The Importance of Bcl-x_L in the Survival of Human RPE Cells

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Purpose. In normal eyes and in diseases such as age-related macular degeneration (AMD) and proliferative vitreoretinopathy (PVR), retinal pigment epithelial (RPE) cell survival is critically important. Bcl-x_L has been shown to be among the most highly expressed survival factors in cultured human RPE cells. In the current study the effect of Bcl-x_L blockade on human RPE cell survival was determined under normal conditions and after induced oxidative stress.

Methods. Cultured human RPE cells from three different donors were transfected with modified, 2′-*O*-methoxyethoxy Bcl-x_L–mismatched control antisense oligonucleotides (ASOs), Bcl-x_L-specific ASOs, and Bcl-x_L splice switching oligonucleotides (SSOs), which shift the splicing pattern of Bcl-x pre-mRNA from Bcl-x_L into Bcl-x_S, a proapoptotic factor. RNA and protein were harvested at various time points after transfection. Bcl-x_L and Bcl-x_S mRNA transcript levels were analyzed using gene-specific primers with reverse transcription–polymerase chain reaction. Bcl-x_L protein levels were analyzed using Western blot. Cell viability was measured by WST-1 and lactate dehydrogenase (LDH) assays. The mode of cell death was determined with a cell death ELISA and an M30 assay. To study the effects of oxidative stress, the cells were stimulated after transfection with various concentrations of H_2O_2. Cell viability was analyzed by WST-1 (Roche, Indianapolis, IN) and LDH assays.

Results. After Bcl-x_L-specific ASO and SSO transfections, Bcl-x_L mRNA and protein levels were significantly reduced. Bcl-x_S levels were increased after transfection with SSO. By day 8 after plating, the cells transfected with Bcl-x_L-specific ASO had significantly decreased viability, which was further reduced by day 10. The SSO had an even more potent effect. Cell viability was reduced on day 4 after plating and by day 10, less than 10% of the cells were viable. Apoptotic cell death occurred as early as day 4 after plating. H_2O_2, used as a model oxidant, further enhanced cell death induced by Bcl-x_L-specific ASO and SSO.

Conclusions. Bcl-x_L plays an important role in human RPE cell survival under normal conditions and when cells are exposed to oxidative stress. Treatment strategies that enhance Bcl-x_L expression and/or prevent conversion of Bcl-x_L to Bcl-x_S may be useful in preventing RPE cell death in AMD. Treatments that reduce Bcl-x_L and enhance Bcl-x_S may be useful in inhibiting unwanted RPE cell proliferation in PVR. (*Invest Ophthalmol Vis Sci.* 2007;48:3846–3853) DOI:10.1167/iovs.06-1145

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in adults over the age of 65 in the United States and currently affects more than 1.75 million individuals.[1] The exact etiology of AMD is unknown and is probably multifactorial. However, it has been hypothesized that cumulative oxidative stress throughout life and associated RPE cell injury play an important role. In normal eyes, there is very little RPE cell turnover, and most RPE cells survive for an individual's lifetime. In geographic atrophy, an advanced form of AMD, RPE cells die by apoptotic and nonapoptotic mechanisms.[2,3] RPE cell death is accompanied by underlying choriocapillaris atrophy and overlying retinal thinning, ultimately resulting in decreased visual acuity.[4]

Proliferative vitreoretinopathy (PVR), is the leading cause of retinal detachment surgery failure and is a potentially blinding condition. Unlike geographic atrophy in AMD, this condition is characterized by pathologic survival of RPE cells that migrate and proliferate onto the retinal surface and undersurface and into the vitreous cavity.[5,6] The factors that lead to long-term RPE cell survival in normal eyes, to RPE cell demise in conditions such as geographic atrophy and to pathologic RPE cell survival in diseases such as PVR are not well understood.

In our laboratory, we have examined the effect of nuclear transcription factor (NF)-κB inhibition on tumor necrosis factor (TNF)-α–induced apoptosis in human RPE cells.[7] NF-κB is a transcription factor that controls a wide range of genes, including genes that regulate apoptosis.[8] TNF-α is a cytokine that regulates a variety of RPE cell activities, including cell attachment, spreading, chemotaxis, migration, and proliferation.[9,10] In many cells, TNF-α induces apoptosis, particularly when NF-κB is blocked.[11] However, we found that RPE cells are resistant to TNF-α–induced apoptosis, even after NF-κB expression is specifically blocked.[12] These data led us to hypothesize that specific survival proteins protect RPE cells from apoptosis in an NF-κB-independent manner.

Bcl-2 and Bcl-x_L are key antiapoptotic members of the Bcl-2 family that regulate the intrinsic apoptosis pathway.[13] Specifically, high Bcl-x_L levels are expressed in RPE cells in vitro and in situ.[14] It has been shown that the stimulation of de novo Bcl-x_L is an essential mediator of fibroblast growth factor-2 (FGF2) survival signaling in bovine RPE cells.[15] Recently, we have shown that Bcl-x_L is the most highly expressed survival factor in situ among 10 tested. In the present study, we sought to understand the factors that regulate RPE cell survival in an in vitro model. We hypothesized that Bcl-x_L is a key protein that promotes RPE cell survival normally and under conditions of oxidative stress.

