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Promoter demethylation of *Keap1* gene in human diabetic cataractous lenses

Periyasamy Palsamy [a], Masahiko Ayaki [b], Rajan Elanchezhian [a], Toshimichi Shinohara [a,*]

[a] Department of Ophthalmology and Visual Sciences, University of Nebraska Medical Center, Omaha, NE, United States
[b] Shizuoka National Hospital, Saitama, Japan

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ABSTRACT

Age-related cataracts (ARCs) are the major cause of visual impairments worldwide, and diabetic adults tend to have an earlier onset of ARCs. Although age is the strongest risk factor for cataracts, little is known how age plays a role in the development of ARCs. It is known that oxidative stress in the lens increases with age and more so in the lenses of diabetics. One of the central adaptive responses against the oxidative stresses is the activation of the nuclear transcriptional factor, NF-E2-related factor 2 (Nrf2), which then activates more than 20 different antioxidative enzymes. Kelch-like ECH associated protein 1 (*Keap1*) targets and binds to Nrf2 for proteosomal degradation. We hypothesized that hyperglycemia will lead to a dysfunction of the Nrf2-dependent antioxidative protection in the lens of diabetics. We studied the methylation status of the CpG islands in 15 clear and 21 diabetic cataractous lenses. Our results showed significant levels of demethylated DNA in the *Keap1* promoter in the cataractous lenses from diabetic patients. In contrast, highly methylated DNA was found in the clear lens and tumorized human lens epithelial cell (HLEC) lines (SRA01/04). HLECs treated with a demethylation agent, 5-aza-2′deoxycytidine (5-Aza), had a 10-fold higher levels of *Keap1* mRNA, 3-fold increased levels of *Keap1* protein, produced higher levels of ROS, and increased cell death. Our results indicated that demethylation of the CpG islands in the *Keap1* promoter will activate the expression of *Keap1* protein, which then increases the targeting of Nrf2 for proteosomal degradation. Decreased Nrf2 activity represses the transcription of many antioxidant enzyme genes and alters the redox-balance towards lens oxidation. Thus, the failure of antioxidant protection due to demethylation of the CpG islands in the *Keap1* promoter is linked to the diabetic cataracts and possibly ARCs.

1. Introduction

The theory for the development of age-related cataracts (ARCs) is similar to the "free-radical theory of aging" [1]. Increases in oxidative stress during chronological aging is at the center of the pathogenesis of ARCs [2,3]. Oxidative stress is composed of three basic elements; (1) generation of reactive oxygen species (ROS), (2) scavenging of ROS, and (3) removal of oxidized macromolecules. ARCs including diabetic cataracts are the major visual impairment in the world, and more than 50 million ARC patients undergo cataract surgery every year. The cost to Medicare for the cataract surgery is one of the highest expenses in the USA (Global initiative for the elimination of avoidable blindness. Fact sheet number 213, Geneva: WHO, 2011, available from, http://www.who.int/mediacentre/factsheets/fs213/en/).

The development of ARCs is closely associated with many environmental stresses such as electrophilic reactive species, xenobiotics, drugs, inflammation, ionizing radiation, sunlight, atomic oxygen, and diabetes [4,5]. Among the ARCs, the diabetic cataracts have been extensively studied, and the ROS levels or glycoxidation can increase markedly under hyperglycemia stress [6]. These changes can also induce the unfolded protein response (UPR) [7,8]. We realized that most stresses known to cause cataracts in humans also induce the UPR [9]. The UPR is a protective mechanism that eliminates toxic ROS in most organisms. Interestingly, chronic ER stress also generates the UPR and production of ROS, and cell death. In such chronic stress antioxidant defense protective mechanisms must be failed.

One of the main antioxidant protection mechanisms is the activation of NF-E2-related factor 2 (Nrf2). Nrf2 is a transcriptional activator, and it binds to the antioxidant response element (ARE).

Abbreviations: ARCs, age-related cataracts; ARE, antioxidant response element; 5-Aza, 5-aza-2′ deoxycytidine; BGS, bisulfite genomic DNA sequencing; EthD, ethidium homodimer-III; ER, endoplasmic reticulum; FCS, fetal calf serum; HLECs, human lens epithelial cells; *Keap1*, Kelch-like ECH associated protein 1; Nrf2, NF-E2-related factor 2; PBS, phosphate buffered saline; ROS, reactive oxygen species; UPR, unfolded protein response; H_2-DCFH-AD, 2′,7′-dichlorodihydrofluorescein diacetate.

* Corresponding author. Address: Department of Ophthalmology and Visual Sciences, University of Nebraska Medical Center, Omaha, NE 68198-5840, United States. Fax: +1 402 559 3869.
E-mail address: tshinohara@unmc.edu (T. Shinohara).

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This then leads to the transcription of approximately 200 protective genes including 20 antioxidant associated enzymic genes [10,11].

There is growing evidence that oxidative stress stimulates DNA modification including DNA methylation [12–15]. We hypothesize that the Nrf2-dependent antioxidant protection is dysfunctional in diabetic lenses under chronic UPR. We initially studied the status of DNA methylation of the Nrf2 and *Keap1* genes. We found significant levels of demethylation in the *Keap1* gene in diabetic cataractous lenses but not in the clear lenses. We shall present evidence that the lenses of diabetic subjects can become cataractous induced by a failure of antioxidative processes against oxidation in the lens.

