Effect of miR-23a on Oxidant-Induced Injury in Human Retinal Pigment Epithelial Cells

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PURPOSE. Micro(mi)RNAs negatively regulate a wide variety of genes through degradation or posttranslational inhibition of their target genes. The purpose of this study was to investigate the role of miR-23a in modulating RPE cell survival and gene expression in response to oxidative damage.

METHODS. The expression level of miR-23a was measured in macular retinal pigment epithelial (RPE) cells of donor eyes with age-related macular degeneration (AMD) and age-matched normal eyes by using qRT-PCR. Cultured human ARPE-19 cells were transfected with miR-23a mimic or inhibitor. Cell viability was assessed by the MTT assay. Apoptosis was determined by incubating cells with hydrogen peroxide (H2O2) or t-butylhydroperoxide (tBH). Caspase-3 activity and DNA fragmentation were measured by enzyme-linked immunosorbent assays. The protein relevant to apoptosis, such as Fas expression level, was analyzed by Western blot analysis.

RESULTS. miR-23a expression was significantly downregulated in macular RPE cells from AMD eyes. H2O2-induced ARPE-19 cell death and apoptosis were increased by an miR-23a inhibitor and decreased by an miR-23a mimic. Computational analysis found a putative target site of miR-23a in the 3′UTR of Fas mRNA, which was verified by a luciferase reporter assay. Forced overexpression of miR-23a decreased H2O2 or tBH-induced Fas upregulation, and this effect was blocked by downregulation of miR-23a.

CONCLUSIONS. The protection of RPE cells against oxidative damage is afforded by miR-23a through regulation of Fas, which may be a novel therapeutic target in retinal degenerative diseases. (Invest Ophtalmol Vis Sci. 2011;52:6308–6314) DOI: 10.1167/iows.106652

Micro(mi)RNAs are small, noncoding molecules that have emerged as critical regulators of gene expression via translational repression or mRNA degradation.1–3 Studies have shown that miRNAs control diverse aspects of eye development and differentiation.3,5 Since miRNAs are involved in controlling various pathways, they have been regarded as novel therapeutic targets for various diseases such as cancer and cardiovascular and regenerative eye diseases.2,6–8 Numerous studies have demonstrated that miRNAs are involved in regulation of cell survival in response to oxidative stress.9,10 High levels of reactive oxygen species (ROS)–induced RPE cell damage play an important role in the pathogenesis of AMD.11–14 Exposure of cells to oxidative generators, such as H2O2 or tBH, elicits changes in expression of multiple genes, and these changes are responsible for ROS-mediated RPE cell death and apoptosis.14–17 The extrinsic apoptosis pathway is activated by apoptosis-inducing ligands, such as the Fas ligand (FasO).18,19 The increased Fas expression in AMD photoreceptors has been found in eyes with exudative AMD and in those with geographic atrophy.19

miR-23a is a key factor in the regulation of oligodendroglia development and myelin formation.20 Inhibition of miR-23a downregulates cell growth.21 In addition, miR-23a regulates retinoic-acid–induced neuronal differentiation of NT2 cells through regulation of Hes1 expression at the posttranscriptional level.22 Recent studies have suggested that miR-23a regulates mitochondrial glutamine metabolism and is associated with ROS in human P-493 B lymphoma cells and PC3 prostate cancer cells.23 However, the role of miR-23a in oxidative stress of RPE cells remains unclear. In the present study, the miR-23a expression level in AMD was evaluated, and the interaction between miR-23a and apoptotic factor, such as Fas in oxidative stress, was determined.

MATERIALS AND METHODS

Antibodies against Fas were purchased from Upstate Biotechnology (Lake Placid, NY). An miRNA isolation kit (mirVana) and a qRT-PCR miRNA detection kit were purchased from Ambion (Austin, TX). Human ARPE-19 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). MTT cell respiration assay kit was purchased from R&D System (Minneapolis, MN). Sources of other reagents are indicated in the text.

