Effect of NF-κB Inhibition on TNF-α–Induced Apoptosis in Human RPE Cells

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Purpose. In many cell types, tumor necrosis factor (TNF)-α–induced apoptosis is prevented by production of TNF-α–induced antiapoptotic protein, a process mediated by nuclear transcription factor (NF)-κB. TNF-α is widely expressed in proliferative vitreoretinopathy (PVR) membranes and is present in the vitreous of eyes with PVR. To understand mechanisms responsible for RPE cell survival and death in this disease, this study was conducted to determine whether specific NF-κB blockade by mutant inhibitory (I)-κB (IκB) affects TNF-α–induced cell death.

Methods. Cultured human RPE cells and T-98G cells were infected with adenovirus encoding either β-galactosidase or mutant IκB and then treated with TNF-α or interleukin (IL)-1β. IκB, inhibitor of apoptosis protein (IAP)-1, and cellular Fas-associated death domain–like interleukin-1β–converting enzyme-like inhibitory protein (c-FLIP) expression was determined by Western blot. Functional NF-κB activation was examined by luciferase reporter assay. Cell viability was evaluated by trypan blue exclusion assay. Caspase-3 activity and DNA fragmentation was measured with an enzyme-linked immunosorbent assay.

Results. Mutant IκB expression blocked cytokine-induced IκB degradation and NF-κB transcriptional activity in RPE cells and T-98G cells. RPE cells were resistant to TNF-α–induced apoptosis, even after NF-κB activation was specifically blocked. In contrast, TNF-α dramatically induced apoptosis in T-98G cells after NF-κB inhibition. c-IAP1 expression was not affected by TNF-α or mutant IκB, and mutant IκB abolished TNF-α–induced c-FLIP induction in RPE cells.

Conclusions. RPE cells are resistant to TNF-α–induced cell death, even after NF-κB activation is specifically blocked. RPE cell resistance to apoptotic signals present in eyes with PVR, mediated by survival factors such as c-FLIP and c-IAP1, may help to explain unwanted and unchecked cell proliferation in this disease. (*Invest Ophthalmol Vis Sci.* 2004;45:2438–2446) DOI:10.1167/iovs.03-0805

Proliferative vitreoretinopathy (PVR) remains the principal cause for the failure of retinal reattachment surgery and is a potentially blinding disease. PVR is characterized by uncontrolled cell proliferation and migration into the subretinal space and vitreous cavity and onto the retinal surface and undersurface.[1,2] Cell-mediated contraction of the fibrocellular scar causes traction on the retina, leading to retinal detachment and subsequent loss of vision.[3]

Apoptosis is a form of programed cell death that plays a fundamental role in many normal biological processes, as well as in diseases such as PVR and age-related macular degeneration (AMD).[4,5] The targeted induction of apoptosis is a novel therapeutic approach to control the unlimited growth of proliferating cells.[6]

Tumor necrosis factor (TNF)-α, originally described for its antitumor activity, is widely expressed in PVR membranes[7,8] and choroidal neovascular membranes (CNVMs)[9] and is present in the vitreous of eyes with PVR.[7,10,11] Retinal pigment epithelial (RPE) cells are found in surgically removed PVR epiretinal membranes[1,12,13] and CNVMs.[9] RPE cell proliferation is thought to contribute to membrane formation in PVR.[1,12,13] TNF-α is a major regulator of RPE activation responses, including cell attachment, spreading, chemotaxis, migration, and proliferation.[14,15]

Nuclear transcription factor (NF)-κB is a pivotal regulator of many different genes, including multiple inflammatory cytokine genes and apoptosis-related genes.[16] In many cells types, TNF-α–induced apoptosis is prevented by parallel TNF-α–induced production of antiapoptotic proteins, such as cellular inhibitor of apoptosis protein (c-IAP) and cellular Fas-associated death domain (FADD)-like interleukin-1β-converting enzyme-like inhibitory protein (c-FLIP), a process mediated by NF-κB.[17–20] RPE cells are normally resistant to TNF-α–mediated apoptosis, and in fact proliferate in response to this cytokine.[15] NF-κB inhibition results in apoptosis in a variety of cell types originally resistant to TNF-α–induced apoptosis, implying anti-NF-κB therapies could be a new tool for the treatment of inflammatory diseases and cancers.[21–24] However, the effect of specific NF-κB inhibition on RPE cell apoptosis is unknown. To understand mechanisms responsible for RPE cell survival and death in PVR, we transduced a NF-κB super-repressor, a mutant form of the inhibitor κB (IκB) gene with substitutions by alanine at serines 32 and 36, into human RPE cells to determine whether specific NF-κB inhibition affects TNF-α–induced RPE cell apoptosis.

Materials and Methods

Cell Culture

Human donor eyes were obtained from the North Carolina Organ Donor and Eye Bank, in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. RPE cells were harvested from eyes, as previously described.[25] T-98G

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glioma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were chosen as a positive control because they are known to express TNF receptor (TNF-R)1[26] and are sensitive to TNF-α–induced apoptosis after NF-κB blockade.[23] Cells were grown in Eagle's minimum essential medium (MEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and 1× antibiotic–antimycotic (Invitrogen) at 37°C in a humidified environment containing 5% CO_2.

Detection of LacZ Expression

To determine transduction efficiency, cells were infected with an adenovirus encoding β-galactosidase (LacZ) at a variety of multiplicities of infection (MOIs) ranging from 10 to 300. Forty-eight hours after infection, LacZ expression was examined by staining the cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution. Blue cell staining was detected by phase-contrast microscopy. A masked observer determined the percentage of cells with blue staining in each of three microscopic fields (200×).

Adenoviral Infection and Stimulation

RPE cells or T-98G cells (1×10^5) were seeded in six-well plates (Costar, Corning Inc., Corning, NY). Twenty-four hours later, cells were incubated with fresh medium for an additional 24 hours. Cells were infected with adenovirus encoding either LacZ or mutant IκB[27] (University of North Carolina at Chapel Hill Gene Delivery Core, Chapel Hill, NC) in MEM containing 1% FBS (RPE cell MOI = 10, T-98G cell MOI = 300). Twenty-four hours later, cells were treated for various times with TNF-α (1.1×10^3 U/mL; R&D Systems Inc., Minneapolis, MN) or IL-1β (5 U/mL; Becton Dickinson Labware, Bedford, MA) in MEM containing 1% FBS. We chose IL-1β and TNF-α concentrations that we have shown to stimulate cytokine gene expression and cause IκB degradation in RPE cells.[28,29]

