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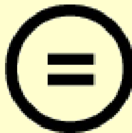

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Role of miR-146a in the regulation of inflammation in an in vitro model of Graves' orbitopathy

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Role of miR-146a in the regulation of inflammation in an in vitro model of Graves' orbitopathy

Directed by Professor Jin Sook Yoon

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Sun Young Jang

June 2016

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ABSTRACT

Role of miR-146a in the regulation of inflammation in an in vitro model of Graves' orbitopathy

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(Directed by Professor Jin Sook Yoon)

Purpose: To investigate the role of miR-146a in the regulation of inflammation in an *in vitro* model of Graves' orbitopathy (GO).

Materials and Methods: The orbital adipose tissues of GO patients (n=16) and control group (n=12) were used in this study. The level of miR-146a expression was compared between GO and control group by quantitative real-time PCR (qPCR). The effects of IL-1β on miR-146a expression were analyzed in orbital fibroblasts by qPCR. To investigate the molecular mechanism underlying IL-1β-induced miR-146a expression, the effects of inhibitors of NF-κB, MEK-1/2, JNK-1/2, p38 MAP kinase, and PI3-K were analyzed. The effects of miR-146a mimics and inhibitors on IL-1β-induced IL-6 release were examined by ELISA and Western blotting.

Results: The level of miR-146a expression was significantly higher in GO orbital adipose tissue than in non-GO ($p<0.001$). IL-1β induced a time- and concentration-dependent increase in miR-146a expression. IL-1β (10 ng/mL, 16 hours) induced an approximately 17.5-fold increase in miR-146 expression. The increase in miR-146a expression by IL-1β was significantly inhibited by NF-κB, JNK-1/2, and PI3K

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inhibitors (1.94 ± 0.25, 5.28 ± 0.34 and 9.73 ± 2.32-fold, respectively, $p<0.05$ compared to IL-1β-induced miR-146 expression, independent *t* test). IL-1β-induced IL-6 protein production was further decreased by miR-146a mimics, but not by inhibitors of miR-146a.

Conclusions: miR-146a was upregulated by inflammatory stress in orbital fibroblasts. Our results indicated that miR-146a had a positive effect on the anti-inflammatory process. miR-146a may play a role in the regulation of inflammation in orbital fibroblasts, and may participate in the pathogenesis of GO.

Key words : miR-146a; inflammation; Graves' orbitopathy

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Role of miR-146a in the regulation of inflammation in an in vitro model of Graves' orbitopathy

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I. INTRODUCTION

Graves' orbitopathy (GO) is an inflammatory autoimmune disorder of the orbit. Previous studies have indicated that the thyroid stimulating hormone (TSH) receptor, which is expressed on orbital fibroblasts, is the autoimmune target of GO.[1-4] Binding of autoantibodies to TSH receptors, expressed on orbital fibroblasts, activates the T cell-dependent inflammatory process. Thus, GO is believed to be related to T cell-mediated autoimmunity to an antigen present in orbital fibroblasts. Activated $CD4^+$ T cells secrete IL-1, IFN-γ, and TNF-α, inducing the expression of TSH receptor and CD40 on the surface of orbital fibroblasts, which promote the secretion of IL-6, -8, fibronectin, type 1 collagen, and glycosaminoglycans.[5-7] Interaction with $CD4^+$ T cells enhances orbital fibroblast activation, proliferation, differentiation, and lipid accumulation.

MicroRNAs (miRNAs) are endogenous, single-stranded, noncoding RNAs, 18 – 24 nucleotides in length that can play important regulatory roles by targeting mRNAs for cleavage or translational repression.[8-10] Thus, they negatively regulate gene expression at the posttranscriptional level. Several recent studies have shown that inflammatory autoimmune diseases, such as rheumatoid arthritis (RA),[11] systemic lupus erythematosus (SLE),[12,13] ulcerative colitis,[14] and psoriasis,[15] have been reported to be associated with altered miRNA expression. Thus, based on reports that inflammatory autoimmune conditions are related to altered miRNA expression, we postulated that specific miRNAs may also be associated with GO.

Located in the LOC285628 gene on human chromosome 5, miR-146a is a

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relatively well known miRNA in inflammatory autoimmune diseases.[10,16] Studies have indicated that miR-146 plays an important role in the pathogenesis of several autoimmune disorders, such as RA,[11,17] SLE,[13] osteoarthritis (OA),[18] and Sjögren's syndrome.[19] miR-146a seems to act through inhibition of the nuclear factor κB (NF-κB) pathway by downregulation of its target genes, such as TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1).[20] This leads to termination or mitigation of an inflammatory response. Based on this background, we focused on miR-146a in an inflammatory cellular model of orbital fibroblast inflammation induced by IL-1β.

In this study, we first compare the expression levels of miR-146a in orbital adipose tissue between GO and non-GO. Then, we determine the role of miR-146a in the regulation of inflammation in an *in vitro* model of GO.

