Cancer Gene Therapy (2004) 11, 681–690

www.nature.com/cgt

Inhibition of the NF-κB pathway enhances TRAIL-mediated apoptosis in neuroblastoma cells

Bahri Karacay,[1] Salih Sanlioglu,[2] Thomas S Griffith,[3] Anthony Sandler,[1,4] and Daniel J Bonthius[1,5,6]

[1]Department of Pediatrics, University of Iowa, Iowa City, Iowa 52242, USA; [2]Human Gene Therapy Unit, Akdeniz University Faculty of Medicine, Antalya, Turkey; [3]Urology, University of Iowa, Iowa City, Iowa 52242, USA; [4]Surgery, University of Iowa, Iowa City, Iowa 52242, USA; [5]Neurology, University of Iowa, Iowa City, Iowa 52242, USA; and [6]Anatomy & Cell Biology, University of Iowa, Iowa City, Iowa 52242, USA.

Neuroblastoma is the most common solid extracranial neoplasm in children and causes many deaths. Despite treatment advances, prognosis for neuroblastoma remains poor, and a critical need exists for the development of new treatment regimens. TNF-related apoptosis-inducing-ligand (TRAIL) induces cell death in a variety of tumors, but not in normal tissues. Moreover, TRAIL is nontoxic, making it a strong antitumor therapeutic candidate. We demonstrate that introduction of the TRAIL gene into neuroblastoma cell lines using an adenoviral vector leads to apoptotic cell death. RT-PCR and flow-cytometric analyses demonstrated that TRAIL's effect is mediated primarily via the TRAIL R2 receptor. As TRAIL can activate the nuclear factor-κB (NF-κB) signaling pathway, which can exert an antiapoptotic effect, we hypothesized that inhibition of NF-κB signaling may augment TRAIL's killing effects. TRAIL-mediated cell death was enhanced when neuroblastoma cells were simultaneously infected with a dominant-negative mutant of IκB kinase, a kinase essential for NF-κB activation. The combination of blockade of NF-κB signaling and expression of TRAIL induced apoptotic death in a greater proportion of SKNSH cells than did either treatment alone. Thus, concurrent inhibition of the NF-κB pathway and the induction of TRAIL-mediated apoptosis may become a useful approach for the treatment of neuroblastoma.

Cancer Gene Therapy (2004) **11,** 681–690. doi:10.1038/sj.cgt.7700749
Published online 27 August 2004

Keywords: neuroblastoma; TRAIL; NF-κB; IKKβ dominant-negative mutant; apoptosis

Neuroblastoma is a pediatric malignancy of neural crest derivatives. It is the most common solid extracranial neoplasm in children and is responsible for 15% of all childhood cancer deaths. Despite advances in treatment modalities, including radiation, surgery, and chemotherapy, many children with advanced neuroblastoma face a poor prognosis. Owing to the harmful effects of radiation and chemotherapy, many neuroblastoma survivors suffer from learning, memory and fine motor disabilities.[1,2] Thus, improved therapy for neuroblastoma is critically needed.

Apoptosis, or programmed cell death, is a key mechanism for homeostasis throughout embryonic and adult life. Genetic aberrations disrupting programmed cell death often underpin tumorigenesis and drug resistance.[3] Identification of mechanisms that activate the apoptotic program in neuroblastoma cells could substantially enhance the therapeutic efficacy in this tumor and other forms of cancer.[4]

Received January 28, 2004.
Address Correspondence and reprint requests to: Dr Bahri Karacay, PhD, Department of Pediatrics, University of Iowa, 2185 Medical Laboratories, Iowa City, Iowa 52242, USA.
E-mail: bahri-karacay@uiowa.edu

Several members of the tumor necrosis factor (TNF) superfamily can induce apoptosis. TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, which also includes TNF, Fas, lymphotoxin alpha (LT-a), and CD40,[5] TNF-alpha, LT-a and Fas ligands can efficiently induce apoptosis in a variety of tumors. However, each can also induce life-threatening toxicities that severely damage the normal tissues.[6] In contrast, TRAIL selectively induces apoptosis in tumor and transformed cells, but not in normal cell lines.[5,7,8] This selectivity of TRAIL makes it a potentially valuable agent for cancer therapy. Whereas intravenous injection of mice with Fas L induces massive hepatocellular degeneration, tissue necrosis, hemorrhage and death, injection of mice with TRAIL induces no observable toxicity.[9] The non-toxic effects of TRAIL have likewise been documented in non-human primates,[10] demonstrating the safety of systemically administered TRAIL.

A problem with the use of TRAIL as an antitumor agent *in vivo* is its short half-life. Therefore, the protein cannot be delivered to cells through conventional methods. As an alternative, an adenoviral vector encoding TRAIL (Ad5-TRAIL) has been developed.[11] Use of this viral vector can potentially circumvent the problem of

TRAIL's short half-life by allowing production of TRAIL at its site of action. A principal goal of this study was to determine whether the apoptotic pathway could be activated within neuroblastoma cells by delivering the death-inducing TRAIL gene to the tumor cells using this adenoviral vector.

Nuclear factor kappa B (NF-κB) is a transcription factor that can prevent cells from undergoing apoptosis via activation of antiapoptotic genes. TRAIL can activate the NF-κB pathway, an effect that limits the ability of TRAIL to induce apoptosis. Thus, inhibition of the NF-κB pathway may increase the sensitivity of tumor cells to TRAIL-mediated apoptosis. Activation of the NF-κB pathway depends upon phosphorylation of inhibitory proteins by specific kinases (inhibitor of κB (IκB) kinase, IKK). IKKβ is one of the catalytic domains of the kinase IKK and is essential for NF-κB activation and for protection from apoptosis *in vivo*.[12] This critical role of IKKβ for activation of the NF-κB pathway makes it a potential target for therapeutic approaches. The second major goal of this study was to determine whether inhibition of the NF-κB pathway at the IKKβ site enhances the sensitivity of neuroblastoma cells to TRAIL-mediated apoptosis.

The concept has recently emerged that viral gene therapy vectors could be used to deliver anticancer agents to tumors. This study explores the efficacy of a dual vector approach in which viral vectors are used to deliver TRAIL and simultaneously to block the NF-κB pathway in neuroblastoma cells.

Materials and methods

In vitro *killing of neuroblastoma cells with recombinant TRAIL protein*

To determine the effect of TRAIL on neuroblastoma cell viability, SKNSH and NB7 cells were exposed to recombinant human TRAIL protein. Microtiter plates were seeded with 2×10^4 cells/well and allowed to adhere for at least 6 hours before treatment with rhTRAIL. Cells were incubated with increasing concentrations of rhTRAIL protein (4.1–1000 mg/ml) for 20 hours. Cell viability was determined by crystal violet staining.