Cell Extracts and Western Blot

Cells in duplicate wells were stimulated with TNF-α or IL-1β for various times. After the medium was removed, the cells were washed twice with cold Hanks' balanced salt solution and lysed with RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were transferred to 1.5-mL microcentrifuge tubes and cleared by centrifugation. Total protein in the supernatants was measured by the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) used to generate the curve, according to the manufacturer's instructions. Protein (20 μg) was electrophoresed on a 12.5% SDS-polyacrylamide gel overlaid with a 3.6% polyacrylamide stacking gel. The proteins were transferred to nitrocellulose membrane (Bio-Rad) using a mini-transblot apparatus (Bio-Rad) according to the manufacturer's directions. Transfers were performed overnight at room temperature (RT). Nonspecific binding sites were blocked by immersing the membrane in 10% fat-free milk powder (SACO Foods Inc, Middleton, WI) for 60 minutes at RT. The blocking step was repeated, and then membranes were washed three times (5 minutes per wash) in Tris-buffered saline containing 0.1% Tween-20 (TBST). The membranes were incubated overnight with rabbit polyclonal antibody directed against IκB (1:2000 in 5% milk; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against c-IAP1 (1:2000 in 5% milk; Santa Cruz Biotechnology) and mouse monoclonal antibody against c-FLIP NF6 (1:500 in 5% milk; Alexis, San Diego, CA) at 4°C. The blots were then washed three times (20 minutes per wash) in TBST and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 in 5% milk; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or anti-mouse IgG conjugated with horseradish peroxidase (1:2500 in 5% milk; Jackson ImmunoResearch Laboratories) at 4°C for 60 minutes. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Transfection and NF-κB–Driven Luciferase Reporter Assay

Transfection was performed with a lipophilic reagent (LipoTAXI; Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Briefly, cells in triplicate wells were washed twice with 3 mL of serum-free, antibiotic-free MEM (MEM-SA) and incubated in MEM-SA at 37°C for 15 minutes. Plasmid DNA (5 μg) encoding firefly luciferase downstream from the NF-κB promoter (Stratagene) and 1 μg of DNA encoding a constitutively expressed *Renilla* luciferase gene (Promega, Madison, WI) were mixed with the transfection reagent and then added to cell cultures in MEM–SA medium. After a 4-hour incubation, 3 mL of 10% FBS-MEM was added to each well. Cells were re-fed 24 hours after transfection and maintained in fresh 10% FBS-MEM for another 24 hours before infection. Cells were stimulated with TNF-α or IL-1β for 4 hours. NF-κB-luciferase reporter assay was performed with a luciferase reporter assay kit (Dual-Luciferase Reporter Assay System; Promega) according to the manufacturer's instructions. Firefly luciferase activity and *Renilla* luciferase activity in the supernatants were measured with a luminometer (LB 9501, EG&G Berghold, Bundoora, Australia), and the ratio of firefly luciferase activity to *Renilla* luciferase activity was calculated.

Assessment of Cell Number

Cells in triplicate wells were stimulated with TNF-α for various times. After the medium was removed, cells were washed with PBS and trypsinized. Cell viability was determined with a trypan blue exclusion assay. Live cells that excluded 0.2% trypan blue dye were counted with a hemocytometer.

Detection of Caspase-3 Activity and DNA Fragmentation

Cells in triplicate wells were stimulated with TNF-α or IL-1β for 8 hours. They were harvested by lysis in buffers provided in caspase-3 and DNA fragmentation kits, respectively, as described later. Lysates were transferred to tubes and cleared by centrifugation. Caspase-3 activity, an early marker of apoptosis,[30,31] and DNA fragmentation, a late marker of apoptosis, were quantified by enzyme-linked immunosorbent assay (ELISA; caspase-3 activity kit: BD Biosciences-Clontech, Palo Alto, CA; DNA fragmentation kit: Roche) according to the manufacturers' instructions.

Statistical Analysis

Data are expressed as the mean ± SD. Student's *t*-test was used to determine whether there were statistically significant differences between treatment groups determined by luciferase assay, the number of cells, caspase-3 activity, or DNA fragmentation assay. $P < 0.05$ was considered to be statistically significant. More than five different experiments were performed with Western blot to show that overexpression of mutant IκB was resistant to cytokine-induced IκB degradation. Assessment of cell number was performed in triplicate and was repeated four times with RPE cells from three different donors. The luciferase reporter assay, a functional assay of NF-κB activation and caspase-3 activity was performed in triplicate and was repeated two times with RPE cells from two different donors. The DNA fragmentation assay, performed in triplicate was repeated two times. Western blot on duplicate samples from each treatment group was performed once to determine c-FLIP and c-IAP1 expression.

RESULTS

Determination of Transduction Efficiency

We have previously reported that an MOI of 10 with the present adenoviral vector was optimal to block NF-κB activation by mutant IκB in RPE cells.[29] With this information, we compared LacZ transduction efficiency in RPE cells to that in T-98G cells by X-gal assay. The percentage of RPE cells infected with adenovirus encoding LacZ at an MOI of 10 was 95%. The percentage in T-98G cells at MOI of 10, 50, 150, and 300 was 30%, 40%, 70%, and 80%, respectively, indicating that there was greater transduction efficiency in RPE cells. We also examined mutant IκB adenoviral infection efficiency in T-98G cells by Western blot. The expression of mutant IκB protein was increased in an MOI-dependent manner in T-98G cells (Fig. 1). Based on our previous studies,[29] our current results, and T-98G cell transduction efficiency reported previously,[23] we chose an MOI of 10 in RPE cells and of 300 in T-98G cells for subsequent experiments.

Resistance to Cytokine-Stimulated IκB Degradation by Mutant IκB Overexpression

We have reported that RPE cells that overexpress mutant IκB are resistant to IL-1β–induced IκB degradation.[29] To evaluate the effect of mutant IκB transgene on TNF-α–induced IκB degradation in RPE cells and on TNF-α– and IL-1β–induced IκB degradation in T-98G cells, IκB protein expression was determined by Western blot analysis. When RPE cells were treated with TNF-α or T-98G cells were treated with TNF-α or IL-1β, endogenous IκB protein was degraded within 30 minutes (Figs. 2A–C). In contrast, mutant IκB was not degraded after cytokine treatment. Endogenous IκB degradation was variable, despite constant overexpression of mutant IκB, a finding that we have observed previously.[29] As a control for cytoplasmic phosphorylation and degradation we chose β-catenin, a protein that is phosphorylated and degraded by the proteasome in response to an NF-κB–independent signal-transduction pathway.[32] In contrast to IκB, β-catenin was not degraded in response to cytokine stimulation in noninfected cells, LacZ-expressing cells, or cells infected to express mutant IκB (Figs. 2D–F). Accordingly, β-catenin also serves as an excellent protein to standardize protein loading (also see Fig. 1C).