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TABLE 1. Primers for qRT-PCR

Gene	Sequence (5′–3′)
GAPDH	F CTG GCA TTG CCC TCA ACG ACC
	R CTT GCT GGG GCT GGT GGT CC
Bcl-x_L	F GCA GGT ATT GGT GAG TCG GAT CGC
	R CAC AAA AGT ATC CCA GCC GCC G

F, forward; R, reverse.

MATERIALS AND METHODS

RPE 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.[16] Retinal pigment epithelial cells from three different donors without known eye diseases, ages 61, 7, and 41 years, respectively, were used for these experiments. 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.

Antisense Oligonucleotide Transfection

RPE cells were seeded in 6-well plates (1.5×10^5 cells), 8-chamber glass slides (5×10^4 cells), or 96-well plates (10×10^3 cells; Costar; Corning Inc., Corning, NY) on day 0, and were allowed to grow for 48 hours in MEM with 10% FBS. On day 2 after plating, the cells were transiently transfected by a liposome-mediated method. The cells were washed twice with serum-free medium (OPTI-MEM; Invitrogen) and then transfected with three different modified 2′-*O*-methoxyethoxy (MOE) antisense oligonucleotides (ASOs): Bcl-x_L specific (**TCCCG**GTGCTCTGA[b]GACAT, ISIS 15999; ISIS Pharmaceuticals, Inc., Carlsbad, CA; bold-MOE residues, all internucleotide bonds are phosphorothioate), Bcl-x_L mismatched control (**CCTCG**ATTCCTGTTA[b]GAGAG, ISIS 355092), or splice switching oligonucleotide (SSO) (**TGGTTCTTACCCAGCCGCCG,** ISIS 105751; 150 or 300 μM) with lipofectin (0.006 mg/mL; Invitrogen).[17,18] The Bcl-x_L-specific ASO has a complementary sequence to Bcl-x_L mRNA. On binding to the target sequence, the RNA-DNA heteroduplex forms a substrate for RNase H, which degrades the RNA component.[19] Bcl-x_L SSO, spanning the proximal 5′ splice site of Bcl-x pre-mRNA of intron 2, does not promote RNase H cleavage and shifts the Bcl-x pre-mRNA splicing pattern from Bcl-x_L to Bcl-x_S, a proapoptotic Bcl-2 family member.[20] On day 3 after plating, after a 24-hour incubation, medium containing ASO or SSO was removed, and fresh serum-free MEM was added. The cells were allowed to recover for an additional 24 hours before experiments were conducted. In this study, experiments were generally conducted starting on day 4 after plating. Unless otherwise specified, the incubation period for the ASOs was 24 hours.

RNA Extraction and Amplification by Real-Time Quantitative PCR and by RT-PCR

Total RNA was isolated and analyzed using real-time quantitative (q) reverse transcription–polymerase chain reaction (RT-PCR) with gene-specific primer pairs (Table 1) on days 4 and 6 after plating as previously described.[14] Each qRT-PCR measurement of Bcl-x_L mRNA expression was performed with duplicate samples collected from two separate wells (iCycler IQ; Bio-Rad, Richmond, CA). Real-time quantification of *Bcl-x_L* gene expression was normalized to the threshold cycle (C_T) of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) where C_T equals the PCR cycle number at which the relative fluorescence units (RFUs) of the amplified product reaches a threshold set at 100. Gene expression of Bcl-x_L in ASO-transfected samples was calculated relative to expression of lipofectin-transfected group: $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = [(C_{T,target} - C_{T,GAPDH})_{ASO\text{-}transfected} - (C_{T,target} - C_{T,GAPDH})_{lipofectin\text{-}transfected}]$.[21] Because PCR product identity was confirmed by both a single melting-temperature peak per primer pair and 2% agarose gel analysis in the previous study,[14] only a melting curve for all products was obtained immediately after amplification in the present study. RT-PCR was performed and products were analyzed after 4-hour incubation with ASO, as previously described.[22] The primers used detected Bcl-x_L and Bcl-x_S as two separate bands, to allow quantification.

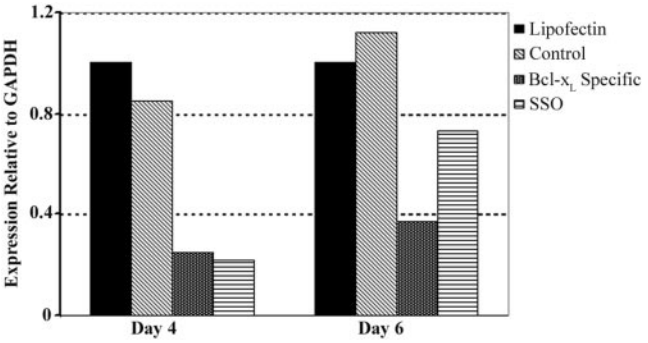

FIGURE 1. Reduced Bcl-x_L mRNA levels in RPE cells after transfection with Bcl-x_L-specific ASO and SSO (300 nM). mRNA was collected in duplicate samples at days 4 and 6 after plating and analyzed by qRT-PCR.