Adenoviral infection

The human neuroblastoma cell line SKNSH was grown in modified Eagle's medium (MEM) supplemented with non-essential amino acids (NEAA), 15% fetal bovine serum (FBS), L-glutamine and penicillin–streptomycin antibiotic. NB7 neuroblastoma cell line was grown in RPMI 1640 supplemented with NEAA, 10% FBS, L-glutamine and penicillin–streptomycin antibiotics. Cells were cultured in complete medium and permitted to adhere for at least 6 hours before adding adenoviral vectors. Immediately prior to infection, the cells were washed with phosphate-buffered saline (PBS). The vectors were then added at a variety of concentrations in serum-free culture medium. After 2 hours of incubation at 37°C, medium containing $2\times$ FBS was added to the cultures to produce a final serum concentration of 15 and 10%, respectively. Viral infections were conducted in the same manner during the *in vitro* killing experiments, with the exception that cells were maintained in complete medium throughout the experiments.

Reporter gene assays for measuring adenoviral infection

Analysis of adenoviral infection efficiency was performed using an adenovirus encoding for enhanced green fluorescent protein (Ad5-EGFP). A total of 6×10^4 SKNSH and NB7 human neuroblastoma cells were plated onto a 24-well plate and permitted to adhere for at least 6 hours before adding adenoviral vectors. The cells were then infected with 0, 1000 or 5000 Ad5-EGFP particles/cell (0, 20 or 100 PFU/cell), as described above. At 24 and 48 hours after the infection, transduction was evaluated by visual observation, and the cultures were photographed using a confocal microscope. To determine quantitatively the proportion of the cells transduced with Ad5-EGFP, 2×10^5 SKNSH or NB7 cells were plated onto a six-well plate and were infected with Ad5-EGFP at 300 plaque-forming unit (PFU)/cell. At 24 hours after the infection, the cells were analyzed by flow cytometry on a FACScanner (Becton Dickinson, San Jose, CA).

Luciferase assays

We have previously described the construction of the NF-κB-responsive luciferase reporter vector, Ad5-NF-κB-Luc.[13] In this construct, the luciferase gene is driven by four tandem copies of the NF-κB consensus sequence attached to a TATA-like promoter from the herpes simplex virus thymidine kinase gene. Ad5-Bgl II is an empty adenoviral vector carrying only a *Bgl*II site. Ad5-IKKβKA is an adenoviral vector carrying a dominant-negative mutant of IKK. As the activation of the NF-κB pathway depends upon the activity of IKK, cellular infection with this vector blocks NF-κB activation.

A total of 2×10^5 SKNSH cells were plated onto a six-well plate and were permitted to adhere for at least 6 hours before adding adenoviral vectors. The cells were then infected with either Ad5-Bgl II or Ad5-IKKβKA at a multiplicity of infection (MOI) of 0, 33, 100 or 300 PFU/cell as described above and incubated at 37°C overnight. At 24 hours after the first infection, the cells were secondarily infected with Ad5-NFκB-luc at an MOI of 100 PFU/cell and incubated for another 24 hours. Cytoplasmic extract was isolated 48 hours after the first infection. Luciferase activity was measured using a commercial assay system (Promega) to assess NF-κB-induced transcriptional activation in a Lumat LB9501 luminometer (Berthold) according to the manufacturer's instructions. All measurements of luciferase activity (relative light units) were normalized to the protein concentration of the harvested cell lysates. Three independent experiments were carried out for each reporter construct.

In vitro *killing of SKNSH and NB7 human neuroblastoma cells with adenoviral vectors*

Neuroblastoma cell sensitivity to Ad5-Bgl II, Ad5-TRAIL, Ad5-IKKβKA and Ad5-TRAIL + Ad5-IKKβKA was assayed using the following procedure. Cells were added to 96-well plates (2×10^4 cells/well) in complete medium and allowed to adhere for at least 6 hours before infection with the various adenoviral vectors. Cells in each group were transduced by 600 total PFU and its threefold serial dilutions (7.4–600 PFU/cell). The control group (Ad5-Bgl II, adenoviral empty vector carrying only a *Bgl*II site) received 600 PFU/cell of Ad5-Bgl II vector. Groups receiving Ad5-TRAIL or Ad5-IKKβKA vectors received 300 PFU/cell of either one or the other of these vectors along with 300 PFU of Ad5-Bgl II to make the total viral load 600 PFU/cell in each individual experiment. The Ad5-TRAIL + Ad5-IKKβKA group received 300 PFU/cell of each vector. Cell death was determined after 22 hours by crystal violet staining, as described previously.[14] Results are presented as the percent of cell death: [1−(OD cells treated per OD cells not treated)] × 100. Three independent experiments were carried out for each reporter construct.

Annexin binding

Although crystal violet staining indicates cell death, it does not discriminate between apoptotic and necrotic cell death. One predominant plasma-membrane alteration that occurs during apoptosis is the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet. This ectopic phosphatidylserine then serves as a recognition molecule for macrophages.[15,16] Annexin V is a calcium-dependent phospholipid-binding protein and has a high affinity for phosphatidylserine.[17,18] Thus, the proportion of cells that are positive for Annexin V binding is also the proportion of cells undergoing apoptosis.[19,20]

Annexin V binding was utilized to determine the proportion of neuroblastoma cells undergoing apoptosis following infection with the various adenoviral vectors, alone or in combination. SKNSH cells were infected with Ad5-TRAIL, Ad5-IKKβKA or Ad5-TRAIL + Ad5-IKKβKA as described above, and apoptotic cell death was measured by flow cytometry using FITC-conjugated Annexin V (R&D Systems, Minneapolis, MN) and propidium iodide (Sigma, St Louis, MO). Uninfected SKNSH cells served as control for flow cytometric analysis of the infected cells.

Regular and quantitative RT-PCR for human TRAIL receptors

TRAIL interacts with four membrane-bound receptors: TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4[21] However, only two of the TRAIL receptors, TRAIL-R1 and TRAIL-R2, contain functional death domains capable of inducing apoptosis.[22] Regular RT-PCR was utilized to determine the expression profile of TRAIL receptor message in neuroblastoma cell lines.

Total RNA was isolated from SKNSH, and NB7 human neuroblastoma cell lines with TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed using Superscript™ II. Primers and conditions for the RT-PCR have been described earlier.[22]

The real-time quantitative RT-PCR primers and probe for TRAIL R1 and TRAIL R2 were designed to cross an intron using published sequences (Human genome project BAC clone RP11-1149023 and RPRP11-875011, respectively). Sequences of the real-time quantitative RT-PCR primers and probes used were: hTRAIL R1-5′, 5′-TGTA CGCCCTGGAGTGACAT-3′; hTRAIL R1-3′, 5′-CACC AACAGCAACGGAACAA-3′, and hTRAIL R1 probe, 5′-6FAM-TGTCCACAAAGAATCAGGCAATGGACAT AAT-TAMRA-3′; hTRAIL R2-5′, 5′-CACTCACTGGA ATGACCTCCTTT-3′; hTRAIL R2-3′, 5′-GTGCAGGG ACTTAGCTCCACTT-3′; and hTRAIL R2 probe 5′-6FA M-TCACACCTGGTGCAGCGCAAGCAG-TAMRA-3′.

