MicroRNA-21 Negatively Regulates Treg Cells Through a TGF-β1/Smad-Independent Pathway in Patients with Coronary Heart Disease

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Key Words
MiR-21 • Treg cells • Coronary heart disease • TGF-β1 • Smad7

Abstract

Background: CD4+CD25+FoxP3+ regulatory T cells (Treg cells) play a protective role against the development and progression of the inflammatory disease atherosclerosis (AS). MicroRNA-21 (miR-21) is expressed in Treg cells and is up-regulated in the context of AS and other inflammatory diseases. Aims: This study aimed to determine the role of miR-21 in Treg cell regulation and gene expression during the development of AS in patients with coronary heart disease (CHD). Methods and Results: MiR-21 expression in peripheral blood mononuclear cells (PBMCs) was significantly up-regulated in patients with CHD (acute myocardial infarction (AMI) group, n=24; unstable angina (UA) group, n=21; stable angina (SA) group, n=24) compared with patients with chest pain syndrome (CPS, n=27), and miR-21 expression showed an increasing trend from SA to UA to AMI patients. Moreover, flow cytometry analysis indicated that the frequencies of circulating Treg cells decreased in a manner proportionate opposite with the level of miR-21. Quantitative real-time PCR (qRT-PCR) revealed a decrease in mRNA expression of forkhead box P3 (foxp3), transforming cell growth factor beta 1(TGF-β1) and smad7 (a known target gene of miR-21).ELISA analysis revealed a decrease in TGF-β1 secreted into the plasma. In addition, we transfected PBMCs with a miRNA negative control (NS-m), a miR-21 mimic (miR-21-m), a miRNA inhibitor negative control (NS-i), or a miR-21 inhibitor(miR-21-i). Up-regulation of miR-21 decreased the frequency of circulating Treg cells, decreased the expression levels of foxp3, TGF-β1 and smad7, and decreased the amount of TGF-β1 secreted into the plasma. Consistent with these observations, miR-21 down-regulation increased the frequency of circulating Treg cells, increased the expression of foxp3, TGF-β1 and smad7, and increased the amount of TGF-β1 secreted into the plasma. Conclusions: Because the smad7 expression pattern was similar to that of TGF-β, our study suggests that miR-21 can negatively regulate the frequency of circulating Treg cells through a TGF-β1/smad-independent signaling pathway in PBMCs.

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Introduction

As the leading cause of death worldwide, atherosclerosis (AS) accounts for approximately 29% of all mortalities. Coronary heart disease (CHD) has become the most common manifestation of AS [1, 2]. AS is thought to involve a variety of immune cell types [3, 4], among which Treg cells are of particular interest [5-7] because they can actively mediate immunologic tolerance and inhibit AS development or progression by down-regulating T cell responses, as shown in both human and rodent models of AS [8-10]. TGF-β is one of the major mediators of Treg cell function in the context of AS [11, 12]. In addition, TGF-β1 maintains Treg cells in the peripheral vasculature by inducing the expression of foxp3, which is essential for Treg development and function [13-15]. Because foxp3 expression is specific to Treg cells, foxp3 is considered the most reliable molecular marker for Treg cells.

MicroRNAs are a class of non-coding RNAs that are approximately 19-25 nucleotides in length. By binding to their target protein-encoding mRNAs, microRNAs regulate nearly 30-50% of all genes. MiR-21 is a major miR component of the human plasma that increases with age and under conditions of inflammation, cardiovascular disease and obesity [16]. Recent studies have shown that miR-21 levels are increased in the peripheral blood of AS patients [17-19]. MiR-21 was also preferentially over-expressed in human Treg cells [20, 21], but the mechanisms of action of miR-21 remain unclear. According to previous studies, miR-21 targets the negative regulator smad7, a downstream signaling molecule of TGF-β1 [22, 23], to enhance effect of TGF-β1 signaling in cancer and CAF (carcinoma-associated fibroblasts) [24]. However, the function of miR-21 in Treg cells in CHD patients remains largely unknown. In our study, we speculated that miR-21 may be involved in regulating Treg cells in the context of CHD and could thus serve as a novel therapeutic target in cardiovascular disease.

