Depletion of Intracellular Zinc and Copper with TPEN Results in Apoptosis of Cultured Human Retinal Pigment Epithelial Cells

Hyae Jung Hyun,1 Joon Hong Sohn,2 Dong Wook Ha,2 Young Ho Ahn,3 Jae-Young Koh,1,4 and Young Hee Yoon2

PURPOSE. Although zinc deficiency may contribute to the pathogenesis of age-related macular degeneration, how it leads to retinal pigment epithelium (RPE) degeneration is unknown. To investigate this, cultured human RPE cells were rendered zinc depleted with a membrane-permeant metal chelator, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), and the resultant cytopathic changes were examined.

METHODS. RPE cell degeneration was examined with light microscopy, TdT-mediated dUTP nick end labeling (TUNEL) staining, Hoechst dye staining, and electron microscopy and quantified with cell counting or lactate dehydrogenase release assay. The effect of sublethal zinc depletion on the vulnerability of RPE cells to UV irradiation or hydrogen peroxide (H2O2) exposure, was studied in cultures without or with pretreatment with low-concentration TPEN.

RESULTS. Exposure to 1 to 4 μM TPEN for 48 hours induced RPE cell death in a concentration-dependent manner. Features of apoptosis such as membrane blebbing, chromatin condensation, nuclear fragmentation, and caspase-3 activation, accompanied the TPEN-induced cell death. Addition of equimolar zinc or copper completely reversed TPEN-induced apoptosis, whereas addition of iron had no effect. As in apoptosis of several other cell types including neurons, a protein synthesis inhibitor as well as caspase inhibitors blocked TPEN-induced apoptosis. On the contrary, at sublethal concentrations, TPEN increased the vulnerability of RPE cells to subsequent UV irradiation but not to H2O2 exposure.

CONCLUSIONS. The present results suggest that depletion of intracellular zinc and copper, but not copper alone, may be harmful to RPE cells, directly inducing apoptosis or indirectly increasing vulnerability of RPE cells to UV injury. The present culture model may be useful for gaining insights into the mechanisms of zinc depletion-associated RPE cell degeneration. (Invest Ophthalmol Vis Sci. 2001;42:460–465)

Retinal pigment epithelium (RPE) serves many supportive functions for the overlying neural retina.1,2 For examples, RPE continually phagocytizes shed rod outer segments (ROSs), which is critical for normal regeneration of the ROSs.3-5 In addition, melanin pigments in RPE absorb light, prevent excessive light scattering, and protect the eye from oxidative stress.6-7 Metabolically, carbonic anhydrase, ion transporters, and ion channels in RPE help maintain acid-base and electrolyte balances of the neural retina.8,9 RPE cells also secrete growth factors essential for proper differentiation of photoreceptors during development.10 Structurally, RPE cells form intercellular tight junctions, which serves as an effective diffusion barrier.8,11

Considering the diverse supportive functions that RPE provides for the retina, it is not surprising that dysfunction or degeneration of RPE causes significant problems in vision. In several forms of hereditary diseases causing RPE degeneration, overlying retinal neurons eventually fail to thrive, resulting in vision loss.1,12-15 RPE dysfunction and degeneration accompanied by neural retinal degeneration, are also seen in ARMD.14,15 which is one of the leading causes of vision loss in the elder population.16 ARMD is classified into two types; nonneovascular and neovascular.17,18 The former is more prevalent, but the latter causes more severe deficits. Common pathologic features in both types are drusen deposition and pigmentary alterations of RPE.

The precise pathogenic mechanism of ARMD is currently unknown. However, photic injury and oxidative stress are proposed as possible contributing mechanisms. In addition to these, for the past three decades, zinc deficiency also has been suspected as a potential risk factor.19-21 First, patients with ARMD have low levels of macular zinc.22,23 Second, although there is counter-evidence,24,25 some studies report that oral zinc supplementation ameliorates the symptoms of ARMD.20,26-28 Thus, the possibility that zinc deficiency contributes to the pathogenesis of ARMD20,22,26,29 may warrant further investigation. If zinc deficiency has a role in the pathogenesis of ARMD, it may be important to know in which way zinc deficiency negatively influences RPE cells, the main cell type affected by ARMD.

It has been demonstrated recently that zinc depletion may increase oxidative stress in RPE cells, possibly by decreasing the activity of antioxidant enzymes such as catalase and glutathione peroxidase.29,30 Also, zinc deficiency may cause deficits in phagocytic and lysosomal functions,31,32 which may further derange the homeostasis of photoreceptors. Besides these functional changes, zinc deficiency may directly induce degeneration of RPE cells. Consistent with this possibility, intracellular zinc chelators have been shown to induce apoptosis of thymocytes and cortical neurons.33-35 In the latter, zinc depletion causes caspase- and macromolecule-synthesis-dependent apoptosis. Therefore, in the present study, we sought to examine whether the cell membrane-permeant zinc chelator N,N,N',N'-tetakis(2-pyridylmethyl) ethylenediamine (TPEN) induces or modulates death of cultured human RPE cells.