The rRNA primers and probes were purchased from PE Applied Biosystems. The rRNA probe was labeled with a second dye to analyze TRAIL Rs and rRNA in the same reaction. cDNA (250 ng) was used as a template for TaqMan assay of both TRAIL R1 and TRAIL R2 message and the internal control ribosomal RNA. The TaqMan PCR reaction was carried out as described previously.[23]

Flow cytometry for TRAIL receptor expression

To assess the expression of TRAIL receptor protein on neuroblastoma cells, SKNSH cells were incubated with unlabeled primary mAbs for TRAIL R1 (HS101, Mouse IgG1), TRAIL R2 (HS201, Mouse IgG1) (Alexis Biochemicals, Carlsbad, CA), nonspecific mouse IgG1 isotype (MOPC-21; Sigma, St Louis, MO) and MHC I (W6/32) in 3% BSA in PBS (PBSA) for 30 minutes on ice. Following two washes with PBSA, cells were incubated for 30 minutes on ice with a phycoerythrin-conjugated, Fc-specific, goat–anti-mouse F(ab′)2 (diluted 1/100 in 3% PBSA, Jackson Immunoresearch, West Grove, PA). Cells were analyzed immediately after staining on a FACScan (Becton Dickinson, San Jose, CA).

Results

Both recombinant TRAIL protein and an adenoviral vector encoding the TRAIL gene induced cell death in neuroblastoma cell lines

The success of adenoviral vector-mediated TRAIL gene therapy relies on the presence of the TRAIL-induced apoptotic pathway in neuroblastoma tumor cells. Thus, it was critical first to demonstrate the sensitivity of neuroblastoma cells to TRAIL protein. SKNSH cells were treated with increasing amounts of soluble recombinant TRAIL protein, and cell death was determined by crystal violet staining. Figure 1 shows that TRAIL

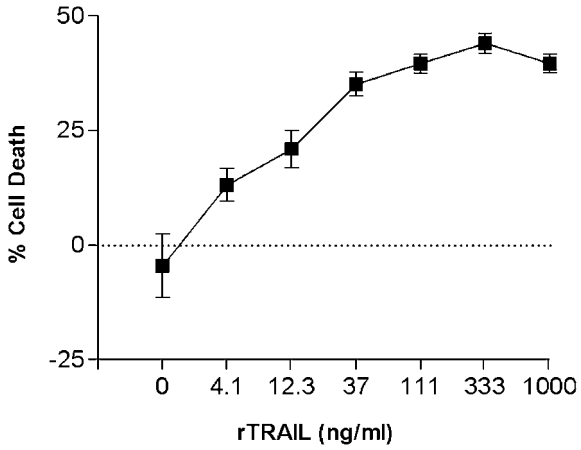

Figure 1 Recombinant human TRAIL protein kills human neuroblastoma cells. Microtiter plates were seeded with 2×10^4 cells/well before the addition of recombinant hTRAIL at the indicated concentrations. Cell viability was determined after 20 hours by crystal violet staining. Each value represents the mean of six independent data points. Three independent experiments were carried out, and similar results were observed. These results demonstrate that TRAIL induces cell death in SKNSH cells in a dose–response manner.

induced the death of SKNSH cells in a dose-dependent manner. Thus, SKNSH cells are sensitive to TRAIL.

The next step was to test neuroblastoma cells for their susceptibility to infection by adenovirus. We examined the receptiveness of SKNSH cells to adenovirus infection by using a reporter vector, an adenovirus carrying the cDNA for enhanced green fluorescent protein (Ad-EGFP). The tumor cells were incubated with Ad5-EGFP at a variety of viral concentrations. Figure 2a shows that adenovirus readily infected the SKNSH cells. By 48 hours postinfection, a large proportion of the SKNSH cells exposed to 20 PFU/cell and virtually all of the SKNSH cells exposed to 100 PFU/cell of Ad5-EGFP were transduced, as determined by visual examination. This finding was confirmed by flow cytometry analysis. As shown in Figure 2b, 99.7% of SKNSH neuroblastoma cells were transduced with (300 PFU/cell) Ad-EGFP within 24 hours of infection. To determine the commonality of the adenovirus infectivity of neuroblastoma cells, we have repeated the experiment with NB7, NB10 and NB16 neuroblastoma cell lines. After 24 hours of infection, 97.7, 99.3 and 97.0% of the NB7, NB10 and NB16 cell, respectively, were positive for reporter gene expression (data not shown).

With the demonstration that adenovirus can successfully infect neuroblastoma cells, subsequent experiments were performed to examine the consequences of Ad5-TRAIL infection. As shown in Figure 3, no cell death was observed when SKNSH cells were infected at progressively greater multiplicities of infection with Ad5-Bgl II. Thus, neuroblastoma cells were not killed by an empty adenoviral vector. In contrast, Ad5-TRAIL infection did kill the neuroblastoma cells, and the proportion of cells

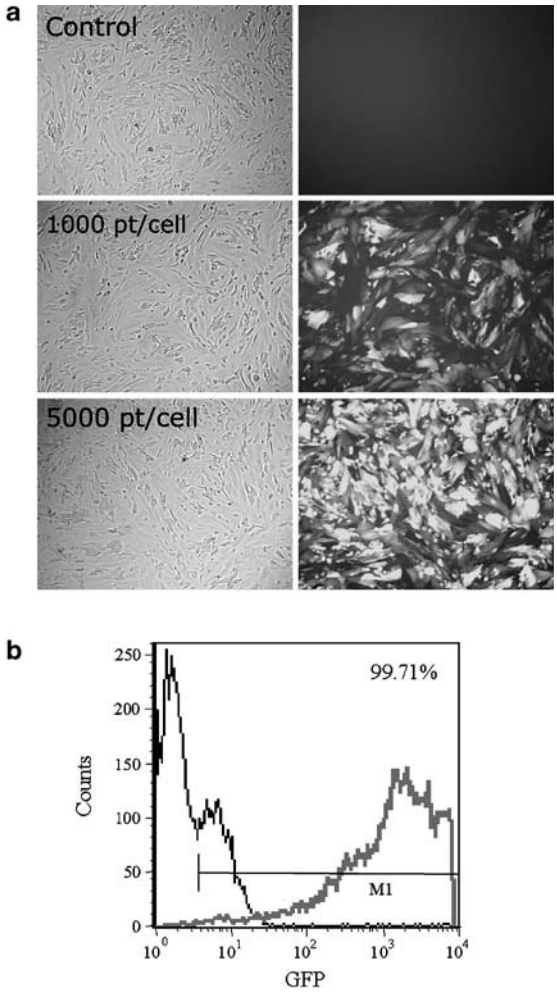

Figure 2 Susceptibility of human neuroblastoma cell lines to adenovirus infection. (**a**) SKNSH cells were infected with Ad5-EGFP at the indicated MOI. The cultures were photographed with a confocal microscope 48 hours after the infection. As shown, a high proportion of the cells exposed to 1000 particle/cell and virtually all of the cells exposed to 5000 particle/cell are infected with the adenovirus. (**b**) Infection efficiency was quantitatively determined by flow cytometry. The histograms represent uninfected (thin line) and Ad5-EGFP-infected (thicker line) cells. Among the cells exposed to Ad5-EGFP, 99.71% were EGFP positive. The histograms each represent 10^4 gated cells, and viability was $>95\%$ for each treatment, as assessed by propidium iodide exclusion. The experiment was repeated three times with similar results.

killed progressively increased as the MOI increased. At 600 PFU/cell, Ad5-TRAIL killed nearly half of the neuroblastoma cells. These results demonstrate that Ad5-TRAIL infection of neuroblastoma cells can induce death of the tumor cells, most likely by producing TRAIL protein.

