Constitutive Nuclear Factor- κ B Activity Is Crucial for Human Retinoblastoma Cell Viability

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Retinoblastoma (Rb) is the most common intraocular malignancy of childhood. Although systemic and intrathecal chemotherapy with local and cranial radiotherapy have improved overall survival, the prognosis for patients with central nervous system involvement is still poor. We investigated the role of the transcription factor nuclear factor (NF)- κ B, which promotes cell survival in several other models, in the pathophysiology of Rb. The human Rb cell lines Y79 and WERI-Rb1 were treated with the cell permeable peptide SN50, that specifically inhibits the transcriptional activity of NF- κ B by blocking its translocation into the nucleus. We found that NF- κ B inhibition up-regulated Bax; down-regulated the anti-apoptotic proteins Bcl-2, A1, and cIAP-2; and induced loss of the mitochondrial transmembrane potential and caspase-independent, calpain-dependent apoptosis in Rb cells. Inhibition of the p38 kinase sensitized cells to SN50-induced cell death, whereas insulin-like growth factor-1 activated NF- κ B and attenuated the proapoptotic effect of SN50. Finally, NF- κ B inhibition sensitized Rb cells to doxorubicin. In conclusion, inhibition of NF- κ B activity in Rb cells leads to loss of mitochondrial transmembrane potential and caspase-independent, calpain-dependent apoptosis. Therapeutic strategies targeting NF- κ B could be beneficial in the clinical management of Rb, either alone or in combination with conventional chemotherapy. (*Am J Pathol* 2002, 161:2229–2240)

Apoptosis is an active process of cellular self-destruction, which is of vital importance for the maintenance of tissue homeostasis.¹ Deregulation of this process contributes to the pathogenesis of a wide spectrum of disorders ranging from autoimmune diseases to cancer. It has been reported that several therapeutic modalities, such as chemotherapy and radiation, operate via the interaction with distinct cellular targets and the induction of

apoptosis in susceptible cancer cells,¹ whereas deficient activation of the apoptotic pathways has been correlated with treatment failure.¹ Although the molecular mechanisms controlling the apoptotic commitment are not fully characterized, protease cascades appear to have a regulatory role. Among these proteases, two categories have the leading role: the caspases² and the calcium-activated proteases calpains.³ Therapy designed to modify the susceptibility of cancer cells to apoptosis will lead to a more efficient therapeutic approach in cancer and is feasible only with the elucidation of the molecular basis of the resistance of cancer cells to apoptosis.

Retinoblastoma (Rb) is the most common intraocular malignancy of childhood.⁴ The tumor originates from a primitive neuroectodermal cell and occurs as a nonhereditary, sporadic form (unilateral) and a hereditary (bilateral) form. Predisposition to the latter form can be transmitted as an autosomal-dominant trait.⁵ Although treatment strategies for Rb have gradually evolved throughout the past decades, salvage of useful vision is possible only in limited cases and the prognosis of the patients with extraocular disease is dismal. Retinoblastoma is characterized by the functional inactivation of both alleles of the tumor suppressor gene Rb, which encodes the 105-kd nuclear phosphoprotein Rb. The latter is a transcription factor that functions at the core of fundamental decisions for cell division, differentiation, and apoptosis in almost all cell types. In its hypophosphorylated state, Rb binds to the activation domain of the transcription factor E2F-1 and actively represses transcription from the promoters of all of the S-phase genes that bear E2F-1 binding sites, leading to cell-cycle arrest.⁶ Absence of the Rb protein in Rb causes the release of free, transcriptionally active E2F-1, thus permitting unrestricted cell proliferation. Deregulated proliferation in these cells is supported by a mitogenic loop that is dependent on insulin-like growth factor-1 (IGF-1) and the type I insulin-like growth factor receptor.⁷ Rb has been reported to inhibit apoptosis, and, in Rb-deficient cells, active E2F-1 seems to be responsible for the progression through the cell cycle and the induction of apoptosis. Therefore it is likely that, in Rb cells, the strong proapop-

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otic signal of the unopposed E2F-1 action must be counterbalanced by anti-apoptotic survival mechanisms.

Experimental evidence suggests a major role for the Rel/nuclear factor (NF)- κ B family of transcription factors in the inhibition of apoptosis in a variety of settings. Disruption of the *relA* locus leads to embryonic lethality at 15 to 16 days of gestation, concomitant with a massive degeneration of the liver by programmed cell death or apoptosis.⁸ Fibroblasts from mice with a deletion of the *RelA* component of NF- κ B are highly sensitive to tumor necrosis factor- α killing, in contrast to the resistance of normal fibroblasts.⁹ Inactivation of endogenous Rel/NF- κ B factors by superrepressor forms of the inhibitory protein I κ B α sensitizes cells to stimulus-induced apoptosis (tumor necrosis factor- α , ionizing radiation, or daunorubicin),^{10,11} whereas overexpression of transcriptionally competent Rel/NF- κ B factors blocks apoptosis.^{12,13} Because the protective activity of NF- κ B is dependent on RNA and protein synthesis, the current notion is that NF- κ B regulates the expression of genes that antagonize cell death. Candidate genes are the well-known caspase inhibitors cIAP-1, cIAP-2, XIAP, and survivin.^{14–16} It was recently demonstrated that NF- κ B also up-regulates in other models prosurvival proteins of the Bcl-2 family, such as Bcl-2,¹⁷ Bcl-xL, and A1.^{18–21} We therefore explored the role of NF- κ B as an inhibitor of apoptosis in Rb. We found that NF- κ B inhibition induces apoptosis in Rb cell lines that is associated with reduced levels of Bcl-2, A1, and cIAP-2, increased Bax expression, and mitochondrial dysfunction, and is not dependent on caspase activation. On the contrary, it involves the protease calpain. We also found that inhibition of the p38 kinase potentiates the proapoptotic effect of NF- κ B inhibition. IGF-1, a well-known growth/survival factor for Rb cells, activated NF- κ B and exerted an anti-apoptotic effect. Finally, NF- κ B inhibition sensitized Rb cells to DNA-damaging anticancer chemotherapy.