II. MATERIALS AND METHODS

1. Subjects and cell culture protocol

Orbital adipose/connective tissue explants were obtained from 19 GO patients and from 17 age- and sex-matched control subjects with no history of GO. All GO patients underwent orbital decompression for proptosis correction and control subjects underwent cosmetic upper and lower blepharoplasty. Informed written consent was obtained from all subjects and this study was approved by the Institutional Review Board of the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine. All GO patients were euthyroid status at the time of surgery and had not been treated with steroids or radiation therapy for at least 3 months.

Orbital fibroblast cell cultures were performed according to the methods described previously.[21-23] Briefly, for primary cell cultures, tissue explants were minced and placed in plastic culture dishes containing Dulbecco's modified Eagle's medium (DMEM):F12 (1:1) (Lonza, Basel, Switzerland), 20% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA), and penicillin–streptomycin (Life Technologies). After orbital fibroblasts had grown out from the explants, monolayers were passaged serially by gentle treatment

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with trypsin/EDTA, and cells were incubated in DMEM with 10% FBS and antibiotics. Cell cultures were grown in a humidified 5% CO_2 incubator at 37°C. Cells were stored in liquid N_2 until needed and used between the third and seventh passage.

2. Microarray analysis

For the microarray analysis, the total RNA in each orbital adipose/connective tissue sample obtained from eight GO patients and six non-GO normal controls was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. The quality and quantity of total RNA were assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Starting with 250 ng of total RNA, the labeling process began by adding a poly(A) tail to each RNA strand using poly(A) polymerase, followed by ligation of biotin-labeled 3DNA dendrimer. Biotinylated RNA strands were hybridized at 48°C for 18 hours on Affymetrix GeneChip miRNA 4.0 Array (Affymetrix, Santa Clara, CA, USA). The GeneChip miRNA 4.0 Array with 2,578 human mature miRNA was washed and stained in an Affymetrix Fluidics Station 450. Amplified fluorescent signals were scanned using an Affymetrix GeneChip Scanner 3000 7G.

The arrays were analyzed using an Agilent scanner with associated software. The miRNA expression levels were calculated with Expression Console 1.4 (Affymetrix) Relative signal intensities for each miRNA were generated using the Robust Multi-Array Average algorithm. The data were processed based on the quantile normalization method using a GeneSpring GX 13.1 (Agilent Technologies). This normalization method aims to achieve a consistent distribution of intensities for each of a set of arrays. The normalized and log-transformed intensity values were then analyzed using GeneSpring GX 13.1 (Agilent Technologies). The fold changes in the miRNA expression between the GO and non-GO samples were calculated from the signal values. miRNA expression was considered significantly different if the fold change exceeded 1.5. Target prediction was performed using a cut-off at the 95% percentile using the

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TargetScan6.2 database (http://www.targetscan.org/).

3. Measurement of miR-146a expression by quantitative real-time PCR

For quantitative real-time PCR (qPCR), the total RNA (1   g) of each orbital adipose/connective tissue sample obtained from another five GO patients and five non-GO normal controls was isolated using a mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA), and reverse-transcribed into complementary DNA using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA was amplified using a thermocycler (ABI StepOnePlus Real Time PCR; Applied Biosystems, Carlsbad, CA, USA) with TaqMan universal PCR master mix (No AmpErase® UNG; Applied Biosystems) and the recommended PCR conditions for quantitative assessment of gene transcript levels in the tissue samples. All PCRs were performed in triplicate. The catalog number of the primers used was 000468 for miR-146a. RNU6B expression was used for normalization, and the results were expressed as relative fold changes of threshold cycle (Ct) value relative to the control group using the $2^{-\Delta\Delta Ct}$ method.[24]

4. Cell stimulation

Orbital fibroblasts were plated onto 6-well plates for assessment of cytokine release and RNA extraction. Cells were stimulated in triplicate in DMEM:F12 with the indicated IL-1β (R&D Systems, Minneapolis, MN, USA) concentration (0, 1, 5, 10, and 20 ng/mL) or with 10 ng/mL of IL-1β for the indicated time (0, 3, 6, 16, and 24 hours).

To assess the molecular mechanism of IL-1β-induced miR-146a expression, the effects of inhibitors of MEK-1/2, JNK-1/2, p38 MAP kinase, and PI3-K were investigated using GO orbital fibroblasts. MEK-1/2 (PD098059; Sigma-Aldrich, St. Louis, MO, USA), JNK-1/2 (SP600125; Sigma-Aldrich), p38 MAP kinase (SB203580; Sigma-Aldrich), and PI3-K (LY294002; Sigma-Aldrich) were used in the present study. To examine the effects on IL-1β-induced miR-146a expression, these inhibitors were added at a concentration of 20   M 60 minutes

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prior to addition of IL-1β (10 ng/mL).

To assess the possible involvement of proinflammatory transcription factors, including the NF-κB pathway, the effects of preincubation with SC-514 (Calbiochem, La Jolla, CA, USA), a selective IκB kinase-2 inhibitor, and dexamethasone were examined.[25] Following 1-hour pretreatment with SC-514 (100 M) and dexamethasone (0.1 M), orbital fibroblasts were stimulated with IL-1β (10 ng/mL) and the expression of miR-146a was determined at 16 hours.

