Inhibitory Effects of Retinoic Acid Receptor Alpha Stimulants on Murine Cataractactogenesis through Suppression of Deregulated Calpains

Nami Nishikiori,1,2 Makoto Osanai,1 Hideki Chiba,1 Takashi Kojima,1 Hiroshi Obguro,2 and Norimasa Sawada1

PURPOSE. To determine whether retinoic acid (RA)—mediated inhibition of deregulated calpains had any effect on the development of cataract given that accumulating evidence has demonstrated a possible relationship between cataractogenesis and inappropriate activation of calpains.

METHODS. The authors examined for Ca2+ influx and expression alternation of calpains in F9 cells with or without RAs, such as all-trans retinoic acid (ATRA), and specific stimulant of retinoic acid receptor α (RARα; Am580) in the presence of oxidative stress, such as mediated by H2O2. They next examined the clinical relevance of RAs by applying these agents to a murine diabetic cataract and observed the development of the disease.

RESULTS. F9 cells constitute a well-established autonomous cell model for investigating retinoid signaling, partially representing the lens epithelial phenotype, as determined by the expression of aquaporin 0, a specific differentiation marker for lens cells. Treatment with ATRA and Am580 significantly decreased the influx of Ca2+ into the cells, causally resulting in decreased mRNA expression and inhibited activation of calpains. In addition, RARα agonists significantly abrogated the upregulation of calpain 2 induced by H2O2, which is a potential etiological contributor to the diabetic cataract, whereas H2O2 had no effect on calpain 1. Importantly, this RA-mediated gene-expression alteration was sufficient for dramatically inhibiting the development of lens opacity in mice with diabetes.

CONCLUSIONS. Results showed that a certain type of RA inhibits Ca2+ elevation and subsequent overactivation of calpains, suggesting the potential feasibility of calpain-targeting therapies mediated by RA for cataract. (Invest Ophthalmol Vis Sci. 2007;48:2224–2229) DOI:10.1167/iovs.06-1222

Cataract is an ever-increasing visual problem that accounts for most cases of treatable blindness among the elderly population throughout the world. Epidemiologic studies have indicated that 50% of the general population older than 65 has cataracts.1 Although clinical morphology studies have proposed three types of age-related cataract—nuclear, cortical, and posterior subcapsular—a growing issue is cataract in patients with diabetes in whom opacities are observed in a number of regions of the lens (for a review, see Biswas et al.2). Diabetic cataracts develop faster, and the incidence of cataract in persons with diabetes is more than 50% higher than in age-matched persons without diabetes.2,3 Oxidative stress is generally accepted as the major mechanism common to different types of cataractogenesis, and sugar-mediated chemical changes of lens proteins and accumulation of glycation end products are responsible for the diabetic cataract.4–6 However, the precise etiologies and underlying pathways that lead to various types of cataract remain to be fully clarified.

Experimental cataracts in several animal models have shown that the lens opacification associated with cataract is primarily due to a loss of Ca2+ homeostasis, subsequent activation of the Ca2+-dependent proteolytic enzymes (i.e., calpains), and major structural modifications of water-soluble lens protein crystallins (for reviews, see Biswas et al.2, Huang and Wang,7 Zatz and Starling,8 Biswas et al.9 and Biswas et al.10,11 Because functional integrity of crystallins is primarily responsible for lens transparency, pathologic insults to change crystallin structure lead to the aggregation of these proteins, eventually contributing to the impaired lens performance associated with cataract. It has become increasingly clear that the aging process and lens insults, such as oxidation and diabetes, can lead to impaired membrane integrity, resulting in pathologically elevated concentrations of lens Ca2+. Elevated levels of Ca2+ can induce crystallin proteolysis by calpains. Therefore, strategies that regulate inappropriate activation of calpains may be an important means of developing effective preventive and therapeutic modalities for cataract.

More than 50 endogenous and exogenous inhibitors of calpains and various other types of anti-cataractogenic agents have been described, whereas no drug has yet been approved for clinical use.2,7–10 Given that a number of reports have demonstrated a mechanistic link between gene regulation of calpains and certain types of retinoic acids (RAs),12,13 we examined whether RA-induced inhibition of deregulated calpains had any effect on cataractogenesis. Here we successfully demonstrate that all-trans retinoic acid (ATRA) and a synthetic retinoid that stimulates retinoic acid receptor (RAR) α modulate the deregulated expression of calpains, which is sufficient for the reduction of lens opacity in the murine diabetic cataract.