Inhibition of Cytokine-Induced NF-κB Transcriptional Activity by Overexpression of Mutant IκB

After IκB degradation, NF-κB is translocated to the nucleus, binds to DNA and activates gene transcription. We have shown previously that expression of mutant IκB protein blocks IL-1β–induced NF-κB transcriptional activity in RPE cells.[29] In the current study, we used a dual-luciferase reporter system to determine the effect of mutant IκB on TNF-α–induced NF-κB transcriptional activity in RPE cells and its effect on TNF-α– and IL-1β–induced transcriptional activity in T-98G cells. TNF-α significantly induced NF-κB–driven luciferase activity in LacZ-expressing RPE cells and T-98G cells. A similar effect was observed in IL-1β–treated T-98G cells. Basal and cytokine mediated NF-κB transcriptional activity was significantly suppressed in both cell types when infected to express mutant IκB (Fig. 3).

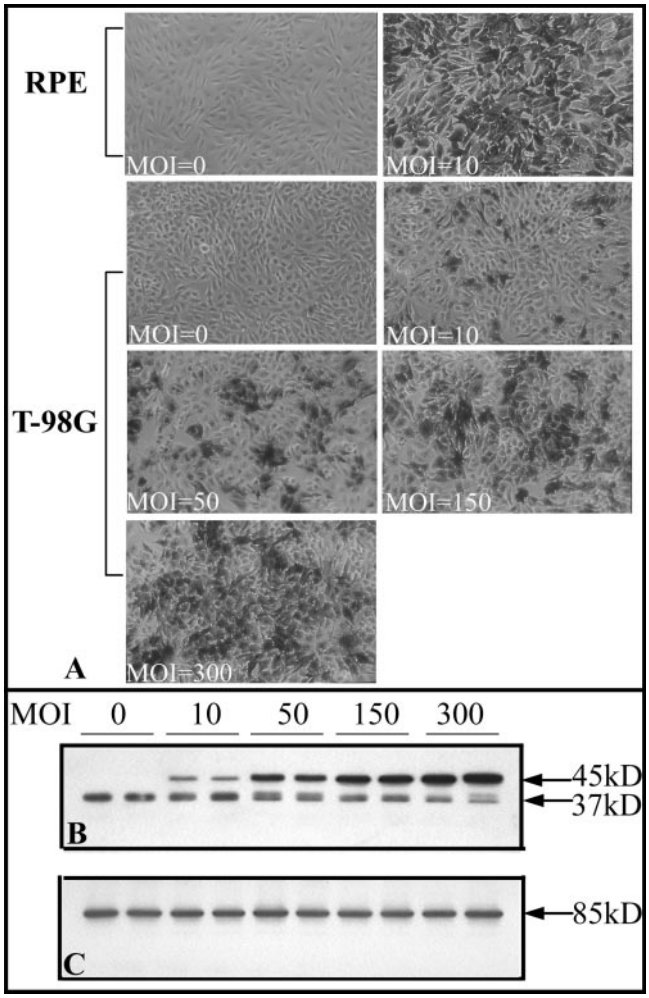

FIGURE 1. Detection of LacZ and mutant IκB expression. (**A**) RPE cells and T-98G cells were infected with an adenovirus containing LacZ at different MOIs. Forty-eight hours after infection, the expression of the LacZ transgene was examined by staining the cells with X-gal. (**B**) T-98G cells in duplicate wells were infected with adenovirus containing mutant IκB at different MOIs. One day after infection, the expression of the mutant IκB transgene was examined by Western blot. Bands at 45 kDa correspond to mutant IκB transgene and bands at 37 kDa to endogenous IκB. (**C**) Blot in (**B**) stripped and reprobed with antibody to β-catenin, a control for gel loading.

Resistance to TNF-α–Induced Apoptosis in RPE Cells that Overexpress Mutant IκB

In many cells, TNF-α–induced NF-κB activation leads to antiapoptotic protein production.[17–20] TNF-α–induced proteins protect cells from TNF-α–driven caspase activity and apoptosis. To determine whether NF-κB activation protects RPE cells from TNF-α–induced apoptosis, we examined the effect of NF-κB blockade on RPE cell number, caspase-3 activity, and DNA fragmentation in the presence and absence of TNF-α. RPE cells infected either with LacZ virus or mutant IκB virus with or without 24-hour TNF-α treatment appeared healthy. However, TNF-α treatment induced a marked increase in T-98G cell death when cells were infected with an adenovirus encoding mutant

