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Anti-apoptotic Activity of Heme Oxygenase-1 Up-regulated by Etoposide in Human Retinal Pigment Epithelial Cells

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The topoisomerase II inhibitor etoposide causes an accumulation of DNA double strand breaks within the nuclei of cells. In this study, we investigated the effect of etoposide on the cell growth and apoptosis of human RPE cells. Etoposide evoked a significant inhibition of cell growth, and also induced DNA fragmentation in ARPE-19 cells. In addition, etoposide significantly up-regulated the expression of heme oxygenase-1 (HO-1), which is a stress-responsive protein and is known to play a protective role against the oxidative injury. And, etoposide-induced HO-1 expression was affected by the ROS scavenger *N*-acetyl cysteine. We also used oligonucleotides interfering with HO-1 mRNA (siRNA) for the inhibition of HO-1 expression. Interestingly, knock-down of the HO-1 gene significantly increased the level of DNA fragmentation in etoposide-treated ARPE-19 cells. In conclusion, these results suggest that up-regulated HO-1 plays as an anti-apoptotic factor in the process of apoptosis of ARPE-19 cells stimulated by etoposide.

Key words – Etoposide, apoptosis, HO-1, ROS, ARPE-19 cells

Introduction

The retinal pigment epithelium (RPE) forms a single cell layer located between the photoreceptors of the neurosensory retina and the vascular-rich choroids. RPE regulates the transport of nutrients to the photoreceptors, phagocytoses shed outer segments and absorbs stray light, and limits access of blood components to the retina [4,41]. In addition to these functions, the RPE is thought to play an important role in immune responses. This is suggested by the ability of RPE cells to express cytokines (IL-1 [19], IL-6 [27], IL-8 [11], MCP-1 [9], GM-CSF [28], and TGF-β2 [20]), HLA class II antigens [7], adhesion molecules [10,29], and FasL [13]. It normally functions under relatively high oxygen tensions in postnatal life. Conversely, dysfunction of RPE may lead to loss of photoreceptors or retinal degeneration [12], which accounts for a major cause of aging-dependent visual impairment and blindness in the developed world.

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The topoisomerase II inhibitor etoposide is an anti-cancer drug that has been widely used to affect DNA damage [24]. Topoisomerase II is a nuclear enzyme that functions during both DNA replication and transcription [14]. Topoisomerase II inhibitors stabilize the complex formed by topoisomerase II and the 5'-cleaved ends of the DNA, thus forming stable protein-linked DNA double strand breaks [5]. Cells are apparently able to recognize such DNA damage and, in turn, to eliminate the injured cells by apoptosis. Here, we report that etoposide shows anti-proliferative and apoptotic activities in human RPE cells, and induces up-regulation of HO-1 expression by ROS produced by etoposide treatment in ARPE-19 cells, resulting in the inhibition of DNA fragmentation. Our results suggest that HO-1 plays as an anti-apoptotic factor in the process of apoptosis of ARPE-19 cells stimulated by etoposide.

Materials and Methods

Materials

Etoposide, *N*-acetyl cysteine (NAC), protease inhibitor

cocktail, dimethyl sulfoxide (DMSO), and trypan blue (0.4%) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Molecular Probes (Eugene, OR, USA). Anti-HO-1 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary horse radish peroxidase (HRP)-conjugated antibody and ECL Western blotting kits were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Fetal bovine serum (FBS), HBSS and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA).

Cell culture

The human RPE cell line, ARPE-19, was purchased from American Tissue Culture Collection (Rockville, MD, USA). Cells were cultured in a 1:1 mixture of DMEM and nutrient mixture F12 (DMEM/F12) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin) at 37°C with 10% CO_2 in air atmosphere. To maintain reproducibility, subconfluent cultures were passaged according to standard trypsinization protocol, and passages 2 through 16 were used for all experiments.

