Mechanisms Underlying Benzyl Alcohol Cytotoxicity (Triamcinolone Acetonide Preservative) in Human Retinal Pigment Epithelial Cells

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PURPOSE. Benzyl alcohol (BA) is the preservative in triamcinolone acetonide (TA) suspensions, which are used in treating vitreoretinal diseases and during surgery. This paper investigates the molecular mechanisms and signaling pathways underlying BA toxicity in human retinal pigment epithelial (RPE) cells.

METHODS. Cultured human RPE cells from the ARPE-19 cell line were exposed to culture medium alone (control) or with BA (0.0225, 0.225, 0.9, 3, or 9 mg/mL) for up to 6 hours. BA toxicity was assessed by TUNEL assay, propidium iodide/annexin V-FITC staining and flow cytometry, caspase activation assay, caspase and apoptosis inhibition assays, mitochondrial transmembrane potential by rhodamine staining and flow cytometry, reactive oxygen species by chemiluminescence, and apoptosis-inducing factor staining.

RESULTS. BA caused RPE cell death not only by necrosis but also by apoptosis, evidenced by exposure to 9 mg/mL BA for 6 hours leading to 19.0% early apoptotic cells and 64.2% apoptotic cells. Apoptotic signaling involved the immediate production of reactive oxygen species, activation of caspase-8, impairment of the mitochondrial transmembrane potential, and further activation of caspase-9 and -3. In addition, BA induced translocation of apoptosis-inducing factor into the nucleus, indicating caspase-independent apoptosis.

CONCLUSIONS. BA leads to necrosis of RPE cells and triggers mitochondrial apoptosis through both caspase-dependent and -independent pathways. Extreme caution is suggested in the intraocular use of TA suspensions and meticulous evaluation before adoption of BA as a preservative in the future development of ophthalmic formulations. (Invest Ophthalmol Vis Sci. 2011;52:4214–4222) DOI:10.1167/iovs.10-6058

Triamcinolone acetonide (TA) suspensions are widely used to treat a variety of vitreoretinal and macular diseases, including proliferative diabetic retinopathy, refractory diabetic or uveitic or pseudophakic macular edema, macular degeneration, retinal vein occlusion, and proliferative vitreoretinopathy.1–10 Instillation of crystalline TA particles also helps in visualizing the vitreous, posterior hyaloid, epiretinal membrane, and internal limiting membrane during vitreoretinal surgery.11–16 Between 2004 and 2009, approximately 700 articles on these topics were added to the MEDLINE database. In addition to TA, commercially available suspensions contain benzyl alcohol (BA) as the preservative and solubilizer, carboxymethylcellulose sodium and polysorbate 80 as the suspending agents, and sodium hydroxide or hydrochloric acid to adjust pH.

Since injection of the preservatives used for crystalline corticosteroids into the rabbit eye were found to cause retinal degeneration, preretinal membrane, or cataract,17 intravitreal injection of commercial TA suspensions with preservatives in the clinic may damage the eye. Commercially available TA formulations, initially developed for intra-articular or intramuscular injection, consist of TA particles suspended in a vehicle containing the preservative, BA. Preservative toxicity is of particular concern during TA-assisted macular hole surgery,18,19 or repair of rhegmatogenous retinal detachment20 because in these clinical scenarios the RPE layer is in direct contact not only with TA but also with vehicle.

Recently, we found that both commercially available TA suspensions and BA alone damage human RPE cells and rabbit corneal endothelial cells by decreasing their viability, impairing mitochondrial function, and damaging cellular ultrastructure, while TA particles without vehicle do not.21–23 In addition, the cytotoxicity is dose- and time-dependent and is mainly caused by necrosis. However, it remains unresolved whether apoptosis is also involved in BA-induced RPE cell damage, and the mechanisms and pathways of RPE apoptosis are also unclear. Therefore, this study aimed to further investigate BA toxicity by assessing cell death and relevant key components in the pathways to apoptosis.

