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Decitabine and Vorinostat Cooperate To Sensitize Colon Carcinoma Cells to Fas Ligand-Induced Apoptosis In Vitro and Tumor Suppression In Vivo

Dafeng Yang,* Christina M. Torres,* Kankana Bardhan,* Mary Zimmerman,* Tracy L. McGaha,†§ and Kebin Liu*‡

The death receptor Fas and its physiological ligand (FasL) regulate apoptosis of cancerous cells, thereby functioning as a critical component of the host cancer immunosurveillance system. To evade Fas-mediated apoptosis, cancer cells often downregulate Fas to acquire an apoptosis-resistant phenotype, which is a hallmark of metastatic human colorectal cancer. Therefore, targeting Fas resistance is of critical importance in Fas-based cancer therapy and immunotherapy. In this study, we demonstrated that epigenetic inhibitors decitabine and vorinostat cooperate to upregulate Fas expression in metastatic human colon carcinoma cells. Decitabine also upregulates BNIP3 and Bik expression, whereas vorinostat decreased Bcl-xL expression. Altered expression of Fas, BNIP3, Bik, and Bcl-xL resulted in effective sensitization of the metastatic human colon carcinoma cells to FasL-induced apoptosis. Using an experimental metastasis mouse model, we further demonstrated that decitabine and vorinostat cooperate to suppress colon carcinoma metastasis. Analysis of tumor-bearing lung tissues revealed that a large portion of tumor-infiltrating CD8+ T cells are FasL+, and decitabine and vorinostat-mediated tumor-suppression efficacy was significantly decreased in FasL−/− mice compared with wild-type mice, suggesting a critical role for FasL in decitabine and vorinostat-mediated tumor suppression in vivo. Consistent with their function in apoptosis sensitization, decitabine and vorinostat significantly increased the efficacy of CTL adoptive transfer immunotherapy in an experimental metastasis mouse model. Thus, our data suggest that combined modalities of chemotherapy to sensitize the tumor cell to Fas-mediated apoptosis and CTL immunotherapy is an effective approach for the suppression of colon cancer metastasis.


The death receptor Fas and its physiological ligand (FasL) regulate apoptosis of cancerous cells, thereby functioning as a critical component of the host cancer immunosurveillance system. To evade Fas-mediated apoptosis, cancer cells often downregulate Fas to acquire an apoptosis-resistant phenotype, which is a hallmark of metastatic human colorectal cancer. Therefore, targeting Fas resistance is of critical importance in Fas-based cancer therapy and immunotherapy. In this study, we demonstrated that epigenetic inhibitors decitabine and vorinostat cooperate to upregulate Fas expression in metastatic human colon carcinoma cells. Decitabine also upregulates BNIP3 and Bik expression, whereas vorinostat decreased Bcl-xL expression. Altered expression of Fas, BNIP3, Bik, and Bcl-xL resulted in effective sensitization of the metastatic human colon carcinoma cells to FasL-induced apoptosis. Using an experimental metastasis mouse model, we further demonstrated that decitabine and vorinostat cooperate to suppress colon carcinoma metastasis. Analysis of tumor-bearing lung tissues revealed that a large portion of tumor-infiltrating CD8+ T cells are FasL+, and decitabine and vorinostat-mediated tumor-suppression efficacy was significantly decreased in FasL−/− mice compared with wild-type mice, suggesting a critical role for FasL in decitabine and vorinostat-mediated tumor suppression in vivo. Consistent with their function in apoptosis sensitization, decitabine and vorinostat significantly increased the efficacy of CTL adoptive transfer immunotherapy in an experimental metastasis mouse model. Thus, our data suggest that combined modalities of chemotherapy to sensitize the tumor cell to Fas-mediated apoptosis and CTL immunotherapy is an effective approach for the suppression of colon cancer metastasis.

colon tissues; however, in human primary colorectal cancer, Fas expression is often diminished, and complete loss of Fas expression is often observed in metastatic human colorectal carcinoma (27, 28). Thus, resistance to Fas-mediated apoptosis is a major obstacle of Fas-based CTL immunotherapy against metastatic human colorectal cancer.