Cell viability and growth analysis

ARPE-19 cells were plated at 1×10^5 cells per 60-mm dishes and incubated for 24 hr. Cells were cultured in presence or absence of different concentrations of etoposide in fresh DMEM/F12 medium supplemented with 10% FBS. After incubation of different time periods, the cells were washed with cold phosphate-buffered saline (PBS), trypsinized, rinsed medium containing FBS and centrifuged at 500 ×g. Cells were resuspended in medium and viable cells and dead cells were countedby the trypan blue dye exclusion method using a hemocytometer.

TUNEL assay for detection of apoptotic cells

Cells were washed with 1% PBS/BSA and fixed in 4% paraformaldehyde for 15 min. Cells were washed with PBS/BSA and permeabilized in 0.1% Triton-X 100 for 5 min on ice. Next, FITC-conjugated dUTP was used to label the cells with DNA nicking, using Apoptosis Detection System kit from Roche Molecular Biochemicals (Mannheim, Germany) according to the manufacturer's instructions.

Transfection of siRNA

Twenty-one nucleotide RNA with 3'-dTdT overhangs was synthesized to interfere exclusively with HO-1 mRNA by Dharmacon (Lafayette, CO, USA) in the "ready-to-use" option. As a negative control, the same nucleotides were scrambled to form a nongenomic combination (controlled by basic local alignment search tool). The mRNA targets were HO-1 target sequence (5'-ACACUCAGCUUUCUGGU GGUU-3'). Cells in exponential phase of growth were plated in 6-well plates at 5×10^5 cells/well, grown for 24 hr, and then transfected with 30 nM of siRNA per well using DharmaFECT™ and DMEM/F12 media according to manufacturer's recommended protocol.

Measurement of intracellular ROS

Intracellular ROS was detected by monitoring changes in the fluorescence of the ROS-sensitive fluorophore H_2DCF-DA, as described previously [2]. The fluorescence was measured after 10 min of incubation with H_2DCF-DA using a Zeiss LSM 510 laser-scanning confocal microscope (Göettingen, Germany). Absolute fluorescence intensities were determined from the same numbers of cells in a randomly selected area.

Western blot analysis

Equivalent amounts of total protein were loaded onto 10% SDS/PAGE. The gels were transferred to nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Richmond, CA, USA) and reacted with each antibody according to standard methods. Bound immunocomplexes were visualized on X-ray film by ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). β-actin was used as an internal control to monitor equal protein sample loading.

Statistical analysis

Three independent assays were performed. Statistical values were expressed as the means ± standard deviation (S.D.) of the means obtained from each independent experiment.

Results

Etoposide induces growth inhibition and apoptosis in human RPE cell line ARPE-19

Initial experiments were designed to determine the effect of etoposide, a topoisomerase II inhibitor, on cell