In the present study, we found that the expression of miR-21 in peripheral blood mononuclear cells (PBMCs) was significantly up-regulated in patients with CHD compared with patients with chest pain syndrome (CPS). Moreover, miR-21 expression showed a gradually increasing trend in stable angina (SA), unstable angina (UA), and acute myocardial infarction (AMI) patients, respectively. Additionally, we found decreased frequencies of circulating Treg cells, down-regulated expression levels of foxp3, TGF-β1 and smad7 mRNAs, and decreased TGF-β1 secretion into the plasma. To verify the relationship between miR-21 and Treg cells, including its functional transcription factor foxp3, we also transfected PBMCs with a miRNA negative control (NS-m), a miR-21 mimic (miR-21-m), a miRNA inhibitor negative control (NS-i), or a miR-21 inhibitor (miR-21-i) and found that miR-21 could reduce the expression of TGF-β1, down-regulate the Treg cell population and foxp3 expression level, and decrease TGF-β1 secretion from Treg cells. Although the trend of smad7 expression dynamics was opposite compared with that of miR-21, smad7 expression followed a pattern similar to that of TGF-β1. In fact, the changes in smad7 expression were not consistent with its negative regulatory function of the TGF-β1/smad-dependent signaling pathway. Our results suggest that these effects caused by miR-21 may occur through a TGF-β1/smad-independent signaling pathway.

Materials and Methods

Study population

This study was approved by the ethical commission of Tongji Medical College of Huazhong University of Science and Technology and was conducted according to the Declaration of Helsinki and its amendments and according to the approved institutional guidelines. Ninety-six patients were enrolled in this study after providing informed consent. The patients were divided into the following 4 groups according to their diagnosis [25, 26]: (1) an AMI group (included both ST-segment-elevated and non-ST-segment-elevated patients, who were verified by clinical blood tests and electrocardiography); (2) a UA group (patients who experienced pain on minor exertion or at rest, ST-segment changes and/or T-wave inversion); (3) an SA group (patients who experienced pain occurring after a relatively constant level of exertion with definite
ischemic electrocardiographic changes: ST-segment depression >1 mm); and (4) a CPS group (patients who experienced chest pain without electrocardiographic changes, coronary stricture, or coronary spasm).

All patients included in this study did not have previous attacks and lacked the following: atrial fibrillation, implanted pacemaker, valvular heart disease, anti-inflammatory treatment, serious liver disease, renal failure, collagen disease, malignant disease, disseminated intravascular coagulation, and thromboembolism.

**Blood processing**

Blood samples were collected by venous puncture using EDTA-coated tubes in patients maintained in a fasted state on the morning following hospital admission. Thus, the time between disease attack and blood collection was within 24 hours in all cases. Blood samples from the four groups were collected before reperfusion therapy. Plasma was collected and stored at -80°C for cytokine detection. The remaining peripheral whole blood sample was subjected to Ficoll density gradient separation to isolate PBMCs to be used for flow cytometry and qRT-PCR.

**Cell transfection and culture**

To investigate the role of miR-21 in Treg cells, we performed miR-21 transfection experiments in PBMCs. Blood samples were collected from healthy volunteers, and PBMCs were isolated by Ficoll density gradient. After isolation, NS-m or miR-21-m, NS-i, or miR-21-i (obtained from RiboBio; Guangzhou, China) was transfected into the PBMCs using a riboFECT CP Transfection Kit (Guangzhou, China). Cells were cultured in a 24-well plate at 3x10^6 cells per ml of RPMI-1640 medium (Gibco, CA, USA) supplemented with 15% fetal calf serum (Gibco, CA, USA) and maintained at 37°C with 5% CO². Finally, cells were harvested for flow cytometry and qRT-PCR analysis, and the supernatants were used for ELISA analysis.

