Published in final edited form as:

Apoptosis. 2009 January ; 14(1): 31–41. doi:10.1007/s10495-008-0285-7.

Involvement of c-Abl, p53 and the MAP kinase JNK in the cell death program initiated in A2E-laden ARPE-19 cells by exposure to blue light

Barbro S. Westlund, Bolin Cai, Jilin Zhou, and Janet R. Sparrow

Department of Ophthalmology, Columbia University, 630 W. 168th Street, New York, NY 10032, USA

Janet R. Sparrow: jrs88@columbia.edu

Abstract

The lipofuscin fluorophore A2E has been shown to mediate blue light-induced damage to retinal pigmented epithelial (RPE) cells. To understand the events that lead to RPE cell apoptosis under these conditions, we explored signaling pathways upstream of the cell death program. Human RPE cells (ARPE-19) that had accumulated A2E were exposed to blue light to induce apoptosis and the involvement of the transcription factors p53 and c-Abl and the mitogen activated protein kinases p38 and JNK were examined. We found that A2E/blue light caused upregulation and phosphorylation of c-Abl, and upregulation of p53. Pretreatment with the c-Abl inhibitor STI571 and transfection with siRNA specific to c-Abl and p53 prior to irradiation reduced A2E/blue light-induced cell death. Gene and protein expression of JNK and p38 was upregulated in response to A2E/blue light. Treatment with the JNK inhibitor SP600125 before irradiation resulted in increase in cell death whereas inhibition of p38 with SB203580 had no effect. This study indicates that c-Abl and p53 are important for execution of the cell death program initiated in A2E-laden RPE cells exposed to blue light, while JNK might play an anti-apoptotic role.

Keywords

Age-related macular degeneration; RPE; A2E; c-Abl; p53; JNK

Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible legal blindness in the developed world [1]. AMD results in progressive loss of central vision due to degenerative and neovascular changes in the macula region of the retina. The pathology of AMD is still poorly understood, but it is generally accepted that it starts with death of the retinal pigmented epithelium (RPE) followed by degeneration of the photoreceptor cells.

Studies indicate that the accumulation of lipofuscin in the RPE cells contributes to development of AMD [2, 3]. The lipofuscin of RPE cells is unusual in that precursors of this material originate in photoreceptor cells' outer segments and are deposited in the RPE through the process of outer segment shedding and phagocytosis [4, 5]. RPE lipofuscin accumulates in membrane-bound organelles of the lysosomal compartment, these lipofuscin-laden organelles being referred to as lipofuscin granules because of their characteristic

microscopic appearance. The fluorophore A2E is a major constituent of RPE lipofuscin that forms via a biosynthetic pathway involving reactions between all-trans-retinal and phosphatidylethanolamine and formation of the intermediate dihydro-A2PE (A2PE-H₂; absorbance maxima: 490 and 330 nm) that undergoes oxidation to generate the immediate precursor A2PE (absorbance maxima: 449 and 342 nm). Phosphate hydrolysis of A2PE within lysosomes releases A2E. A2E is a bisretinoid molecule, that acts as a photosensitizer generating singlet oxygen and superoxide anion upon irradiation with blue light (430 nm) [6, 7]. The singlet oxygen reacts with the carbon-carbon double bonds along the retinoid derived side-arms of A2E and the resulting photooxidized forms of A2E are highly reactive and likely contribute to RPE cell damage and death. We have shown, that the photochemical events initiated by irradiation of A2E can lead to modification of DNA and protein [8, 9]. We have previously reported, that upregulation of Bcl-2 protects against A2E/blue light-induced RPE cell death and that the cell death program involves activation of caspase-3. Moreover, inhibiting caspase-3 can reduce apoptosis by approximately 50% [10]. To uncover additional upstream signaling pathways we explored the roles of c-Abl, p53 and the stress kinase pathway.

c-Abl is a ubiquitously expressed non-receptor tyrosine kinase that is localized at several subcellular sites, including; the plasma membrane, cytoplasm, nucleus, mitochondria and the endoplasmic reticulum. c-Abl activity has been implicated in the regulation of various responses, such as cell differentiation, division, adhesion, and survival [11] as well as cell cycle arrest [12, 13], and induction of apoptosis [14, 15].

The regulation of c-Abl is complex and is still not fully elucidated. c-Abl can be activated in many ways: by phosphorylation, by a change in the molecular structure (displacement of Myristate, or of the SH2- and the SH3 domains), and by interaction with other proteins [16]. The activation of c-Abl is highly regulated and c-Abl is autoinhibited under normal conditions [16, 17]. A variety of different stimuli can cause autophosphorylation of c-Abl, and it appears that autophosphorylation occurs in a stepwise fashion, with phosphorylation at Y412 followed by phosphorylation at Y245. Hantschel et al. [18] have shown that phosphorylation at Y412 and Y245 together account for most of the positive regulatory action of autophosphorylation on c-Abl catalytic activity.

In several cell types including human RPE [19, 20], DNA damage induces apoptosis by mechanisms that require p53 transcription factor, and c-Abl induced apoptosis is reported to be executed by p53-dependent as well as p53-independent mechanisms [21–23]. In large part, p53-initiated apoptosis appears to proceed by way of mitochondria; in particular p53 activates genes encoding mitochondrial proteins of the Bcl-2 family such as Bax [24, 25].

The mitogen-activated protein kinases (MAPK) are part of a phosphorylation cascade that transduces extracellular signals to cytoplasmic or nuclear targets. The MAPK signaling pathways have varying and even opposite functions. For instance, MAPKs can both stimulate and arrest cell proliferation and can be either pro- or anti-apoptotic depending on the stimulus- and cell-type [26, 27]. The MAPK superfamily consists of three major signaling pathways: the extracellular signal-related protein kinases (ERKs), the c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK) and the p38 family of kinases [28]. MAPKs are activated upon dual phosphorylation on threonine and tyrosine residues [29]. ERKs are activated in response to growth stimuli, serum and phorbol esters [28], whereas JNK and p38 are activated by cellular and environmental stresses, e.g., changes in osmolality or metabolism, heat shock, ischemia, pro-inflammatory agents, UV irradiation, DNA damage, and oxidative stress [27, 30–35].

Given that A2E-photooxidation can induce DNA damage and oxidative stress in RPE cells and that c-Abl, p53, JNK and p38 are all known to participate in the cell's response to these perturbations, we sought to evaluate the involvement of c-Abl, p53, JNK and p38 in the cell death program that is initiated in RPE cells that have accumulated A2E and are exposed to blue light.