Materials and Methods

Cell Lines and Tissue Culture

The human Rb cell lines Y79 and WERI-Rb1 were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (FCS) (Life Technologies, Inc., Gaithersburg, MD).

Reagents

The peptide SN50, consisting of the nuclear localization signal (NLS) of p50 (residues 360 to 369) fused to the hydrophobic region of the signal sequence of Kaposi fibroblast growth factor (K-FGF) to provide cell-permeability, specifically inhibits nuclear translocation of NF- κ B.^{22–29} SN50M, a synthetic analogue with a mutated nuclear localization sequence, is inactive and served as a negative control.²² Both peptides were purchased from Biomol (Plymouth Meeting, PA).

Monoclonal antibodies for Bcl-2, Bcl-xL, Bax, and tubulin and polyclonal antisera for A1 and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal antisera raised against XIAP, cIAP-1, and cIAP-2 from R&D Systems (Minneapolis, MN); polyclonal antiserum for FKHL1 from Upstate Biotechnologies (Lake Placid, NY); polyclonal antiserum raised against survivin from Oncogene Research (Cambridge, MA); and the monoclonal antibody for PARP from Biomol.

The caspase inhibitors specific for caspase-3 (DEVD-FMK), caspase-9 (LEHD-FMK), and caspase-8 (IETD-FMK), as well as the pan-caspase inhibitor ZVAD-FMK were purchased from Calbiochem (La Jolla, CA) and used at a concentration of 20 μ mol/L. Calpeptin and the p38 inhibitor PD169316 were from Calbiochem and used at a concentration of 50 μ mol/L and 10 μ mol/L, respectively. Doxorubicin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). IGF-1 was from R&D Systems.

NF- κ B DNA-Binding Assay

The DNA-binding activity of NF- κ B was quantified in the Y79 and WERI-Rb1 cell lines by enzyme-linked immunosorbent assay using the *Trans*-AM NF- κ B p65 Transcription Factor Assay kit (Active Motif North America, Carlsbad, CA), according to the instructions of the manufacturer. Briefly, nuclear extracts were prepared as previously described³⁰ from control and treated cells and incubated in 96-well plates coated with immobilized oligonucleotide (5'-AGTTGAGGGGACTTCCCAGGC-3') containing a consensus (5'-GGGACTTCC-3') binding site for the p65 subunit of NF- κ B. NF- κ B binding to the target oligonucleotide was detected by incubation with primary Ab specific for the activated form of p65 (Active Motif North America), followed by anti-IgG horseradish peroxidase-conjugate and developing solution, and quantified at 450 nm with a reference wavelength of 655 nm. Background binding, obtained by incubation with a 2-nucleotide mutant oligonucleotide (5'-AGTTGAGGC-CACCTTCCCAGGC-3'), was subtracted from the values obtained for binding to the consensus DNA sequence. Results were normalized for nuclear extract protein content.

Detection of Cell Death with the MTT Colorimetric Assay

This assay was used to quantify cell death and performed as described previously.³¹ In brief, cells were treated with the NF- κ B inhibitor SN50 (0 to 30 μ mol/L) or its inactive mutant SN50M or left untreated for 18 hours in serum-free RPMI medium at 37°C. At the end of each treatment, cells were incubated with 1 mg/ml of MTT for 4 hours at 37°C. Then, a mixture of isopropanol and 1 N HCl (24:1, v/v) was added under vigorous pipetting to dissolve the formazan crystals. Dye absorbance (A) in viable cells was measured at 570 nm, with 630 nm as a reference wavelength. Cell survival was estimated as a percentage of the value of untreated controls. All experiments were repeated at least twice and each experimen-

tal condition was repeated at least in quadruplicate wells in each experiment.

