Inhibition of DNA Methylation Sensitizes Glioblastoma for Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Mediated Destruction

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Abstract

Life expectancy of patients affected by glioblastoma multiforme is extremely low. The therapeutic use of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has been proposed to treat this disease based on its ability to kill glioma cell lines *in vitro* and *in vivo*. Here, we show that, differently from glioma cell lines, glioblastoma multiforme tumors were resistant to TRAIL stimulation because they expressed low levels of caspase-8 and high levels of the death receptor inhibitor PED/PEA-15. Inhibition of methyltransferases by decitabine resulted in considerable up-regulation of TRAIL receptor-1 and caspase-8, down-regulation of PED/PEA-15, inhibition of cell growth, and sensitization of primary glioblastoma cells to TRAIL-induced apoptosis. Exogenous caspase-8 expression was the main event able to restore TRAIL sensitivity in primary glioblastoma cells. The antitumor activity of decitabine and TRAIL was confirmed *in vivo* in a mouse model of glioblastoma multiforme. Evaluation of tumor size, apoptosis, and caspase activation in nude mouse glioblastoma multiforme xenografts showed dramatic synergy of decitabine and TRAIL in the treatment of glioblastoma, whereas the single agents were scarcely effective in terms of reduction of tumor mass, apoptosis induction, and caspase activation. Thus, the combination of TRAIL and demethylating agents may provide a key tool to overcome glioblastoma resistance to therapeutic treatments. (Cancer Res 2005; 65(24): 11469-77)

Introduction

Malignant gliomas are brain tumors arising from cells of the astrocytic lineage. Glioblastoma multiforme is the most aggressive malignant glioma (grade 4 astrocytoma; ref. 1) characterized by a median survival of 10 to 12 months. Extensive surgical resection is not curative due to the highly invasive capacity of glioblastoma multiforme cells into normal brain parenchyma. Moreover, glioblastoma multiforme is largely resistant to current treatments based on cytotoxic approaches targeting replicating DNA, such as chemotherapy or radiotherapy. Consequently, only a small minority of glioblastoma multiforme patients achieves long-term survival (2–4).

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Impaired apoptosis contributes to tumor development and resistance to therapy. Mammalian cell apoptosis results from the activation of two major pathways. The intrinsic pathway is generated by multiple signals, including radiation and chemotherapy. This apoptotic route involves the mitochondria-dependent activation of the initiator caspase-9, which in turn activates downstream executive caspases, such as caspase-3. The antiapoptotic members of the Bcl-2 family promote tumor formation and resistance to therapy by preventing the release of apoptogenic factors from mitochondria (5).

The extrinsic death receptor pathway is triggered by death ligands belonging to the tumor necrosis factor (TNF) family, such as CD95 ligand and TNF-related apoptosis-inducing ligand (TRAIL), through the formation of the death-inducing signaling complex (DISC). This complex is composed of aggregated death receptors, the adaptor molecule FADD, and the initiator caspase-8. After DISC formation, the zimogen form of caspase-8 is proteolytically cleaved and activated to initiate the apoptotic signaling (6, 7). Death receptor activation can be blocked by c-FLIP and PED/PEA-15, two inhibitory proteins that compete with caspase-8 for FADD binding and neutralize the extrinsic apoptotic pathway (8, 9).

Death receptor triggering can promote apoptosis independently from the mitochondrial pathway. Therefore, death receptor ligands may kill tumor cells resistant to chemotherapy and radiotherapy. Several defects within the apoptotic machinery have been identified in tumors of different origin. One of the mechanisms responsible for apoptosis resistance in cancer results from silencing of tumor suppressor or proapoptotic genes, occurring by hypermethylation of the CpG-rich sites located in the promoter region of the gene. Methyltransferases may contribute to the development of glioblastomas through the transcriptional inhibition of the carboxyl-terminal modulator protein, which binds Akt and reduces its protein kinase activity (10). The tumorigenic role of methyltransferases in brain tumors is further supported by the repression of caspase-8 expression observed in neuroblastomas and medulloblastomas, where the use of the methyltransferase inhibitor decitabine (5-aza-2-deoxycytidine) results in caspase-8 up-regulation and restoration of apoptosis sensitivity (11–16).

Due to its ability to induce apoptosis preferentially in cancer cells, the apoptotic pathway activated by TRAIL is a very attractive candidate for cancer treatment, currently exploited in several phase I trials through the use of recombinant TRAIL or agonistic anti–TRAIL receptor antibodies (17, 18).

The weak cytotoxic effect of chemotherapeutic drugs on glioblastoma multiforme cells encouraged several investigators to examine the sensitivity of glioblastoma cells to recombinant TRAIL.

Based on its ability to kill some glioblastoma cell lines both *in vitro* and *in vivo*, TRAIL has been proposed for glioblastoma treatment (19–23). However, glioblastoma cell lines exhibited variable sensitivity to TRAIL. In some cases, the combined administration of other compounds has been proposed for increasing TRAIL-induced apoptosis, such as chemotherapeutic drugs or cell-permeable peptides mimicking the mitochondrial release of the proapoptotic protein Smac/Diablo (24–26).

Little is known about primary glioblastoma cells and their response to TRAIL stimulation. Although the extreme variability of glioblastoma cell lines in terms of TRAIL sensitivity might reflect the heterogeneity of the tumors from which the cells have been derived, it cannot be excluded that such variability results from adaptation to *in vitro* growth. Direct analysis of tumors and primary cells is required to obtain more reliable data concerning the glioblastoma multiforme response to TRAIL. In this study, we investigated TRAIL signaling pathway in human glioblastomas. We found that primary glioblastoma cells were completely refractory to TRAIL stimulation. However, treatment with DNA demethylating agents was able to restore the sensitivity to TRAIL-induced apoptosis *in vitro* and *in vivo* through the reconstitution of the early signaling pathway.

