Intratumor Administration of Interleukin 13 Receptor-targeted Cytotoxin Induces Apoptotic Cell Death in Human Malignant Glioma Tumor Xenografts[1]

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Abstract

Apoptosis is not only essential for homeostasis in normal cells but also in cancer cells, in which it is associated with cell death mechanisms caused by novel therapeutics. We have previously reported that interleukin-13 receptors (IL-13R) are constitutively overexpressed on a majority of human malignant glioma cell lines and primary cell cultures. In addition, we have reported that IL-13 cytotoxin, comprised of human IL-13 and a mutated form of *Pseudomonas* exotoxin, is highly and specifically cytotoxic to these cells and can lead to pronounced antitumor activity in malignant glioma tumors in animal models. However, the molecular mechanisms of tumor cytotoxicity induced by IL-13 cytotoxin are poorly understood. In this study, we demonstrate that glioma tumors undergo apoptotic cell death on intratumoral administration of IL-13 cytotoxin. This conclusion was made based on (*a*) time-dependent induction of several proapoptotic molecules, such as caspases (caspase-3, -8, and -9) in tumors; (*b*) cleavage of procaspase-3 and poly(ADP-ribose) polymerase (PARP); and (*c*) the release of cytochrome *c* from mitochondria to the cytosol on injection of IL-13 cytotoxin in U251 glioblastoma tumors established in immunodeficient animals. These indicators of two major pathways of apoptosis were detected in tumors even though IL-13 cytotoxin was no longer present in tumors. In addition, we found that inducible nitric oxide was expressed in tumors in a time-dependent manner with primary localization in infiltrating phagocytes after treatment with IL-13 cytotoxin. These studies demonstrate that IL-13 cytotoxin mediates apoptotic death of glioma cells, resulting in regression of established tumors. Our studies will help unravel the molecular pathways of cell death associated with tumor regression and provide additional insight and define apoptosis as possible surrogate marker of tumor response.

Introduction

We have previously reported that a variety of human tumor cell lines, including those derived from malignant glioma, express receptors for a Th2-derived cytokine, IL[3]-13 (1–4). We have developed an IL-13R-directed recombinant cytotoxin, IL13-PE38QQR, which is composed of IL-13 and a truncated form of PE. This cytotoxin is highly cytotoxic to glioma cell lines *in vitro* and *in vivo*. This cytotoxin also mediates significant antitumor activity in a nude-mice animal model of glioma tumors (5). The molecular mechanisms of cell death mediated by IL-13 cytotoxin *in vivo* are not known.

Apoptosis, or programmed cell death, is a major pathway of cell death described by its morphological characteristics, including chromatin condensation and nuclear fragmentation (6, 7). Recently, two major interacting pathways of apoptosis have been identified. In the first pathway, apoptosis is induced by the engagement of proapoptotic cytokines to specialized cell surface death receptors (CD95/Fas/Apo and tumor necrosis factor receptor; Refs. 8, 9). These death receptors specifically bind to their ligands such as FasL and lead to the activation of FADD (Fas-associated death domain) and then initiator caspases such as caspase-8 and -10 through death effector domain. Caspase-8 then activates downstream effector caspases (caspase-3, -6, and -7) leading to the terminal event, cell death. Another pathway of apoptosis is triggered by changes in the homeostasis of mitochondria (8, 10). Cytochrome *c* is released from the mitochondria into the cytosol after the induction of apoptosis (10–12). The released cytochrome *c* activates apoptosis activating factor-1 and caspase-9 through caspase-recruitment domain. Activated caspase-9 leads to the activation of effector caspases such as caspase-3 (CPP32), which results in proteolytic cleavage of numerous substrates including PARP, a nuclear enzyme involved in DNA repair (13–15).

In addition to the two highly investigated pathways of apoptosis, NO, an important signal transduction molecule, has been implicated in a variety of functions such as vasodilatation, neurotransmission, host defense, and apoptosis (16–18). NO also plays a key role in the cytostatic/cytotoxic function of the immune system. It is endogenously produced

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[3] The abbreviations used are: IL, interleukin; IL-13R, IL-13 receptor; PE, *Pseudomonas* exotoxin; GBM, glioblastoma multiforme; i.t., intratumoral/intratumorally; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; PARP, poly(ADP-ribose) polymerase; *p*NA, *p*-nitroanilide; HSA, human serum albumin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TBS, Tris-buffered saline; TBST, TBS with Tween 20; Ab, antibody.

by NOS, a group of enzymes including three different isoenzymes in mammalian cells (18). Inducible isoenzyme (iNOS) was originally isolated from mouse macrophages, but it is now known to be expressed in a variety of cell types. The iNOS is not constitutively expressed in cells. Although the function of NO has been extensively studied *in vivo*, its role in apoptosis is still poorly understood. It has been shown that lipopolysaccharide or cytokines such as IFN-γ and IL-1β can induce iNOS in target cells, which then release copious amounts of NO in short period of time. NO then interacts with superoxide resulting in cellular cytotoxic activity (19, 20). In contrast, NO has also been reported to be a potent inhibitor of apoptosis through *S*-nitrosylation of the active cysteine residues conserved in all caspases (21, 22).

Because targeted immunotoxins and cytotoxins can mediate apoptosis in breast carcinoma and in head and neck tumor cells (23–25), and we have observed that IL-13 cytotoxin can induce apoptosis in tumor cells *in vitro*, in this study we have examined whether IL-13 cytotoxin can induce apoptosis *in vivo* in malignant glioma tumors. We demonstrate that IL-13 cytotoxin can induce significant apoptosis of glioma cells in a time-dependent manner. Even though IL-13 cytotoxin was rapidly metabolized in tumors and its level was nondetectable, some apoptotic activities continued to be detected, resulting in potent *in vivo* antitumor activity of IL-13 cytotoxin.

Materials and Methods

Recombinant IL-13 Cytotoxin and Cell Culture. Recombinant IL-13 cytotoxin (IL13-PE38QQR), which is composed of human IL-13 and a truncated form of PE, was produced and purified from *Escherichia coli* in our laboratory (26). The endotoxin level in purified IL-13 cytotoxin preparation was 6.4 endotoxin unit/dose. U251, human GBM cell line was obtained from the National Cancer Institute, Frederick Cancer Research Facility, Division of Cancer Treatment Tumor Repository Program (Frederick, MD). A172 and T98G cell lines were purchased from American Type Culture Collection (Manassas, VA). U251 and A172 cells were cultured in RPMI 1640 and T98G cells were cultured in Eagle's modified essential medium (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum, 1 mM HEPES, 1 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker).