MATERIALS AND METHODS

Human RPE Cell Culture

Whole human eyes were obtained from Asan Medical Center (Seoul, Korea) and harvested within 24 hours after the patient’s death. Human
eyes were used in accordance with applicable laws and with the tenets of the Declaration of Helsinki. According to a previously described method, 46 eyes were opened 360° posterior to the ora serrata, and the vitreous and the retinal tissues were removed. The remaining eye cups were rinsed with phosphate-buffered saline (PBS) and incubated in 0.25% trypsin in Dulbecco’s minimum essential medium (DMEM; Gibco, Grand Island, NY) for 30 minutes at 37°C. After trypsinization, the human RPE cells were placed in DMEM supplemented with 10% fetal bovine serum (FBS) and triturated into single cells with gentle pipetting. Cells were transferred to tissue culture flasks (Nunc, Roskilde, Denmark) containing DMEM supplemented with 20% FBS and placed in a 5% CO₂ incubator (37°C). After proliferation, cells were retrypsinized with a 0.1% trypsin-EDTA solution (Sigma, St. Louis, MO) for 5 minutes at 37°C. After triple washes with DMEM, cells were plated in 24-well plates (Nunc) at 2 × 10^4 cells/well and allowed to grow to confluence for 7 to 10 days. Third- or fourth-passage cells were used for experiments.

**Exposure to TPEN and Other Drugs**
TPEN, ZnCl₂, CuCl₂, FeCl₂, trolox, and cycloheximide (CHX) were purchased from Sigma. Carbobenzoxy z-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD-fmk) and z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (DEVd-fmk) were purchased from Enzyme Systems Products (Livermore, CA). Cells were exposed to 0.25 to 4 μM TPEN and other drugs in serum-free culture medium (Eagle’s minimum essential medium, Eagle’s salts, supplied glutamine-free). Before the exposure, preexisting medium was washed out several times and replaced with the serum-free medium. Exposure to TPEN and other drugs was accomplished by the addition of desired volumes of stock solutions to the serum-free exposure medium. Control sister cultures underwent identical media change procedures except exposure to TPEN. Eighteen different populations of cultures were used for experiments.

**UV Irradiation**
Cells were irradiated for 1 to 5 minutes with a UV lamp (UL 200; Hoya–Schott, Tokyo, Japan; main output at 254 nm) with an intensity of 5.28 mW/cm² (calibrated with a UV sensor [VLX-254; CALVLXCA] at the plane of exposure).

**Estimation of Cell Death**
Cell death was morphologically assessed under the phase-contrast microscope. For quantification, dead cells that were stained with trypan blue (0.4%; 20 minutes) and live cells that were not stained were counted in five ×200 fields (area for each field, 0.785 mm²) in each well; the fields were randomly chosen before the counting. Percentage of cell death in each field was calculated by dividing the number of dead cells by the number of total cells (dead and live). In addition, for most experiments, overall cell death was quantified by measuring lactate dehydrogenase (LDH) released from injured cells into the medium. 57 LDH activity in the medium was estimated using an automated microplate reader (UVMax; Molecular Devices, San Francisco, CA) by measuring the rate of decrease in absorbance at 340 nm. 57 All LDH values, after subtraction of background value in sham wash control cultures, were normalized to the mean maximal value (100) in sister cultures exposed for 48 hours to 5 μM TPEN, which causes complete cell death. Two methods, cell count and LDH release assay, were highly correlated.

**TUNEL Staining**
TdT-mediated dUTP nick end labeling (TUNEL) of cultures was performed according to the manufacturer’s protocol (In Situ Cell Death Detection Kit; Rohering–Mannheim, Mannheim, Germany). Briefly, cultures were fixed in 4% paraformaldehyde for 30 minutes at room temperature and then incubated with TUNEL mixtures containing TdT and fluorescein-labeled dNTP for 1 hour at 37°C. Incorporated fluorescein was detected by anti-fluorescein antibody conjugated with horse-radish peroxidase. After substrate reaction, stained cells were analyzed under a light microscope.

