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Apoptosis in human glioblastoma cells produced using embryonic stem cell–derived astrocytes expressing tumor necrosis factor–related apoptosis-inducing ligand

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Object. Embryonic stem (ES) cell–derived astrocytes have several theoretical and practical advantages as gene therapy vectors in the treatment of malignant gliomas. The aim of this study was to test the proapoptotic effects of ES cell–derived astrocytes expressing transgenic *tumor necrosis factor–related apoptosis-inducing ligand* (*TRAIL*) in human malignant glioma cells.

Methods. Mouse ES cells containing a doxycycline-inducible transgene were engineered with human TRAIL (hTRAIL) and then directed to differentiate into astrocytes. The ES cell-derived–TRAIL-expressing astrocytes were cocultured with human malignant glioma cells. Reverse transcriptase polymerase chain reaction, immunocytochemistry, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling, and flow cytometry were used to quantify results.

In vitro coculture of ES cell–derived astrocytes expressing *hTRAIL* with A172 human malignant glioma cells after doxycycline induction caused a significant decrease in cell viability from 85 ± 2% at baseline to 8 ± 2% posttreatment ($p < 0.001$). Labeling with apoptotic markers showed that cell death occurred by means of apoptosis. A significant increase in apoptotic rate (88 ± 3%) from baseline (4 ± 2%) was found in A172 cells after doxycycline induction ($p < 0.005$). This effect was superior to the apoptotic rate seen after treatment with recombinant *TRAIL* (57 ± 2%). A decrease in cell viability and an increase in the apoptotic rate were not found in *TRAIL*-expressing–ES cell-derived astrocytes after induction with doxycycline or in A172 cells exposed to doxycycline alone.

Conclusions. Engineering of transgenic *hTRAIL* by using ES cell–derived astrocytes induced apoptosis in human malignant glioma cells while sparing nontumor astrocytes. The apoptotic effects of transgenic *hTRAIL* are greater than those of recombinant *hTRAIL*. Analysis of these results suggests that *hTRAIL*-expressing–ES cell-derived astrocytes should be considered in the development of new in vivo strategies to treat malignant human gliomas.

Key Words • tumor necrosis factor–related apoptosis-inducing ligand • embryonic stem cell • astrocyte • malignant glioma • gene therapy

The resistance of malignant gliomas to current treatment remains a major concern in cancer therapy. Therapeutic strategies for malignant gliomas are a major focus in the field of neurooncology. Surgical, radio-, and chemotherapeutic treatment of malignant astrocytomas has been shown to yield to a median survival time of less than 12 months.[16] Viral vector–based antitumor gene therapy has been attempted for these highly aggressive tumors,[9,14] but its efficacy is limited in part by the fact that malignant glioma cells infiltrate deeply into the normal brain parenchyma surrounding the main tumor mass, whereas viral vectors do not. In addition, episomal viral vectors may have a limited duration of transgene expression, whereas viral vectors that insert into the host cell chromosome may themselves be tumorigenic.[17]

Cellular vectors may circumvent some of these problems. For example, NPCs transfected with viral vectors to express antitumor transgenes migrate through brain parenchyma to reach malignant tumor cells that otherwise would be inaccessible to therapy.[1,5,10] We recently reported the production of highly purified, ES cell–derived transgenic astrocytes.[6] Like NPCs, astrocytes have the potential to migrate through the brain parenchyma following transplantation.[11,32] Unlike NPCs, astrocytes have undergone full differentiation and cannot generate neurons or other cell types that could interfere with existing neural circuits. In addition, therapeutic transgenes may be permanently and precisely inserted

Abbreviations used in this paper: DAPI = 4′,6-diamidino-2-phenylindole; ES = embryonic stem; FCS = fetal calf serum; GFAP = glial fibrillary acidic protein; hTRAIL = human tumor necrosis factor–related apoptosis-inducing ligand; IMDM = Iscove modified Dulbecco medium; NPC = neural precursor cell; PBS = phosphate-buffered saline; PE = phycoerythrin; RT-PCR = reverse transcriptase polymerase chain reaction; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; 7AAD = 7-amino-actinomycin D.

into the chromosome of ES cell–derived astrocytes, avoiding some of the problems associated with viral vectors.

In this report, we describe the engineering of mouse ES cells that contain a doxycycline-inducible–transgene encoding TRAIL. A member of the tumor necrosis factor–α family, TRAIL induces apoptosis in various tumor cell types, including malignant glioma cell lines, while sparing most normal cells.[37] In the present study, we show that *TRAIL*-expressing ES cells can be directed to differentiate into astrocytes, and these astrocytes can induce apoptosis in malignant human glioma cells in vitro.

Materials and Methods

Cell Lines, Culture, and Cocultures

Mouse ES cells were passaged and maintained according to previously published protocols.[6] The Ainv-18 embryonic stem cells (gift from Michael Kyba) constitutively express a doxycycline-binding transcriptional activator fusion protein and include a doxycycline-responsive promoter upstream of a Lox cloning site.[22] Genes cloned into the Lox site by using Cre-assisted recombination[31] are then expressed when the cells are exposed to doxycycline. The ES cells were directed to differentiate into astrocytes by using protocols recently developed in our laboratory.[6]

The A172 human glioma cell lines were used to test the effects of *TRAIL*-expressing–ES cell-derived astrocytes. Jurkat cells were used as positive controls because the proapoptotic effects to recombinant TRAIL are well known.[25] Jurkat and A172 cell lines were maintained with IMDM supplemented with 10% FCS at 37˚C incubator supplemented with 5% CO_2.