Detection of Apoptosis with Annexin V Labeling

Quantification of early apoptosis was determined with the Annexin V-propidium iodide detection kit (Immunotech/Beckman Coulter, Miami, FL). Annexin V has high affinity for phosphatidylserine, which translocates from the inner to the outer leaflet of the plasma membrane during early apoptosis. Briefly, 10^6 Y79 cells before and after treatment with SN50 (20 μ mol/L for 6 hours) were labeled with fluorescein-conjugated Annexin V and propidium iodide and analyzed by dual-color cytometry using an EPICS-XL-MCL flow cytometer (Coulter, Hialeah, FL). Cells that were propidium iodide-negative (ie, with intact cellular membrane) and Annexin V-positive were considered as early apoptotic cells.³¹

Detection of Apoptotic Nuclei with the Terminal dUTP Nick-End Labeling (TUNEL) Method

Y79 cells were treated with or without 20 μ mol/L of SN50 for 16 hours, in the presence or absence of the caspase-3 inhibitor DEVD-FMK, the caspase-9 inhibitor LEHD-FMK, or the caspase-8 inhibitor IETD-FMK, as well as the pan-caspase inhibitor ZVAD-FMK (all used at a concentration of 20 μ mol/L). At the end of the incubation, the plates were centrifuged at 2000 rpm for 10 minutes so that the cells attach to the plate. After brief washes in phosphate-buffered saline (PBS), the cells were permeabilized with a 1:2 mixture of acetic acid: EtOH for 10 minutes at -20°C . After washes in PBS, the cells were labeled with the *in situ* cell death kit, Fluorescence (Boehringer Mannheim, Indianapolis, IN), following the instructions of the manufacturer as previously described,³² and were viewed with a Leica MZ FLIII microscope (Leica Microsystems Inc., Deerfield, IL). The images were captured on an Apple G4 Computer (Apple, Cupertino, CA) and analyzed using Openlab software (Improvision Inc., Lexington, MA).

Western Blot Analysis

The levels of protein expression of several modulators of apoptosis were evaluated by Western blotting, performed as described previously.³² Briefly, Y79 cells were treated with SN50 (20 μ mol/L for 3 and 6 hours) or left untreated, and subsequently lysed for 30 minutes on ice in lysis buffer (50 mmol/L of Tris-HCl, pH 8, with 120 mmol/L of NaCl and 1% Nonidet P-40), supplemented with the Complete-TM mixture of proteinase inhibitors. The samples were cleared by microcentrifugation (14,000 rpm, 30 minutes, 4°C) and assessed for protein concentration. Thirty μ g of protein/sample were electrophoresed in a 12% sodium dodecyl sulfate-polyacrylamide gel, and electroblotted onto nitrocellulose membranes. After a 1-hour incubation in blocking solution [20% IgG-free normal horse serum, in phosphate-buffered saline (PBS)],

the membranes were exposed overnight at 4°C to the respective primary antibody. After washing in PBS, the respective secondary peroxidase-labeled antibody was applied at 1:10,000 dilution for 1 hour at room temperature. The proteins were visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Piscataway, NJ).

The potential involvement of caspases in SN50-induced apoptosis and the kinetics of caspase activation were evaluated in Y79 cells before and after treatment with SN50 (20 μ mol/L for 3, 6, and 18 hours). Thirty μ g of protein extracted at the above time points were electrophoresed, electroblotted, and immunostained with antibodies against caspase-9 and PARP. The proteins were visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated with the Trizol reagent (Life Technologies, Inc.) according to the instructions of the manufacturer. Two μ g of RNA were reverse-transcribed into cDNA with the Superscript kit (Life Technologies, Inc.) using random hexamers. We performed multiplex RT-PCR using sets of primers for Bcl-2, Bax (R&D Systems), and as an internal standard, for the 18S RNA (QuantumRNA 18S internal standards; Ambion, Austin, TX). By mixing 18S primers with increasing amounts of 18S competitors (Ambion), the overall PCR amplification efficiency for the 18S RNA was reduced to match that of the less abundant transcripts. We used a 3:7 ratio of primers to competitors, which was found to be suitable for comparison to moderately expressed transcripts, such as Bcl-2 and Bax. Two μ l of the reverse transcription reaction were subjected to quantitative multiplex PCR using the above primers. The PCR cycles were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 5 minutes. The PCR products were subjected to agarose gel electrophoresis and visualized under UV light.

Caspase-3 Activity Assay (PhiPhiLux)

The activity of caspase-3 was monitored in Y79 cells after treatment with SN50 (20 μ mol/L) for 8 and 16 hours with the Caspase-3 Intracellular Activity Assay kit (Calbiochem, La Jolla, CA), according to the instructions of the manufacturer. The assay depends on the detection of the cleavage of the peptide PhiPhiLux, which is mediated by caspase-3. When the peptide is cleaved, it provides an intense fluorescent signal.

Mitochondrial Transmembrane Potential

The mitochondrial transmembrane potential was quantified with the rhodamine 123 method. Y79 cells were treated with SN50 (20 μ mol/L for 8 hours) or left untreated. Subsequently, they were incubated with rhodamine 123 (10 μ g/

Figure 1. **A:** NF-κB DNA-binding activity in the Y79 and WERI-Rb1 Rb cell lines treated without or with SN50 (20 μmol/L) for 4 hours, as quantified by enzyme-linked immunosorbent assay using the *Trans*-AM NF-κB p65 Transcription Factor assay kit (Active Motif North America, Carlsbad, CA), according to the instructions of the manufacturer. Results were normalized for nuclear extract protein content and expressed as mean ± SD. Rb cell lines possess constitutive NF-κB DNA-binding activity that is blocked by SN50. **B and C:** Cell survival (mean ± SD), as evaluated by MTT, of Y79 (**B**) and WERI-Rb1 (**C**) cell lines treated with various concentrations of SN50 (open squares) or its inactive analog SN50M (filled squares) for 16 hours. SN50, but not SN50M, induces concentration-dependent cell death in Rb cell lines.

ml) for 30 minutes at 37°C, washed twice in PBS and analyzed on an EPICS-XL-MCL flow cytometer.