5. Transfection with miR-146a mimics and inhibitors

Orbital fibroblasts were transfected with miR-146a mimics, inhibitors, and each control according to the respective manufacturer's protocol. Three separate experiments were performed using cells from three different individuals. The miR-146a mimics were obtained from Ambion/Applied Biosystems (Ambion) and miR-146a inhibitors were obtained from Exiqon (Vedbaek, Denmark). Lipofectamine 2000 Reagent (Life Technologies) was used as a negative control. Orbital fibroblasts were plated onto 6-well plates for assessment of cytokine release. Cells were transfected with the indicated miR146a mimics and inhibitors at concentrations of 0, 10, 30, or 100 nM using Lipofectamine and RNAiMAX Reagent.

6. Measurement of IL-6 secreted by ELISA

The effects of miR-146a mimics and inhibitors on IL-1β-induced IL-6 release expression were analyzed using a human cytokine ELISA kit (R&D Systems) according to the manufacturer's protocol for three GO and three non-GO orbital fibroblasts from different individuals.

Transfected cells were plated into 6-well plates and left to adhere overnight. The cells were then starved for 6 hours prior to stimulation with 10 ng/mL IL-1β. Supernatants were removed at 24 hours and IL-6 levels were determined by ELISA.

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7. Western blotting

The effects of miR-146a mimics on IL-1β-induced IL-6, COX-2, and ICAM-1 release in GO orbital fibroblasts were analyzed by Western blotting. Transfected cells were washed with ice-cold PBS, and whole-cell lysates were obtained by incubation on ice for 30 minutes in cell lysis buffer (20 mM HEPES, pH 7.2, 10% (vol/vol) glycerol, 10mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 g/mL leupeptin, 1 g/mL pepstatin, and 1% (vol/vol) Triton X-100). Reagents were purchased from Sigma-Aldrich. Lysates were centrifuged at 12,000 × *g* for 10 minutes and the cell homogenate fractions were stored at –70°C until use.

Protein concentrations were determined by the Bradford assay (21, 22). Equal amounts of protein (50 g) were boiled in sample buffer and resolved by 10% (wt/vol) SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Billerica, MA, USA). The samples were probed overnight with primary antibodies (IL-6, COX-2, and ICAM-1) in Tris-buffered saline containing Tween 20 (TBST), and washed three times with TBST. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody and developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposed to x-ray film (Amersham Pharmacia Biotech). The immunoreactive bands were quantified by densitometry and normalized relative to the β-actin level in the same sample.

8. Data analysis

All experiments were performed at least three times independently, and using at least three cell cultures harvested from different individuals. The results are presented as means ± standard deviation (SD). Differences between groups were assessed by independent and paired *t* tests. In all analyses, $p<0.05$ was taken to indicate statistical significance.

III. RESULTS

1. miR-146a expression in GO

To determine which miRNAs are involved in the pathogenesis of GO, we performed microarray analysis using GO (n = 8) and non-GO (n = 6) orbital connective tissue. Microarray analyses showed that 38 miRNAs were upregulated and 7 miRNAs were downregulated in orbital connective tissue from GO patients compared to healthy controls (Figure 1). The heat map represents the results using a color intensity scale with the highest and lowest expression levels corresponding to bright red and bright blue, respectively (Figure 1 A).

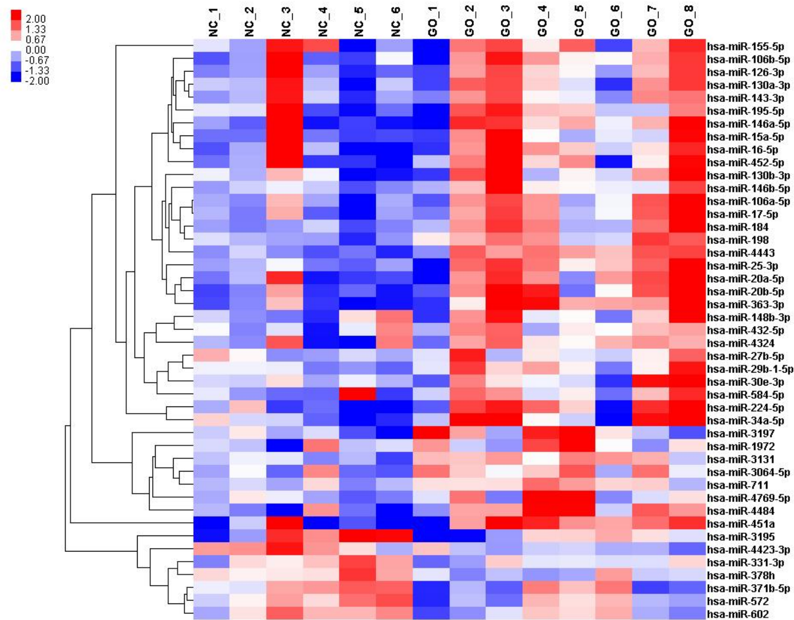

Figure 1. microRNA expression profiling in Graves' orbitopathy (GO). The heat map represents the expression value of each specimen (eight GO and six non-GO) using color intensity. Gene names of microRNA, showing a more than 1.5-fold change, are listed to the right. In total, 38 miRNAs were upregulated and 7 miRNAs were downregulated in orbital connective tissue from GO patients, compared to healthy normal controls. Six of the eight GO subjects had higher miR-146a expression level than the mean control value. NL, normal; GO, Graves' orbitopathy; miR, microRNA.

