Immunopathologic Processes in Sympathetic Ophthalmia as Signified by MicroRNA Profiling

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PURPOSE. Recent discovery of microRNAs and their negative gene regulation have provided new understanding in the pathogenesis of inflammatory diseases. This study demonstrated microRNA expression profiling and their likely role in sympathetic ophthalmia, using formalin-fixed, paraffin embedded samples.

METHODS. Two groups of four enucleated globes (total eight globes) from patients with clinical and histopathological diagnosis of SO (experimental samples) and one group of four age-matched, noninflamed enucleated globes (control samples) were used. Human genome-wide microRNA PCR array was performed and results were subjected to bioinformatics calculation and *P* values stringency tests. The targets were searched using the recently published and periodically updated miRWalk software. Quantitative real-time PCR and immunohistochemical staining were performed to confirm the validated targets in the mRNA and in the protein levels, respectively.

RESULTS. No microRNA was significantly upregulated in SO, but 27 microRNAs were significantly downregulated. Among these, four microRNAs (hsa-miR-1, hsa-let-7c, hsa-miR-9, and hsa-miR-182) were known to be associated with the inflammatory signaling pathway. Only hsa-miR-9 has the validated targets, tumor necrosis factor- α , and nuclear factor kappa B1, which have been previously shown to be associated with mitochondrial oxidative stress-mediated photoreceptor apoptosis in eyes with SO.

CONCLUSIONS. Identification of altered levels of microRNAs by microRNA expression profiling may yield new insights into the pathogenesis of SO by disclosing specific microRNA signatures. In the future these may be targeted by synthetic microRNA mimic-based therapeutic strategies. (*Invest Ophthalmol Vis Sci.* 2012;53:4197–4204) DOI:10.1167/iops.12-9465

Sympathetic ophthalmia (SO) is a bilateral, granulomatous uveitis that usually occurs after a penetrating injury or intraocular surgery to one eye.^{1–3} The injured eye is termed the

exciting or sympathogenic eye, and the contralateral eye is termed the sympathizing eye.¹ Both the exciting and the sympathizing eye show similar pathologic features of diffuse granulomatous inflammation in the uvea with preservation of the choriocapillaris and retina.¹ Treatment for SO consists of high-dose systemic corticosteroids supplemented by immunosuppressive agents as needed. Even though management of SO has improved, up to one-third of affected patients may eventually become legally blind.³ The pathogenesis of SO is yet to be completely elucidated. But previous immunohistopathologic studies have shown that T cells predominantly infiltrate the choroid¹; therefore, SO is believed to relate to a T cell-mediated autoimmunity to an antigen present in melanocytes. However, the mechanism of visual loss in the absence of recognizable retinal damage and retinal inflammatory cell infiltration has been an enigma.

MicroRNAs are approximately 18- to 24-nucleotide-long, single-stranded, noncoding RNAs that bind to partially complementary sequences within the 3'-untranslated region of target mRNAs. In general, they negatively regulate gene expression posttranscriptionally in eukaryotic cells.⁴ The microRNAs are thought to regulate approximately one-third of the protein-coding genes of the human genome, and individual microRNAs target hundreds of mRNAs rather than just one specific gene.⁵ In recent years, several studies have revealed that microRNAs act as key regulators in a wide variety of biological processes, such as cell proliferation, cell differentiation, cell fate determination, apoptosis, and signal transduction.^{6,7} Therefore, altered microRNA expression indicates a strong association in the pathogenesis of diverse human diseases, such as muscular disease,⁸ heart disease,⁹ cancer,¹⁰ and schizophrenia.¹¹ Most recently, inflammatory and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, primary biliary cirrhosis, ulcerative colitis, and psoriasis have been reported to be associated with altered microRNA expression.^{12,13} Thus, microRNA may represent an important target for potential therapeutic and diagnostic agents.^{14,15} However, the ability of inflammatory cytokines to modulate microRNA expression and the role of microRNAs in the development of immune response, especially in the eye, are just beginning to be explored.

In routinely processed formalin-fixed, paraffin-embedded (FFPE) tissues, DNA and RNA were found to be preserved and can be extracted for analysis by use of PCR.^{16,17} MicroRNA expression profiles in FFPE human tissue sections have been recently shown to be virtually identical to those of frozen fresh tissues.¹⁸ New methods for gene retrieval from FFPE samples have demonstrated much-improved recoveries for microRNAs. Therefore, FFPE tissue may serve as an invaluable tool for microRNA profiling in a variety of diseases.

