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Ceramide-Induced Cell Death in Cultured Rat Retinal Pigment Epithelial Cells

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Tomita, H., Abe, T. and Tamai, M. *Ceramide-Induced Cell Death in Cultured Rat Retinal Pigment Epithelial Cells.* Tohoku J. Exp. Med., 2000 **190** (3), 223-229 —— We investigated whether retinal pigment epithelial (RPE) responds to ceramide, a known second messenger of apoptosis. RPE cells were isolated by 6-8 day old Long Evans rat eye. We used MTS assay for viability test, and used Hoechst 33552 and propidium iodide for apoptic cell staining. In cultured rat RPE cells, the addition of membrane-permeable ceramide induced apoptosis-like cell death rapidly. RPE cell death was dependent on C2-ceramide concentration. The effective dose (ED_{50}) of C2-ceramide was 23.64 μM. Ceramide-induced RPE cell death was inhibited by zVAD-fmk, a CPP32-like protease inhibitor. Our findings indicated that ceramide in RPE cell death functions upstream of CPP32-like proteases. —— retinal pigment epithelial cell; apoptosis; CPP32
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Phagocytosis of shed rod outer segment is an important role of retinal pigment epithelial (RPE) cells. The dysfunction or cell death of RPE leads to retinal degeneration. It is thought that some cases of age-related macular degeneration (ARMD) may be caused by the degeneration of RPE. The mechanisms of the dysfunction or cell death of RPE are not clearly understood and may involve multiple factors such as degenerative changes in Bruch's membrane, damage to the choroidal vasculature, and oxidative injury (Tso 1985; Young 1987).

Apoptosis is one of two major mechanisms by which cells die; the second is necrosis. Apoptosis plays a crucial role in many pathologic processes (Arends et al. 1990), including neuro-degenerative disorders such as Parkinson's disease (Hartley et al. 1994). It has been reported that apoptosis in stromal RPE cells, endothelial cells, and occasional macropharges is shown in highly vasculized choroidal neovascular membrane related to ARMD. Apoptosis is associated with prominent Fas and FasL expression (Trauth et al. 1989; Watanabe-Fukunaga et al. 1992; Hanabuchi et al. 1994; Kneitz et al. 1995).

The Fas antigen is a member of a family of cell surface receptors that includes

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tumor necrosis factor-receptor (TNF-R) (Smith et al. 1994), and nerve growth factor receptor (p75) (Berg et al. 1991; Chao 1994). The members of apoptosis-induced receptor family share a cytoplasmic "death domain" (Itoh and Nagata 1993), which is critical for coupling to the caspase cascade (Nagata 1997). Ceramide has recently emerged as a second messenger molecule that induces multiple cellular responses such as cell cycle arrest, differentiation, and apoptosis (Kim et al. 1991; Bielawska et al. 1992, Obeid et al. 1993). Ceramides may participate in mediating some of the actions of extracellular agents such as TNF (Dressler et al. 1992; Dbaibo et al. 1993; Tepper et al. 1995) and Fas (Cifone et al. 1994; Tepper et al. 1995). However, the function of ceramide in RPE cells has not been investigated. In this study, we investigated the effect of ceramide on RPE.

Materials and Methods

All experiments conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of animals in Ophthalmic and Vision Research.

Cell preparation and cell culture

RPE cells were isolated using a method previously described (Yamaguchi et al. 1993). In brief, the eyes of Long Evans rats (6 to 8 days old) were enucleated, and the whole eyeballs were incubated at 37°C in two enzyme-containing solutions. The first incubation was for 40 minutes in Hank's balanced salt solution (HBSS, pH 7.0), containing 78 U/ml of collagenase (Wako, Tokyo) and 38 U/ml of hyaluronidase (Wako). The second incubation was for 50 minutes in HBSS containing 0.1% trypsin (1 : 250; Sigma, St. Louis, MO, USA). Eyes were placed in growth medium containing Ham's F12 (Gibco BRL, Bethesda, MD, USA) supplemented with 20% fetal bovine serum (FBS) and antibiotics (100 units penicillin, 0.1 mg streptomycin, and 0.25 μg amphotericin B per milliliter of medium). The anterior segment, lens, and vitreous were removed, and then the retina with the attached RPE was peeled away from the posterior eye segment. The retina-adherent RPE was incubated in growth medium at 37°C for 1 hour. The RPE was collected into a conical centrifuge tube, centrifuged at 1000 $\times$ g, and washed with growth medium. The RPE was dissociated mechanically into a single cell suspension by trituration using a Pasteur pipette. The medium was changed every 3 days. The cells were passaged by 0.125% trypsine/0.01% EDTA digestion. Subconfluent cells were used for all experiments. Serum-free F12 medium was used for all experiments of ceramide effect on RPE cells.