Decitabine is a cytidine analog that inhibits DNA methyltransferase activity upon incorporation into replicating DNA and is an approved agent for myelodysplastic syndrome. Decitabine was initially used at or near the maximally tolerated dose (MTD), at which it has a cytotoxic effect, to treat solid tumors, but was found to be associated with severe toxicity and minimal efficacy (29). It was later observed that decitabine, at a dose well below its MTD, is effective in the inhibition of DNA methylation and achieves long-term tolerance and improved clinical efficacy in patients with myelodysplastic syndrome and solid tumors (30, 31). Vorinostat is a histone deacetylase (HDAC) inhibitor and is an approved agent for the treatment of cutaneous T cell lymphoma. Vorinostat, at or near its MTD, is also associated with severe toxicity and exhibits minimal efficacy in solid tumors when used as a single agent (32). Although the expression of many genes is modulated by vorinostat, vorinostat alone is often ineffective in the induction of expression of hypermethylated genes (33). Therefore, vorinostat and decitabine are often combined to achieve maximal activation efficacy of epigenetically silenced genes in cancer cells (31, 33–35).

Pioneer studies demonstrated that decitabine and vorinostat can overcome apoptosis resistance in various types of cancers (35–37). These epigenetic inhibitors were shown to either reactivate the expression of death receptor Fas in tumor cells (36–38) or target the Fas-mediated apoptosis-signaling pathways to induce tumor cell apoptosis (33–35, 39, 40). Based on these observations, we hypothesized that epigenetic mechanism-based chemotherapy may be combined with CTL immunotherapy to overcome tumor cell Fas resistance to increase the efficacy of CTL immunotherapy. This idea is analogous to a “one-two punch” strategy. First, cancer cells are treated with apoptosis-sensitizing drugs to activate Fas and/or sensitize tumor cells to Fas-mediated apoptosis. Once “sensitized,” tumors are treated with Fasl,” tumor-specific CTLs that promote Fas-mediated apoptosis to destroy the tumors. To test this hypothesis, we performed this proof-of-concept study and identified that epigenetic inhibitors decitabine and vorinostat regulate the expression of Fas, BNIPI3, Bik, and Bcl-xL, to cooperatively sensitize the metastatic human colon carcinoma cells to FasL-induced apoptosis. Furthermore, we demonstrated that decitabine and vorinostat-mediated tumor suppression depends, at least in part, on Fasl in vivo. Overall, our results indicate that combined Fas-based chemotherapy and Fasl-dependent CTL immunotherapy is effective in suppressing colon carcinoma metastasis, and it holds great promise for further development for the treatment of metastatic human colorectal cancer.

Materials and Methods

Mice

Fasl (Cpt.C3-FastgldfJ), BALB/cByJ mice were obtained from The Jackson Laboratory. BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). All mice were used at age ≥6 wk. Experiments and care/welfare were in agreement with federal regulations and an approved protocol by the Georgia Health Sciences University Animal Care and Use Committee.

Reagents

Decitabine was obtained from Sigma (St. Louis, MO). Vorinostat (Merck) was provided by the Cancer Treatment and Evaluation Program, National Cancer Institute/National Institutes of Health. Fasl (Mega-Fas Ligand, kindly provided by Drs. Steven Butcher and Lars Damstrup, Topotarget, Copenhagen, Denmark) is a recombinant fusion protein that consists of three human Fasl extracellular domains linked to a protein backbone comprising the dimer-forming collagen domain of human adipoctone. The Mega-Fas Ligand was produced as a glycoprotein in mammalian cells using a Good Manufacturing Practice-compliant process at Topotarget.

RT-PCR analysis

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen, San Diego, CA) and used for real-time RT-PCR analysis of gene expression, as described (41, 42). The PCR primer sequences are as follows: mouse Fas: forward: 5′-ATTATGCTGCAAAAAGTAT-3′, reverse: 5′-TACATGGTCCTCTCAGGTCTGAC7-3′; mouse Fasl: forward: 5′-CTCTGGCTCCTCCAGGGTCGT-3′, reverse: 5′-TCTCCCTCATAGCACCAGAC-3′; and β-actin: forward: 5′-ATGGTAAACACGTGGAGCAGCATG-3′, reverse: 5′-CTTCCATGAGTAGTCTGATGTC-3′.