For coculture experiments, differentiated ES cell-derived–hTRAIL-expressing astrocytes were cultured in monolayer on gelatin-coated tissue culture six-well 35-mm plates. Jurkat cells and A172 cells were plated on top of the ES cell–derived astrocytes in a ratio of 10:1 between tumor cell and ES cell–derived astrocytes (10^3). The A172 and ES cell–derived astrocytes coculture was maintained with IMDM-ES (IMDM, 20% FCS, 0.5% LIF, 10% CCE Lena conditioned media). Doxycycline induction was performed by adding doxycycline (1 μg/ml) to the coculture 24 hours after coplating. In controls, doxycycline induction was omitted. The effects of coplating experiments were assessed 24 hours after doxycycline induction.

Construction of Ainv-hTRAIL ES Cells

Complementary DNA containing the entire coding sequence of hTRAIL[20] (gift from B. Fang) was cloned into the pLox vector (gift from M. Kyba)[22] and sequenced (Mount Sinai School of Medicine Core Sequencing Facility, New York, NY). The hTRAIL–pLox plasmid DNA and Cre recombinase–expressing helper plasmid DNA were prepared using a column purification system (Qiagen, Valencia, CA) and electroporated into appropriately prepared Ainv-18 mouse ES cells. Electroporated Ainv-18 cells were plated onto drug-resistant embryonic feeder cells, and recombinant clones were selected with increasing concentrations of G418. Individual G418-resistant colonies were selected and expanded. Insertion of transgene into the Lox cloning site was confirmed using PCR from a genomic DNA template (forward primer 5′-GTG GCTGTA ACT TAC GTG TA-3′; reverse 5′-TTG ATA GAT GGA ATA GAG TC-3′; conditions 35 cycles, 2 minutes at 95˚C denature; 15 seconds at 95˚C denature; 30 seconds at 60˚C annealing; 60 seconds at 72˚C extension; 10 minutes at 72˚C elongation).

Analysis of Gene Expression and RT-PCR

The Ainv-hTRAIL ES cells or astrocytes were cultured for 24 hours in the presence or absence of 1 μg/ml doxycycline (Sigma, St. Louis, MO) and harvested for RT-PCR analysis. Total cellular RNA from cultured cells was purified using the RNeasy kit (Qiagen), with on-column deoxyribonuclease treatment. All RNA preparations were spectrophotometrically quantified and examined for degradation by using gel electrophoresis prior to RT analysis. The RT assay was performed on 1 μg of total RNA, using the Omniscript RT kit (Promega, Madison, WI) and PDN6 random hexamer primers (Pharmacia, Piscataway, NJ) in a total volume of 20 μl. The 12.5-μl PCR mixtures each contained 0.5 μl of the complementary DNA template. All PCR amplifications were conducted for 35 cycles. Primer sequences and annealing temperatures were previously published.[15] The PCR products were examined on 1.8% agarose gels and photographed using an Eagle Eye II Imager (Stratagene, La Jolla, CA). Each experiment was repeated three times.

Immunohistochemistry and Detection of In Vivo Apoptosis

Differentiated cells were grown on glass cover slips (Fisher Scientific, Hampton, NH) coated with poly-D-lysine (Sigma), whereas ES cells were grown on glass coverslips coated with gelatin (Sigma). For immunofluorescent labeling, cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature, washed, preincubated in 10% normal goat serum (Sigma) for 30 minutes at room temperature, and then placed in primary antibody for 1 hour at room temperature. Primary antibodies and dilutions were as follows: monoclonal anti-GFAP 1:200 (Sigma) and monoclonal anti–TRAIL antibody 1:200 (Chemicon International, Temecula, CA). Secondary antibodies and dilutions were as follows: Alexa 488 anti–mouse antibody (1:400) and CY3 (1:250) anti–rabbit for double labeling (Chemicon International). The cells were then washed and mounted in Vectashield medium (Vectashield, Burlingame, CA) with added DAPI (Vector Laboratories, Burlingame, CA) and examined using the Openlab fluorescent microscopy imaging system (Improvision, Lexington, MA). A negative control was established by omitting the primary antibody.

For detection of in vivo apoptosis, cells were grown on poly-D-lysine–coated coverslips, and the apoptosis signal was detected using the ApopTag Plus fluorescein in situ apoptosis detection kit (Chemicon International); all procedures were performed according to the kit protocol. Counterstaining was performed using DAPI. After completing the experiments, the cells were mounted on slides with Vectashield mounting medium, and images were taken using a fluorescence microscope (Leica, Bannockburn, IL).