Viability test

One day before the cell viability test, rat cultured RPE cells were resuspended in 20% FBS/F12 medium and seeded in a 96-well plate at a viable cell density

of 1×10^4 cells/well. Confluent cells were washed with serum-free F12 medium and were exposed to various concentrations of C2-ceramide (Cayman Chemical, Ann Arbor, MI, USA) in serum-free F12 medium containing 1% DMSO. We performed on the cell viability test at 24 hours after C2-ceramide application.

RPE cells were preincubated for 3 hours in various concentrations of CPP32-like inhibitor zVAD-fmk (Enzyme System Products, Livermore, CA, USA). RPE cells were washed, and then 100 μM C2-ceramide was applied with zVAD-

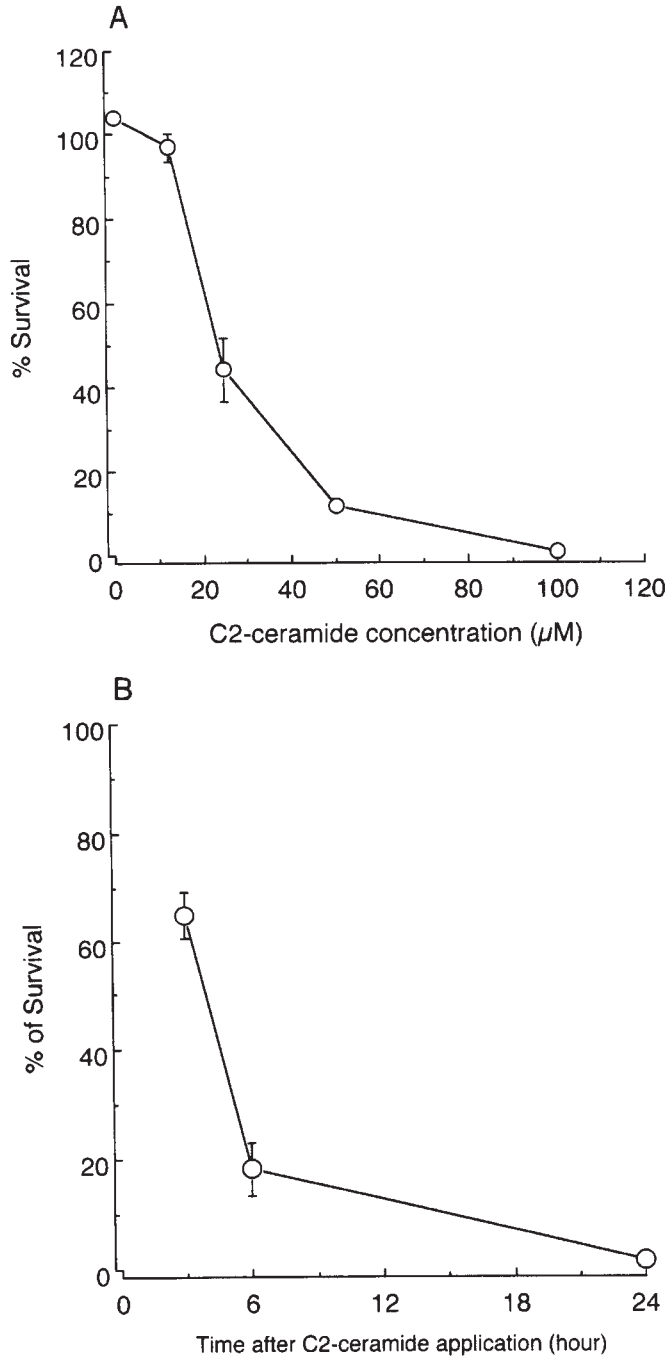

Fig. 1. RPE cell death was caused by ceramide application. A, concentration-dependent RPE cell cytotoxicity of ceramide at 24 hours after application. B, Time course of RPE cell death after application of ceramide. MTS assay was used for cell viability test. Data show mean $\pm$ S.D. of each 6 samples.

fmk. A viability test was performed at 6 hours after C2-ceramide application.

We used MTS† assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) for determing the number of viable cells. At the indicated time after the C2-ceramide application, 20 μl of one solution reagent was added to each well of the 96-well plate. After incubation at 37°C in humidified 5% CO_2 atmosphere for 1 hour, we recorded the absorbance at 490 nm using a 96-well plate reader.