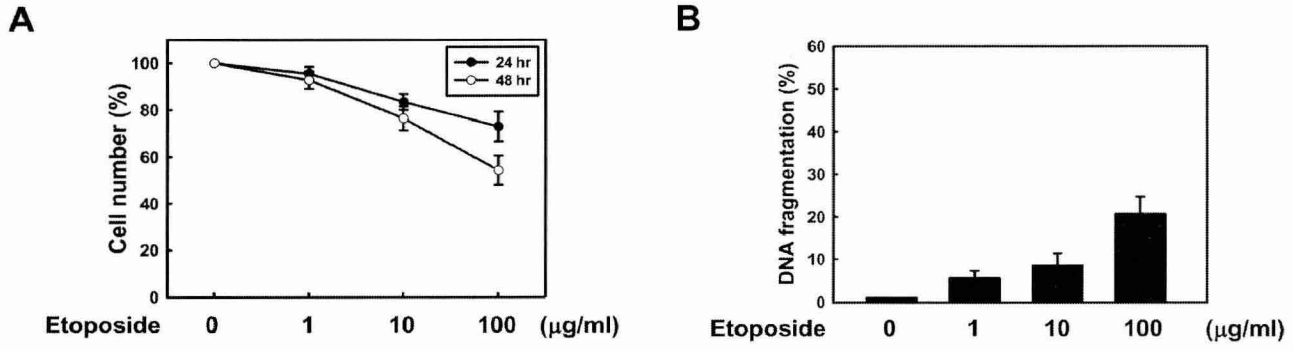

Fig. 1. Effect of etoposide on cell growth and DNA fragmentation in human RPE cells. ARPE-19 cells were plated at 1×10^5 cells per 60-mm dishes, incubated for 24 hr and treated with different concentrations of etoposide for 24 and/or 48 hr, as indicated. (A) Measurement of viable cell number. The viable cells were scored by hemocytometer counts of trypan blue-excluding cells. (B) TUNEL assay. After 48hr of stimulation, TUNEL positive cells were counted under an epifluorescence microscope with 250-300 cells counted for each condition. Values were determined as a percentage to total cells. Three independent experiments were performed and data shown are means ± S.D. obtained from triplicate of each experiment.

growth and apoptosis of ARPE-19 cells. Cells were treated with dose-dependent concentrations of etoposide (1, 10, and 100 μg/ml) and collected at different time point after treatment. The amount of viable cells was measured using the trypan blue dye exclusion method (Fig. 1A). Exposure of etoposide to ARPE-19 cells resulted in a significant decrease of cell proliferation. And, measurement of DNA fragmentation using TUNEL assay was used to evaluate the apoptotic response of the control and cells treated with etoposide (Fig. 1B). When cells were incubated with 100 μg /ml etoposide for 48 hr, approximately 25% of them were TUNEL-positive.

Etoposide stimulates up-regulation of HO-1 in ARPE-19 cells

Next, to determine whether apoptosis induced by etoposide is correlated with HO-1 expression, we analyzed the amount of expressed HO-1 by western blotting. As shown in Fig. 2, etoposide significantly induced the expression of HO-1 in a dose-dependent manner.

Knock-down of HO-1 expression induces etoposide-induced apoptosis in ARPE-19 cells

We used oligonucleotides interfering with HO-1 mRNA (siRNA) for the inhibition of HO-1 expression. siRNA, directed against HO-1, efficiently silenced the HO-1 gene and reduced the level of HO-1. The siRNA to HO-1 gene significantly inhibited HO-1 expression in etoposide-stimulated ARPE-19 cells (data not shown). Surprisingly, knock-down of the HO-1 gene significantly increased the population of TUNEL-positive cells in etoposide-treated ARPE-19 cells (Fig. 3). This result suggests that up-regulated HO-1 plays as an anti-apoptotic factor in the process

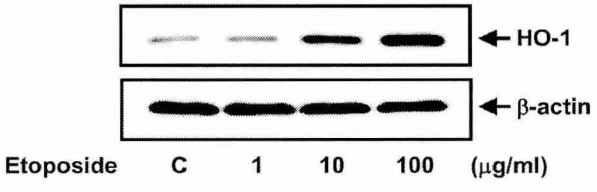

Fig. 2. Effect of etoposide on the expression of p53 in ARPE-19 cells. Cells were treated with 1, 10, and 100 μg /ml etoposide for 24 hr. Whole cell extracts were analyzed by western blotting using an anti-HO-1 antibody. β-actin was used as an internal control to monitor equal protein loading.

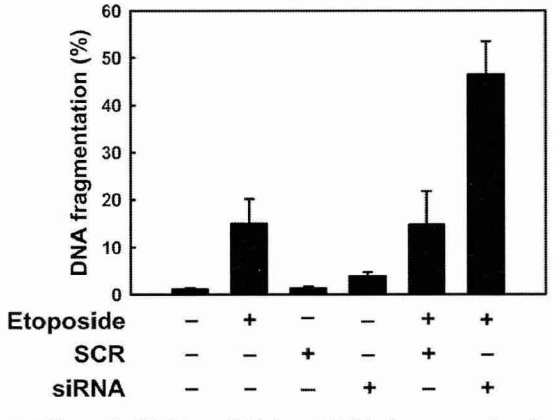

Fig. 3. Effect of siRNA to HO-1 on DNA fragmentation in etoposide-stimulated ARPE-19 cells. Cells were transfected with siRNA for the silencing of the HO-1 gene, and incubated in the presence or absence of 100 μg/ml etoposide. After 24 hr of etoposide treatment, TUNEL assay was performed. Values are means ± S.D. of three independent cell preparations.

of apoptosis of ARPE-19 cells stimulated by etoposide.