Among the miRNAs overexpressed in GO, we noted that miR-146a, which is a relatively well known miRNA in inflammatory autoimmune diseases, was increased by 3.15-fold compared with normal controls. Six of the eight GO subjects had miR-146a expression higher than the mean control value (Figure 1). To confirm the results of microarray analysis, we performed qPCR of miR-146a expression in RNA samples obtained from another five GO patients and five non-GO control subjects. miR-146a was significantly highly expressed in orbital adipose tissue from GO (53.30 ± 35.67-fold increase in miR-146a expression levels) compared with non-GO (1.70 ± 1.14-fold) ($p=0.032$, independent *t* test) (Figure 2).

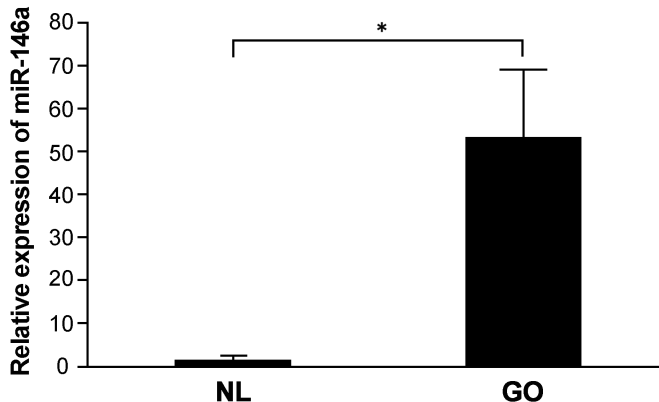

Figure 2. miR-146a expression in Graves' orbitopathy (GO). miR-146a was significantly highly expressed in orbital adipose tissue from GO (53.30 ± 35.67-fold increase in miR-146a expression level) compared with non-GO (1.70 ± 1.14-fold) (* $p < 0.05$). The results are expressed as the means ± standard deviation of five individual samples and the graphs are representative of three independent experiments. NL, normal; GO, Graves' orbitopathy; miR, microRNA.

2. Effects of IL-1β on miR-146a expression

Orbital fibroblasts from three GO and three non-GO patients were used to measure miR-146a expression following exposure to IL-1β. In an experiment using GO orbital fibroblasts, IL-1β (10 ng/mL) caused a 5-fold increase in miR-146a

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expression level at 6 hours (4.96 ± 0.73-fold, p=0.019 compared to control, paired *t* test), which continued to rise to 17.05 ± 1.38-fold at 16 hours and 18.63 ± 1.41-fold at 24 hours (Figure 3A). IL-1β (16 hours) induced a concentration-dependent increase in miR-146a expression. IL-1β caused an increase in miR-146a expression level by about 12-fold at 1 ng/ml (12.38 ± 1.18-fold, p<0.001 compared with the control, paired *t* test), which continued to rise to 14.35 ± 2.10-fold at 5 ng/mL, 17.54 ± 2.70-fold at 10 ng/mL, and 18.75 ± 2.42-fold at 20 ng/mL (Figure 3B). IL-1β induced a time- and concentration-dependent increase in miR-146a expression.

The same experiments were performed using orbital fibroblasts from non-GO patients. The results showed a similar response to GO orbital fibroblasts, but higher levels of miR-146a expression were observed in non-GO cells under some conditions (10 ng/mL: 16 hours and 24 hours; 16 hours: 1, 5, 10 and 20 ng/mL), compared to GO orbital fibroblasts (independent *t* test, p<0.05) (Figure 3).

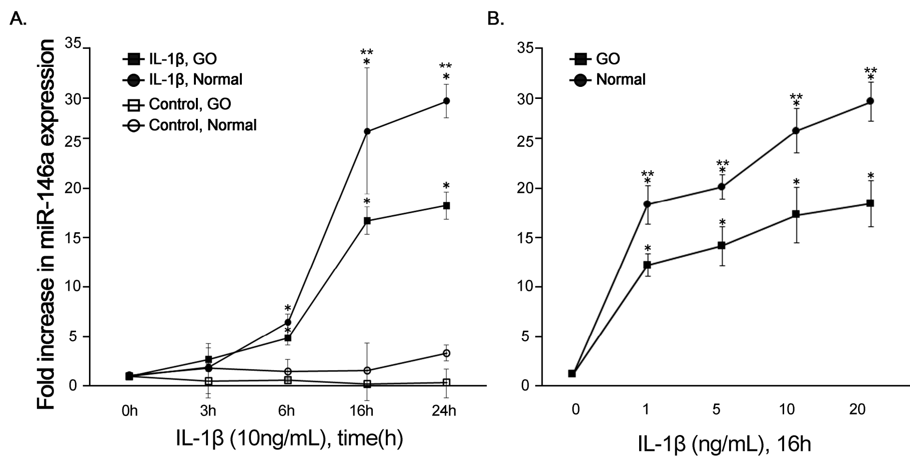

Figure 3. Effects of IL-1β on miR-146a expression in Graves' orbitopathy (GO) and non-GO orbital fibroblasts. IL-1β induced a time- and concentration dependent increase in miR-146a expression in orbital fibroblasts from both GO and non-GO specimens (* p<0.05, versus time and concentration-matched controls).
Non-GO cells showed a similar response as GO orbital fibroblasts, but higher levels of miR-146a expression were observed in non-GO cells under some

