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Inflammatory Cytokines Regulate MicroRNA-155 Expression in Human Retinal Pigment Epithelial Cells by Activating JAK/STAT Pathway

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Abstract

Inflammatory response of the retinal pigment epithelium plays a critical role in the pathogenesis of retinal degenerative diseases such as age-related macular degeneration. Our previous studies have shown that human retinal pigment epithelial (HRPE) cells, established from adult donor eyes, respond to inflammatory cytokines by enhancing the expression of a number of cytokines and chemokines. To investigate the role of microRNA (miRNA) in regulating this response, we performed microarray analysis of miRNA expression in HRPE cells exposed to inflammatory cytokine mix (IFN- γ + TNF- α + IL-1 β). Microarray analysis revealed ~11-fold increase in miR-155 expression, which was validated by real-time PCR analysis. The miR-155 expression was enhanced when the cells were treated individually with IFN- γ , TNF- α or IL-1 β , but combinations of the cytokines exaggerated the effect. The increase in miR-155 expression by the inflammatory cytokines was associated with an increase in STAT1 activation as well as an increase in protein binding to putative STAT1 binding elements present in the *MIR155* gene promoter region. All these activities were effectively blocked by JAK inhibitor 1. Our results show that the inflammatory cytokines increase miR-155 expression in human retinal pigment epithelial cells by activating the JAK/STAT signaling pathway.

Keywords

Retinal Pigment Epithelium; microRNA-155; TNF-alpha; IL-1beta; IFN-gamma; JAK/STAT pathway

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1. Introduction

Retinal pigment epithelium (RPE), a monolayer of cells located behind the neuroretina, is an essential component of the visual system. RPE is needed for the regeneration of the visual chromophore 11-*cis*-retinal, for providing nutrients to photoreceptors, and for removing shed photoreceptor outer segments by phagocytosis [1]. It also maintains the immune privilege of the retina by constituting in part the blood/retina barrier and by secreting immunosuppressive factors. A normal functioning RPE is indispensable for vision, and its impaired function resulting from chronic inflammation is an important factor in the pathogenesis of age-related macular degeneration [2,3]. Ocular inflammation is often associated with the infiltration of lymphocytes and macrophages to the posterior compartment of the eye and their secretion of inflammatory mediators such as IFN- γ , TNF- α and IL-1 [4,5]. These inflammatory cytokines can target RPE and seriously impair its many critical functions, thus, eventually causing retinal degeneration [2-4]. Human RPE (HRPE) cells in culture respond to IFN- γ , TNF- α and IL-1 β by increasing the expression of cytokines and chemokines [6-8].

MicroRNAs, single-stranded noncoding small (~22 nucleotides) RNA molecules and posttranscriptional regulators of gene expression by their ability to target messenger RNAs for degradation or translational repression, are known to regulate inflammatory responses [9]. Inflammatory mediators such as lipopolysaccharide, TNF- α and IFN- γ can induce the expression of miR-155 in macrophages [10,11]. Many genes including *BACH1*, *SHIP1*, *CEBPB* and *IKK ϵ* are targeted for translational repression by miR-155 [11-14]. This miRNA and its precursor transcript BIC are encoded by *MIR155* gene, which is localized to the human chromosome band 21q21.3 [15].

The potential role of miR-155 or other miRNAs in modulating the inflammatory response of the human RPE or other retinal cells has not been elucidated yet. Therefore, we employed microarray analysis to investigate the miRNA expression in HRPE cells in response to treatment with inflammatory cytokines IFN- γ , TNF- α and IL-1 β . Here, we show that miR-155 is predominantly targeted for regulation by the inflammatory cytokines in HRPE cells. We also provide evidence for the first time that the JAK (Janus family kinases)/STAT (signal transducers and activators of transcription) signaling pathway could be directly involved in the regulation of miR-155 expression.