**Flow cytometry analysis**

PBMCs were isolated from patients and cultured after transfection as described above. Aliquots of 2 x 10^6 cells were washed once in flow cytometry staining buffer (staining buffer) and resuspended in 100 µl of staining buffer. For surface staining, cells were incubated with FITC-anti-human CD4 and APC-anti-human CD25 antibodies for 40 minutes at 4°C in darkness. After surface staining, cells were fixed, permeabilized and stained with PE-anti-foxp3 antibody according to the protocol recommended by the manufacturer. All of the antibodies used for flow cytometry were purchased from eBioscience (San Diego, CA, USA). Isotype control antibodies were included to enable correct compensation and to confirm antibody specificity. Stained cells were detected using a FACSCalibur flow cytometer (BD, CA, USA) and were analyzed using the FlowJo 7.6.1 software.

**RNA isolation and qRT-PCR analysis**

Total RNA was isolated from cells using TRIzol total RNA extraction reagent according to protocol recommended by the manufacturer. The purity and concentration of the total RNA were quantified using a UV spectrophotometer. MicroRNA cDNA was synthesized using a miR-21-specific Bulge-Loop™ RT primer (RiboBio; Guangzhou, China) and a reverse transcription system. The reaction was incubated at 70°C for 10 min, 4°C for 2 min, 42°C for 60 min and 70°C for 10 min. qRT-PCR was performed using the Bulge-Loop™ qRT-PCR primer and SYBR Green PCR Master Mix Kit (Takara Biotechnology, Dalian, China). Sample data were normalized to U6 expression. cDNA for the mRNAs was prepared using PrimeScript RT Reagent Kit and was amplified using a SYBR Green PCR Master Mix Kit (Takara Biotechnology, Dalian, China) with the mRNA primers shown below. Expression of GAPDH was used to normalize the sample data. mRNA primers are as follows:

TGF-β1: Forward: 5’-ACA CCA ACT ATT GCT TCAG-3’
Reverse: 5’-TGT CCA GCC TCC AAATG-3’

Smad7: Forward: 5’-TTT GTG TAT TTA TTT CTT TCT CTC-3’
Reverse: 5’-CAC TCT GCT CTT CTC CTC-3’

Foxp3: Forward: 5’-GAG AAG CTG AGT GCC ATGCA-3’
Reverse: 5’-AGA GCC CCT GTC GGA TGAT-3’

GAPDH: Forward: 5’-GCT GCC GCT GAG TAC TGC GTG GAGT-3’
Reverse: 5’-CAG AGT CCT CTG GGT GCC AGT GATG-3’
ELISA assay

The levels of TGF-β1 in the plasma of the patients and in the cell culture supernatants were detected using a Human TGF-β1 ELISA kit (Neobioscience, China) following the protocol recommended by the manufacturer without cross-reactivity.

Statistical analysis

PASW Statistics 18 was used for statistical analyses. Values are presented as the means ± SD in the text and figures. One way ANOVA was used to analyze differences between the groups, and p<0.05 was considered statistically significant.

Results

Clinical characteristics of the study population

This study included 96 patients in total. No significant differences were observed regarding age, gender or CHD risk factors when comparing the AMI, UA, SA and CPS groups. However, the numbers of diseased vessels and coronary artery stents in patients with AMI, UA, and SA were significantly higher than those in patients with CPS. All of the clinical characteristics of the study populations are summarized in Table 1.

Circulating Treg cell frequencies in patients with CHD and CPS

Flow cytometry analysis of the PBMCs from patient blood samples revealed no significant differences in the frequencies of circulating CD4+CD25+ T lymphocytes between the four groups (Table 2, Fig. 1). However, the frequency of circulating Treg cells was reduced significantly in the AMI group (1.6 ± 0.3%, n=24) and the UA group (2.4 ± 0.3%, n=21) compared with that of the CPS group (3.4 ± 1.0%, n=27) (P<0.01), respectively. We also found that the frequencies of circulating Treg cells in the AMI and UA groups were lower than that of the SA group (3.2 ± 0.5%, n=24) (P<0.05). Moreover, the frequency of circulating Treg cells in the AMI group was slightly lower than that of the UA group, but this difference was not statistically significant (P>0.05). (Table 2, Fig. 1) These results suggest that the frequency of circulating Treg cells in the human peripheral blood samples decreased progressively during the progression of AS. In another words, AS progress gradually from SA to UA and AMI accompanied with a decreased protection offered by Treg cells.