Cell Viability and Apoptosis Assays for Flow Cytometry

Cell viability and apoptotic rate were quantified using flow cytometry after staining with vital dye 7AAD. Apoptosis was detected using Annexin V–PE (BD Biosciences, San Diego, CA), used according to manufacturer's instructions. With this technique, four kinds of cells can be identified. Cells that are Annexin V and 7AAD negative are viable; cells that are Annexin V positive and 7AAD negative are those in the early stage of apoptosis; cells that are Annexin V positive and 7AAD positive are apoptotic dead cells. Cells that are 7AAD positive are necrotic dead cells. This method of detecting cell death has advantages over the use of vital dyes alone (such as trypan blue).[36] Specifically, apoptosis can be distinguished from other types of cell death, and apoptosis and total cell death can be rapidly and accurately quantified using flow cytometry.

Control Jurkat cells and A172 cells were tested 24 hours after the addition of 30 ng/ml of recombinant soluble TRAIL protein (Peprotech, Rocky Hill, NJ). Cocultured cells were tested after 24 hours. Controls included samples in which recombinant TRAIL and doxycycline induction were omitted.

Flow Cytometry Analysis

Cells growing in monolayer or coculture were trypsinized and then washed with medium containing FCS. Cells were then centrifuged, resuspended in wash buffer (PBS, 10% FCS, and 0.01% NaN_3), and counted. For flow cytometry, cells sequentially labeled with primary antibodies and appropriate secondary antibodies or detection reagents were counted with a flow cytometer fluorescence-activated cell sorter scanner (Calibur; Becton Dickinson, San Jose, CA) that was running the Cell Quest software package (BD Biosciences). A gating technique, with appropriate measurement of forward and side scatter, was used to exclude dead cells and cell aggregates from the sorting, and 5000 to 10,000 cells were counted per analysis.

Statistical Analysis

Cell viability and the apoptotic rate were quantified using flow cytometric analysis and expressed as a mean percentage of total cells ± standard deviation. The Student t-test was used to compare baseline and posttreatment data. A probability value of less than 0.05 was considered to reflect a significant difference.

Results

Recombinant hTRAIL–Induced Apoptosis in Human Malignant Glioma Cells but not in ES Cell–Derived Astrocytes

Twenty-four hours after exposure to recombinant *hTRAIL*, the A172 human glioma cell viability was significantly decreased (from 89 ± 3% at baseline to 15 ± 2% after exposure; $p < 0.0002$; Fig. 1A). This finding was similar to the decrease observed in Jurkat control cells in which the baseline viability of 78 ± 12% decreased to 12 ± 3% after exposure ($p < 0.001$). Cell viability in ES cell–derived astrocytes, however, did not change significantly from baseline.

Apoptosis was detected by TUNEL in A172 cells 24 hours after exposure to recombinant hTRAIL (Fig. 1B). Data were quantified using flow cytometric analysis for apoptosis. The apoptotic rate in A172 cells increased from 5 ± 4% at baseline to 58 ± 3% after treatment ($p < 0.003$; Fig. 1C). Similarly, the apoptotic rate in Jurkat cells increased from a baseline level of 10 ± 2% to a postexposure level of 84 ± 4%. In ES cell–derived astrocytes, the apoptotic rate did not exceed 10% after exposure to recombinant *TRAIL* (Fig. 1C). The apoptotic rate was not dose or time dependent (data not shown; *TRAIL* dose range 3–300 ng/ml; time range 24–72 hours).

Doxycycline-Inducible hTRAIL*–Transgene Expression by ES Cell–Derived Astrocytes*

Expression of the inserted *TRAIL* transgene was assayed using immunohistochemistry, RT-PCR, and flow cytometric analysis in ES cell–derived astrocytes. The RT-PCR analysis (Fig. 2A) showed a readily detectable *TRAIL* expression 24 hours following doxycycline induction. Immunohistochemistry (Fig. 2B) demonstrated the presence of *TRAIL* protein after doxycycline induction. In the absence of doxycycline, *TRAIL* expression was not detected. Flow cytometric analysis (Fig. 2C) showed that 93 ± 5% of the ES cells were positive for *TRAIL* after doxycycline induction. The ES cell–derived astrocytes also showed *TRAIL* expression following doxycycline treatment as shown by RT-PCR, immunohistochemistry, and flow cytometric analysis (73 ± 2% positivity; Fig. 3). Analysis of these data indicated that Ainv-*hTRAIL*–ES cells and ES cell–derived astrocytes strongly express the *hTRAIL* transgene under the tight control of a doxycycline-sensitive promoter.

The TRAIL-Expressing ES Cells and ES Cell–Derived Astrocytes Induce Apoptosis in Human Malignant Glioma Cells

Human glioma A172 cells were cocultured with ES cells or ES cell–derived astrocytes expressing *TRAIL* before and after induction with doxycycline for 24 hours. Cell viability in A172 cells decreased from 85 ± 2% to 8 ± 2% after