MATERIALS AND METHODS

Cell Culture

Human RPE cells (ARPE-19), obtained from a nontransformed cell line (American Type Culture Collection, Manassas, VA) with structural and functional properties characteristic of RPE cells in vivo,24 were cultured in DMEM/Ham’s F12 medium (1:1) containing 10% fetal bovine...
serum (Invitrogen, Grand Island, NY) at 37°C and 5% CO2. The following substances were added: 0.01 g/L transferrin, 0.01 g/L insulin, 0.91 g/L sodium bicarbonate, 100 U/mL penicillin G potassium, 0.1 mg/mL streptomycin sulfate, 5.0 mg/mL gentamicin sulfate, 3.58 g/L HEPES, and 1.75 g/L D-glucose.72,23,25 The cells propagated rapidly before passage 5; they were seeded at a density of 5000 cells/mL and grown to 80% confluence before treatment to prevent contact inhibition.26

Test Solutions

By diluting commercial BA (99.8%; Riedel-deHäen, Seelze, Germany) with the culture medium, we prepared five concentrations of BA test solutions: 0.0225, 0.225, 0.9, 3, or 9 mg/mL. The rationale for selecting these concentrations was as follows. Most commercially available TA suspensions used for the eye (e.g., Kenalog; Bristol-Myers Squibb, Princeton, NJ; Kanacort-A; Bristol-Myers Squibb, Taipei, Taiwan), contain 40 mg TA in a 1-mL vial preserved with 9 mg/mL BA. During TA-assisted macular hole surgery, if the TA suspension is injected without removing its vehicle, BA-preserved TA particles are sprayed onto the macula and allowed to settle for 1 minute, so the localized concentration of BA directly contacting the naked RPE is as high as 9 mg/mL. As for intravitreal injection of TA for exudative age-related macular degeneration and diabetic macular edema, if 0.1 mL of the preserved TA suspension is injected into a 4-mL vitreous cavity, the final diluted intravitreal concentration of BA is 0.225 mg/mL. We further tested its 1/10 dilution (0.0225 mg/mL).

The culture medium was removed from the RPE cell cultures, and a sufficient amount of new culture medium (control) or a BA test solution was added to overlay the cell monolayer, incubated at 37°C. Then the following assays were used to evaluate the cytotoxic effects and mechanisms of RPE cell death.

TUNEL Assay

RPE cultures in four-slide chambers were exposed to the culture medium (control) or BA solutions for 2 or 6 hours, washed with the culture medium, fixed in 4% paraformaldehyde, and then processed for TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridine–biotin nick-end labeling; Clontech Laboratories, Mountain View, CA) to identify DNA fragmentation or strand breaks. DAPI (4',6-diamidino-2-phenyl indole; Vector Laboratories, Burlingame, CA) was used as a nuclear counterstain, with little or no cytoplasmic labeling.

Propidium Iodide/Annexin V-FITC Staining and Flow Cytometry

For morphologic examination, RPE cultures in 3-cm dishes were exposed to the culture medium or 9 mg/mL BA for 2 or 6 hours, washed with the culture medium, and, without trypsinization or fixation, annexin V-FITC working solution (BD Biosciences, San Jose, CA) containing propidium iodide (PI, Sigma, St. Louis, MO) and Hoechst 33258 (Sigma) was added. After incubation at room temperature in the dark for 15 minutes, the cells were gently washed with phosphate-buffered saline and observed under a fluorescence microscope.