Expression levels of miR-21, foxp3, TGF-β1 and smad7 in PBMCs from patients with CHD and CPS

Because miR-21 is preferentially overexpressed in human Treg cells, we sought to determine whether circulating Treg cells and corresponding miR-21 levels could impact the expression of foxp3, TGF-β1 and smad7 in PBMCs isolated from patients with AMI (n=24),
Table 2. Circulating frequencies of Treg cells reduced in the progression of CHD. Values are expressed as mean ± SD, or number. AMI: acute myocardial infarction; UA: unstable angina; SA: stable angina; CPS: chest pain syndrome.

|                  | AMI (n=24) | UA (n=21) | SA (n=24) | CPS (n=27) |
|------------------|------------|-----------|-----------|------------|
| CD4+CD25+/ CD4+ lymphocytes (%) | 4.9±1.6    | 4.3±1.1   | 4.4±1.0   | 4.8±1.4    |
| CD4+CD25+Foxp3+/ CD4+ lymphocytes (%) | 1.6±0.3*#  | 2.4±0.3*# | 3.2±0.5   | 3.4±1.0    |

Fig. 1. Flow cytometric analysis of circulating Treg cell frequencies in patients with CHD and CPS. PBM-Cs isolated from patients were labeled with CD4-FITC, CD25-APC and foxp3-PE antibodies. Cells were gated for CD4+T cells, after which the cells were gated for CD4+CD25+T cells and CD4+CD25+foxp3+T cells from samples obtained from AMI, UA, SA and CPS patients. The percentages of CD4+CD25+T cells and CD4+CD25+foxp3+T cell subsets in each group are shown in the panels. CHD: coronary heart disease (includes AMI, UA and SA); AMI: acute myocardial infarction; UA: unstable angina; SA: stable angina; CPS: chest pain syndrome; Treg cells: CD4+CD25+foxp3+T cells.

UA (n=21), SA (n=24) and CPS (n=27). We found that the levels of miR-21 were increased in AMI, UA and SA patients compared with CPS patients. Moreover, the expression level of miR-21 in the AMI and UA groups was higher than that of the SA group. However, no significant differences were observed between the AMI and UA groups (Fig. 2A). These results suggest that the expression of miR-21 progressively increases from CPS to SA to UA to AMI patients, and this trend negatively correlates with the frequency of circulating Treg cells in each group. Moreover, the expression levels of foxp3, TGF-β1 and smad7 decreased significantly in patients with CHD compared with those in the CPS group (Fig. 2B, C, D); these levels also showed a decreasing trend among the AMI, UA and SA patients. The expression of foxp3 was higher in SA patients than in AMI and UA patients; this trend is consistent with that observed for the plasma TGF-β1 levels, as determined by ELISA (Fig. 2E). No significant differences
were found between the AMI and UA groups. These data are consistent with the change in circulating Treg frequency observed in patients with CHD when compared with CPS patients. These results suggest that not only the frequency of circulating Treg cells but also the level of the transcription factor foxp3 decreased during the progression of AS.

**MiR-21 regulates the frequencies of Treg cells and the expression of foxp3 in PBMCs**

To further determine the relationship between miRNA-21 and Treg cells, we transfected PBMCs isolated from healthy volunteers with miR-21 NS-m, miR-21-m, miR-21 NS-I, or miR-21-i for 48 hours. A significant change in miR-21 expression levels was confirmed by qRT-PCR (Fig. 4A; Fig. 6A). When compared with NS-m-transfected or non-transfected cells, cells overexpressing miR-21 exhibited decreases in the frequency of circulating Treg cells (Fig. 3B, D) and the expression of foxp3 (Fig. 4B). Similarly, miR-21 down-regulation increased the frequency of circulating Treg cells and increased foxp3 mRNA expression levels. (Fig.