Materials and methods

Reagents

The c-Abl inhibitor STI571 was a gift from Novartis, the specific JNK inhibitor SP600125 was purchased from Biosource, Camarillo, CA and the specific p38 inhibitor, SB203580 from Alexis Biochemicals, San Diego, CA. The following antibodies were used: p53 (Oncogene Research Products, Boston, MA), Bax (Trevigen, Gaithersburg, MD), c-Abl (BD Biosciences), c-Abl-phospho Y245 (Abcam, Cambridge, MA), β -actin (ab6276, Abcam, Inc., Cambridge, MA), p38 (Abcam, Inc., Cambridge, MA), and JNK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA). Small interfere RNA (siRNA) of c-Abl was purchased from Dharmacon Inc., Dallas, p53 siRNA plasmid from IM-GENEX Corporation, San Diego, CA and TransIT-TKO reagent from Mirus, Madison, WI. G418 sulfate was purchased from Gibco-Life Technologies.

A2E accumulation and irradiation

Cells were treated as previously described [7, 36, 37]. In short, A2E was synthesized from all-*trans*-retinal and ethanolamine (2:1) [38]. Human adult RPE cells (ARPE-19 American Type Culture Collection, Manassas, VA) lacking endogenous A2E [36] were grown to confluence in either 35 mm dishes or in 8-well slide chambers for at least 4 weeks. Subsequently, cells were incubated with synthesized A2E in cell culture media at a concentration of 10 μ M to allow A2E accumulation [7, 36, 37].

Culture medium was replaced with phosphate-buffered saline containing calcium, magnesium and glucose, and cells were irradiated with blue light (430 ± 30 nm) delivered to the entire area of a 35 mm dish (1 mW/cm²) or to a 8-well-chamber (8 mW/cm²).

Immunoblotting

A2E-laden ARPE-19 cells were irradiated with blue light for 12 or 20 min and incubated for 6 or 8 h. Cells were harvested and whole cell lysate was prepared using commercially obtained cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, and 1 μ g/ml leupeptin (Cell Signaling Technology, Beverly, MA). 1 mM PMSF was added to the lysis buffer prior to use.

For detection of c-Abl, p53 and Bax, the cell lysates were immuno-precipitated with antibodies to, respectively, p53, Bax, c-Abl, c-Abl phospho Y245, and β -actin using protein A/G-agarose matrix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Cell lysates were electrophoresed on 10% SDS-poly-acrylamide gels, and the proteins were electrotransferred onto nitrocellulose membranes (Novex Invitrogen, Carlsbad, CA). Antibody binding was visualized using reagents of the Vectorstain ABC kit (Vector, Burlingame, CA) and detection by ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ). Membranes were then stripped and reprobed with antibody to β -actin. The molecular mass of the protein bands was estimated by comparison to commercially obtained

protein standards (Cell Signaling Technology, Beverly, MA). Quantitative densitometry was performed using Molecular Dynamics Personal Densitometer and ImageQuant software.

To test the specificity of the c-Abl-phospho Y245 antibody to the c-Abl phosphorylation site, a peptide competition experiment was performed. Prior to use, the c-Abl-phospho Y245 antibody was pre-incubated with c-Abl phosphopeptide immunogen (Biosource, Camarillo, CA) in 1:16 ratio at room temperature for 30 min. A nonphosphopeptide was used as control. Three identical nitrocellulose strips blotted with protein immunoprecipitated by monoclonal antibody to c-Abl were probed with c-Abl-phospho Y245 antibody, or c-Abl-phospho Y245 antibody pre-incubated with specific c-Abl-phospho-peptide or nonspecific non-phospho-peptide.

Quantitative RT-PCR

A2E-laden ARPE-19 cells grown for a minimum of 4 weeks were irradiated at 430 nm (7 and 12 min) followed by a 3 or 5 h incubation. Control groups included untreated ARPE-19 cells and A2E-laden ARPE-19 cells that had not been exposed to blue light. Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantitative RT-PCR was performed on a LightCycler system (Roche Molecular Biochemicals, Indianapolis, IN) using the LightCycler RNA amplification SYBR Green I one-step kit, (Roche Diagnostics GmbH, Germany). Table 1 shows primer sequences used. Reactions were performed in duplicates. To account for differences in the amount of total RNA added to each reaction, the housekeeping genes β 2-microglobulin (β 2MG), human 18s rRNA (18s), and GAPDH was used as internal amplification controls. The levels of c-Abl mRNA were normalized to that of β 2MG, whereas the levels of p38 mRNA were normalized to the average of the housekeeping genes β 2MG, 18s, GAPDH, and that of JNK1 and JNK2 to 18s. PCR amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide to visualize single products of the appropriate size.

Treatment with inhibitors

Cells were treated with a specific inhibitor of c-Abl (STI571; 5, 10 and 20 μ M for 48 h) [39], and with specific inhibitors of JNK (SP600125; 0.2, 2, 10 and 20 μ M) and p38 (SB203580; 0.2, 2, 10 and 20 μ M) for 1 h [40, 41] prior to irradiation at 430 nm. Control samples included untreated cells, cells treated only with the inhibitor, and A2E-laden cells that had not been exposed to 430 nm.

Transfection

ARPE-19 cells were allowed to accumulate A2E for the c-Abl transfection. The cells were replated at a density of 70–80% confluence. The c-Abl siRNA oligonucleotide was incubated with the TransIT-TKO reagent and serum free DMEM at room temperature for 10 min. The formed complexes were delivered to the cells at a concentration of 500 nM in DMEM and incubated for 24 h. Medium containing transfections complexes were then removed and cells were incubated with complete culture medium (DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids solution and 10 μ g/ml gentamicin sulfate) for an additional 24 h. The cells were then washed with PBS containing calcium, magnesium and glucose and exposed to blue light as described above.

For p53 transfection, the p53 antisense oligonucleotides (5'-CGCTAGGATCTGACTGC-3') and sense (5'-GCAGTCAGAGCCTAGCG-3') were synthesized and HPLC purified (Invitrogen Life Technologies, Frederick, MD). The oligonucleotides were transfected into the cells using Plus Reagent and LipofectAmine (Life Technologies, Inc., Paisley United Kingdom) according to the manufacturer's instructions. In brief, oligonucleotides were

incubated with the enhancer reagent (Plus Reagent) in serum- and antibiotic-free DMEM, before addition of the cationic lipid reagent (LipofectAmine) diluted in DMEM. The formed oligonucleotide-enhancer-lipid complexes were delivered to A2E-laden cells (70–80% confluent) in serum/antibiotic-free DMEM and incubated for 8 h; subsequently DMEM supplemented with 20% FBS was added without removing the transfection mixture. After additional 24 h the mixture was replaced with complete culture medium and maintained for another 24 h before blue light exposure.