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conditions (10 ng/mL: 16 hours and 24 hours; 16 hours: 1, 5, 10, and 20 ng/mL), compared to GO orbital fibroblasts (** $p<0.05$, comparison between GO and healthy non-GO with IL-1β treatment). The results are expressed as the means ± standard deviation of three individual samples and the graphs are representative of three independent experiments. GO, Graves' orbitopathy; miR, microRNA.

3. Effects of inhibitors of MEK-1/2, JNK-1/2, p38 MAP kinase, PI3-K and NF-κB on IL-1β-induced miR-146a expression

To investigate the molecular mechanism underlying IL-1β-induced miR-146a expression, the effects of inhibitors of MEK-1/2, JNK-1/2, p38 MAP kinase, and PI3-K were analyzed using GO orbital fibroblasts. IL-1β (10 ng/mL, 16 hours) induced an increase of about 17.5-fold in miR-146 expression. This increase in miR-146a expression induced by IL-1β was significantly inhibited by JNK-1/2 (5.28 ± 0.34-fold, p=0.030 compared to IL-1β-induced miR-146 expression, independent *t* test) and PI3K inhibitors (9.73 ± 2.32-fold, p=0.039), but not by those of MEK-1/2 and p38 MAP kinase (Figure 4).

To assess the involvement of the NF-κB pathway, samples were pretreated with SC-514 and the miR-146a expression was examined. The SC-514 pretreatment significantly decreased the level of miR-146a expression by (1.94 ± 0.25-fold) compared to the IL-1β-induced miR-146 expression (17.54 ± 3.84-fold, p=0.019).

Glucocorticoids have powerful anti-inflammatory actions. Therefore we examined whether dexamethasone affects the IL-1β-induced miR-146a expression. Dexamethasone pretreatment significantly decreased the level of miR-146a expression (4.95 ± 0.48-fold) compared with the IL-1β-induced miR-146 expression (17.54 ± 3.84-fold, p=0.029).

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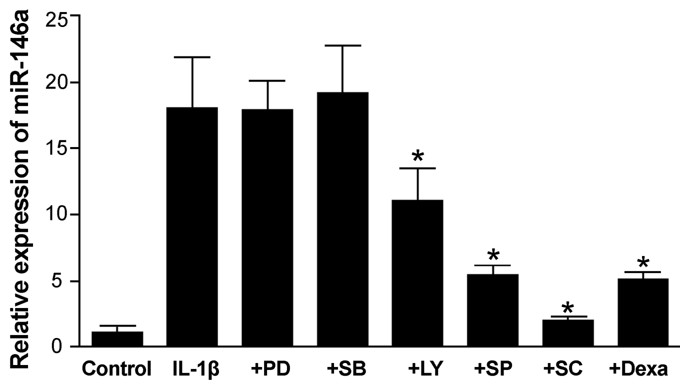

Figure 4. Effects of inhibitors of MEK-1/2, JNK-1/2, p38 MAP kinase, PI3-K, NF-κB and dexamethasone on IL-1β-induced miR-146a expression in Graves' orbitopathy (GO) orbital fibroblasts. The increase in miR-146a expression induced by IL-1β (17.54 ± 3.84-fold) was inhibited by JNK-1/2 (5.28 ± 0.34-fold, p=0.030) and PI3K inhibitors (9.73 ± 2.32-fold, p=0.039), but not by MEK-1/2 or p38 MAP kinase inhibitors (*p<0.05, versus IL-1β-induced miR-146 expression). The level of miR-146a expression was significantly decreased by pretreatment with SC-514 (1.94 ± 0.25-fold) and dexamethasone (4.95 ± 0.48-fold) compared to the IL-1β-induced miR-146 expression (17.54 ± 3.84-fold, p=0.019 and 0.029, respectively). The results are expressed as the means ± standard deviation of three individual samples and the graphs are representative of three independent experiments. PD, MEK-1/2 inhibitor; SB, p38 MAP kinase inhibitor; LY, PI3-K inhibitor; SC, NF-κB inhibitor; SP, JNK-1/2 inhibitor; Dexa, dexamethasone.