Cell Culture

Eyes of aged normal (60–80 years) and AMD (60–80 years) donors were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA), the Lions Eye Bank of Texas (Houston, TX), or the Minnesota Lions Eye Bank (St. Paul, MN), in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. The eyes were received, and the globes were dissected under sterile conditions. An 8-mm sterile trephine was used to remove a disc from the macula. The RPE cells were loosened after trypsin digestion (30 minutes at 37°C) and were then collected and spun at 1000 rpm in a centrifuge at 4°C for 5 minutes. The pellet was resuspended in fresh cell growth medium. Test for purity of the cultures using cytokeratin antibody staining showed 97% RPE cells at the time of isolation.
Determination of miR-23a Expression Level
Primary cultured macular RPE cells were seeded at 1 x 10⁶ per 100-mm plate and incubated until 80% to 90% confluent. RPE cells were then harvested at passage 3. Total RNA was extracted from RPE cells (TriReagent; Invitrogen, Carlsbad, CA). Enrichment of small RNA was performed (mirVana miRNA Isolation Kit; Ambion) and quantified (mirVana qRT-PCR miRNA Detection Kit; Ambion), as described before.24 For real-time PCR, SYBR Green (SYBR Green I: Invitrogen) was used for quantification of miRNA transcripts, according to the manufacturer’s instructions. The appropriate cycle threshold (Ct) was determined using the automatic baseline determination feature. Reactions containing qRT-PCR primer sets were specific for human miR-23a. As an internal control, U6 was used for miR-23a template normalization. The relative gene expression was calculated by comparing cycle times for miR-23a.25,26 RPE cells from four different donors per group were used.

Northern Blot Analysis
Northern-blot analyses were performed with a kit (Signosis, Sunnyvale, CA) per the manufacturer's instructions. Briefly, the total RNA (40 µg) was run on a 15% polyacrylamide-urea gel, transferred to a nitrocellulose membrane (Hybond-N; Amersham Pharmacia Biotech Inc) with a semidry apparatus (BioRad, Hercules, CA) and UV crosslinked (Stratalinker; Stratagene). Membranes were exposed using a chemiluminescence imaging system (Ultralum, Inc., Claremont, CA). The normalization of the result was done by stripping the blot and probing it for U6 expression.

miRNA Transfection
ARPE-19 cells were maintained in the growth medium for 24 hours to reach 70% confluence, and transfection was performed (Endofectin; GeneCopoeia, Rockville, MD). For miR-23a upregulation, precursor miR-23a (sequence: ggccggcugggguuccuggggaugggauuugcuuccu-gucacaaaucacauugccagggauuuccaaccgacc; GeneCopoeia) was added directly to the transfection complexes at a final concentration of 0.5 µg/mL. For the miR-23a knockdown, miR-23a inhibitor plasmid (GeneCopoeia) was added at the final concentrations of 25, 50, 100, and 150 ng/mL. Cells were incubated at 37°C with 5% CO₂ for 24 hours with antibiotic-free medium. miR-23a levels were measured (mirVana qRT-PCR miRNA Detection Kit; Ambion). Scrambled miRNA controls were applied.

Cell Viability Assays
After transfection, medium was replaced with MEM supplemented with 0.5% FBS. ARPE-19 cells were exposed to 100, 300, or 600 µM H₂O₂ for 16 hours. The viability of ARPE-19 cells was measured in an MTT assay. Briefly, the cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL) for 4 hours at 37°C. The attached cells were lysed in 2-isopropanol containing 0.04 M HCl, and the amount of metabolized MTT was determined with a microplate reader.

Caspase-3 Activity
Caspase-3 activity was determined with a colorimetric assay kit (BioSource, Hopkinton, MA) per the manufacturer’s instructions. In brief, samples were lysed, and the assay was performed by incubating 200 µg cell lysate in 100 µL of reaction buffer containing 5 µL of caspase-3 substrate (4 mM DEVD-pNA). Caspase-3 activity was evaluated by spectrophotometry at 405 nm.