Transfection of Bcl-2 Anti-Sense Oligonucleotide

To delineate the role of Bcl-2 as a negative regulator of apoptosis in Y79 cells, phosphorothioate single-stranded anti-sense oligonucleotide (ASO) directed against the human bcl-2 translation initiation site and the subsequent 15 bases (sequence: 5'-TCTCCCAGCGTGCGCCAT-3'), as well as a 2-base mismatch control oligonucleotide (CO) (sequence: 5'-TCTCCCAGCATGTGCCAT-3') were purchased from Biomol Research Labs, Inc. (Plymouth Meeting, PA), and transfected into Y79 cells as previously described³³ with the help of Oligofectamine (Life Technologies, Inc., Gaithersburg, MD). Forty-eight hours later, cell survival was quantified by MTT as described above.

Statistical Analysis

Statistical significance was examined by analysis of variance, followed by Duncan's post hoc test. A *P* value of <0.05 was considered consistent with statistical significance, in all analyses.

Results

The Peptide SN50 Inhibits NF-κB DNA-Binding Activity in Human Rb Cell Lines

We first evaluated the effect of SN50, a cell-permeable peptide derived from the nuclear localization sequence of p50, which inhibits the nuclear translocation of NF-κB,^{22,23} on the DNA-binding activity of NF-κB in human Y79 and WERI-Rb1 Rb cell lines. We found that human Rb cell lines possess constitutive NF-κB activity and that

treatment with SN50 for 4 hours results in significant inhibition (Figure 1A). The specificity of this effect was verified by finding that the nuclear translocation of another transcription factor, namely forkhead (FKHRL1) was not decreased after treatment with SN50 in Y79 and WERI-Rb1 cell lines (data not shown).

The NF-κB Inhibitor SN50, But Not Its Mutant Analog SN50M, Kills Human Rb Cell Lines

We then evaluated the effect of SN50, and its mutant analog SN50M, on human Rb cell survival *in vitro*. We found that the cell permeable NF-κB inhibitor SN50, but not its inactive mutant counterpart SN50M, induced cell death in both Rb cell lines tested (Y79 and WERI-Rb1), in a concentration-dependent manner (Figure 1, B and C). This suggests that constitutive NF-κB activity is crucial for Rb cell survival.

Figure 2. Evaluation of the externalization of phosphatidylserine, detected by Annexin V-FITC labeling, in the Y79 cell line treated with the NF-κB inhibitor SN50 (20 μmol/L) for 6 hours (**B**) or in control cells (**A**). SN50 resulted in detection of Annexin V-FITC in propidium iodide-negative cells (ie, cells with intact cellular membrane), suggesting the externalization of phosphatidylserine and presence of apoptosis.

Figure 3. TUNEL staining in the Y79 cell line. **A:** Control cells. **B–F:** Cells treated with the NF- κ B inhibitor SN50 (20 μ mol/L) for 16 hours in the absence of caspase inhibitors (**B**) or in the presence of the pan-caspase inhibitor ZVAD-FMK (**C**), the caspase-3 inhibitor DEVD-FMK (**D**), the caspase-9 inhibitor LEHD-FMK (**E**), or the caspase-8 inhibitor IETD-FMK (**F**), respectively. Caspase inhibition had no protective effect on apoptosis induced by NF- κ B inhibition.

Cell Death Induced in the Rb Cell Line Y79 by SN50 Is Apoptotic in Nature

Subsequently, we investigated whether SN50-induced cell death is indeed apoptosis. The apoptotic nature of this death was verified in SN50-treated Y79 cells by the early externalization of phosphatidylserine, that was detected with Annexin V labeling (Figure 2) and by the detection of numerous positive nuclei by the TUNEL method (Figure 3, A and B). The control peptide SN50M did not induce the appearance of TUNEL-positive nuclei (not shown).

Lack of Involvement of Caspases in SN50-Induced Apoptosis in the Rb Cell Line Y79

We subsequently assessed the functional role of caspases in SN50-induced apoptosis. We found that neither the caspase-3 inhibitor DEVD-FMK, the caspase-9 inhibitor LEHD-FMK, the caspase-8 inhibitor IETD-FMK, nor the pan-caspase inhibitor ZVAD-FMK, had any inhibitory effect on SN50-induced apoptosis, as evidenced by TUNEL (Figure 3) and quantified by MTT (Figure 4). Moreover, no activation of caspase-3 was detected in SN50-treated Y79 cells, after 8 or 16 hours (Figure 5, A and B) with the PhiPhilux assay.