As a further approach to p53 knockdown, a plasmid-based system containing p53 siRNA was utilized. The pSuppressorNeo p53 plasmid DNA (IMG-803, IMGENEX, San Diego, CA) was incubated with ARPE-19 cells using Plus Reagent and LipofectAmine as described above. A plasmid (IMG-800-6, IMGENEX) containing a scrambled sequence with no significant homology to the mouse and human sequence were used as negative control. Three days after transfection, the cells were passaged 1:10 into selective medium containing 10% FBS and 0.7 mg/ml of the antibiotic G418 sulfate. After three weeks, G418-resistant cells were replated into 35 mm dishes (for immunoblotting) and 8-well chambers (for cell viability assays) and allowed to accumulate A2E. After 24 h quiescence, the A2E-laden selectants with p53 siRNA expression and p53 negative DNA plasmid were irradiated at 430 nm (12 min) followed by 8 h incubation. Cells were either evaluated for viability or protein was extracted for immunoblotting.

Cell viability assays

Cell viability was assessed by 3,4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) reduction assay (Roche Diagnostics, Mannheim, Germany) as previously described [42]. Cell viability was also quantified after labeling nuclei of nonviable cells with the membrane impermeant dye Dead Red (Molecular Probes, Eugene, OR) and the nuclei of all cells with 4',6'-diamino-2-phen-ylindole (DAPI), as previously described [7].

Statistical analysis

Data were analyzed by one-way ANOVA and the Newman Keul Multiple Comparison test (Prism, GraphPad Software, San Diego, CA). Values were considered statistical significantly different when $P < 0.05$.

Results

The aim of the present study was to investigate the signaling pathways mediating cell death initiated by the photooxidation of A2E. For these experiments we utilized confluent cultures of ARPE-19 cells, a cell-line that we have shown does not contain endogenous A2E [36], and we introduce synthesized A2E to the cells. While RPE lipofuscin consists of a mixture of bisretinoid pigments, the use of this cellular system allowed us to study events initiated by the photooxidation A2E specifically. With this design, we also included as important controls, cells that had not accumulated A2E. Of added advantage, the cells do not express melanin (unless grown for many weeks after reaching confluence). The absence of melanin in the cultures eliminates the problem of variations in melanin concentration that could confound studies on light damage.

c-Abl protein is upregulated and phosphorylated in ARPE-19 cells that have accumulated A2E and irradiated at 430 nm

By Western blotting, we explored the expression of the c-Abl protein in response to irradiation of A2E-laden ARPE-19 cells. ARPE-19 cells that had accumulated A2E were irradiated at 430 nm (A2E/430 nm) for 20 min, harvested after 8 h and probed on immunoblots with antibodies to c-Abl and β -actin. The immunodetected protein band at 145

kDa was the size expected for c-Abl (Fig. 1a). Densitometric analysis of the protein bands with normalization to β -actin revealed a 1.5-fold increase in c-Abl in A2E-laden ARPE-19 cells irradiated at 430 nm as compared to untreated ARPE-19 cells and to A2E-laden ARPE-19 cells that had not been exposed to blue light.

Phosphorylation of the c-Abl protein at tyrosine 245 is indicative of an increase in kinase activity [16, 19]. Thus, to determine whether c-Abl was activated in response to A2E-photooxidation, A2E-laden cells were irradiated at 430 nm for 20 min and harvested after 6 h. Phosphorylation was determined by immunoprecipitating cell lysates with c-Abl antibody and immunoblotting with c-Abl-phospho Y245 antibody in addition to antibody to c-Abl and β -actin. As shown in Fig. 1c, after A2E-laden ARPE-19 cells were irradiated, the protein band at 145 kDa indicative of c-Abl was recognized by the c-Abl-phospho Y245 antibody. Immunodetection was prevented by pre-absorption of the c-Abl-phospho Y245 antibody with specific phosphopeptide, but not by pre-incubated with non-specific peptide, confirming that the c-Abl-phospho Y245 antibody reacted specifically with the phosphorylated form of c-Abl (Fig. 1b).

Expression of c-Abl is upregulated in ARPE-19 cells that have accumulated A2E and are irradiated at 430 nm; inhibition of c-Abl reduces cell death

To determine whether blue light exposure of A2E-laden ARPE-19 cells leads to upregulation of c-Abl gene expression, we exposed A2E-laden RPE cells to 430 nm light for 7 min, harvested the cells after 3 h and measured c-Abl mRNA by quantitative RT-PCR. As shown in Fig. 2a, blue light exposure in the presence of A2E increased c-Abl mRNA levels by 6.4-fold ($P < 0.01$), as compared to A2E-laden cells that had not been exposed to blue light.

To test for the involvement of c-Abl in A2E/430 nm-induced apoptosis, A2E-laden ARPE-19 cells were transfected with siRNA targeted to c-Abl prior to irradiation. In control cells treated for 24 h with Cy3-labeled siRNA targeted to Lamin A/C and then examined by fluorescence microscopy, we observed internalization of the siRNA and 95% transfection rate. Cy3-labeling was specific for internalized siRNA since cell-associated fluorescence labeling did not occur in the absence of the transfection reagent (data not shown). As shown in Fig. 2a and b, siRNA specific to c-Abl prevented upregulation of c-Abl mRNA and protein, as confirmed by quantitative RT-PCR and immunoblotting, respectively. Cell viability assessed by MTT assay revealed that knockdown of c-Abl increased cell survival by 29% (Fig. 2c). In addition, co-labeling of the nuclei of non-viable cells with a membrane impermeable dye and DAPI, demonstrated that treatment with c-Abl siRNA resulted in a 34% decrease in cell death associated with A2E accumulation and 430 nm irradiation (data not shown).

To further test for the involvement of c-Abl in apoptosis induction, A2E-laden ARPE-19 cells were pre-treated with the c-Abl inhibitor STI571 for 48 h prior to irradiation with blue light and cell viability was assessed by MTT assay. Control samples included untreated cells, cells treated only with STI571 and cells that had only accumulated A2E. Accordingly, treatment of the cells with the c-Abl inhibitor at 5, 10 and 20 μ M concentrations resulted in increased cell survival by 22, 54 and 27%, respectively, as compared to A2E-laden cells irradiated with blue light in the absence of the inhibitor (Fig. 2d).

A2E-laden cells irradiated with blue light upregulate p53 protein expression

Examination of cell extracts by Western blotting revealed a 3-fold increase in p53 protein expression in ARPE-19 cells that had accumulated A2E and were irradiated (Fig. 3a and b). p53-induced apoptosis under some conditions has been shown to involve transcriptional

upregulation of Bax [24] along with translocation of Bax from the cytoplasm to the mitochondrial membrane where it induces the release of cytochrome *c* [25, 43–45]. While in our experiments we observed cytochrome *c* release into the cytoplasm (unpublished data), we found that expression of Bax protein was unchanged (Fig. 3a).

siRNA targeting p53 prevent cell death

We reasoned that if p53 plays a role in the cell death pathway, silencing of p53 would reduce cell death. To inhibit the expression of p53, ARPE-19 cells were transfected with a specific oligonucleotide and also by siRNA targeted to p53. To demonstrate that p53 protein expression was efficiently knocked down, A2E-laden ARPE-19 cells that had been transfected with a specific oligonucleotide (Fig. 4a) or siRNA (Fig. 4b) targeting p53, were irradiated at 430 nm and protein expression was analyzed by Western blotting. As shown in Fig. 4a and b, p53 protein expression was suppressed as compared to non-transfected, irradiated A2E-laden ARPE-19 cells.