Detection of Apoptosis
The degree of intracellular DNA fragmentation (apoptosis) was quantified by cell viability ELISA (Cell Death Detection ELISA plus kit; Roche, Welwyn Garden City, UK). After transfection, cells in triplicate wells were stimulated with H₂O₂ for 16 hours. The assay was based on a quantitative sandwich enzyme-immunoassay directed against cyto-plasmic histone-associated DNA fragments and was performed according to the manufacturer's instructions.

Estimation of Mitochondrial Cytochrome c and AIF Release
The release of mitochondrial cytochrome c into the cytosol was determined by ELISA (MBL International, Woburn, MA), per the manufacturer's instructions. The change in color was monitored at a wavelength of 450 nm using a plate reader (Molecular Devices, Sunnyvale, CA). Measurements were performed in duplicate, and the cytochrome c content was expressed as OD₄₅₀ µg protein. AIF (apoptosis-inducing factor) in the cytosol was detected in the extracted mitochondria-free cytotoxic protein fraction by immunoblot assay.

 Luciferase Reporter Assay
The 3'UTR of human Fas, with or without miR-23a binding site mutation, was cloned into the cloning site of the pMir-Luc-target vector (OriGene Technologies, Inc., Rockville, MD), and the precursor of the miR-23a expression clone (miR-23a) was constructed in a CMV promoter and fused with an eGFP system (GeneCopoeia). The miR-23a expression clone (miR-23a) was constructed in a CMV promoter, and a luciferase assay was performed (Luc-Pair miR Luciferase Assay Kit; GeneCopoeia). The cells were plated in six wells until 70% confluent and transfected with (1) 1.0 µg Fas 3'UTR; (2) Fas 3'UTR +1.4 µg sc-miR (miRNA scramble control); (3) Fas 3'UTR +1.4 µg miR-23a; (4) Fas 3'UTR +1.4 µg miR-23a + 100 ng sc-inh (miRNA inhibitor scramble control); (5) Fas 3'UTR +1.4 µg miR-23a + (20, 50, or 100 ng) inh-23a; and (6) Fas 3'UTR with miR-23a seed-matching mutation (Fas
3′UTRmt) + miR-23a. The cells were transferred to a 96-well plate 18 hours after transfection and cultured for another 24 hours. Both firefly luciferase and Renilla luciferase activities were determined in the HEK293 cell line. Firefly luciferase activity was then normalized with Renilla luciferase activities in the same well.

Western Blot Analysis

Samples were homogenized in lysis buffer (in mM): 25 Tris HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, 1 Na<sub>4</sub>VO<sub>4</sub>, 1% Triton X-100, 2% SDS and 1% protease inhibitor cocktail. Protein (70 μg) was fractionated by SDS-PAGE (4%-20% polyacrylamide gels) and transferred to PVDF membranes (Millipore, Bedford, MA). Samples were incubated with anti-Fas antibodies. Bound antibodies were detected using the chemiluminescent substrate (NEN Life Science Products, Boston, MA). Protein signals were quantified with an image-scanning densitometer and normalized to the corresponding β-actin signal.

LDH Release Assay

ARPE-19 cells were transfected with miR-23a precursor, inhibitor, or scramble control. To trigger apoptosis, 300 μM H<sub>2</sub>O<sub>2</sub> or 300 μM thimerosal were then added. Intracellular LDH release was measured by using an LDH assay kit (Cayman Chemical, Ann Arbor, MI). Protein signals were quantified with an image-scanning densitometer and normalized to the corresponding β-actin signal.

Statistical Analysis

Data are presented as the mean ± SD. Analysis of variance (ANOVA) with the Sidak correction for multiple comparisons was applied to compare the different groups. *P < 0.05 was considered statistically significant.