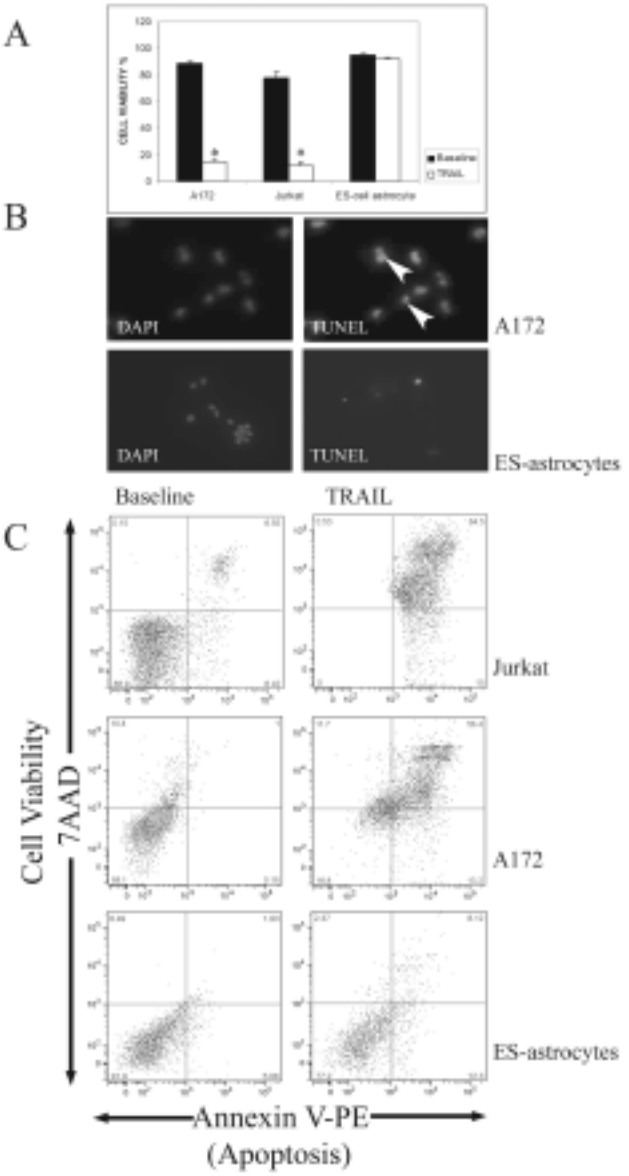

FIG. 1. Examples showing that recombinant *hTRAIL* induces apoptosis in malignant human glioma cells but not ES cell–derived astrocytes. A: Bar graph demonstrating cell viability of A172 tumor cells and ES cell–derived astrocytes 24 hours after exposure to recombinant *TRAIL* (3 ng/ml). Note the significant decrease in cell viability in A172 cells ($p < 0.001$) and in Jurkat cells ($p < 0.005$). B: Immunofluorescence photomicrographs of malignant glioma A172 cells and ES cell–derived astrocytes 24 hours after exposure to recombinant *TRAIL* (3 ng/ml). A significant number of A172 cells were TUNEL positive after exposure to TRAIL *(arrowheads)*. DAPI nuclear stain *(left panels)* and TUNEL stain *(right panels)*, original magnification × 40. C: Flow cytometric analysis of Jurkat cells, used as positive control *(upper panels)*, A172 human glioma cells *(center panels)*, and ES cell–derived astrocytes *(lower panels)* at baseline *(left column)* and 24 hours after exposure to *TRAIL* (3 ng/ml) *(right column)*. The apoptotic rate in Jurkat cells increased from 6.5 to 84%, in A172 cells from 1 to 58%, and in ES cell–derived astrocytes from 2 to 8%. Labeling: 7AAD, Annexin V–PE.