To evaluate the effect of p53 knockdown on cell viability, transfected and non-transfected cells were irradiated at 430 nm (20 min) followed by 8 h of incubation. Co-labeling nuclei with a membrane-impermeable dye and DAPI showed a decrease in non-viable cells from 20.8% to 11.3% in non-transfected, irradiated A2E-laden ARPE-19 cells as compared to A2E-laden ARPE-19 cells irradiated at 430 nm transfected with oligonucleotides (Fig. 4c). Transfection with siRNA resulted in a decrease in cell death from 15.6% to 2.8% (Fig. 4d).

A2E-laden ARPE-19 cells exposed to blue light upregulate JNK1 gene and protein expression and inhibition of JNK augments cell death

To determine the involvement of JNK in A2E/430 nm-induced apoptosis we examined gene expression of JNK1 and JNK2 5 hours after exposure of A2E-laden ARPE-19 cells to blue light (12 min). Blue light significantly increased JNK1 mRNA expression levels by 2.5-fold, when compared to untreated cells or A2E-laden cells that had not been exposed to blue light. Levels of JNK2 gene expression were not significantly altered.

Protein expression was assessed by Western blotting using antibodies that specifically recognize JNK1 and JNK2. Membranes were stripped and reprobed with antibody to β -actin. We found that protein expression of JNK1 increased 1.5-fold within 6 h in A2E-laden ARPE-19 cells irradiated at 430 nm as compared to controls (Fig. 5a and b), whereas a significant increase in JNK2 protein expression was not observed (Fig. 5a and b).

Several roles have been ascribed to JNK, including modulation of cell injury [46, 47]. To determine whether inhibition of the JNK pathway would alter the survival of cells, we pre-treated A2E-laden cells with the specific JNK inhibitor SP600125, at different concentrations (0.2, 2, 10 and 20 μ M) for 1 h before irradiation.

Cell viability measured by MTT assay revealed that cell death was augmented as the concentration of the inhibitor was increased. To test the cytotoxicity of the inhibitor, we treated ARPE-19 cells with the inhibitor, in the absence of blue light (Fig. 5c). There were no significant differences between SP600125 treated and untreated ARPE-19 cells.

A2E-laden ARPE-19 cells exposed to blue light upregulate p38 gene- and protein expression but inhibition of p38 has no effect on cell survival

To investigate whether blue light exposure of A2E-laden ARPE-19 cells leads to a change in p38 gene expression, A2E-laden ARPE-19 cells were irradiated at 430 nm (7 min) and after 3 h p38 gene expression was measured by quantitative RT-PCR. Irradiation with blue light

in the presence of intracellular A2E significantly increased p38 mRNA levels 2-fold, when compared with untreated cells or A2E-laden cells that had not been exposed to blue light.

To determine protein levels, cells were irradiated for 12 min and harvested after 6 h for immunoblotting. Expression of p38 protein was increased 1.5-fold in A2E-laden ARPE-19 cells irradiated with blue light, when compared to untreated ARPE-19 cells or A2E-laden ARPE-19 cells that had not been exposed to blue light (Fig. 6a and b).

Using the same experimental design as for the JNK inhibitor, we also studied the specific p38 inhibitor SB203580. We found no significant difference in cell death between treated and untreated A2E-laden ARPE-19 cells irradiated with blue light (Fig. 6c).

Discussion

It has long been speculated that accumulation of lipofuscin in the RPE cells may contribute to development of AMD. Studies concerned with examining associations between RPE lipofuscin and RPE cell death, have shown that a major constituent of RPE lipofuscin, the bisretinoid fluorophore A2E and its photoisomer iso-A2E [38], can mediate blue light damage to RPE [37, 48]. We have previously shown that the cell death program in RPE initiated by A2E/430 nm involves activation of caspase-3 and protection by bcl-2 [10].

The molecular basis for A2E/430 nm-induced RPE cell death has remained unclear. Here we show that apoptosis induced in A2E-laden RPE cells by blue light was inhibited by approximately 30% in cells transfected with siRNA specific to c-Abl and we demonstrated up to 50% increased survival in the presence of the c-Abl inhibitor, STI571. c-Abl is a latent tyrosine kinase that becomes activated in response to numerous extra- and intra-cellular stimuli. A variety of cellular processes are tightly regulated by c-Abl kinase activity and/or by interactions between c-Abl and other signaling molecules. These processes include differentiation, cell growth arrest, adhesion, DNA repair, death and responses to stress. The factors that determine cell fate due to a given stimulus are largely unknown.

Kharbanda et al. have observed that ionizing irradiation and other DNA-damaging agents induce activation of c-Abl in proliferating cells whereas the same stimuli fails to activate c-Abl in confluent and growth quiescent cells, indicating that the proliferative state and/or cell-cell contact might regulate activation of c-Abl [49].

A role for c-Abl in oxidative stress- and DNA-damage induced apoptosis in cell types other than RPE cells is well established [50–54]. We have previously shown that illumination of A2E-containing RPE cells at 430 nm leads to DNA injury, the extent of which is proportional to duration of exposure. Here we demonstrate that inhibition of c-Abl kinase by STI571 treatment or suppression of c-Abl with siRNA specific for c-Abl attenuates the apoptotic response induced in the presence of A2E and 430 nm light.

p53-initiated apoptosis may involve the mitochondrial protein Bax. While some investigators suggest that Bax is the primary target for p53-induced apoptosis [55], others conclude that no single gene is chiefly responsible and that apoptosis probably represents a cumulative response to the activation of several genes [56]. Here we demonstrate that blue light induced sensitization of A2E lead to increased expression of p53 protein. As a further indication of p53 involvement in A2E/430 nm-induced RPE cell death, we showed that inhibition of p53 with specific siRNA attenuated cell death. The observation that expression of Bax protein was unchanged, indicates that the involvement of p53 in A2E/430 nm-induced apoptosis is executed by a mechanism independent of Bax.

Inhibition of neither c-Abl nor p53 was sufficient to completely abolish the A2E/430 nm-induced cell death. These findings suggest that the role of these molecules may be to modulate cell death and that several pathways may be involved in A2E/430 nm-induced cell death.