Detection of Apoptosis *in Vitro* by Bis-Benzimide Staining. Apoptotic cells were assessed morphologically by fluorescent microscopy. U251, A172, or T98G cells (2×10^6) were plated in 100-mm Petri dishes and exposed to medium only (control), IL-13 cytotoxin (100 ng/ml), or IL-13 (100 ng/ml) for 48 h. Cells were then washed and fixed with 1% glutaraldehyde, and stained with 1 mM bis-benzimide (Hoechst no. 33342; Sigma Chemical Co., St. Louis, MO). The cells were examined by fluorescent microscopy under UV filter.

Flow Cytometry. Cells (8×10^5) were treated with control media or IL-13 cytotoxin (100 ng/ml) in 60-mm Petri dishes and harvested by trypsinization at 0, 6, 12, 24, 48, 72, and 96 h and fixed with cold 70% ethanol. Then, cells were washed with PBS and incubated with 0.5% RNase for 30 min at room temperature and stained with 25 μg/ml propidium iodide. DNA population was analyzed by flow cytometry.

Animals and Treatments. Four-week-old (body weight, ~20–22 g) male athymic *nu/nu* mice were obtained from Frederick Cancer Center Animal Facilities (National Cancer Institute, Frederick, MD). The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. Animal care was in accordance with the guidelines of the NIH Animal Advisory Committee. U251 cells were implanted by s.c. injection of 5×10^6 cells in 150 μl of PBS with 0.2% HSA into the dorsal surface of mice. The tumors were developed in 4–5 days to ~100 mm^3 volume and then i.t. administration with excipient only (0.2% HSA/PBS) or with IL-13 cytotoxin at a concentration of 100 μg/kg was performed. After administration of IL-13 cytotoxin, mice were killed at 0.5, 1, 2, 4, 6, 24, 48, and 72 h, and tumors were excised for lysates and tissue sections.

Apoptosis Detection by H&E or TUNEL Staining *in Vivo*. Tumors that developed in nude mice were excised and sectioned to 5-μm thickness. IL-13 cytotoxin (100 μg/kg) or 0.2% HSA/PBS for control had been previously injected i.t. Apoptotic cells in tumor sections were identified and counted after staining with H&E or with the TUNEL-based ApopTag *in situ* hybridization detection kit (Intergen, Purchase, NY). Previously established cytomorphological criteria were used to assess apoptosis. These include chromatin condensation, cytoplasmic budding to form membrane-bound fragments (apoptotic bodies), and nuclear fragmentation (6, 7). Apoptotic cells in tumors were counted with a light microscope and percentages were calculated. Cancer cells ($n = 1000$) were counted under $\times$400 magnification in 10 chosen fields from tissue sections derived from entire tumor tissue. The number of apoptotic cell counts was expressed as a percentage of 100 viable tumor cells.

Caspase Activity Assay. The excised tumors were homogenized in lysis buffer (BioVision Inc., Palo Alto, CA) by grinding with a pestle and then passing several times through 22-gauge needles. The whole tumor lysates were kept on ice for 10 min and were clarified by centrifugation at 10,000 rpm for 1 min at 4°C. Caspase activity in the supernatant was determined using a caspase colorimetric assay kit (BioVision) as described previously (27, 28). Briefly, 100 μg of total protein as determined by the Bio-Rad protein assay (Bio-Rad, Richmond, VA) in the tumor lysate, was incubated with 5 μl of each caspase substrate (4 mM; BioVision), DEVD (*N*-acetyl-Asp-Glu-Val-Asp)-*p*NA (caspase-3), IETD (*N*-acetyl-Ile-Glu-Thr-Asp)-*p*NA (caspase-8), and LEHD (*N*-acetyl-leu-Glu-His-Asp)-*p*NA (caspase-9) in a total 100 μl of caspase assay reaction buffer (final concentration of substrate, 200 μM). After incubation at 37°C for 2 h, caspase activity was determined by measuring the cleavage of these substrates. The light absorption was monitored using a MRX II microplate reader (DYNEX, Chantilly, VA) at 405 nm, which measures *p*NA cleaved from the labeled substrates by activated caspases.

Preparation of Subcellular Fractions. The procedure described in previous reports was adopted with modifications (29, 30). Treated cells or tumors were homogenized in isotonic sucrose buffer containing 250 mM sucrose, 10 mM

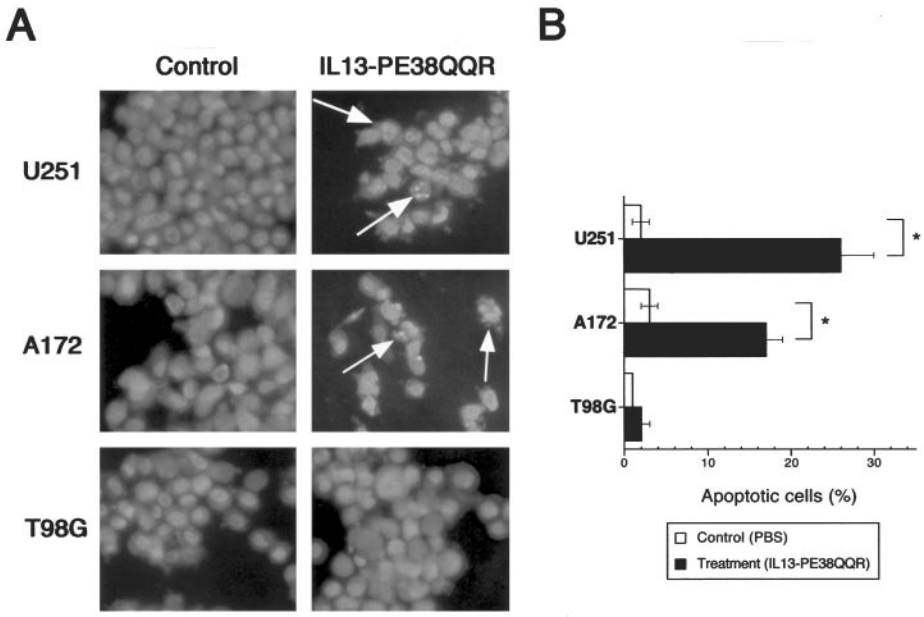

Fig. 1. *A*, detection of apoptosis in human GBM cell lines. GBM cell lines (U251, A172, and T98G) were incubated with only PBS for control or with IL-13 cytotoxin (100 ng/ml) for 48 h, fixed with 1% glutaraldehyde, and stained with 1 mM bis-benzimide. The pictures shown were taken with a UV microscope (×400). *Arrows*, apoptotic cells showing nuclear condensation. *B*, percentages of apoptotic cells were calculated at 48 h after incubation with IL-13 cytotoxin. *Bars*, mean ± SD of triplicate determinations.

HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA (pH 7.1) plus 0.025% digitonin and complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN) with 1% BSA. After removing nuclear proteins by centrifugation, lysates were centrifuged then supernatant was designated "cytosolic fraction." The pellet was resuspended in isotonic sucrose buffer containing 0.5% Triton X-100 and complete protease inhibitor mixture and was kept for 10 min on ice, after which the Triton X-100-insoluble fraction (including mitochondria) was separated by centrifugation. Fractions were resolved in 15% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with rabbit antihuman cytochrome *c* Ab (Santa Cruz Biotechnology).

Western Analysis. Whole-cell extracts from tumor tissues were mixed with SDS sample buffer and heated for 5 min at 70°C. Proteins (20–50 μg/lane) were separated by 4–12% SDS/PAGE and transferred onto a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA). Membranes were blocked for 1 h in TBST [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% Tween 20] and 5% skim milk. After blocking, membranes were incubated for 1 h with mouse antihuman procaspase-3 monoclonal Ab (Transduction Laboratories, Lexington, KY), rabbit antihuman PARP monoclonal Ab (Boehringer Mannheim, Indianapolis, IN), rabbit anti-PE polyclonal Ab (Sigma), or rabbit antimouse iNOS polyclonal Ab (Santa Cruz Biotechnology) in TBST. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary Ab in TBST for 30 min and were developed by using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia).

Antitumor Activity *in Vivo*. Mice with established tumors, by s.c. injection of 5×10^6 U251 cells in 150 μl of PBS plus 0.2% HSA, received either the excipient (0.2% HSA in PBS) or the IL-13 cytotoxin i.t. (30 μl) using a 27-gauge needle. Tumor sizes were carefully measured by a Vernier caliper. The tumor volume on a given day was calculated by the formula: (length of the tumor) × (width of the tumor)^2/2. Each treatment group consisted of seven mice.

Immunohistochemistry. Frozen sections (5-μm) were prepared and fixed in acetone at −20°C for 5 min. Nonspecific binding was blocked by treatment with 10% goat serum for 1 h followed by incubation with Ab or isotype control. Slides were stained with 1:500 rabbit antimouse NOS2 polyclonal Ab (M-19; Santa Cruz Biotechnology) and developed using a DAB (3,3′-diaminobenzidine) substrate biotinylated peroxidase kit (Vector Laboratories, Inc., Burlingame, CA). Tissues were counterstained with hematoxylin (Sigma, St. Louis, MO).

Statistical Analysis. The statistical significance of tumor regression and various parameters was calculated by Student's *t* test.

Results

IL-13 Cytotoxin Causes Apoptosis in GBM Cells *in Vitro*. To investigate whether IL-13 cytotoxin causes apoptosis in human GBM tumor cells *in vitro*, three GBM cell lines (U251, A172, and T98G) were incubated with IL-13 cytotoxin, and morphological changes, including chromatin condensation, were examined by bis-benzimide staining. As shown in Fig. 1*A*, after treatment with IL-13 cytotoxin, apoptotic cells were detected in U251 and A172 cell lines that overexpressed IL-13R, whereas T98G cells not expressing high levels of IL-13R and all control cell lines incubated with PBS did not show apoptosis. The percentage of apoptotic cells significantly increased as a result of the IL-13 cytotoxin treatment in U251 and A172 cell lines compared with respective untreated control cells ($P < 0.05$; Fig. 1*B*). In contrast, no increase in apoptotic cells was observed in the T98G cell line. Furthermore, IL-13 (100 ng/ml) did not cause apoptosis in any of the three cell lines (data not shown).