MATERIALS AND METHODS

Cell Culture and Treatment

F9 murine teratocarcinoma cells were originally provided by Pierre Chambon (Institute of Genetics and Molecular and Cellular Biology, Illkirch-Cedex, France).14 F9 cell lines were known to display different RA sensitivities, depending on origin. Our preliminary experiments demonstrated that F9 cells expressed RARα, RARβ, and RARγ mRNA (Nishikiori N, manuscript in press), which corresponded with F9-1,2,4

From the Departments of 1Pathology and 2Ophthalmology, Sapporo Medical University School of Medicine, Chuo-ku, Sapporo, Japan. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Japan Diabetes Foundation. Submitted for publication October 11, 2006; revised November 16, 2006; accepted February 15, 2007.

Disclosure: N. Nishikiori, None; M. Osanai, None; H. Chiba, None; T. Kojima, None; H. Obguro, None; N. Sawada, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Nami Nishikiori, Department of Ophthalmology, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo, Japan; nami751978@sapmed.ac.jp.
cells, as described in a previous study. Cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Cell Culture Technologies, Lugano, Switzerland) or charcoal-dextran-treated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells (5 × 10⁵ cells/24-well) were treated with 100 nM ATRA, 10 nM cis-RA (9cRA), or 10 nM various synthetic RAs such as 4-[(5,6,7,8-tetrahydro-5,8,9-trimethyl-2-naph-thaleny]carboxamido]benzoic acid (Am580) and 4-[(2,3-(2,5-dimethyl-2,5-hexanoyl) dibenzob[b,f] [1,4]thiazepin-11-yl]benzoic acid (Hx630) for up to 24 hours in combination with 20 μM H₂O₂ as a default concentration, unless otherwise specified.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA (1 μg) extracted using reagent (TRizol; Invitrogen, Carlsbad, CA) was reverse-transcribed with poly-T oligonucleotide and M-MulV reverse transcriptase (Invitrogen). For gene expression analysis, the gene of interest was amplified from dilutions of cDNA using specific sense and antisense primers for up to 30 cycles at 94°C for 15 seconds, 55°C for 15 seconds, and 72°C for 30 seconds. We examined various cycling parameters for each PCR experiment to define optimal conditions for linearity to allow for semiquantitative analysis of signal intensity. To provide a qualitative control for reaction efficiency, PCR reactions were performed with primers coding for GAPDH (5'-AAAGTGATGTCGGAGGA-3' and 5'-GTAGGTGCGGATCTGAGT-3'). PCR primers used in this study were as follows: aquaporin 0 (AQP0; GenBank accession number, BC082567) 5'-AGGAAACCTACTGGCT-CAACA-3' and 5'-ATTGGAGTCTGGTCTGG-3', calpain 1 (BC050276), 5'-AGTGGAAAGGACCCTGGAGT-3' and 5'-ACTCCGGGT-TGTCATATGCT-3', calpain 2 (NM_009794), 5'-AAGTCGACCGCT-TCAGATC-3' and 5'-GAAACTCTACCGCAAGG-3'. Samples were separated by electrophoresis in ethidium bromide-impregnated 2% agarose gels. For densitometric analysis, signals in RT-PCR analysis were quantitated (Image 1.62; Scion Corporation, Frederick, MD).

**Ca²⁺ Influx**

Ca²⁺ content in the cells was measured (Radiometer ABL 625; Diamond Diagnostics, Holliston, MA). Ca²⁺ influx into cells was defined as the subtraction of Ca²⁺ contents in conditioned culture medium from that in F9 cells.

**Caseinolytic Activity**

Protein contents of whole lysates extracted from F9 cells and mouse lenses were measured with a BCA protein assay kit (Pierce, Rockford, IL). Protein homogenates (40 μg) were subjected to electrophoresis without heating under nonreducing conditions in 0.1% casein-containing 10% polyacrylamide gels. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 hour to remove the SDS; this was followed by overnight incubation at 37°C in Tris buffer (50 mM Tris-HCl, 200 mM NaCl, and 10 mM CaCl₂ [pH 7.4]). Gels were then stained for 30 minutes with 0.5% Coomassie brilliant blue in 30% methanol/10% acetic acid. Clear bands appear on the blue background in the areas of caseinolytic activity.