The exact role of the JNK pathway in apoptosis is highly controversial and there has been much speculation as to whether the JNK pathway is pro- or anti-apoptotic or does not play a role [47]. The consequence of JNK activation depends on cell- and stimulus- type, co-activation of additional pathways and the duration of activation. Thus, the JNK pathway is not simply a pro- or an anti-apoptotic pathway. The role for JNK and p38 in RPE cell death is still poorly understood. Ho et al. found that H₂O₂ exposure markedly increased JNK and p38 activity in ARPE-19 cells and that H₂O₂-mediated cell death was reduced by treatment with inhibitors of JNK and p38 [57]. Qin et al. also found that H₂O₂-induced apoptosis lead to phosphorylation of JNK and p38, but in contrast, inhibition of JNK and p38 did not influence the extent of cell death. Further, they demonstrated that treatment with the cyclopentenone 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (dPGJ₂)—an endogenous PPAR γ agonist that can protect RPE cells from oxidative injury—[58] prolonged the H₂O₂-induced phosphorylation of JNK and p38, and the protective effect of dPGJ₂ was dependent on activation of JNK and p38. This is consistent with our data showing that the specific JNK inhibitor SP600125 afforded increased cell death. We found an upregulation of protein and mRNA for both JNK and p38. However, we were not able to establish a role for p38 in executing the apoptosis program. Specifically, using the p38 inhibitor SB20358 and a treatment paradigm reported previously to be effective [59], we did not observe protection against cell death. Activation of p38 has been shown to act under a variety of conditions such as inflammation, cell proliferation, apoptosis and stabilization of COX2 mRNA [60, 61]. Hence, in the setting of intracellular accumulation of A2E and blue light irradiation, p38 may play a role unknown to us at this time.

c-Abl is considered a key regulator of p53, and can inhibit degradation of p53 [62] and enhance p53-transcriptional activity and accumulation of p53 in the nucleus upon activation [63]. On the other hand, p53 can also act independently of c-Abl [22]. Likewise, c-Abl can be activated by p53-dependent as well as p53-independent mechanisms. The ability of c-Abl to promote apoptosis can occur via mechanisms that depend on activation of JNK as well as through ways independent of JNK activation. Thus, the interplay between c-Abl, p53 and JNK is very complex and further studies are required to evaluate the cross talk between these three pathways.

Conclusion

The evidence for upregulation of c-Abl and p53 together with the observation that inhibition of c-Abl and p53 attenuate the frequency of apoptosis, indicates that exposure of RPE to blue light in the setting of intracellular A2E initiates a cell death program that is at least in part executed by p53 and modulated by c-Abl. Further, our results suggest that JNK can modulate the cell death program in A2E/430 nm-induced RPE cell death. RPE cells play an essential role for the function of retina, and these finding might be important for better understanding of the mechanisms involved in RPE cell death.

Acknowledgments

We thank Ms. A. Hung for technical help. This work was supported by National Eye Institute Grant EY 12951 to JRS, a gift from Dr. Gertrude Neumark Rothschild, a grant from Research to Prevent Blindness to the Department of Ophthalmology Columbia University and grants from Danish Medical Society, Fabrikant Einar Willumsens Mindelegat, Lars Andersens Legat, Grosserer Chr. Andersen og hustru Ingeborg Andersen, f. Schmidts legat (fond) oprettet af deres datter frk. Lilli Ellen Andersen, Kristine Petrea—Marius Claus—og Erik Feldthusens Fond, Mette

Warburgs Fond, and Hotelejer Edvard Johnsens Legat. JRS is the recipient of a Research to Prevent Blindness Senior Investigator Award.

References

1. Gehrs KM, Anderson DH, Johnson LV, et al. Age-related macular degeneration-emerging pathogenetic and therapeutic concepts. *Ann Med*. 2006; 38:450–471. doi:10.1080/07853890600946724. [PubMed: 17101537]
2. Holz FG, Bellman C, Staudt S, et al. Fundus autofluorescence and development of geographic atrophy in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2001; 42:1051–1056. [PubMed: 11274085]
3. von RA, Fitzke FW, Bird AC. Fundus autofluorescence in age-related macular disease imaged with a laser scanning ophthalmoscope. *Invest Ophthalmol Vis Sci*. 1997; 38:478–486. [PubMed: 9040481]
4. Sparrow JR, Boulton M. RPE lipofuscin and its role in retinal pathobiology. *Exp Eye Res*. 2005; 80:595–606. doi:10.1016/j.exer.2005.01.007. [PubMed: 15862166]
5. Sparrow, JR. RPE lipofuscin: formation, properties and relevance to retinal degeneration. In: Tombran-Tink, J.; Barnstable, CJ., editors. *Retinal degenerations: biology, diagnostics and therapeutics*. Totowa, NJ: Humana Press; 2007.
6. Ben-Shabat S, Itagaki Y, Jockusch S, et al. Formation of a nonaoxirane from A2E, a lipofuscin fluorophore related to macular degeneration, and evidence of singlet oxygen involvement. *Angew Chem Int Ed Engl*. 2002; 41:814–817. doi:10.1002/1521-3773(20020301)41:5<814::AID-ANIE814>3.0.CO;2-2. [PubMed: 12491345]
7. Sparrow JR, Zhou J, Ben-Shabat S, et al. Involvement of oxidative mechanisms in blue-light-induced damage to A2E-laden RPE. *Invest Ophthalmol Vis Sci*. 2002; 43:1222–1227. [PubMed: 11923269]
8. Sparrow JR, Zhou J, Cai B. DNA is a target of the photodynamic effects elicited in A2E-laden RPE by blue-light illumination. *Invest Ophthalmol Vis Sci*. 2003; 44:2245–2251. doi: 10.1167/iops.02-0746. [PubMed: 12714667]
9. Sparrow JR, Vollmer-Snarr HR, Zhou J, et al. A2E-epoxides damage DNA in retinal pigment epithelial cells. Vitamin E and other antioxidants inhibit A2E-epoxide formation. *J Biol Chem*. 2003; 278:18207–18213. doi:10.1074/jbc.M300457200. [PubMed: 12646558]
10. Sparrow JR, Cai B. Blue light-induced apoptosis of A2E-containing RPE: involvement of caspase-3 and protection by Bcl-2. *Invest Ophthalmol Vis Sci*. 2001; 42:1356–1362. [PubMed: 11328751]
11. Pendergast AM. The Abl family kinases: mechanisms of regulation and signaling. *Adv Cancer Res*. 2002; 85:51–100. doi: 10.1016/S0065-230X(02)85003-5. [PubMed: 12374288]
12. Sawyers CL, McLaughlin J, Goga A, et al. The nuclear tyrosine kinase c-Abl negatively regulates cell growth. *Cell*. 1994; 77:121–131. doi:10.1016/0092-8674(94)90240-2. [PubMed: 7512450]
13. Daniel R, Cai Y, Wong PM, et al. Deregulation of c-abl mediated cell growth after retroviral transfer and expression of antisense sequences. *Oncogene*. 1995; 10:1607–1614. [PubMed: 7731715]
14. Van Etten RA. Cycling, stressed-out and nervous: cellular functions of c-Abl. *Trends Cell Biol*. 1999; 9:179–186. doi:10.1016/S0962-8924(99)01549-4. [PubMed: 10322452]
15. Wang JY. Regulation of cell death by the Abl tyrosine kinase. *Oncogene*. 2000; 19:5643–5650. doi:10.1038/sj.onc.1203878. [PubMed: 11114745]
16. Hantschel O, Nagar B, Guettler S, et al. A myristoyl/ phosphotyrosine switch regulates c-Abl. *Cell*. 2003; 112:845–857. doi: 10.1016/S0092-8674(03)00191-0. [PubMed: 12654250]
17. Nagar B, Hantschel O, Young MA, et al. Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell*. 2003; 112:859–871. doi:10.1016/S0092-8674(03)00194-6. [PubMed: 12654251]
18. Hantschel O, Superti-Furga G. Regulation of the c-Abl and Bcr-Abl tyrosine kinases. *Nat Rev Mol Cell Biol*. 2004; 5:33–44. doi: 10.1038/nrm1280. [PubMed: 14708008]