Inhibition of the NF-κB pathway enhances the sensitivity of neuroblastoma cells to TRAIL

As NF-κB signaling can inhibit TNF-induced apoptosis,[24] and because TRAIL can activate the NF-κB

pathway,[25] we hypothesized that inhibition of the NF-κB pathway will enhance TRAIL-mediated apoptosis in neuroblastoma cells. As a first step, we established that the adenoviral vector carrying a dominant-negative mutant of IKK (Ad5-IKKβKA) can block the NF-κB pathway in neuroblastoma cells. As shown in Figure 4, infected control group of SKNSH cells exhibited a high level of NF-κB activity. Infection of the cells with the Ad5-IKKβKA vector resulted in a marked dose-dependent inhibition of NF-κB activity. The empty vector (Ad5-Bgl II) did not cause a decrease in luciferase activity. These results confirmed that the IKK dominant-negative vector could effectively inhibit NF-κB activity in neuroblastoma cells.

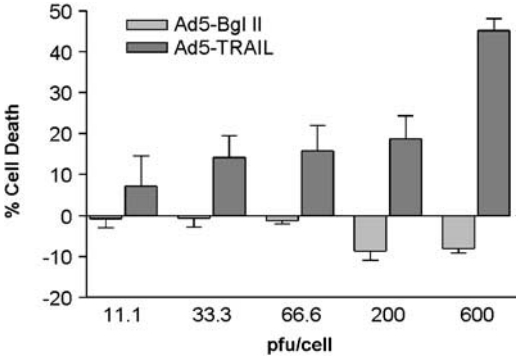

Figure 3 Induction of cell death in human neuroblastoma cells infected with an adenoviral vector carrying human TRAIL cDNA. SKNSH cells were infected with adenoviral vectors Ad5-TRAIL or Ad5-Bgl II (empty vector) at the indicated MOI (PFU/cell). Cell viability was determined after 20 hours by crystal violet staining. Each value represents the mean of three wells. The Ad5-TRAIL vector induced cell death in a dose–response manner. In contrast, the Ad5-Bgl II vector induced no cell loss. Three independent experiments were carried out, and similar results were observed with each replication.

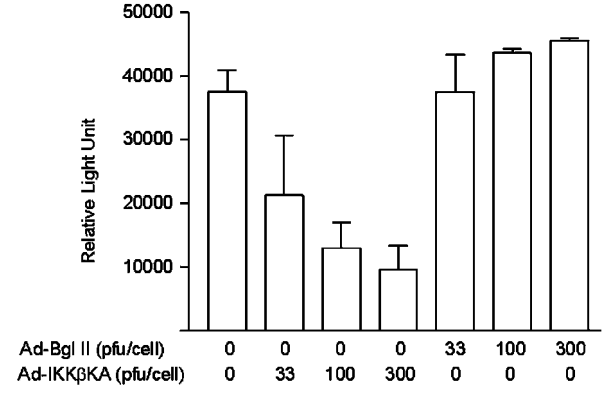

Figure 4 Adenovirus encoding the dominant-negative IKK mutant (Ad5-IKKβKA) blocks NF-κB activation in SKNSH neuroblastoma cells. SKNSH cells were infected with either Ad5-Bgl II (empty vector) or Ad5-IKKβKA at an MOI of 0, 33, 100 or 100 PFU/cell. After 24 hours, cells were infected with Ad5-NFκB-luc at an MOI of 100 PFU/cell. Luciferase activity, which reflects NF-κB activity, is expressed as relative light units (RLU) per microgram of protein. As the MOI of Ad5-IKKβKA increased, NF-κB activity diminished markedly. Data represent the mean of three independent experiments. Error bars represent standard error of the mean.

Next, SKNSH cells were infected with Ad5-TRAIL, Ad5-IKKβKA or with a combination of these two viruses to assess the effect of TRAIL, NF-κB pathway inhibition and the combination of these effects on cellular survival. Infection with the negative control vector, Ad5-Bgl II, did not cause any cell death, as determined by crystal violet staining (Fig 5a). On the contrary, in this control group, cells continued to proliferate, resulting in negative cell loss rates on the survival curve. Delivery of the TRAIL-expressing vector into SKNSH cells did cause cell death, and the magnitude of cell loss increased as the MOI increased. At the highest vector concentration (600 PFU/cell), the cell death rate reached 20.2% (Fig 5a). Ad5-IKKβKA vector alone caused no cell loss at an MOI of 200 PFU/cell and below. At the highest MOI (600 PFU/cell), the Ad5-IKKβKA vector alone induced cell death at the modest rate of 9.5%.

In contrast, the combination of the two vectors very effectively induced the death of SKNSH cells. At an MOI of 66.6 and above, the combination of Ad5-TRAIL and Ad5-IKKβKA induced the death of a greater proportion of SKNSH cells than either vector alone or than the sum of the effects of either vector used alone. This synergistic effect of the two vectors was most evident at the highest MOI, where the combination of vectors induced the death of 50.6% of the cells, while the summed effects of the individual vectors reached only 29.7%. These results demonstrate that inhibition of the NF-κB pathway synergistically enhances the cell death caused by TRAIL, presumably by reducing the antiapoptotic effect of the NF-κB pathway.

To determine whether the observed effects are a general phenomenon, rather than a cell-line-specific phenomenon, we exposed another neuroblastoma cell line, NB7, to recombinant TRAIL, and to the same combination of viral vectors as tested with SKNSH cell line. Recombinant TRAIL induced cell death in NB7 cells in a dose-dependent manner (data not shown). Infection with the negative control vector, Ad5-Bgl II, did not cause any cell death, as determined by crystal violet staining (Fig 5b). Similar to SKNSH cells, the NB7 cells in this control group continued to proliferate, resulting in negative cell loss rates on the survival curve. Delivery of the TRAIL-expressing vector into NB7 cells did cause cell death, and the magnitude of cell loss increased as the viral titer increased. At the highest vector concentration (600 PFU/cell), the cell death rate reached 15.7% (Fig 5b). Whereas Ad5-IKKβKA vector alone induced some cell death at the highest MOI (600 PFU/cell) in SKNSH cells, the Ad5-IKKβKA vector alone did not induce cell death in NB7 cells, even at highest MOI. Although cell death was not observed in the NB7 cells treated with Ad5-IKKβKA, cell proliferation was substantially reduced, relative to the NB7 cells that received the negative control vector, Ad5-Bgl II (Fig 5b).