Cell treatments

For treatment with decitabine, cells were cultured in its presence for 3 d. For vorinostat treatment, cells were cultured in its presence for 2 d.

Sodium bisulfite treatment and DNA-methylation analysis

Genomic DNA was isolated using a DNeasy Tissue Kit (Qiagen). Sodium bisulfite treatment of genomic DNA was carried out using a CpGenome Universal DNA Modification Kit (Chemicon, Temecula, CA). Methylation-sensitive (MS)-PCR was carried out as previously described (43). The primer sequences are as follows: BNIPI3: unmethylation: forward: 5′-TGGTITTGTTTTAAGGGAGGAGATT-3′, reverse: 5′-CAAAAAACAAAAAACCCTACAATACAC-3′, methylation: forward: 5′-TTACAGTGGTTTTTTAAAGGAGAGATT-3′, reverse: 5′-GAAAAAACAAAAAACCCTACATGACG-3′, and Bik: unmethylation: forward: 5′-GTAAGGAGGATGAGATGGATTGG-3′, reverse: 5′-GTAACAGCGACGTGGATAGATGTCGG-3′, reverse: 5′-TAAAACAAAAACCCCAACCGACG-3′. The bisulfite-modified genomic DNA was also used as an template for PCR amplification of the modified genomic DNA fragments of the human Fas promoter regions. The PCR primers are as follows: region 1: forward: 5′-GGATAGGAATGGTTTATTGTTGTA-3′, reverse: 5′-CTAAAACACTCAACACCTCTCCCC-3′, region 2: forward: 5′-GGAGGATTGTTTTAATATTTGTGGG-3′, reverse: 5′-CTTAAAAACTCACAACACTCTCCTCCCC-3′. The PCR fragments were cloned to pCR2.1 vector (Invitrogen), and plasmid was purified from individual clones and sequenced.

Western blotting analysis

Western blotting analysis was performed as previously described (44). The following primary Abs were obtained from Cell Signaling Biotech (Danvers, MA): Bak, Bik, Bid, FLIP, CHAP1, XIAP, Bad, Bok, and PUMA. The following primary Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): Bax, survivin, McI-1, and BNIPI3. The following Abs were obtained from BD Biosciences: Bcl-2 and Bcl-xL. Anti–β-actin was obtained from Sigma (St. Louis, MO).

Apoptosis assays

Cells were either stained with propidium iodide (PI) (Trevigen, Gaithersburg, MD) or PI plus Annexin V-Alexa Fluor 647 (BioLegend, San Diego, CA) and analyzed by flow cytometry.

Cell surface protein analysis

Tumor cells were stained with anti-Fas (BD Biosciences) mAb. Isootype-matched control IgG (BD Biosciences) was used as a negative control. The stained cells were analyzed by flow cytometry. For Fasl protein analysis, mouse lungs were digested in collagenase solution to create a single-cell suspension. The cell suspension was stained with PE-conjugated Fasl (BD Biosciences), FITC-conjugated CD8 mAb (BioLegend), and both mAbs and analyzed by flow cytometry.

Gene silencing

Tumor cells were transiently transfected with scramble small interfering RNA (sRNA; Dharmacon, Lafayette, CO) and human Bcl-xL–specific siRNA (Santa Cruz Biotechnology), respectively. Cells were then harvested and cultured in 24-well plates overnight in the absence or presence of Fasl, before analysis for apoptosis.
Tumor cell transfection

Tumor cells were transiently transfected with the pEGFP control vector and pEGFP-hBik plasmid, respectively. Cells were then harvested and cultured overnight in 24-well plates in the absence or presence of FasL before analysis for apoptosis.

Liver-toxicity analysis

Decitabine (0.1 mg/kg body weight) and vorinostat (25 mg/kg body weight) were injected i.v. into BALB/c mice either alone or in combination. Serum was collected from mice 3 d later and measured for complete liver profile (Table I) at Georgia Laboratory Animal Diagnostic Service (Athens, GA).