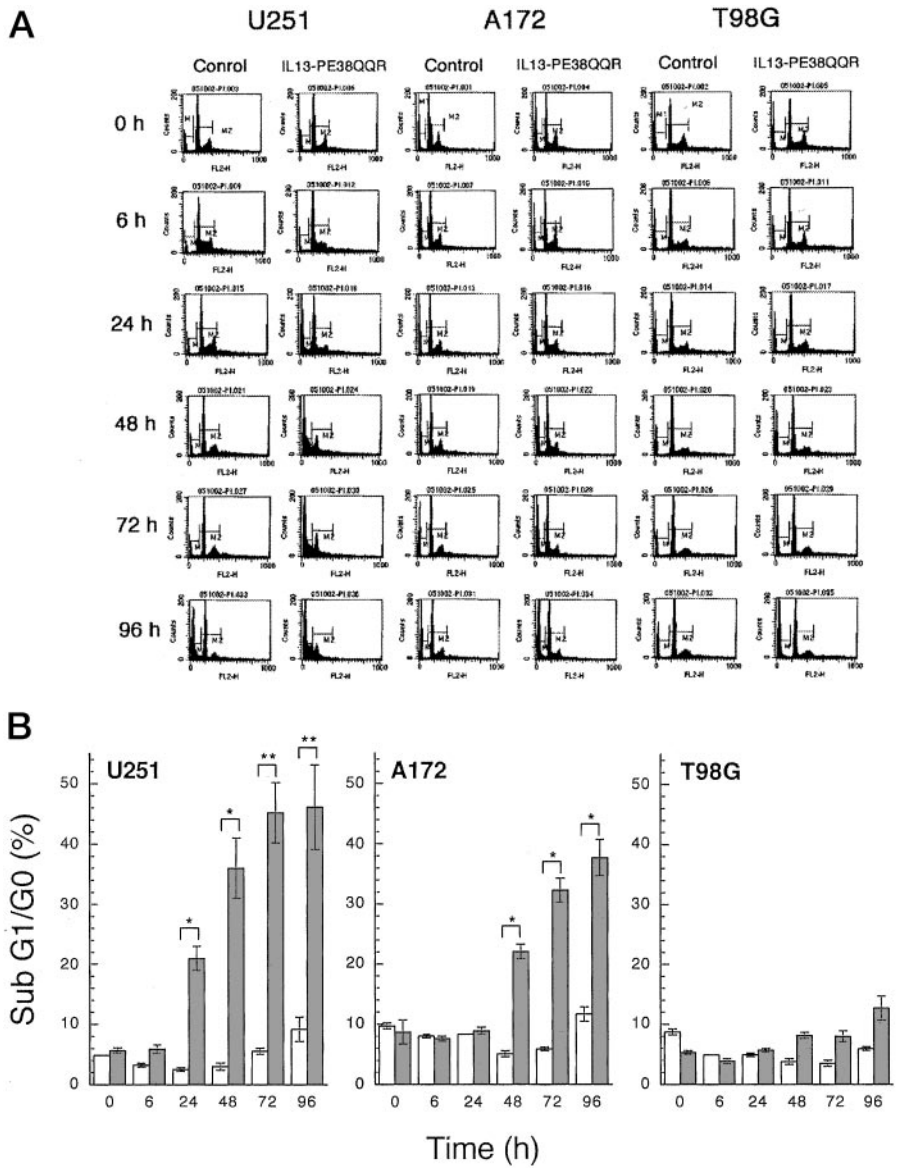

Fig. 2. Increase in hypodiploid DNA in U251 and A172 cell lines after IL-13 cytotoxin treatment. *A*, U251, A172, or T98G cell lines were incubated with PBS for control or IL-13 cytotoxin (100 ng/ml) for 0–96 h, and cells were assessed by flow cytometric analysis. The sub-G_1-G_0 cell populations (M1 region) were gated, and then percentages were determined. Data are representative of two independent experiments with comparable results. *B*, the results are presented as the mean ± SE of four independent experiments as histograms. □, control (PBS); ▨, treatment (IL-13-PE38QQR); *, significantly different from control at $P < 0.05$; **, significantly different from control at $P < 0.01$.

Increase in Sub-G_1-G_0 Population of GBM Cells after Treatment with IL-13 Cytotoxin *in Vitro*. To assess the rate of apoptosis, we measured the sub-G_1-G_0 cell population in U251, A172, and T98G GBM tumor cells by flow cytometry after incubation with IL-13 cytotoxin (Fig. 2*A*). The sub-G_1-G_0 cell population represents cells with fragmented DNA. We found that the sub-G_1-G_0 cell population (represented by the M1 region) increased in a time-dependent manner in U251 and A172 cells treated with IL-13 cytotoxin but not in T98G IL-13 cytotoxin-treated cells (Fig. 2, *A* and *B*). The sub-G_1-G_0 cell population was monitored over time, and data were expressed as a histogram (Fig. 2*B*). After IL-13 cytotoxin incubation, the sub-G_1-G_0 cell population significantly increased by 8.3-fold (24 h) and 11.9-fold (48 h; $P < 0.05$) and 8.1-fold (72 h) and 4.6-fold (96 h; $P < 0.01$) in U251 cell lines and by 4.3- (48 h), 5.4- (72 h), and 3.2- (96 h) folds ($P < 0.05$) in A172 cell lines, respectively, compared with control (untreated) group at each time point. On the other hand, T98G cell line showed only a small increase in the sub-G_1-G_0 cell population.

i.t. Administration of IL-13 Cytotoxin Causes Apoptosis in U251 Tumors in a Xenograft Model. To assess whether IL-13 cytotoxin can induce apoptosis *in vivo*, U251 tumors were harvested from each treatment group (0, 0.5, 1, 2, 4, 6, 24, 48, and 72 h) after i.t. administration of IL-13 cytotoxin at a dosage of 100 μg/kg. Tissue sections (5 μm) were examined after H&E or TUNEL staining. Tissue sections derived from IL-13 cytotoxin-treated tumors (after 24 h) showed apoptotic cells (*arrows* or *green fluorescence*; Fig. 3*A*), whereas control sections treated with only excipient (0.2% HSA/PBS) did not show any apoptotic cells by either assay (Fig. 3*A*). The same tissue sections stained with H&E or TUNEL were also subjected to the counting of apoptotic cells, and the percentage of apoptotic cells in total tumor cell fractions