Protein Extracts and Western Blot

At days 4, 6, 8, and 10 after plating, both floating and adherent cells in duplicate wells were collected for protein extraction by lysing cells in RIPA buffer (50 mM Tris-HCl [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). Protein concentration of the cell extracts was measured by Bradford assay (Bio-Rad). An equal amount of protein (30 μg) was electrophoresed and transferred to a nitrocellulose membrane (Bio-Rad) by a mini-transblot apparatus (Bio-Rad), as previously described.[7] Briefly, the membranes were incubated overnight with rabbit polyclonal antibody directed against Bcl-x_L (1:1000 in 2.5% milk; Cell Signaling, Danvers, MA) and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 in 2.5% milk; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature for 60 minutes. Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) fluorescence detection kit (GE Healthcare, Piscataway, NJ).

Cell Viability Detection

The number of surviving cells was measured by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Roche, Indianapolis, IN) in 96-well plates in five replicates on days 4, 6, 8, and 10 after plating. The dye absorbance was measured at 440 nm with a reference wavelength of 690 nm. At day 8 after plating, the viability of RPE cells treated with 150 nM ASO did not differ

TABLE 2. Levels of Bcl-x_L mRNA* in Cells after Transfection on Postplating Day 4 in Representative Experiments on Each Cell Line

Cell Line	Lipofectin Alone	Control-Transfected	Bcl-x_L-Transfected	SSO-Transfected
1	1.00	0.85	0.25	0.22
2	1.00	1.74	0.44	0.47
3	1.00	1.15	0.57	0.93

* Expressed as average change in expression (*x*-fold) of Bcl-x_L relative to GAPDH, analyzed by real-time qRT-PCR.

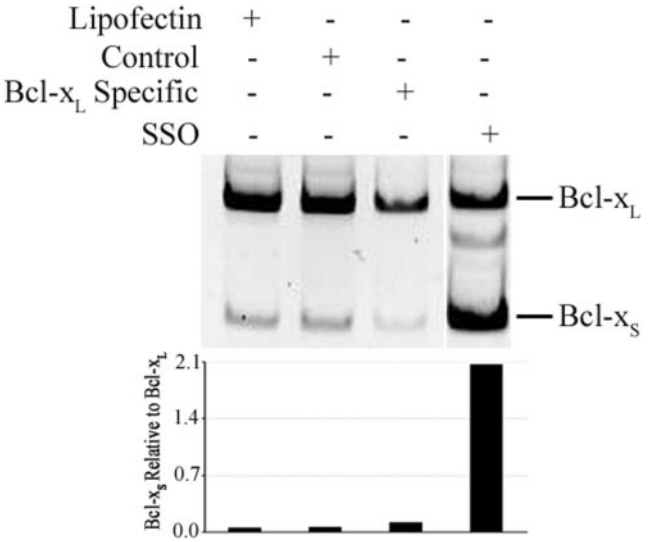

FIGURE 2. Increased Bcl-x_S mRNA levels in RPE cells after transfection with SSO. mRNA was collected on postplating day 4 after 4-hour transfection with 300 nM SSO and analyzed by RT-PCR. The primers used detected both Bcl-x_S and Bcl-x_L as two separate bands on the gel. Levels of Bcl-x_S mRNA in human RPE cells relative to levels of Bcl-x_L mRNA are shown below each lane.

significantly from those treated with 300 nM ASO. Additional WST-1 assays were conducted with 150 nM ASO.

Cell Death Detection

Lactate dehydrogenase (LDH) was measured as an indicator of cell death by using a cytotoxicity detection kit (Roche), as previously described.[23] Briefly, cell medium was collected from lipofectin-, control oligonucleotide–, Bcl-x_L-specific ASO-, and SSO-transfected cells 4 to 10 days after plating. Cells treated with lipofectin alone served as the "low" control. LDH activity was measured in a 96-well plate with five replicates in each group at an absorbance of 490 nm with a reference wavelength at 690 nm. As shown below, cell death did not differ significantly in 150 and 300 nM control oligonucleotide–, Bcl-x_L-specific ASO-, and SSO-transfected groups on day 4 after plating. Additional LDH assays were conducted with 150 nM ASO.

Detection of DNA Fragmentation

DNA fragmentation was measured as an indicator of apoptosis. On day 4 after plating, cells in triplicate wells were harvested using the lysis buffer provided in the DNA fragmentation kit and DNA fragmentation activity was quantified by enzyme-linked immunosorbent assay (ELISA; DNA fragmentation kit, Roche) according to the manufacturer's instructions.

Immunofluorescent Detection of M30

In addition to the DNA fragmentation assay, an M30 assay was performed to detect RPE cell apoptosis in cells, as previously described,[23] at days 3 and 4 after plating with lipofectin, control oligonucleotides, Bcl-x_L-specific ASO, and SSOs. Fluorescent stain was observed with a light microscope with an epifluorescence attachment. A masked observer determined the number of cells with M30-positive stain, of the total number stained with 4′,6′-diamino-2-phenylindole (DAPI), in three microscopic fields, each containing approximately 300 cells, for each treatment group plated in triplicate wells.

H_2O_2 Treatments

To establish a sublethal H_2O_2 concentration, cells in triplicate wells were transfected with lipofectin only, control oligonucleotides, Bcl-x_L-specific ASO, or SSO in 96-well plates. On day 4 after plating, H_2O_2 was added to the wells in concentrations varying from 50 to 150 μM. After an 8-hour incubation, H_2O_2 was removed, and WST-1 reagent was added. After a 2-hour incubation, the plates were assayed as described for immunofluorescence. As shown later, 50 μM H_2O_2 was found to be a sublethal concentration. The cells in five replicate wells were treated with 50 μM H_2O_2 for 18 hours on day 4 after plating. The medium was removed at the end of stimulation and assayed for LDH activity as described earlier. Three independent experiments were performed on the cells in five replicate wells, with reproducible results.