Experimental lung metastasis mouse model and CTL immunotherapy

Tumor-specific CTLs were generated from perforin-deficient BALB/c mice, as previously described (45). The experimental lung metastasis mouse model and CTL adoptive-transfer immunotherapy were carried out as previously described (45). Decitabine was used at a dose of

FIGURE 1. Decitabine and vorinostat cooperate to upregulate Fas expression and sensitize the metastatic human colon carcinoma cells to FasL-induced apoptosis. LS411N cells were treated with decitabine for 3 d (A) or vorinostat for 2 d (B) at the indicated doses, stained with Fas-specific mAb, and analyzed by flow cytometry for cell surface Fas protein level. Gray area: IgG isotype-control staining; solid line: Fas-specific staining. Lower panels, The Fas protein level was quantified as mean fluorescence intensity (MFI). Column: mean; bar: SD. (C) LS411N cells were treated with decitabine (0.5 μM, 3 d), vorinostat (0.5 μM, 2 d), or both decitabine and vorinostat and analyzed for Fas protein level by flow cytometry (left and middle panels). The Fas protein level is quantified as MFI (middle panel). Column: mean; bar: SD. Fas mRNA level was measured by real-time RT-PCR (right panel). The Fas mRNA level in control cells was arbitrarily set at 1. (D) Tumor cells were treated as in (C), followed by incubation with FasL (200 ng/ml) overnight, staining with PI, and analysis by flow cytometry (left panels). The percentage of FasL-induced cell death was quantified as percentage of PI+ cells in the presence of FasL – percentage of PI+ cells in the absence of FasL (right panel). Column: mean; bar: SD.
0.1 mg/kg body weight, and vorinostat was used at a dose of 20 mg/kg body weight.

**Statistical analysis**

Where indicated, data are represented as the mean ± SD. Statistical analysis was performed using a two-sided t test, with p values < 0.05 considered statistically significant.

**Results**

Decitabine and vorinostat cooperate to upregulate Fas expression and sensitize metastatic human colon carcinoma cells to Fas-L-induced apoptosis

It was shown that vorinostat activates Fas gene expression in tumor cells (36, 37, 46), whereas Fas promoter DNA methylation has been observed in certain colon carcinoma cells (47). Based on these observations, we reasoned that inhibition of DNA methylation and HDAC activity may upregulate Fas expression in metastatic human colon carcinoma cells. To test this notion, the metastatic human colon carcinoma cell line LS411N was treated with decitabine and vorinostat and analyzed for Fas expression. Both decitabine and vorinostat increased Fas protein level on the tumor cell surface in a dose-dependent manner, and the increase reached a plateau at a dose of ∼0.75 μM (Fig. 1A, 1B). Interestingly, combined decitabine and vorinostat treatment resulted in a significantly higher level of Fas protein than did either agent alone (Fig. 1C). Decitabine and vorinostat also increased the Fas mRNA level, but combined decitabine and vorinostat did not further increase the Fas mRNA level compared with either agent alone (Fig. 1C).

Metastatic human colon carcinoma LS411N cells are highly resistant to Fas-L-induced apoptosis. To determine whether the increased Fas expression leads to increased sensitivity of the tumor cells to Fas-L-induced apoptosis, LS411N cells were treated with decitabine and vorinostat, either alone or in combination, and then incubated with Fas-L protein. Analysis of cell death revealed that decitabine or vorinostat treatment alone increased the tumor cell sensitivity to Fas-L-induced apoptosis (Fig. 1D). However, consistent with the Fas protein level, combined treatment with the two agents rendered the metastatic human colon carcinoma cells more sensitive to Fas-L-induced apoptosis than did either treatment alone (Fig. 1D). Taken together, our data suggest that inhibition of both DNA methylation and HDAC activity is an effective approach to overcome apoptosis resistance in metastatic human colon carcinoma cells.

**Fas promoter DNA is sporadically methylated in metastatic human colon carcinoma cells**

To determine whether decitabine upregulates Fas expression through inhibition of the Fas promoter DNA methylation, we analyzed the Fas promoter DNA methylation status in the metastatic human colon carcinoma cell lines LS411N and SW620. Analysis of the human Fas gene revealed that the human Fas gene promoter contains multiple classical CpG islands surrounding the transcription-initiation site (Fig. 2A). However, analysis of the genomic DNA sequence in two regions of the Fas promoter indicated that the Fas promoter is not methylated in LS411N cells (Fig. 2B). In SW620 cells, we observed that only 1–3 cytosines of the 34 CpGs analyzed are methylated (Fig. 2B). Therefore, we conclude that Fas upregulation by decitabine is unlikely to occur through inhibition of Fas promoter DNA methylation.