2. Materials and methods

2.1. Cell culture and treatment

Human retinal pigment epithelial (HRPE) cell cultures were prepared from adult donor eyes as described before and used between passages 7 and 10 [7]. Cell culture media and fetal bovine serum were obtained from Invitrogen, Carlsbad, CA. Human recombinant TNF- α and IFN- γ were purchased from Roche Applied Science, Indianapolis, IN while IL-1 β was from R&D Systems, Minneapolis, MN. JAK inhibitor 1 was obtained from Calbiochem, San Diego, CA. Cells were grown to confluence in 100 mm dishes or 6 well plates using minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids and antibiotic-antimycotic mixture. The confluent cultures were washed with serum free medium (medium described above without FBS) and then treated with the inflammatory cytokine mix containing TNF- α (10 ng/ml), IL-1 β (10 ng/ml), and IFN- γ (100 u/ml) for 16 h, unless otherwise stated. JAK inhibitor 1, when used, was added to the cultures 30 min prior to the cytokine treatment.

2.2. Microarray analysis of miRNA expression

HRPE cell cultures derived from two different donor eyes were employed for microarray analysis. Cells were treated with inflammatory cytokine mix for 16 h and total RNA including miRNA was extracted and size fractionated (Ambion mirVana miRNA Isolation Kit, Applied Biosystems). The control and treated RNA samples were then labeled with Cy3 and Cy5, respectively, and hybridized to chips containing miRNA probes (LC Sciences, Houston, TX; <http://lcsclences.com>). Data was normalized by the LOWESS method after subtracting the background. Transcripts with low signals (< 500) were not considered for further analysis. The signal differences were analyzed using Student *t* test, and a *p* < 0.05 was used to denote significant difference between controls and treated.

2.3. Analysis of secreted cytokines and chemokines

HRPE cells were treated with inflammatory cytokine mix for 16 h, and the culture supernatants were collected. The amounts of CCL5, IL-6, CXCL9 and CSF2 secreted into the medium were estimated using ELISA kits purchased from R&D Systems.

2.4. Polymerase Chain Reaction

Real-time RT-PCR analysis of miRNAs and BIC transcript in total RNA fractions obtained from HRPE cells was performed on an Applied Biosystems 7500 using default thermal cycling conditions and reagents from Applied Biosystems (Foster City, CA). Relative quantification ($\Delta\Delta CT$) method was employed. Analysis of miRNA expression was done using TaqMan MicroRNA Reverse Transcription Kit, individual TaqMan MicroRNA Assays (miR-155, miR-125b, miR-181d, miR-30b or miR-455-3p) and TaqMan Universal PCR Master Mix, No AmpErase. RNU48 was used as the endogenous control and manufacturer's default thermal cycling conditions were followed. For analyzing BIC transcript, reverse transcription and real-time PCR analysis was performed using High Capacity cDNA Archive Kit, TaqMan Universal PCR Master Mix, and specific TaqMan probe and primers for *BIC* gene (assay ID Hs01374570_m1). Human *GAPDH* (part number: 4352934E) gene was used as the endogenous control. Expression of BIC transcript was also tested by regular RT-PCR using oligo-dT primer, Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and HotStar Taq Mastermix Kit (Qiagen, Valencia, CA). Primers 5'-CAAGAACAACCTACCAGAGACCTTACC and 5'-TGATAAAACAAACATGGGCTTGA were used to generate a 475 bp amplification product. The amplification conditions used were: 15 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 50°C and 1 min at 72 °C; 10 min at 72°C. The cycles were reduced to 25 to generate a 597 bp product for GAPDH, used as a control, with primers 5'-CCACCCATGGCAAATTCCATGGCA and 5'-TCTAGACGGCAGGTCAGGTCCACC.