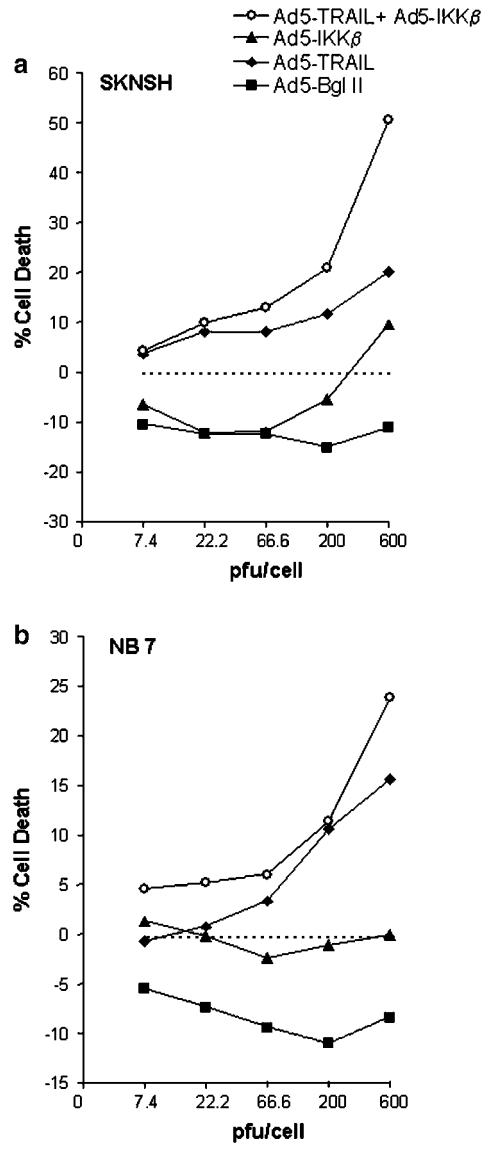

Figure 5 Inhibition of the NF-κB pathway enhances the apoptotic effect of TRAIL in human neuroblastoma cells. SKNSH (**a**) and NB7 cells (**b**) were infected with Ad5-Bgl II, Ad5-TRAIL, Ad5-IKKβKA, or with a combination of Ad5-TRAIL and Ad5-IKKβKA. Cell viability was determined after 20 hours by crystal violet staining. The Ad5-Bgl II vector induced no cell loss, indicating that infection with empty vector alone does not impair SKNSH and NB7 cell viability. Ad5-IKKβKA induced cell death, but only to a modest extent and only at the highest MOI in SKNSH cells. Ad5-TRAIL induced more cell death than did Ad5-IKKβKA, but the extent was still modest. In contrast, the combination of Ad5-TRAIL and Ad5-IKKβKA induced substantial amounts of cell death and to a much greater extent than did either vector alone. Each value represents the mean of three wells. Standard error bars were omitted from the graph for clarity but were less than 10% for all data points. Experiments were performed at least three separate times with similar results.

The combination of the two vectors induced the death of many NB7 cells, just as the combination did in SKNSH cells. At all MOI applied, the combination of Ad5-TRAIL and Ad5-IKKβKA induced the death of a greater proportion of NB7 cells than did either vector alone or than the sum of the effects of either vector alone. This synergistic effect of the two vectors was most evident at the highest MOI, where the combination of vectors induced the death of 23.8% of the cells, while the summed effects of the individual vectors reached only 15.6%. These results demonstrate that, in both cell lines of neuroblastoma tested, inhibition of the NF-κB pathway synergistically enhances the cell death caused by TRAIL, presumably by reducing the antiapoptotic effect of the NF-κB pathway.

Infection with the Ad5-TRAIL and Ad-IKKβKA viral vectors induced apoptosis in neuroblastoma cells

To confirm that the tumor cell death following infection with the viral vectors was mediated through an apoptotic mechanism, SKNSH cells infected with Ad5-TRAIL, Ad5-IKKβKA, alone or in combination, were analyzed for Annexin V binding. As shown in Figure 6 (panel a), uninfected SKNSH cells exhibited only a low level (2.3%) of Annexin V positivity, reflecting a low background rate of apoptosis. Infection of SKNSH cells with Ad5-TRAIL alone increased Annexin V positivity to 21.2% (panel b), while 9.3% of cells were Annexin V positive following infection with Ad5-IKKβKA alone (panel c). These results suggest that each vector alone induced apoptosis in a modest proportion of the infected cells. However, when SKNSH cells were infected with the combination of Ad5-TRAIL and Ad5-IKKβKA, the Annexin V labeling rate rose to 44.3% (panel d), which was substantially higher than the summed labeling rates of these vectors used alone.

These results suggest that the SKNSH cell death induced by infection with the Ad5-TRAIL and Ad5-IKKβKA vectors was apoptotic. This was further confirmed by light microscopy, in which morphological changes, including membrane blebbing and the release of apoptotic bodies, were observed in cells infected with Ad5-TRAIL (data not shown). Furthermore, these results obtained with Annexin V binding corroborate the results obtained from crystal violet staining. Both techniques demonstrated that Ad5-IKKβKA and Ad5-TRAIL infections could individually induce cell death in modest proportions of SKNSH cells, and that simultaneous infection with the two vectors substantially enhanced this effect.

Neuroblastoma cell lines expressed high levels of the TRAIL R2 receptor, but not the other TRAIL receptors

The demonstration that neuroblastoma cells undergo apoptosis when treated with either recombinant TRAIL or an adenoviral vector carrying human TRAIL cDNA suggested that these tumor cells express at least one of the TRAIL receptors on their surfaces. We used receptor-specific oligonucleotide primers[22] and RT-PCR to determine which TRAIL receptor mRNAs are expressed by SKNSH cells. As shown in Figure 7a, SKNSH cells expressed predominantly the message for the TRAIL R2