Materials and Methods

Tumor cell isolation and characterization. Primary cultures of glioblastoma were established from specimens obtained from consenting patients undergoing surgery at the Department of Neurosurgery, Catholic University, Rome, Italy (Table 1). The institutional review board at the Catholic University approved this study. Tissues were mechanically disrupted in the presence of HBSS. Cell suspension was recovered, passed through 100-μm nylon cell strainers, and subjected to Ficoll gradient centrifugation. Cells were then cultured in DMEM/F-12 complete medium supplemented with 10% fetal bovine serum (FBS). Phenotypic characterization of isolated primary cells in comparison with a panel of glioblastoma cell lines was done by flow cytometry and real-time PCR. Cells isolated from all tumor samples homogeneously expressed the neural progenitor cell marker nestin and the glial fibrillary acidic protein (GFAP), whereas these proteins were differently expressed in the four cell lines. In agreement with literature data, both primary cultures and cell lines constantly expressed the neuron-specific enolase (27). Whereas the fibroblast antigen Thy1 was present in three of four glioblastoma cell lines, the absence of contaminant endothelial cells and fibroblasts in primary glioblastoma cells was confirmed by negativity for von Willebrand factor and Thy1, respectively (data not shown). Thus, primary cultures derived from tumor samples were virtually pure glioblastoma cultures.

The human glioblastoma cell lines T98G, U87MG, U251, and TB10 (28) were grown in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FBS (Life Technologies, Inc., Grand Island, NY), 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mmol/L L-glutamine (Life Technologies, Inc., Rockville, MD) and maintained in 5% CO_2 at 37°C.

Flow cytometric analysis. One hundred thousand cells were used for flow cytometric analysis. Cells were washed with cold PBS and incubated with control or specific antibodies. Mouse anti-Thy1 antibodies (PharMingen, Inc., San Diego, CA), goat anti–TRAIL receptor antibodies (R&D Systems, Minneapolis, MN), phycoerythrin-conjugated anti-goat secondary antibodies (Chemicon, Temecula, CA), and FITC-conjugated anti-mouse antibodies (Molecular Probes, Eugene, OR) were used. Labeled cells were washed twice with PBS and fluorescence intensity was evaluated by FACScan (Becton Dickinson, San Jose, CA).

Detection of apoptosis and caspase activation. Decitabine (Sigma, St. Louis, MO) was dissolved in DMSO 100 mmol/L, and 1-μL aliquots were stored at −20°C. Single aliquots were thawed immediately before use and diluted in complete medium. Cells were grown in the presence of 0.1 to 1 μmol/L decitabine for 6 days to be used for DNA demethylation experiments. Decitabine-containing medium was replaced daily. Cells were treated with leucine zipper TRAIL (LZ-TRAIL; kindly provided by Dr. Henning Walczak, Heidelberg, Germany) in complete medium for apoptosis induction. Cell viability was then analyzed by Cell Titer 96 assay (Promega, Madison, WI) and caspase activation was measured by Apo1 Caspase-3/7 Assay kit (Promega). Colorimetric or fluorimetric assays were analyzed by Victor 2 plate reader (Wallac, Turku, Finland).

Western blotting and real-time PCR analyses. For immunoblotting studies, mouse monoclonal anti-FADD/MORT1 antibody was purchased from Becton Dickinson Transduction (Los Angeles, CA) and mouse monoclonal anti-caspase-8 (clone 5F7) was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-PED/PEA-15 antibody was a kind gift of G. Condorelli (Naples, Italy). Mouse monoclonal anti-β-tubulin antibody was purchased from Sigma. Bands were detected with Super Signal West Pico chemiluminescent substrates (Pierce, Rockford, IL) and quantified using Scion Image software (Scion Corp., Frederick, MA). For real-time PCR, total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA (1 μg) was reverse transcribed into cDNA by using SuperScript II RT with oligo(dT) as primers (Invitrogen, Grand Island, NY) according to the manufacturer's protocol.

Table 1. Clinical features and immunohistochemical pattern of glioblastoma tumors

Case code	Age/sex	Tumor location	Immunohistochemistry			
			GFAP	Ki-67 (%)	p53	Epidermal growth factor receptor
GBM2	60/F	Temporal	+	10	Wild-type	+
GBM3	55/F	Parietal	+	30	Mutant	+
GBM5	62/F	Frontal	+	10	Wild-type	+
GBM6	40/M	Parietal	+	15	Mutant	−
GBM7	35/M	Temporal	+	15	Mutant	−
GBM8	55/F	Frontal	+	10	Mutant	+
GBM9	66/F	Temporal	+	15	Mutant	+
GBM10	67/M	Frontal	+	20	Mutant	+
GBM11	73/M	Frontal	+	18	Wild-type	+

NOTE: Immunohistochemical pattern of glioblastoma tumors. Expression analysis of specific proteins was done by immunohistochemistry.

Real-time PCR was done with ABI Prism 7900HT Sequence Detection System and all reagents were from Applied Biosystems (Foster City, CA) following the manufacturer's instructions. Assays-on-Demand for TRAIL receptor-1 (TRAIL-R1), PED/PEA-15, and caspase-8 were used.

Retroviral gene transfer. Caspase-8 coding sequence and antisense PED/PEA-15 sequence were cloned into PINCO retroviral vector. TRAIL-R1 cDNA was cloned into a green fluorescent protein (GFP)–defective PINCO vector. Retroviral particle generation and tumor cell infection was done as described (29). The evaluation of infection efficiency was done by flow cytometry based on the expression of the GFP reporter protein or TRAIL-R1 immunostaining preceded flow cytometric analysis in the case of GFP-defective vector. The percentage of infected cells was >95%. Caspase-8 overexpression and PED/PEA-15 down-modulation were evaluated by Western blotting analysis.

Animal studies and immunohistochemistry of tumor tissues. One million U87MG glioblastoma cells were injected s.c. into one flank of 6-week-old athymic *nu/nu* mice (Charles River Laboratory, Wilmington, MA). The use and care of experimental animals was approved by the ethical committee of the Catholic University School of Medicine (Rome, Italy). Mice were kept under pathogen-free conditions and observed daily for the visual appearance of tumors at injection sites. Tumor diameter was measured using calipers and calculated as the mean value between the shortest and the longest diameters. When tumors reached ~6 mm in mean diameter (~3-4 weeks postinjection), treatment of mice with decitabine was started. Decitabine (3.75 mg/kg) was administered i.p. twice daily for 6 days. Two injections of 2 μg LZ-TRAIL at the tumor site were done after 4 and 6 days of decitabine treatment. Mice were maintained up to 11 days without any further treatment, except for measurement of tumor masses. Control animals were injected with equal volumes of saline either i.p. or at the tumor site.