**Animals**

C57BL/6 male mice (5 weeks old; Clea Japan, Tokyo, Japan) were housed in a specific-pathogen-free facility and were maintained on standard mouse chow and water. Previous reports have demonstrated that various etiological factors are associated with cataractogenesis, though it is generally accepted that diabetes is a well-documented determinant of its pathogenesis. We thus used diabetic mice to induce cataracts in clinically a relevant setting in which the disease was induced by intraperitoneal injections of 40 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO) dissolved in 0.01 M trisodium citrate buffer (pH 4.5) for 5 consecutive days. Control animals were injected with 250 μL vehicle (trisodium citrate buffer [pH 4.5]) for 5 consecutive days. Mice were screened for diabetes by tests of urinary sugar (Bayer, Leverkusen, Germany) and blood sugar (Arkray, Kyoto, Japan) and verified hyperglycemic state 6 weeks after the initial administration of STZ.

**RESULTS**

**Downregulation of Calpains by ATRA**

Mouse F9 teratocarcinoma cells constitute a well-established autonomous cell model for investigating retinoid signal-14,15,17,18 ing. To elucidate the roles played by multiple retinoid receptors in response to RA treatment and the consequences of RA-induced differentiation, we used F9 cells in this study. We first determined the cellular character of F9 cells and showed that the cells partially represented the lens epithelial phenotype, as determined by the expression of AQP0 (Fig. 1A), a specific differentiation marker for lens cells. After con-19,20 firming that our RT-PCR analysis had quantitativeness (Fig. 1B), we examined whether RAs had competent effects that could change mRNA expression of calpains. Although expression of calpain 1 and calpain 2 was not changed in the media supplemented with endogenous RA-depleted (i.e., charcoal-dextran-treated) FBS, ATRA treatment resulted in a significant reduction of mRNA expression in both calpains (Fig. 1C), suggesting that ATRA-induced gene-expression alteration required a pharmacologic dose of ATRA. We also demonstrated that the ATRA-induced decrease of the calpain mRNA showed dose and time dependency (Figs. 1D, 1E). Calpain expression was decreased most significantly at the concentrations of 100 nM for calpain 1 and 50 nM for calpain 2 (Fig. 1D), and 4-hour treatment with ATRA was sufficient to cause the maximal reductions of calpain expression (Fig. 1E).

**Preferential Effect on Calpains by RARα Stimulants**

ATRA acts through specific RA nuclear receptors RARα, RARβ, and RARγ and their heterodimeric partners, the retinoid-X-receptors (RXRα, RXRβ, and RXRγ), to positively or negatively regulate the expression of target genes. To identify RA nuclear receptor subtypes responsible for the reduction of calpain expression, we examined the calpain mRNA by treatment with various synthetic retinoids. Treatment with ATRA, 9cRA, and the RARα-selective compound Am580 satisfactorily reduced mRNA expression of calpains, whereas the RXR pan-agonist Hx630 did not have a significant effect on calpains (Fig. 2A). In addition, the enzyme activities of calpains were also significantly decreased by treatment with ATRA and Am580 (Fig. 2B). Our observations suggest that RARα is, at least in part, involved in the gene-expression alterations of calpains.
Suppression of H₂O₂-Induced Activation of Calpains by RARα Stimulants

Previous studies have demonstrated that oxidative stress has promoting effects on cataractogenesis in vitro and in vivo.⁴–⁶

We next examined the expression alterations of calpains by treatment with H₂O₂, which is an effective inducer of oxidative stress. H₂O₂ induced a remarkable increase in Ca²⁺ influx (Fig. 3A), whereas treatment with RARα agonists such as ATRA and Am580 significantly reduced the influx of Ca²⁺ into F9 cells (Fig. 3A) and consequently abrogated the upregulation of calpain 2 induced by H₂O₂ (Fig. 3B). In addition, treatment with H₂O₂ induced a significant increase in caseinolytic activity, which was efficiently suppressed by the treatments with ATRA and Am580 (Fig. 3C). On the other hand, H₂O₂ had no effect on calpain 1 in F9 cells (data not shown).

Inhibitory Effect of RARα Agonists on Murine Diabetic Cataractogenesis

Based on our observation that RARα stimulants effectively antagonized the oxidative stress-induced upregulation of calpains in vitro, we examined whether RARα agonists had any effect on lens transparency in animals with cataracts. We used diabetic mice to induce cataracts in a more clinically relevant condition because oxidative stress is thought to be an important contributing factor to the lens in diabetes.⁴–⁶ Surprisingly, treatment with RA reduced the Ca²⁺ concentration in the whole lenses of diabetic mice (Fig. 4A), and resulted in dramatic inhibition of the development of lens opacity (Fig. 4B). This effect was potentially associated with the inhibition of enzymatic activities of calpains by treatment with RA (Fig. 4C).

