Therapeutic Modulation of Akt Activity and Antitumor Efficacy of Interleukin-12 Against Orthotopic Murine Neuroblastoma


Background: Patients with advanced neuroblastoma have a poor prognosis. The antiapoptotic protein Akt has been implicated as a possible mediator of the resistance of human neuroblastoma cells to apoptosis; the proapoptotic protein Bid, is inhibited by activated Akt. Neuroblastoma has demonstrated responsiveness to immunotherapeutic approaches in preclinical studies, prompting investigation of new therapeutic strategies based on potentiation of the host immune response, including the use of systemic cytokines. Methods: We examined the antitumor efficacy and mechanisms of action of the central immunoregulatory cytokine interleukin-12 (IL-12) in mice bearing established orthotopic neuroblastoma tumors derived from murine TBJ and Neuro-2a cells. Cohorts of mice (10 mice/group) bearing established orthotopic neuroblastoma tumors were injected intraperitoneally with IL-12 or vehicle and monitored for survival. IL-12–induced apoptosis within the tumor microenvironment was investigated using ribonuclease protection assays, nuclear staining, and electron microscopy. Protein expression was determined via Western blot analysis and enzyme-linked immunosorbent assays. Confocal microscopy was used to examine the distribution of overexpressed Bid–enhanced green fluorescent protein fusion protein (Bid-EGFP) in TBJ cells. All statistical tests were two-sided. Results: IL-12 induced complete tumor regression and long-term survival of 8 (80%) of 10 mice bearing established neuroblastoma tumors compared with 1 (10%) of 10 control mice (P =.0055) and profound tumor cell apoptosis in vivo despite the fact that TBJ and Neuro-2a cells were resistant to receptor-mediated apoptosis in vitro. These cells expressed high levels of phosphorylated Akt, a key prosurvival molecule, and Akt inhibitors sensitized neuroblastoma cells to apoptosis mediated by IL-12–inducible cytokines including tumor necrosis factor-α and interferon-γ in vitro. IL-12 increased the expression of proapoptotic genes and decreased Akt phosphorylation within established TBJ tumors in conjunction with activation and subcellular translocation of Bid. Conclusions: Our results suggest that IL-12 overcomes a potentially critical mechanism of tumor self-defense in vivo by inhibiting Akt activity and imply that IL-12 may possess unique therapeutic activity against tumors that express high levels of activated Akt.

Neuroblastoma is the most common extracranial solid tumor in children (1–3). The poor prognosis of patients with advanced neuroblastoma, despite advances in existing therapeutic modalities, has prompted intense interest in new therapeutic strategies for these patients, including antibody-, vaccine-, and/or cytokine-mediated approaches that potentiate the host immune response. Interleukin-12 (IL-12) is a central immunoregulatory cytokine that provides a key mechanistic link between innate immunity and the development of an adaptive, T-cell–mediated immune response (4–8). IL-12 also mediates potent antitumor activity in preclinical tumor models (9–14) and has been evaluated as a single therapeutic agent in adults with advanced solid tumors (15,16). In murine tumor models, the antitumor activity of IL-12 is mediated by effecter cell subsets that may include T cells, natural killer cells, or natural killer T cells (9,10,13,17–21), by the induction of endogenous interferon-γ (IFN-γ) production (10,13,22,23), and, in some instances, by activation of receptor-mediated apoptosis pathways, including those mediated by Fas (CD95)/FasL (13,17,24) and/or tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and its receptor TRAIL-R (25), within the tumor microenvironment. IL-12–inducible cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) can directly modulate these receptor-mediated apoptosis pathways as well. In tumor cells, IFN-γ increases expression of proapoptotic genes encoding Fas (26–28), TNF receptors (29), and caspase-8 (30–33), sensitizes cells to apoptosis induced by Fas/FasL (26–28,30,31,34), TRAIL (30–32,34), or TNF-α (30,34), and decreases expression of genes encoding antiapoptotic prosurvival factors such as Bcl-2 (34) and inhibitor of apoptosis protein-2 (35). IFN-γ also increases the expression of Fas (36,37) in endothelial cells and enhances the sensitivity of these cells to apoptosis induced by Fas/FasL (36,37) or TNF-α. Thus, IL-12 and downstream cytokines such as IFN-γ and TNF-α are potent modulators of receptor-mediated apoptosis pathways.

Molecular alterations that confer a resistance to apoptosis may contribute to the poor responsiveness of some neuroblastomas to conventional therapeutic modalities. These alterations include those that result in a deficiency of key proteases involved in apoptosis such as caspase-3 (38) or caspase-8 (38–41); in an absence or decreased expression of programmed cell death receptors such as Fas (28,42) or TRAIL-R (32); or in the overexpression or activation of prosurvival molecules, including survivin (43,44),...
FLICE-inhibitory protein (FLIP) (45), Bcl-2 (45), and Akt (46). Akt mediates the resistance of various human neuroblastoma cell lines to chemotherapeutic agents (46–48) and to TRAIL-mediated (49) apoptosis in vitro.

The present studies were undertaken to investigate the antitumor activity and mechanisms of action of IL-12 in mice bearing established orthotopic intra-adrenal neuroblastoma tumors.

**Materials and Methods**

**Reagents and Antibodies**

Recombinant murine IL-12 was generously provided by Genetics Institute (Cambridge, MA; specific activity $7 \times 10^6$ U/mg) or was purchased from Peprotech (Rocky Hill, NJ; specific activity $1 \times 10^7$ U/mg) and was administered as described previously (11). Recombinant murine TNF-α (specific activity $1 \times 10^7$ U/mg) and recombinant murine IFN-γ (specific activity $1 \times 10^7$ U/mg) were purchased from Peprotech. Soluble human recombinant (rhs) Super FasL and rhsTRAIL were obtained from Alexis Biochemicals (San Diego, CA). The pan-caspase inhibitor z-vad.fmk (z-vad) was purchased from BD Pharmingen. Recombinant murine TNF-α (speci