For *in situ* apoptosis detection in tumor xenografts, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction was done using *In situ* Cell Death Detection AP kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Apoptotic nuclei appeared as blue dots. Counterstaining of cytoplasm was done using eosin.

Immunofluorescence staining was done on 6-μm-thick paraffin-embedded tumor xenograft sections. Tissue samples were deparaffinized and hydrated. For antigen unmasking, sections were heated in 10 mmol/L sodium citrate buffer (pH 6.0) and washed in distilled H_2O for 5 minutes. Slides were then incubated for 5 minutes in 1% H_2O_2, washed, and exposed to 1% bovine serum albumin (BSA)–containing TBS for 10 minutes to reduce unspecific staining. Excess BSA was removed and samples were incubated with active caspase-8-specific rabbit polyclonal antibody or isotype-matched control antibody (Cell Signaling Technology, Beverly, MA) overnight at 4°C. After two washes in TBS, sections were exposed to rhodamine-conjugated goat anti-rabbit immunoglobulins (Molecular Probes). Nuclei were labeled with Hoechst 33342 (Molecular Probes).

For immunohistochemical staining, 5-μm-thick paraffin-embedded sections were incubated with TBS/BSA for 10 minutes to reduce unspecific staining. Tissue sections were then exposed to goat polyclonal antibody against caspase-8 (N19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or isotype-matched control antibody for 1 hour. After two washes in TBS, sections were exposed to anti-goat biotinylated antibody, washed again, and incubated with streptavidin-conjugated peroxidase (Vectastain, Universal Quick kit, Vector Laboratories, Inc., Burlingame, CA). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole as a substrate. Counterstaining of tissue sections was done using aqueous hematoxylin.

Results

Low levels of caspase-8 and high levels of PED/PEA-15 correlated with resistance of primary glioblastoma cells to tumor necrosis factor–related apoptosis-inducing ligand–induced death. To determine whether the cancer cells forming human glioblastoma multiforme can be killed by TRAIL, isolated primary glioblastoma cells from surgical specimens were compared with four glioblastoma cell lines for sensitivity to TRAIL-induced apoptosis. As expected, glioblastoma cell lines displayed variable responses to TRAIL stimulation. T98G, U87MG, and U251 cells were partially sensitive to TRAIL-induced apoptosis, whereas TB10 cells were completely resistant (Fig. 1*A*). Surprisingly, primary cells from all the nine patients analyzed were refractory to TRAIL stimulation and did not undergo apoptosis even if exposed to high doses of recombinant TRAIL (Fig. 1*B*).

To explore the mechanisms responsible for TRAIL resistance, we investigated whether an altered expression of key elements of the proximal TRAIL pathway could be involved in impaired transmission of the apoptotic signal. We first analyzed by flow cytometry the expression levels of TRAIL receptors both in cell lines and in primary glioblastoma cells. Whereas TRAIL-R1 was weakly expressed, TRAIL receptor-2 (TRAIL-R2) was present at higher levels in all the cells analyzed (Fig. 1*C*). The expression levels of TRAIL-R1 and TRAIL-R2 were similar in both TRAIL-sensitive and TRAIL-resistant cells, indicating that the levels of TRAIL receptors may be unrelated to the different TRAIL susceptibility. We therefore investigated the expression of other TRAIL-related apoptotic and antiapoptotic proteins. FADD was consistently expressed in all the cell lines analyzed, ruling out its possible involvement in the different response to TRAIL stimulation (Fig. 1*D*). In contrast, caspase-8 immunoblot analysis showed that caspase-8 was expressed at very low levels in all the primary cells analyzed and in the TRAIL-resistant cell line TB10, whereas the partially sensitive cell lines expressed higher levels of caspase-8, suggesting that TRAIL resistance of glioblastoma cells resulted from insufficient caspase-8 activation (Fig. 1*D*). Moreover, the levels of the caspase-8 inhibitory protein PED/PEA-15 were higher in resistant than in sensitive cells, whereas the antiapoptotic protein c-FLIP was expressed at very low levels in all the cells analyzed (Fig. 1*D*; data not shown). Thus, the sensitivity to TRAIL-induced apoptosis in glioblastoma cells seems to be related to the ratio between the levels of caspase-8 and PED/PEA-15, which was considerably higher in sensitive cells (Fig. 1*D*).

To exclude the possibility that the low caspase-8 expression detected in glioblastoma primary cultures was dependent on *ex vivo* manipulation of glioblastoma cells, we investigated the expression of caspase-8 directly in tumors obtained at surgery from patients. In agreement with *in vitro* results, immunohistochemical analysis has clearly proven that glioblastomas display very low levels of caspase-8 and high expression of PED/PEA-15 (Fig. 1*E*), confirming the data obtained in cells from disaggregated tumors. Thus, the death receptor pathway is not functional in glioblastomas.

Decitabine treatment of primary glioblastoma cells results in sensitization to tumor necrosis factor–related apoptosis-inducing ligand–induced death. The expression of caspase-8 can be impaired by the activity of methyltransferases (11–16). Therefore, we investigated whether treatment with the methylation-defective cytidine analogue decitabine could restore the sensitivity to TRAIL-induced death in primary glioblastoma cells. Exposure to decitabine resulted in a considerable dose-dependent growth reduction of glioblastoma cells (Fig. 2*A*). Cell death analysis revealed that, although in the presence of 0.1 μmol/L decitabine cell viability was preserved, a significant number of dead cells were detectable at higher doses (data not shown). To reduce the unspecific toxicity, 0.1 μmol/L decitabine was used in all subsequent *in vitro* studies. As observed previously in other tumors (30, 31), decitabine-mediated growth inhibition correlated with

higher levels of p21 (Fig. 2*B*), a methyltransferase-regulated cell cycle inhibitor that binds to cyclin/cyclin-dependent kinase complexes and blocks cell proliferation (32).

Following exposure to decitabine, all partially sensitive cell lines displayed increased induction of cell death on TRAIL receptor stimulation. More importantly, those glioblastoma cells that were completely resistant acquired substantial sensitivity to TRAIL killing (Fig. 2*C*; data not shown). The restored TRAIL-induced apoptosis in primary glioblastoma cells treated with decitabine was associated with a considerable increase in caspase activation, which was barely detectable in the absence of methyltransferase inhibition (Fig. 2*D*). Thus, treatment with decitabine primes primary glioblastoma cells for TRAIL-mediated apoptosis.