For quantitative measurements, the cultures in 3-cm dishes were exposed to the culture medium or BA solutions 0.0225–9 mg/mL for 2 or 6 hours and washed with the culture medium. Then 2 × 10⁶ cells were resuspended in 200 μL binding buffer, and PI and annexin V-FITC (BD Biosciences) were added, followed by incubation on ice in the dark for 15 minutes and analysis on a flow cytometer (FACScan; BD Biosciences) for calculating the proportions of necrotic versus apoptotic cell death.27

Caspase Activation Assay

Cellular caspase activation was determined (Caspase-Family Colorimetric Substrate Set Plus; BioVision, Mountain View, CA) for caspases 1, 2, 3, 5, 6, 8, and 9. The optical density (OD) at 405 nm was measured using an ELISA plate reader (Pharmacia Biotech, Uppsala, Sweden). RPE cultures in 24-well plates were exposed to the control solution or 9 mg/mL BA for 30 minutes or 1, 2, 4, or 6 hours. The OD ratio of BA/control represented the fold of caspase induction.

Caspase and Apoptosis Inhibition Assay

Apoptosis, characterized by DNA fragmentation, was analyzed by PI staining and flow cytometry.29–31 In brief, after exposure to the culture medium or 9 mg/mL BA for 2 hours, the RPE cells were trypsinized, fixed with 70% ethanol in phosphate buffer solution, stained with PI/RNase working solution in PBS containing 40 μg/mL PI and 100 μg/mL RNase A (Sigma) for 30 minutes at room temperature, and then analyzed using flow cytometry. Apoptotic cells displayed a hypodiploid sub-G0/G1 (M1) peak, which was discriminated from the peak of living cells with normal (diploid) DNA content.

Whether the activated caspases played a role in the mechanism of cell death was studied by pretreatment with caspase inhibitors.29–31 Before exposure to 9 mg/mL BA, the cultures were pretreated for 1 hour with 2 μM of caspase inhibitors (BioVision): z-YVAD-fmk (caspase-1 inhibitor), z-VDVAD-fmk (caspase-2 inhibitor), z-DEVD-fmk (caspase-3 inhibitor), z-VEID-fmk (caspase-6 inhibitor), z-IETD-fmk (caspase-8 inhibitor), z-LEHD-fmk (caspase-9 inhibitor), or z-VAD-fmk (broad-spectrum caspase inhibitor). The percentages of apoptotic cells in the pretreated cultures were compared with nonpretreated cultures.

Similarly, to determine the roles of mitochondria and reactive oxygen species (ROS) in the apoptotic pathway, 10 or 20 μM cyclosporine A (a stabilizer of mitochondria; Sigma) and 10 μM diphenylethenidonium (DPI, an inhibitor of ROS; Sigma) were used for pretreatment before exposure to 9 mg/mL BA and measurement of the percentages of apoptotic cells.

Production of ROS

Immediately after addition of the culture medium or 9 mg/mL BA, the RPE cultures were put in the chamber of a luminol-enhanced chemiluminescence (CL) analyzer (Tohoku Electronic Industrial Co., Miyagi, Japan) to measure the production of superoxide.32 Background CL was recorded during the first 10 minutes, and then luminol (Sigma) was added at a final concentration of 0.2 mM to react with superoxide and generate CL. The CL counts were recorded every 10 seconds for 30 minutes.

Mitochondrial Transmembrane Potential Assay

RPE cells after exposure to the culture medium or 3 or 9 mg/mL BA for 2 hours were incubated with rhodamine 123 (Sigma) at a final concentration of 5 μM for 30 minutes, washed with phosphate buffer solution, and analyzed using flow cytometry to represent the mitochondrial transmembrane potential (ΔΨ̅m).29–31 The percentage of ΔΨ̅m reduction indicated the severity of mitochondrial dysfunction.

To further determine the effects of decreasing the ΔΨ̅m reduction, cultures were pretreated with DPI, z-IETD-fmk, or z-VAD-fmk, before exposure to 9 mg/mL BA.

Apoptosis-Inducing Factor/DAPI Staining

After exposure to the medium or 9 mg/mL BA for 2 or 6 hours, the cultures were fixed and treated with rabbit anti-apoptosis-inducing factor (AIF) polyclonal antibody (BioVision), goat polyclonal secondary antibody to rabbit IgG (DyLight 488; Abcam Inc., Cambridge, MA), and DAPI. Fluorescence microscopy was used to assess the translocation of AIF from the cytoplasm to the nucleus.