2.5. Transcription factor assay

Nuclear extracts were prepared from HRPE cells exposed to inflammatory cytokine mix for 2, 6 or 16 h (Nuclear Extract Kit from Active Motif, Carlsbad, CA) as described [6]. The STAT1 activation was estimated using Panomics Transcription Factor ELISA Kit (Affymetrix, Inc., Fremont, CA)

2.6. Electrophoretic mobility shift assay

Confluent cultures of HRPE cells were treated with cytokine mix for 6 h in the presence or absence of JAK inhibitor 1. Nuclear extracts were prepared according to the manufacturer's instructions (Active Motif). Electrophoretic mobility shift assays (EMSA) were performed using the LightShift chemiluminescent EMSA kit (Pierce, Rockford, IL). The probes were prepared by annealing complimentary oligonucleotides with their 3'-end labeled with biotin. The forward sequences of the oligonucleotides used were, 5'-

CTGTAGGTTCCAAGAACAGGCAGGAG and 5'-TCGACTTTTCCTTTAAAAAGAA. The DNA/Protein binding was performed for 20 min at room temperature in a final volume of 20 μ l containing 1X binding buffer (10 mM Tris, pH 7.5, 1 mM DTT, 50 mM KCl), 5% glycerol (v/v), 5 mM MgCl₂, 0.05% NP-40, 0.05 μ g of poly (dI-dC), 8 pmol of double-stranded biotinylated probe, and 2 μ g of nuclear extract. For the competition assay, 100X concentrated unlabeled probe were included in the binding reaction. The DNA-protein complexes were subjected to electrophoretic separation on a non-denaturing 6% polyacrylamide gel at 100 V in 0.5 X TBE buffer. DNA-protein complexes in gel were transferred to Hybond-N⁺ nylon membrane (GE Health Care) and UV cross-linked to the membrane. The biotin labeled DNA-protein complexes were incubated with streptavidin-horseradish peroxidase, and visualized by enhanced chemiluminescence.

3. Results

3.1. Inflammatory cytokines increase miR-155 expression in HRPE cells

Microarray hybridization analysis (GEO accession number GSE23979) demonstrated that the expression of miR-155 in HRPE cells is highly increased when these cells were treated with TNF- α , IL-1 β and IFN- γ for 16 h (Fig. 1A). The microarray result was validated by real-time PCR analysis, which showed that the inflammatory cytokines increased the miR-155 expression by ~8-fold (Fig. 1B). To ascertain the actions of inflammatory cytokines on HRPE cells under these treatment conditions, we analyzed the secretion of a number of cytokines and chemokines by the cells. The treatment resulted in a marked increase in the secretion of CCL5 (RANTES), IL-6, CXCL-9 (MIG) and CSF2 (GM-CSF) from HRPE cells (Fig. 1C).

The ability of TNF- α , IL-1 β and IFN- γ to alter miR-155 expression in HRPE-cells individually or in combination was investigated. Real-time PCR analysis showed that all three cytokines when tested individually increased the miR-155 expression modestly (Fig. 2A). However, combination of any two cytokines exaggerated the effect, and the highest increase was observed when all three cytokines were present. The response of miR-155 expression to inflammatory cytokines was time-dependent (Fig. 2B) as well as concentration-dependent (Fig. 2C). Even at the lowest concentration of TNF- α (0.1 ng/ml), IL-1 β (0.1ng/ml) and IFN- γ (1 u/ml), there was ~4-fold increase in miR-155 expression. These results suggest that the induction of miR-155 in HRPE cells by inflammatory cytokines may reflect the pathophysiological condition within the retinal microenvironment.

We also studied the effect of inflammatory cytokine mix on the expression of BIC, the primary transcript of miR-155. RT-PCR analysis showed that BIC transcript increased in HRPE cells in response to cytokine treatment (Fig. 2D). Real-time PCR analysis shows that this increase in the expression of BIC transcript was dependent on the concentration of TNF- α , IL-1 β and IFN- γ , similar to that observed for miR-155 (Fig. 2E).

3.2. JAK/STAT pathway inhibitor blocks the increase in miR-155 expression by inflammatory cytokines

IFN- γ is known to regulate gene expression by activating JAK/STAT signaling pathway [16]. Therefore, we studied the role of this signaling pathway in mediating the regulation of miR-155 expression in HRPE cells by IFN- γ in combination with TNF- α and IL-1 β . JAK inhibitor 1, a known blocker of JAK/STAT pathway, effectively reduced the induction of miR-155 expression by the inflammatory cytokines (Fig. 3A).