Etoposide-induced HO-1 up-regulation is associated with ROS generation in ARPE-19 cells

Next, we investigated whether etoposide could contribute to the generation of intracellular ROS in ARPE-19 cells. Cells were treated with various concentrations of etoposide (1, 10 and 100 μg/ml) for 12 hr and the amount of ROS was determined quantitatively by confocal microscopy. As shown in Fig. 4, there was significant increase in DCF fluorescent intensity from ARPE-19 cells treated with etoposide. We further examined whether etoposide evokes the induction of HO-1 expression by regulation of ROS generation. ROS scavenger NAC was utilized to inhibit the generation of etoposide-stimulated ROS. Cells were pretreated with NAC (0.5 or 2 mM) for 30 min prior to etoposide stimulation. Interestingly, NAC inhibited the up-regulation of HO-1 stimulated by etoposide (Fig. 5). This result suggests that etoposide induces the up-regulation of HO-1 by ROS-dependent mechanism in ARPE-19 cells.

Discussion

Chemotherapeutic drugs are commonly used to induce cell cycle arrest and apoptosis in the treatment of proliferating diseases such as proliferative vitreoretinopathy (PVR) and cancer. Among the approaches for the treatment of PVR, some involve the use of anti-proliferative and cell cycle perturbation agents which are DNA damaging agents that cause apoptosis in dividing RPE cells [36,38]. Unfortunately chemotherapeutic drugs have been toxic and lacked specificity [16,32]. We have been studying to reduce proliferation of RPE cells as it pertains to both the basic drug mechanism and the potential therapeutics of the pathologic conditions accompanying retinal pigment cell proliferation [26,39,40].

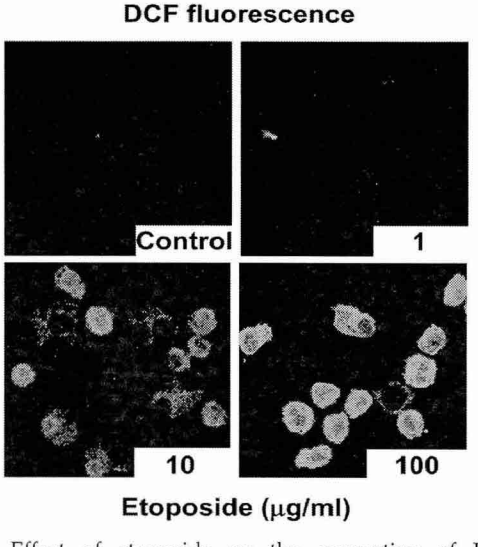

Fig. 4. Effect of etoposide on the generation of ROS in ARPE-19 cells. Cells (1×10^6) were incubated for 24 hr in the presence or absence of etoposide, as indicated. Then, the cells were incubated with 10 μM H_2DCF-DA for an additional 10 min. Cells were washed twice with PBS, and the intracellular levels of ROS were analyzed by confocal microscope.

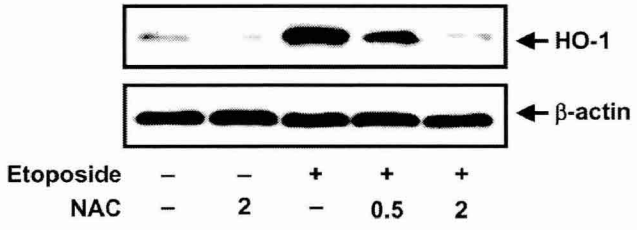

Fig. 5. Effect of NAC on the etoposide-induced HO-1 up-regulation. ARPE-19 cells were pretreated with 0.5 or 2 mM of NAC for 30 min and then incubated with 100 μg /ml etoposide for 24 hr. Whole cell extracts were analyzed by western blotting using an anti-HO-1 antibody. β-actin was used as an internal control to monitor equal protein loading.