Statistical Analysis

For PI/annexin V-FITC staining and flow cytometry, caspase and apoptosis inhibition assay, ROS production, and ΔΨ̅m assay, five dishes/wells were exposed to each test/control solution for each time period; for the caspase activation assay, four wells were used. Results were entered into a worksheet (Excel, Office 2003; Microsoft Corp., Redmond, WA), and statistical differences between the study and control
groups were evaluated using an unpaired Student’s t-test with the level of significance set at $P < 0.05$.

**RESULTS**

**BA-Induced Apoptosis in RPE Cells Indicated by TUNEL Staining**

On TUNEL staining, no apoptosis was found among cells exposed to culture medium (control) or 0.0225–0.225 mg/mL BA. In contrast, some of the cells exhibited faint TUNEL-positive, after exposure to 0.9 mg/mL BA for 6 hours or 3 mg/mL BA for 2 or 6 hours. Moreover, most of the cells exposed to 9 mg/mL BA for 2 or 6 hours exhibited strong TUNEL-positive (Fig. 1).

**Apoptosis and Necrosis Assessed by Propidium Iodide/Annexin V-FITC Staining and Flow Cytometry**

On morphologic examination of PI/annexin V-FITC/Hoechst triple staining, annexin V-FITC was present in the cultures exposed to 9 mg/mL BA for 2 or 6 hours but was absent from the control cultures (Fig. 2), indicating that the cells exposed to BA underwent early apoptosis.

PI/annexin V-FITC flow cytometry showed that 96.5% of cells in the culture medium remained alive, and only minor proportions were single PI-positive (necrotic; 1.7%), single annexin V-FITC-positive (early apoptotic; 0.5%), or double staining-positive (apoptotic necrotic; 1.2%). Exposure to concentrations of BA ≤3 mg/mL for 2 or 6 hours only mildly increased the proportion of damaged cells; not >4.6% (necrotic), 3.8% (early apoptotic), and 5.2% (apoptotic necrotic). However, 9 mg/mL BA led to 10.3% necrotic cells after 2 hours, and 19.0% early apoptotic cells after 6 hours, and 64.2–71.4% apoptotic necrotic cells after 2 to 6 hours of exposure (Fig. 3), indicating that BA induced both necrosis and apoptosis.

**BA-Activated Caspase-8**

BA at 9 mg/mL significantly increased the activity of caspase-8 in the RPE cultures, leading to 1.23-fold induction after 30 minutes of exposure, and lasting up to 6 hours of exposure (Fig. 4), indicating the involvement of caspase-8 in BA-induced apoptosis.

**Roles of Caspases, ROS, and Mitochondria Revealed by Caspase and Apoptosis Inhibition Assays**

From the flow cytometry of PI-stained cells, the percentage of sub-$G_0/G_1$ (apoptotic) cells was 14.0% after exposure to 9 mg/mL BA for 2 hours, compared to 2.2% for medium alone ($P < 0.05$). The percentages of BA-induced apoptotic cells were statistically reduced by pretreatment with the inhibitors of caspases 1, 2, 3, 8, and 9, or with the broad-spectrum caspase inhibitor. Among these, z-IETD-fmk (caspase-8 inhibitor) and z-VAD-fmk (broad-spectrum inhibitor) greatly decreased the percentages of apoptotic cells, indicating a key role of caspase-8 in caspase-dependent apoptosis (Fig. 5).

The percentages of apoptotic cells were also reduced by pretreatment with DPI and cyclosporin A (CSA), indicating the involvement of ROS and mitochondria in cell death.