**Cell Lines and Mice**

We used two murine neuroblastoma tumor cell lines that are syngeneic to A/J mice: Neuro-2a cells (purchased from ATCC, Rockville, MD) and TBJ cells, a metastatic subclone of Neuro-2a cells (a generous gift from Dr. Moritz Ziegler, Children's Hospital, Boston, MA). Mouse hemangioendothelioma EOMA cells were purchased from ATCC. TBJ and Neuro-2a cells were both maintained via serial orthotopic intra-adrenal in vivo passage in A/J mice. Cell cultures were maintained in vitro in RPMI-1640 (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) in a humidified atmosphere with 5% CO2 at 37 °C. We used FuGENE reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions to transfect TBJ cells with the pd4EGFP-Bid expression vector, and cell clones that stably overexpressed the Bid-EGFP fusion protein (TBJ-Bid-EGFP cells) were subsequently selected by their ability to grow in medium containing geneticin (600 μg/mL) (Invitrogen). Expression of the Bid-EGFP fusion protein by the clones was confirmed by fluorescence microscopy and by Western blot analysis of tumor cell lysates using a rat monoclonal anti-mouse Bid antibody that also cross-reacts with human Bid protein.

Male A/J mice were purchased from Charles River Laboratories (Frederick, MD), maintained under pathogen-free conditions, and generally used at 8–10 weeks of age. Mice were cared for in accordance with procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publ No 86-23, 1985). This study was approved by the Animal Care Users Committee, NCI-Frederick, Frederick, MD.

**Tumor Models**

We used an orthotopic model of transplantable intra-adrenal murine neuroblastoma that was developed in our laboratory (52, 53). Briefly, mice were anesthetized in a chamber containing a 5% isoflurane/oxygen mixture and then fitted with a nose cone that administered 5% isoflurane for surgery. A 10- to 20-mm incision was made in the flank using a scalpel, and the kidney was manually pushed through the opening to expose the adrenal gland. A Tridak Stepper microinjector (Indicon Inc., Brookfield Center, CT) equipped with a 29-gauge needle was used to inject $5 \times 10^4$ TBJ or Neuro-2a cells in 30–50 μL Hanks’ balanced salt solution (HBSS, Invitrogen) directly into the adrenal gland. After tumor cell implantation, the kidney and adrenal gland were pushed back into the abdomen, and 9-mm Autoclips were used to close the incision. Therapy was initiated 10–12 days later, after the implanted tumor cells had formed well-established (52, 53) primary tumors in the adrenal gland. For some experiments, mice were injected subcutaneously in the mid-flank with TBJ tumor cells (1 × 106 cells/mouse in 0.2 mL of HBSS), and palpable (approximately 100 mm3) subcutaneous primary tumors were allowed to form for approximately 7 days before therapy was initiated. Mice were monitored daily for evidence of toxicity, and tumor dimensions in each mouse were measured two times per week. To investigate the effect of IL-12 on the induction of an immunologic memory response, one cohort of 10 mice cured of their original subcutaneous TBJ tumors by treatment with IL-12 and a second cohort of 10 age-matched naïve control mice that had not been previously exposed to either TBJ cells or IL-12 were injected subcutaneously with 1 × 106 TBJ cells/mouse, and then monitored for survival. To investigate the effect of IL-12 on the growth of established neuroblastoma metastases, 2.5 × 105 TBJ cells were injected intrasplenically into two cohorts of mice (10 mice/group) and were allowed to grow in the liver for 4 days.
before therapy was initiated. Mice were randomly assigned to treatment groups at the time of tumor cell injection.

In Vivo Treatment Regimens

Survival studies. Cohorts of mice (10 mice/group) bearing established orthotopic TBJ (or Neuro-2a) neuroblastoma cell-derived tumors were injected intraperitoneally with IL-12 (0.5 μg) in vehicle (0.2 mL of phosphate-buffered saline [PBS] containing 0.1% A/J mouse serum) or vehicle alone once daily on days 12–16, 19–23, and 26–30 after tumor cell implantation and then monitored for survival. Mice bearing established subcutaneous TBJ cell-derived tumors were injected intraperitoneally once daily with IL-12 (0.5 μg) or vehicle alone on days 7–11, 14–18, and 21–25 after tumor cell implantation. Cohorts of mice (10 mice/group) treated with IL-12 were also selectively depleted of CD4+ or CD8+ T cells by injection with 790 μg of rat anti-mouse CD4 or 163 μg of mouse anti-mouse CD8 monoclonal antibodies, respectively, on days 4, 7, 9, 11, 14, 16, 18, 21, 23, and 25 after tumor cell implantation and monitored for survival. Cohorts of mice (10 mice/group) bearing TBJ cell-derived liver metastases as described above were injected intraperitoneally once daily with IL-12 (0.5 μg) or vehicle alone on days 4–8, 11–15, and 18–22 after tumor cell implantation and then monitored for survival. For survival studies, mice were killed when the estimated tumor volume in a given mouse reached a minimum of 8000 mm3 and/or when signs of paralysis, a decrease in body temperature, and general malaise were observed. Mice were killed by cervical dislocation or CO2 asphyxiation.

Modulation of IFN-γ production and apoptosis pathways in vivo. Cohorts of mice (20 mice/group) bearing established orthotopic intra-adrenal TBJ cell-derived tumors were injected intraperitoneally once daily with IL-12 (0.5 μg) or vehicle alone on days 12–16 and 19–22 after tumor cell implantation. Five mice from each of these groups were killed by cervical dislocation or CO2 asphyxiation on day 16, and another five mice were killed on day 23 after tumor cell implantation (i.e., on days 4 and 11, respectively, of therapy). Whole blood was collected from each mouse via cardiac puncture for an enzyme-linked immunosorbent assay (ELISA) determination of the serum concentration of IFN-γ as described below. Tumors from the remaining 10 mice/group were resected on day 23 post tumor implantation and processed for analysis of local gene expression using ribonuclease protection assay, immunohistochemical staining to characterize the infiltration of T cells within the local tumor site, and/or electron microscopy to investigate alterations in the ultrastructural histology of TBJ cell-derived tumors, as described below. Similarly designed experiments were conducted to generate serum and/or tumor specimens from TBJ tumor-bearing mice treated with either IL-12 (0.5 μg) or vehicle alone as outlined above for subsequent ELISA assay of serum TNF-α (day 17 after tumor implantation) and soluble FasL (sFasL) levels (day 21 after tumor implantation) and investigation of alterations in the local expression of Akt (days 16, 21, and 22 after tumor implantation) and Bid (day 21 after tumor implantation) within TBJ tumors via Western blot. To investigate the effect of systemic IL-12 administration on the subcellular localization of Bid in TBJ tumors in vivo, cohorts of mice (10 mice/group) bearing established subcutaneous tumors derived from TBJ-Bid-EGFP cells were treated with IL-12 (0.5 μg) or vehicle on days 9–16 after tumor cell implantation. We killed five mice from each group on days 13 and 17 after tumor cell implantation (i.e., on days 4 and 9, respectively, of therapy) and immediately perfused them with 25 mL of cold PBS followed by 25 mL of cold 4% paraformaldehyde in PBS for in situ fixation of tumors. Tumors were resected and processed as outlined below for imaging of Bid-EGFP localization by confocal microscopy. Similarly designed experiments were performed to generate TBJ parental tumor specimens (day 23 after tumor implantation) from control and IL-12–treated mice, with subsequent staining with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclear morphology and assess the extent of apoptosis within these tumor sections.