The present study was designed to investigate microRNA expression and the likely role of microRNAs in modulating the inflammatory cytokines in the pathogenesis of SO.

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MATERIALS AND METHODS

FFPE Samples of Human Globes with SO and Control

This study was performed in accordance with the tenets of the Declaration of Helsinki.

The institutional review board of the University of Southern California approved our use of human specimens. Eight FFPE eyes of eight patients with a clinical and histopathological diagnosis of SO were randomly grouped into two experimental groups (E1 and E2) of four eyes each, and four FFPE eyes of four age-matched patients without intraocular inflammation made up the control group (C). All cases were retrieved from the archives of the A. Ray Irvine, Jr., Pathology Laboratory of the Doheny Eye Institute, Los Angeles, CA. The patients ranged in age from 28 to 76 years, with a mean age of 55.0 ± 13.2 years. All eight SO eyes had been enucleated because the patients had no light perception and the eyes were painful. The duration between onset of symptoms related to SO and the enucleation ranged from 2 days to 3 years; however, the histopathological findings were similar among them. All eight SO patients had received topical and/or systemic corticosteroid treatment prior to enucleation. All four control eyes were part of exenterated orbital contents, and the exenterations were performed for management for orbital tumors (however with no intraocular invasion or inflammation). Each experimental group (E1 and E2) comprised eyes with similar histopathological findings of SO, and this grouping was performed in order to obtain statistically significant overlapping microRNAs when compared to controls (C) without uveitis.

RNA Extraction from FFPE Sample

From each FFPE block, 10- μ m-thick paraffin sections were cut and mounted on plain glass slides. Under microscopic observation, each section was then carefully microdissected with the aid of a sterile needle, and the unwanted tissue (anterior segment, sclera, and optic nerve) was scraped off with a blade (preincubated with RNase). Four samples (only retina and choroid) of each group (E1, E2, and C) were pooled and then respectively collected into separate Eppendorf tubes. These samples were used immediately after sectioning to avoid the disruption of RNA by prolonged exposure to the air. RNA was extracted from each group using the RNeasy FFPE extraction kit (SABiosciences/QIAGEN, Frederick, MD) as previously reported.^{19–21}

MicroRNA PCR Array and Assay

Human genome-wide microRNA PCR Array (SABiosciences/QIAGEN) was performed twice, to compare groups E1 to C and E2 to C, as follows: 200 ng of enriched small RNA was converted into complementary DNA (cDNA) using RT² microRNA First Strand Kit (SABiosciences/QIAGEN). The cDNAs were mixed with $2 \times$ RT² SYBR Green qPCR Master Mix (SABiosciences/QIAGEN) and dispersed into a 384-well human genome microRNA PCR Array with 10 μ L/well reaction volume. MicroRNA PCR Array was used to monitor the expression of a panel of primer sets for 376 human microRNAs, with four housekeeping genes (U6, SNORD 44, 47, and 48) for small nuclear RNA as the endogenous controls to normalize raw data. Duplicate reverse transcription controls to test the efficiency of the microRNA reverse transcription reaction and duplicate positive PCR controls to test the efficiency of the PCR itself were also included in the array. The average of four housekeeping genes was used for normalization. Normalization of microRNA expression was conducted using SABiosciences Online PCR Array Data Analysis Web Portal. The $2^{-\Delta\Delta Ct}$ values from the original expression profiles were obtained.

Target Search

To search the validated and predicted targets for the altered microRNAs and to prioritize the list of targets and determine inflammatory signaling

pathways that are most significantly affected by the microRNAs in SO, the recently published and periodically updated miRWalk software (<http://www.umm.uniheidelberg.de/apps/zmf/mirwalk/>) was used.