19. Jin GF, Hurst JS, Godley BF. Hydrogen peroxide stimulates apoptosis in cultured human retinal pigment epithelial cells. *Curr Eye Res.* 2001; 22:165–173. doi:10.1076/ceyr.22.3.165.5517. [PubMed: 11462152]
20. Hueber A, Esser P, Heimann K, et al. The topoisomerase I inhibitors, camptothecin and beta-lapachone, induce apoptosis of human retinal pigment epithelial cells. *Exp Eye Res.* 1998; 67:525–530. doi:10.1006/exer.1998.0544. [PubMed: 9878214]
21. Levav-Cohen Y, Goldberg Z, Zuckerman V, et al. C-Abl as a modulator of p53. *Biochem Biophys Res Commun.* 2005; 331:737–749. doi:10.1016/j.bbrc.2005.03.152. [PubMed: 15865930]
22. Yuan ZM, Huang Y, Fan MM, et al. Genotoxic drugs induce interaction of the c-Abl tyrosine kinase and the tumor suppressor protein p53. *J Biol Chem.* 1996; 271:26457–26460. doi: 10.1074/jbc.271.43.26457. [PubMed: 8900110]
23. Yuan ZM, Huang Y, Ishiko T, et al. Regulation of DNA damage-induced apoptosis by the c-Abl tyrosine kinase. *Proc Natl Acad Sci USA.* 1997; 94:1437–1440. doi:10.1073/pnas.94.4.1437. [PubMed: 9037071]
24. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell.* 1995; 80:293–299. doi:10.1016/0092-8674(95)90513-8. [PubMed: 7834749]
25. Dubrez L, Coll JL, Hurbina A, et al. Caffeine sensitizes human H358 cell line to p53-mediated apoptosis by inducing mitochondrial translocation and conformational change of BAX protein. *J Biol Chem.* 2001; 276:38980–38987. doi:10.1074/jbc.M102683200. [PubMed: 11489880]
26. Suzuki K, Hino M, Kutsuna H, et al. Selective activation of p38 mitogen-activated protein kinase cascade in human neutrophils stimulated by IL-1beta. *J Immunol.* 2001; 167:5940–5947. [PubMed: 11698472]
27. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* 2001; 81:807–869. [PubMed: 11274345]
28. Pearson G, Robinson F, Beers GT, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev.* 2001; 22:153–183. doi:10.1210/er.22.2.153. [PubMed: 11294822]
29. Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev.* 2004; 68:320–344. doi:10.1128/MMBR.68.2.320-344.2004. [PubMed: 15187187]
30. Chen Z, Gibson TB, Robinson F, et al. MAP kinases. *Chem Rev.* 2001; 101:2449–2476. doi: 10.1021/cr000241p. [PubMed: 11749383]
31. Han J, Lee JD, Bibbs L, et al. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science.* 1994; 265:808–811. doi:10.1126/science.7914033. [PubMed: 7914033]
32. Derijard B, Hibi M, Wu IH, et al. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell.* 1994; 76:1025–1037. doi:10.1016/0092-8674(94)90380-8. [PubMed: 8137421]
33. Mizukami Y, Yoshioka K, Morimoto S, et al. A novel mechanism of JNK1 activation. Nuclear translocation and activation of JNK1 during ischemia and reperfusion. *J Biol Chem.* 1997; 272:16657–16662. doi:10.1074/jbc.272.26.16657. [PubMed: 9195981]
34. Mizukami Y, Yoshida K. Mitogen-activated protein kinase translocates to the nucleus during ischaemia and is activated during reperfusion. *Biochem J.* 1997; 323(Pt 3):785–790. [PubMed: 9169613]
35. Li DQ, Luo L, Chen Z, et al. JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp Eye Res.* 2006; 82:588–596. doi:10.1016/j.exer.2005.08.019. [PubMed: 16202406]
36. Sparrow JR, Parish CA, Hashimoto M, et al. A2E, a lipofuscin fluorophore, in human retinal pigmented epithelial cells in culture. *Invest Ophthalmol Vis Sci.* 1999; 40:2988–2995. [PubMed: 10549662]
37. Sparrow JR, Nakanishi K, Parish CA. The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. *Invest Ophthalmol Vis Sci.* 2000; 41:1981–1989. [PubMed: 10845625]