Decitabine treatment of primary glioblastoma cells results in up-regulation of tumor necrosis factor–related apoptosis-inducing ligand receptor 1 and caspase-8 and down-modulation of PED/PEA-15. We then explored the mechanism of decitabine-induced TRAIL sensitization of primary glioblastoma cells. Reportedly, treatment of glioblastoma cell lines with chemotherapeutic agents resulted in increased TRAIL-R2 expression (33). Therefore, we first evaluated the possible modulation of those receptors for TRAIL able to transduce apoptotic signals.

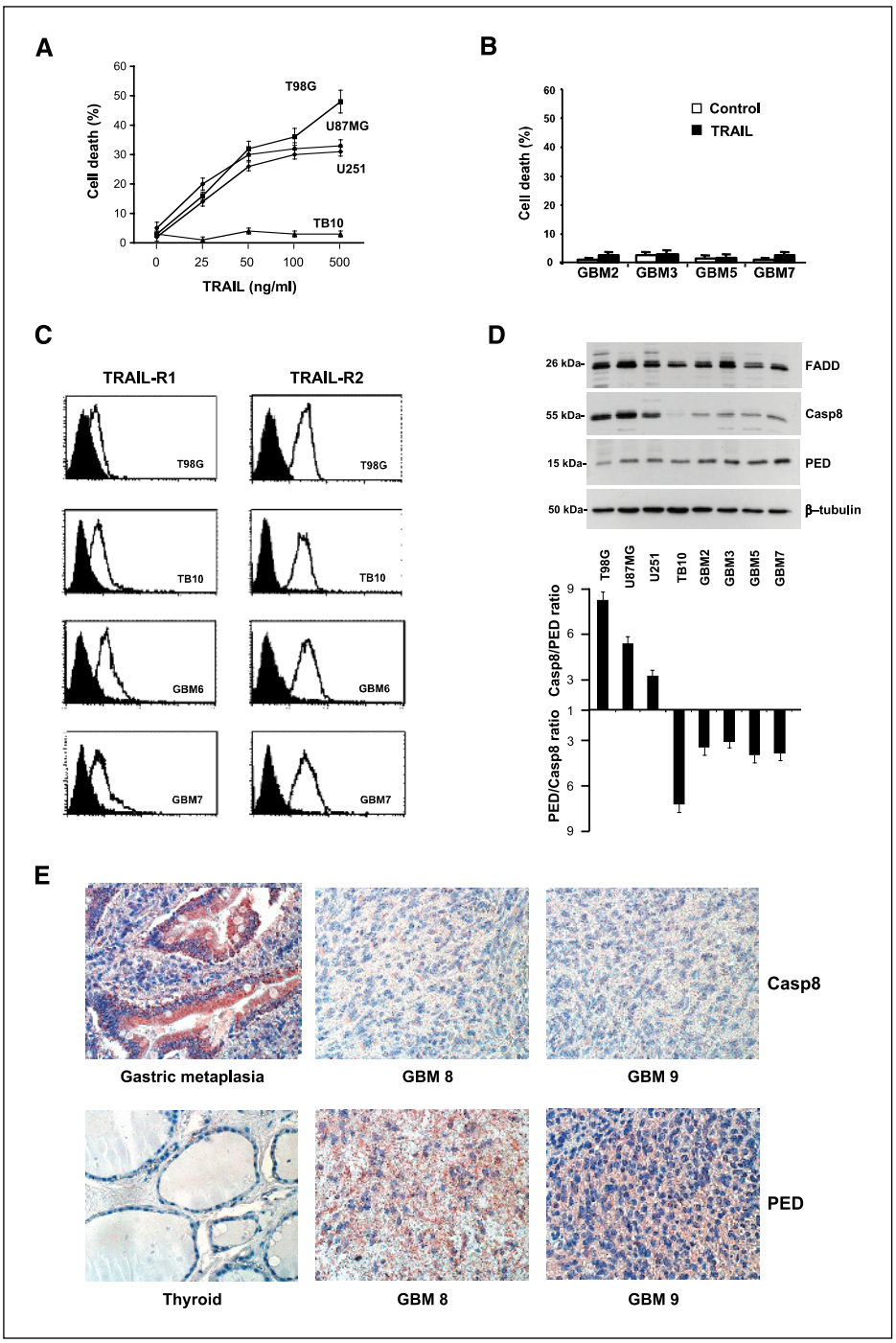

Figure 1. Sensitivity of glioblastoma multiforme to TRAIL-mediated cytotoxicity. Evaluation of cell death in glioblastoma cell lines (*A*) or primary glioblastoma cells (indicated as GBM followed by a number) from four different patients (*B*) exposed to different doses (*A*) or 500 ng/mL LZ-TRAIL (*B*). *Points,* mean of three independent experiments; *bars,* SD (*A*). *Columns,* mean of three independent experiments; *bars,* SD (*B*). *C,* flow cytometric analysis of TRAIL-R1 and TRAIL-R2 expression in the indicated cell lines and primary glioblastoma cells. *White histograms,* specific TRAIL receptor staining; *black histograms,* fluorescence controls. *D,* immunoblot analysis of caspase-8 (*Casp8*), FADD, and PED/PEA-15 (*PED*) expression in the glioblastoma cells analyzed in (*A* and *B*). β-Tubulin was used as loading control (*top*). *Columns,* mean absorbance of immunoblot band ratios obtained in four different experiments; *bars,* SD (*bottom*). *E,* immunohistochemical analysis of caspase-8 and PED/PEA-15 in two representative of four glioblastoma multiforme from different patients. Gastric metaplasia and nonneoplastic thyroid specimens were used as positive and negative controls, respectively. Representative of similar independent experiments with cells from nine patients (*B-E*).