Quantitative Real-Time PCR

To detect tumor necrosis factor alpha (TNF- α) and nuclear factor kappa B1 (NFkB1), and inducible nitric oxide synthase (iNOS) transcripts, quantitative real-time RT-PCR was performed in triplicate and analyzed in each group (E1, E2, and C) by the LightCycler 480 system (Roche Applied Science, Mannheim, Germany). Briefly, a cDNA library was generated using a reverse transcription system (Promega, Madison, WI) with 1 μ g of the total RNA extracted from tissue samples with SO and control samples. The cDNA was used to amplify TNF- α , NFkB1 and iNOS genes, and GAPDH gene was used as an endogenous control for the PCR. PCR primers were TNF- α sense, 5'-GGAGAAGGGTGACCGACTCA-3', and TNF- α antisense, 5'-TGCCCAGACTCGGCAAAG-3'; NFkB1 sense, 5'-GGCTACACCGAAGCAATTGAA-3', and NFkB1 antisense, 5'-CAGCGAGTGGGCCTGAGA-3'; iNOS sense, 5'-TCCTTGCATCCTCATCGGGCC-3', and iNOS antisense, 5'-TCGTGATAGCGCTTCTGGCTC-3'; GAPDH sense, 5'-AACTGCTTAGCACCCCTGGC-3', and GAPDH antisense, 5'-ATGACCTTGCCACAGCCTT-3'. All primers were purchased from Eurofins MWG Operon (Huntsville, AL). PCR was performed under the following conditions: 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds, 60°C for 10 seconds, 72°C for 10 seconds. There was no significant difference in the result obtained from E1/C and E2/C for TNF- α , NFkB, and iNOS, and thus we used the averages of E1/C and E2/C for analysis.

Statistical Analysis

MicroRNA and mRNA expression between SO and control samples were compared using Student's *t*-test as previously reported,^{22,23} and a *P* value <0.05 was considered statistically significant. A positive fold-change value indicated upregulation of microRNA expression in SO patient samples, while a negative fold-change value indicated downregulation. A fold-change cutoff of ≥ 2 for upregulation (or ≤ -2 for downregulation) was applied in addition to the *P* value cutoff (<0.05) to determine microRNAs with significant expression alterations.

Immunohistochemistry Staining

Five-micron-thick sections cut from the SO globes and the age-matched control globes were deparaffinized in xylene and rehydrated in graded alcohol. Specimens were subjected to antigen retrieval by covering the sections with a 10 mM sodium citrate buffer (pH 6.0) and heating them in a microwave for 1 minute. Slides were then cooled at room temperature for 20 minutes, rinsed with PBS, and blocked with 5% bovine serum albumin for 30 minutes at room temperature. Sections were incubated overnight at 4°C with mouse monoclonal TNF- α antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal NFkB antibody (1:100; Santa Cruz Biotechnology), and rabbit iNOS antibody (1:50; Santa Cruz Biotechnology), respectively, as the primary antibody. Sections were washed three times with PBS and incubated in the dark for 45 minutes at room temperature with donkey anti-mouse immunoglobulin G (IgG) conjugated with Cy2 (1:200; Jackson Laboratory, Bar Harbor, ME), donkey anti-goat IgG conjugated with FITC (1:200; Santa Cruz Biotechnology), and donkey anti-rabbit IgG conjugated with Cy2 (1:200; Jackson Laboratory), respectively, as secondary antibody. The sections were then washed with PBS and mounted with medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

RESULTS

Histopathologic Features of Investigated SO Globes

All the SO eyes showed the typical histopathologic feature of diffuse, nonnecrotizing, granulomatous inflammation of the

FIGURE 1. Microscopic aspect of a representative eye with sympathetic ophthalmia from the experimental group. Note the diffuse nonnecrotizing granulomatous inflammation in the choroid, with preservation of the choriocapillaris. The retina is preserved (hematoxylin-eosin; $\times 200$).

uvea and markedly thickened choroid infiltrated by lymphocytes associated with nests of macrophages, epithelioid cells, and giant cells. The choriocapillaris was spared, and no extension of the inflammatory cells into the retina was detected (Fig. 1). None of the eyes were phthisical prior to enucleation.

MicroRNA Expression in SO

MicroRNA expression was initially evaluated using microarray analysis. There was some overlapping between E1/C and E2/C groups in microRNA expression, as follows: five microRNAs were upregulated (fold change >2 ; for reference: hsa-miR-21, -142-3p, -142-5p, -146b-5p, and -150); on the other hand, 79 microRNAs were downregulated (fold change <-2) in both E1/C and E2/C groups. Among these, bioinformatics showed that 27 microRNAs were significantly downregulated (P value < 0.05 by Student's t -test) in SO samples compared with control samples. These microRNAs were hsa-miR-1, hsa-let-7e, hsa-miR-9, -23b, -30b, -30d, -95, -99a, -100, -125a-5p, -129-3p, -130b, -139-5p, -181a, -181b, -181d, -182, -183, -190b, -203, -211, -335, -338-3p, -376a, -379, -504, and -551b (Table 1). In contrast, no microRNA was significantly upregulated after bioinformatics analysis. After investigating the association between these 27 significantly downregulated microRNAs and T cell-mediated inflammatory pathways based on the recently published miRWalk software, we have further selected four microRNAs (hsa-miR-1, hsa-let-7e, hsa-miR-9, and hsa-miR-182), which were downregulated by 43.59-, 3.91-, 6.81-, and 45.19-fold, respectively, as the important signature of SO pathogenesis (Table 2).

mRNA Expression of TNF- α , NF κ B1, and iNOS in SO

The expression of TNF- α , NF κ B1, and iNOS mRNA was significantly upregulated (P value <0.05), by 2.38-, 1.62-, and 6.52-fold, respectively, compared with control samples (Fig. 2).