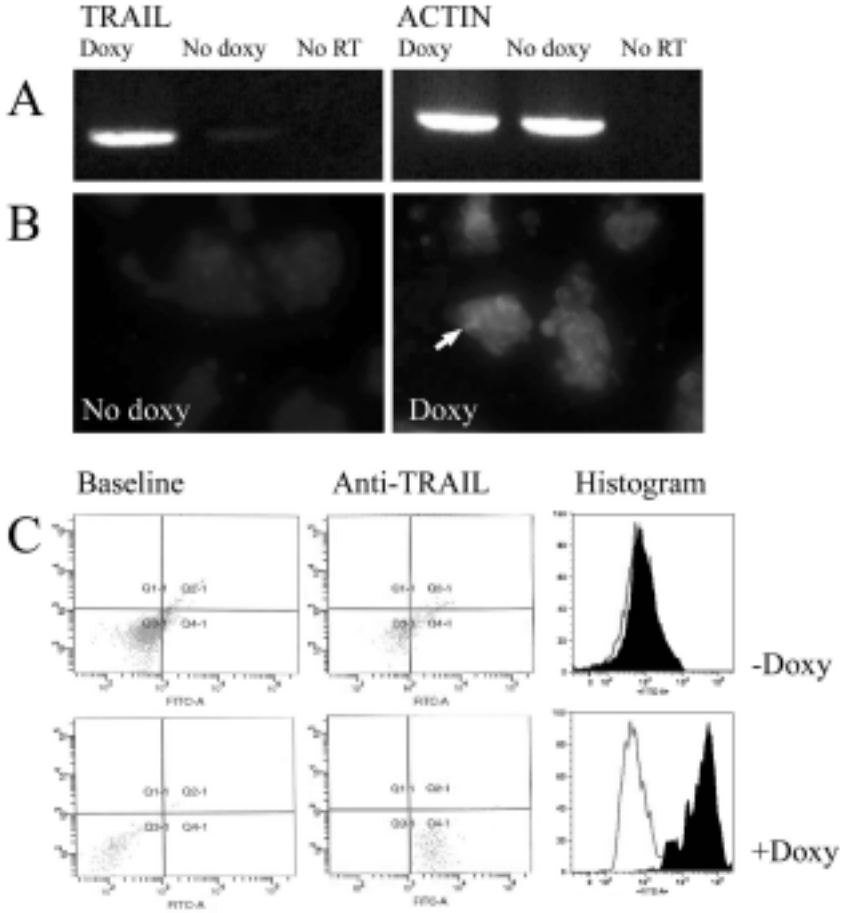

FIG. 2. Expression of the *hTRAIL* transgene by ES cells. A: The RT-PCR analysis shows *hTRAIL* expression 24 hours after doxycycline induction in *TRAIL*-expressing ES cells. Actin and absence of RT (no RT) are used as positive and negative controls, respectively. B: Immunofluorescence photomicrographs of *TRAIL*-expressing ES cells before and 24 hours after induction of doxycycline (doxy) labeled with anti–TRAIL antibody. The TRAIL labeling was detected on the cell membrane *(arrow*, *right panel)* after doxycycline induction. It was not detected in absence of doxycycline induction *(left panel)*. Alexa 488, original magnification × 40. C: Flow cytometric analysis showing *TRAIL* expression in ES cells. The *left column* represents baseline control samples without anti–TRAIL antibody, the *center column* shows cells after labeling with anti–TRAIL antibody. Flow cytometric analysis demonstrates that up to 95% of ES cells expressed *TRAIL* after doxycycline induction *(lower center panel)*. Minimal expression is noted in the absence of doxycycline *(upper center panel)*. Histograms are shown in the *right column*. The area edged in gray indicates cells at baseline and the black area indicates cells after anti–TRAIL antibody.

a 24-hour coculture with *TRAIL*-expressing–ES cell-derived astrocytes induced by doxycycline. Similar results were found when coculturing undifferentiated ES cells and A172 glioma cells. A decrease in viability was not observed when doxycycline was omitted.

Apoptosis was detected by TUNEL in A172 cells cocultured with *TRAIL*-expressing undifferentiated ES cells (Fig. 4A) and ES cell–derived astrocytes (Fig. 4B) 24 hours after doxycycline induction. Apoptosis was not observed in the absence of doxycycline induction. Although it was possible to distinguish A172 cells and ES cells based on their differing morphological features, the difference between the tumor cells and the ES cell–derived astrocytes was not dramatic. Both cell types express GFAP and other astrocytic markers and their morphology is very similar. Therefore, to determine which cell population was undergoing apoptosis, double labeling for TUNEL and *TRAIL* was undertaken. Analysis of these data showed that *TRAIL*-positive cells (ES cell–derived astrocytes) were TUNEL negative. Similarly, *TRAIL*-negative cells (A172 cells) were TUNEL positive (Fig. 4C).

Flow Cytometric Analysis of Cocultured Astrocytes and A172 Cells

To further quantify the apoptotic rate of A172 glioma cells in coculture, flow cytometry with fluorescence-activated cell-sorter analysis was used. The images in Fig. 5A show that A172 glioma cells had slightly larger cytoplasm than that of ES cell–derived astrocytes. Thus, cells were gated using forward-scattered and side-scattered profiles based on size (Fig. 5B).

After the induction of doxycycline, cocultured A172 glioma cells exhibited a significant increase in apoptotic rate compared with that at baseline (from 4 ± 2% to 88 ± 3%). This was not observed in ES cell–derived astrocytes (Fig. 5C). These data corroborate the evidence that A172 cells