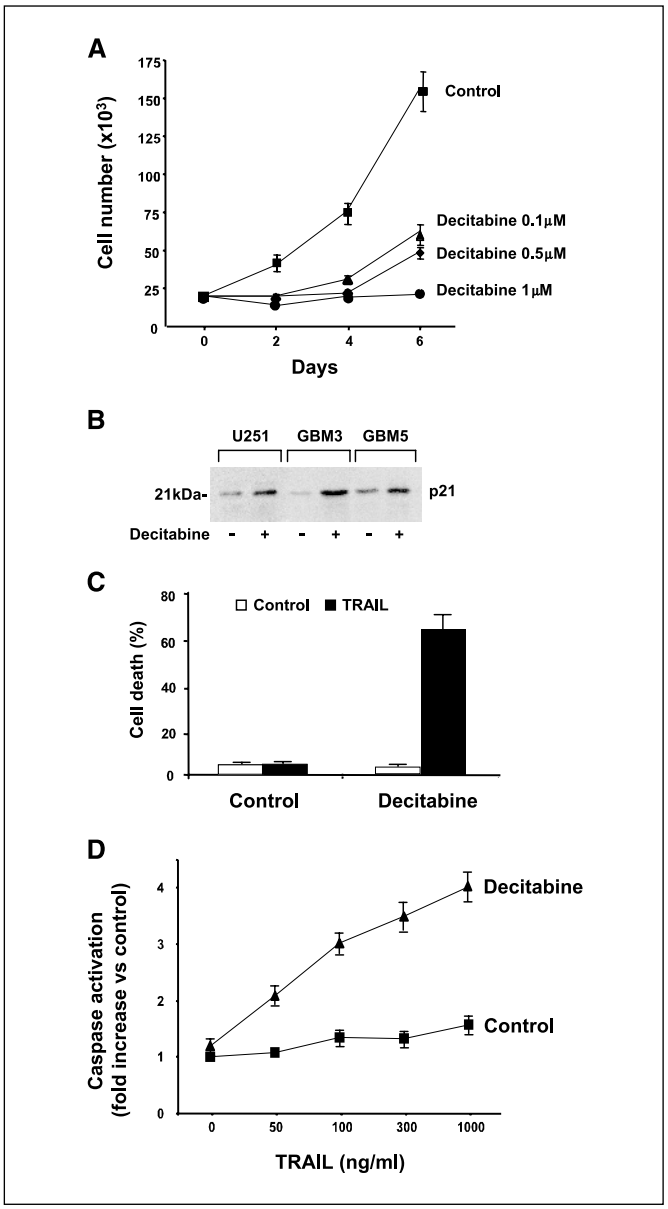

Figure 2. Decitabine-mediated sensitization to TRAIL in glioblastoma cells. *A,* primary glioblastoma cell growth in the presence of different doses of decitabine. *B,* immunoblot analysis of p21 expression in glioblastoma cells untreated (-) or treated (+) with decitabine. *C,* percentage of cell death in primary glioblastoma multiforme cells treated with 500 ng/mL LZ-TRAIL. *D,* LZ-TRAIL-induced caspase activation in control or decitabine-treated primary glioblastoma cells as determined by fluorescent caspase substrate cleavage. Caspase activation and cell death were measured 48 hours after LZ-TRAIL stimulation. *Points,* mean of five independent experiments with primary cells from seven different patients; *bars,* SD.

Control and decitabine-treated cell lines and primary glioblastoma cells were compared by flow cytometry for TRAIL-R1 and TRAIL-R2 expression. Although TRAIL-R1 was weakly expressed in untreated glioblastoma cells, its expression was considerably increased in decitabine-treated cells, suggesting that DNA methylation events might regulate TRAIL-R1 transcription in glioblastoma. In contrast, TRAIL-R2 was expressed at high levels in control cells and did not undergo any significant variation after decitabine treatment (Fig. 3*A*). We next investigated whether inhibition of DNA methylation could result in modulation of the intracellular components of the TRAIL pathway altered in glioblastoma. Primary glioblastoma multiforme cells were treated with decitabine and analyzed by immunoblot analysis for expression of caspase-8 and PED/PEA-15. Decitabine treatment of both cell lines and primary cells resulted in marked caspase-8 up-regulation and down-regulation of PED/PEA-15, whereas FADD expression was not significantly modified (Fig. 3*B*). The correlation between caspase-8 up-regulation, PED/PEA-15 down-regulation, and increased sensitivity to TRAIL suggested that decitabine treatment allowed efficient DISC formation after TRAIL stimulation.

To evaluate whether variations in the protein levels observed after decitabine treatment were dependent on transcriptional events, we measured caspase-8, TRAIL-R1, and PED/PEA-15 mRNA by real-time PCR analysis. Control and decitabine-treated TRAIL-resistant TB10 cells were compared with TRAIL-sensitive T98G cells. Whereas decitabine-treated samples displayed increased TRAIL-R1 and caspase-8 mRNA levels, PED/PEA-15 levels in the control samples were not significantly different from those in the treated samples (Fig. 3*C*). In particular, caspase-8 mRNA levels increased by 5.6-fold. A similar pattern was observed in primary glioma cells from three patients, where decitabine treatment resulted in a significant increase ($\sim 4.8 \pm 0.5$–fold) in caspase-8 mRNA (data not shown). Thus, whereas TRAIL-R1 and caspase-8 expression is regulated at the transcriptional level by decitabine treatment, the decrease in PED/PEA-15 levels may not be directly dependent on transcriptional regulation, possibly resulting from altered protein stability (34).

Caspase-8 up-regulation is the major event responsible for decitabine-mediated tumor necrosis factor–related apoptosis-inducing ligand sensitization. To evaluate the relevance for TRAIL sensitization of single proteins modulated by decitabine, we reproduced these protein level modifications in primary glioblastoma cells using retroviral vectors. Overexpression of TRAIL-R1 was not able to sensitize primary glioblastoma cells to TRAIL (Fig. 4*A* and *B*), suggesting that decitabine-mediated up-regulation of this receptor is unable to promote TRAIL cytotoxicity in the absence of substantial levels of caspase-8. Similarly, antisense cDNA-mediated reduction of PED/PEA-15 at levels comparable with those obtained with decitabine treatment did not prime these cells for TRAIL killing (Fig. 4*C* and *D*). In contrast, exogenous expression of caspase-8 was sufficient to promote caspase activation and apoptosis in primary glioblastoma cells exposed to TRAIL (Fig. 4*D* and *E*), indicating that caspase-8 up-regulation is a major event in decitabine-induced TRAIL sensitization.