Immunolocalization of TNF- α in SO

No expression of TNF- α was detected in the control retina (Fig. 3B), whereas increased expression of TNF- α was detected in the inner segments of the photoreceptors and inner and outer plexiform layers of SO retina (Fig. 3C). The SO retina revealed no staining when the primary antibody was replaced by isotype immunoglobulin or PBS (figures not shown). The localization of TNF- α was seen in all the SO retinas.

Immunolocalization of iNOS in SO

No expression of iNOS was detected in the control retina (Fig. 3D), whereas increased expression of iNOS was detected in the inner segments of the photoreceptors of SO retina (Fig. 3E). The SO retina revealed no staining when the primary antibody was replaced by isotype immunoglobulin or PBS (figures not shown). The localization of iNOS was seen in all the SO retinas.

Immunolocalization of NF κ B1 in SO

Mild and diffuse expression of NF κ B1 was detected in the cytoplasm, whereas no expression was detected in the nuclei of the control retina (Fig. 3F without DAPI stain; Fig. 3G with DAPI).²⁴ Increased expression of NF κ B1 was detected in the nuclei of the outer nuclear layer and the inner segments of photoreceptors; in addition, mild expression was detected in

TABLE 1. Significantly Downregulated MicroRNAs in Sympathetic Ophthalmia (SO) Samples Compared with Control Samples, Using MicroRNA PCR Array

MicroRNA	P Value	Fold Change
hsa-miR-1	0.0003	-43.59
hsa-let-7e	0.0128	-3.91
hsa-miR-9	0.0471	-6.81
hsa-miR-23b	0.0100	-5.37
hsa-miR-30b	0.0071	-7.95
hsa-miR-30d	0.0044	-10.85
hsa-miR-95	0.0161	-6.01
hsa-miR-99a	0.0200	-6.91
hsa-miR-100	0.0189	-4.49
hsa-miR-125a-5p	0.0440	-5.18
hsa-miR-129-3p	0.0033	-18.24
hsa-miR-130b	0.0204	-2.01
hsa-miR-139-5p	0.0086	-5.33
hsa-miR-181a	0.0189	-4.92
hsa-miR-181b	0.0044	-3.33
hsa-miR-181d	0.0347	-2.35
hsa-miR-182	0.0040	-45.19
hsa-miR-183	0.0191	-42.67
hsa-miR-190b	0.0074	-7.93
hsa-miR-203	0.0355	-4.18
hsa-miR-211	0.0017	-10.88
hsa-miR-335	0.0160	-9.53
hsa-miR-338-3p	0.0123	-4.22
hsa-miR-376a	0.0026	-2.03
hsa-miR-379	0.0330	-2.87
hsa-miR-504	0.0362	-4.24
hsa-miR-551b	0.0001	-6.09

the nuclei of the inner nuclear layer of SO retina (Fig. 3H without DAPI; Fig. 3I with DAPI).²⁴ The SO retina revealed no staining when the primary antibody was replaced by isotype immunoglobulin or PBS (figures not shown). The localization of NFκB1 was seen in all the SO retinas.

DISCUSSION

In this study, among the 27 significantly downregulated microRNAs, hsa-miR-1, hsa-let-7e, hsa-miR-9, and hsa-miR-182 were found to be interrelated with the inflammatory signaling pathway and to be an important signature of SO pathogenesis. In contrast, there was no significant upregulated microRNA. Hsa-miR-9, in particular, has the potential to target a significant number of genes involved in the TNF-α and NFκB signaling pathways. A previous study has shown that overexpression of hsa-miR-9 by transfection with pre-microRNA mimics significantly attenuates interleukin-1β-induced TNF-α protein production by 40% in human primary osteoarthritis chondrocytes. Moreover, locked nucleic acid inhibitor of hsa-miR-9 marginally elevates basal TNF-α protein production compared with a control.²⁵ With the luciferase reporter assay, NFκB1 mRNA is