ELISA to Quantify Serum Concentrations of IFN-γ, TNF-α, and sFASL

We collected whole blood from individual mice via cardiac puncture into red-top serum separator tubes (Becton-Dickinson, San Jose, CA). The separated serum was analyzed with Quantikine-M murine immunoassay kits (R & D Systems) according to the manufacturer’s instructions to determine the concentrations of IFN-γ, TNF-α, and sFasL.

Tumor Studies

Ribonuclease protection assay. Total cellular RNA was isolated from snap-frozen tumors obtained from mice treated with IL-12 or vehicle using TRIzol reagent (Invitrogen) and subjected to ribonuclease protection assays of local gene expression using a RiboQuant mouse APO-3 multiprobe template set (BD Pharmingen) according to the manufacturer’s instructions.

Western blot analysis. Total cellular proteins were extracted from snap-frozen tumors obtained from mice treated with either IL-12 or vehicle using cell lysis buffer (100 mM NaCl, 1% Igepal, 30 mM Tris-HCl [pH 7.5], 30 mM NaF, and 1 mM EDTA) containing freshly added 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor (Roche Diagnostics). We used the same protein extraction method as described above to prepare protein lysates from snap-frozen adrenal glands obtained from un.injected, untreated A/J mice as well as from 2 × 106 TBJ, Neuro-2a, and EOMA cells cultured in vitro. TJB cells were similarly seeded at 3 × 105 cells in 60 mm dishes and incubated with the Akt inhibitor SH-5 for 48 hours. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal quantities of protein samples (60 μg/lane) were resolved on 8% or 4%–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride (0.2-μm pore size) membranes, which were then probed with rabbit polyclonal anti-mouse Akt antibody or phosphor-specific (serine 473) rabbit polyclonal anti-mouse Akt antibody (1:1000 dilution) or rat monoclonal anti-mouse Bid antibody (1 μg/mL), followed by washing and probing with either HRP-conjugated goat anti-rabbit IgG (1:3000 dilution) or HRP-conjugated goat anti-rat (1:5000 dilution) antibodies, respectively. The immunoreactive bands were detected using an ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK).

Electron microscopy, DAPI staining, and immunohistochemistry. TBJ cell-derived tumors from mice treated with IL-12 or vehicle were fixed in 4% paraformaldehyde and 2% glutaraldehyde in PBS and processed for electron microscopy as previously described (13). For DAPI staining, TBJ cell-derived...
tumors fixed in situ by paraformaldehyde perfusion were resected and incubated in 4% paraformaldehyde overnight at 4 °C with gentle agitation, followed by washing with PBS and incubation in 18% sucrose–PBS at 4 °C for 24–48 hours. Tumors were then embedded in Tissue Tek OCT medium (VWR, West Chester, PA), frozen on dry ice, cut into 10-μm thick sections on a cryostat, and then counterstained with DAPI for imaging of nuclear morphology. A Nikon Eclipse E400 equipped with an epi-fluorescence illumination was used for high power (100–400× magnification) fluorescence microscopy. For DAPI imaging, excitation was produced at 355 nm, and emitted fluorescence was collected through a 400-nm filter. For immunohistochemistry, 5-μm formalin-fixed paraffin-embedded tissue sections were processed by standard techniques (14). Sections were then incubated with rabbit anti-human CD3 primary antibody or rabbit anti-human chloramphenicol acetyltransferase as an irrelevant isotype control antibody as described previously (14) to characterize the infiltration of T cells within TBJ cell–derived tumors from mice treated with IL-12 or vehicle.

Confocal Microscopy. TBJ-Bid-EGFP cell–derived tumors were fixed in situ by paraformaldehyde perfusion and were then resected and processed as described above. Tissue sections were counterstained with DAPI for imaging nuclear morphology. Subcellular localization of Bid-EGFP was determined by using an LSM 410 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). A 488 nm Ar/Kr laser with a 500- to 550-nm band pass emission filter was used. The objective used was an LD-Apostigmat 20× dry lens with a numerical aperture of 0.30.

In Vitro Modulation of Tumor Cell Apoptosis

Death receptor expression. EOMA endothelial and TBJ or Neuro-2a neuroblastoma cells were cultured for various times in RPMI-1640 medium that contained or lacked 100 IU/mL IFN-γ and 50 ng/mL TNF-α (hereafter referred to as IFN-γ + TNF-α). Cells were harvested in PBS containing 1 mM EDTA and stained with PE-labeled hamster anti-mouse Fas antibody (Jo-2), PE-labeled hamster anti-mouse TNFR-p55, PE-labeled hamster anti-mouse TRAIL-R2 (MD5–1), or an appropriate isotype control antibody and then analyzed for antibody binding on a FACScan flow cytometer using CellQuest software (BD Biosciences).