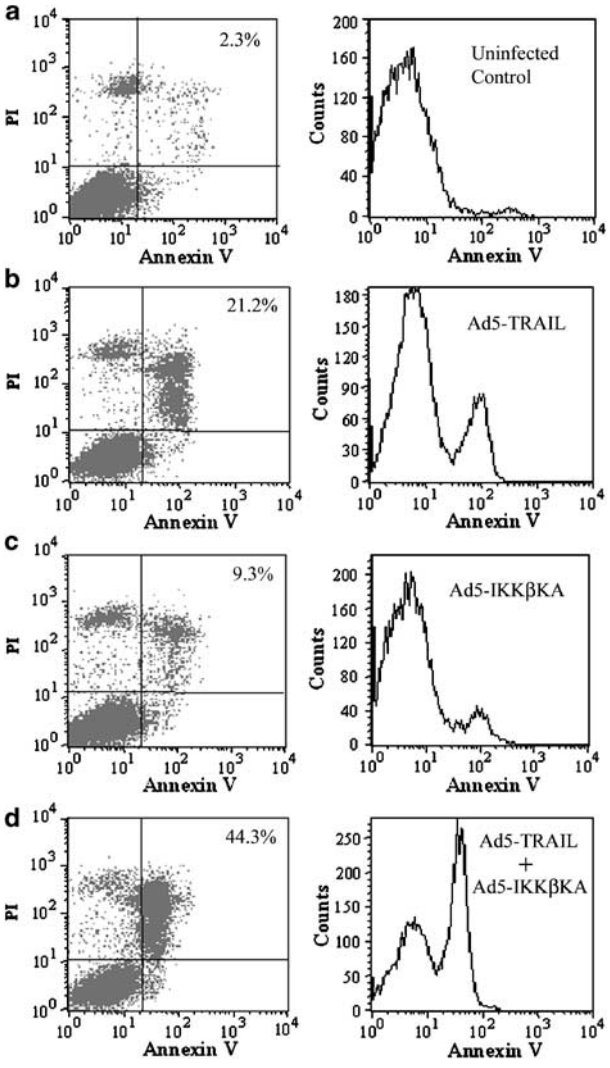

Figure 6 Simultaneous expression of TRAIL and inhibition of the NF-κB signaling pathway induce more Annexin V binding than either treatment alone. SKNSH cells were infected with Ad5-TRAIL alone, Ad5-IKKβKA alone or with a combination of Ad5-TRAIL and Ad5-IKKβKA. Cells were then stained with FITC–Annexin V and propidium iodide, and analyzed by flow cytometry. Uninfected cells served as a negative control. Histograms represent 10^4 gated SKNSH cells. (**a**) Only 2.3% of the SKNSH cells exhibited Annexin V binding, indicating that only a small proportion of cells underwent apoptosis in culture. (**b**) Inhibition of the NF-κB signaling pathway via infection with Ad5-IKKβKA alone induced Annexin V binding in 9.3% of the SKNSH cells. (**c**) Infection of the cells with an adenovirus encoding TRAIL induced Annexin V binding in 21.2% of the SKNSH cells, indicating that expression of TRAIL alone induces apoptosis in a moderate proportion of neuroblastoma cells. (**d**) The combination of TRAIL expression and NF-κB signaling inhibition induced Annexin V binding in a substantial proportion (44.3%) of the cells. Thus, inhibition of NF-κB signaling augmented TRAIL in inducing apoptosis in the SKNSH cells.

receptor. The TRAIL R1 receptor gene was also expressed, but at much lower levels. Specific oligonucleotides designed for the TRAIL R3 and TRAIL R4

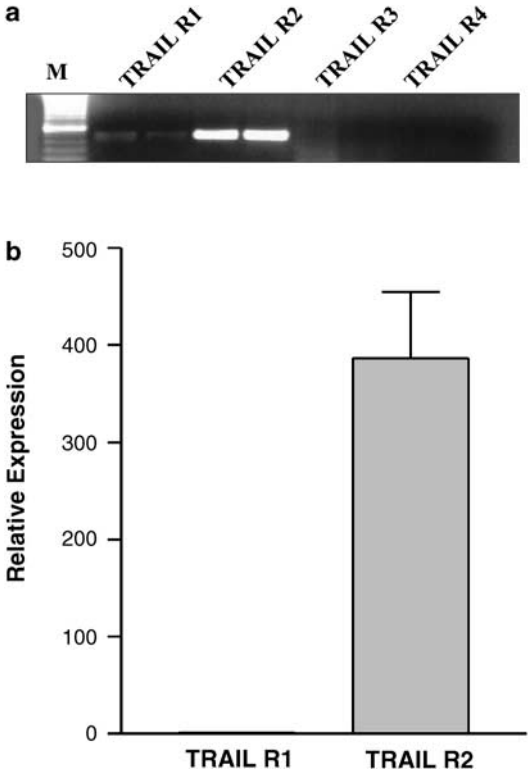

Figure 7 TRAIL R2 is the dominant TRAIL receptor expressed by SKNSH neuroblastoma cells. Regular (**a**) and quantitative (**b**) RT-PCR was carried out to determine the expression of TRAIL receptors by SKNSH cells. As shown in (**a**), TRAIL R1 was expressed at much lower levels than TRAIL R2. TRAIL R3 and TRAIL R4 were not expressed at detectable levels. The first lane contains the 1 kb molecular size marker. (**b**) Shows that TRAIL R2 was expressed at 386-fold greater levels than was TRAIL R1. Thus, the apoptosis-inducing effect of TRAIL is likely mediated via the TRAIL R2 receptor in SKNSH neuroblastoma cells.

receptors did not amplify the messages of the corresponding receptors, indicating that SKNSH cells do not express these decoy receptors.

Next, we quantitated the messages of the TRAIL R1 and TRAIL R2 receptors by quantitative RT-PCR. Figure 7b shows that the TRAIL R2 receptor was expressed at a level several hundred fold higher than that of the TRAIL R1 receptor in SKNSH cells. TRAIL receptor expression was also assessed by flow cytometry (Fig 8), which revealed that neuroblastoma cells expressed TRAIL R2 but not TRAIL R1. These data confirm the regular and quantitative PCR results and further suggest that TRAIL R2 mediates the effect of TRAIL in the SKNSH cell line. To determine the commonality of the TRAIL R expression profile among different neuroblastoma cell lines, we analyzed three additional cell lines: NB7, NB 10 and NB16, using the TRAIL R-specific oligonucleotides. The TRAIL R2 receptor was expressed at much higher levels than any of the other TRAIL receptors in each of these cell lines (data not shown).

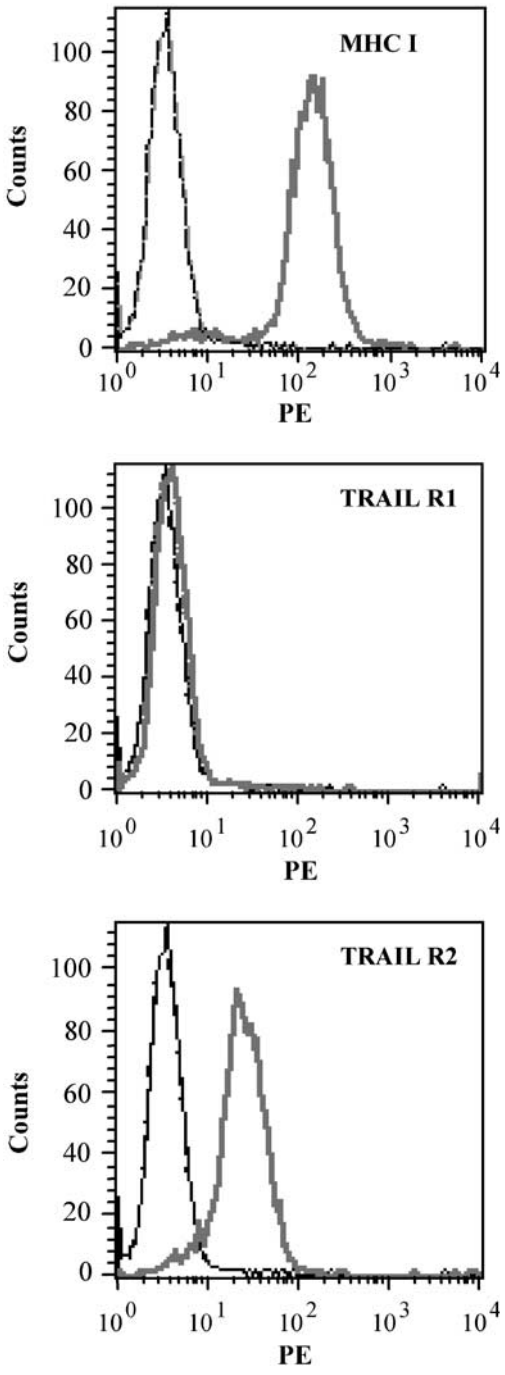

Figure 8 Surface analysis of SKNSH cells for TRAIL receptor expression by flow cytometry. The thin dotted line in each histogram represents staining with isotype control mAb. The thick solid gray line in each histogram represents staining by anti-MHC I mAb (positive control), anti-TRAIL R1 mAb or anti-TRAIL R2 mAb. Each histogram represents 10^4 gated SKNSH cells.