By immunoblotting, cleavage of the initiator caspase caspase-9 was not observed by 4, 8, or 16 hours of treatment (Figure 5C). As a positive control, the proteasome inhibitor MG132 induced apoptosis of Y79 cells (not shown) and cleavage of caspase-9 (Figure 5C). Caspase-8, another initiator caspase, was not cleaved on SN50 treatment either (not shown).

PARP cleavage was detected after 16 hours of SN50 treatment in Y79 cells. However, the pattern of cleavage was atypical ($\sim$ 60 kD) and different from in MG132-treated cells (where the classical 85-kD fragment appeared), suggesting a different mechanism of cleavage (Figure 5C). Collectively, these data show that caspases do not play a major role in mediating SN50-induced apoptosis in Rb cells.

Figure 4. Cell survival (mean $\pm$ SD), as evaluated by MTT, of the Y79 cell line. **A:** Control cells. **B–F:** Cells treated with the NF- κ B inhibitor SN50 (20 μ mol/L) for 16 hours in the absence of caspase inhibitors (**B**) or in the presence of the pan-caspase inhibitor ZVAD-FMK (**C**), the caspase-3 inhibitor DEVD-FMK (**D**), the caspase-9 inhibitor LEHD-FMK (**E**), or the caspase-8 inhibitor IETD-FMK (**F**), respectively. Caspase inhibition had no protective effect on apoptosis induced by NF- κ B inhibition.

Figure 5. A and B: Caspase-3 activity assay (PhiPhilux assay) in the Y79 cell line treated without (open curve) or with (filled curve) the NF- κ B inhibitor SN50 (20 μ mol/L) for 8 hours (A) or 16 hours (B). **C:** Immunoblotting analysis of the Y79 cell line treated with the NF- κ B inhibitor SN50 (20 μ mol/L for 3, 6, and 18 hours) for caspase-9 and PARP. Caspase-9 was not cleaved in the Y79 cell line treated with SN50, but was cleaved in cells treated with the proteasome inhibitor MG132 (4 μ mol/L for 18 hours), that induced apoptosis and served as a positive control. PARP was cleaved in Y79 cells treated with SN50, resulting in an ~60-kd fragment that was different from the classical 85-kd fragment that appeared in MG132-treated cells.

Effect of SN50 on Expression of Apoptosis Modulators in the Rb Cell Line Y79

We then investigated the effect of NF- κ B inhibition on the expression of a panel of apoptosis inhibitors in Rb cell lines. We found that SN50 down-regulated the levels of the mitochondrial anti-apoptotic proteins Bcl-2 and A1, as well as those of cIAP-2, but not those of BclxL, cIAP-1, survivin, or XIAP (Figure 6A). On the other hand, SN50 up-regulated the protein levels of the proapoptotic molecule Bax.

We subsequently investigated the mechanism of the modulation of Bcl-2 and Bax expression by SN50. Quantitative RT-PCR analysis revealed that SN50 treatment decreased bcl-2 and increased bax mRNA levels, suggesting a transcriptional mechanism of regulation (Figure 6, B and C).

Effect of NF- κ B Inhibition on Mitochondrial Transmembrane Potential

Bcl-2 and A1 are mitochondrial proteins that confer cell survival by stabilizing the mitochondrial transmembrane

Figure 6. A: Immunoblotting analysis for the apoptosis inhibitors Bcl-2, BclxL, A1, cIAP-1, cIAP-2, XIAP, and survivin, as well as the proapoptotic molecule Bax, in the Y79 cell line treated with the NF- κ B inhibitor SN50 (20 μ mol/L) for 3 or 6 hours. NF- κ B inhibition down-regulated the protein levels of Bcl-2, A1, and cIAP-2, and up-regulated Bax expression. **B and C:** Quantitative RT-PCR for Bcl-2 (B), Bax (C) and 18S RNA in Y79 cells treated with or without SN50 (20 μ mol/L for 3 hours). NF- κ B inhibition down-regulates Bcl-2 and up-regulates Bax mRNA levels.

potential, whereas Bax antagonizes their effects. We hypothesized that their modulation by SN50 could lower mitochondrial transmembrane potential, leading to cell death. Indeed, we found that SN50 treatment induced loss of the mitochondrial transmembrane potential in Y79 cells (Figure 7).

Transfection of an Anti-Sense Bcl-2 Oligonucleotide Results in Loss of Viability in the Rb Cell Line Y79

Having demonstrated that SN50-induced apoptosis is preceded by down-regulation of Bcl-2 protein levels, we investigated whether forced Bcl-2 down-regulation could replicate this apoptotic effect. We, thus, transfected Y79 cells with an anti-sense oligonucleotide directed against the human Bcl-2 translation initiation codon, or a scrambled control oligonucleotide. We found that Bcl-2 anti-sense treatment resulted in a significant loss of cell viability (Figure 8).