 Detecting Induction of Apoptosis. EOMA endothelial cells (1–2 × 10^5 cells/60-mm dish) were cultured in medium containing IFN-γ + TNF-α for 6, 24, or 48 hours and then stained with annexin V–FITC to characterize cell surface staining as an indicator of apoptosis by flow cytometry. TBJ or Neuro-2a neuroblastoma cells (1 × 10^5 cells/30-mm well) were incubated in medium containing IFN-γ + TNF-α with or without rhsFasL (10–100 ng/mL) or rhsTRAIL (50–500 ng/mL) for 48 hours and then stained with annexin V–FITC and subjected to flow cytometry as described above to characterize cell surface staining as an indicator of apoptosis. To examine whether murine neuroblastoma tumor cells overexpress an inhibitory factor(s) that blocks receptor-induced apoptosis, Neuro-2a cells plated as above were incubated for 1 hour in medium containing 0.5 μg/mL cycloheximide and were then incubated for 72 hours in fresh medium that contained rhsTRAIL (300 ng/mL), rhsFasL (100 ng/mL), or IFN-γ + TNF-α. TBJ cells plated as above were incubated for 1 hour in medium containing 0.5 μg/mL cycloheximide that contained or lacked the pan-caspase inhibitor z-vad (25 μM) and were then incubated for an additional 24 hours in fresh medium that contained rhsTRAIL (300 ng/mL), rhsFasL (100 ng/mL), or IFN-γ + TNF-α. Cells were harvested, stained with annexin-V–FITC and propidium iodide, and analyzed by flow cytometry. To directly address whether the Akt pathway is functionally active, TBJ cells were plated as described above and cultured for 6 hours in medium that contained various concentrations of the Akt inhibitor SH-5 (5–20 μM) followed by incubation for 48 hours in fresh medium containing IFN-γ + TNF-α. The cells were harvested, stained with annexin V–FITC and propidium iodide, and then analyzed by flow cytometry. In subsequent studies, we cultured TBJ cells for 6 hours in medium containing 20 μM SH-5, followed by incubation for 48 hours in fresh medium containing rhsFasL (100 ng/mL), rhsTRAIL (300 ng/mL), or IFN-γ + TNF-α. Cells were harvested, and apoptotic responses were assayed by annexin V–FITC and propidium iodide staining as described above.

Statistical Analysis

Kaplan–Meier curves were plotted to analyze survival. The proportion of mice achieving a complete response (defined as complete tumor regression and long-term survival) in the respective treatment groups were compared where indicated by using Fisher’s exact test. The survival times of the groups were compared with the log-rank test. Serum IFN-γ, TNF-α, and sFasL levels were compared using the nonparametric Mann-Whitney U test. All other data were first analyzed by a two-way analysis of variance (ANOVA). Appropriate follow-up tests for statistical significance were then performed and consisted of pairwise contrasts using Bonferroni adjustment, the Games Howell procedure, and the Tukey honestly significant difference method (54). All P values were two-sided and were considered statistically significant at P<.05. Where indicated 95% confidence intervals (CIs) were calculated. The general strategy for the calculation of 95% confidence intervals for mean differences among treated and untreated groups was to use the t statistic with degrees of freedom computed by the Welch modified approach (55). Because many testing scenarios involved the problem of heteroskedasticity (unequal variances across the groups to be compared), this strategy gave an interval estimate that conserves the nominal α level of 5%. For the purposes of stabilizing variances across groups, some analyses were performed using the common (base 10) logarithms of the dependent variable instead of the raw values. However, all 95% confidence intervals are presented in terms of the raw values.

Results

Effect of IL-12 on Established Murine Neuroblastoma Tumors and Activation of the Host Immune Response In Vivo

To investigate the effect of IL-12 on the survival of mice bearing neuroblastoma tumors, mice bearing 12-day established orthotopic TBJ tumors were treated by intraperitoneal injection with IL-12 or vehicle (control) and monitored for survival. IL-12 induced complete tumor regression and long-term (>90 days) survival in 8 (80%) of 10 mice compared with 1 (10%) of 10 control mice (Fig. 1A, P = .0055). IL-12 also induced long-term survival and tumor regression in mice bearing well-established subcutaneous TBJ or Neuro-2a tumors (data not shown). In addition, IL-12 induced complete tumor regression...
and long-term survival in 10 (100%) of 10 mice bearing TBJ cell–derived liver metastases compared with 1 (10%) of 10 control mice (Fig. 1B, *P*<.001). Statistical analyses of complete response rates and survival distributions (log-rank analysis) for the respective groups were in complete interpretative agreement. We next examined the effect of IL-12 on the survival of mice that bore established subcutaneous TBJ cell-derived tumors and were treated concurrently with antibodies to CD8 or CD4 to deplete them of CD8+ or CD4+ T cells, respectively (Fig. 1C). Long-term survival and complete tumor regression was achieved by 9 (90%) of 10 mice treated with IL-12 alone, by 9 (90%) of 10 mice treated with IL-12 and the anti-CD4 antibody, by 0 (0%) of 10 mice treated with IL-12 plus the anti-CD8 antibody, and by 1 (90%) of 10 control mice treated with vehicle alone (IL-12 alone versus IL-12 plus anti-CD8: *P*<.001; IL-12 alone versus IL-12 plus anti-CD4: *P* = 1.0; IL-12 versus control: *P* = .001). Statistical analyses of complete response rates and survival distributions (log-rank analysis) for the respective groups were in complete interpretative agreement. We also compared survival of the respective control groups and the respective IL-12 treated groups from the experiments shown in Figures 1A and C using log-rank analysis and found no statistically significant interexperiment variability for either experiment. Among mice bearing TBJ cell–derived tumors, those treated with IL-12 had higher mean circulating levels of IFN-γ and TNF-α (IFN-γ: 225 pg/mL; TNF-α: 32 pg/mL) than control mice (IFN-γ: 2 pg/mL; TNF-α: 3 pg/mL; difference [IFN-γ]: = 223 pg/mL [95% CI = 104 to 342 pg/mL], *P*<.001; difference [TNF-α]: = 29 pg/mL [95% CI = −21 to 36 pg/mL], *P* = .008). Mice treated with IL-12 also had more infiltrating T cells within their TBJ tumors than control mice (data not shown), and a potent immunologic memory response was generated in mice cured of their original tumors by IL-12. Specifically, 10 (100%) of 10 mice cured of their original subcutaneous TBJ tumors by treatment with IL-12 rejected a subsequent tumor rechallenge, compared with only 1 (10%) of 10 naive control mice that had not been exposed previously to either TBJ tumor cells or treated with IL-12 (*P*<.001). Collectively, these observations suggest that administration of IL-12 potently activates the immune system in vivo and induces CD8+-T cell–dependent regression of TBJ tumors in conjunction with the generation of an immunologic memory response.