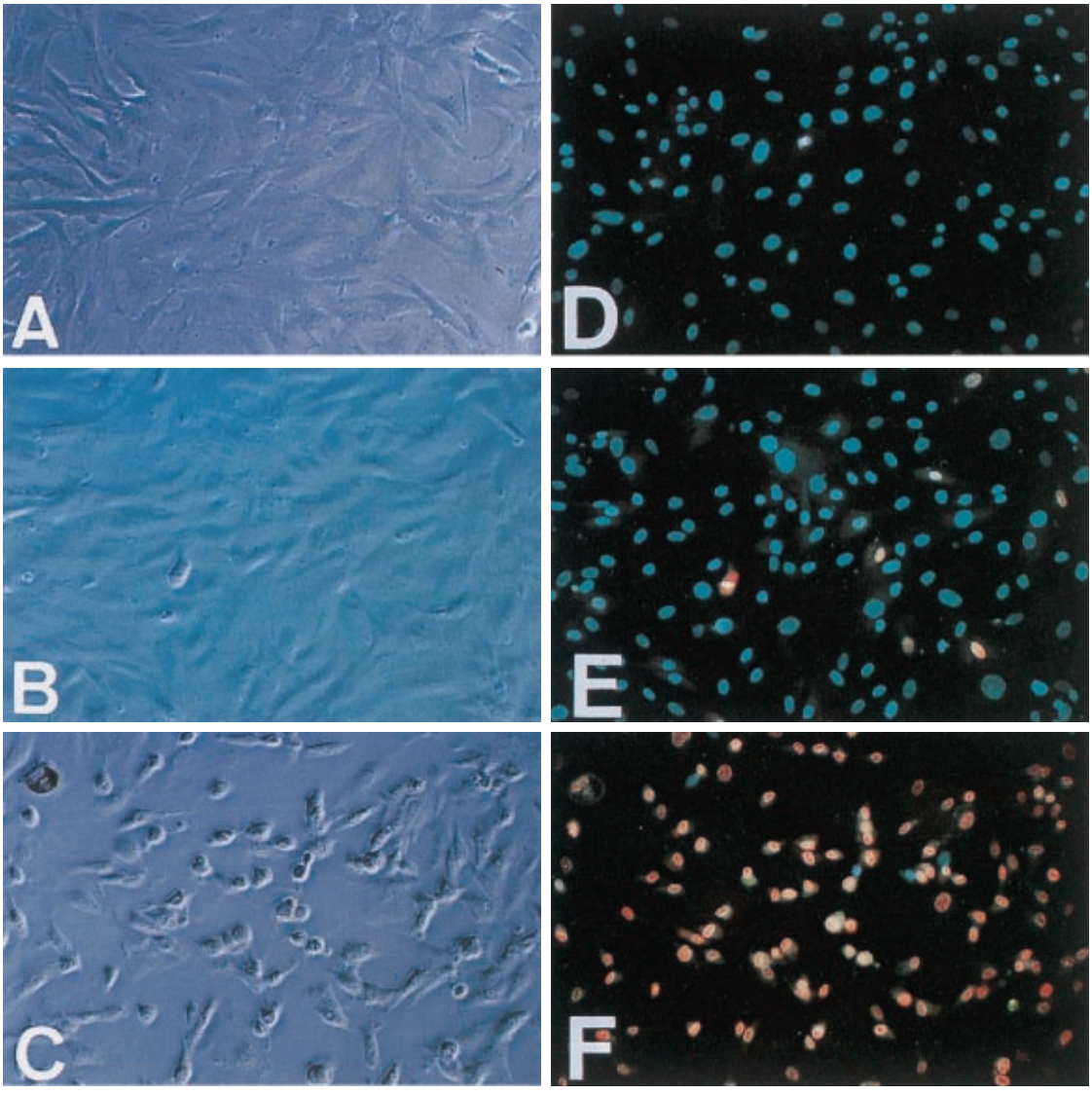

Fig. 2. Photographs of cultured rat RPE cells exposed to ceramide for 3 (B, E) or 6 hours (C, F) and control cells (A, D). A, B, and C were photographed using phase contrast microscopy. D, E, and F were stained with Hoechst 33552 and propidium iodide and photographed using fluorescent microscopy.

