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RESEARCH ARTICLE

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Role of TLR4 and miR-155 in peripheral blood mononuclear cell-mediated inflammatory reaction in coronary slow flow and coronary arteriosclerosis patients

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Background: To study the role of toll-like receptor 4 (TLR4) and MicroRNA-155 (miR-155) in the peripheral blood mononuclear cell (PBMC)-mediated inflammation in coronary slow flow (CSF) and coronary arteriosclerosis.

Methods: Patients were divided into acute coronary syndrome (ACS), stable angina pectoris (SAP), CSF, and healthy control (HC) groups. The isolated PBMCs were treated with lipopolysaccharide (LPS)/and antagomiR-155. TLR4, miR-155, and the concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, and IL-10 were measured.

Results: Before LPS intervention, the TLR4, TNF-α, IL-1, and IL-6 levels were higher and the level of miR-155, IL-10 was lower in the ACS group compared with the SAP, CSF, and HC groups. After exposure to LPS, the levels of TLR4, miR-155, TNF-α, IL-1, and IL-6 were increased and the level of IL-10 was decreased in the SAP and CSF groups compared with the HC group. But when over-expressing antagomiR-155 into PBMCs from SAP and CSF groups, the miR-155 expression, and TNF-α, IL-6, and IL-1 secretion increase induced by LPS were restrained.

Conclusions: TLR4, miR-155, and inflammatory cytokines may be closely related to ACS. LPS can induce TLR4, miR-155 expression and inflammatory response in SAP and CSF patients. AntagomiR-155 can inhibit this inflammatory response.

KEYWORDS

acute coronary syndromes, coronary slow flow, inflammatory cytokine, microRNA-155, toll-like receptor 4

1 | INTRODUCTION

Inflammation is crucial for the initiation and progression of atherosclerosis from the initial lesions to end-stage complications, participating in the progression of a stable plaque to a vulnerable one, which leads to acute coronary syndrome (ACS). Toll-like receptor 4 (TLR4) is an innate immune receptor expressed in immune cells and the heart that plays an important role in promoting atherosclerosis by expressing inflammatory proteins.[1,2] TLR4 signaling is closely related to plaque destabilization. MicroRNA-155 (miR-155), a typical multi-functional miRNA, has recently emerged as a novel component of inflammatory signal transduction in the pathogenesis of atherosclerosis and the onset of ACS.[3] However, the mechanisms by which miR-155 functions as a putative pro-instability plaque microRNA are largely unknown.

The coronary slow flow (CSF) phenomenon is not a rare finding, and CSF is characterized by angiographically normal or near-normal coronary arteries with delayed distal angiogram. Sometimes only according to clinical symptoms, it can be difficult to distinguish CSF from ACS, and therefore, we propose that the underlying pathological mechanisms for these conditions are related. Possible underlying mechanisms of CSF are endothelial dysfunction, chronic inflammation, microvascular dysfunction, and diffuse atherosclerosis. However, little is known about the inflammatory reaction and the role of PBMCs-expressed TLR4 and miR-155 in CSF patients. Therefore, this study

aims to analyze the effect of TLR4 and miR-155 in the PBMCs inflammatory response in coronary arteriosclerosis disease and CSF patients as well as the underlying pathological mechanisms for ACS and CSF.

2 | PATIENTS AND METHODS

2.1 | Study population

The study population consisted of 489 consecutively admitted Chinese patients who underwent diagnostic coronary angiography for suspected coronary atherosclerosis disease at the First Affiliated Hospital of Nanchang University from January 2015 to December 2015. The study was guided by the Declaration of Helsinki and its amendments and also conformed to the approved institutional guidelines. Informed consent was obtained from each patient. The patients were further divided into four groups according to the diagnostic criteria and exclusion criteria: ACS group (n=302), stable angina pectoris (SAP) group (n=98), CSF group (n=39), and healthy control (HC) group (n=50). The inclusion criteria for ACS patients were typically chest pain and ST-segment elevation or depression in at least two contiguous leads on standard ECG, and angiographically confirmed coronary artery disease. SAP was based on symptoms, specifically typical pre-cordial chest pain during exercise (the presence of effort angina for >3 months) and no episodes of angina at rest and documented stenosis of >50% in one or more principal coronary arteries on coronary angiography. CSF was defined as delayed opacification of coronary vessels during angiography without any evident obstructive disease. HC had angiographically normal coronary arteries. Participants with acute infection, active chronic inflammatory diseases (eg, inflammatory bowel disease, rheumatic diseases, upper and lower respiratory tract diseases), diabetes, tumors, liver diseases, and renal insufficiency as well as those with current use of beta-blockers, ACEI, ARB, a lipid-lowering statin, or immunosuppressive drugs were excluded.