Combined treatment with decitabine and tumor necrosis factor–related apoptosis-inducing ligand results in dramatic reduction of tumor growth and induction of tumor cell apoptosis in human glioblastoma xenografts. To evaluate the *in vivo* effectiveness of decitabine-mediated TRAIL sensitization of glioblastoma multiforme cells, we compared the antitumor activity of decitabine, TRAIL, or the combination of both agents in U87MG s.c. xenograft mouse model system (35). When tumors reached ~ 6 mm in size, mice were treated with decitabine alone or in combination with LZ-TRAIL. Decitabine was administered i.p. for 6 days and LZ-TRAIL was injected locally at days 4 and 6 of decitabine treatment. As a control, PBS was injected i.p. and i.t. Tumors were measured daily up to day 11, when the mice were killed to allow microscopic analysis of the

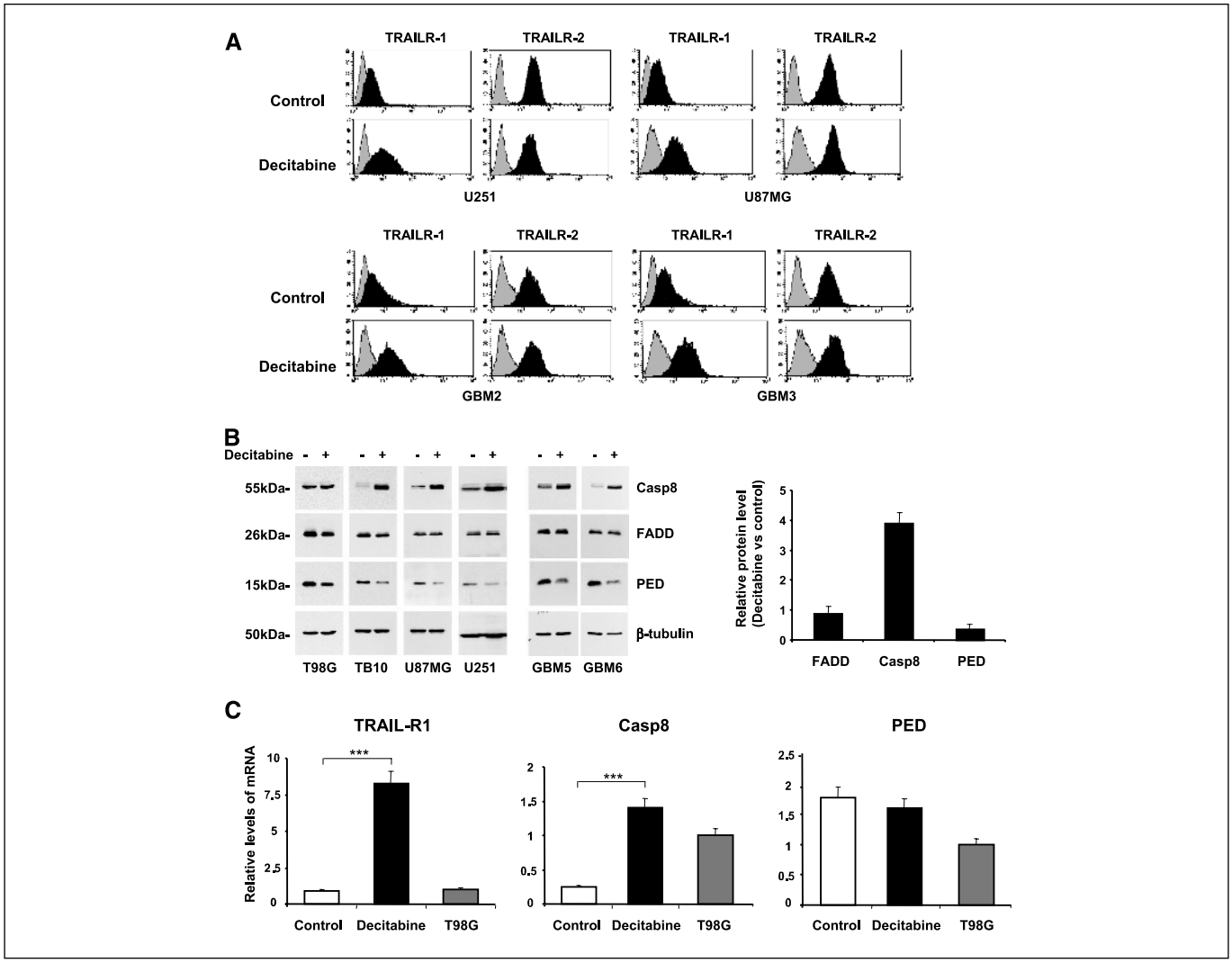

Figure 3. Up-regulation of TRAIL-R1 and caspase-8 and down-regulation of PED/PEA-15 by decitabine treatment. *A,* detection of TRAIL-R1 and TRAIL-R2 expression by flow cytometry in control or decitabine-treated primary glioblastoma cells. *Black histograms,* specific TRAIL receptor staining; *gray histograms,* fluorescence controls. *B,* immunoblot analysis of caspase-8, FADD, and PED/PEA-15 expression in glioblastoma cells untreated (-) or treated (+) with decitabine. β-Tubulin was used as loading control. *Columns,* mean of four experiments evaluating absorbance of immunoblot band ratios between treated and untreated primary cells from four different patients; *bars,* SD. Data were normalized for β-tubulin expression. *C,* real-time PCR analysis of TRAIL-R1, caspase-8, and PED/PEA-15 expression in TRAIL-resistant TB10 cells untreated (*Control*) or treated with decitabine. TRAIL-sensitive T98G glioblastoma cell line cDNA was used as positive control. ***, $P < 0.0001$.

tumor tissue. In agreement with *in vitro* experiments, decitabine treatment promoted a considerable up-regulation of caspase-8 in tumor xenografts (Fig. 5*A*). Macroscopic analysis showed that treatment with either decitabine or TRAIL alone resulted in modest reduction of final tumor size (Fig. 5*B*) possibly secondary to the transient inhibition of tumor cell proliferation rather than induction of apoptosis as suggested by the low number of apoptotic cells observed *in vivo* (Fig. 5*C*). In contrast, the combined administration of decitabine and TRAIL was able to induce a marked and consistent reduction of tumor size (Fig. 5*B*) and induction of apoptosis (Fig. 5*C*). Accordingly, whereas active caspase-8 was rarely detected in tumor xenografts treated with either single agent, the treatment with decitabine and TRAIL resulted in massive caspase-8 activation (Fig. 5*D*), confirming the effective triggering of the TRAIL death pathway. Moreover, H&E staining revealed that tumors of mice treated with decitabine and TRAIL were largely degenerated, showing massive postapoptotic and necrotic areas not observed in tumors treated with either single agent (data not shown). These results indicate that the combined administration of decitabine and TRAIL is able to induce a remarkable antitumor effect *in vivo* through the inhibition of cell growth and the induction of apoptosis in glioblastoma multiforme cells.