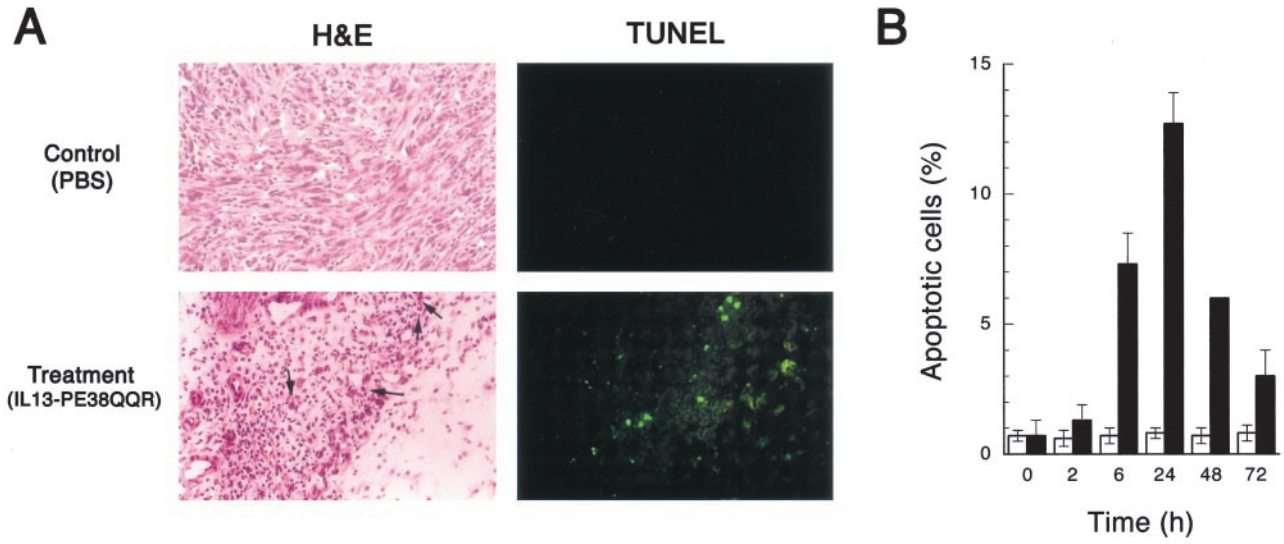

Fig. 3. Incidence of apoptosis in U251 tumor xenografts after a single i.t. administration of IL-13 cytotoxin *in vivo*. *A*, U251 tumors developed by s.c. implantation of 5×10^6 cells in nude mice that were treated with excipient only (0.2% HSA/PBS) for control or with IL-13 cytotoxin (100 μg/kg) by single i.t. injection. Tumors were excised at several time points. Tissue sections were stained with H&E or TUNEL ($\times$400). Apoptotic cells (*arrows* or *green fluorescence*) are seen in the sections 24 h after injection of IL-13 cytotoxin. *B*, apoptotic cells were counted at several time points after single i.t. injection of PBS only for control or of IL-13 cytotoxin. □, control (PBS); ■, treatment (IL-13-PE38QQR); *bars*, mean ± SD of apoptotic cells in 10 tissue fields.

including viable and dead cells was calculated (Fig. 3*B*). In control animals, which received only excipient, or in animals in which IL-13 cytotoxin was i.t. injected and tumors examined after 2 h, few apoptotic cells were detected. However, 6 h after injection, the incidence of apoptotic cells began to increase from 7.3 ± 1.2% to a peak of 12.7 ± 1.2% ($P < 0.05$) at 24 h compared with that in control tumors. After 24 h, although the percentage of apoptotic cells decreased, these cells still persisted for up to 72 h after a single injection of IL-13 cytotoxin. At 72 h, an ~3-fold higher number of apoptotic cells were detected in IL-13 cytotoxin-treated tumors compared with tumors at time 0.

Activation of Caspases Induced by Single i.t. Administration of IL-13 Cytotoxin in U251 Tumors. To evaluate the intracellular modulation of proapoptotic molecules, we measured the activities of caspase-3, -8, and -9 in U251 tumors at various time periods (0, 0.5, 1, 2, 4, 6, 24, 48, and 72 h) after a single i.t. administration of IL-13 cytotoxin. Control xenograft tumors received excipient only (0.2% HSA/PBS) by i.t. administration, and separate control tumors were analyzed at each time point. As a result of IL-13 cytotoxin treatment, all three of the caspases were activated, reaching a maximum level between 2–4 h after IL-13 cytotoxin administration (Fig. 4*A*). The activity of caspase-8 and -9 returned to basal levels 72 h after IL-13 cytotoxin administration. However, caspase-3 activity was 1.5 times higher, compared with control tumors ($P < 0.05$). Because caspase-3 is an effector caspase, its persistence 72 h after IL-13 cytotoxin administration suggests the continuation of some apoptotic process. Control excipient-treated tumors at each time point did not show any change in caspase activity.

Cleavage of Procaspase-3 and PARP by i.t. Injection of IL-13 Cytotoxin in U251 Tumors. Cellular homogenates from excipient or IL-13 cytotoxin-treated tumors (Fig. 4*B*) were analyzed by Western blot to determine whether procaspase-3 and PARP were cleaved. Cleavage was documented by a decrease in intensity of the procaspase-3 band or conversion of the M_r 116,000 PARP band to M_r 89,000 and M_r 24,000 bands. Procaspase-3 was cleaved rapidly, showing a visual decrease in the M_r 32,000 band within 30 min and an additional decrease in band intensity at 2 h. The procaspase-3 band intensity subsequently increased, reaching the basal level 24–48 h after IL-13 cytotoxin administration. Although PARP concentration was lower in the control group, it appeared to be rapidly cleaved by caspase-3 between 6 and 72 h after IL-13 cytotoxin administration (Fig. 4*C*). Control excipient-treated tumors did not show cleavage of procaspase-3 or PARP.

Induction of Release of Cytochrome *c* from Mitochondria to the Cytosol in U251 Cells by IL-13 Cytotoxin *in Vitro* and *in Vivo*. To investigate whether IL-13 cytotoxin-induced apoptosis involves mitochondrial homeostasis, we studied the release of cytochrome *c* from mitochondria to cytosol in U251 tumors after the incubation of tumor cell lines with IL-13 cytotoxin *in vitro* or i.t. injection of IL-13 cytotoxin *in vivo*. As shown in Fig. 5*A*, incubation of U251 tumor cells with IL-13 cytotoxin (100 ng/ml) caused a release of cytochrome *c* into the cytosol that was clearly detected after 24 h of incubation. Cytochrome *c* release continued to increase up to 72 h in a time-dependent manner. The mitochondrial fraction of treated U251 cells did not show any change in cytochrome *c*. Similarly, on a single i.t. administration of IL-13 cytotoxin (100 μg/kg) *in vivo*, cytochrome *c* release into the cytosol was detected by 2 h and increased to a maximum level at 6 h and maintained this level up to 72 h after IL-13 cytotoxin administration (Fig. 5*B*). These results suggest that IL-13 cytotoxin can use the mitochondrial pathway of programmed cell death in glioma tumor cells *in vitro* and *in vivo*.