Statistical Analysis

Experiments were performed on the cells in triplicate or five replicate wells (as indicated), and data are expressed as the mean ± SD. The Student's *t*-test was used to determine whether there were statistically

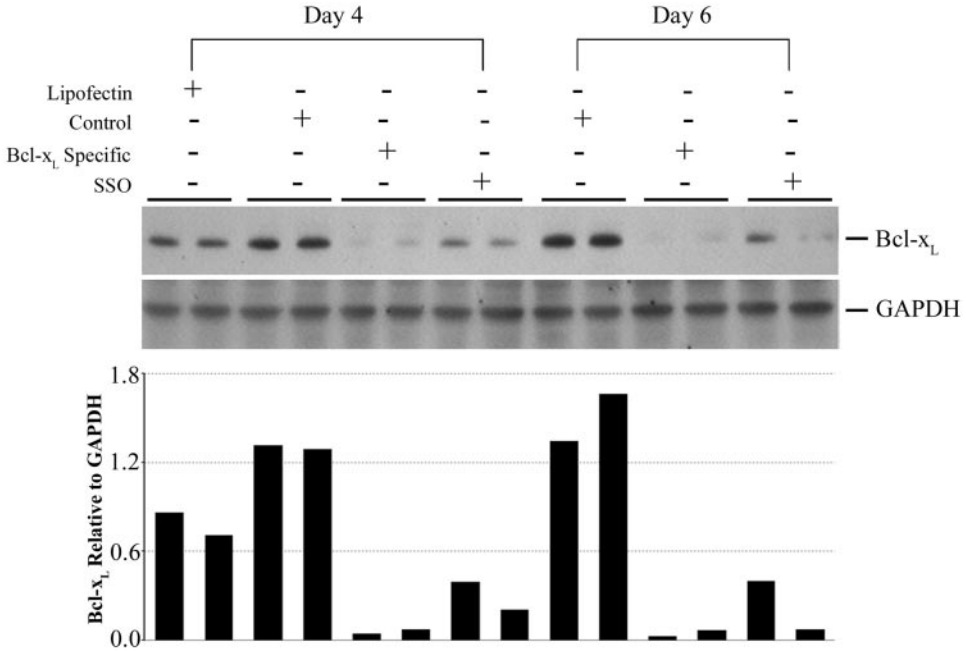

FIGURE 3. Reduced Bcl-x_L protein levels in RPE cells transfected with Bcl-x_L-specific ASO and SSO. RPE cells were transfected with 300 nM Bcl-x_L-specific ASO and SSO. Cytoplasmic protein (30 μg) was collected from RPE cells in duplicate wells on days 4 and 6 after plating. *Top*: Western blot probed with antibody to Bcl-x_L. *Bottom*: blot shown at *top* was stripped and reprobed with antibody to GAPDH, a control for gel loading. Densitometry to determine the relative quantity of proteins in each lane was normalized to the densitometry of corresponding GAPDH proteins and is shown below each lane.

TABLE 3. Levels of Bcl-x_L Protein* in Cells after Transfection on Postplating Day 4 in Representative Experiments from Each Cell Line

	Lipofectin Alone		Control-Transfected		Bcl-x_L-Transfected		SSO-Transfected	
Cell Line	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	0.86	0.71	1.32	1.29	0.04	0.07	0.39	0.20
2	0.49	0.50	0.76	0.90	0.11	0.06	0.09	0.08
3	0.67	1.06	0.84	1.41	0.08	0.11	0.20	0.33

* Expressed as densitometry of Bcl-x_L relative to densitometry of GAPDH and displayed in duplicate for each group.

significant differences between treatment and control groups, analyzed by cell death assessment, DNA fragmentation assay, and M30 assay. $P < 0.05$ was considered to be statistically significant. Three separate experiments were conducted to show that transfection with Bcl-x_L-specific ASO and SSO decreased Bcl-x_L mRNA and protein levels. The RT-PCR experiment to show Bcl-x_S levels after a 4-hour transfection with SSO was performed once. Three independent experiments were also performed to demonstrate the effect of Bcl-x_L-specific ASO and SSO on cell death. The DNA fragmentation assay was performed on cells in triplicate wells and was repeated twice. The M30 assay was repeated four times. All the above experiments were conducted on cells from donor 1 (61 years old). The following key experiments were repeated on cells from donors 2 and 3: qRT-PCR and Western blot analysis, to measure Bcl-x_L mRNA and protein levels, respectively; LDH assay and DNA fragmentation ELISA to measure necrotic and late apoptotic cell death and apoptosis, respectively, after ASO and SSO treatment.