**Effects of IL-12 on Vascularity and Apoptosis Within TBJ Tumors**

We observed marked reductions in overall vascularity and large areas of coagulative necrosis and/or apoptosis in hematoxylin-eosin–stained sections of TBJ tumors from mice treated with IL-12 versus those from mice treated with vehicle (data not shown). We also stained tumor sections with DAPI to visualize nuclear morphology and observed that tumors from mice treated with vehicle alone had little evidence of nuclear condensation (Fig. 2, A and B) whereas tumors from mice treated with IL-12 had large areas in which the tumor cells displayed marked evidence of nuclear condensation, consistent with apoptosis (Fig. 2, C and D). Electron microscopy was used to more directly investigate the ultrastructural histology of tumor and endothelial cells (identified by morphology) within tumors from IL-12 treated and control mice. In tumors resected on day 11 of therapy, we observed substantial nuclear condensation and fragmentation as well as vacuolization within TBJ tumor cells from mice treated with IL-12 compared with those from control mice treated with vehicle alone (Fig. 2, E and G). We also observed nuclear condensation and mitochondrial swelling, ultrastructural changes that are consistent with vascular endothelial...
Materials and Methods

Tumors from mice treated with vehicle or IL-12 were resected and processed as outlined in “Materials and Methods.” Tumors were resected and processed using electron microscopy as described in “Materials and Methods.” Representative images at 200× (panels A and B) and 400× (panels C and D) magnifications are shown. Arrows point to apoptotic TBJ tumor cells with condensed/fragmented chromatin. To investigate the impact of IL-12 on the ultrastructural histology of both tumor and endothelial cell populations within tumors from mice treated with vehicle or IL-12, respectively, fixative was resected and processed as outlined in “Materials and Methods.” Representative images are shown at 200× (panels A and B) and 400× (panels C and D) magnification, respectively. Arrows point to apoptotic TBJ tumor cells with condensed/fragmented chromatin. To investigate the impact of IL-12 on the ultrastructural histology of both tumor and endothelial cell populations within tumors from mice treated with vehicle or IL-12, respectively, fixative was resected and processed as outlined in “Materials and Methods.” Representative images are shown at 200× (panels A and B) and 400× (panels C and D) magnification, respectively. Arrows point to apoptotic TBJ tumor cells with condensed/fragmented chromatin.

Effect of IL-12 on Expression of Proapoptotic Genes Within TBJ Tumors

We next investigated whether IL-12 could modulate specific molecular pathways capable of mediating tumor and/or endothelial cell apoptosis within established tumors by examining the effects of IL-12 on the expression of genes involved in apoptosis. By using a ribonuclease protection assay to detect specific messenger RNA transcripts in total RNA isolated from TBJ tumors, we observed that tumors from mice treated with IL-12 had increased expression of proapoptotic genes encoding Fas/FasL, TNFR-p55, TRAIL, and caspase-8 compared with tumors from control mice (Fig. 3A). IL-12 also induced large increases in the serum level of sFasL protein; the mean serum concentrations of sFasL were 426 pg/mL for mice treated with IL-12 versus 25.6 pg/mL for mice treated with vehicle alone (difference = 400.4 pg/mL [95% CI = 349.5 to 451.3 pg/mL] P = .008, Fig. 3, B). These observations suggest that administration of IL-12 may strongly activate receptor-mediated apoptosis pathways within the tumor microenvironment.

Evaluation of Apoptosis in TBJ and Neuro-2a Neuroblastoma Cells

Neither murine endothelial (EOMA) nor neuroblastoma (TBJ, Neuro-2a) cells express functional IL-12 receptors (Khan T and Wigginton J, data not shown). Thus, it is likely that the biologic and therapeutic effects of IL-12 on the tumors derived from these cells that we observed were mediated indirectly. Accordingly, we examined the effects of inducible cytokines that act downstream of IL-12, such as IFN-γ and TNF-α, on tumor and endothelial cell apoptosis in vitro. We found that IFN-γ + TNF-α induced apoptosis in EOMA microvascular endothelial cells, as assessed by annexin-V staining (data not shown). We also found that EOMA cells constitutively express cell surface Fas and are highly sensitive to Fas-mediated apoptosis (data not shown) and that treatment of EOMA cells with IFN-γ + TNF-α enhanced Fas expression as well as their sensitivity to Fas-induced apoptosis (data not shown).

By contrast, TBJ neuroblastoma cells expressed TNFR-p55 and IFN-γ receptors but not Fas and only negligible amounts of TRAIL-R2, either constitutively or after exposure to IFN-γ + TNF-α (data not shown). In addition, TBJ cells were resistant to apoptosis induced by FasL (Fig. 4, A) or TRAIL (Fig. 4, B), either alone or in combination with IFN-γ + TNF-α, as determined by annexin V staining. Neuro-2a cells expressed death receptors, including Fas, TRAIL-R2, and TNFR-p55 (data not shown), and treatment of cells with IFN-γ + TNF-α further increased Fas expression compared with untreated control cells at all time points assayed (Fig. 4, C; P<.001 for control versus treated at each time point). Nonetheless, treatment of Neuro-2a cells with IFN-γ + TNF-α alone (Fig. 4, D) or in combination with FasL (Fig. 4, E) or TRAIL (data not shown) did not increase the percentage of annexin V-positive (i.e., apoptotic) cells compared with untreated control cells.