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**Fig. 2.** Expression levels of miR-21, foxp3, TGF-β1 and smad7 in PBMCs and the levels of TGF-β1 in serum samples from patients with CHD and CPS. Levels of miR-21 (A), foxp3 (B), TGF-β1 (C) and smad7 (D) in PBMCs were analyzed by qRT-PCR, and serum TGF-β1 levels (E) were measured by ELISA. *P<0.05 vs. CPS; #P<0.01 vs. SA. CHD: coronary heart disease (includes AMI, UA and SA); AMI: acute myocardial infarction; UA: unstable angina; SA: stable angina; CPS: chest pain syndrome.
MiR-21-regulated expression of smad7 in PBMCs and expression of TGF-β1 in PBMCs and cell culture supernatants

To identify signaling pathways that may contribute to the regulation of Treg cells by miR-21, we analyzed the expression of smad7 in PBMCs and the expression of TGF-β1 in PBMCs and in cell culture supernatants (Fig. 4C, D; Fig. 6C, D). The results of these analyses showed that the mRNA expression levels of smad7 and TGF-β1 in PBMCs and the level of TGF-β1 secreted into the cell culture supernatants were significantly down-regulated by miR-21-m and up-regulated by miR-21-i compared with the control cells. (Fig. 4E; Fig. 6E). Several
studies have shown that smad7 is targeted by miR-21. Our study is consistent in showing that miR-21-m decreases smad7 expression and that miR-21-i increases smad7 expression.

**Discussion**

miR-21 has been shown to play a key role in the induction and resolution of inflammatory responses [27], especially in the regulation of adaptive and innate immunity [28-30]. More recently, miR-21 has received significant attention with respect to its role in cardiovascular diseases because miR-21 is up-regulated in the human peripheral blood of AS patients [31]. In this study, we showed that miR-21 expression in PBMCs gradually increases from SA to UA to AMI patients, which suggests that miR-21 expression levels increase as the pathology of AS becomes more severe. Moreover, miR-21 plays a positive role in inflammation and may serve as a biomarker of inflammation [19, 32, 33], a condition in which Treg cells play a key role [34]. We speculated that changes in miR-21 levels in PBMCs of CHD patients could contribute to the decrease in the frequency of circulating Treg cells and the expression of foxp3 mRNA.
To test our hypothesis, we transfected miR-21 NS-m or miR-21-m and miR-21-NS-i or miR-21-i into PBMCs isolated from healthy volunteers. Overexpression of miR-21 decreased the frequency of circulating Treg cells and the expression level of foxp3 in PBMCs. Similarly, miR-21 down-regulation increased the frequency of circulating Treg cells and mRNA expression level of foxp3 in PBMCs. Together, these data suggest that miR-21 functions to diminish the
population of circulating Treg cells, down-regulate foxp3 expression in PBMCs from both healthy individuals and CHD patients, and ultimately play a pro-inflammatory role in the progression of AS.

We could not identify a miR-21 target sequence in the foxp3 mRNA transcript [21, 35]. However, TGF-β signaling could promote a rapid increase in the expression of mature miR-21 by facilitating the processing of primary transcripts of miR-21 (pri-miR-21) into precursor miR-21 (pre-miR-21) [36]. Moreover, milk exosomal TGF-β can induce Treg and Th17 cell differentiation [37, 38]. To further investigate the regulatory mechanism between miR-21 and Treg cells, we analyzed the expression of TGF-β1 and smad7 (a miR-21 target gene that can also inhibit the TGF-β1/Smad signaling pathway) \textit{in vitro} and \textit{in vivo}. The results of our \textit{in vivo} analysis showed that the expression levels of TGF-β1 and smad7 in PBMCs decreased in CHD patients relative to CPS patients. Moreover, the levels of plasma TGF-β1 decrease in correlation with the decrease in gene expression. The results of our \textit{in vitro} analysis showed that the expression of TGF-β1 and smad7 in PBMCs and the TGF-β1 level in cell culture supernatants decreased in response to miR-21 overexpression and increased in response to miR-21 down-regulation.