Discussion

Despite increasing efforts for improved therapies, the prognosis for patients with neuroblastoma remains poor. Some patients survive neuroblastoma due to current therapeutic modalities, but acquire treatment-induced permanent cognitive and motor disabilities. Thus, improved therapy for this pediatric nervous system tumor is of paramount importance. Gene therapy holds great promise as an emerging therapy for neuroblastoma, and viral vectors offer a potentially effective means of delivering therapeutic genes to the tumor. This study describes the use of an adenoviral vector expressing a functional human TRAIL gene[6,11] as a potential therapeutic agent for the treatment of neuroblastoma. To promote apoptosis and enhance the sensitivity of neuroblastoma cells to TRAIL, we applied a dual vector approach resulting in simultaneous inhibition of the NF-κB pathway and induction of TRAIL expression.

Delivery of TRAIL via an adenoviral vector induces apoptosis in neuroblastoma cells

As the success of adenoviral vector-mediated TRAIL gene therapy relies on the susceptibility of neuroblastoma cells to infection with adenoviral vectors, it was critical to demonstrate that neuroblastoma cells can take in the adenoviral vectors with high efficiency. All of the neuroblastoma cell lines tested demonstrated a high level of infectability by adenoviral vectors.

We then delivered the TRAIL gene to the tumor cells via adenoviral vector and demonstrated that TRAIL can induce the death of neuroblastoma cells and that the death is apoptotic. These results demonstrate that adenoviral delivery of TRAIL to neuroblastoma can be an effective antitumorigenic agent. The fact that injection of either human or murine TRAIL resulted in no observable toxic effects in mice[9] or primates[10] along with our observation that TRAIL can induce apoptosis in neuroblastoma cells makes TRAIL an excellent candidate for exploration of its therapeutic effects on neuroblastoma *in vivo*.

Two of the four surface receptors for TRAIL, namely TRAIL R1 and TRAIL R2, trigger a downstream intracellular apoptotic cascade. Both PCR and flow cytometric analyses demonstrated that the TRAIL R2 receptor is expressed at high levels, while the TRAIL R1 receptor is either absent or expressed at very low levels in multiple neuroblastoma cell lines. These results suggest that the TRAIL R2 receptor mediates the apoptosis-inducing effect of TRAIL in neuroblastoma cells.

The SKNSH and NB7 cells used in this study were sensitive to both recombinant TRAIL protein and TRAIL expressed from the adenoviral vector. However, other reports have indicated that several neuroblastoma cell lines are resistant to TRAIL-induced apoptosis.[26] Variation among neuroblastoma cell lines in sensitivity to TRAIL may be due to differences in expression levels of certain components of the TRAIL pathway.[27] For example, several neuroblastoma tumors and cell lines fail to express caspase-8.[28] Alternatively, the differences in sensitivity may be due to epigenetic factors, such as the methylation of caspase-8,[26,28,29] which may substantially alter the ability of TRAIL to induce apoptosis. However, lack of TRAIL receptor expression prevented

sensitization of neuroblastoma cells to TRAIL, even when caspase-8 expression was restored,[27] suggesting that integrity of the entire TRAIL pathway is necessary for its function.

Inhibition of the NF-κB signaling pathway promotes apoptosis in neuroblastoma cells

NF-κB is a transcription factor composed of two subunits sequestered in the cytoplasm in an inactive form. Upon activation of the NF-κB pathway, inhibitory proteins are released from the complex, due to phosphorylation by specific kinases (IKK). NF-κB is then translocated to the nucleus, where it acts as a transcription regulator and can function as an antiapoptotic factor. It can suppress TNF-α-induced cell death.[24,30] In contrast, NF-κB can paradoxically play a proapoptotic role.[31,32]

Utilizing a luciferase reporter gene, we found that SKNSH neuroblastoma cells express a high level of NF-κB activity. This observation is consistent with the previously reported finding that NF-κB is constitutively active in S-type neuroblastoma cells and required for their survival.[33] Several other tumors including ovarian,[34] thyroid,[35] prostate,[36] melanoma,[37] breast[38] and colon[39] also exhibit abnormal NF-κB activation. As IKKβ is required for NF-κB activation, this catalytic domain of IKK is a potential target for gene therapy. We found that inhibition of the NF-κB pathway at the IKKβ site, using a dominant-negative mutant of IKKβ, induced death in a proportion of the SKNSH cells and, to a lesser extent, of the NB7 cells. In both the SKNSH and NB7 cell lines, the dominant-negative mutant of IKKβ inhibited cell proliferation. The inhibitory effect on cell proliferation was more obvious in the NB7 cells than in the SKNSH cells, possibly due to the much higher proliferation rate of NB7 cells. As the MOI increased, proliferation was abolished in both cell lines and cell death was induced in the SKNSH cell line. These results suggest that cells with a higher proliferation rate (NB7) require greater inhibition of the NF-κB pathway than do cells with a lower proliferation rate (SKNSH) to exhibit the same level of apoptosis.

Constitutive expression of NF-κB leading to an antiapoptotic effect in many cancer types makes this important transcription factor a therapeutic target in neuroblastoma and other forms of cancer.

The precise mechanism by which inactivation of the NF-κB pathway triggers apoptosis in neuroblastoma is unknown. Composition and activity of NF-κB in tumor cells can play a role in determining the effect of NF-κB on expression of TRAIL receptors or survival proteins and their susceptibility to apoptosis after interacting with TRAIL.[40] Whereas the c-Rel subunit of NF-κB induces the expression of TRAIL R1 and R2, cytokine-mediated activation of the RelA subunit of NF-κB increases the expression of the antiapoptotic gene Bcl-xl and protects cells from TRAIL.[40] Several other antiapoptotic genes are also regulated by NF-κB, including AIP1, AIP2, XAIP, BFL-1/A1 and A20.[41] The cell death induced by NF-κB inactivation may be tied to dysregulation of these protective genes rather than its effect on expression of TRAIL receptors. NF-κB inactivation may also trigger cell death by inducing collapse of the mitochondrial transmembrane gradient and activation of caspase-9.[33]

NF-κB inhibition and TRAIL expression synergistically induce apoptosis in neuroblastoma

Inhibition of the NF-κB signaling pathway induced neuroblastoma cell death, but in only a relatively small proportion of the cells. Expression of TRAIL induced the death of a greater proportion of neuroblastoma cells, yet the effect of TRAIL alone was still modest. However, the combination of NF-κB inhibition and TRAIL expression very effectively induced apoptosis and diminished neuroblastoma cell survival.