Thus, the finding that Neuro-2a and TBJ cells express death receptors involved in apoptosis but appear to be intrinsically resistant to receptor-mediated apoptosis suggests that these cells
may overexpress a molecule(s) that blocks apoptosis. To investigate this possibility, we incubated TBJ and Neuro-2a cells for 1 hour in medium that contained or lacked the protein synthesis inhibitor cycloheximide (CHX), followed by incubation for 72 hours in medium containing various apoptosis-inducing ligands (i.e., TRAIL, FasL, or IFN-γ + TNF-α) and annexin V staining to identify apoptotic cells. We found higher percentages of annexin V-positive Neuro-2a cells in cultures treated with CHX and death-receptor ligands than in cultures treated with the respective ligand alone, CHX alone, or medium alone (annexin V-positive Neuro-2a cells: medium = 17%, CHX = 23%, FasL = 12%, FasL + CHX = 39%, TRAIL = 13%, TRAIL + CHX = 32%, IFN-γ + TNF-α = 20%, and IFN-γ + TNF-α + CHX = 62%; medium versus CHX: difference = 6% [95% CI = 4.5% to 8.4%], P = .1087; TRAIL versus TRAIL + CHX: difference = 19% [95% CI = 6.8% to 31.2%], P < .001; FasL versus FasL + CHX: difference = 27% [95% CI = 91.6% to 37.5%], P < .001; IFN-γ + TNF-α versus IFN-γ + TNF-α + CHX: difference = 42% [95% CI = 18.8% to 66.3%], P < .001) (Fig. 5, A). In addition, we observed a higher percentage of annexin V-positive cells in TBJ cell cultures pretreated with CHX followed by IFN-γ + TNF-α than in cultures treated with CHX or IFN-γ + TNF-α (annexin V-positive TBJ cells: CHX = 10%, IFN-γ + TNF-α = 8%, IFN-γ + TNF-α + CHX = 64%; IFN-γ + TNF-α versus IFN-γ + TNF-α + CHX: difference = 56% [95% CI = 53.9% to 57.8%], P < .001; CHX versus IFN-γ + TNF-α + CHX: difference = 54% [95% CI = 51% to 58%], P = .001) (Fig. 5, B). The ability of CHX to sensitize TBJ cells to TNF-α + IFN-γ was completely ablated by the pan-caspase inhibitor z-vad (annexin V-positive TBJ cells: IFN-γ + TNF-α + CHX + z-vad = 20%; IFN-γ + TNF-α + CHX versus IFN-γ + TNF-α + CHX + z-vad: difference = 44% [95% CI = 41.5% to 47.3%], P < .001) (Fig. 5, B). Collectively, these results suggest that both TBJ and Neuro-2a cells have intact apoptosis-inducing machinery as well as an active inhibitor(s) that can block apoptosis induced by Fas/FasL, TRAIL/TRAIL-R, and IFN-γ + TNF-α.

Expression of Phosphorylated Akt in TBJ and Neuro-2a Neuroblastoma Cells

In human neuroblastomas, several anti-apoptotic proteins, including Akt, have been implicated as possible mediators of the resistance of tumor cells to apoptosis (43–49). Akt is a serine/threonine protein kinase whose activity is regulated by site-specific phosphorylation, with phosphorylation at serine 473 required for maximal activity (56–58). We therefore examined whether Akt could be involved in the intrinsic resistance of murine neuroblastoma tumor cells to receptor-induced apoptosis by comparing the levels of total and serine-phosphorylated (i.e., activated) Akt in protein lysates of TBJ and Neuro-2a neuroblastoma cells and of normal mouse adrenal gland. Western blot analysis revealed that compared with normal adrenal gland, both Neuro-2a and TBJ cells expressed higher levels of serine 473 phosphorylated Akt protein (Fig. 6, A). Next, we examined the levels of serine 473 phosphorylated Akt protein in TBJ cells that had been incubated for 48 hours in the presence or absence of the Akt inhibitor SH-5. Dose titration studies demonstrated that SH-5 concentrations of 10 μM and higher inhibited Akt phosphorylation with optimal inhibition observed at concentrations of 20 μM or greater (Fig. 6, B). We also examined the effect of SH-5 pretreatment on the percentage of TBJ cells that were annexin V-positive after subsequent incubation in the presence or absence of IFN-γ + TNF-α. TBJ cells treated with 20 μM SH-5 followed by IFN-γ + TNF-α had more than double the percentage of apoptotic cells than TBJ cells treated with 20 μM SH-5 only (annexin V-positive TBJ cells: medium = 4.5%, SH-5 (5 μM) = 6.3%, SH-5 (10 μM) = 5.8%, SH-5 (20 μM) = 8%, IFN-γ + TNF-α = 8.6%,
Effect of IL-12 on Serine Phosphorylation of AKT and Bid Localization in TBJ Cell–Derived Tumors in Vivo

The above observations led us to hypothesize that IL-12 suppresses Akt activity in vivo and that this suppression contributes to the profound tumor cell apoptosis we observed in TBJ cell–derived tumors from mice treated with IL-12. To investigate this possibility, we used Western blot analysis to examine the levels of total and phosphorylated Akt in protein lysates of orthotopic intra-adrenal TBJ cell–derived tumors harvested from mice treated with IL-12 or vehicle alone (control). Compared with control tumors, most tumors from mice treated with IL-12 had substantially less serine 473–phosphorylated Akt (Fig. 7). The decline in the level of serine-phosphorylated Akt was observed as early as day 4 of IL-12 treatment, and by day 9 of treatment, three of four tumors from mice treated with IL-12 had substantially reduced expression of phosphorylated Akt compared with one of three control tumors. Tumors from treated and untreated mice had similar levels of total Akt protein. Because phosphorylation of Akt on serine 473 is required for maximal Akt activity (56–58), this observed decrease in serine 473 phosphorylation suggests a decline in Akt function in TBJ tumors from mice treated with IL-12.

We next examined whether the observed decrease in phosphorylated Akt in tumors from mice treated with IL-12 was associated with functional activation of the proapoptotic protein Bid, a downstream target molecule of Akt that is normally suppressed by activated Akt. During receptor-mediated apoptosis, Bid is cleaved by activated caspase-8 to an active form that is translocated from the cytosol to the mitochondria, where it amplifies and sustains mechanisms that mediate apoptotic cell death (59,60). Activated Akt inhibits the cleavage of Bid by activated caspases (61,62). Western blot analysis of protein lysates of TBJ cell–derived tumors revealed that three of four mice treated with IL-12 had a Bid antibody-positive band whose size...
was consistent with the size of the Bid cleavage product; by contrast, no such band was detected in lysates of three tumors from control mice treated with vehicle (Fig. 8, A).