4. Effects of miR-146a mimics and inhibitors on IL-1β-induced IL-6 protein production

We transfected three GO and three non-GO orbital fibroblasts with 0, 10, 30, and 100 nM miR-146a mimics and inhibitors. In an experiment using GO orbital fibroblasts, IL-1β induced IL-6 protein production (526.59 ± 19.55 pg/mL) compared with baseline (86.82 ± 3.01 pg/mL). IL-1β-induced IL-6 protein production was decreased by miR-146a mimics (480.59 ± 17.80 pg/mL at 30 nM and 418.15 ± 15.83 pg/mL at 100 nM, p=0.017 and 0.001, respectively compared to control mimics). miR-146a mimics induced a

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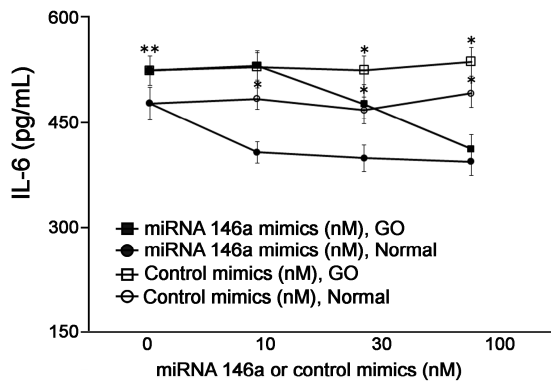
concentration-dependent decrease in IL-1β-induced IL-6 protein production, whereas miR-146a inhibitors did not induce any changes in IL-6 protein production (Figure 5).

The same experiments were performed using non-GO orbital fibroblasts. The results showed a similar response to GO orbital fibroblasts. miR-146a mimics induced a decrease in the IL-1β-induced IL-6 protein production at concentrations of 10, 30, and 100 nM, whereas miR-146a inhibitors did not induce any change in the IL-6 protein production.

Of note, IL-1β induced less IL-6 production in non-GO orbital fibroblasts (481.05 ± 20.01 pg/mL) than in GO orbital fibroblasts (526.59 ± 19.55 pg/mL, p=0.048). In an experiment using non-GO orbital fibroblasts, IL-1β-induced IL-6 protein production was significantly decreased by 10 nM miR-146a mimics (413.39 ± 15.34 pg/mL, p=0.011), whereas 10 nM miR-146a mimics did not induce any changes in IL-6 protein production in GO orbital fibroblasts (Figure



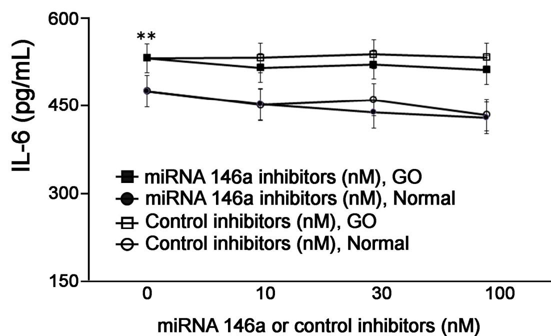


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Figure 5. Effects of miR-146a mimics and inhibitors on IL-1β-induced IL-6 protein production expression in Graves' orbitopathy (GO) and non-GO orbital fibroblasts. IL-1β induced less IL-6 production by non-GO orbital fibroblasts (481.05 ± 20.01 pg/mL) than GO orbital fibroblasts (526.59 ± 19.55 pg/mL, **p=0.048). IL-1β-induced IL-6 protein production was decreased by miR-146a mimics (30 and 100 nM, *$p<0.05$ compared to control mimics), whereas miR-146a inhibitors did not induce any changes in IL-6 protein production in an experiment using GO orbital fibroblasts. IL-1β-induced IL-6 protein production was decreased by miR-146a mimics (10, 30 and 100 nM, *$p<0.05$ compared to control mimics), whereas miR-146a inhibitors did not induce any changes in IL-6 protein production in an experiment using non GO orbital fibroblasts. The results are expressed as the means ± standard deviation of three individual samples and the graphs are representative of three independent experiments.

5. Effects of miR-146a mimics on IL-1β-induced IL-6, COX-2, and ICAM-1 protein production

We further investigated the effects of miR-146a mimics on the expression of inflammatory proteins, such as IL-6, COX-2, and ICAM-1, by Western blotting using orbital fibroblasts from GO. Stimulation with IL-1β increased the levels of IL-6, COX-2, and ICAM-1 expression. The increases in IL-6 and ICAM-1 were inhibited by miR-146a mimics (30 nM), but no significant effect was observed on COX-2 expression (Figure 6).