RESULTS

Inhibition of Bcl-x_L mRNA and Protein Expression by Bcl-x_L ASOs

After transfection with Bcl-x_L-specific ASO and SSO, Bcl-x_L mRNA expression and protein levels were measured. In cells that were transfected with Bcl-x_L-specific ASO, steady state Bcl-x_L mRNA levels measured by qRT-PCR were reduced by 78% and 60%, respectively, compared with lipofectin- and control-transfected cells on day 4 after plating. This inhibitory effect lasted until day 6 after plating. In SSO-transfected cells, on day 4 after plating, Bcl-x_L mRNA levels were reduced by 79% and 63% respectively, when compared with lipofectin- and control-transfected cells; however, only a 27% to 39% reduction was present on day 6 after plating (Fig. 1). Similar reduction of Bcl-x_L mRNA levels in Bcl-x_L-specific ASO- and SSO-transfected cells on day 4 after plating were obtained in cells from donors 2 and 3 (Table 2). The Bcl-x_S mRNA levels in SSO-transfected cells detected by RT-PCR were increased by 61%, compared with lipofectin- and control-transfected cells on day 4 after plating. In contrast, Bcl-x_S mRNA levels in cells that were transfected with the Bcl-x_L-specific ASO were increased by only 5%, compared with mRNA levels in cells transfected with lipofectin and control ASO (Fig. 2). We next examined Bcl-x_L protein expression levels by Western blot analysis. After Bcl-x_L-specific ASO and SSO transfection, Bcl-x_L protein levels were significantly decreased as early as day 4 after plating, and this inhibition was sustained until day 6 (Fig. 3). On days 8 and 10, Bcl-x_L protein levels were still lower than that in control-transfected cells, though higher than on days 4 and 6 (not shown). Bcl-x_L protein levels decreased similarly in cells from donors 2 and 3 that were transfected with Bcl-x_L-specific ASO and SSO on day 4 after plating (Table 3).

Effect of Inhibition of Bcl-x_L Expression on Cell Viability and Cell Death

After experiments to show that both Bcl-x_L-specific ASOs and SSOs reduced the expression of Bcl-x_L in cultured human RPE cells, we determined the effect of reduced Bcl-x_L on RPE cell viability. On postplating day 4, after Bcl-x_L-specific ASO and SSO transfection, RPE cells were rounded, detached from the plates, and floated in the medium, an effect that was more conspicuous in the SSO-transfected groups. A WST-1 colorimetric assay was used to quantify this effect. Starting on day 8 after plating, the cells transfected with both Bcl-x_L-specific ASO and SSO had significantly reduced cell viability compared with control-transfected cells (Fig. 4). This effect lasted until day 10 ($P < 0.001$; not shown).

An LDH assay was next used to quantify the amount of late apoptotic and necrotic cell death. Transfection with Bcl-x_L-specific ASO and, especially, SSO induced significantly more cell death compared with control-transfected cells starting from day 4 after plating (Fig. 5), an effect that persisted until day 10 ($P < 0.001$; not shown). The LDH assay was repeated on cells from donors 2 and 3, and similar results were observed (Table 4).

Apoptotic Cell Death after Bcl-x_L Blockade

To investigate the mode of cell death after Bcl-x_L levels are reduced, we examined DNA fragmentation in RPE cells. As soon as day 4 after plating, significant DNA fragmentation was detected in cells transfected with Bcl-x_L-specific ASO and SSO, indicating that reduced Bcl-x_L levels were associated with apoptotic cell death, an effect that was most pronounced after SSO transfection (Fig. 6). In cells from donor 3 that were transfected with Bcl-x_L-specific ASO and SSO, the number of

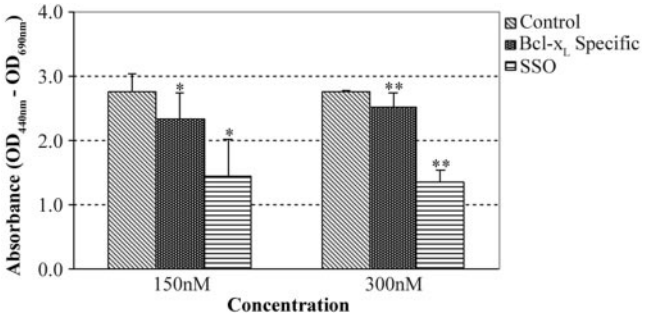

FIGURE 4. Reduced viability of RPE cells treated with Bcl-x_L-specific ASO and SSO on day 8 after plating. RPE cells in five replicate wells were transfected with control oligonucleotides, Bcl-x_L-specific ASO, or SSO at two different concentrations, 150 and 300 nM. Results are expressed as the mean ± SD. *$P < 0.05$ versus control-transfected cells at 150 nM. **$P < 0.05$ versus control-transfected cells at 300 nM. Cell viability was the same, whether cells were transfected with 150 or 300 nM oligonucleotides.

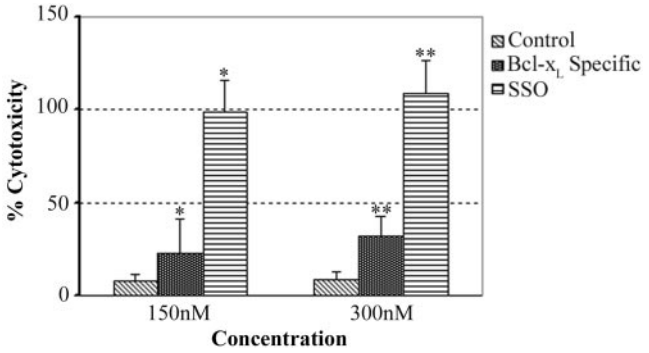

FIGURE 5. Increased death of Bcl-x_L-specific ASO- and SSO-treated RPE cells on day 4 after plating. RPE cells in five replicate wells were transfected with lipofectin (served as low control), control oligonucleotides, Bcl-x_L-specific ASO, or SSO (150 and 300 nM). Results are expressed as percentage of cells showing cytotoxicity ± SD. Percent cytotoxicity = (experiment − low control)/(high control − low control) × 100%. $^*P < 0.05$ versus control-transfected cells at 150 nM. $^{**}P < 0.0001$ versus control-transfected cells at 300 nM. Cell death was the same, whether the cells were transfected with 150 or 300 nM oligonucleotides.