RESULTS

miR-23a Expression in AMD and Its Response to Oxidative Stress

To evaluate the expression levels of miR-23a in RPE cells from the macula of AMD and normal age-matched donor eyes, qRT-PCR was used. miR-23a was significantly downregulated in AMD compared with normal (Fig. 1A). The results were confirmed by Northern blot analysis (Fig. 1B). Collectively, aberrant expression of miR-23a was a remarkable characteristic in the RPE cells of human AMD, suggesting the possibility that it could function as a mediator in the process of AMD development. 4-(4-hydroxyphenyl)-retinamide (4HPR), a retinoic acid, has been reported to induce ROS generation and increase the expression level of miR-23a in ARPE-19 cells. Our results showed that the expression level of miR-23a in primary cultured RPE cells increased when exposed to H<sub>2</sub>O<sub>2</sub> at 100 to 200 μM, but decreased at 300 to 500 μM (Fig. 1C). The same pattern was followed in ARPE-19 cells (Fig. 1D). Previous studies suggest that 200 μM H<sub>2</sub>O<sub>2</sub> does not affect ARPE-19 cell viability. Persistent elevation of phosphorylated-Akt induced by 200 μM H<sub>2</sub>O<sub>2</sub> may enhance the ARPE cell’s ability to resist the damaging effects of low level oxidative stress. Preconditioning the cells by low dose of H<sub>2</sub>O<sub>2</sub> induces resistance to killing, while high doses of H<sub>2</sub>O<sub>2</sub> produce toxicity through oxidative stress. Different enzyme activities or signaling pathways are triggered in cells treated with different doses of H<sub>2</sub>O<sub>2</sub>. Our results suggest that H<sub>2</sub>O<sub>2</sub> causes differential expression of miR-23a based on the dose. The increased level of miR-23a induced by 200 μM in our study indicated that miR-23a may also play a role in cell survival. However, higher concentrations of H<sub>2</sub>O<sub>2</sub> may induce destruction of RPE cells. The combined effect of apoptosis and necrosis may decrease the level of miR-23a.

The Effect of miR-23a on Cell Viability in ARPE-19 Cells Subjected to Oxidative Injury

To assess the functional consequences of up and downregulation of miR-23a, antisense inhibitor (inh-23a) and pre-miR-23a (miR-23a) were transfected or co-transfected into human ARPE-19 cells. The expression levels of miR-23a were increased approximately 3.5-fold by 0.5 μg/mL of miR-23a compared with the control (Fig. 2A). In contrast, miR-23a levels were decreased by the
specific inhibitor, inh-23a (25, 50, 100, 150 ng/mL), in a dose-dependent manner. Maximum inhibition was reached at a concentration of 150 ng/mL. Endogenous miR-23a was inhibited by inh-23a at 100 and 150 ng/mL. Scrambled controls (Sc-23a, Sc-inh) had no effect. Overexpression of miR-23a had no effect on cell viability, whereas inhibition of miR-23a reduced cell growth (Fig. 2B). Cell viability, as assessed by MTT assay, was reduced at 300 μM H₂O₂ (Fig. 2C). In cells subjected to H₂O₂ exposure, overexpression of miR-23a increased cell viability, and this effect was abolished by co-transfection of inhibitor (inh-23a). miR-23a attenuated the cell death induced by 300 μM H₂O₂, and this effect was abolished by inh-23a (Fig. 2D).