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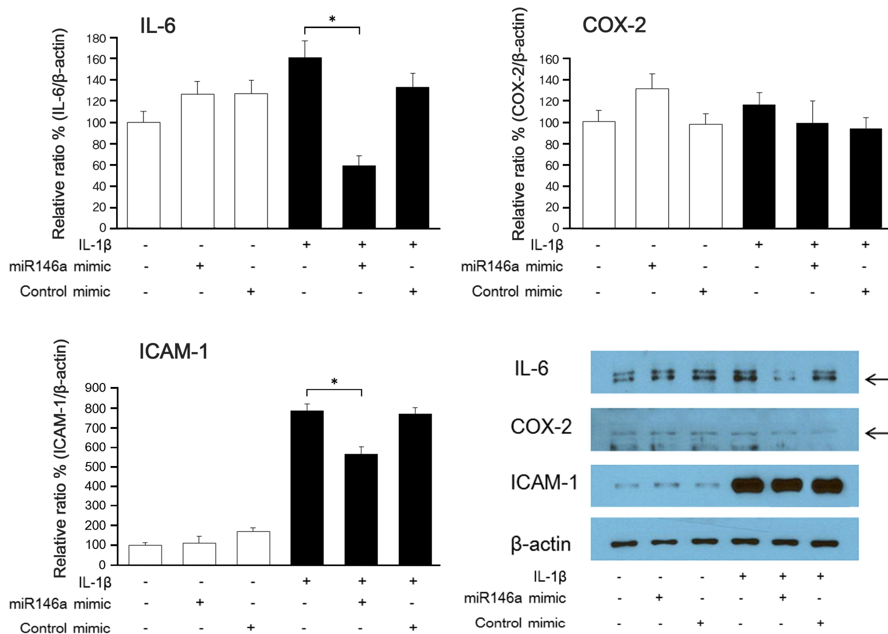

Figure 6. Effects of miR-146a mimics on IL-1β-induced IL-6, COX-2, and ICAM-1 protein production in Graves' orbitopathy (GO) orbital fibroblasts. The increases in IL-6 and ICAM-1 were inhibited by miR-146a mimics (30 nM) (* $p<0.05$, versus IL-1β-induced IL-6 and ICAM-1 production). However, COX-2 did not show significant results. The results are expressed as the means ± standard deviation of three individual samples and the graphs are representative of three independent experiments.

IV. DISCUSSION

This study investigated the role of miR-146a in regulation of inflammation in an *in vitro* model of GO. We compared the expression level of miR-146a between GO and non-GO orbital adipose tissue. The level of miR-146a expression showed an approximately 30-fold increase in GO (53.30 ± 35.67-fold increase in miR-146a expression level) compared to non-GO (1.70 ± 1.14-fold) ($p<0.001$). GO is an inflammatory autoimmune disease, and thus miR-146a seems to be upregulated by inflammatory stress, as in GO. Similarly, there have been several reports that

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expression of miR-146a was increased in diseased tissue.[11,15,17,18] Nakasa et al.[11] reported that miR-146a was highly expressed in RA synovial tissue, and Yamasaki et al.[18] reported strong miR-146a expression in cartilage in low-grade OA. Sonkoly et al. reported that miR-146a was significantly overexpressed in psoriatic lesional skin compared with healthy skin.[15]

Several recent pharmacological studies investigated the mechanism of IL-1β-induced miR-146a expression using different cell lines.[26,27] Larner-Svensson et al.[27] reported that IL-1β induced a time-dependent, 100-fold induction in miR-146a expression in primary human airway smooth muscle cells. Perry et al.[26] also reported that IL-1β induced a time- and concentration-dependent increase in miR-146a expression in human lung alveolar epithelial cells. In this study, we found that IL-1β induced time- and concentration-dependent increases in miR-146a expression in both GO and non-GO orbital fibroblasts. Interestingly, the expression of miR-146a was upregulated to a lesser extent in GO orbital fibroblasts than in non-GO orbital fibroblasts after stimulation with IL-1β ($p<0.05$). This result is in line with studies that showed that GO orbital fibroblasts produced significantly lower levels of IL-1 receptor antagonist (IL-1RA) compared to non-GO orbital fibroblasts.[28] Because IL-1RA acts as a competitive inhibitor of IL-1α and IL-1β and reduces the inflammatory process in GO orbital tissues,[28-30] we believe that these findings support the hypothesis that GO orbital fibroblasts have a reduced capacity to control the inflammatory response.

Inhibitors of MEK-1/2, JNK-1/2, p38 MAP kinase, and PI3-K were used to investigate which signal cascade controls the increase in miR-146a expression by IL-1β. The results indicated that the increase in miR-146a expression induced by IL-1β was inhibited by JNK-1/2 and PI3K inhibitors, suggesting that activation of JNK and PI3K pathways was required for IL-1β-induced miR-146a expression in orbital fibroblasts. Larner-Svensson et al.[27] reported similar experimental results indicating that IL-1β-induced miR-146a was regulated by MEK-1/2 and JNK-1/2 in human airway smooth muscle cells. In their report, the extent of the decrease in miR-146a expression was greater for JNK-1/2 than

MEK-1/2. In the present study, the JNK-1/2 pathway, but not MEK1/2, was associated with IL-1β-induced upregulation of miR146a. As the function and mechanism of action of miR-146a are dependent on the cell type,[27] we assumed that this discrepancy was due to the different cell lines used in these studies. The observation that the PI3K pathway was required for IL-1β-induced miR-146a expression in orbital fibroblasts can be explained. Recently, a cAMP-independent cascade was shown to increase PI3K activity, a signaling pathway that plays a central role in the pathogenesis of GO.[31] Kumar et al.[31] reported that a stimulatory TSH receptor antibody enhanced adipogenesis via PI3K activation in GO, and suggested that inhibition of PI3K signaling may represent a potential novel therapeutic approach in GO.