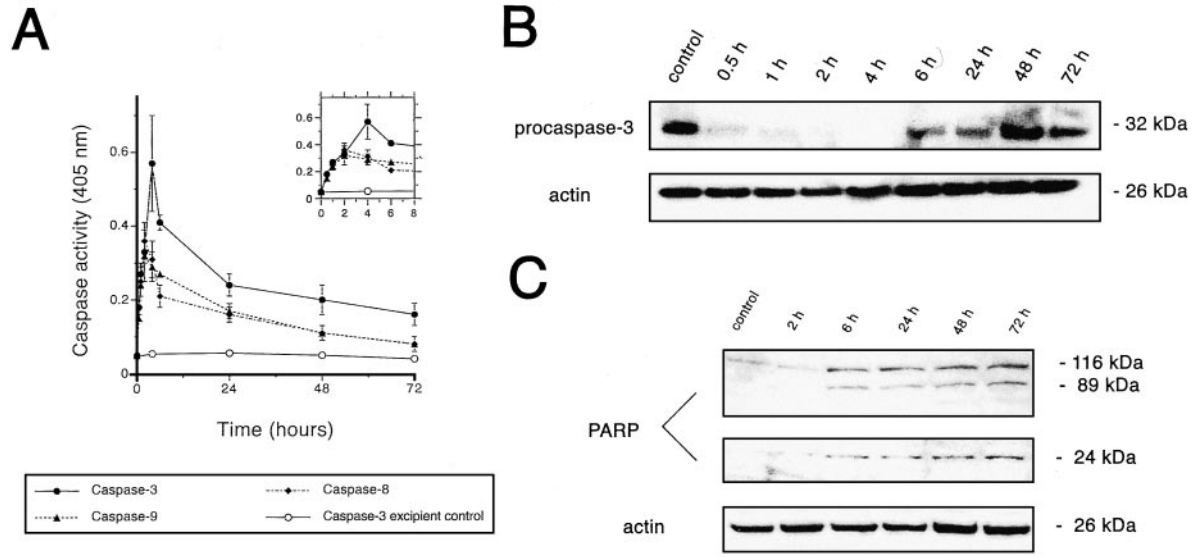

Fig. 4. Activation of caspase in U251 tumors after i.t. injection of IL-13 cytotoxin. *A*, after single i.t. injection of excipient only or of IL-13 cytotoxin, tumors were excised and caspase activities in the tumor lysates determined by colorimetric methods by measuring the cleavage of each caspase substrate. A total of 100 μg of total cellular protein was used at each time point. *Error bars*, the mean $\pm$ SE of two independent experiments. *Insert graph*, the magnified portion of full graph to show changes at early time points. *B*, Western blot analysis to detect cleavage of procaspase-3 (*32 kDa*) after single i.t. injection of IL-13 cytotoxin in U251 tumors. *C*, PARP was cleaved to M_r 89,000 and M_r 24,000 fragments after i.t. treatment with IL-13 cytotoxin. Actin protein served as an internal control in both assays (*B* and *C*). *kDa*, M_r in thousands.

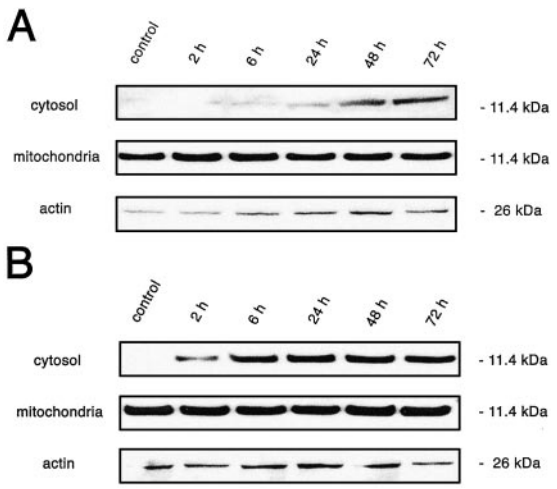

Fig. 5. Induction of cytochrome *c* release from mitochondria to the cytosol after IL-13 cytotoxin treatment *in vitro* and *in vivo*. *A*, Western blot analysis of cytochrome *c* in cytosolic and mitochondrial fractions isolated from U251 tumor cells incubated with IL-13 cytotoxin (100 ng/ml). *B*, U251 tumors, treated with single i.t. administration of IL-13 cytotoxin, were homogenized, and Western blot analysis of cytochrome *c* in cytosol and mitochondrial fractions was performed. Actin protein served as an internal control. *kDa*, M_r in thousands.

i.t. Treatment with IL-13 Cytotoxin Causes Regression of Established U251 Tumors. To determine a possible temporal relationship between activation of proapoptotic molecules and tumor response, s.c. established U251 tumors in immunodeficient mice were treated with IL-13 cytotoxin. Tumor-bearing mice were divided into three control groups and three treatment groups: (*a*) control mice receiving a single i.t. injection of excipient; (*b*) control mice receiving three i.t. injections of excipient on alternate days (qod), (*c*) control mice receiving three i.t. injections of excipient once a day every 3 days; (*d*) mice receiving a single i.t. injection of IL-13 cytotoxin (100 μg/kg); (*e*) mice receiving three i.t. injections of IL-13 cytotoxin (100 μg/kg) on alternate days (qod); and (*f*) mice receiving three i.t. injections of IL-13 toxin (100 μg/kg) once a day every 3 days. Tumor volumes were measured every day during the treatment, and then twice a week (Fig. 6). The tumor volumes in three control groups showed no statistically significant difference among each other throughout the experiment. In contrast, U251 tumors began to regress after one or three injections of IL-13 cytotoxin. The single i.t. injected tumors began to grow again, and on day 44, the reduction of tumor volume was 32% compared with control tumors (3371.1 $\pm$ 318.7 mm^3 control *versus* 2289.4 $\pm$ 428.2 mm^3 treated group; $P < 0.005$). Interestingly, the animals receiving three i.t. injections of IL-13 cytotoxin by either of the two different schedules continued to show tumor regression throughout the 44 days of follow-up. Three of seven animals in both of the treated groups showed complete regression of their tumors. These animals remained complete responders until the last day of the experiment. The remaining four animals in both of the treated groups showed regrowth of tumors; however, tumor volumes remained statistically smaller compared with the control group. The reduction in tumor volumes in groups e and f on day 44 was 98.2% (61.7 mm^3; $P < 0.0005$) and 97.7% (73.7 mm^3; $P < 0.0005$), respectively, compared with tumors in the each control group (3456 $\pm$ 398.5, and 3216 $\pm$ 329.7 mm^3, respectively). Tumor volumes in groups e and f showed no statistically significant differences with each