**BA Triggered the Production of ROS**

In the CL analysis, 9 mg/mL BA greatly increased the production of ROS, mainly within 15 minutes with a peak at 3 minutes. However, this induction was significantly reduced by pretreatment with the ROS inhibitor, DPI (Fig. 6).
Mitochondrial Involvement Downstream to ROS and Caspase-8 Indicated by the Mitochondrial Transmembrane Potential Assay

Exposure to 3 and 9 mg/mL BA for 2 hours led to 25.7% and 55.6% (both \( P < 0.05 \)) reduction in a dose-dependent manner, compared to 14.1% after exposure to the medium alone (Fig. 7). Furthermore, DPI, the caspase-8 inhibitor, and the broad-spectrum caspase inhibitor were similarly effective in greatly decreasing the \( \Delta \Psi_m \) reduction (29.9%, 37.0%, and 37.2%, respectively; all \( P < 0.05 \)), indicating mitochondrial involvement downstream to ROS and caspase-8 in the pathway of BA-induced cell death.

BA-Induced Nuclear Translocation of Apoptosis-Inducing Factor

In the cells exposed to culture medium alone, AIF was distributed in the cytoplasm (Fig. 8 and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6058/-/DCSupplemental). However, in the cells exposed to 9 mg/mL BA for 2 hours, green fluorescence was seen within the nuclei. In the cells exposed for 6 hours, green fluorescence was marked within the nuclei, indicating translocation of AIF from the cytoplasm into the nuclei.

DISCUSSION

Our previous study showed dose- and time-dependent cytotoxicity of BA in cultured human RPE cells by decreasing cell viability, impairing mitochondrial dehydrogenase function, and inducing ultrastructural changes including organelle swelling and BA accumulation within the cells. In the present study, we further demonstrated that BA caused RPE cell death not only by necrosis but also by apoptosis. Moreover, we showed that BA induced immediate production of ROS, activated the caspases,
impaired the mitochondrial transmembrane potential, and led to the translocation of AIF into the nucleus. To our knowledge, these findings have never been reported and have significant clinical implications for the safe use of intravitreal TA suspensions.

Preservatives in Ophthalmic Formulations

Ophthalmologists have long been familiar with preservatives commonly used in commercial eye drops, such as benzalkonium chloride, chlorobutanol, methyl paraben, sodium perborate, and thimerosal, which provide antimicrobial effects in the bottle to prevent contamination, to maintain the potency, and to prolong the half-life of the drug by reducing biodegradation. In fact, all these preservatives have been reported to be more or less toxic to cultured corneal or conjunctival epithelial cells. BA has not been used as the preservative in topical ophthalmic solutions because it is irritating, has slow activity, and dissolves polystyrene. Actually, the commercially available TA formulations used in the eye were initially developed for intra-articular or intramuscular injection and not manufactured specifically for ocular use. Because TA, formulated as suspended particles, is a long-lasting synthetic corticosteroid with good anti-inflammatory, anti-proliferative, anti-angiogenesis, and anti-permeability effects, it is now used in a variety of vitreoretinal/macular diseases or surgery, mainly administered by intravitreal or sometimes sub-Tenon's capsule injection. This novel application raises the concern of ocular toxicity by its added preservative, BA.