TABLE 2. MicroRNAs Associated with Inflammatory Signaling Pathway and Their Validated and Predicted Targets

MicroRNA	Validated Targets	Predicted Targets
hsa-miR-1		CD4
hsa-let-7e		NFκB
hsa-miR-9	TNF-α, NFκB1	
hsa-miR-182		Fas, Fas ligand

FIGURE 2. There was significant upregulation of TNF-α, NFκB1, and iNOS in SO samples compared with control samples using quantitative real-time PCR analysis (* $P < 0.05$, ** $P < 0.01$). iNOS, inducible nitric oxide synthase.

also found to be directly regulated by hsa-miR-9 in human peripheral monocytes and polymorphonuclear neutrophils.²⁶ These results indicate that TNF-α and NFκB1 are validated targets of hsa-miR-9. The other significantly downregulated microRNAs seem to indirectly involve the inflammatory pathways. Hsa-miR-1 is significantly downregulated in CD4⁺ T cells of relapsing-remitting multiple sclerosis patients after stimulation by anti-CD3/CD28 antibodies, compared with before stimulation.²⁷ All hsa-let-7 family members inhibit the interleukin-6-dependent signaling pathway both directly through its 3' untranslated region and indirectly by an interaction with Ras that leads to a reduction in NFκB activity.²⁸ Forkhead box O1, which is a validated target of hsa-miR-182,²⁹ is shown to be associated with increased expression of the Fas/Fas ligand system in the mitochondrial apoptotic pathway.³⁰ Under normal conditions, these microRNAs repress the translation of target mRNAs posttranscriptionally in order to keep the expression levels low in maintaining homeostasis. Conversely, in disease conditions, the downregulation of these microRNAs leads to overexpression of proinflammatory proteins such as TNF-α and NFκB.

TNF-α is a multifunctional cytokine secreted by the activated T cells, monocytes, and other lymphocytes³¹ and is known to induce iNOS, which is responsible for the production of nitric oxide (NO).³² Mitochondria is a copious source of superoxide (O_2^-), which is generated at the sites of complexes I and III of the electron transport chain.³³ The concomitant generation of NO and O_2^- at a localized site is known to trigger the generation of peroxynitrite ($ONOO^-$), a major cytotoxic agent that initiates tissue damage in many neurodegenerative and inflammatory diseases.³⁴ Previous study shows a significant elevation of ocular and systemic TNF levels in patients with SO.³⁵ Our previous immunohistochemical study also reports that increased expression of TNF-α was detected in the photoreceptor nuclear layer and that TNF receptor-1 (TNFR1), iNOS, and oxidative stress markers such as nitrotyrosine and acrolein were detected in the inner segments of photoreceptors of SO in the absence of inflammatory cell infiltration.³⁶ Furthermore, most apoptosis cells were colocalized with nitrotyrosine in SO retina.³⁶ These findings indicate that mitochondrial oxidative stress induced by TNF-α and iNOS may lead to photoreceptor apoptosis in SO.³⁶ In accordance with these findings, in the present study, the significant elevation of TNF-α and iNOS was observed in the mRNA level, compared with control samples (Fig. 2), and the immunolocalization was observed in the inner segments of photoreceptors of SO (Figs. 3C, 3E).

FIGURE 3. Immunolocalization of TNF- α , NF κ B1, and iNOS in SO retina. (A) Showing hematoxylin-eosin-stained SO retina. (B) Immunostain for TNF- α . No expression of TNF- α was detected in the control retina. (C) Immunostain for TNF- α . Expression of TNF- α was detected in the inner segments of the photoreceptors and inner and outer plexiform layers of SO retina. (D) Immunostain for iNOS. No expression of iNOS was detected in the control retina. (E) Immunostain for iNOS. Expression of iNOS was detected in the inner segments of the photoreceptors of SO retina. (F) Without DAPI. (G) With DAPI. Immunostain for NF κ B1. Mild expression of NF κ B1 was detected in the cytoplasm, whereas no expression was detected in the nuclei of the control retina. (H) Without DAPI. (I) With DAPI. Immunostain for NF κ B1. Increased expression of NF κ B1 was detected in the nuclei of the outer nuclear layer and inner segments of photoreceptors and mild expression was also detected in the nuclei of the inner nuclear layer of SO retina. The SO retina revealed no staining in all the cases when the primary antibody was replaced by isotype immunoglobulin (figures are not shown). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment of photoreceptors.