38. Parish CA, Hashimoto M, Nakanishi K, et al. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc Natl Acad Sci USA*. 1998; 95:14609–14613. doi:10.1073/pnas.95.25.14609. [PubMed: 9843937]
39. Dai Y, Rahmani M, Corey SJ, et al. A Bcr/Abl-independent, Lyn-dependent form of imatinib mesylate (STI-571) resistance is associated with altered expression of Bcl-2. *J Biol Chem*. 2004; 279:34227–34239. doi:10.1074/jbc.M402290200. [PubMed: 15175350]
40. Bian ZM, Elnor SG, Yoshida A, et al. Differential involvement of phosphoinositide 3-kinase/Akt in human RPE MCP-1 and IL-8 expression. *Invest Ophthalmol Vis Sci*. 2004; 45:1887–1896. JID-7703701. [PubMed: 15161854]
41. Zhuang S, Demirs JT, Kochevar IE. p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J Biol Chem*. 2000; 275:25939–25948. doi:10.1074/jbc.M001185200. [PubMed: 10837470]
42. Zhou J, Gao X, Cai B, et al. Indirect antioxidant protection against photooxidative processes initiated in retinal pigment epithelial cells by a lipofuscin pigment. *Rejuvenation Res*. 2006; 9:256–263. doi:10.1089/rej.2006.9.256. [PubMed: 16706653]
43. Dewson G, Cohen GM, Wardlaw AJ. Interleukin-5 inhibits translocation of Bax to the mitochondria, cytochrome c release, and activation of caspases in human eosinophils. *Blood*. 2001; 98:2239–2247. doi:10.1182/blood.V98.7.2239. [PubMed: 11568012]
44. Soucie EL, Annis MG, Sedivy J, et al. Myc potentiates apoptosis by stimulating Bax activity at the mitochondria. *Mol Cell Biol*. 2001; 21:4725–4736. doi:10.1128/MCB.21.14.4725-4736.2001. [PubMed: 11416148]
45. Jurgensmeier JM, Xie Z, Deveraux Q, et al. Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci USA*. 1998; 95:4997–5002. doi:10.1073/pnas.95.9.4997. [PubMed: 9560217]
46. Strniskova M, Barancik M, Ravingerova T. Mitogen-activated protein kinases and their role in regulation of cellular processes. *Gen Physiol Biophys*. 2002; 21:231–255. JID-8400604. [PubMed: 12537349]
47. Lin A, Dibling B. The true face of JNK activation in apoptosis. *Aging Cell*. 2002; 1:112–116. doi: 10.1046/j.1474-9728.2002.00014.x. [PubMed: 12882340]
48. Schutt F, Davies S, Kopitz J, et al. Photodamage to human RPE cells by A2-E, a retinoid component of lipofuscin. *Invest Ophthalmol Vis Sci*. 2000; 41:2303–2308. [PubMed: 10892877]
49. Yoshida K, Yamaguchi T, Natsume T, et al. JNK phosphorylation of 14-3-3 proteins regulates nuclear targeting of c-Abl in the apoptotic response to DNA damage. *Nat Cell Biol*. 2005; 7:278–285. doi:10.1038/ncb1228. [PubMed: 15696159]
50. Kharbanda S, Ren R, Pandey P, et al. Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. *Nature*. 1995; 376:785–788. doi:10.1038/376785a0. [PubMed: 7651539]
51. Kharbanda S, Yuan ZM, Weichselbaum R, et al. Determination of cell fate by c-Abl activation in the response to DNA damage. *Oncogene*. 1998; 17:3309–3318. JID-8711562. [PubMed: 9916993]
52. Li B. c-Abl in oxidative stress, aging and cancer. *Cell Cycle*. 2005; 4:246–248. [PubMed: 15655364]
53. Raina D, Pandey P, Ahmad R, et al. c-Abl tyrosine kinase regulates caspase-9 autocleavage in the apoptotic response to DNA damage. *J Biol Chem*. 2005; 280:11147–11151. doi: 10.1074/jbc.M413787200. [PubMed: 15657060]
54. Shaul Y, Ben-Yehoyada M. Role of c-Abl in the DNA damage stress response. *Cell Res*. 2005; 15:33–35. doi:10.1038/sj.cr.72.90261. [PubMed: 15686624]
55. Wu X, Deng Y. Bax and BH3-domain-only proteins in p53-mediated apoptosis. *Front Biosci*. 2002; 7:d151–d156. doi: 10.2741/wu1. [PubMed: 11779719]
56. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell*. 2001; 7:683–694. JID-9802571. [PubMed: 11463392]
57. Ho TC, Yang YC, Cheng HC, et al. Activation of mitogen-activated protein kinases is essential for hydrogen peroxide-induced apoptosis in retinal pigment epithelial cells. *Apoptosis*. 2006; 11:1899–1908. doi:10.1007/s10495-006-9403-6. [PubMed: 16927023]

58. Qin S, McLaughlin AP, De Vries GW. Protection of RPE cells from oxidative injury by 15-deoxy-delta12, 14-prostaglandin J2 by augmenting GSH and activating MAPK. *Invest Ophthalmol Vis Sci.* 2006; 47:5098–5105. doi:10.1167/iovs.06-0318. [PubMed: 17065531]
59. Nagineni CN, Samuel W, Nagineni S, et al. Transforming growth factor-beta induces expression of vascular endothelial growth factor in human retinal pigment epithelial cells: involvement of mitogen-activated protein kinases. *J Cell Physiol.* 2003; 197:453–462. doi:10.1002/jcp.10378. [PubMed: 14566975]
60. Li X, Udagawa N, Takami M, et al. p38 Mitogen-activated protein kinase is crucially involved in osteoclast differentiation but not in cytokine production, phagocytosis, or dendritic cell differentiation of bone marrow macrophages. *Endocrinology.* 2003; 144:4999–5005. doi:10.1210/en.2003-0166. [PubMed: 12960069]
61. Kumagai T, Nakamura Y, Osawa T, et al. Role of p38 mitogen-activated protein kinase in the 4-hydroxy-2-nonenal-induced cyclooxygenase-2 expression. *Arch Biochem Biophys.* 2002; 397:240–245. doi:10.1006/abbi.2001.2601. [PubMed: 11795877]
62. Sionov RV, Moallem E, Berger M, et al. c-Abl neutralizes the inhibitory effect of Mdm2 on p53. *J Biol Chem.* 1999; 274:8371–8374. doi:10.1074/jbc.274.13.8371. [PubMed: 10085066]
63. Sionov RV, Coen S, Goldberg Z, et al. c-Abl regulates p53 levels under normal and stress conditions by preventing its nuclear export and ubiquitination. *Mol Cell Biol.* 2001; 21:5869–5878. doi:10.1128/MCB.21.17.5869-5878.2001. [PubMed: 11486026]

Fig. 1.

Exposure of A2E-laden ARPE-19 cells to blue light induces increased expression and phosphorylation of c-Abl protein. **a** ARPE-19 cells that had accumulated A2E were non-irradiated (A2E) or irradiated with blue light (A2E 430 nm) for 20 min. Control lane was untreated cells. Whole cell lysates were separated, blotted and probed with antibodies to c-Abl and β-actin. Ratio of c-Abl to β-actin was determined by densitometry. **b** To demonstrate phospho-antibody specificity, cell lysates from A2E-laden ARPE-19 cells, harvested 6 h after irradiation, were immuno-precipitated with antibody to c-Abl. Protein was blotted on three separate membranes and probed with antibody to c-Abl-phospho Y245 or with c-Abl-phospho Y245 antibody pre-absorbed with either specific c-Abl-phospho-

peptide or non-specific non-phospho-peptide. **c** Cells that had accumulated A2E were irradiated at 430 nm for 20 min (A2E 430 nm). Controls included A2E-free cells irradiated at 430 nm (430 nm) and cells that had only accumulated A2E (A2E). After 6 h cell homogenates were immuno-precipitated with antibodies to c-Abl and β -actin. The immunoprecipitates were blotted and probed with the same antibodies and the antibody to c-Abl-phospho Y245. Ratio of phosphorylated c-Abl to β -actin was determined by densitometry

Fig. 2.