The mechanism underlying this synergistic effect is not known for certain. It is possible that they act independently to induce cell death through additive effects. However, the synergism of NF-κB inhibition and TRAIL expression may be due to linkage of their signaling pathways. The activation of TRAIL receptors R1, R2 and R3 can activate the NF-κB signaling pathway,[25,42,43] which can upregulate the expression of antiapoptotic factors. We hypothesize that blockade of the NF-κB signaling pathway enhances the cell death-inducing effects of TRAIL by preventing expression of protective antiapoptotic genes.

This study shows the feasibility of using gene therapy vectors as antitumor agents in neuroblastoma. Furthermore, the dual vector approach produced a synergistic effect, suggesting that such an approach may have potential use in the treatment of this devastating disease.

Acknowledgments

This work was supported by Grants from the Children's Miracle Network (to BK), Akdeniz University Scientific Research Division Administration (to SS), and National Cancer Institute, Holden Comprehensive Cancer Center of the University of Iowa, and Howard Hughes Medical Research Institute Collaborative Grant (to DJB).

References

1. Humpl T. Neuroblastoma. *World J Urol*. 1995;13:233–239.
2. De Bernardi B, Conte M, Nigro M, et al. Neuroblastoma: update on clinical aspects and therapy. *Pediatr Med Chir*. 1994;16:7–14.
3. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell*. 2002; 108:153–164.
4. McGill G. Apoptosis in tumorigenesis and cancer therapy. *Front Biosci*. 1997;2:d353–d379.
5. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science*. 1998;281:1305–1308.
6. Griffith TS, Broghammer EL. Suppression of tumor growth following intralesional therapy with TRAIL recombinant adenovirus. *Mol Ther*. 2001;4:257–266.

7. French LE, Tschopp J. The TRAIL to selective tumor death. *Nat Med*. 1999;5:146–147.
8. Griffith TS, Lynch DH. TRAIL: a molecule with multiple receptors and control mechanisms. *Curr Opin Immunol*. 1998;10:559–563.
9. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med*. 1999;5:157–163.
10. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol*. 1999;11:255–260.
11. Griffith TS, Anderson RD, Davidson BL, et al. Adenoviral-mediated transfer of the TNF-related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. *J Immunol*. 2000;165:2886–2894.
12. Li ZW, Chu W, Hu Y, et al. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med*. 1999;189:1839–1845.
13. Sanlioglu S, Luleci G, Thomas KW. Simultaneous inhibition of Rac1 and IKK pathways sensitizes lung cancer cells to TNFalpha-mediated apoptosis. *Cancer Gene Ther*. 2001;8:897–905.
14. Flick DA, Gifford GE. Comparison of *in vitro* cell cytotoxic assays for tumor necrosis factor. *J Immunol Methods*. 1984;68:167–175.
15. Fadok VA, Savill JS, Haslett C, et al. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol*. 1992;149:4029–4035.
16. Fadok VA, Voelker DR, Campbell PA, et al. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*. 1992;148:2207–2216.
17. Thiagarajan P, Tait JF. Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. *J Biol Chem*. 1990;265: 17420–17423.
18. Raynal P, Pollard HB. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim Biophys Acta*. 1994;1197:63–93.
19. Koopman G, Reutelingsperger CP, Kuijten GA, et al. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*. 1994;84:1415–1420.
20. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med*. 1995;182:1545–1556.
21. Abe K, Kurakin A, Mohseni-Maybodi M, et al. The complexity of TNF-related apoptosis-inducing ligand. *Ann NY Acad Sci*. 2000;926:52–63.
22. Griffith TS, Chin WA, Jackson GC, et al. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol*. 1998;161:2833–2840.
23. Karacay B, O'Dorisio MS, Summers M, et al. VIP receptor 1 (VPAC1) promoter targets the expression of a reporter gene to cerebellum and adrenal medulla in transgenic mice. *Regul Pept*. 2003;116:1–12.
24. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*. 1996;274:782–784.
25. Chaudhary PM, Eby M, Jasmin A, et al. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. *Immunity*. 1997;7:821–830.
26. Hopkins-Donaldson S, Bodmer JL, Bourloud KB, et al. Loss of caspase-8 expression in neuroblastoma is related to malignancy and resistance to TRAIL-induced apoptosis. *Med Pediatr Oncol*. 2000;35:608–611.
27. Yang X, Thiele CJ. Targeting the tumor necrosis factor-related apoptosis-inducing ligand path in neuroblastoma. *Cancer Lett*. 2003;197:137–143.
28. Teitz T, Wei T, Valentine MB, et al. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat Med*. 2000;6:529–535.
29. Eggert A, Grotzer MA, Zuzak TJ, et al. Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. *Cancer Res*. 2001;61: 1314–1319.
30. Van Antwerp DJ, Martin SJ, Kafri T, et al. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science*. 1996;274:787–789.
31. Lipton SA. Janus faces of NF-kappa B: neurodestruction *versus* neuroprotection. *Nat Med*. 1997;3:20–22.
32. Ryan KM, Ernst MK, Rice NR, et al. Role of NF-kappaB in p53-mediated programmed cell death. *Nature*. 2000;404:892–897.
33. Bian X, Opipari Jr AW, Ratanaproeksa AB, et al. Constitutively active NFkappa B is required for the survival of S-type neuroblastoma. *J Biol Chem*. 2002;277: 42144–42150.
34. Bours V, Dejardin E, Goujon-Letawe F, et al. The NF-kappa B transcription factor and cancer: high expression of NF-kappa B- and I kappa B-related proteins in tumor cell lines. *Biochem Pharmacol*. 1994;47:145–149.
35. Visconti R, Cerutti J, Battista S, et al. Expression of the neoplastic phenotype by human thyroid carcinoma cell lines requires NFkappaB p65 protein expression. *Oncogene*. 1997;15:1987–1994.
36. Herrmann JL, Beham AW, Sarkiss M, et al. Bcl-2 suppresses apoptosis resulting from disruption of the NF-kappa B survival pathway. *Exp Cell Res*. 1997;237:101–109.
37. Devalaraja MN, Wang DZ, Ballard DW, et al. Elevated constitutive IkappaB kinase activity and IkappaB-alpha phosphorylation in Hs294T melanoma cells lead to increased basal MGSA/GRO-alpha transcription. *Cancer Res*. 1999;59:1372–1377.
38. Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene*. 1999;18:6938–6947.
39. Dejardin E, Deregowski V, Chapelier M, et al. Regulation of NF-kappaB activity by I kappaB-related proteins in adenocarcinoma cells. *Oncogene*. 1999;18:2567–2577.
40. Ravi R, Bedi GC, Engstrom LW, et al. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. *Nat Cell Biol*. 2001;3:409–416.
41. Hersey P, Zhang XD. How melanoma cells evade trail-induced apoptosis. *Nat Rev Cancer*. 2001;1:142–150.
42. Hu WH, Johnson H, Shu HB. Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways. *J Biol Chem*. 1999;274:30603–30610.
43. Mayo MW, Baldwin AS. The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. *Biochim Biophys Acta*. 2000;1470:M55–M62.