NF κ B consists of five subunits, RelA, RelB, cRel, NF κ B1, and NF κ B2, and these form several homodimers and heterodimers. NF κ B plays an important role in transcription of the gene encoding many proinflammatory cytokines, chemokines, and

enzymes that generate reactive oxygen species and prostaglandins (e.g., iNOS and cyclooxygenase) and regulates inflammatory responses, apoptosis, cell proliferation, and cell migration.³⁷ NF κ B1 in unstimulated resting cells is restricted

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FIGURE 4. The downregulation of these microRNAs in SO could lead to the overexpression of proinflammatory cytokines and proteins, and finally causes mitochondrial oxidative stress mediated photoreceptor apoptosis. *Bold line* indicates the validated targets, and *dashed line* indicates the predicted targets.

to the cytoplasm with RelA by the inhibitor kappa B alpha (IκBα), which subsequently prevents them from entering the nucleus. When these cells are stimulated, IκB kinases phosphorylate IκBα, leading to its rapid degradation by proteasome with the release of NFκB1/RelA and their passage into the nucleus.^{24,37} In addition, NFκB1 and NFκB2 are upregulated by TNF-α stimulation at the mRNA and protein levels.³⁸ In the present study, NFκB1 was significantly increased at the mRNA level, compared with control samples (Fig. 2), and the immunolocalization was seen within the nucleus of the inner and outer nuclear layers of the SO retina (Figs. 3F–I). These findings indicate that NFκB1 may activate specific target gene expression, such as iNOS, in the nuclear layers of SO retina.

Experimental autoimmune uveoretinitis (EAU) is a highly reproducible animal model for human endogenous uveitis that has been used in our previous studies.^{39–42} Similar to SO, gene expression levels of TNF-α and iNOS are significantly upregu-

lated in the absence of retinal inflammatory cell infiltration at postimmunization day 5 (the early stage) of EAU; and immunohistochemical study shows that iNOS is primarily colocalized with the reactive oxidants in the photoreceptor mitochondria.³⁹ Our earlier laboratory studies report that ONOO[−] mediates selective nitration of mitochondrial DNA and protein in the early stage of EAU.^{40–42} The inflammatory signals and the subsequent mitochondrial oxidative stress damage appear to begin in the retina in the early phase of EAU as well as in SO, even though there is no histological or clinical evidence for uveitis.

Traditionally, deciphering the immunopathologic processes involved in inflammatory diseases has required demonstration of inflammatory cell infiltration, including T cells and macrophages. These effects are then related to the consequential tissue damages. In the retina of SO, the downregulated microRNAs in the present study may serve as triggers of inflammatory signalings, such as TNF-α and iNOS, and then

induce mitochondrial oxidative stress-mediated photoreceptor apoptosis (Fig. 4). This hypothesis may be related to the mechanism of the visual loss in the absence of recognizable retinal damage and inflammatory cell infiltration in SO, even though our data may present the effect of choroidal inflammatory cell infiltration as well.

The recently elaborated microRNA methodology can pinpoint the hitherto unrecognized disease signature by revealing the disease-induced gene level alterations,^{8–13} leading to a better understanding of the pathways of this disease's progress and, therefore, to the possibility of selective treatment. Our data, however, provide only a cross-sectional analysis of the process. Further studies on these pathways, including microRNA kinetics, are necessary to address their precise roles in the pathogenesis of SO and their potential as diagnostic or therapeutic targets. This may be easier in the area of cancer research,¹⁰ in which samples are easier to obtain and thus a good number of validated targets have been detected.

In conclusion, we have demonstrated that the identification of altered levels of microRNAs by microRNA expression profiling may yield new insights into the pathogenesis of SO. Combining the validated target search and recent information on the biological functions of these microRNAs, we have further elaborated the disease processes of SO beyond the past recognition of merely a T cell-mediated inflammatory disease. Among these proinflammatory cytokines, the upregulation of TNF- α and of NF κ B1 seem to be the crucial signaling factors in the pathogenesis of SO. Recently, a translational research possibility using synthetic microRNA mimic-based intervention has been reported in other diseases.^{43,44} Our study identified a microRNA signature in SO that may, in the future, be amenable to modification by a synthetic microRNA mimic-based therapeutic strategy. This study is, to the best of our knowledge, the first report of microRNA expression profiling on human ocular samples of SO and has thus raised numerous questions and opened up several new avenues for moving forward with the future study of this disease.

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