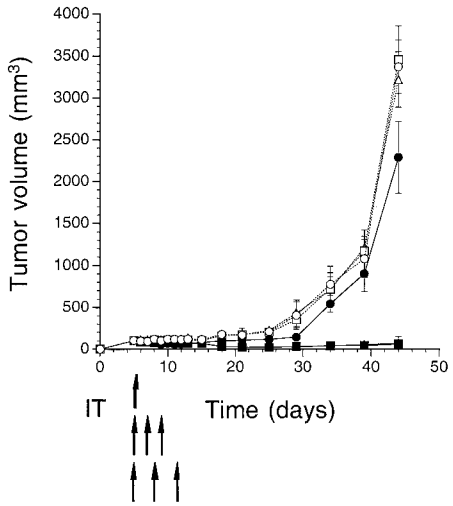

Fig. 6. Antitumor activity of IL-13 cytotoxin in U251 tumors after i.t. administration. After implantation of 5×10^6 U251 cells (at day 0), nude mice received 1 or 3 i.t. injections (two different schedules) of excipient only or of IL-13 cytotoxin (100 μg/kg) on day 5; days 5, 7, and 9; or days 5, 8, 11. ○, control (*IT* day 5); ●, treatment (*IT* day 5); □, control (*IT* days 5, 7, and 9); ■, treatment (*IT* days 5, 7, and 9); △, control (*IT* days 5, 8, and 11); ▲, treatment (*IT* days 5, 8, and 11); *arrows*, the days of injections. Tumor volumes were carefully measured by a vernier caliper. *Tumor volumes*, means ± SD of 7 mice/group. Some *symbols* and *error bars* are overlapped. The experiment shown is representative of two experiments.

other ($P = 0.7$). These results suggest that IL-13 cytotoxin mediates significant antitumor activity by two schedules of i.t. administration.

IL-13 Cytotoxin Disappeared Early after i.t. Administration in U251 Tumors. To investigate the persistence of IL-13 cytotoxin within tumors after a single i.t. administration, and to determine whether its persistence was associated with apoptotic changes in U251 tumors, we determined M_r 50,000 IL-13 cytotoxin levels in U251 tumors by Western blot analysis using an Ab to PE. The maximum levels of IL-13 cytotoxin were detected 30 min after administration, then declined over the course of 4 h; beyond this time, no IL-13 cytotoxin was detected (Fig. 7). Levels of actin control protein remained consistent throughout the 72-h time period. Taken together, our results suggest that the apoptosis process continued even after IL-13 cytotoxin was degraded, absorbed, or undetectable by this assay.

Expression of iNOS in U251 Tumors Treated with IL-13 Cytotoxin. To investigate other molecular determinants that may play a role in regressing tumors, we looked at expression of iNOS in U251 tumors after a single i.t. administration of IL-13 cytotoxin. By immunohistochemical staining for iNOS, we found that infiltrating cells, most likely phagocytes surrounding dying tumor cells, were strongly positive when stained with anti-iNOS Ab (Fig. 8*A*). Control excipient-treated tumors did not show infiltration of phagocytes or staining with iNOS Ab. Induction of iNOS activity in IL-13 cytotoxin-treated tumors was confirmed by Western blot analysis (Fig. 8*B*). Within 30 min after a single i.t. administration of IL-13 cytotoxin, iNOS activity was induced to a maximum level, which was maintained for up to 48 h before becoming slightly decreased by 72 h.

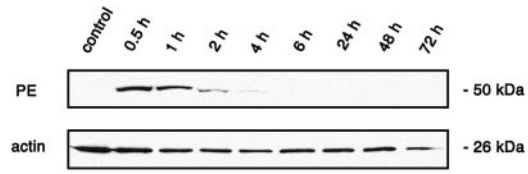

Fig. 7. Persistence of IL-13 cytotoxin after single i.t. administration. Single i.t. injections of IL-13 cytotoxin (100 μg/kg) were made into U251 tumors, and tumors were excised at various time points. Total protein (20 μg/*lane*) isolated from excipient control and IL-13 cytotoxin-treated tumors was subjected to SDS-PAGE. A M_r 50,000 IL-13 cytotoxin band was visualized by immunoblot with an anti-PE Ab. Actin protein served as an internal control. *kDa*, M_r in thousands.

Discussion

In this study, we demonstrate that IL-13 cytotoxin can induce apoptosis in IL-13R overexpressing GBM tumors *in vitro* and *in vivo*. Several proapoptotic molecules were induced in glioma tumor xenografts *in vivo*. We have previously reported that a majority of human GBM cell lines express high numbers of IL-13Rs on their cell surface and that IL-13 cytotoxin mediates specific cytotoxicity *in vitro* and *in vivo* (3, 5, 31). Now, we demonstrate that IL-13 cytotoxin-induced cytotoxicity may be mediated, at least in part, by apoptosis *in vitro* and *in vivo*.

IL-13 cytotoxin induced several morphological changes in A172 and U251 cell lines that expressed high levels of IL-13R. These changes included chromatin condensation and nuclear fragmentation. However, the T98G cell line, which expresses low numbers of IL-13R, showed few morphological features, even after treatment with 100 ng/ml IL-13 cytotoxin. Because the T98G cell line does not express the IL-13Rα2 chain and only demonstrates low or no sensitivity to IL-13 cytotoxin ($IC_{50} = 200$ to >1000 ng/ml; Refs. 5, 32), it is possible that IL-13 cytotoxin will induce apoptosis when the IL-13Rα2 chain is inserted in these cells. Thus, as seen in previous studies using PE-based immunotoxin/cytotoxins, IL-13 cytotoxin also seems to cause cell death by apoptosis (23–25). Although the percentage of the hypodiploid cell population increased in a time-dependent manner as determined by fluorescence-activated cell sorting analysis, nevertheless, the percentage did not reach 50%, even though cells were treated with IL-13 cytotoxin for 96 h. These findings suggest that IL-13 cytotoxin causes cell death not only by apoptosis but also by necrosis.

IL-13 cytotoxin also induced apoptosis in U251 tumor xenografts *in vivo*. A single i.t. administration of IL-13 cytotoxin induced both caspase-8 and cytochrome *c* pathways of apoptosis. Activities of caspase-3, -8, and -9 reached maximum levels within 4 h, whereas optimum mitochondrial cytochrome *c* release to the cytosol seemed to occur between 6 and 48 h. Interestingly, activity of caspase-8 and -9 returned to baseline; however, effector caspase-3 activity remained somewhat higher than baseline, even 72 h after the i.t. injection of IL-13 cytotoxin. These results suggest that the apoptotic process continues for a prolonged period of time (at least 72 h), even though peak apoptotic cells were detected at 24 h. Sensitivity of assays between TUNEL and Western blot analyses may account for these differences.