3.3. STAT1 activation by inflammatory cytokines

Activation of STAT1 transcription factor is a key step in the activation of JAK/STAT pathway by IFN- γ . Therefore, we analyzed whether STAT1 activation is associated with the increase in miR-155 expression by the inflammatory cytokines. Activation of STAT1 was observed when HRPE cells were treated with a mixture of TNF- α , IL-1 β and IFN- γ for different time intervals (Fig. 3B). Also, this activation was effectively blocked by JAK inhibitor 1.

3.4. Putative STAT1 binding elements in miR-155 promoter

The proximal region of BIC/miR-155 promoter was analyzed for the possible presence of STAT1 binding sequences. As shown in Fig. 3C, two putative STAT1 binding elements depicted as STAT1BE-1 and STAT1BE-2 are detected upstream of the TATA box and AP-1 elements.

3.5. Inflammatory cytokines increase protein binding to putative STAT1 binding elements

To determine whether transcription factors can bind to the putative STAT1 binding element STAT1BE-1, electrophoretic mobility shift assay was performed (Fig. 3D). When a biotinylated oligonucleotide probe containing STAT1BE-1 sequence was incubated with nuclear extracts from untreated cells, a strong DNA-protein binding was observed (lanes C). A marked increase in this binding was observed when nuclear extracts were from cells treated with inflammatory cytokines (lanes T). This increase was effectively blocked when the treatment was done in the presence of JAK inhibitor-1 (lane I). Competition experiments using excess of corresponding unlabeled oligonucleotide completely abolished the complex formation (lane U). Although multiple bands are observed, the one indicated by arrow appears to be specific since this band is the only one completely eliminated by competition with excess of unlabeled oligo.

The electrophoretic mobility shift assay performed with the biotinylated oligonucleotide probe containing STAT1BE-2 sequence also showed similar results (Fig. 3E). The DNA-protein binding observed with nuclear extract (lanes C) was increased when cells were treated with inflammatory cytokines (lanes T). This increase was blocked by JAK inhibitor-1 (lane I). Excess of corresponding unlabeled oligonucleotide abolished the complex formation (lane U).

4. Discussion

MiRNAs are known to influence many cellular activities in normal and disease states due to their ability to regulate gene expression posttranscriptionally by controlling the translation and/or stability of target mRNAs. A large number of miRNAs are expressed in human retinal pigment epithelial cell line, ARPE-19 [17]. The expression of miR-9 is regulated in these cells during apoptosis induced by N-(4-hydroxyphenyl)retinamide. Mir-204 and Mir-211, two of many miRNAs expressed in fetal human retinal pigment epithelium, play important role in maintaining epithelial barrier function and physiology [18]. To understand the role of miRNAs in retinal inflammatory and degenerative diseases, we performed microarray analysis to evaluate the global miRNA expression profiles in HRPE cells treated with a mixture of inflammatory cytokines (TNF- α + IL-1 β + IFN- γ). These cytokines are secreted during inflammatory reactions by leucocytes such as macrophages, lymphocytes, and neutrophils typically present at the site of retinal inflammation and/or infection. The results presented in this report show that miR-155 is increased by several fold in HRPE cells following treatment with inflammatory cytokines. The observed response is pathophysiologically relevant since significant increase in miR-155 expression is observed at very low concentrations of the cytokines mimicking in vivo situations (Fig. 2).

MiR-155 is implicated in a variety of cellular functions including oncogenesis, infection and inflammation. The induction of miR-155 in response to inflammatory stimuli has been reported in murine macrophages, human THP-1 monocytes and human synovial fibroblasts [10,19,20]. This miRNA is thought to play a protective role during the pathogenesis of rheumatoid arthritis, an inflammatory disease, by reducing the expression of the matrix metalloproteinase MMP3 in synovial tissue [19]. Mir-155 could also control the inflammatory process by its ability to target genes such as *BACH1*, *SHIP1*, *CEBPB* and *IKKε* for translational repression [11-14]. RPE cell perturbation resulting from chronic inflammation is thought to be a major factor in the development of age-related macular degeneration [2]. Thus, miR-155, due to its inherent ability to regulate inflammatory process, could play a key role in the pathogenesis of age-related macular degeneration.