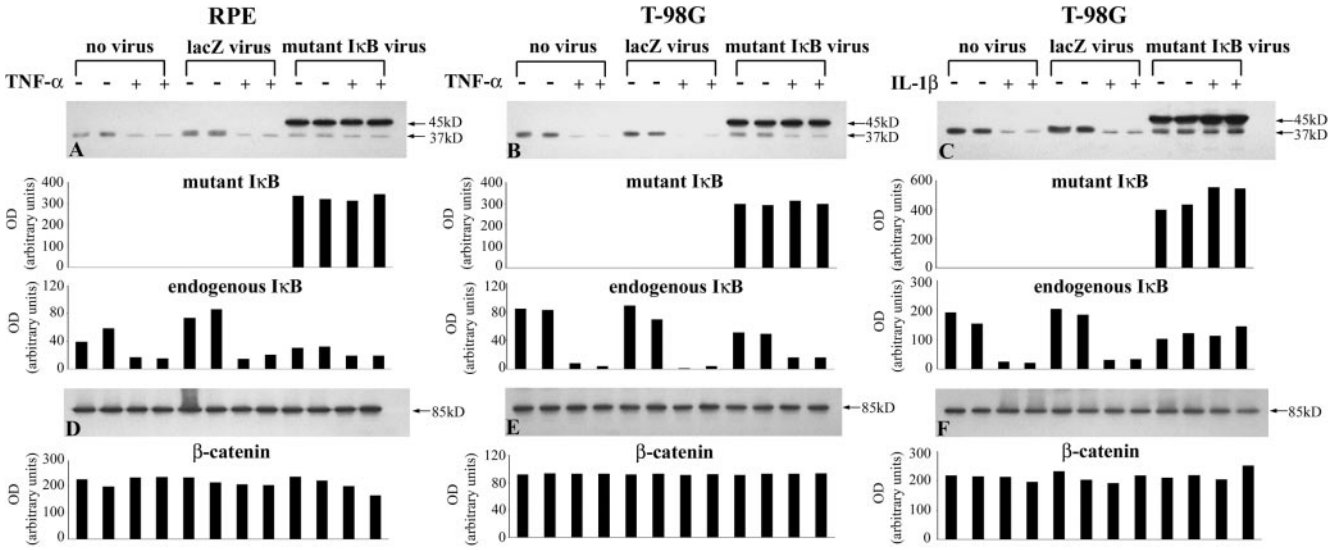

FIGURE 2. Resistance to cytokine-induced IκB degradation by mutant IκB overexpression. RPE cells and T-98G cells were infected with adenovirus containing LacZ or mutant IκB. One day after infection, cells were treated with medium alone, TNF-α (1.1×10^3 U/mL), or IL-1β (5 U/mL) for 30 minutes, and cytoplasmic proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. (**A–C**) Western blot probed with antibody to IκB. Bands at 45 kDa correspond to mutant IκB transgene and bands at 37 kDa to endogenous IκB. The relative quantities of endogenous and mutant IκB proteins, determined by densitometry, are shown separately *below* each lane. (**D–F**) Blots in (**A**), (**B**), and (**C**), respectively, separately stripped and reprobed with antibody to β-catenin, a control for gel loading. The relative quantity of β-catenin protein is shown *below* each lane.

IκB, but not when they were infected by an adenovirus encoding LacZ. T-98G cells infected to express mutant IκB were rounded, detached from plates, and floated in the medium at 24 hours after TNF-α stimulation (Fig. 4). IL-1β had no effect on RPE cell or T-98G cell morphology regardless of whether cells were not infected, infected with adenovirus encoding LacZ, or infected with adenovirus encoding mutant IκB.

The number of viable RPE cells was not reduced within 48 hours when cells were infected with LacZ virus or mutant IκB virus, either with or without TNF-α treatment. In contrast, the number of T-98G cells infected to express mutant IκB was markedly decreased in a time-dependent manner, whereas the number of viable noninfected or LacZ-infected T-98G cells was unchanged (Fig. 5A). To investigate further whether RPE cells undergo TNF-α–mediated cell death after NF-κB blockade, but at a slower rate than T-98G cells, the viable cell number was determined at 0, 24, 48, and 72 hours. Noninfected RPE cells or RPE cells infected with adenovirus were resistant to TNF-α–induced cell death at all time points, even after NF-κB blockade (Fig. 5B).

An MOI of 10 in RPE cells produced an infection efficiency similar to that produced by an MOI of 300 in T-98G cells. To further confirm that the differential effect of TNF-α and NF-κB blockade on cell death was specific and not related to different MOIs in the two cell types, an additional experiment was performed.

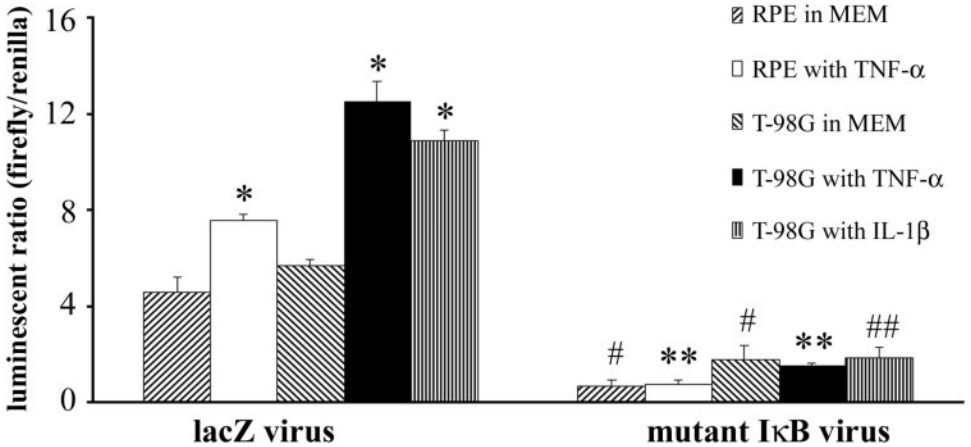

FIGURE 3. Inhibition of NF-κB transcriptional activity by mutant IκB overexpression. RPE cells in triplicate wells were cotransfected with 5 μg plasmid DNA encoding firefly luciferase driven by the NF-κB promotor and 1 μg of DNA encoding a constitutively expressed *Renilla* luciferase gene. The firefly luciferase plasmid contains a luciferase reporter gene driven by a tandem repeat of NF-κB binding elements and therefore serves as an experimental reporter of NF-κB activation. *Renilla* luciferase serves as the control reporter to normalize the activity of the experimental reporter based on transfection efficiency and cell viability. Two days after transfection, the cells were infected with adenovirus containing LacZ or mutant IκB. One day after infection, cells were exposed to medium alone, TNF-α (1.1×10^3 U/mL) or IL-1β (5 U/mL) for 4 hours. Cells were harvested, and lysates were tested for luciferase activity using a dual-luciferase assay system. Relative luminescence denotes the ratio of firefly luciferase activity (NF-κB-dependent) to *Renilla* luciferase (control) activity to standardize for transfection efficiency. Results are expressed as mean ± SD ($n = 3$). $^*P < 0.002$ versus untreated cells; $^{**}P < 0.0001$ versus TNF-α–treated cells in the LacZ virus group; $\#P < 0.0003$ versus untreated cells in LacZ virus group; $\#\#P < 0.00001$ versus IL-1β–treated cells in LacZ virus group.

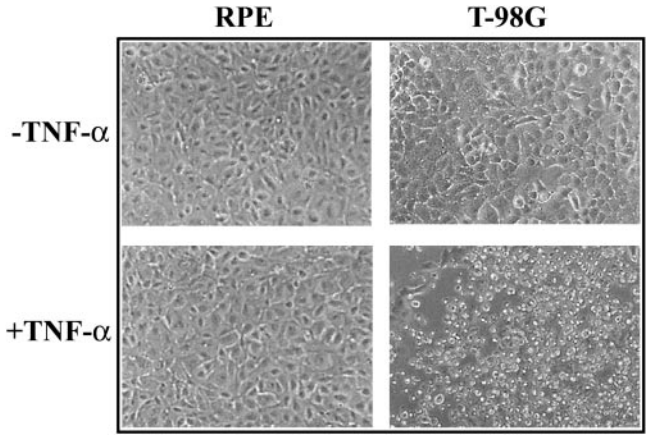

FIGURE 4. Effect of mutant IκB overexpression on TNF-α–induced cell death. RPE cells and T-98G cells were infected with adenovirus containing mutant IκB. One day after infection, cells were treated with TNF-α (1.1×10^3 U/mL) for 24 hours. Cells were observed under a phase-contrast microscope. Original magnification, ×100.