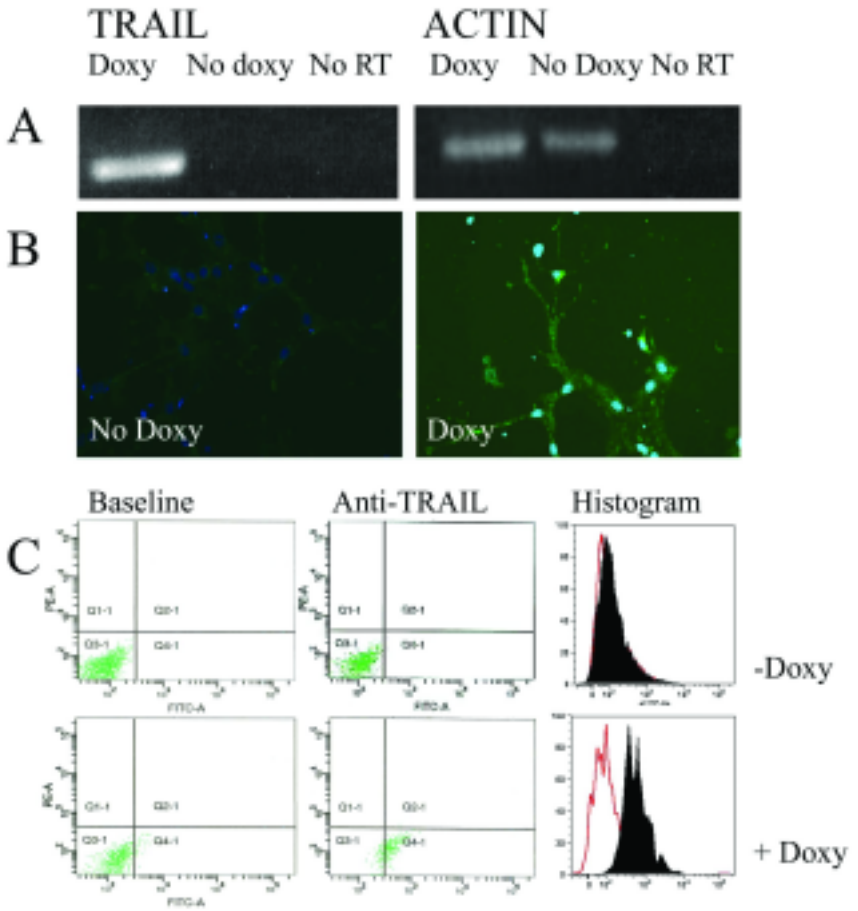

FIG. 3. Expression of *hTRAIL* by ES cell–derived astrocytes. A: The RT-PCR analysis shows *hTRAIL* expression after doxycycline induction in TRAIL-expressing–ES cell-derived astrocytes. Actin and absence of RT (no RT) are used as positive and negative controls, respectively. B: Immunofluorescence photomicrographs of TRAIL-expressing–ES cell-derived astrocytes before *(left)* and after *(right)* induction of doxycycline. Note the strong *TRAIL* expression after doxycycline induction. Alexa 488, original magnification × 40. C: Flow cytometric analysis showing *TRAIL* expression. Baseline control without anti–TRAIL antibody *(left column)* and after labeling with anti–TRAIL antibody *(center column)*. Flow cytometric analysis shows that up to 75% of ES cell–derived atrocytes expresses *TRAIL* after doxycycline induction *(lower center)*. Minimal expression is noted in absence of doxycycline *(upper center)*. Histograms are shown in the *right column*. The area edged in red indicates the baseline control and the black area cells after labeling with anti–TRAIL antibody. A right shift indicated *TRAIL* expression after induction with doxycycline *(lower right)*; no shift was detected without induction *(upper right)*.

undergo apoptosis in presence of TRAIL-expressing–ES cell-derived astrocytes after doxycycline induction.

Discussion

Advantages of ES Cell–Derived Astrocytes as Brain Tumor Gene Therapy Vectors

Astrocytes have several theoretical advantages as vectors for delivery of genetic materials to the brain.[28] Astrocytes are a cell type native to the central nervous system and therefore should survive and function well after transplantation into the brain. They normally support and protect neuronal survival and function.[7] Finally, they have considerable migratory capacity.[11,32] Although astrocytes for use as gene therapy vectors could be obtained from several sources, including neural stem cell cultures or autologous brain tissue expanded ex vivo, only the use of ES cells as a source of astrocytes allows permanent transgene insertion without the use of potentially hazardous viral vectors. In addition, ES cell–derived astrocytes allow production of unlimited numbers of cells for transplantation. We previously described a new protocol for directed differentiation of transgenic mouse ES cells into astrocytes.[6] In this study, we proved that these astrocytes were able to kill human malignant glioma cells in vitro.

Importance of Conditional Antitumor Transgene Expression

Most putative therapeutic transgenes have potential adverse effects. For example, although the authors of early studies of *TRAIL* administration in rodent and primate models suggested that toxicity was absent,[21] subsequent investigators suggested that some hepatotoxicity and neurotoxicity activity was present.[34] Whereas most of this toxicity is

probably due to heterologous peptide "tags" inserted into recombinant *TRAIL* proteins,[34] even wild-type *TRAIL* protein may be neurotoxic in some conditions.[30] For this reason, conditional, highly regulated expression of *TRAIL* and other potentially therapeutic transgenes will be desirable. In our study, we documented that transgene expression could be regulated and readily induced using doxycycline, a safe and commonly used antibiotic agent. Flow cytometric analysis showed that 93 ± 5% of ES cells and 73 ± 2% ES cell–derived astrocytes with transgenic *TRAIL* insertion were expressing *TRAIL* after doxycycline induction. These data indicate that ES cell–derived astrocytes can express transgenes under tight control of a doxycycline-sensitive promoter. A decrease in *TRAIL* expression that was noted when cells differentiated from ES into astrocytes was not surprising, and it is a reported fact in embryonic transgenic expression. The ability to cause transgenic *TRAIL* expression with doxycycline has a significant impact in the clinical setting, as it is a well tolerated oral antibiotic agent.