† MTS: 3-(4, 5-dimenthylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

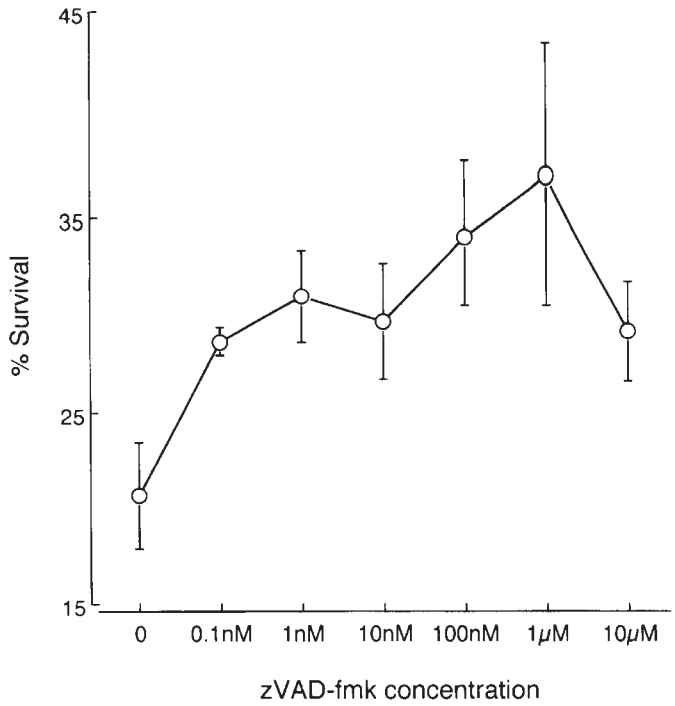

Fig. 3. The effect of CPP32-like protease inhibitor on RPE cells. RPE cells were preincubated for 3 hours in various concentrations of CPP32-like inhibitor zVAD-fmk. At 6 hours after ceramide application, cell viability was assayed using the MTS assay. Data show mean ± S.D. of each 6 samples.

Viable and apoptic cell staining

We used Hoechst 33552[††] and propidium iodide for viable and apoptic cell staining. In brief, at 3 or 6 hours after 100 μM C2-ceramide application, RPE cells were washed with PBS, and 200 μl of D-PBS containing 1 μg of Hoechst 33552 and 1 μg of propidium iodide were added. RPE cells were incubated at room temperature for 15 minutes in the dark. After incubation, cells were washed and fixed in 2% paraformaldehyde/0.1 M PB. We observed the cells under a fluorescence microscope.

Results

C2-ceramide induced RPE cell death. Cell death was dependent on C2-ceramide concentration (Fig. 1A). Viability of RPE cells was 0.98 ± 0.98 (% of control) by application of 100 μM C2-ceramide for 24 hours. The effective dose (ED_{50}) of C2-ceramide was 23.64 μM. Viability of RPE cells at 3 and 6 hours after 100 μM C2-ceramide application was 64.96 ± 4.33 and 18.29 ± 4.88(%), respectively (Fig. 1B). The exposure of RPE cells to C2-ceramide resulted in the detachment of the majority of cells from the adherent monolayer (Fig. 2A). The adherent RPE cells exhibited chromatin condensation consistent with apoptosis

[††] Hoechst 33552: Bisbenzimide H33342 Fluorochrome, Trihydrochloride (HOE33342:2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2, 5′-bi-1H-benzimidazole, HCl) (Calbiochem, San Diego, CA, USA).

on propidium iodide staining (Fig. 2B). C2-ceramide-induced cell death was inhibited by zVAD-fmk concentration in dose dependent manner.

Discussion

It is reported that human RPE cells express CD95 (Fas/Apo-1) but are rather resistant to agonistic CD95 antibody (Esser et al. 1995). Resistance to CD95 antibodies is overcome by preincubation with cytokines, TNF-α, or interferon-γ, or by coexposure to CD95 antibodies and inhibitors of RNA or protein synthesis. Ceramide is thought to be a second messenger of Fas-induced apoptosis, which causes cell death rapidly. On the other hand, the fact that ceramide is not an upstream messenger in Fas-mediated apoptosis have been reported (Sillence and Allan 1997). In this study, we indicated that RPE cell death caused by ceramide was due to the activation of CPP32-like proteases and that ceramide in RPE cell death functions upstream of CPP32-like proteases.

RPE cells in culture show a weak expression of Fas that is increased by cytokine stimulation (Esser et al. 1995). Choroidal neovascular membrane in ARMD shows apoptosis in RPE endothelial cells, which are associated with Fas and FasL expression(Hinton et al. 1998). If RPE dysfunction in ARMD is related to Fas-induced apoptosis, CPP32-like protease inhibitor may inhibit RPE cell death.

To our knowledge, this is the first report to investigate the response of RPE cells to ceramide. We believe that it provides a clue to the mechanism of RPE cell death in RPE-related disorders such as ARMD and proliferative vitroretinopathy.

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