Systemic and Ocular Toxicity of BA

BA, an organic compound with the formula C₆H₅CH₂OH, is used as a solvent for inks, paints, lacquers, and epoxy resin coatings due to its polarity. It is also a precursor to a variety of esters, used in the soap, perfume, and flavor industries. In some medications, BA is added as the preservative and solvent, and, when injected into the body, it is normally oxidized to benzoic acid and conjugated with glycine in the liver, and then excreted as hippuric acid. Clinically, the previously adopted but nowadays abandoned use of 0.9% BA-preserved intravenous saline flush solutions in premature infants was reported to cause a fetal toxic syndrome, gasping syndrome, intraventricular hemorrhage, neurologic handicap, and even 16 neonatal deaths, due to the immature metabolizing function of the neonatal liver, leading to accumulation and prolonged detrimental effects of BA. BA was also found to cause neurotoxicity in some experimental animal studies, in which cisterna magna injection damaged the central nervous system in dogs, and intrathecal injection caused demyelination and axonal degeneration in the cauda equina of rats. When BA-preserved TA was used for vitreoretinal diseases, it was presumed to...
contribute to noninfectious endophthalmitis in 1.1%–23.8% of patients after intravitreal injection.40–42 This was supported by two large-scale studies in which none of the patients developed ocular inflammation after intravitreal injection of BA-free TA suspension.43,44 Because the vitreous cavity is a gel within a closed space without active venous or lymphatic drainage, we speculated that BA injected here may damage ocular tissues and induce the secretion of cytokines and inflammation within the eye. We therefore suggest removal of the vehicle before intraocular use of TA by any of the three methods, standstill/sedimentation, density-gradient centrifugation, or filtration, as previously described.21–23

In Vivo Retinal Necrosis and Apoptosis Caused by BA

Our previous and present work focused on BA toxicity in RPE cells because their survival and health are essential for maintaining the normal function of photoreceptors. In fact, whether BA is toxic to other types of retinal cells such as photoreceptors, bipolar cells, or ganglion cells is another important issue, since BA molecules are small (molecular weight 108 Da), lipophilic, and can easily diffuse through the entire retina after intravitreal injection. This concern is supported by several rabbit studies, in one of which Morrison et al.45 reported that intravitreal injection of 1 mg BA led to retinal whitening and hemorrhage, photoreceptor shortening, and RPE proliferation. Similarly, Macky et al.46 reported a rabbit study in which intravitreal injection of ~1 mg BA produced severe electroretinographic reduction as well as structural damage to the ganglion cells, inner and outer nuclear layers, and photoreceptors. Li et al.47 reported that rabbit eyes intravitreally injected with TA vehicles containing 1–2 mg BA resulted in retinal necrosis and atrophy and ultrastructural damage to the photoreceptors, including nuclear pyknosis/karyolysis (suggesting photoreceptor apoptosis) and swelling or rupture of mitochondria and discs. These animal studies support our in vitro findings and indicate that BA is detrimental to various kinds of retinal cells.48 On the other hand, Ruiz-Mereno et al.49 have reported no morphologic or functional changes in rabbit eyes intravitreally injected with ~1 mg BA.

Molecular Mechanisms of BA in Apoptotic Signaling Pathways

Although both BA and the TA vehicle have been shown to damage cultured RPE cells,22,23,26,50,51 relevant research regarding their molecular mechanisms and signaling pathways is scanty. We previously found that BA is toxic to various types of retinal cells such as photoreceptors, bipolar cells, or ganglion cells. However, our in vitro findings and in vivo studies support the notion that BA is detrimental to various kinds of retinal cells.21–23 We previously reported that intravitreal injection of 1 mg BA led to retinal necrosis and atrophy and ultrastructural damage to the photoreceptors, including nuclear pyknosis/karyolysis (suggesting photoreceptor apoptosis) and swelling or rupture of mitochondria and discs. These animal studies support our in vitro findings and indicate that BA is detrimental to various kinds of retinal cells.48 On the other hand, Ruiz-Mereno et al.49 have reported no morphologic or functional changes in rabbit eyes intravitreally injected with ~1 mg BA.

Being lipophilic, BA changes the fluidity of cell membranes in leukemic T-lymphocytes and myeloid cell lines, increases ceramide generation, and further triggers apoptosis mediated by caspases 8 and 3.52 Barak et al.53 showed that ceramide is a potential mediator of RPE apoptosis induced by oxidants or reactive oxygen intermediates. Although RPE cells have physiological antioxidants and enzymes against the oxidative damage that occurs in diseases such as age-related macular degeneration, our results showed that the high level of ROS induced by BA occurred within a very short time, thus leading to acute disruption of cell membranes and organelles, and cell death (i.e., necrosis), as shown in our previous ultrastructural studies.21,23 We found that in some of the cells that escaped acute
cellular damage, ROS triggered apoptosis through sequential activation of caspases.