Discussion

The poor prognosis of malignant gliomas calls for intensive molecular and preclinical investigations to develop new and effective therapies. Current nonsurgical cancer treatments are essentially based on radiotherapy or chemotherapy, which exploit the intrinsic apoptotic pathway to destroy the tumor. Therefore, the majority of therapy-resistant cancers have an upstream or downstream defect involving the intrinsic apoptotic pathway. The possibility to exploit the extrinsic pathway in cancer

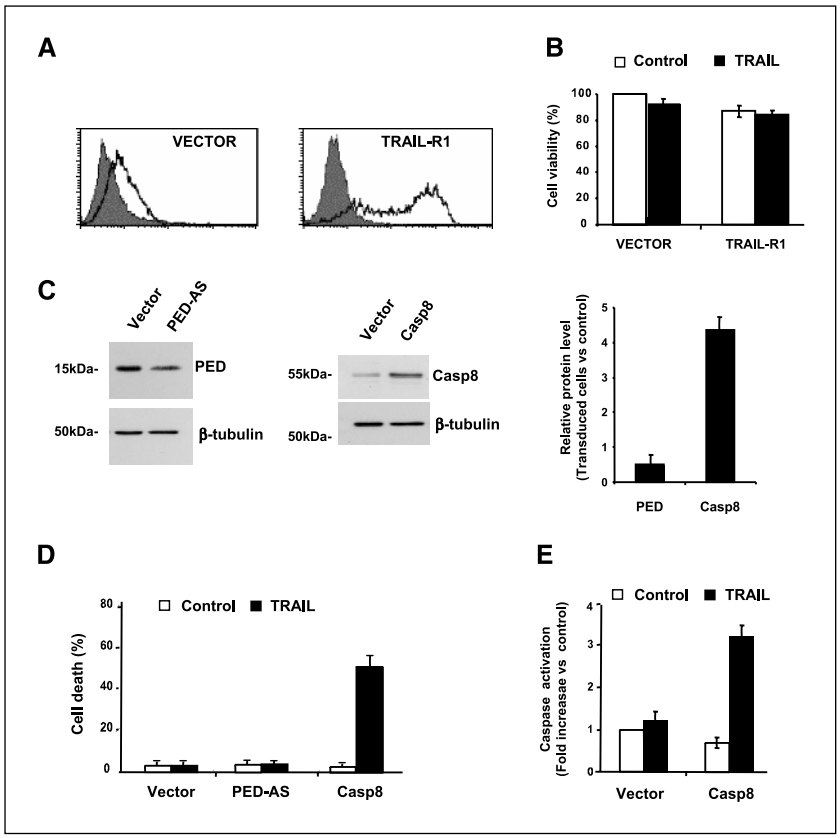

Figure 4. Effect of exogenous gene expression on TRAIL-sensitivity. *A,* flow cytometric analysis of TRAIL-R1 in primary glioblastoma cells transduced with empty vector or TRAIL-R1 cDNA. *B,* percentage of viability in cells transduced as in (*A*) and treated with LZ-TRAIL. *C,* PED/PEA-15 and caspase-8 expression in primary glioblastoma cells transduced with empty vector, PED/PEA-15 antisense (*PED-AS*), or caspase-8. Relative protein levels were quantified by densitometry. LZ-TRAIL induced cell death (*D*) and caspase activation (*E*) of primary glioblastoma cells transduced as indicated. Cell death and caspase activation were measured 48 hours after LZ-TRAIL stimulation. *Columns,* mean of three independent experiments with GBM6, GBM7, and GBM8 primary cells; *bars,* SD.

treatment has become feasible after the discovery that TRAIL may be administered to patients based on its ability to preferentially induce apoptosis in cancer while sparing normal cells (17, 18).

Several studies have shown that TRAIL is able to kill different glioma cell lines (20, 21, 23). Following the inability of chemotherapy and radiotherapy to improve patient prognosis, TRAIL has been proposed as an attractive candidate for glioblastoma treatment. However, some cell lines were reported to be resistant to TRAIL-induced apoptosis. Such resistance has been proposed to depend on high levels of antiapoptotic protein PED/PEA-15 expression (23).

In this study, we showed that primary glioblastoma multiforme cells are completely refractory to TRAIL-mediated apoptosis. However, treatment with decitabine was able to restore their responsiveness to TRAIL stimulation through caspase-8 and TRAIL-R1 up-regulation and down-regulation of PED/PEA-15. The synergistic activity of decitabine and TRAIL was confirmed *in vivo* using a mouse xenograft model, which showed massive apoptotic regression of treated tumors.

In agreement with other studies, we found a variable sensitivity to TRAIL in glioma cell lines (20, 21, 23). In contrast, all primary cells analyzed were invariably resistant to TRAIL-mediated apoptosis. It is likely that results achieved with the use of primary cells are more reliable than those obtained with cell lines as shown by the extremely low levels of caspase-8 and high PED/PEA-15 expression consistently observed by immunohistochemistry in tumors. The heterogeneity of glioblastoma cell lines was evident even during the phenotypical analysis of basic glioma markers. Whereas the four cell lines analyzed displayed significant variability in terms of antigen expression with GFAP, neuron-specific enolase, nestin, and the fibroblast antigen Thy1 being expressed at different levels, primary cells were rather homogeneous.

In agreement with the studies analyzing glioma cell lines (23), we observed that high levels of the antiapoptotic protein PED/PEA-15 correlated with increased resistance to TRAIL. The balance between the expression of caspase-8 and its inhibitor PED/PEA-15 seems extremely relevant for determining the susceptibility to TRAIL-mediated apoptosis. Inhibition of DNA methylation in glioblastoma multiforme cells resulted in a modification of this balance in favor of caspase-8, thus increasing apoptosis susceptibility. Although methyltransferase-mediated caspase-8 silencing occurs in other cancers, there is a lack of agreement concerning the identification of the promoter responsible for epigenetic regulation of caspase-8 expression. Therefore, the significance of direct caspase-8 promoter demethylation or transacting factors acting on this promoter in decitabine-induced caspase-8 up-regulation remains to be determined.