In the present study, we demonstrate that etoposide shows anti-proliferative and apoptotic activities in human RPE cells, and induces up-regulation of HO-1 expression by ROS produced by etoposide treatment in ARPE-19 cells. We also used HO-1 siRNA to determine whether up-regulation of HO-1 induced by etoposide is correlated with apoptotic activity in ARPE-19 cells. As shown in Fig. 3, knock-down of the HO-1 gene significantly increased the population of TUNEL-positive cells in etoposide-treated ARPE-19 cells. This result suggest that up-regulated HO-1 plays as an anti-apoptotic factor in the process of apoptosis of ARPE-19 cells stimulated by etoposide.

ROS have been implicated in the processes of aging and degenerative diseases of the eye [34,35,37]. The RPE cells are exposed to relatively chronic oxidative stress from high oxygen tensions. As a result, these cells possess high levels of the enzymes required to detoxify ROS. Thus, RPE cells are an excellent model for studying the molecular mechanisms of the oxidative adaptive response. In fact, RPE cells have high catalase activity and contain high concentrations of nonenzymatic antioxidants [33,34]. However, RPE cell catalase and other antioxidant activities decrease with in-

creasing age in humans [22,33]. Therefore, it is necessary to elucidate intracellular antioxidant molecules involved in the regulatory mechanisms of antioxidant defenses, leading to DNA damage mediated by ROS, in RPE cells.

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism, which leads to the generation of biliverdin, free iron, and carbon monoxide [23,25]. Three distinct isoforms have been identified and cloned. Among them, the HO-1 isoform is distributed ubiquitously and is strongly induced by a variety of oxidative stimuli [3,18]. Recently, several investigators have investigated the functional significance of HO-1 induction, usually by observing the ability of cells to resist different stress insults when HO-1 is under- or overexpressed [6,8,30]. RPE expresses various enzymes that are important in protection against oxidative stress, including HO-1, thereby coping with large amounts of ROS generated by light exposure and during active phagocytosis of shed outer segments [17,31]. Several reports have shown that HO-1 is induced in retina by visible light [21] and in cultured RPE cells by oxidative stresses [1,15,17].

In conclusion, the results presented in this report demonstrate that etoposide induces growth inhibition and apoptosis of human RPE cell line ARPE-19. The results also show that etoposide induces up-regulation of HO-1 by ROS-dependent mechanism in ARPE-19 cells, which may be associated with anti-apoptotic activity of HO-1 in response to oxidative DNA damage in cells.

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초록 : Etoposide에 의한 인간 망막색소상피세포인 ARPE-19 세포의 아폽토시스 과정에서 Heme oxygenase-1의 항아폽토시스 기능에 대한 연구

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Totopoisomerase II 저해제인 etoposide는 핵안에 DNA double strand breaks를 일으키므로써 세포의 DNA에 손상을 초래한다. 본 연구에서는 인간 망막색소상피세포인 ARPE-19 세포에서의 세포성장 및 아폽토시스에서 etoposide의 역할을 살펴보았다. Etoposide는 세포의 성장을 크게 감소시켰으며 TUNEL에서 아폽토시스를 나타내는 DNA fragmentation의 증가를 유도하였다. 게다가, etoposide는 산화적 손상에 대해 세포나 조직을 보호하는 역할을 하는 것으로 알려진 세포내 항산화효소인 heme oxygenase-1 (HO-1)의 발현을 크게 증가시켰다. Etoposide에 의한 HO-1 발현증가는 항산화물질 NAC에 의해 억제되었는데, 이는 etoposide에 의한 세포내 ROS의 증가가 HO-1 발현에 중요한 역할을 한다는 것을 의미한다. 또한 HO-1 발현을 억제하기 위하여 HO-1 siRNA 방법을 사용하였다. 흥미롭게도, HO-1 유전자의 knock-down은 etoposide에 의해 유도되는 DNA fragmentation의 정도를 증가시켰다. 이들 결과를 종합해볼 때, etoposide에 의해 자극되어진 ARPE-19 세포에서 발현증가된 HO-1은 etoposide에 의한 아폽토시스 유발과정에서 세포를 보호하는 항아폽토시스의 기능을 한다는 것을 시사한다.