**Transmission Electron Microscopy**
 Cultures were fixed in 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer (pH 7.4). Cells were postfixed in 2% buffered osmium tetroxide. After staining en bloc in 0.4% uranyl acetate, cultures were dehydrated serially through increasing concentrations of ethanol and embedded in Epon resin (EMbed812; Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections (70 nm) were prepared on a microtome (Ultracut J; Reichart–Jung, Vienna, Austria), picked up on collodion-coated copper grids, and double stained with 0.4% uranyl acetate and 2% lead citrate. After carbon coating, the samples were photographed under an electron microscope (1200EX-II; JEOL, Tokyo, Japan).

**Western Blot Analysis for Caspases**
Polyclonal rabbit antibodies to caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Cells were washed with ice-cold PBS and lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein levels were measured by a protein assay kit (Bio-Rad, Richmond, CA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 15% PAGE) and transferred onto polyvinylidene difluoroide (PVDF) membrane (Schleicher & Schuell, Dassel, Germany). Membrane was blocked with 5% nonfat dry milk for 1 hour and incubated with primary antibody for overnight. The secondary antibody was goat anti-rabbit IgG (Amersham–Pharmacia Biotech, Uppsala, Sweden) conjugated to horseradish peroxidase. Enhanced chemiluminescence (ECL; Amersham-Pharmacia Biotech) was used for the detection of protein signals. Enhanced luminescence of luminol by peroxidase-catalyzed oxidation was detected by autoradiography (Hyperfilm ECL; Amersham–Pharmacia Biotech).

**RESULTS**
Approximately 24 hours after the onset of exposure to 2 μM TPEN, many of cultured human RPE cells changed the cell morphology from broad, flat shapes (Fig. 1A) to more fibrous shapes (Fig. 1B). At 48 hours, most cells lost their membrane integrity (Fig. 1C) and were stained with trypan blue (Fig. 1D). The concentration–cell death relationship was obtained by cell counting and LDH release assay, as described earlier, after 48 hours’ exposure to varying concentrations of TPEN (Fig. 1E). The percentage of cell death estimated by LDH release assay correlated well with that estimated by cell counting, although LDH assay tended to slightly underestimate the death compared with the cell counting assay. In both cases, however, 0.5 μM TPEN induced less than 20% of cell death by both cell counting and LDH release assays, whereas 2 μM TPEN induced approximately 80% to 90% cell death. The half maximal lethal concentration (LC₅₀) of TPEN with 48 hours of exposure was estimated to be 1.5 μM with the LDH assay.

Next, we examined whether TPEN-induced death of RPE cells occurred by apoptosis or necrosis. Staining of nuclei with Hoechst dye 33342 (Molecular Probes, Eugene, OR) revealed that the nuclei of degenerating RPE cells were condensed and fragmented (Fig. 2B compared with 2A). Furthermore, TUNEL staining showed that TPEN induced DNA fragmentation in RPE cells (Fig. 2D). Electron microscopic findings were also consistent with apoptosis. Chromatin condensation, nuclear fragmentation, and cytoplasmic compaction were seen in TPEN-treated cultures (Fig. 2F versus 2E).

**Retinal Pigment Epithelial Cell Apoptosis by TPEN**
461
On several occasions, apoptosis has been found to be sensitive to inhibitors of macromolecule synthesis. Consistently, TPEN-induced apoptosis of RPE cells was also markedly attenuated by CHX (Fig. 3A). Addition of an irreversible inhibitor of glutathione synthesis, BSO, did not reverse the CHX protection, indicating that the effect was not mediated by increases in glutathione levels. Addition of caspase inhibitors, DEVD-fmk and zVAD-fmk, almost completely blocked TPEN-induced cell death (Fig. 3B), suggesting that caspase mediates TPEN-induced apoptosis. Moreover, 32-kDa procaspase 3 was found cleaved into the active 20-kDa form (Fig. 3C).

In cell biology studies, TPEN is used mainly as a zinc-specific chelator. However, it also chelates other endogenous metals such as iron and copper. To find out whether zinc depletion in particular is the cause of TPEN-induced apoptosis, cells were exposed to TPEN (2 μM) with the addition of equimolar ferrous, zinc, or copper ions. As depicted in Figure 4, addition of zinc and copper completely abrogated TPEN toxicity, whereas iron had no protective effect. Based on the affinities of metal to TPEN (Cu²⁺, 5 × 10²⁰ M⁻¹; Zn²⁺, 4 × 10⁻¹⁵ M⁻¹; Fe²⁺, 4 × 10¹⁴ M⁻¹), these results are consistent with the idea that chelation of zinc, and possibly copper, is the mechanism of TPEN-induced apoptosis in RPE cells.