**FIGURE 2.** The Fas promoter DNA methylation status in metastatic human colon carcinoma cells. *Upper panel,* The human Fas gene promoter, showing CpG islands (gray areas). The vertical bars under the line indicate the locations of CpG dinucleotides. The locations of the CpG islands relative to the Fas transcription initiation site (+1) are also indicated under the line. *Lower panel,* Methylation status of the human Fas gene promoter in LS411N and SW620 cells. The indicated regions of bisulfite-modified genomic DNA isolated from LS411N and SW620 cells were cloned and sequenced. ○, Unmethylated CpG; ●, methylated CpG.

**FIGURE 3.** Decitabine and vorinostat alter the level of apoptosis-related proteins. (A) Western blotting analysis of both pro- and antiapoptosis proteins. LS411N cells were treated with decitabine, vorinostat, or both agents as in Fig. 1 and analyzed for the indicated proteins. Methylation status of BNIP3 (B) and Bik (C) promoters in colon carcinoma cells. BNIP3 (B) and Bik (C) promoter structure (*left panels*). The CpG islands are indicated by the gray-filled areas. Methylation status of BNIP3 (B) and Bik (C) promoter DNA (*right panels*). Bisulfite-modified genomic DNA was analyzed by MS-PCR to detect DNA methylation. M, Methylated; U, unmethylated.
Decitabine and vorinostat regulate the expression of BNIP3, Bik, and Bcl-xL

Sensitivity to Fas-mediated apoptosis is mediated at both the death receptor Fas level and within its downstream signaling pathway (48–50). Therefore, we next analyzed the key mediators of the Fas-signaling pathway in decitabine and vorinostat-treated metastatic human colon carcinoma cells. Western blotting analysis revealed that protein levels of BNIP3 and Bik were increased

FIGURE 4. Decitabine and vorinostat sensitize colon carcinoma cells to FasL-induced apoptosis, in part through altering Bcl-xL and Bik expression. (A) Silencing Bcl-xL increased tumor cell sensitivity to FasL-induced apoptosis. LS411N cells were transfected with scramble and Bcl-xL-specific siRNAs for ~24 h. Transfected cells were then incubated with FasL (200 ng/ml) overnight, stained with Annexin V and PI, and analyzed for apoptosis by flow cytometry. Number in each plot indicates the percentage of PI+ and Annexin V+ cells. (B) Apoptotic cell death was quantified as percentage of PI and Annexin V double-positive cells in the presence of FasL. Column: mean; bar: SD. (C) Overexpressing Bik increased tumor cell sensitivity to FasL-induced apoptosis. LS411N cells were transiently transfected with vector (pEGFP) or Bik-expressing vector (pEGFP.hBik) and analyzed for sensitivity to FasL-induced apoptosis as in (A). (D) Apoptotic cell death was quantified as in (B).
following decitabine treatment, whereas Bcl-xL protein was decreased by vorinostat treatment (Fig. 3A). Thus, inhibition of DNA methylation and HDAC activity altered the expression levels of multiple apoptosis-related mediators. Analysis of the BNIP3 and Bik promoters revealed that there are classical CpG islands in these two promoter regions around the transcription-initiation sites (Fig. 3B, 3C). MS-PCR analysis indicated that both BNIP3 and Bik promoter DNA was hypermethylated in the metastatic human colon carcinoma cell lines LS411N and SW620 (Fig. 3B, 3C). Thus, our data demonstrated that the pro-apoptotic BNIP3 and Bik gene promoters are silenced by DNA methylation in metastatic human colon carcinoma cells and that decitabine inhibits DNA methylation to reactivate BNIP3 and Bik.

