottom left, structure of decitabine. B, regions of the TRAIL promoter analyzed by bisulfite sequencing. Exon1 (white/gray box), the TSS (arrow), a CpG-island 2 kb upstream (dark gray box), and the localization of amplified sequences with individual CpGs (circles) numbered in 5’ to 3’ direction are shown. Bisulfite sequencing of the exon1/intron1 TRAIL promoter region and 2 regions covering a CpG-island 2 kb upstream of the TSS with the methylation rate for each individual CpG in HA1E (gray bars) and HA1ER (black bars); n = numbers of clones analyzed. C, top, RT-qPCR for TRAIL mRNA in HA1E after retroviral infection with a HRASG12V- and a HRASG12V/S35-expressing pBabe-plasmid (black bars) and an empty control vector (gray bar), n = 2. Bottom, Western blot for TRAIL, phospho-p42/p44 MAPK, and p42/p44 MAPK. D, left: RT-qPCR for TRAIL mRNA after treatment of HA1E and HA1ER with the MAPKK inhibitor U0126 or decitabine or combination of both drugs, n = 3. Right, Western blot for TRAIL in HA1ER. Data are means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
xenografts in immunoincompetent mice with HRASG12V-transformed human HA1ER cells. Treatment of HA1ER xenografted mice with decitabine significantly reduced tumor growth. Normalized weight profiles showed the absence of overt toxicity at the given concentration of decitabine (Fig. 6A). The expression pattern for apoptosis regulators in decitabine-treated tumors, including significant induction of TRAIL, DR5, XAF1, and STAT1 (Fig. 6B), was virtually identical to the one observed in vitro (Fig. 5A). Concomitant with the induction of TRAIL intratumoral PARP cleavage revealed apoptosis induction by decitabine (Fig. 6C). Notably, decitabine induced TRAIL and DR5 in C26 mouse colon carcinoma cells and in tumors of transplanted syngenic BALB/c mice, leading to significant reduction of tumor growth without toxicity (Supplementary Fig. S4A and B).

This shows that TRAIL induction by decitabine is operative in the context of an intact murine immune system without side effects. These data suggested that treatment of tumors with decitabine for reactivation of the tumor-selective TRAIL apoptosis pathway is a valuable option.

Discussion

It is generally accepted that the development of human cancer is a multi-step process that involves 6 types of essential functional alterations in cell physiology, which...
Figure 5. Decitabine modulates the expression of apoptosis regulators to trigger execution of TRAIL-induced apoptosis. A, RT-qPCR for genes involved in (i) putative regulation of TRAIL by interferon signaling or (ii) regulation of apoptosis after treatment with decitabine or IFNγ in HA1ER, n = 3. B, Western blot for TRAIL, DR5, DR4, BCL2, FLIP, IRF1, STAT1, CASP8, and cleaved PARP after treatment with decitabine in HA1ER. C, RT-qPCR for the genes treated with decitabine in BJELR and HMLE/PR, n = 3. In A and C, the fold induction is relative to the corresponding untreated cell line (set to 1). Note logarithmic scale of y-axis. D, left, dose-dependent induction of TRAIL and DR5 mRNA after treatment with decitabine for 96 hours, n = 3. Right, top, measurement of APO2.7-positive BJELR by fluorescence-activated cell sorting after treatment with TRAIL for 72 hours 1 day after transfection with BID siRNA and the corresponding scrambled RNA. Right, bottom, Western blot for BID after transfection with BID siRNA. Data are means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
together drive malignant growth (32). Some of these features can be mimicked in a cell-autonomous manner by stepwise tumorigenesis models (20). We observed that TRAIL expression was very low in tumor cells while normal primary cells displayed higher TRAIL levels and reasoned that TRAIL is attenuated during tumorigenesis. Indeed, there is accumulating evidence for TRAIL silencing, as (i) intense immunohistochemical staining for TRAIL was reported in 35% of low-grade cervical lesions or cervical intraepithelial neoplasia (CIN) but only in 3% of high-grade CINs (33), (ii) expression of TRAIL was decreased upon colon adenoma to carcinoma progression (34), and (iii) loss of TRAIL expression accompanied oral cancer progression (35).

However, in the above cases, it is impossible to discern the cell-autonomous action of TRAIL and its contribution to immune surveillance. We found that at the transition from immortalization to HRASG12V-mediated transformation TRAIL is silenced. That the TRAIL locus can be epigenetically silenced puts it in line with other tumor suppressor genes such as CDKN2A, CDKN2B, ARHGAP, or APC (24). TRAIL is not the only gene of the TRAIL regulon that is epigenetically silenced. DNA methylation of both apoptogenic and decoy receptors of TRAIL and CASP8 has been reported for glioblastoma and small cell lung carcinomas (36–38). Moreover, gene-selective epigenetic silencing upon transformation by oncogenic Ras has been previously observed (39–41); and among these genes is PAR-4, which is required for TRAIL-induced apoptosis (42). Although the mechanistic link between oncogenic transformation and gene-selective epigenetic silencing has remained elusive, it is important to point out the pleiotropic action of decitabine.

Our data support a model in which decitabine increases the apoptogenic potential of the TRAIL signaling pathway concomitantly at several key positions to attain threshold levels, which can then be boosted with exogenous TRAIL or TRAIL sensitizers. TRAIL-induced apoptosis displays cell-to-cell variability based on its control by multivariate factors that are constituted by the protein levels of apoptosis regulators (43). Those levels differ between individual cells in a population. There is evidence that TRAIL-mediated apoptosis is crucially dependent on activation of the mitochondrial death pathway as the increase of BID levels correlated with TRAIL-mediated apoptosis induction (43). Our data confirm that BID is indispensable for TRAIL-mediated apoptosis. BID is increased and BCL2 decreased in all...
transformed cell lines after treatment with decitabine. Thus co-drugging reduces the impact of cell-to-cell variability and increases the efficiency of TRAIL-mediated killing of cancer cells thereby lowering the critical threshold for efficient killing (44). Our data show that decitabine activates the TRAIL pathway to attain this threshold.

We show a link between HRASG12V-mediated activation of the MAPK pathway and TRAIL repression. In transformed cells, this repression is subordinated to epigenetic silencing of TRAIL and Par-4. RAS activates the MAPK pathway leading to suppression of TRAIL expression. Relief of TRAIL expression by MAPKK inhibition is subordinated to the epigenetic silencing and can be seen in tumor cells only after combinatorial treatment with decitabine and U0126. Decitabine regulates several genes facilitating TRAIL-induced apoptosis. Blue, genes upregulated by decitabine; red, genes downregulated. DISC, death-inducing signaling complex.

Figure 6. (Continued) D, model of the TRAIL regulon affected by Ras transformation. Transformation by HRASG12V increases DNA methylation in regulatory modules (I, III) of the TRAIL promoter. The PAR-4 genomic loci whose gene product and its chaperone GRP78 are both required for TRAIL-induced apoptosis was reported to be silenced by hypermethylation in RAS-transformed cells. Decitabine treatment relieves the epigenetic repression of TRAIL and Par-4. RAS activates the MAPK pathway leading to suppression of TRAIL expression. Relief of TRAIL expression by MAPKK inhibition is subordinated to the epigenetic silencing and can be seen in tumor cells only after combinatorial treatment with decitabine and U0126. Decitabine regulates several genes facilitating TRAIL-induced apoptosis. Blue, genes upregulated by decitabine; red, genes downregulated. DISC, death-inducing signaling complex.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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