At an MOI of 300, RPE cell morphology was altered in both the LacZ virus group and mutant IκB virus group. Some cells in both groups were rounded, detached from the plates, and floated in the medium. Surviving cells appear enlarged, elongated, and less densely packed. However, TNF-α did not further reduce the number of surviving adenovirus-infected cells compared with non–TNF-α–treated adenovirus-infected cells. In contrast, even at an MOI of 10, when the infection efficiency of T-98G cells was only 30%, TNF-α still induced significant cell death compared with cells that were not treated with TNF-α (Table 1).

To evaluate the effect of NF-κB blockade on RPE cell apoptosis after cytokine stimulation, we measured caspase-3 activity, an early marker of apoptosis, in both RPE cells and T-98G cells. Caspase-3 was not activated in noninfected RPE cells, LacZ-expressing RPE cells, or RPE cells infected to express mutant IκB 8 hours after TNF-α treatment. However, caspase-3 activity was strongly induced in T-98G cells infected to express mutant IκB, but not in noninfected or LacZ-expressing T-98G cells after TNF-α treatment (Fig. 6). Even at 6 hours, TNF-α significantly induced caspase-3 activity in T-98G cells infected to express mutant IκB compared to untreated group ($P < 0.01$) (not shown). IL-1β had no effect on caspase-3 activity in T-98G cells infected to express mutant IκB (not shown).

We next measured DNA fragmentation, a late marker of apoptosis, in RPE cells and T-98G cells to confirm further our observation that RPE cells, unlike T-98G cells, were resistant to TNF-α–induced apoptosis after NF-κB inhibition. Neither TNF-α nor IL-1β induced DNA fragmentation in RPE cells infected with either LacZ or mutant IκB. In contrast, after TNF-α treatment for 8 hours, significant DNA fragmentation was detected in T-98G cells infected to express mutant IκB, but not in LacZ-expressing T-98G cells. Notably, IL-1β did not cause DNA fragmentation in T-98G cells infected to express mutant IκB (Fig. 7).

To verify that RPE cells were truly resistant to TNF-α–induced apoptosis after NF-κB blockade and did not simply undergo apoptosis more slowly than T-98G cells, we repeated the RPE cell DNA fragmentation assay at 8, 24, 48, and 72 hours. In this experiment, DNA fragmentation in T-98G cells was used as a positive apoptosis control. DNA fragmentation could not be measured accurately in these cells at later time points, because of massive cell death. DNA fragmentation was not induced by TNF-α in RPE cells infected to express mutant IκB at any time points (Fig. 8).

Effect of NF-κB Activation on c-FLIP and c-IAP1 Expression

NF-κB regulates the expression of a number of genes whose products can inhibit apoptosis.[33] c-FLIP is a protein that is

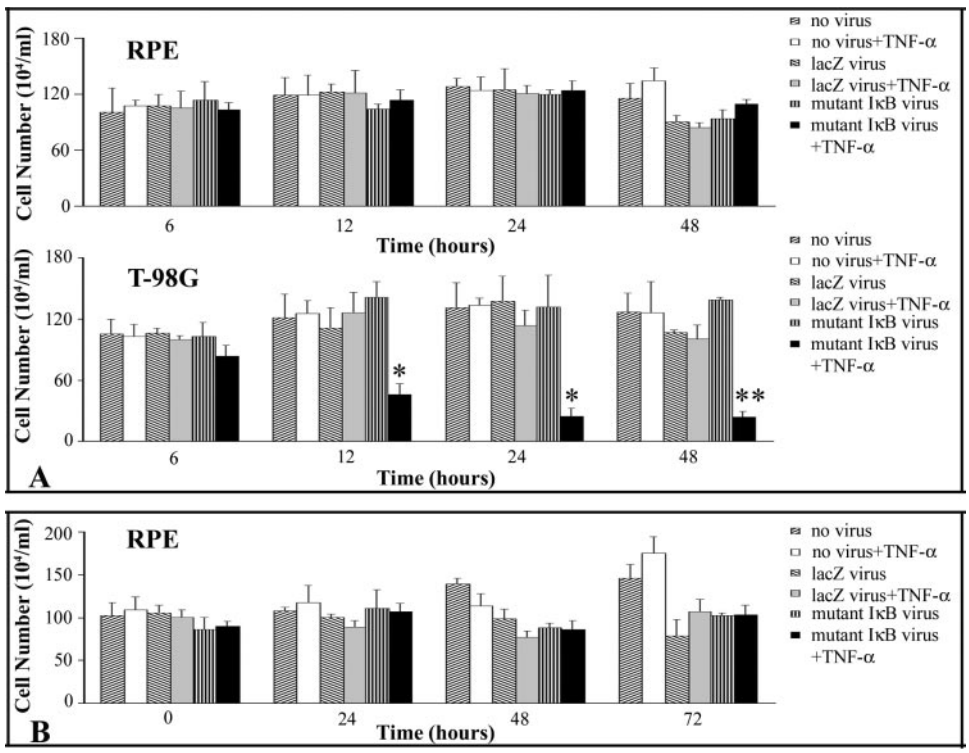

FIGURE 5. Resistance to TNF-α–induced cell death in RPE cells that overexpress mutant IκB. (**A**) RPE cells and T-98G cells in triplicate wells were infected with adenovirus containing LacZ or mutant IκB. One day after infection, cells were treated with medium alone or TNF-α (1.1×10^3 U/mL) for varying times. Cell viability was evaluated by trypan blue exclusion assay. Cell Number represents live cells that excluded trypan blue. Results are expressed as the mean ± SD ($n = 3$). $^*P < 0.004$ versus untreated cells in mutant IκB virus group; $^{**}P < 0.000005$ versus untreated cells in mutant IκB virus group. (**B**) RPE cells in triplicate wells were infected with adenovirus containing LacZ or mutant IκB and then treated with medium alone or TNF-α (1.1×10^3 U/mL) for varying times 1 day after infection. Cell viability was evaluated by trypan blue exclusion assay. Cell Number represents live cells that excluded trypan blue. Results are expressed as the mean ± SD ($n = 3$).

TABLE 1. The Effect of Adenovirus Transduction on TNF-α–Induced Cell Death

	RPE Cells		T-98G Cells	
	LacZ Virus	Mutant IκB Virus	LacZ Virus	Mutant IκB Virus
MEM	58 ± 16	65 ± 7	161 ± 9	179 ± 7
TNF-α	85 ± 24	71 ± 10	153 ± 17	124 ± 20*

Data represent number of viable cells in triplicate wells treated with TNF-α (1.1×10^3 U/mL) for 24 hours 1 day after infection. An MOI of 300 was used in RPE cells and an MOI of 10 in T-98G cells.