**BNIP3 and Bik, Bcl-xL mediate apoptosis resistance in metastatic human colon carcinoma cells**

The above observations that decitabine and vorinostat alter BNIP3, Bik, and Bcl-xL protein levels suggest that these three proteins might play critical roles in the regulation of apoptosis in metastatic human colon carcinoma cells. The function of BNIP3 in regulating apoptosis in metastatic human colon carcinoma cells was demonstrated recently (51). To determine whether Bik and Bcl-xL function in the apoptosis of colon carcinoma cells, we silenced Bcl-xL in LS411N cells and analyzed the tumor cell sensitivity to Fas-mediated apoptosis. Analysis of apoptotic cell death indicated that silencing Bcl-xL significantly increased tumor cell sensitivity to FasL-induced apoptosis (Fig. 4A, 4B). We also overexpressed Bik in LS411N cells and observed that restoration of Bik expression significantly increased LS411N cell sensitivity to FasL-induced apoptosis (Fig. 4C, 4D). Therefore, BNIP3, Bik, and Bcl-xL play critical roles in apoptosis in metastatic human colon carcinoma cells.

**Decitabine and vorinostat cooperate to suppress colon carcinoma development in vivo**

To determine whether the above observations can be extended to in vivo colon carcinoma suppression, we used the colon carcinoma CT26 experimental lung metastasis mouse model. We first analyzed the CT26 responses to decitabine and vorinostat and observed that, like metastatic human colon carcinoma cells, CT26 cells responded to decitabine and vorinostat to upregulate Fas and became sensitive to FasL-induced apoptosis (Supplemental Fig. 1). Next, CT26 cells were transplanted to syngeneic BALB/c mice. These tumor-bearing mice were then treated with decitabine and vorinostat, either alone or in combination, and examined for lung metastasis. Both decitabine and vorinostat exhibited tumor-suppression effects individually. However, a much greater tumor-suppression effect was observed when decitabine and vorinostat were used in combination (Fig. 5A). Fas-mediated apoptosis is initiated by FasL binding to the Fas receptor. To identify the source of FasL, we first extracted total RNA from lungs derived from tumor-free control mice or tumor-bearing mice and analyzed FasL mRNA levels. Real-time RT-PCR analysis indicated that both tumor-free and tumor-bearing lung cells express FasL, and tumor-free lung tissues express higher levels of FasL than do the tumor-bearing lung tissues (Fig. 5B). Next, we sought to determine which types of cells in the lung express FasL. Lungs from tumor-bearing mice were digested with collagenase to make single-cell suspensions and were analyzed for FasL protein levels on the cell surfaces of CD8+ T cells and non-CD8+ cells in the lung. CD8+ T cells accounted for ~8% of the total lung cells (Fig. 5Cb), and ~10% of lung cells expressed FasL (Fig. 5Ce). Approximately 24.8% of lung tissue-infiltrating CD8+ T cells expressed FasL (Fig. 5Cc, 5D), whereas ~12.7% of non-CD8+ cells expressed FasL (Fig. 5Cf, 5D). Taken together, our data suggest that, although a significant portion of tumor-infiltrating CD8+ T cells is FasL+ cells, both tumor-infiltrating CD8+ T cells and CD8- lung cells are the sources of FasL in the tumor microenvironment.

**FasL plays a critical role in suppression of metastatic colon carcinoma in vivo**

The above observations suggest that FasL is expressed on tumor-infiltrating immune cells, as well as other lung cells. To determine the role of FasL in tumor suppression in vivo, CT26 cells were transplanted to wild-type (wt) and Fasgld mice. In the absence of any treatment, no significant difference in lung tumor burden was observed between wt and Fasgld mice (Fig. 6A). However, combined decitabine and vorinostat treatment exhibited significantly greater tumor-suppression efficacy in wt mice than in Fasgld mice (Fig. 6B). Therefore, our results suggest that decitabine and vorinostat sensitize colon carcinoma cells to FasL-mediated tumor suppression in vivo.

**Low-dose decitabine and vorinostat exhibit no significant liver toxicity in vivo**

Toxicity, especially liver toxicity, is the major limitation for the use of DNA methylation inhibitors and HDAC inhibitors in human cancer therapy (29, 31, 52). To determine the toxicity of decitabine and vorinostat at the dose used in this study, we injected the two drugs i.v. into BALB/c mice, either alone or in combination. Three days later, blood was collected, and serum was analyzed for complete liver enzyme profiles. Aspartate aminotransferase level was decreased ~2-fold by vorinostat treatment (Table I). Decitabine and vorinostat did not significantly alter liver enzyme leakage into the peripheral blood. Taken together, our data suggest that decitabine and vorinostat exert effective tumor-suppression activity at a dose that is not toxic in mice.