These results suggest that the overexpression of miR-21 could decrease the expression of TGF-β1 mRNA, which further down-regulates the frequency of circulating Treg cells and foxp3 mRNA levels [39]. In turn, the decrease in Treg cell frequency and the down-regulation of foxp3 result in less TGF-β1 secretion, suggesting an inhibition of the anti-inflammatory effect of Treg cells. In contrast, down-regulation of miR-21 resulted in an increase in the expression of TGF-β1 mRNA, which further increased circulating Treg cell frequency and up-regulated foxp3 mRNA levels. As more TGF-β1 is secreted, the anti-inflammatory effects of the Treg cells become enhanced.

Several studies have shown that TGF-β1 binds to TGF-βR (TGF-β receptor) and exerts its biological role in part through smad-dependent signaling pathways. For example, TGF-β1 promotes the phosphorylation of R-smads by binding to the TGF-βR. In turn, the inhibitory smad7, a target of miR-21, can negatively regulate the phosphorylation of R-smads through the TGF-βR [40, 41, 42]. Whether TGF-β1 increases the Treg cell population and expression of foxp3 by a TGF-β1/smad-dependent signaling pathway or TGF-β1/smad-independent signaling pathways remains unknown [43, 44, 45]. In our study, miR-21 overexpression significantly decreased smad7 expression, which should enhance TGF-β1/smad-dependent signaling. However, in response to miR-21 overexpression, the expression of TGF-β1, the frequency of circulating Treg cells, and the expression of foxp3 mRNA decreased. Similarly, miR-21 down-regulation increased smad7 expression, which should inhibit TGF-β1/smad-dependent signaling. However, in response to miR-21 down-regulation, the expression of TGF-β1, the frequency of Treg cells and the expression of foxp3 mRNA increased. Taken together, these data suggest that miR-21 decreases the Treg cell population and the expression level of foxp3 through a TGF-β1/smad-independent signaling pathway. Could other signaling mechanisms have been affected by miR-21 in the regulation of Treg cells? Studies have shown that miR-21 enhances mTORC1 signaling by inhibiting IGFBP3, Sprouty, PTEN, FoxO1, and PDCD4 [46, 47]. Additionally, miR-21 has been shown to increase AKT/mTORC1 signaling by inhibiting Treg differentiation [48, 49]. MiR-21 has also been shown to regulate PI3K/AKT/mTOR signaling by targeting TGFβI gene expression [50]. Thus, miR-21 may also regulate Treg cell function through the regulation of the TGF-β/AKT/mTORC1 signaling pathway.

Contrary to our results, Rouas et al. published a paper showing that miR-21 acts as a positive but indirect regulator of foxp3 expression [21]. However, there are two important differences between this study and ours. First, Rouas et al. used human umbilical cord T cells, whereas the current study used human PBMCs. Human umbilical cord blood contains a distinct population of CD25+ Treg cells that are less heterogeneous than those in adult peripheral blood. In addition, the majority of umbilical cord CD4+CD25+T cells express foxp3. Thus, there is likely a higher ratio of CD4+CD25+Foxp3+T cells after stimulation of CD4+CD25-T cells derived from umbilical cord blood than in those derived from human
PBMCs. The second difference is that Rouas et al. used CD4+CD25-T cells stimulated with IL-2 and PHA, whereas our study used no stimulation. T cell activation can augment the expression of some microRNAs, especially miR-21, and cytokines in CD4+CD25-T cells and CD4+CD25+Foxp3+T cells, which could indirectly affect the expression of foxp3.

In conclusion, the results from our experiments demonstrate that in human PBMCs, increased expression of miR-21 partially contributes to a diminished Treg cell population, decreased expression of foxp3, and decreased TGF-β1 secretion into the plasma through a TGF-β1/smad-independent pathway.

Acknowledgments

This work was supported by the National Science Foundation of China to Dr. Jiangjiao Xie (No.81100195) and Dr. Shaolin He (No.81500338).

Disclosure Statement

None.

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