The effect of TNF- α , IL-1 β and IFN- γ on the induction of miR-155 expression in HRPE cells was synergistic, maximum induction being observed when all three cytokines were combined. Both TNF- α and IL-1 β could elicit their effect by activating either JNK (c-Jun N-terminal kinase) signaling pathway or NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway since the promoter regions of human and mouse genes encoding miR-155 are reported to contain both AP1 and NF- κ B sites [10,21]. The increase in miR-155 expression induced by B-cell receptor activation is mediated through JNK pathway which promotes the binding of JunB and FosB transcription factors to the AP1 site [21]. The induction of miR-155 expression in mouse macrophages by TNF- α and poly(I:C) is also mediated by JNK pathway [10]. Inhibition of either JNK or NF- κ B signaling pathway effectively decreased the induction of miR-155 in HRPE cells by the inflammatory cytokines, thus, indicating that these signaling pathways also regulate the miR-155 expression in HRPE cells (data not shown).

Although IFN- γ is known to elicit its action via JAK/STAT signaling pathway, the direct involvement of this pathway in regulating miR-155 expression is not yet demonstrated. IFN- γ is reported to increase the miR-155 expression in mouse macrophages by activating TNF- α autocrine/paracrine signaling, leading to the suggestion that canonical JAK/STAT pathway may not be involved in this case [10]. However, it is quite unlikely that IFN γ can increase miR-155 expression in HRPE cells through TNF- α autocrine/paracrine signaling or via IL-1. The production of TNF- α or IL-1 is not observed in these cells either constitutively or upon exposure to IFN- γ or other cytokines (data not shown). Therefore, we analyzed the *MIR155* gene promoter region for the presence of STAT1 binding elements in order to understand the role of JAK/STAT signaling pathway in mediating the induction of miR-155 by inflammatory cytokines in HRPE cells. We detected two putative binding sequences (STAT1BE-1 and STAT1BE-2; Fig. 3) based on the DNA binding specificity attributed to STAT1 [22,23]. Oligonucleotides containing these elements showed ability to bind protein(s) present in the nuclear extracts of HRPE cells in our electrophoretic mobility shift assays. Interestingly, the increase in the expression of miR-155 in HRPE cells on exposure to inflammatory cytokines was associated with a parallel increase in this binding activity. Also, the increases in both binding activity as well as miR-155 expression elicited by the inflammatory stimuli were effectively blocked by JAK inhibitor 1. Thus, our studies indicate that the JAK/STAT signaling pathway is directly involved in the regulation of miR-155 expression in HRPE cells by the inflammatory cytokines.

Our results clearly show that the inflammatory cytokines (TNF- α + IL-1 β + IFN- γ) markedly increased the expression of miR-155 in human retinal pigment epithelial cells. We have also provided evidence for the first time that the JAK/STAT signaling pathway could be directly involved in the regulation of miR-155 expression. Mir-155 has the potential to modulate the response of the RPE cells to inflammatory stimuli and, therefore, this

microRNA may serve as a target for therapeutic intervention in retinal diseases such as uveitis and age-related macular degeneration.

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Abbreviations

RPE	Retinal pigment epithelium
HRPE	Human retinal pigment epithelial
JAK	Janus family kinases
STAT	Signal transducers and activators of transcription
JNK	c-Jun N-terminal kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

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Fig. 1.

The effect of inflammatory cytokines on miRNA expression and cytokine secretion by HRPE cells. The cells were treated with a mixture of TNF- α (10 ng/ml), IL-1 β (10 ng/ml), and IFN- γ (100 u/ml) for 16 h. (A) Analysis of miRNA expression profile by microarray hybridization shows that the expression of miR-155 is highly increased when cells were treated with inflammatory cytokines. Differentially expressed miRNAs and fold changes in their expression are shown. (B) Real-time PCR analysis validates that miR-155 expression is increased in cells exposed to inflammatory cytokines. (C) HRPE cells respond to inflammatory cytokines by markedly increasing the secretion of CCL5, IL-6, CXCL9 and CSF2. The concentrations of cytokines and chemokines in the culture supernatants were estimated by ELISA.