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**FIGURE 6.** Decitabine and vorinostat-mediated tumor suppression is FasL-dependent in vivo. (A) CT26 cells were injected into wt and Fasgld mice at doses of 7.5 × 10⁶ cells/mouse (upper panel) or 5 × 10⁶ cells/mouse (lower panel) and examined for lung tumor growth. The number of lung tumor nodules was enumerated as in (right panel). Each circle represents total tumor counts from a single mouse. The horizontal lines in the plot box represent mean tumor nodule number. (B) CT26 cells were injected into wt and Fasgld mice at doses of 7.5 × 10⁶ cells/mouse. The tumor-bearing mice were treated with decitabine and vorinostat, as in Fig. 5A. Shown are tumor-bearing lungs of wt and Fasgld mice. The number of lung tumor nodules was enumerated as in (A) (right panel).
A CT26 tumor-specific, perforin-deficient, lung metastases. The tumor-bearing mice were then treated with transplanted into syngeneic mice for 7 d to establish extensive carcinoma metastasis. To test this hypothesis, CT26 cells were munion therapy is an effective therapy for the suppression of colon carcinoma metastasis in vivo. Because CD8+ T cells express Fasl and use Fasl as one of their primary effector mechanisms (9–12), we reasoned that combined chemotherapy with decitabine and vorinostat and tumor-specific CTL-adoptive immunotherapy is an effective therapy for the suppression of colon carcinoma metastasis. To test this hypothesis, CT26 cells were transplanted into syngeneic mice for 7 d to establish extensive lung metastases. The tumor-bearing mice were then treated with CT26 tumor-specific, perforin-deficient, pfpCTLs or pfpCTLs plus decitabine and vorinostat. The use of pfpCTLs eliminates the perforin-mediated cytotoxicity to allow better evaluation of the Fasl-induced cytotoxicity. The prediction is that if decitabine and vorinostat can overcome apoptosis resistance of the tumor cells in vivo, then combinational therapy should exhibit greater antitumor efficacy than CTL-adoptive immunotherapy alone. Indeed, although combined decitabine and vorinostat treatment (Fig. 5A) and pfpCTLs treatment (Fig. 7) alone showed significant tumor-rejection efficacy, combination chemotherapy plus CTL immunotherapy exhibited significantly enhanced tumor-rejection efficacy against the established colon carcinoma lung metastases compared with CTL immunotherapy or decitabine and vorinostat chemotherapy alone (Figs. 5A, 7). In summary, our data suggest that chemotherapy with decitabine and vorinostat in combination with CTL adoptive immunotherapy is effective for the intervention of colon carcinoma metastasis in vivo.

Discussion
It is well-established in the literature that decitabine and vorinostat exert direct cytotoxicity to induce tumor cell death, in part through inducing cell cycle arrest and DNA damage response to activate the intrinsic apoptosis pathway (34, 53, 54). This mechanism may explain the decitabine and vorinostat-induced cell death in the absence of Fasl observed in this study (Fig. 1D). Previous studies also convincingly demonstrated that vorinostat modulates Fas and other apoptosis-related genes to mediate tumor cell apoptosis (36–38). However, Fas is a death receptor, and it initiates apoptosis only after engagement by its ligand, Fasl. Therefore, an increase in Fas alone and alteration of apoptosis-related genes are not sufficient to initiate Fas-mediated apoptosis in tumor cells in the absence of Fasl. Nevertheless, increased Fas expression level induced by decitabine and vorinostat is apparently associated with increased sensitivity of human colon carcinoma cells to Fasl-induced apoptosis (Fig. 1). More importantly, combined treatment with decitabine and vorinostat effectively overcomes metastatic human colon carcinoma cell resistance to Fasl-mediated apoptosis, a hallmark of metastatic human colorectal cancer (27, 28).

Although a large portion of the tumor-infiltrating CD8+ cells express Fasl, CD8+ cells in the lung tissue also express Fasl (Fig. 5C). RT-PCR analysis revealed that the Fasl mRNA level is higher in the lungs of tumor-free mice than in those of tumor-bearing mice (Fig. 5B). The majority of cells in lungs of tumor-bearing mice is tumor cells (Fig. 5A). Although tumor-infiltrating CD8+ T cells express Fasl, these T cells only consist of a small portion of totals lung cells. This may explain the high Fasl mRNA level in the tumor-free mice. The type of Fasl+ lung cells and their function in Fasl-mediated apoptosis require further study.