A Calpain Inhibitor Protects Y79 Cells from Apoptosis Induced by NF- κ B Inhibition

Our finding of lack of functional involvement of caspases in SN50-induced apoptosis led us to search for other

Figure 7. Evaluation of the mitochondrial transmembrane potential in the Y79 cell line treated without (open curve) or with (filled curve) the NF- κ B inhibitor SN50 (20 μ mol/L) for 8 hours. NF- κ B inhibition results in collapse of the mitochondrial transmembrane potential.

potential mediators. As calpain has been implicated in cell death induced by mitochondrial dysfunction, and because we detected loss of mitochondrial transmembrane potential in SN50-treated Y79 cells, we hypothesized that calpain could have a role in our model. Indeed, we found that a calpain inhibitor exerted a protective effect in SN50-treated Y79 cells (Figure 9). Collectively, these data raise the possibility that NF- κ B inhibition results in mitochondrion-dependent, caspase-independent, calpain-dependent Rb cell apoptosis.

Figure 8. Cell survival (mean $\pm$ SD) of the Y79 cell line transfected with Bcl-2 anti-sense (A/S) or scrambled oligonucleotides, as evaluated by MTT 48 hours after transfection. Results are expressed as a percentage of the value of cells transfected with the scrambled oligonucleotide.

Figure 9. TUNEL staining of the Y79 cell line left untreated (A) or treated with the NF- κ B inhibitor SN50 (20 μ mol/L, B), calpeptin (50 μ mol/L, C) or both (D) for 18 hours. Calpeptin protected Y79 cells from apoptosis induced by NF- κ B inhibition.

The p38 Inhibitor PD169316 Sensitizes Rb Cell Lines to Apoptosis Induced by NF- κ B Inhibition

As the pathway activated by the p38 kinase has been implicated in the regulation of cell survival³⁴ and the activation of NF- κ B,³⁵ we investigated the role of p38 in SN50-induced apoptosis. We found that the p38 inhibitor PD169316 increased the sensitivity of Y79 cells to SN50-induced apoptosis (Figure 10).

IGF-1 Activates NF- κ B and Protects from SN50-Induced Apoptosis

Rb cells express IGF receptor I and produce IGF-1, that together mediate an autocrine growth mechanism that stimulates proliferation.⁷ The growth/survival factor IGF-1

Figure 10. Cell death (mean $\pm$ SD), as evaluated by MTT, of the Y79 cell line treated with the p38 inhibitor PD169316 (10 μ mol/L) or the NF- κ B inhibitor SN50 (5 μ mol/L) or both for 16 hours. Inhibition of p38 strongly sensitized Y79 cells to apoptosis induced by NF- κ B inhibition.

Figure 11. A: NF-κB DNA binding activity in the Y79 cell line treated for 4 hours without or with IGF-1 (200 ng/ml) in the absence or presence of SN50 (20 μmol/L) and PD169316 (10 μmol/L) (1 hour pretreatment). IGF-1 up-regulated the NF-κB activity in the Y79 cell line. Results were normalized for nuclear extract protein content. Both SN50 and the p38 inhibitor PD169316 down-regulated both constitutive and IGF-1-induced NF-κB activity. **B:** Cell survival (mean ± SD), as evaluated by MTT, of Y79 cells treated with increasing concentrations of the NF-κB inhibitor SN50 for 16 hours, in the absence (filled bars) or presence (open bars) of IGF-1 (200 ng/ml).

has been reported to exert an anti-apoptotic function in a variety of models.^{36,37} We, therefore, investigated the effect of IGF-1 in our model. We found that IGF-1 activated NF-κB DNA-binding activity in Y79 cells (Figure 11A). This finding suggests NF-κB as a potential mediator of the anti-apoptotic effects of IGF-1. Moreover, both SN50 and the p38 inhibitor PD169316 down-regulated both constitutive and IGF-1-induced NF-κB activity (Figure 11A). Our result that p38 inhibition down-regulates NF-κB activity explains the synergistic effect of the combination of SN50 with PD169316 and identifies NF-κB as a downstream target of p38 in Rb cells.

These findings also suggest that IGF-1 and NF-κB inhibition exert opposite effects on Rb cell survival. We thus investigated the effect of IGF-1 on SN50-induced apoptosis. We found that IGF-1 protected Y79 cells from SN50-induced apoptosis (Figure 11B).

NF-κB Inhibition Sensitizes Rb Cell Lines to Anti-Cancer Chemotherapy

The anti-apoptotic effects of NF-κB raise the possibility that NF-κB inhibitors could be used clinically in the treatment of Rb, alone or in combination with current modalities. We thus investigated the effect of the combined use of SN50 with doxorubicin, a chemotherapeutic drug commonly used in the treatment of Rb. We found that NF-κB inhibition sensitized Y79 cells to doxorubicin (Figure 12).

Discussion

Rb is a highly malignant retinal childhood tumor. In this study, we demonstrate that the transcription factor NF-κB is constitutively active in human Rb cell lines and that its inhibition results in caspase-independent, calpain-dependent apoptosis, associated with reduced levels of anti-apoptotic proteins, such as Bcl-2, A1, and cIAP-2, and mitochondrial dysfunction. Additionally, the growth/survival factor IGF-1 activates NF-κB and protects Rb cells from apoptosis. Finally, NF-κB inhibition acts synergistically with inhibition of p38 kinase and anti-cancer

Figure 12. Cell death (mean ± SD), as evaluated by MTT, of Y79 cells treated with doxorubicin (0.1 μg/ml) or the NF-κB inhibitor SN50 (5 μmol/L) or both. SN50 was added to the cells 24 hours after doxorubicin and the cells were incubated for an additional 24 hours, resulting in a total of 48 hours of treatment with doxorubicin. Inhibition of NF-κB increased the sensitivity of Y79 cells to doxorubicin-induced apoptosis.

chemotherapeutic drugs, such as doxorubicin, to promote apoptosis in Rb cells.