* $p < 0.05$ compared to controls in (B) and (C).

Fig. 2.

The expression of miR-155 and its primary transcript BIC in HRPE cells is dependent on treatment time and concentration of TNF- α , IL-1 β and IFN- γ . (A) Real-time PCR analysis shows that an increase in the expression of miR-155 is noticeable when the cells are exposed to IFN- γ (100 u/ml), TNF- α (10 ng/ml) or IL-1 β (10 ng/ml) for 16 h. Combination of any two of these cytokines exaggerates the effect, and highest induction is observed when all three are present. (B) Induction of miR-155 expression by inflammatory cytokines is time dependent. HRPE cells were treated with a combination of IFN- γ (100 u/ml), TNF- α (10 ng/ml) and IL-1 β (10 ng/ml) for indicated time intervals and the miR-155 expression was analyzed by real-time PCR. (C) Induction of miR-155 expression is dependent on the concentration of inflammatory cytokines. The miR-155 expression was analyzed by real-time PCR after treating cells with indicated concentrations of the cytokines. (D) RT-PCR analysis shows that BIC transcript (upper panel) is increased in HRPE cells when exposed to a combination of IFN- γ (100 u/ml), TNF- α (10 ng/ml) and IL-1 β (10 ng/ml). The cells were treated for 12 or 24 hours. GAPDH (lower panel) was used as the endogenous control. DNA size markers seen on lane 1 from top are 1353, 1078, 872, 603 and 310 bp; lane 2, control 12 h; lane 3, control 24 h; lane 4, treated 12 h; lane 5, treated 24 h. (E) Real-time PCR analysis shows that the expression of BIC transcript is dependent on the concentration of inflammatory cytokines.

* $p < 0.05$ compared to controls in (A), (C) and (E).

Fig. 3.

JAK/STAT pathway mediates the induction of miR-155 expression in HRPE cells by inflammatory cytokines. (A) Real-time PCR analysis shows that the increase in the miR-155 expression in HRPE cells treated with cytokine mix (TNF- α , 10 ng/ml; IL-1 β , 10 ng/ml; IFN- γ , 100 u/ml) for 16 h is effectively blocked by JAK inhibitor 1. * $p < 0.05$ compared to control; ** $p < 0.05$ compared to cytokines treated in the absence of JAK inhibitor-1; $n = 4$. (B) STAT1 transcription factor is activated when HRPE cells are treated with cytokines for indicated time intervals. The STAT1 activation is also effectively blocked by JAK inhibitor 1. Results shown are representative of three separate experiments. (C) Two putative STAT1 binding elements, labeled STAT1BE-1 and STAT1BE-2, are detected upstream of TATAA box and AP-1 element in the BIC/miR-155 promoter sequence (all elements are shown in bold letters). Underlined are two oligonucleotide sequences employed for electrophoretic mobility shift assays. (D, E) Electrophoretic mobility shift assay shows that the protein binding to putative STAT1 binding elements is increased when HRPE cells are treated with cytokines. This increase is effectively blocked by JAK inhibitor 1. The assay was performed with biotin-labeled oligonucleotides containing STAT1BE-1 (panel D) and STAT1BE-2 (panel E) using nuclear extracts prepared from HRPE cells. Complex formation is noticed in extracts from control cells (lane C) compared with probe alone (lane P). Extracts from cells treated with cytokines for 6 h shows greatly increased complex formation (lane T). This increase is effectively blocked when the treatment is done in the presence of JAK inhibitor 1 (lane I). A 50-fold molar excess of the corresponding unlabeled oligonucleotide inhibited the complex formation observed with extracts from treated cells (lane U). The arrow identifies the protein binding.