Although direct and fulminant apoptosis induced by severe zinc depletion may provide clues to the cytopathic mechanisms of ARMD, effects of less severe zinc depletion may be more relevant for chronic diseases such as ARMD. Therefore, we examined the possibility that mild zinc depletion alters the vulnerability of RPE cells to other injury mechanisms proposed to be relevant in ARMD, such as UV irradiation and oxidative stress. Indicating that two injuries may be qualitatively different, addition of the antioxidant trolox markedly attenuated oxidative injury induced by hydrogen peroxide exposure (800 μM, 48 hours), whereas it did not alleviate even mild 1-minute UV irradiation injury (Fig. 5A). Exposure of RPE cells to 0.5 μM TPEN for 48 hours induced little cell death by LDH release assay (Fig. 1E). However, subsequent exposure of TPEN-treated cells to UV irradiation induced markedly increased death (Fig. 5B). In control cultures, UV irradiation for 1 minute induced only approximately 20% cell death, whereas TPEN-treated cultures, the identical exposure induced more than 50% cell death. By contrast, pre-exposure to 0.5 μM TPEN did not alter the vulnerability of RPE cells to H₂O₂ exposure (Fig. 5C).

## DISCUSSION

The central findings of the present study are that a membrane-permeant metal chelator TPEN, at lethal concentrations (≥1
mM), directly induces apoptosis of cultured human RPE cells in 48 hours and at sublethal concentrations (0.5 mM), augments the vulnerability of RPE cells selectively to UV-induced damage. Although TPEN can chelate all the endogenous transition metals such as zinc, iron, and copper, TPEN-induced apoptosis is most likely caused by chelating and thus depleting intracellular zinc, and possibly copper. This conclusion can be deduced from the result that addition of equimolar zinc or copper, but not iron, blocked TPEN-induced apoptosis, and from the affinities of metals with TPEN (copper > zinc > iron).35 If the responsible metal were copper alone, then addition of zinc should not have been protective. If the responsible metal were mainly iron, then addition of iron should have reversed toxicity. However, the addition of zinc or copper, but not iron, abrogated TPEN toxicity, which indicates that depletion of zinc is probably the mechanism responsible for TPEN toxicity. However, the possibility that concomitant chelation of zinc and copper underlies TPEN toxicity cannot be ruled out by the results of the current experiments.

Because zinc depletion has been proposed as a contributing factor for ARMD, the zinc and copper depletion-triggered apoptosis of RPE cells, direct or indirect, may be an injury mechanism relevant for ARMD. Of note, a recent study found evidence for extensive apoptosis of RPE cells in ARMD.41 Zinc depletion induces apoptosis of retinal neurons, photoreceptors,42 and RPE cells, all of which are also affected in ARMD, suggesting that zinc depletion may be a common apoptosis-triggering mechanism for all the cellular elements affected in ARMD.

Although direct apoptosis of RPE cells induced by severe zinc depletion by micromolar TPEN can provide clues to the injury mechanism, more subtle zinc deficiency may be more relevant in the pathogenesis of ARMD. In this regard, it is interesting that sublethal zinc and copper depletion by nanomolar TPEN markedly increased the vulnerability of RPE cells to UV irradiation injury, another proposed risk factor for ARMD. This injury potentiation seems somewhat specific, because straightforward oxidative stress injury induced by hydrogen peroxide exposure was not at all augmented by sublethal
zinc depletion. This result is different from the report by Tate et al., who discovered increased susceptibility of RPE cells to oxidative stress including H$_2$O$_2$ toxicity. This difference may have originated from differences in the method of culture or of zinc depletion. For example, relatively brief exposure to TPEN was used to lower intracellular zinc levels in the present study, whereas exposure to low zinc media for a prolonged time was used by Tate et al. In addition to this, as discussed earlier, TPEN toxicity may involve both zinc and copper chelation. Regardless, in the current cell culture and experimental conditions, TPEN and UV irradiation injury may act synergistically to trigger RPE cell death. A possible mechanism for this is the known effect of zinc deficiency on the DNA structure, probably making it more susceptible to UV-induced DNA damage.

Although no definite cause for ARMD is currently known, zinc depletion has been proposed as an important risk factor. In the present study we have demonstrated that zinc depletion alone or in combination with copper depletion, either directly induces apoptosis or enhances the probability of apoptosis after UV irradiation, in cultured human RPE cells. This culture model may be useful in elucidating molecular events associated with zinc and copper depletion injury, which may be relevant in ARMD.

References
40. Shumaker DK, Vann LR, Goldberg MW, Allen TD, Wilson KL. TPEN, a Zn(II)/Fe(III) chelator with low affinity for Ca(II), inhibits lamin assembly, destabilizes nuclear architecture and may independently protect nuclei from apoptosis in vitro. Cell Calcium. 1998;23:151–164.