Next we investigated whether administration of IL-12 induces activation and subcellular translocation of Bid specifically within TBJ tumor cells that stably expressed a Bid-GFP fusion protein (TBJ-Bid-EGFP). In viable non-apoptotic TBJ-BID-EGFP cells, diffuse green fluorescence corresponding to overexpressed Bid-EGFP localized to the cytosol was observed. We and others have shown previously that exposure of tumor cells to agents that induce Bid activation (i.e., doxorubicin and PS-341) results in translocation and subcellular localization of Bid-EGFP in a punctate pattern of fluorescence consistent with colocalization in mitochondria (53,63). We used confocal microscopy to analyze Bid-EGFP localization in sections from subcutaneous TBJ-Bid-EGFP cell-derived tumors resected from mice treated in vivo with IL-12 or vehicle alone. The majority of the tumor cells in TBJ-BID-EGFP cell-derived tumors from mice treated with vehicle alone displayed a diffuse pattern of fluorescence, which

![Fig. 5. Effect of cycloheximide on receptor-mediated apoptosis in Neuro-2a and TBJ neuroblastoma cells. Neuro-2a cells were incubated for 1 hour in the presence (CHX) or absence (Control) of cycloheximide (0.5 μg/mL) and then incubated in fresh medium with or without recombinant tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) (300 ng/mL), FasL (100 ng/mL), or interferon-γ (IFN-γ) (100 IU/mL) + tumor necrosis factor-α (TNF-α) (50 ng/mL) for 72 hours as described in “Materials and Methods.” Cells were then harvested, and cell surface annexin V expression was determined by fluorescence-activated cell sorter (FACS) analysis (A). TBJ cells were pretreated with CHX (0.5 μg/mL) in the presence or absence of z-vad.fmk (z-vad) for 4 hours before incubation in fresh medium with or without recombinant TRAIL (300 ng/mL), FasL (100 ng/mL), or TNF-α + IFN-γ for 24 hours as described in “Materials and Methods.” Cells were then harvested, and cell surface annexin V staining was determined by FACS analysis (B). Data are shown as mean values of duplicate samples with error bars representing ±2 standard errors of the mean.

![Fig. 6. Akt expression and activity in neuroblastoma tumor cells. A) Levels of total and serine-473 (Ser 473)–phosphorylated Akt protein in TBJ and Neuro-2a (Nu-2a) tumor cells and in normal mouse adrenal gland were examined by Western blot analysis. B) Effect of the Akt inhibitor SH-5 on Akt serine 473 phosphorylation in TBJ tumor cells. TBJ cells were treated with SH-5 for 48 hours and levels of total and phosphorylated AKT in cell lysates were determined by Western blot analysis. C and D) Effect of SH-5 inhibition of Akt on receptor-mediated apoptosis induction in TBJ cells. TBJ cells were pretreated with various concentrations of SH-5 for 6 hours followed by incubation in fresh medium that contained or lacked (Control) interferon-γ (IFN-γ) (100 IU/mL) + tumor necrosis factor-α (TNF-α) (50 ng/mL) for 48 hours (C). In subsequent studies, TBJ cells were pretreated with an 20 μM SH-5 for 6 hours, followed by incubation for 48 hours in medium that contained or lacked IFN-γ (100 IU/mL) + TNF-α (50 ng/mL), FasL (100 ng/mL) or tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) (300 ng/mL) (D). Cell surface annexin V staining was determined via fluorescence-activated cell sorter analysis. Data are shown as mean values of duplicate samples with error bars representing ±2 standard errors of the mean.]
suggests that the Bid-EGFP fusion protein was distributed diffusely throughout the cytoplasm (Fig. 8, B and D). By contrast, tumor cells in sections from TBJ-Bid-EGFP cell-derived tumors from mice treated with IL-12 displayed a punctate pattern of green fluorescence, which is consistent with a mitochondrial translocation of Bid-EGFP (Fig. 8, C and E). These changes in Bid-EGFP localization were observed as early as day 4 of treatment (data not shown) and by day 9 of treatment, most tumor cells from mice treated with IL-12 exhibited the punctate distribution of Bid-EGFP fluorescence (Fig. 8, C and E). Collectively, these results demonstrate that both cleavage of Bid and subcellular translocation of Bid specifically in TBJ tumor cells are observed in mice treated with IL-12 but not in TBJ tumor cells from control mice treated with vehicle alone.

**DISCUSSION**

We found that systemic administration of IL-12 induced complete regression of established orthotopic intra-adrenal neuroblastoma tumors and marked apoptosis of both tumor and endothelial cell populations in vivo. These effects occurred in conjunction with the inhibition of Akt phosphorylation within neuroblastoma tumors as well as the activation and intracellular redistribution of Bid within the tumor cell compartment. These findings suggest that IL-12 may have therapeutic activity against tumors that overexpress activated Akt, such as neuroblastoma.

The development of new approaches for the treatment of neuroblastoma has been difficult because of the complex mechanisms by which neuroblastoma tumor cells resist programmed cell death. For example, we previously reported that administration of IL-12/pulse IL-2 therapy induces complete regression of well-established primary and/or metastatic murine renal cell carcinoma (Renca) tumors (11). The IL-12/pulse IL-2 combination also inhibits the vascularization of Renca tumors, induces apoptosis of both vascular endothelial and tumor cell populations within the tumor microenvironment (13), and mediates these antiangiogenic/antivascular and overall antitumor effects via mechanisms that are critically dependent on both IFN-γ and the presence of an intact Fas/FasL pathway in vivo (13). IL-12-inducible cytokines such as IFN-γ and TNF-α also directly increase cell surface Fas expression on Renca cells, and sensitize these cells to Fas-mediated apoptosis in vitro (27). In turn, chemokines (i.e., IFN-γ–inducible apoptosis protein 10 and monokine induced by IFN-γ) can mediate both the antiangiogenic (64) and the overall antitumor effects (65,66) of IL-12 in preclinical models.