Mitochondria are very important in the regulation of apoptosis because they are not only involved in the production of ROS through electron carriers in the respiratory chain but also very susceptible to oxidative stress as evidenced by lipid peroxidation, protein oxidation, and mitochondrial DNA mutations. In other words, mitochondria play dual roles as the generators and the targets of oxidative stress. Subsequent release of cytochrome c from the intermembrane space into the cytoplasm and opening of the permeability transition pore are critical events in the apoptotic cascade. In our study, DPI (an inhibitor of ROS) significantly, although not completely, inhibited the production of ROS and decreased the level of reduction, indicating that ROS induced by BA damaged the mitochondria. This mitochondrial injury, however, could induce further production of ROS, leading to a vicious cycle of cell death.

Pathways to apoptotic cell death are distinguished based on the involvement (caspase-dependent) or exclusion (caspase-independent) of activated caspase enzymes. The activation of initiator caspase-8 or -9 stimulates the activation of effector caspase-3, which further induces cleavage of poly (ADP-ribose) polymerase, proteolytic degradation of various cellular targets, and activation of endonucleases, ultimately leading to cell death. In this study, we found that suppression of caspase activity with inhibitors decreased the RPE apoptosis induced by BA, suggesting the caspase-dependent pathway was involved. Furthermore, ROS and mitochondria participate in this process of cell death.

AIF, an apoptogenic protein normally distributed in mitochondria, is specific to caspase-independent apoptosis and helps to discriminate between the caspase-dependent and -independent pathways, since they share common key features of apoptotic death, such as rearrangement of cell architecture, loss of ΔΨm, and DNA fragmentation. We demonstrated that BA triggered the release of AIF from the cytoplasm and its translocation into the nucleus, where it initiates DNA cleavage and chromatin condensation. However, some cross-talk exists between the caspase-dependent and -independent pathways. For example, the release of AIF affects the barrier function of mitochondria, thus facilitating cytochrome c release and activation of caspase-9.

Taking our previous and current studies together, we present a summary of the proposed molecular mechanisms and signaling pathways of BA-induced RPE cell death (Fig. 9).

**Model of BA-Induced RPE Cell Death**

Cellular resistance to various kinds of stress (such as oxidation, heat, and toxic agents) is influenced by the type of cell and the duration of culture. The 80% confluent culture used in this study prevents contact inhibition and is suitable for the analysis of cell proliferation or cell death, but the cellular stability may be lower than that in the in vivo environment. In contrast, cultured RPE cells in a monolayer usually become more stable after reaching total confluence with a longer cultivation time and theoretically are more resistant to stress. To investigate the effect of confluence on the BA-induced toxicity, we did a similar experiment with PI/annexin V-FITC staining and flow cytometry on totally confluent cultures. The results were consistent with and not significantly different from those with 80% confluent cultures (data not shown).

**CONCLUSIONS**

This study showed that BA induced immediate production of ROS, which not only led to necrosis of RPE cells but also triggered mitochondrial apoptosis through both caspase-dependent and -independent mechanisms, the latter involving...
translocation of ALF into the nucleus. The limitation of this study is that in vitro BA cytotoxicity cannot be directly extrapolated to clinical practice, but cultured cells offer the advantage of precise manipulation of the environment, thus providing a good model system for defining RPE cell responses to BA. The most effective strategies for a safe administration of intravitreal TA will emerge from a thorough understanding of the molecular events associated with the multiple signals that lead to necrotic and apoptotic cell death. We suggest extreme caution in intraocular use of TA suspensions, further research regarding the mechanisms of in vivo BA toxicity in the retina, and meticulous evaluation before adoption of BA as a preservative in the future development of ophthalmic formulations.

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