2. Materials and methods

2.1. Human lens epithelial cell (HLECs) culture

HLECs were cultured in high glucose, Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and antibiotics at 37 °C under 20% atmospheric oxygen. For the experiments, HLECs were pre-cultured overnight in low glucose DMEM (Invitrogen, Grand Island, NY) with 10% FCS and antibiotics under 1% of atmospheric oxygen.

2.2. Bisulfite conversion

The genomic DNA of epithelial cells from clear human lenses and from diabetic cataractous lenses (NDRI, Philadelphia, PA) was subjected to bisulfite conversion by EZ DNA Methylation-Direct™ kit (Zymo Research Corporation, Orange, CA). HLECs from the area of capsulotomy were isolated from the center of the anterior surface of clear lenses and of diabetic cataractous lenses under a dissection microscope. The human lenses were fixed in 4% formaldehyde for 1 h at room temperature and then stained with Coomassie blue for 10 min. The bisulfite converted DNA was then used for bisulfite genomic DNA sequencing (BGS).

2.3. Bisulfite genomic DNA sequencing

The bisulfite-modified DNAs were amplified by bisulfite sequencing PCR using Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) with primers specific to human *Keap1* promoter (Table S1). The primers were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems Inc. Foster City, CA). The PCR products were purified by gel extraction using the Zymoclean™ Gel DNA recovery kit (Zymo Research Corporation, Orange, CA), then cloned into pCR®4-TOPO vectors using TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). The recombinant plasmids were transformed into One Shot® TOP10 chemically competent *Escherichia. coli* (Invitrogen, Carlsbad, CA) using the regular chemical transformation method as described in the manufacturer's instructions. Plasmid DNA were prepared from about 10 independent clones of each amplicon with PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA) and sequenced (High-Throughput DNA Sequencing and Genotyping Core Facility, University of Nebraska Medical Center, Omaha, NE) to determine the status of CpG methylation. Clones with an insert with > 99.5% bisulfite conversion, i.e., non-methylated cytosine residues to thymine were included in this study, and the remaining was excluded. Then the sequenced data of each clone was compared with the in silico reference human *Keap1* promoter bisulfite converted DNA sequence derived from methyl primer express software (version 1.0), and a schematic diagram of CpG methylation status was produced.

2.4. 5-Aza-2′-deoxycytidine treatment

HLECs were cultured in low glucose DMEM (Invitrogen, Grand Island, NY) along with 10 μmole/L 5-Aza-2′-deoxycytidine (5-Aza; Sigma–Aldrich, St. Louis, MO) for 7 days. The culture medium was changed every 2 days. At the end of the experiment, the cells were harvested and used for cell death assay, intracellular ROS production, bisulfite genomic DNA sequencing, real-time RT-PCR and western blotting.

2.5. Cell death staining

HLECs treated with/without 5-Aza were stained with ethidium homodimer-III (EthD) as included in the Cytotoxicity Assay kit (Biotium Inc., Hayward, CA) for 30 min as described in the manufacturer's protocol. Then the cells were washed twice with phosphate buffered saline (PBS) and examined under a fluorescent microscope (Nikon, Eclipse TE2000-U) with a red filter (for dead cells).

2.6. Intracellular ROS staining

HLECs treated with/without 5-Aza were stained by adding 1 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFH-AD) (Invitrogen, Carlsbad, CA) in PBS for 30 min at 20 ° C. Then the cells were washed twice with PBS, and examined under a fluorescent microscope with a green filter (Nikon, Eclipse TE2000-U).

2.7. Real-time PCR

Total RNA was extracted from the HLECs treated with/without 5-Aza with Quick-RNA™ MicroPrep solution (Zymo Research Corporation, Orange, CA) following the manufacturer's instructions. Then the purified total RNA was reverse transcribed by iScript™ Reverse Transcription Supermix for real-time PCR (Bio-Rad, Hercules, CA) following the manufacture's protocol. The reverse transcribed RNA was analyzed by real-time PCR using the SsoFast™ EvaGreen® supermix (Bio-Rad, Hercules, CA). The primer sequences were designed using the OligoPerfect™ Designer software with the instructions of Invitrogen for optimal primer design and were synthesized commercially. The primer sequences for *Keap1* and *β-actin* are given in Table S2. Each reaction was carried out in triplicate and three independent experiments were run. A standard curve was prepared using a serial dilution of a reference sample and was included in each real-time run to correct for possible variations in product amplification. The relative copy numbers were obtained from the standard curve and were normalized to the values obtained for *β-actin*, the internal control. The fold change in expression was then obtained by the $2^{-\Delta\Delta CT}$ method.

2.8. Protein blot analysis

At the conclusion of the experiment, HLECs were harvested and lysed with 200 μl of RIPA buffer (Cell Signaling Technology, Inc. Beverly, MA). The lysates were centrifuged (12,000 × *g*; 10 min, 4 °C), and the protein content of the supernatant was determined by the Bradford method [16]. The soluble proteins, 10–20 μg, were loaded and separated by 10% SDS–PAGE and blotted onto a polyvinylidene fluoride membrane. Then, the membranes were blocked with 5% nonfat dry milk (in 1× TTBS buffer) for 1 h at room temperature before an overnight incubation with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C. After rinsing the membranes, they were incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and the immunostained bands was made more visible by enhanced chemiluminescence (Pierce, Thermo, Rockford, IL). The