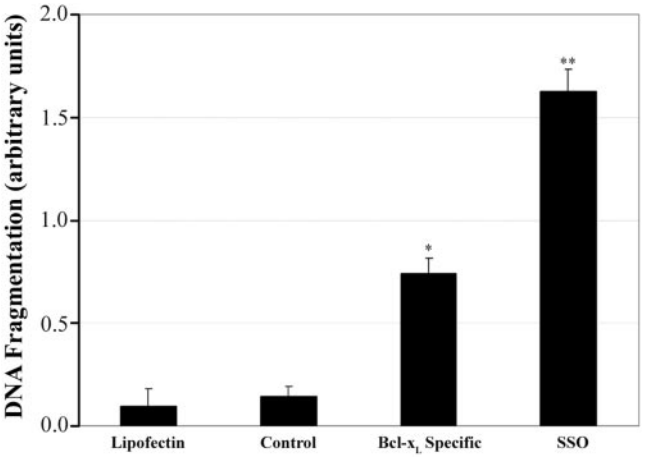

FIGURE 6. Increased DNA fragmentation in cells transfected with 300 nM Bcl-x_L-specific ASO or SSO. DNA fragmentation was measured on day 4 after plating using RPE cells in triplicate wells. Results are expressed as the mean ± SD $^*P < 0.0001$ versus control-transfected cells. $^{**}P < 0.00001$ versus control-transfected cells.

apoptotic cells was significantly increased compared with cells transfected with control oligonucleotides (Table 5). In cells from donor 2, there was significantly increased apoptosis in SSO-transfected cells compared with control-transfected cells. We also observed increased apoptosis in Bcl-x_L-specific ASO-transfected cells, although the difference was not statistically significant (Table 5).

To further confirm apoptotic RPE cell death, an M30 assay was used to measure cytokeratin cleavage as an indicator of early apoptosis. We stained cells for this early apoptotic event at two different time points, days 4 and 5 after plating. There was a greater than twofold increase in the percentage of M30-positive cells after Bcl-x_L-specific ASO transfection ($P = 0.06$) and a significantly higher percentage of M30-positive cells after SSO-transfection relative to control-transfected cells (Fig. 7).

H_2O_2 Enhancement of Cell Killing after Bcl-x_L Blockade

After RPE cells were treated for 8 hours with a sublethal H_2O_2 concentration (50 μM) on day 4 after plating, there was no significant difference in cell viability, measured by WST-1 assay, between the control- and Bcl-x_L-specific ASO-transfected cells ($P > 0.5$; not shown). However, after 8 hours of incubation with 100 or 150 μM of H_2O_2, Bcl-x_L-specific-ASO–transfected cells had significantly reduced cell viability compared with control-transfected cells ($P < 0.01$; not shown). Of importance, as measured by LDH assay, sublethal H_2O_2 treatment for 18 hours led to significantly increased death of Bcl-x_L-specific-ASO–transfected cells compared with control-transfected cells (Fig. 8).

DISCUSSION

In the present study, both types of ASOs, one that specifically targets Bcl-x_L and another that converts Bcl-x_L to Bcl-x_S by alternative splicing sites on pre-mRNA,[20] knocked down Bcl-x_L mRNA expression, and decreased Bcl-x_L protein levels in cultured human RPE cells. Reduced Bcl-x_L led to decreased cell viability and increased apoptotic and nonapoptotic cell death. Using H_2O_2 as a model oxidant, we demonstrated that after Bcl-x_L expression was blocked by Bcl-x_L-specific ASOs, an otherwise sublethal oxidant exposure induced significant cell death. The protective effect of Bcl-x_L on cultured RPE cells appears to be a general phenomenon; although the magnitude of the effects varied, the primary results were consistent across cells obtained from three different donors of various ages.

ASOs are widely used to investigate cell biological and molecular biological mechanisms and to identify potential therapeutic targets.[24,25] For example, in nonocular cells, ASO-mediated downregulation of Bcl-x_L expression enhances apoptosis and augments the response to chemotherapy.[26,27] We found that the Bcl-x_L-specific ASOs and SSOs targeted the RPE cell *Bcl-x$_L$* gene with high efficiency and specificity and thus downregulated the gene product. After transfection with SSO, the level of Bcl-x_L mRNA inhibition was less on day 6 than on day 4 after plating. We speculate that Bcl-x_L was upregulated in surviving cells, to account for these findings. With Bcl-x_L-

TABLE 4. Average Levels of Percent Cytotoxicity* in Cells after Transfection on Postplating Day 4 in Representative Experiments from Each Cell Line

Cell Line	Control-Transfected (± SD)	Bcl-x_L-Transfected (± SD)	*P*†	SSO-Transfected (± SD)	*P*‡
1	8.41 ± 3.26	22.74 ± 18.44	= 0.01	98.37 ± 18.06	<0.00001
2	3.90 ± 0.49	17.19 ± 3.00	<0.00001	39.49 ± 8.30	<0.00001
3	0.36 ± 2.04	13.17 ± 4.71	<0.001	21.22 ± 4.57	<0.00001

* Percentage of cytotoxicity measured by LDH release as a percentage of that obtained from fully lysed cells.
† *P* of percent cytotoxicity of Bcl-x_L-transfected cells versus that of control-transfected cells.
‡ *P* of percent cytotoxicity of SSO-transfected cells versus that of control-transfected cells.