Involvement of c-Abl in cell death induced by blue light irradiation of ARPE-19 cells that have accumulated A2E. **a** c-Abl mRNA was measured by quantitative RT-PCR 3 h after irradiation (7 min) of A2E-laden ARPE-19 cells (A2E + 430 nm) that were transfected or untransfected with c-Abl siRNA. Control cultures were non-irradiated (A2E) or A2E-free. Values are mean $\pm$ SEM of 3 experiments. * $P < 0.01$ as compared to controls. **b** Blue light irradiation of A2E-laden ARPE-19 cells and protein expression of c-Abl. ARPE-19 cells that had accumulated A2E and were irradiated at 430 nm (A2E BL) or that had accumulated A2E only (A2E) or were A2E-free (–) were transfected with c-Abl siRNA or were untransfected. siRNA controls were A2E-free and transfected with transfection reagent only

(1), lamin A/C siRNA (2), and non-targeting siRNA (3). Cells were harvested 8 h after irradiation and blotted protein was probed with antibody to c-Abl and β -actin. **c** A2E-laden ARPE-19 cells were transfected with siRNA specific to c-Abl and irradiated at 430 nm for 20 min. Cells were incubated for 24 h after irradiation and cell viability was quantified by MTT assay. Data were normalized to untreated cells; a decrease in absorbance (570 nm) of reduced MTT is indicative of diminished cellular viability. Values are mean $\pm$ SEM of 6 experiments. **d** A2E-laden ARPE-19 cells were incubated with and without STI571 at the indicated concentrations for 48 h and were irradiated (A2E + 430 nm) and non-irradiated (A2E). Control samples included untreated cells (1), cells that had only accumulated A2E (2) and cells that had not accumulated A2E (A2E-free) and were treated with STI571 only (3). Viability was assayed by MTT assay and is presented as absorbance at 570 nm. Values were normalized to untreated cells. Bar height is positively correlated with cell viability. * P < 0.01 as compared to untreated cells; ** P < 0.01 for A2E + 430 nm with versus without STI571 inhibitor. Mean $\pm$ SEM of 8 experiments. BL: blue light, β 2 M: β 2-microglobulin

Fig. 3. Blue light exposure of A2E-laden ARPE-19 cells increases p53-, but not Bax-, protein expression. **a** A2E-laden ARPE-19 cells were irradiated for 20 min (A2E + 430 nm). Controls were A2E-free cells irradiated at 430 nm (430 nm) and cells that had only accumulated A2E (A2E). Whole cell lysates were analyzed by Western blotting with antibodies to p53, Bax and β -actin. Protein bands corresponding to p53 **b** and Bax (data not shown) were quantified by densitometry and are expressed as a ratio with β -actin. * $P < 0.05$ as compared to A2E-free cells irradiated at 430 nm (430 nm). Values are mean $\pm$ SEM of 4 experiments

Fig. 4.

Silencing p53 by transfection with specific oligonucleotide and siRNA protects ARPE-19 cells against cell death induced by blue light irradiation of A2E-laden ARPE-19 cells. **a** A2E-laden ARPE-19 cells (A2E) were untransfected or transfected with a specific p53 oligonucleotide (p53 antisense) or non-targeting p53 oligonucleotide (p53 sense). An additional control included A2E-free (–) cells. All cells were irradiated at 430 nm. Whole cell lysates were separated, blotted and probed with antibodies to p53 and β-actin. **b** A2E-laden ARPE-19 cells were untransfected or transfected with p53 siRNA or control siRNA. Controls included cells that had not accumulated A2E (–). All cells were irradiated at 430 nm. Immunoblots were probed with antibodies to p53 and β-actin. **c** and **d** Percent nonviable cells was determined by co-labeling nuclei with a membrane-impermeable dye and DAPI. Values are mean ± SEM of 3 experiments

Fig. 5.

Inhibition of JNK augments A2E/blue light-induced cell death **a** A2E-laden ARPE-19 cells irradiated at 430 nm for 12 min (A2E 430 nm) were harvested after 6 h and analyzed by Western blotting with antibodies to JNK1, JNK2 and β-actin. ARPE-19 cells that were untreated (UT) or had accumulated A2E only (A2E), served as controls. **b** Ratio of JNK1 and JNK2 to β-actin was determined by densitometry. Data are normalized to untreated ARPE-19 cells. Values are mean ± SEM of 3 experiments. * $P < 0.05$ as compared to untreated cells. **c** A2E-laden ARPE-19 cells were pre-treated with the specific JNK inhibitor SP600125 at the indicated concentrations for 1 h prior to irradiation at 430 nm for 20 min (A2E + 430 nm). Cell viability was assayed by MTT assay after 24 h. Decrease in

absorbance (570 nm) of reduced MTT is indicative of diminished cellular viability. To test for cytotoxicity of the inhibitor, ARPE-19 cells were treated with SP600125 for 1 h at the indicated concentration, incubated for 24 h and assayed using MTT. Values are normalized to untreated cells; means $\pm$ SEM of 4 experiments. * $P < 0.05$ as compared to A2E-laden ARPE-19 cells irradiated with blue light. ** $P < 0.05$ as compared to untreated cells

Fig. 6.

A2E-laden ARPE-19 cells exposed to blue light upregulate p38 protein expression. **a** ARPE-19 cells that had accumulated A2E were irradiated at 430 nm for 12 min (A2E 430 nm) and harvested 6 h after light exposure. Controls were untreated ARPE-19 cells and cells that had accumulated A2E but were not irradiated (A2E). Whole cell lysates were separated, blotted and probed with antibody to p38 and to β -actin. **b** Ratio of p38 to β -actin was determined by densitometry; values are normalized to untreated cells. **c** ARPE-19 cells that had accumulated A2E were irradiated at 430 nm (20 min) (A2E + 430 nm) and after 24 h cell viability was determined by MTT assay. Cells were either pre-treated with the specific p38 inhibitor SB203580 at the indicated concentrations for 1 h or were not treated with

inhibitor. Controls included untreated cells and cells that had only accumulated A2E (A2E). Decrease in absorbance (570 nm) of reduced MTT is indicative of diminished cellular viability. Values are normalized to untreated control cells. The cytotoxicity of SB203580 was tested by treating ARPE-19 cells at indicated concentration for 1 h, followed by MTT assay after 24 h. Mean $\pm$ SEM of 4 experiments. * P < 0.01 (**b**) and * P < 0.05 (**c**) as compared to untreated cells

Table 1

Primers used for quantitative RT-PCR

	Forward	Reverse
c-Abl	5'-CGGGTCTTAGGCTATAATCAC-3'	5'-CCCTCCCTTCGTATCTCA-3'
p38	5'-CTCGTTGGAACCCAG-3'	5'-CATGTGCAAGGGCTTG-3'
JNK1	5'-CTGCCCCCGTATAACTC-3'	5'-CTGCCCCCGTATAACTC-3'
JNK2	5'-CCTGGGTATGGGCTAC-3'	5'-CGCAGAGCTTCGTCTA-3'
β2MG	5'-TGCCTGCCGTGTGAAC-3'	5'-AGCCCTCCTAGAGCTAC-3'
18 s	5'-CCCGAAGCGTTTACTTTG-3'	5'-CGACGGTATCTGATCGT-3'
GAPDH	5'-CTGAGCTGAACGGGAAG-3'	5'-GGGTGTCGCTGTTGAA-3'