Our studies indicated that the Fasl promoter is only sporadically methylated in metastatic human colon carcinoma cells (Fig. 2). Therefore, decitabine-mediated Fasl upregulation is unlikely to

| Table I. Liver toxicity profiles in mice after decitabine and vorinostat treatment |
|---------------------------------|---------|---------|----------|----------------|
| Serum Enzyme/Protein Level      | Control | Decitabine | Vorinostat | Decitabine and Vorinostat |
| ALP (U/l)                      | 129     | 120      | 106       | 126            |
| ALT (U/l)                      | 31      | 34       | 18        | 21             |
| AST (U/l)                      | 137     | 140      | 54        | 89             |
| Cholesterol (mg/dl)            | 78      | 78       | 76        | 72             |
| Albumin (g/dl)                 | 3.6     | 3.5      | 3.5       | 3.5            |
| Total bilirubin (mg/dl)        | 0.1     | 0.1      | 0.1       | 0.1            |
| Total protein (g/dl)           | 4.8     | 4.6      | 4.5       | 4.4            |

Serum from three mice of each treatment group was pooled for liver profile analysis.

ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate phosphatase.
occur through direct inhibition of the Fas promoter DNA methylation. NF-κB and p53 are prominent regulators of Fas expression, and chemotherapeutic agents were shown to upregulate Fas through NF-κB- and p53-dependent mechanisms (55, 56) Therefore, it is possible that decitabine and vorinostat upregulate Fas expression through NF-κB- and p53-dependent mechanisms in colon carcinoma cells, but it remains to be determined.

We also demonstrated in this study that BNI3P and Bik promoter DNA are methylated in metastatic human colon carcinoma cells (Fig. 3), and decitabine effectively reactivates BNI3P and Bik expression in metastatic human colon carcinoma cells. Furthermore, vorinostat decreased Bcl-xL expression in metastatic human colon carcinoma cells. Because silencing Bcl-xL expression or overexpressing Bik only altered the tumor cell sensitivity to Fas-L–induced apoptosis to a small degree (Fig. 4), it is likely that decitabine and vorinostat cooperate to alter the expression of multiple targets, including Fas, BNI3P, Bik, and Bcl-xL, which additively contribute to the greater degree of apoptosis induction in vitro (Fig. 1D) and enhance tumor suppression in vivo (Fig. 5A).

One of the major obstacles in cancer immunotherapy is immune suppression. Although tumor-specific FasL+ CTLs are potentially effective anticancer agents (11, 57, 58), the target tumor cells often induce immune suppression to suppress CTLs in the tumor microenvironment (59–61). CTLs target tumor cells primarily through two cell contact-dependent cytotoxic mechanisms. The first cytolytic mechanism depends on the polarized secretion of perforin and granzymes. The second effector mechanism involves the interaction of FasL on activated CTL surfaces with its receptor Fas on the target tumor cells (9, 10, 62, 63). Although it was shown that regulatory T cells (Tregs) can inhibit clonal expansion of activated T cells in vitro (64), recent studies indicate that Tregs do not inhibit CD8+ T cell activation and proliferation in vivo but rather selectively inhibit granule exocytosis of CTLs, thereby selectively impairing the perforin effector mechanism of CTLs without inhibiting CTL activation and clonal expansion (65, 66). Therefore, the Fas-mediated cytotoxicity of the tumor-specific CTLs should still be effective and is particularly important in CTL-mediated antitumor activity under immunosuppressive conditions. Furthermore, it was shown that Tregs are highly sensitive to Fas-mediated apoptosis, whereas effector T cells are resistant to Fas-mediated killing (67, 68). Thus, Fas-based cancer therapy may not only induce tumor cell apoptosis, it may also induce Treg apoptosis to eliminate Treg-mediated immune suppression. In summary, our data suggest that chemotherapy with decitabine and vorinostat, in combination with CTL immunotherapy, is an effective strategy for the suppression of colon carcinoma metastasis and holds great promise for further development to treat metastatic colon cancer in human patients.

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Disclosures
The authors have no financial conflicts of interest.

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