TABLE 5. Average DNA Fragmentation* in Cells after Transfection on Postplating Day 4 in Representative Experiments from Each Cell Line

Cell Line	Control-Transfected (±SD)	Bcl-x_L-Transfected (±SD)	*P*†	SSO-Transfected (±SD)	*P*‡
1	0.15 ± 0.05	0.74 ± 0.08	<0.001	1.63 ± 0.11	<0.0001
2	0.36 ± 0.08	0.47 ± 0.10	=0.23	1.48 ± 0.09	<0.001
3	0.34 ± 0.04	0.78 ± 0.18	=0.01	1.28 ± 0.06	<0.0001

* Expressed as OD units ± SD.
† *P* of OD reading of Bcl-x_L-transfected cells versus OD reading of control-transfected cells.
‡ *P* of OD reading of SSO-transfected cells versus OD reading of control-transfected cells.

specific ASOs, significantly decreased RPE cell viability was not observed until 8 or 10 days after plating. In contrast, the effect of the SSOs on cell death was more rapid and profound. The splice switching ASOs target the Bcl-x pre-mRNA to shift the splice variant from Bcl-x_L, an antiapoptotic factor, to Bcl-x_S, a proapoptotic factor. It is thought that the relative balance of anti- and proapoptotic factors determine whether cells survive or die.[28] Therefore, by replacing antiapoptotic Bcl-x_L with proapoptotic Bcl-x_S, the splice-switching oligonucleotide shifts the balance toward death more than it does by downregulating Bcl-x_L alone.[20] Together, our data suggest that the Bcl-x_L-specific ASO is useful in the selective study of the mechanism and function of Bcl-x_L in RPE cells, whereas the SSO is more useful when the goal is to induce the maximum apoptotic cell death.

Bcl-x_L protects against apoptosis and necrosis in different cell types.[29,30] Similarly, we found that in cultured RPE cells, Bcl-x_L protects against both apoptosis and necrosis. Although apoptosis and necrosis are two distinctive types of cell death with differing morphologies, it is thought that apoptosis and some forms of necrosis share common steps.[31] For example, disruption of mitochondrial potential and volume homeostasis are thought to be key common steps in apoptosis and necrosis.[32] Bcl-x_L generally promotes cell survival by regulating the electrical and osmotic homeostasis of mitochondria.[32] Bcl-x_L may form small ion channels that assume a mostly closed conformation, selective for passage of cations, thereby protecting mitochondria from osmotic damage.[33] In addition, Bcl-x_L predominantly localizes at the outer mitochondrial membrane and inhibits the release of cytochrome *c*, obstructing the downstream events that lead to apoptosis, such as Apaf-1-mediated caspase-9 and -3 activation. Therefore, by protecting the outer mitochondrial membrane's integrity, Bcl-x_L promotes cell survival.[34] Other proposed mechanisms of the antiapoptotic property of Bcl-x_L include suppression of death-inducing signal complex (DISC) formation and associated caspase-8 activation.[35] The exact mechanism(s) by which Bcl-x_L protects against RPE cell apoptotic and necrotic death is not known. Additional studies to resolve this question are under way in our laboratory.

The relative importance of Bcl-x_L in cell survival is cell-type specific and depends on the activation state of the cell. We have shown that Bcl-x_L is the most highly expressed RPE cell survival protein in situ, among 10 that were evaluated, and is constitutively expressed by cultured RPE cells.[14] The data from the current report demonstrate that constitutively expressed Bcl-x_L is a key survival protein in cultured RPE cells grown in basal media and those exposed to oxidative insult. Similarly, Bcl-x_L is a crucial apoptosis regulator in mouse macrophages.[36] In contrast, in T cells, Bcl-2, but not Bcl-x_L, is constitutively expressed under basal conditions. Bcl-x_L does not become an important T-cell survival protein until the cells are activated by CD28.[37]

The pathogenesis of AMD is not yet well understood, although oxidative stress is thought to play an important role.[38–40] RPE cells are normally exposed to higher levels of oxidative stress than are many other types of cells: in addition to age-related oxidation, RPE cells are constantly under the burden of reactive-oxygen intermediates generated by phagocytosis of photoreceptors and the resultant lipid peroxidation of polyunsaturated fatty acids. RPE cells are subjected to oxidative stress throughout an individual's lifetime and yet have a very low turnover rate,[41] suggesting potent prosurvival protective mechanisms. We hypothesized that these protective mechanisms include survival proteins such as Bcl-x_L, in addition to known endogenous enzymes and chemical antioxidants. We found that Bcl-x_L exerts a protective effect on RPE cells ex-