* $P < 0.01$ versus MEM in mutant IκB virus group.

thought to be an important determinant of cell survival. It competes with caspase-8 binding to FADD and thereby blocks apoptosis at the apex of the caspase cascade.[17,34] We determined whether RPE cell c-FLIP expression could account for RPE cell resistance to TNF-α–driven apoptosis after NF-κB blockade. TNF-α upregulated the 55-kDa long FLIP ($FLIP_L$) form and short FLIP ($FLIP_S$) form expression in noninfected or LacZ-expressing RPE cells. NF-κB inhibition abolished upregulation of cellular long and short c-FLIP protein. Thus c-FLIP upregulation by TNF-α could help to explain why RPE cells are resistant to TNF-α–induced apoptosis. However, the complete blockade of c-FLIP induction by mutant IκB indicates that c-FLIP does not account for RPE cell resistance to apoptosis after TNF-α stimulation and NF-κB blockade (Fig. 9).

To investigate further the mechanism underlying the survival of RPE cells in response to TNF-α, we examined the expression of c-IAP1, another important cell survival factor.[19] First, we examined the kinetics of c-IAP1 expression in noninfected RPE cells in the presence of TNF-α or IL-1β. The expression of c-IAP1 protein, however, was not affected by TNF-α or IL-1β treatment at any time period tested (Fig. 10A). We investigated the effect of NF-κB inhibition on TNF-α–mediated c-IAP1 induction by examining c-IAP protein expression in RPE cells after TNF-α treatment. NF-κB inhibition failed to block c-IAP1 expression after 6 hours in the presence or absence of TNF-α treatment (Fig. 10B).

DISCUSSION

In the present study, a mutant IκB protein expressed in human RPE cells by viral transduction was resistant to cytokine-stimulated degradation and inhibited cytokine-induced NF-κB transcriptional activity. RPE cells were resistant to TNF-α–induced cell death, caspase-3 activity, and DNA fragmentation, even after NF-κB activation was specifically blocked by mutant IκB. IL-1β had no effect on apoptosis in RPE cells after NF-κB suppression. We identified c-FLIP and c-IAP1 as antiapoptotic factors that could help to protect RPE cells from TNF-α–induced apoptosis and c-IAP1 as a protein that may promote RPE cell survival after NF-κB blockade.

We used T-98G cells as a positive control for TNF-α–induced apoptosis after NF-κB blockade. Our initial experiments were conducted to determine an MOI for T-98G cells that would provide transgene expression efficiency similar to that obtained with an MOI of 10 in RPE cells. Our results, which indicated an optimal MOI of 300 in T-98G cells, are similar to those reported previously.[23] The reason for the more efficient transgene transduction in RPE cells than in T-98G cells is unknown.

The effect of NF-κB activation on apoptosis is very cell-type specific. Depending on the cell, NF-κB may have an antiapoptotic or proapoptotic effect.[33,35–39] Constitutive NF-κB activity, seen in Hodgkin's disease cells, protects the cells against apoptosis, and NF-κB inhibition leads to cell death.[40] In many cells, NF-κB activation protects against TNF-α–driven apoptosis, and NF-κB blockade causes TNF-α–induced cell death.[17–20] For example, human glioma cells are relatively resistant to apoptotic stimuli.[41] Like RPE cells, T-98G and U251 glioma cells express TNF-R1 receptors[26,42] and do not undergo apoptosis when treated with TNF-α alone; however, as indicated earlier, these cells undergo apoptosis after TNF-α exposure when they are first infected with an IκB deletion mutant.[23,43] In contrast, as shown in the current study, RPE cells do not undergo TNF-α–mediated apoptosis after NF-κB blockade. Similarly, human HCT116 colon carcinoma cells, OVCAR-3 ovarian carcinoma cells, and HPB lymphoid T cells are resistant to TNF-α–stimulated apoptosis after NF-κB inhibition.[44] In other experimental systems—for example, after serum withdrawal from 293 cells and in Sindbis virus–infected cells during glutamate-induced neuronal cell cytotoxicity, NF-κB activation is

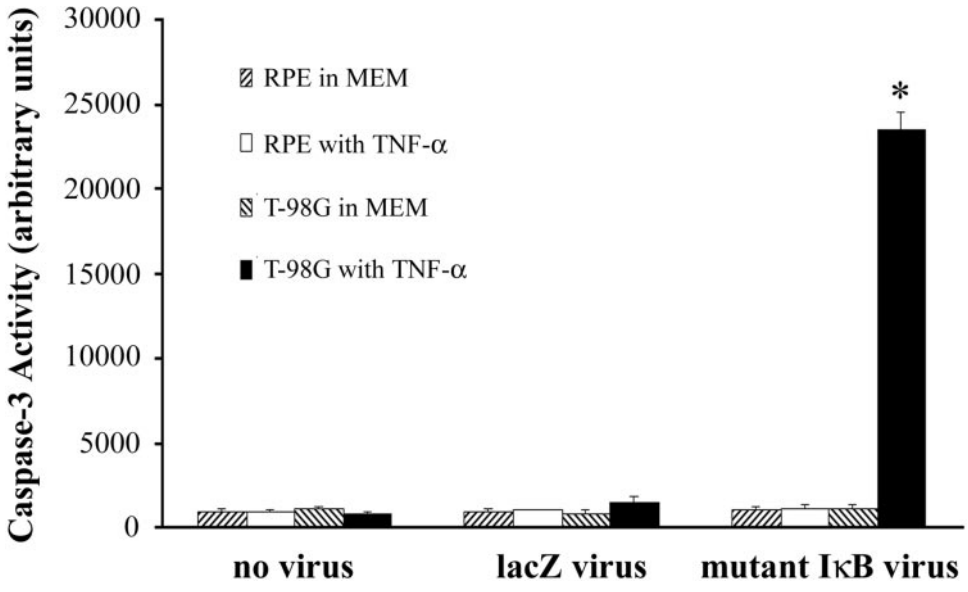

FIGURE 6. Resistance to TNF-α–induced caspase-3 activity in RPE cells that overexpress mutant IκB. RPE cells and T-98G cells in triplicate wells were infected with adenovirus containing LacZ or mutant IκB. One day after infection, cells were treated with medium alone or TNF-α (1.1×10^3 U/mL) for 8 hours. Caspase-3 activity was measured by ELISA. Results are expressed as the mean ± SD ($n = 3$). *$P < 0.00003$ versus untreated T-98G cells in the mutant IκB virus group.