There is growing evidence implicating Rel/NF-κB family members in the emergence of neoplasias and in the protection of tumor cells against apoptotic death induced by a variety of stimuli.^{11,38–45} We have now detected constitutive NF-κB activity in human Rb cell lines. Retinoblastoma cells lack Rb protein, which sequesters and inhibits the E2F-1 transcription factor and negatively regulates the transition from G₁ to S cell-cycle phase as well as entry into the apoptotic pathway. It has been reported that E2F-1 overexpression is sufficient to promote neuronal apoptosis,⁴⁶ and that endogenous E2F-1 modulates the apoptotic death of a variety of cells in different settings.⁴⁷ This unopposed E2F-1 activity and the concurrent presence of functional p53 could render these cells particularly susceptible to apoptosis. Therefore the constitutive expression of NF-κB could be an adaptational response required to sustain Rb cell viability.

We found that the induction of apoptosis in Rb cells after NF-κB inhibition is associated with the down-regulation of the caspase inhibitor cIAP-2, but not cIAP-1, survivin or XIAP. The family of cellular inhibitors of apoptosis (IAPs) includes the proteins cIAP-1, cIAP-2, survivin, and XIAP, that are known to interfere with the transmission of intracellular death signals and have been reported to be regulated by NF-κB in other models.^{14,15,48,49} Our finding of the differential response of cIAP-2 to NF-κB inhibition correlates with the results of Chu and colleagues,⁴⁸ who found that tumor necrosis factor-α induces cIAP-2 but not cIAP-1 expression in Jurkat cells through the activation of NF-κB.

Apoptosis induced by NF- κ B inhibition in Rb cell lines is characterized by an early loss of the mitochondrial potential and reduced levels of the anti-apoptotic proteins Bcl-2 and A1, yet is not mediated through the activation of caspases. It is currently known that the expression of anti-apoptotic Bcl-2 family members, such as Bcl-2,¹⁷ A1,^{18–21,50} and Bcl-xL^{50–54} is directly regulated by NF- κ B via distinct κ B-binding elements in their promoter, although the effect on Bcl-xL seems to be tissue-specific. Although, in our model, NF- κ B inhibition did not affect Bcl-xL levels, Bcl-2 and A1 were down-regulated and Bax was up-regulated. The pivotal role of Bcl-2 down-regulation in SN50-induced apoptosis is highlighted by our finding that specific down-regulation of Bcl-2 protein levels by an anti-sense oligonucleotide resulted in Rb cell death as well. It is expected that in Rb tissues, the absence of functional Rb will leave unopposed the activity of the p53 protein, which reportedly is rarely mutated in Rbs,⁵⁵ resulting in high levels of Bax protein, a known p53 target and proapoptotic member of the Bcl-2 family.⁵⁶ It is also possible that NF- κ B partially inhibits the transactivation of the bax promoter by p53, as reported in carcinoma cells.⁵⁷ Decrease in Bcl-2 and/or increase in Bax expression has been previously reported in studies with other agents that induce apoptosis in Rb cells, such as sodium butyrate^{58–60} and phenylbutyrate⁶¹ and the topoisomerase inhibitors camptothecin, etoposide, and amsacrine.⁶² The changes observed in our model after NF- κ B inhibition will shift the balance of Bcl-2/Bax toward the proapoptotic side. Bax induces pore formation in the mitochondrial membrane, reduces the mitochondrial potential as demonstrated in the present study, and ultimately facilitates the release of cytochrome c to the cytoplasm.⁶³ The cytochrome c is known to bind with the adaptor molecule Apaf-1 and form the apoptosome that activates caspase-9, a caspase that cleaves and activates caspase-3.^{64,65} Although, in our model, the mitochondria are clearly involved in apoptosis induced by NF- κ B inhibition, caspase-3 and caspase-9 were not activated. Moreover, the broad inhibitor of caspases ZVAD-FMK, as well as specific inhibitors of caspase-3 and caspase-9, did not prevent the apoptotic death induced on NF- κ B inhibition in Rb cells, indicating that a caspase-independent pathway is preferred.

This is consistent with the recent work of Kolenko and colleagues,²⁵ who demonstrated that NF- κ B inhibition in T lymphocytes induces apoptotic death that does not involve activation of the caspase-1 and caspase-3. Although there is still the possibility that an unknown caspase is involved that is not inhibited by ZVAD-FMK, the most likely scenario is that a caspase-independent pathway is preferred. Bax overexpression may also result in apoptotic death, evidenced by loss of mitochondrial potential, DNA condensation, and cytoplasmic vacuolation, via a pathway that is not dependent on caspases and not inhibited by ZVAD-FMK.^{66,67} Similarly, staurosporine⁶⁸ and the hybrid polar compound hexamethylene bisacetamide⁶⁹ induce caspase-independent cell death mediated by mitochondrial membrane disruption. The fall in the mitochondrial potential can result in the formation of selected reactive oxygen species that lead

to the apoptotic demise of the cell. This caspase-independent pathway is possibly preferred in our model because of the constitutive expression of known caspase inhibitors, such as XIAP and survivin, that are not down-regulated on NF- κ B treatment.