Apoptotic Effects of Recombinant TRAIL and Transgenic TRAIL *on Human Malignant Glioma Cells*

The proapoptotic effects of recombinant TRAIL have been well described in the recent literature.[2,15,19,24] Tumor necrosis factor–related apoptosis-inducing ligand is a member of the tumor necrosis factor–α family and induces apoptosis in various tumor cell types, including malignant glioma cell lines,[26] while sparing most normal cells.[27,29,39] The fact that messenger RNA for both *TRAIL* and its receptors is often expressed in the same cell[12] hampered the enthusiasm that TRAIL could be used selectively to kill only tumor cells while sparing normal cells. As the recent literature shows, however, authors have indicated that this concern should not discourage the use of *TRAIL* in therapeutic approaches to tumors because exposure to *TRAIL* in combination with other chemotherapeutic agents results in selectively sensitizing malignant glioma cells.[3,13,35]

Various investigators have shown the antitumor effects of *hTRAIL* following systemic administration of soluble *TRAIL* alone or in combination with chemotherapeutic agents;[4,8,38] however, the requirement of a large amount of *TRAIL* and its short half-life are the major hindrances of such a treatment paradigm.

Previous researchers have shown the feasibility of delivering *TRAIL* by using a herpes simplex virus[33] or adenovector (Ad.*TRAIL*)[23] vectors to transduce normal brain cells. In the latter, mice harboring glioblastoma showed significantly increased survival when treated with the *TRAIL* adenovector.[23] Our previous clinical experience with adenoviral vectors,[14] corroborating the experience of others, underlined the limitations of viral vectors, including lack of tissue penetration.[9] These limitations encouraged us to develop a different approach for translation. As described, ES cell–derived astrocytes were a logical choice, and among the various genes, *TRAIL* seemed to be the most promising for apoptotic induction in human glioma cells while sparing the normal astrocytes. The data obtained in this study confirm our hypothesis. Transgenic *TRAIL* caused a decreased viability in human malignant glioma cells to 18% with an increased apoptotic rate up to 88%. It is important to note that *TRAIL*-expressing astrocytes offered a more powerful apoptotic action than recombinant *TRAIL*. This can be explained by the fact that recombinant *TRAIL* is membrane bound. Thus, direct cell contact obtained in coculture is more powerful than exposure to purified protein. Additionally, cell vectors might maximize appropriate posttranscriptional translational modification of protein. These aspects are attractive features when considering the use of our transgenic construct to treat in situ malignant brain tumors.

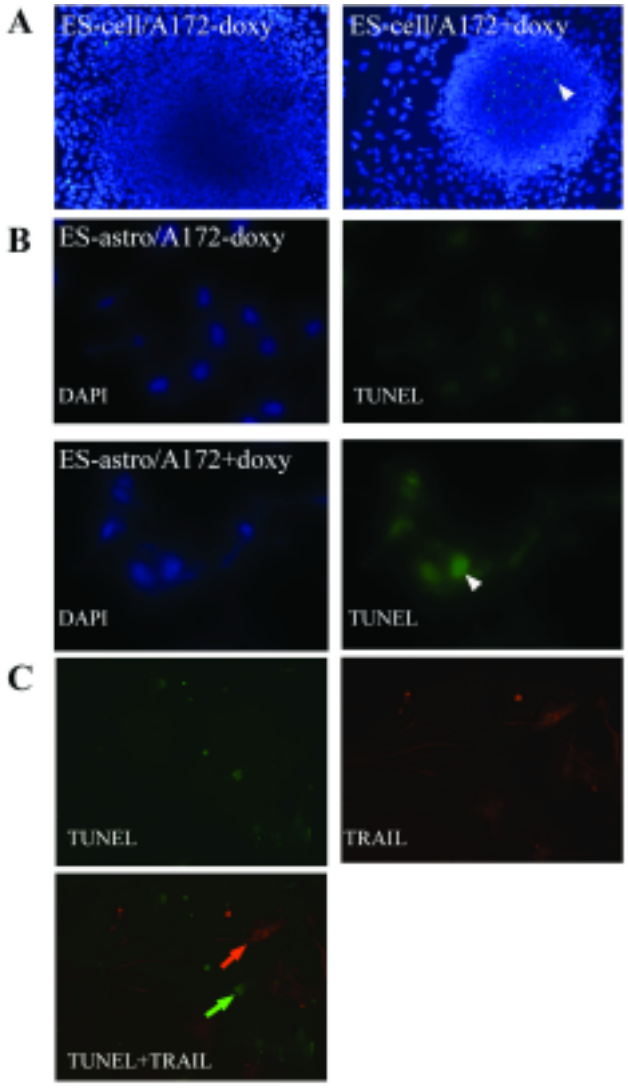

FIG. 4. Evidence that *TRAIL*–expressing ES cells and ES cell–derived astrocytes induce apoptosis in human malignant glioma cells. A: Photomicrographs of in vitro cocultures of *TRAIL*–expressing ES cells before *(left)* and 24 hours after exposure to doxycycline *(right)*. The TUNEL-positive cells *(arrowhead)* are seen after doxycycline induction. Original magnification × 40. B: Photomicrographs of in vitro cocultures of TRAIL-expressing–ES cell-derived astrocytes and A172 cells before *(upper)* and after *(lower)* induction with doxycycline. The TUNEL-positive cells are seen after doxycycline induction *(arrowhead)*. Nuclear stain DAPI *(left)* and TUNEL *(right)*, original magnification × 40. C: Immunohistochemistry labeling with TUNEL, TRAIL, and TUNEL/TRAIL. The TRAIL-positive cells *(red arrow)* are TUNEL negative and TUNEL-positive cells are TRAIL negative *(green arrow)*. Antibody visualization for TRAIL: CY3, original magnification × 40.