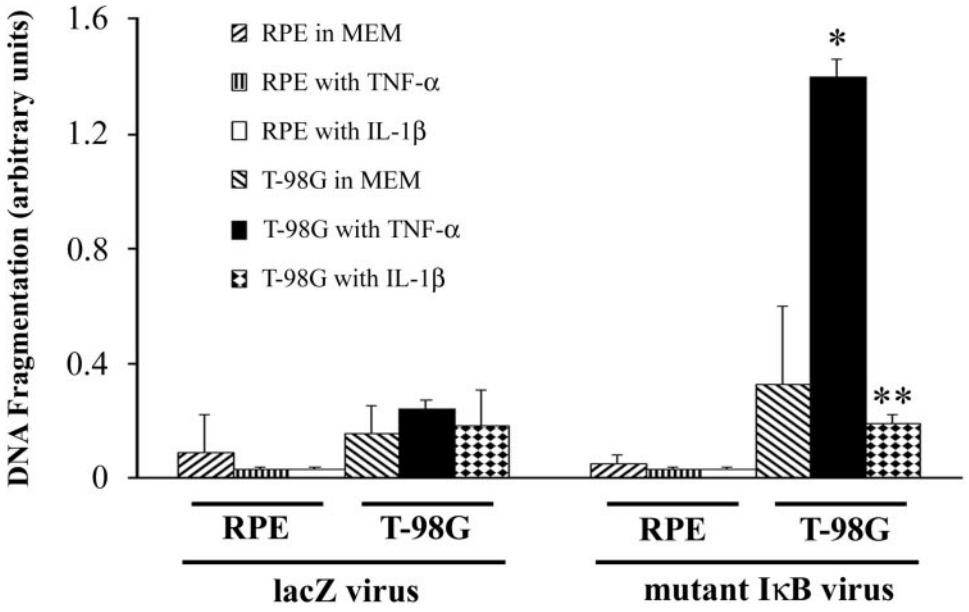

FIGURE 7. Resistance to TNF-α–induced DNA fragmentation in RPE cells that overexpress mutant IκB. RPE cells and T-98G cells in triplicate wells were infected with adenovirus containing LacZ or mutant IκB. One day after infection, cells were treated with medium alone, TNF-α (1.1×10^3 U/mL) or IL-1β (5 U/mL) for 8 hours. DNA fragmentation was measured by ELISA. Results are expressed as the mean ± SD ($n = 3$). $^*P < 0.003$ versus untreated cells in the mutant IκB virus group; $^{**}P < 0.00001$ versus TNF-α–treated cells in the mutant IκB virus group.

necessary for initiation of apoptosis.[36–39] The varying apoptotic response to NF-κB activation may provide organ-specific cell survival or death after physiological stimuli and may contribute to pathologic cell survival or death in diseases such as cancer and PVR.

We originally hypothesized that NF-κB blockade might be a potentially valuable PVR treatment strategy by virtue of its effect as an inducer of RPE cells apoptosis, and its role as an inhibitor of inflammatory cytokine gene expression. The results of the present experiments suggest that NF-κB blockade would not induce RPE cell apoptosis. Regardless, it is very likely that NF-κB inhibition would downregulate multiple cytokines thought to be important in the initiation and perpetuation of PVR.[7,8,10,11] Nonetheless, because NF-κB plays a central role as a transcription regulator of multiple genes, there may be unintended and/or unwanted gene transcription effects. NF-κB blockade as a method to inhibit PVR warrants further investigation.

In the present study, the antiapoptotic factor c-FLIP was upregulated by TNF-α in an NF-κB–dependent manner. Similarly, other investigators have demonstrated that NF-κB activation mediates c-FLIP expression.[17,45] c-FLIP is a potent antiapoptotic factor that binds to the protein Fas-associated death domain (FADD).[46] Two splice variants of c-FLIP have been identified.[47,48] The full-length 55-kDa c-FLIP form, c-FLIP$_L$, has structural homology to caspase-8, and contains two death effector domains (DEDs) that bind to FADD and an inactive caspaselike domain. An alternately spliced short c-FLIP form, c-FLIP$_S$, contains only the two DEDs. Both forms inhibit apoptosis, although the relative antiapoptotic potency of the two forms depends on the specific cell.[45,49] It is thought that the c-FLIP/procaspase-8 ratio determines whether apoptosis is activated through the effector caspases downstream of caspase-8.[46] We found that TNF-α markedly enhanced both the c-FLIP$_L$ and c-FLIP$_S$ RPE protein levels. Accordingly, we hypothesize that c-FLIP may be an important factor that protects RPE cells from TNF-α–induced cell death. NF-κB blockade completely abolished the TNF-α–stimulated c-FLIP$_L$ and c-FLIP$_S$ upregulation. However, under these conditions, RPE cells were still resistant to TNF-α–mediated cell death. Thus, c-FLIP expression cannot fully explain RPE cell resistance to TNF-α–mediated RPE cell apoptosis.

In our experiments, cultured human RPE cells expressed another important antiapoptotic factor, c-IAP, which inhibits apoptosis by binding directly to effector caspases such as caspase-3 and -7, thereby inactivating them.[50,51] In addition,

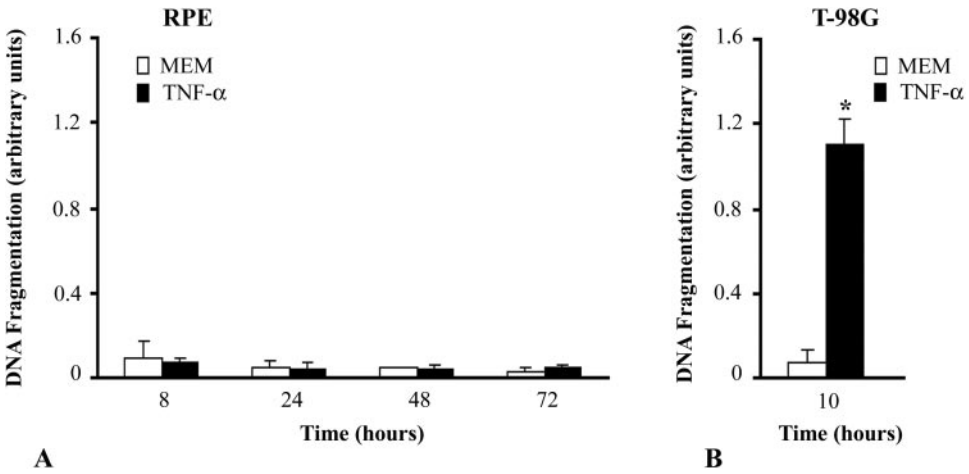

FIGURE 8. Resistance to TNF-α–induced DNA fragmentation at various time points in RPE cells that overexpressed mutant IκB. RPE cells and T-98G cells in triplicate wells were infected with adenovirus containing mutant IκB. One day after infection, cells were treated with medium alone or TNF-α (1.1×10^3 U/mL). DNA fragmentation was measured by ELISA. Results are expressed as the mean ± SD ($n = 3$). $^*P < 0.001$ versus untreated cells in mutant IκB virus group. (**A**) RPE cells; (**B**) T-98G cells.