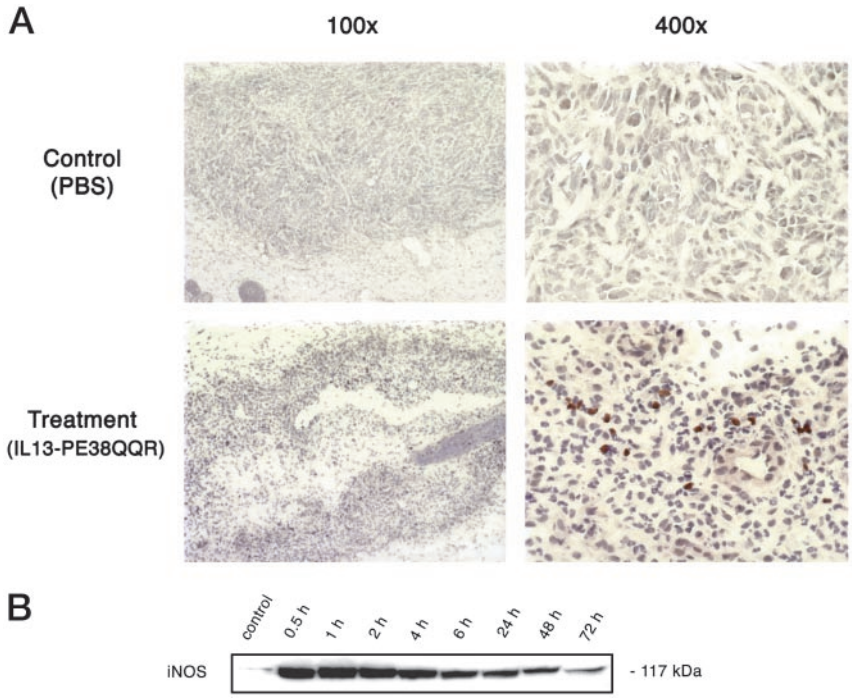

Fig. 8. Expression of iNOS in U251 tumors treated with i.t. IL-13 cytotoxin injections. *A*, immunohistochemistry of tissue sections was performed using rabbit antimouse iNOS Ab (*100*×, *400*×). IL-13 cytotoxin treatment induced infiltration of strongly brown iNOS positive macrophages within tumor nodules. *B*, Western blot analysis showing expression of iNOS in treated tumors at various time points. *kDa*, M_r in thousands.

Alternatively, apoptotic cells were rapidly phagocytized after peaking at 24 h. Finally, the induction of caspase activity correlated with the cleavage of the caspase substrates, procaspase-3 and PARP. Both were cleaved at similar time periods (within 4–6 h) when caspase-3, -8, and -9 were activated.

It is of interest to note that after a single administration of IL-13 cytotoxin in tumors *in vivo*, the IL-13 cytotoxin is rapidly degraded, absorbed, or undetectable by this assay. Four h after injection, levels of IL-13 cytotoxin were barely detectable. This observation is similar to that in our previous studies, in which IL-13 cytotoxin was administered in AIDS-associated Kaposi's sarcoma tumors (33). Nevertheless, in the present study, apoptosis pathways were still active beyond the disappearance of IL-13 cytotoxin from the tumor. This is important information that can help develop the rationale for schedules of IL-13 cytotoxin administration for optimum glioma therapy.

Although, after a single i.t. administration, IL-13 cytotoxin induced the cascade of events leading to apoptosis, a single dose was not sufficient for prolonged and pronounced antitumor activity. This could be attributable to suboptimal drug or distribution and/or suboptimal apoptosis of a limited number of tumor cells. Two additional injections of IL-13 cytotoxin on alternate days produced remarkable antitumor effects. Approximately 40% (3 of 7) of the animals showed complete disappearance of their tumors. Two additional injections once a day every 3 days also showed similar tumor responses. These results suggest that, in clinical situations, one can dose the patient with a more tolerated schedule. A once-a-day-every-3-days schedule may be better tolerated and practical than an every-alternate-day schedule of administration. Because the i.t. administration of IL-13 cytotoxin by two schedules did not produce complete regression in all of the animals, it is possible that complete antitumor activity can be achieved in all of the animals by continuous administration of IL-13 cytotoxin, using Alzet micropumps. These studies are currently ongoing in our laboratory.

We also estimated the numbers of apoptotic cells in tumor sections as a measure of biological activity of IL-13 cytotoxin. By 24 h after IL-13 cytotoxin administration *in vivo*, the maximum number of apoptotic cells was detected. Their numbers had decreased after 72 h of treatment, but still remained ~3-fold higher compared with the control groups. These observations suggest that IL-13 cytotoxin-induced early events (caspase activation) of apoptosis lead to morphological changes at a later time point. This observation can also help guide the design of clinical trials of glioma therapy in regard to the frequency of repeat administration of IL-13 cytotoxin.

To elucidate other cell death mechanisms of IL-13 cytotoxin-induced tumor regression, we investigated expression of iNOS producing NO in U251 tumors. NO has been shown to be associated with several pathways, including signal transduction, immune regulation, and regulation of cell death having two opposite effects; cell killing and the prevention of apoptosis (19, 20). We found iNOS was induced rapidly by IL-13 cytotoxin in U251 tumors. Interestingly, expression of iNOS appeared to be localized in phagocytes that surrounded dying cells after the administration of IL-13 cytotoxin. The expression of iNOS was maintained for 72 h after a single i.t. administration of IL-13 cytotoxin. Because NO is most commonly produced by activated macrophages (16), it is possible that infiltrating macrophages may play a role in tumor regression. Alternatively, it is possible that NO-producing macrophages were recruited by dying tumor cells

(34). Additional studies are needed to elucidate the role of NO that is produced by infiltrating phagocytes.

In conclusion, we demonstrate that IL-13 cytotoxin can cause apoptosis in brain tumor cells *in vitro* and *in vivo*. IL-13 cytotoxin modulated several proapoptotic molecules *in vivo* involving two apoptotic pathways. IL-13 cytotoxin continued to mediate apoptosis and tumor regression even after it had disappeared from tumors. Furthermore, phagocytes expressing iNOS were induced and may play a role in the tumor regression caused by IL-13 cytotoxin.

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