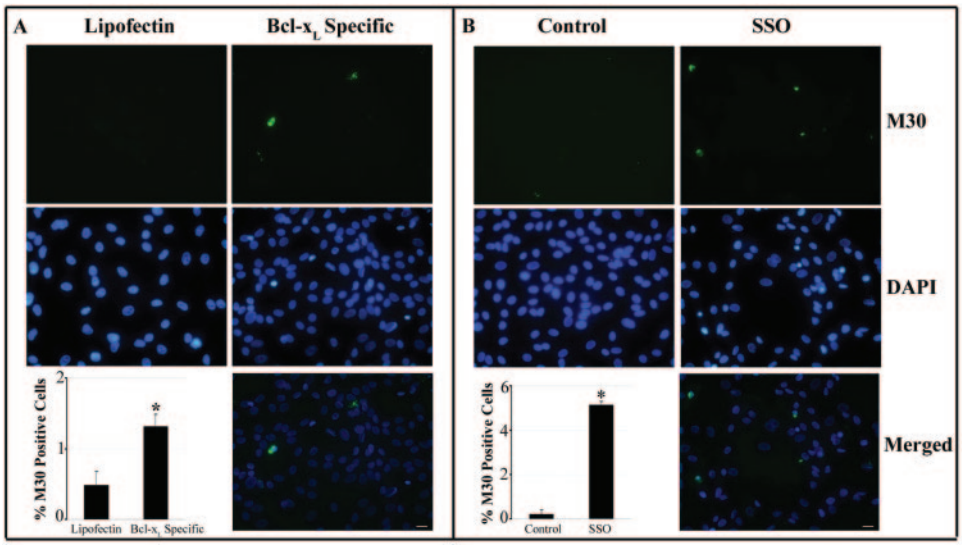

FIGURE 7. Increased apoptosis after Bcl-x_L-specific ASO and SSO transfection. RPE cells in triplicate wells were transfected with lipofectin, 300 nM control oligonucleotides, Bcl-x_L-specific ASO, or SSO. Apoptotic cells appeared *green* when stained with M30 antibody (*top*). Nuclei were stained with DAPI (*middle*). M30-positive cells were expressed as mean ± SD. Bar = 10 μm. (**A**) On day 4 after plating, cells in the lipofectin- and Bcl-x_L-specific ASO-transfected groups were immunostained with antibody against M30, to detect apoptotic cells. **P* = 0.06 versus lipofectin-transfected cells. (**B**) On day 3 after plating, cells in control- and SSO-transfected groups were immunostained using identical procedures as in (**A**). **P* < 0.0001 versus control-transfected cells.

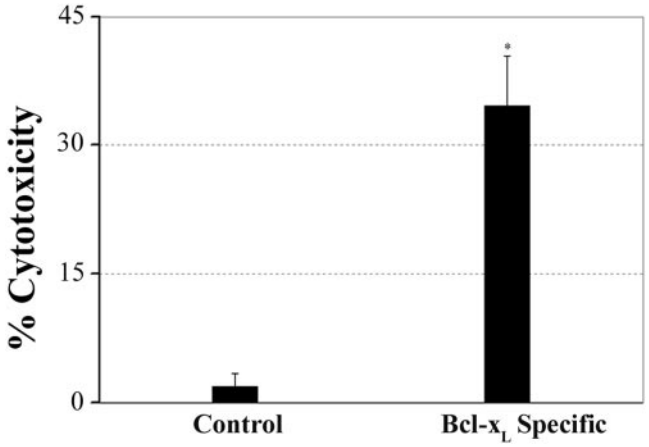

FIGURE 8. Effect of sublethal H_2O_2 on cell death after Bcl-x_L-specific ASO transfection. RPE cells in five replicate wells were transfected with lipofectin (used as low control), control, or Bcl-x_L-specific ASO (150 nM). On day 4 after plating, cells were treated with 50 μM of H_2O_2 for 18 hours. Culture medium was then removed from wells and LDH assay reaction mixtures were added (100 μL reaction mixture to 100 μL medium). Samples were assayed after a 30-minute incubation. Results are expressed as the percentage of cells showing cytotoxicity ± SD. Percent cytotoxicity = (experiment − low control)/(high control − low control) × 100%. *$P < 0.00001$ versus control-transfected cells.

posed to H_2O_2 used as a model oxidant. H_2O_2 impairs mitochondrial respiratory function and preferentially damages mitochondrial DNA.[42] We and others have shown that H_2O_2 induces RPE cell intracellular reactive oxygen intermediate (ROI) accumulation.[23] However, in other cells, it has been shown that Bcl-x_L protects cells from oxidative stress by regulating mitochondrial functions and not by scavenging exogenous ROI.[43] We speculate that Bcl-x_L similarly protects RPE cells from oxidant injury by preserving mitochondrial integrity.

In summary, Bcl-x_L plays an important role in cultured human RPE cell survival normally and when cells are exposed to oxidative stress. Clearly, one cannot directly extrapolate our in vitro experiments to in vivo physiology or pathology. However, based on our data, we hypothesize that decreased Bcl-x_L and a shift in the balance toward proapoptotic factors may contribute to RPE cell death in diseases such as AMD. If this holds true in vivo, treatment strategies that promote expression of Bcl-x_L and prevent conversion of Bcl-x_L to Bcl-x_S may be useful to prevent RPE cell death normally and in AMD. Treatments that reduce Bcl-x_L and enhance Bcl-x_S may be useful methods to inhibit unwanted RPE cell proliferation in diseases such as proliferative vitreoretinopathy. Experiments to test these hypotheses are currently under way in our laboratory.

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