We found that DNA demethylation also resulted in increased TRAIL-R1 expression. Differently from caspase-8, epigenetic silencing of TRAIL-R1 has not been observed in other neurologic tumors. However, low expression of TRAIL receptors in some cancers seems to be involved in resistance to TRAIL. Decitabine-induced TRAIL-R1 expression could represent a possible sensitization strategy to treat these types of cancer. For instance, low levels of TRAIL-R1 expression seem to be associated with TRAIL resistance in non–small cell lung carcinoma cells. A combined treatment with decitabine and IFN-γ was reported to increase both TRAIL-R1 levels and apoptosis sensitivity of these cells,

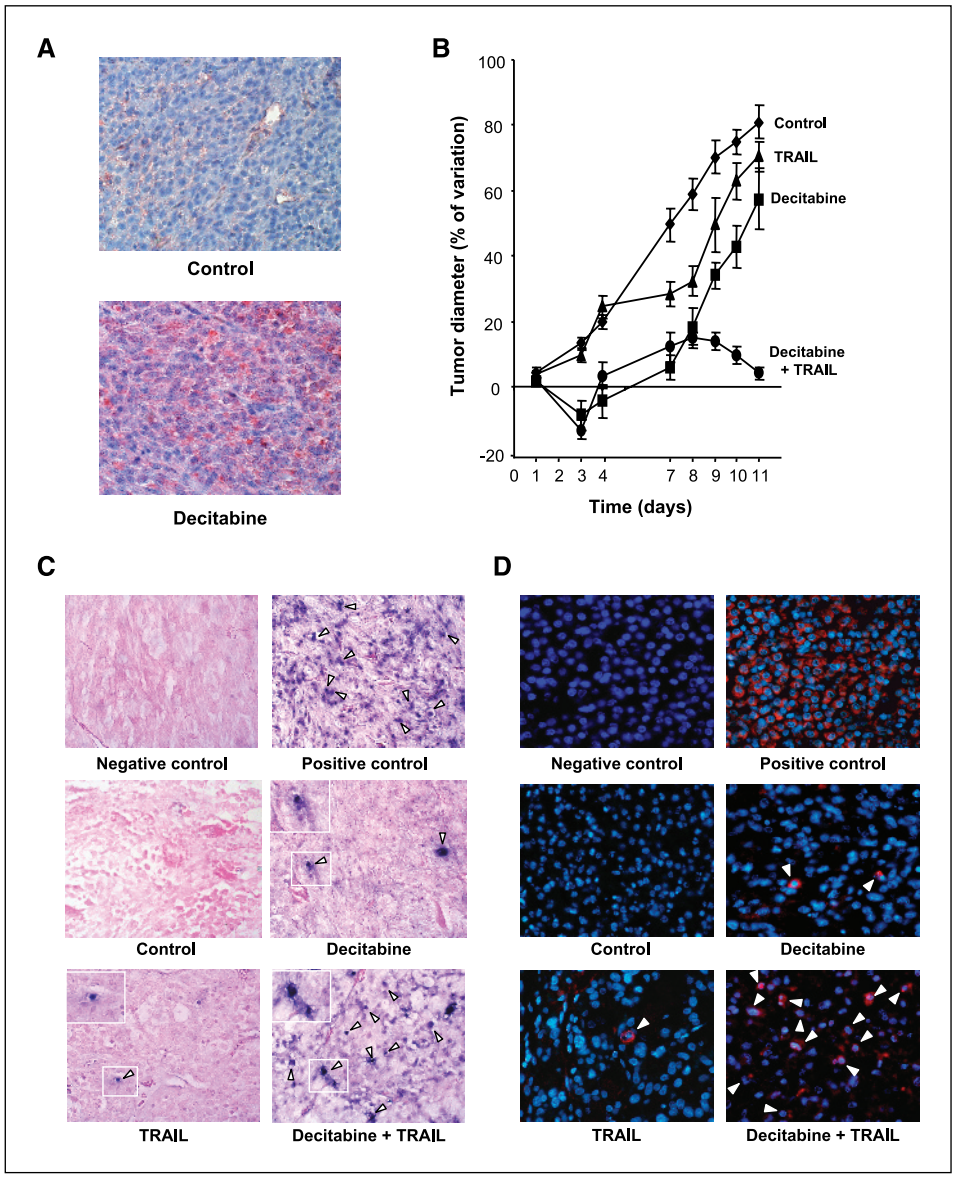

Figure 5. Effect of decitabine and TRAIL treatment on human glioblastoma xenografts. *A,* immunohistochemical analysis of caspase-8 expression done on U87MG xenografts derived from control or decitabine-treated mice. *B,* variation of tumor diameter at the indicated time points versus tumor diameter before decitabine treatment (day 0) in control mice or in mice treated with LZ-TRAIL, decitabine, or both compounds. *Points,* mean of two independent experiments, each experiment being composed by five mice per group (total of 40 animals); *bars,* SE. Tumors from mice treated with decitabine and LZ-TRAIL were significantly smaller than tumors from any other group (day 11; $P < 0.001$). *C, in situ* apoptosis detection in tumor xenografts by TUNEL reaction. Tumors were obtained at day 11 from mice untreated or treated with LZ-TRAIL, decitabine, or the combination of both compounds (*Decitabine + TRAIL*). *Arrowheads,* representative apoptotic cells stained in *dark blue. D,* immunofluorescence analysis of active caspase-8 of tumors obtained as in (*C*). Isotype-matched or active caspase-8-labeled tonsil sections were used as negative and positive controls, respectively.

indicating that epigenetic control of TRAIL-R1 transcription might occur in some other cancers (36).

Decitabine has been used in humans for the treatment of myelodysplastic syndromes, leukemia, and solid tumors. Phase I and II trials showed that decitabine is well tolerated and moderately effective in some types of cancer (37–39). To date, no data are available for clinical toxicity of TRAIL and agonist TRAIL receptor antibodies, which are currently undergoing phase I and II studies. However, experimental data are very promising in terms of antitumor activity and lack of toxicity. Here, we provide preclinical evidence for the efficacy of decitabine and TRAIL combination. Intense effort is required to assess the possible clinical use of decitabine and TRAIL combination for the treatment of glioblasvtoma given the high malignancy and low life expectancy of these patients.

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