Discussion

Herein we demonstrated that RARα stimulation inhibited Ca²⁺ influx into the lens and causally suppressed the overactivation of calpains, which was sufficient for the reduction of lens opacity in murine diabetic cataract, suggesting the possibility of effective preventive and therapeutic modalities for human diabetic cataracts (Fig. 5).

Figure 1. ATRA decreases calpain expression in F9 cells. (A) Expression of AQP0 was examined by RT-PCR. (B) The concentration of total RNA in the template shows an inverse relation to the cycle threshold parameter. Cycle threshold is determined by the PCR cycles to saturate the band intensity. Representative gel image of various amounts of total RNA amplified in 26 cycles. (C) RT-PCR analysis of calpain 1 and calpain 2 treated with ATRA for 4 hours in medium supplemented with endogenous RA-depleted (+) or nondepleted (i.e., normal; −) FBS. Expression of calpains treated with various concentrations of ATRA for 4 hours (D) and at the indicated time points up to 24 hours in the presence of ATRA (E). Densitometric analysis is also shown below each gel image. Signal intensity of the control (cells without treatment) is defined as 100%. *P < 0.05 and **P < 0.01 versus cells without treatment.

Figure 2. RARα is preferentially involved in the gene-expression alterations of calpains. (A) RT-PCR analysis of calpain 1 and calpain 2 treated with various synthetic retinoids for 4 hours. ATRA, RAR pan-agonist; 9cRA, RAR and RXR pan-agonist; Am580, RARα agonist; H6630, RXR pan-agonist. (B) Casein zymograms showing the activity of calpains in F9 cells after 6-hour treatment with ATRA and Am580. Densitometric analysis is also shown below each gel image. Signal intensity of the control (cells without treatment; Ctrl) is defined as 100%. *P < 0.05 and **P < 0.01 versus cells without treatment.
A number of calpains have been identified in the lens, including calpain 2, calpain 1, calpain 10, and two splice variants of calpain 3, Lp82 and Lp85. Consistent with our observation, previous studies have demonstrated that calpain 2 is a major calpain implicated in murine diabetic cataract and the strongest candidate for a role in human types of cataractogenesis. On the other hand, some of the mechanism for cataracts is partially responsible for the impaired membrane integrity of the lens cells, leading to the loss of water homeostasis caused by the hydrolysis of AQP0 by calpain 2. Considering that lens cells contain a number of independent protease systems and nonenzymatic mechanisms, it is interesting to speculate that strategies that inhibit various enzymatic activities may be an important means of decreasing the sensitivity of lens cells to cataract-inducing stimuli. RAs block a wide variety of activated cellular responses involved in the pathogenesis of several types of inflammatory disease and cancer, suggesting the potential feasibility of RA-mediated therapies for cataract.

Calpain inhibitors, designed for the topical administration of ophthalmic solutions, pave the way to serve as nonsurgical alternatives for treating cataract. A number of calpain inhibitors have been synthesized. One of the most promising is a novel peptide aldehyde, SJA6017, which has shown improved membrane permeability and has been shown to inhibit both calpains and cataract formation in rat lens cell culture. In parallel, at least in conjunction with calpain-blocking activities by RA, RA is a good candidate to inhibit cataractogenesis as a complement to conventional diabetes therapy. Given the pleiotropic effects of RA on cellular functions, it is not surprising that certain RAs are effective for preventing the progression of murine cataract. When we consider the use of RAs for differentiation chemotherapy for leukemia, RAs may be an effective therapeutic strategy for human cataract because it is possible that they maintain the membrane integrity of the lens cells to modulate the cellular differentiation machinery. In addition, the dramatic effectiveness of RA on cataract is supported by the concept that RA is a ligand of RA nuclear receptors that can easily access the inside of the cell because of its lipophilic nature to show a high concentration of RA in the aqueous humor and lens cell.

This study focused on the mechanisms of RA-mediated protection of lens cells during cataractogenesis. There appears to be a logical gap between in vitro experiments carried out with F9 cells and the in vivo study on the murine diabetic cataract. Numerous studies have demonstrated that F9 cells are stem cells widely used to study RA-mediated cell differentiation and are an excellent model to investigate retinoid signaling. Controversy remains, however, regarding whether the data obtained from F9 cells are applicable to the lens epithelium. In F9 cells, constitutive expressions of AQP0 and calpains, which are essential components of lens cell biology, were observed in the media that contain physiological concentrations of RAs, showing that the cells partially represented the lens epithelial phenotype in the presence of RA. In addition, we should note here that the commercially available

**Figure 3.** RA agonists abrogate oxidative stress-mediated upregulation of calpain 2. The influx of Ca^{2+} (A) and expression of calpain 2 (B) in F9 cells treated for 4 hours in the presence (+) or absence (−) of H_{2}O_{2} with or without ATRA or Am580. (C) Casein zymograms showing the activity of calpains in F9 cells treated for 6 hours in the presence (+) or absence (−) of H_{2}O_{2} in combination with ATRA or Am580. Densitometric analysis is also shown below each gel image. Signal intensity of the control (cells without treatment) is defined as 100%. *P < 0.05 and **P < 0.01 versus cells without treatment with H_{2}O_{2}, and ***P < 0.05 versus cells without RA treatment.