c-IAP1, along with c-IAP2, TRAF-1, and TRAF-2, is recruited to death domains on TNF receptor 1 to form the death-inducing signaling complex (DISC).[50,51] This complex functions to inhibit apoptosis at the apex of the TNF-α/caspase–signaling cascade. Thus, c-IAP, along with c-FLIP, could serve to protect RPE cells from TNF-α–mediated cell death. In contrast to c-FLIP, high c-IAP levels were expressed under basal conditions, and levels were not influenced significantly by TNF-α–stimulation or NF-κB blockade. The regulation of cellular c-IAP is very cell-type specific. In several cell types, c-IAP expression is enhanced by NF-κB, an effect that is prevented by NF-κB blockade.[19] In many other cells, as in RPE cells, c-IAP protein is expressed constitutively, and levels are not influenced by NF-κB activation.[52,53] Constitutive c-IAP1 protein expressed by RPE cells could help to explain why RPE cells are resistant to TNF-α–mediated apoptosis, despite NF-κB blockade.

Our results indicate that RPE cells are resistant to TNF-α–mediated cell death, both by NF-κB–dependent and NF-κB–independent mechanisms. In PVR, RPE cells that have left their normal monolayer survive for extended times in the subretinal space, in the vitreous cavity, and on the retinal surface and undersurface. Experiments are currently under way in our laboratory to clarify further the role of antiapoptotic factors such as c-FLIP and c-IAP1 in PVR.

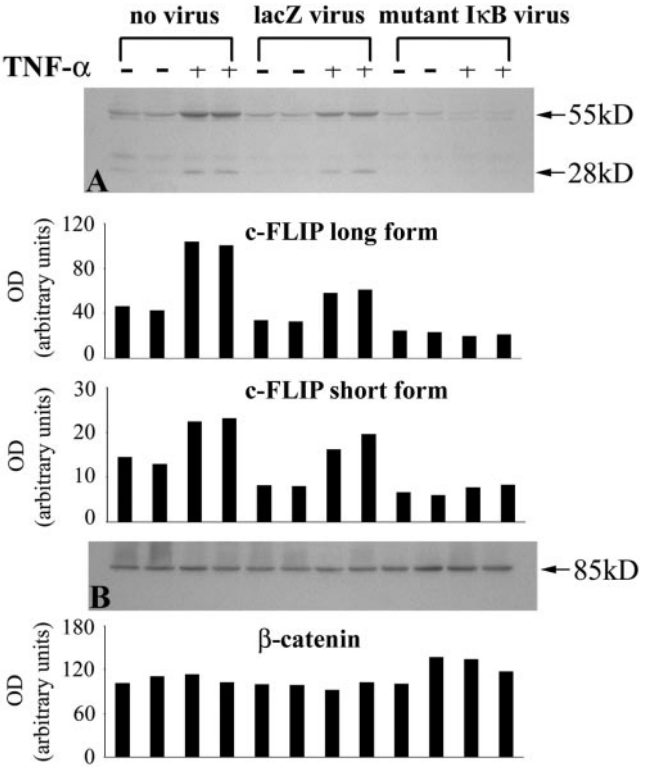

FIGURE 9. Blockade of c-FLIP protein expression by mutant IκB overexpression. RPE cells in duplicate wells were infected with adenovirus containing LacZ or mutant IκB. One day after infection, cells were treated with medium alone or TNF-α (1.1×10^3 U/mL) for 6 hours, and cytoplasmic proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. (**A**) Western blot probed with antibody to c-FLIP. Bands at 55 kDa correspond to the long c-FLIP form and bands at 28 kDa to the short c-FLIP form. The relative quantity of long and short c-FLIP protein forms, determined by densitometry, is shown separately *below* each lane. (**B**) Blot in (**A**) stripped and reprobed with antibody to β-catenin, a control for gel loading. The relative quantity of β-catenin protein is shown *below* each lane.

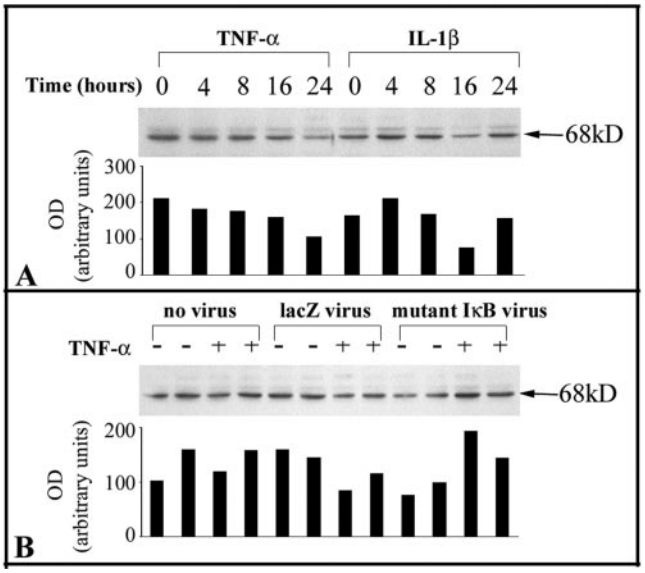

FIGURE 10. c-IAP1 protein expression after NF-κB blockade. Noninfected RPE cells were treated with TNF-α (1.1×10^3 U/mL) or IL-1β (5 U/mL) for various times. In addition, RPE cells in duplicate wells were infected with adenovirus containing LacZ or mutant IκB. One day after infection, cells were treated with medium alone, or TNF-α (1.1×10^3 U/mL). Cytoplasmic proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. (**A**) Kinetics of the c-IAP1 expression after TNF-α or IL-1β treatment for various times. Bands at 68 kDa correspond to c-IAP1. The relative quantity of c-IAP1 proteins, determined by densitometry, is shown separately *below* each lane. (**B**) Effect of mutant IκB overexpression on TNF-α–mediated c-IAP1 expression. Cells were treated with TNF-α for 6 hours. Bands at 68 kDa correspond to c-IAP1. The relative quantity of c-IAP1 proteins, determined by densitometry, is shown separately *below* each lane.

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