The Effect of miR-23a on Caspase-3 Activity and Apoptosis Induced by H₂O₂

To evaluate the effect of miR-23a on RPE cell apoptosis, we measured caspase-3 activity (an early marker of apoptosis) and DNA fragmentation, a late marker of apoptosis. Caspase-3 activity was strongly induced in RPE cells after exposure to 300 μM H₂O₂ for 12 hours, but decreased in cells transfected with miR-23a (Fig. 3A). As early as 6 to 9 hours, 0.5 μg/mL miR-23a significantly reduced the caspase-3 activity that was induced by H₂O₂. Co-transfection of inh-23a reversed the effect. We next measured DNA fragmentation in RPE cells to confirm our observation. As shown in Figure 3B, significant DNA fragmentation was detected in cells challenged with 300 μM H₂O₂. H₂O₂ induced DNA fragmentation was inhibited in cells overexpressing miR-23a, but not in cells co-transfected with the inhibitor inh-23a. Co-transfection of scramble control Sc-inh had no effect. In addition, we performed an assay for apoptosis (Chromatin Condensation/Dead Cell Apoptosis Kit; Invitrogen) which was based on fluorescence detection of the compacted state of the chromatin in apoptotic cells. The representative picture for H₂O₂-treated cells showed strong bright blue fluorescence (apoptotic nuclei; Hoechst 33342 dye) and red fluorescence (dead cells; propidium iodide) (Fig. 3C). For quantification, the number of apoptotic cells was counted in at least six randomly selected fields (magnification, ×40) under the microscope with four independent samples and was plotted in a graph (Fig. 3D). Results showed that miR-23a significantly decreased the oxidative stress-induced apoptosis compared with control. These results clearly indicate that miR-23a plays an important role in cytoprotection of RPE cells by prevention of apoptosis.

Mitochondrial Cytochrome c and AIF Release and Nuclear Translocation

Cytochrome c (Cyt-c) and AIF are apoptotic factors normally located in the mitochondria. Translocation of mitochondrial AIF to the nuclei has been suggested to be a caspase-independent event in apoptosis. The leakage of Cyt-c from mitochondria...
into the cytoplasm is known to activate caspases and initiate apoptosis. We therefore examined leakage of Cyt-c in our experiment after oxidative stimulation. The protein content of Cyt-c from the mitochondria-free cytosolic fraction increased in a dose-dependent manner after 16 hours of exposure to H2O2 (Fig. 4A). The elevated Cyt-c was decreased after transfection of miR-23a; the effect was reversed by co-transfection of inh-23a. The AIF protein content measured in the mitochondria-free cytosolic fraction was increased when exposed to 300 to 500 μM H2O2 for 16 hours (Fig. 4B). Transfection with miR-23a significantly prevented AIF protein release into the cytosol.

**Fas Is the Target of miR-23a for Inhibition of Apoptosis**

The targets of miR-23a were identified through computational (TargetScan 5.1) and bioinformatics approaches hosted by the Wellcome Trust Sanger Institute (Hinxton, UK). Fas was identified as one of the highly potential miR-23a targets. The predictive binding sites of miR-23a on 3′UTRs of human Fas are shown in Figure 5A. Fas 3′UTRs with an miR-23a binding site or with seed-matching mutation were fused to a luciferase reporter gene. miR-23a significantly repressed luciferase activity, whereas non-targeted controls had no effect (Fig. 5B). This suppression effect was blocked by co-transfection of various amounts of inhibitor against miR-23a in a dose-dependent manner. At the highest dose, the reporter activity is higher than the control. This finding may be attributable to the fact that this vector-based inhibitor blocked the regulatory effect of endogenous miR-23a, which would result in increased translational activity of miR-23a 3′-UTR transcript.

Since Fas has an important role in ROS-mediated apoptosis, we assessed its functional involvement in RPE cell survival and death in response to oxidative damage. Fas expression decreased in response to a lower dose of H2O2 (200 μM; Fig. 5C). Fas expression increased in 300 μM H2O2 or 300 μM tBH-treated ARPE-19 cells. Importantly, forced overexpression of miR-23a reduced Fas expression. The effect was reversed by abrogation of miR-23a, indicating a pivotal role of miR-23a in RPE survival in response to oxidant injury. Notably the inhibition of Fas by miR23a inhibitor was only partial after oxidative injury, indicating multiple signaling pathways were involved in Fas regulation. Oxidative stress is a major stimulus in eliciting Fas and Fasl expression, and modulation of Fas and Fasl expression is regulated by the existence of cAMP. Here, we demonstrate that Fas expression is regulated by miR-23a at posttranslational levels. Furthermore, treatment with 400 μM H2O2 or 300 μM tBH led to an increase in cell damage, as evaluated by LDH release assay (Fig. 5D). miR-23a reduced cell injury when compared with that of the scrambled control. ARPE-19 cells were co-transfected with an antisense miR-23a-specific inhibitor (inh-23a) to block miR-23a expression. Inh-23a abolished the miR-23a-induced cytoprotective effects. Taken together, these results demonstrated that Fas is the target of miR-23a expression for inhibition of apoptosis.