In murine neuroblastoma tumors, however, the antitumor mechanisms used by IL-12 appear to be even more complex. For example, here we have shown that systemic administration of IL-12 induced complete regression of both primary and metastatic murine neuroblastoma tumors and did so in conjunction...
with marked increases in circulating levels of IFN-γ, TNF-α, and sFasL, induction of both tumor and endothelial cell apoptosis, and increased expression of proapoptotic genes encoding Fas/FasL, TNFR-p55, TRAIL, and caspase-8 within the tumor microenvironment. In addition, we found that, unlike Renca tumor cells, Neuro-2a and TBJ tumor cells are intrinsically resistant to receptor-mediated apoptosis induced by FasL, TRAIL, or IFN-γ + TNF-α in vitro. These observations suggested that a molecule(s) capable of inhibiting receptor-mediated apoptosis could be expressed and/or activated in these neuroblastoma cells. We addressed this possibility directly by incubating TBJ or Neuro-2a tumor cells first with the protein synthesis inhibitor cycloheximide and then with various inducers of receptor-mediated apoptosis. We found that prior exposure of TBJ and Neuro-2a cells to cycloheximide sensitized these cells to receptor-mediated apoptosis, most notably to apoptosis induced by exposure to TNF-α + IFN-γ. These observations suggest that TBJ and Neuro-2a cells express a short-lived prosurvival factor that mediates resistance to apoptosis in vitro. We suggest that suppression of this pathway could contribute to the induction of tumor cell apoptosis and overall tumor regression by IL-12 in vivo.

Several distinct prosurvival factors, including Bcl-2 (67), FLIP (68), survivin (43,44,69,70), and Akt (46–49,61,62) are overexpressed or constitutively activated in tumor cells, making such cells resistant to apoptosis. Akt is a cytosolic protein that requires phosphorylation for maximal activation (56–58), and the activation status of Akt is regulated by inhibitory signals provided by PTEN (71,72) and by positive signals provided by phosphatidylinositol-3-hydroxy kinase (PI3K) (73), among others. Akt can mediate the resistance of tumor cells to apoptosis induced by TRAIL (61,62), radiation (74), and chemotherapeutic drugs (46–48). By contrast, PI3K/Akt inhibitors sensitize tumor cells to apoptosis induced by radiation (74), chemotherapy (75), and receptor ligands including anti-Fas antibody (Jo-2), TRAIL, and TNF-α (62,76). More specifically, inhibitors of the PI3K/Akt pathway effectively sensitize human neuroblastoma cells to apoptosis induced by TRAIL (47) or chemotherapy (48), suggesting that this pathway may play an important role in the pathobiology of neuroblastoma.

We found that both TBJ and Neuro-2a cells express high levels of activated, serine-phosphorylated Akt compared with normal mouse adrenal gland. The adrenal gland is a common site of origin for neuroblastoma tumors in children. We also found that SH-5, a targeted small-molecule inhibitor of Akt, sensitized these cells to apoptosis induced by IFN-γ + TNF-α, confirming that Akt is a functionally active prosurvival factor in murine neuroblastoma tumor cells. Furthermore, we found that systemic administration of IL-12 inhibited the expression of serine-phosphorylated Akt within established TBJ tumors. These observations, coupled with the finding that in vitro treatment of TBJ cells with IL-12–inducible cytokines such as IFN-γ and TNF-α has no direct effect on the expression of serine phosphorylated Akt (Khan TK, unpublished observations), suggests that yet-to-be-identified factors mediate the inhibition of AKT phosphorylation by IL-12. To our knowledge, these studies provide the first demonstration that an immunotherapeutic regimen such as IL-12 can potently suppress activation of the Akt pathway within tumor cells in vivo.

To investigate the functional consequences of IL-12–mediated inhibition of Akt phosphorylation, we examined whether proapoptotic pathways that are normally inhibited by Akt are currently activated within tumor cells upon administration of IL-12. Akt blocks apoptosis and promotes cell survival by inhibiting the expression and/or activity of several proapoptotic molecules, including Bad (77), caspase-9 (78), Fasl (79), glycogen synthase kinase-3 (80), and Bid (36,37,61,62). In particular, we focused on Bid, given its important role in immune-mediated apoptosis. Following cleavage by caspase-8 during receptor-induced/immune-mediated apoptosis (59,60,63), Bid is activated and translocated to the mitochondria, where it induces oligomerization of Bcl-2 family proteins BAX and BAK, resulting in the release of cytochrome c and the potentiation of cell death (59,60,81). Here we have shown for the first time, to our knowledge, that IL-12 not only inhibits Akt phosphorylation within TBJ tumors but also that this inhibition is accompanied by enhanced cleavage and subcellular translocation of Bid. These observations further suggest that a mitochondria-dependent pathway facilitates the induction of tumor cell apoptosis by IL-12 in these mice. These observations also provide the first direct evidence to implicate mitochondria-dependent pathways in the mediation and/or potentiation of the proapoptotic and overall antitumor effects of IL-12 in mice bearing established tumors.

These observations raise several interesting questions for future investigation. In particular, it will be important to evaluate whether these observations can be extended to other tumor types that express high levels of activated Akt or to other prosurvival molecules such as survivin or Bcl-2 family members. In addition, it will be instructive to investigate whether other immunotherapeutic cytokines can regulate the Akt pathway or whether this mechanism is restricted more specifically to IL-12. Further, these observations suggest that targeted inhibition of the Akt pathway might also warrant investigation as an approach to potentiate the antitumor activity of immunotherapeutic cytokines such as IL-12.

Collectively, the present results demonstrate that IL-12 mediates dramatic antitumor activity in mice bearing well-established orthotopic murine neuroblastoma tumors and thus provide an important preclinical rationale for investigating the efficacy of IL-12 in children with neuroblastoma. Further, these studies provide potentially important new insights into the mechanisms by which immunotherapeutic interventions such as IL-12 can overcome mechanisms of tumor self-defense to induce tumor regression and suggest that IL-12 may possess unique efficacy against tumors that express activated Akt, including neuroblastoma.

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NOTES

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