Dexamethasone, a corticosteroid, attenuates the actions of multiple proinflammatory transcription factors, including NF-κB. We found that IL-1β-induced miR146a expression was inhibited after treatment with dexamethasone. Moreover, we found that the level of miR-146a expression was significantly decreased by SC-514, which is a selective NF-κB inhibitor. According to a previous report,[20] miR-146a acts through inhibition of the NF-κB pathway by downregulating its target genes, such as TRAF6 and IRAK1. This leads to the termination or mitigation of the inflammatory response.

In this study, we found that IL-1β-induced IL-6 protein production was further decreased by miR-146a mimics, confirming the positive effect of miR-146a on the anti-inflammatory process. We further investigated whether miR-146a mimics could affect the levels of other inflammatory cytokines induced by IL-1β and found that increases in IL-6 and ICAM-1 expression by IL-1β were inhibited by miR-146a mimics (30 nM). The increase in COX-2 expression by IL-1β was decreased by miR-146a mimics, although the effect was not statistically significantly. Recently, Wei et al.[32] reported significantly lower circulating serum levels of miR-146a in GO patients compared to controls, and suggested that weakened miR-146a activity caused an increase in inflammation. The authors further showed that the serum levels of miR-146a were significantly

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correlated with clinical activity score, which indicates the disease inflammatory activity of GO.

Attenuation of miR-146a activity using an miR-146a inhibitor had no significant effect on IL-1β-induced IL-6 release in this study. According to a previous review,[37] there are multiple and complex pathways for the release of IL-6 by cells, and cells use different pathways, organelles, carriers, and molecules to control the release of cytokines. As the molecular mechanism of IL-1β-induced IL-6 release in orbital fibroblasts has not yet been fully elucidated, further physiological studies are needed to determine why miR-146a inhibitors did not induce any changes in IL-1β-induced IL-6 release. Although several questions could not be answered by the results of this study, we showed for the first time that miR-146a may play a role in the regulation of inflammation in orbital fibroblasts from GO and that it participates in the pathogenesis of GO.

V. CONCLUSION

In conclusion, miR-146a seems to contribute to GO pathogenesis by modulating inflammatory protein expression and cellular functions in orbital fibroblasts. miR-146a was upregulated by inflammatory stress, such as IL-1β, and our results indicated a positive effect of miR-146a on the anti-inflammatory process. Further studies are required to examine the potential of miR-146a as a target for therapeutic strategies and as a biomarker.

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ABSTRACT(IN KOREAN)

갑상샘눈병증의 in vitro 모델에서 마이크로 RNA-146a가
염증조절에 미치는 영향

<지도교수 윤 진 숙>

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장 선 영

목적: 갑상샘눈병증의 in vitro 모델에서 마이크로 RNA-146a가 염증조절에 미치는 영향을 알아보고자 하였다.

재료 및 방법: 갑상샘눈병증 환자 (n=19) 와 정상 대조군 (n=17) 의 안와 지방 조직을 이용하여 실험하였다. 마이크로 RNA-146a의 발현 정도를 quantitative real-time PCR (qPCR)을 통해 비교하였다. 일차 배양된 안와섬유모세포를 이용하여 IL-1β가 마이크로 RNA-146a 발현 정도에 미치는 영향을 분석하고, IL-1β가 유도한 마이크로 RNA-146a 발현변화에 NF-κB, MEK-1/2, JNK-1/2, p38 MAP kinase, PI3-K 억제제가 미치는 영향을 조사하였다. IL-1β에 의해 증가되는 IL-6 단백질 발현에 마이크로 RNA-146a의 자극제와 억제제가 미치는 영향을 ELISA 와 Western blot을 이용하여 분석하였다.

결과: 마이크로 RNA-146a의 발현은 갑상샘눈병증 환자의 안와지방조직에서 유의하게 발현이 증가되어 있었다 ($p < 0.001$). 안와섬유모세포에서 IL-1β의 노출 시간과 농도가 증가함에 따라 마이크로 RNA-146a의 발현이 증가되었다. IL-1β (10 ng/mL, 16 hours) 는 약 17.5 배의 마이크로 RNA-146a의

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발현 증가를 유도하였고, 이 증가는 NF-κB, JNK-1/2 와 PI3K 억제제에 의해 억제되었다 (1.94 ± 0.25, 5.28 ± 0.34 and 9.73 ± 2.32-fold, respectively, $p < 0.05$). IL-1β에 의해 증가되는 IL-6 단백질의 발현은 마이크로 RNA-146a의 자극제에 의해 감소되었으나, 억제제에 의한 영향은 없었다.

결론: 마이크로 RNA-146a 는 안와섬유모세포에서 염증 스트레스에 의해 발현이 증가하였다. 본 연구는 마이크로 RNA-146a가 항염증 과정에 긍정적인 역할을 할 것이라는 가설을 지지하는 결과들을 보여준다. 마이크로 RNA-146a는 안와모세포의 염증조절에 중요한 역할을 하고 있는 것으로 보이며 또한 갑상샘안병증의 병리과정에도 관여하고 있는 것으로 생각된다.

핵심되는 말 : 마이크로RNA-146a, 염증, 갑상샘눈병증