Further evidence for a caspase-independent mechanism of apoptosis in our model arises from the study of PARP cleavage. Contrary to apoptosis induced by the proteasome inhibitor MG132, that activates caspase-3⁵⁹ and results in classic cleavage of PARP to a ~85-kd fragment, SN50 resulted in the appearance of an atypical ~60-kd fragment. This pattern of PARP cleavage is reminiscent of that observed in human breast carcinoma cells treated with the cytotoxic agent β -lapachone, which is mediated by the protease calpain.⁷⁰ In support, the calpain inhibitor calpeptin inhibited the apoptotic death induced by NF- κ B inhibition in our model, demonstrating that calpain is activated in our experimental setting. Calpain exists in the cytosol of the majority of cells as an inactive proenzyme, procalpain,⁷¹ which translocates to the cell membrane where it is activated autocatalytically in the presence of micromolar concentrations of Ca⁺⁺. Mitochondria play a major role in Ca⁺⁺ homeostasis, and their dysfunction results in increased cytosolic Ca⁺⁺ and activation of calpain, that functions as an executional death protease.⁷² Therefore, calpain seems to be part of a signal transduction mechanism that mediates caspase-independent death after inhibition of NF- κ B. The recent discovery that calpain cleaves Bax into a more potent 18-kd proapoptotic fragment⁷³ suggests that a positive feedback loop may exist between calpain and Bax activation.

It is well known that Rb cells produce and secrete IGF-1 and IGF-2 into their conditioned medium,⁷ and that IGF-1 and its receptor mediate an autocrine growth loop.⁷ IGF-1 protects a broad range of cells from a variety of proapoptotic challenges.^{36,37} This protective effect may be mediated by various intracellular signaling pathways, such as PI3K/Akt,³⁷ and, as we demonstrated in this study, activation of NF- κ B. Therefore, the IGF-1/IGF-1R mitogenic/anti-apoptotic loop in Rb cells could contribute to the constitutive activity of NF- κ B, which in turn could mediate part of their anti-apoptotic actions. Consequently, NF- κ B-inhibitory agents represent an appealing approach that could attenuate at least part of the growth/survival effects of IGF-1.

It has been reported that Akt can activate NF- κ B via p38.³⁵ P38/MAPK appears to be constitutively active in Rb cells, as we found that its inhibition lowers NF- κ B activity by itself. Given that IGF-1 is a well-known inducer of p38/MAPK, it is possible that the constitutive activity of p38/MAPK is the effect of the IGF-1/IGFR-I stimulatory loop that exists in Rb cells. Therefore, IGF-1 can be the center of a cycle of p38 activation, NF- κ B activation and subsequent induction of prosurvival members of the Bcl-2 family, such as Bcl-2 and A1. Indeed, we found that inhibition of p38 partially attenuated IGF-1-induced NF- κ B activation. Conflicting results have been published regarding the involvement of p38/MAPK in the regulation of apoptosis in a variety of cells.^{34,74} P38/MAPK has been reported to mediate cell death induced by γ -irradiation,

growth factor deprivation, and B cell receptor cross-linking.^{74–76} On the other hand, p38 has been reported to attenuate apoptosis induced by Fas ligation and UV irradiation.³⁴ We found that p38 inhibition has a synergistic effect with NF- κ B inhibition in inducing apoptosis in Rb cells. Therefore, the inhibition of NF- κ B and p38 in our model can synergistically tilt the mitochondrial balance of Bcl-2/Bax toward Bax via the down-regulation of anti-apoptotic Bcl-2 family members and induce caspase-independent cell death.

Although the treatment for Rb varies depending on tumor load and localization, chemotherapy is a usual approach and depends on combinations of doxorubicin, vincristine, and cyclophosphamide.⁷⁷ The ability of the chemotherapeutic reagents to induce apoptosis in Rb cells is enhanced by the absence of the anti-apoptotic effect of the Rb protein, and the activation of unopposed apoptotic pathways, such as those governed by the p53 protein. As several studies in other models identified NF- κ B as a modulator of chemotherapy cytotoxicity,¹¹ we evaluated the effects of SN50 on doxorubicin-induced apoptosis in human Rb cells. We found that SN50 and doxorubicin had a synergistic effect on the Y79 cell line. These findings suggest that combination of NF- κ B inhibitors with standard chemotherapeutic agents could result in clinical benefit for Rb patients.

In conclusion, our study demonstrates that inhibition of NF- κ B activity in Rb cell lines induces apoptosis that is associated with reduced levels of the anti-apoptotic Bcl-2 family members and cIAP-2, activation of mitochondria, and caspase-independent death. Therefore, NF- κ B inhibiting strategies could be a novel therapeutic approach for the treatment of Rb.

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