**DISCUSSION**

The novel findings of this study are summarized as follows. First, we identified that miR-23a expression was downregulated in macular RPE cells from AMD patients compared to normal donors. Second, we showed that forced overexpression of miR-23a reduced the cell death induced by H2O2. The protective effect of miR-23a was blocked by inh-23a inhibitor. Finally, we identified that miR-23a binds to 3′UTR of Fas, an apoptotic factor involved in ROS-mediated cell death. Downregulation of Fas by miR-23a could help to protect RPE cells from oxidative damage. For the first time, we showed an antiantioxidant effect of miR-23a against oxidative injury and the expression changes of miR-23a could be very important in ROS-mediated cell death/survival and gene expression.

miR-23a plays a critical role in the regulation of development,20 differentiation,21 cell growth, and apoptosis.22 Our results indicated that miR-23a was downregulated in development,22 differentiation,21 cell growth, and apoptosis.22 Our results indicated that miR-23a was downregulated in primary cultured RPE and ARPE-19 cells at a higher dose of H2O2. ROS-mediated oxidative damage is thought to play a crucial role in AMD.14,18,39 Our results showed that with overexpression of miR-23a, RPE cells were resistant to oxidative stress-induced cell death, caspase-3 activity, and DNA fragmentation. Oxidative injury is associated with apoptosis. Cytochrome c and AIF normally located in the mitochondria and their release into the cytosol are initiated by apoptosis.34 Cytochrome c and AIF protein release into the cytosol was elevated in oxidative conditions, but decreased after transfection of miR-23a. This result indicated that an alternative pathway of programmed cell death, independent of caspase activation, was inhibited by miR-23a. Furthermore, forced overexpression of miR-23a in ARPE-19 cells markedly reduced cell damage induced by H2O2 or tBH, as evaluated by LDH release assay (Fig. 5D). Inhibition of miR-23a resulted in a significant increase in cell damage. Chabra et al. showed
that overexpression of the miR-23a, 27a, 24-2 cluster in HEK293T cells induces caspase-dependent and -independent apoptosis. However, expression of miR-23a was very low, whereas the expression levels of miR24-2 and miR27a were significantly higher after transfection of the cluster. The induction of apoptosis may be due to the upregulation of miR-27a and miR-24-2. FADD protein, the target of miR-23a, is a key death receptor adaptor molecule. In our study, miR-23a clearly played an important role in cytoprotection of ARPE-19 cells. Nevertheless, the physiological and pharmacological significance of miR-23a in H2O2-mediated injury responses in vivo should be verified in future studies.

Oxidative, particularly photo-oxidative, processes are critical in pathogenesis and development of eye disease such as retinal degeneration.15,47,48 More recently, regulation of gene expression at the posttranscriptional level, such as translational regulation by miRNA, are currently uncertain. The activation of the Fas death receptor and apoptotic pathway is stimulated by oxidants.50 Interaction between Fasl and an agonistic anti-Fas antibody to Fas initiates a signal transduction pathway for Fas-induced apoptosis.51,52 Through computational analysis, 3’UTR of Fas has the putative miR-23a binding sequence. Coincidently, increased Fas and FasL expression were detected in AMD, mimicking the phenomenon of miR-23a downregulation in parallel with upregulation of Fas, suggesting that a possible pathologic cell death pathway may contribute to the development of AMD.

Our results indicated that Fas was indeed a functional target gene of miR-23a that is involved in miR-23a-mediated protective effects on RPE cell injury elicited by H2O2. Our novel findings may have extensive implications for the diagnosis and treatment of a variety of eye diseases related to ROS, such as AMD.

References