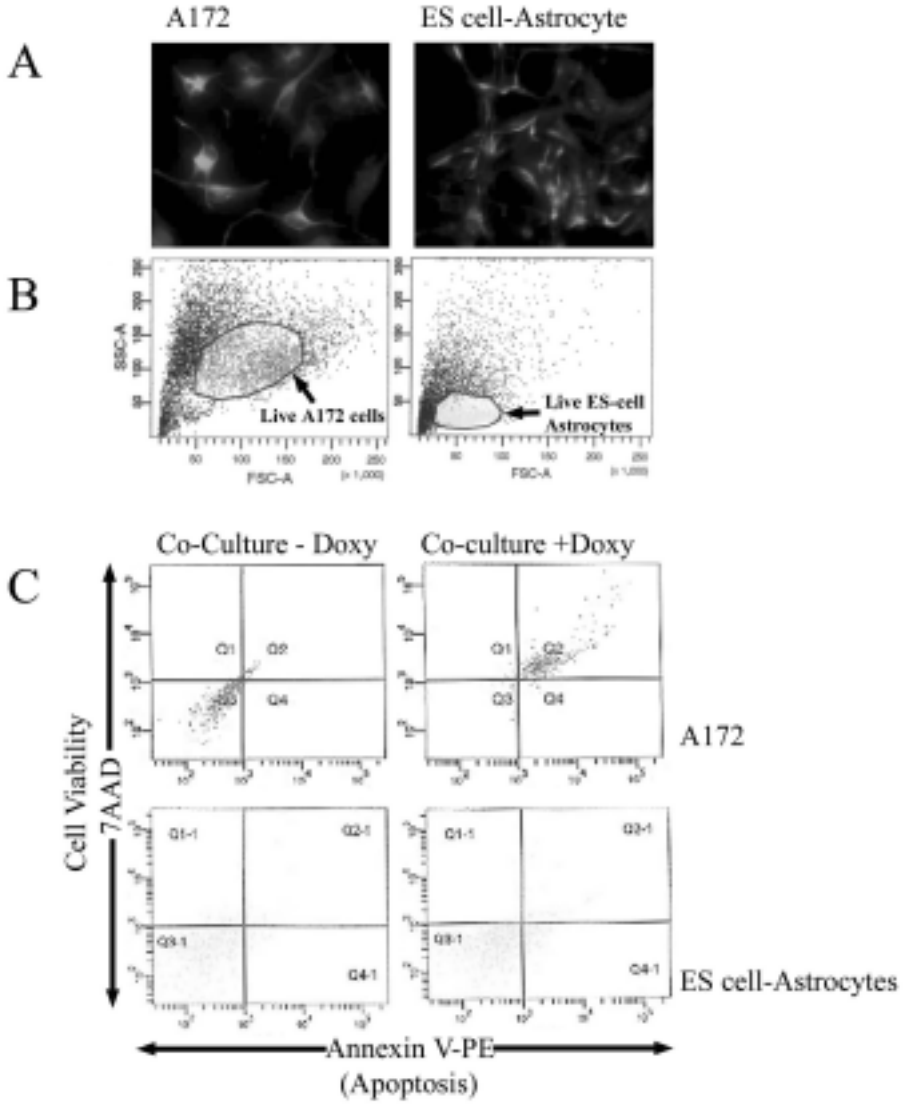

FIG. 5. Fluorescence-activated cell sorter analysis based on morphological gating of coculture *TRAIL*-expressing–ES cell-derived astrocytes and A172 tumor cells. A: Immunofluorescence photomicrographs of malignant A172 glioma cells and ES cell–derived astrocytes showing GFAP expression labeled with monoclonal anti-GFAP antibody and counterstained with DAPI. The A172 cells are larger, more spherical in shape, and exhibit fewer processes than ES cell–derived astrocytes. Antibody visualization: Alexa 488, original magnification × 40. B: Flow cytometric analysis of each cell population at baseline gated for size (forward scatter and side scatter). C: Gated flow cytometric analysis of A172 glioma cells with *TRAIL*-expressing–ES cell-derived astrocytes before *(left column)* and after *(right column)* doxycycline induction. Cocultured A172 glioma cells show a shift into Annexin V–PE-positive quadrant consistent with apoptosis. No significant effects are observed in the ES cell–derived astrocytes in similar conditions.

Finally, our data corroborate the evidence that transgenic *TRAIL* does not induce apoptosis in nontumoral astrocytes, supporting previous work.[18,26]

Conclusions

The paucity of tumor-specific apoptotic genes makes *TRAIL* an appealing choice for adjuvant treatment of malignant brain tumors. In this study, we showed that the engineering of transgenic *TRAIL* involving ES cell–derived astrocytes induced apoptosis in human malignant glioma cells while sparing nontumoral astrocytes. We also showed that the apoptotic effects of transgenic *TRAIL* are greater than those of recombinant *TRAIL*. These results suggest that our transgenic approach should be used in the development of new in vivo strategies to treat malignant human gliomas.

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