**Figure 4.** RAs agonists inhibit cataractogenesis in diabetic mice. Diabetic mice with cataract were treated with ATRA or Am580. Excised lenses were measured for the Ca^{2+} concentration (A), representative developed cataracts and cataract levels (B), and caseinolytic activities (C). Note the significant dense opacification in the diabetic animals, whereas ATRA and Am580 maintained lens translucency in diabetic mice. Lens opacity in control animals (mice treated with solvent alone; Ctrl) is defined as 1, and densitometric analyses are shown below the photographs. *P < 0.05 versus mice treated with solvent alone. **P < 0.01 versus mice without RA treatment.
lens epithelial cell lines are immortalized with simian virus 40 large tumor (SV40 T) antigen. SV40 T antigen is an oncogenic protein in a variety of cell types both in vitro and in vivo, and it is well known that the gene involving carcinogenesis significantly affects the normal cell physiology essential for cellular differentiation. This is also supported by the fact that the transformed cells can hardly be said to faithfully mimic normal biology; in addition, their genomes are notoriously diverse and poorly characterized. Importantly, in commercially available lens epithelial cells, prolonged incubation for more than 20 days is required to observe an appreciable amount of endogenous expressions of crystallin proteins, suggesting that this type of cell does not show the highly differentiated phenotype to lens epithelial cells. Moreover, the preparation of primary culture of lens epithelial cells is labor intensive and time consuming, though the cellular phenotype of this type of cell is highly conserved in vitro. Therefore, one would likely accept that F9 cells, rather than the cells transfected with oncogene and in primary culture, were appropriate for this study.

We could not exclude the possible participation of RXRs and 9cRA pathways in cataractogenesis, based on the evidence that exogenously applied ATRA can upregulate RXR mRNA expression in F9 cells and that calpain is able to digest 54 kDa RXRα, one of the physiological subtypes of RXRα. In addition, the transactivation of RAR/RXR heterodimer depends critically on the ligand-dependent transcriptional transactivation function of AF-2 of RARs, such as ATRA, to cause differential activation of the RA-responsive gene pathway. Another example is presented by our method of incubation of F9 cells with ATRA for 24 hours. It is likely that isomerization of some part of ATRA to 9cRA occurred in usual culture conditions in F9 cells and is a physiologically relevant mechanism for the formation of 9cRA from ATRA. Moreover, it has been found that the interaction between ATRA and 9cRA can be complex. For example, ATRA and 9cRA compete for binding to RARs, and the ligand-mediated gene expression appeared to depend on cell type, ligand pharmacokinetics, and extent of competition or cross-talk among nuclear transcription factors. We also used synthetic retinoid (Am580) and retinoid synergist (HX630), which exert complex actions in the cells and have an apoptosis-promoting capability. Similarly, ATRA may exert its effect by suppressing the activator protein 1 pathway through the inhibition of c-fos and c-jun expression, and calpain is shown to be involved in apoptotic machinery through RAR/RXR-independent pathways. Although we cannot exclude the participation of RXRs responsible for inhibiting diabetes-induced cataract, RARα agonists are, at least in part, clearly involved in the pathogenesis of cataract.

RAs have been shown to affect lens biology in vertebrates, but no previous studies have confirmed the role of RAs in human cataractogenesis. We cannot conclude from our present study how RAs affect lens epithelial integrity; however, our observations may support the plausible hypothesis that strategies that increase RA bioavailability in the lens microenvironment may be promising to reduce the development of lens opacification in a variety of disorders to induce cataract, in which elevated levels of Ca2+ result in deregulated activation of calpains. Studies along this line will help us to understand the pathogenesis of diseases characterized by the disruption of lens epithelial integrity.

Acknowledgments

The authors thank Kim Barrymore for help with the manuscript, the staff at the Animal Care Facility of Sapporo Medical University School of Medicine, and Hiroyuki Kagechika (School of Biomedical Sciences, Tokyo Medical and Dental University, Tokyo, Japan) for the gifts of Am580 and Hx630.

References