2.2 | Separation of PBMCs and treatment groups

Twenty milliliters of whole blood was collected from each potential participant into sodium citrate anticoagulation tubes and then separated the peripheral blood mononuclear cells (PBMCs) using Lymphocyte Separation Medium (Solarbio Beijing Biotechnology Co., Ltd., Beijing, China). PBMCs was stored at −80°C until further analysis.

Prior to intervention, the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, and IL-10 in plasma as well as the expression of TLR4 and miR-155 on PBMCs were detected, as described below. Then, PBMCs of the HC, SAP, and CSF groups were treated with lipopolysaccharide (LPS, 1 mg/L; Solarbio Beijing Biotechnology Co., Ltd. China) for 24 hours. Additional samples of PBMCs in the SAP and CSF groups were pre-treated with antagomiR-155 (Shanghai Chemical Technology Co., Ltd., Shanghai, China) for 2 hours, then treated with LPS (1 mg/L) for 24 hours. The culture medium was collected for quantification of TNF-α, IL-6, IL-1, and IL-10 concentrations, and cells were harvested for quantification of TLR4 protein and miR-155 expression.

2.3 | Recombinant adenoviral antagomiR-155 over-expression

Stable recombinant adenoviral antagomiR-155 was over-expressed, the entire human antagomiR-155 gene open reading frame was obtained by RT-PCR, cloned into the CMV-MCS-EGFP GV135 vector, and ligated into a shuttle plasmid. Adenoviral backbone plasmid was co-transfected into HEK-293A cells to produce the recombinant adenoviral vector Ad-antagomiR-155. Another identical vector without antagomiR-155 cDNA was used to generate empty viruses as controls.

2.4 | Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood mononuclear cells (1×10^6) were plated into 24-well plates, and after 24 hours in culture in RPMI 1640 culture medium containing 10% calf serum (both from Beijing TransGen Biotech, Beijing, China), the cells were incubated with LPS/and Ad-antagoniR-155 as described above. The concentrations of TNF-α, IL-1, IL-6, and IL-10 in the collected media samples were detected using commercial sandwich ELISA kits (Boster Wuhan Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instructions.

2.5 | Flow cytometric analysis

Flow cytometric analysis was performed according to standard procedures. Briefly, 100 μL aliquots of cells were stained with 5 μL phycoerythrin-labeled mice anti-human TLR4 monoclonal antibody (eBioscience, San Diego, CA, USA) at 37°C for 30 minutes in the dark and then washed twice with 1% phosphate-buffered saline (PBS). After centrifugation (1500 *g* for 3 minutes at 4°C), the cells were resuspended in 300 μL of 1% PBS. Cells were analyzed using a flow cytometer (Beckman Coulter, Miami, FL, USA).

2.6 | Quantitative real-time reverse transcription-polymerase chain reaction

RNA was isolated from cultured cells using Trizol-based methods, and then reverse transcribed into cDNA. MiR-155 upstream primer: 5′-GGAGGTTAATGCTAATCG TGATAG-3′ downstream primer: 5′-GTGCAGGGTCCGAGGT-3′. U6 upstream primer: 5′-CTCGCTTCGGCAGCACA-3′, downstream primer: 5′-AACGCTTCA CGAATTTGCGT-3′. Conditions for qRT-PCR were as follows: In the first cycle, reagent mixes were incubated at 95°C for 10 minutes, then at 95°C for 15 seconds, and finally at 60°C for 60 seconds. This cycle was repeated 40 times. The internal normalized reference was U6 snRNA. The relative expression values of miR-155 in each group were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7 | Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses, and the results are shown as means±standard deviation

TABLE 1 Clinical characteristics of study participants

Characteristic	ACS (n=302)	SAP (n=98)	CSF (n=39)	HC (n=50)
Age (y)	64.6±9.8	66.2±8.3	60.5±6.8	60.7±9.4
Male/female, n	192/110	59/39	29/10	31/19
Smokers, n	218	63	26	21
Alcohol users, n	198	54	31	14
BMI (kg/m^2)	25.6±4.3	24.7±4.5	23.8±4.7	24.1±3.2
Dyslipidemia, n	112	39	11	(—)
Hypertension, n	194	69	19	(—)

TABLE 2 Toll-like receptor 4 expression and inflammatory cytokine concentrations before intervention

		Inflammatory cytokine levels (pg/mL)			
Group	TLR4 (%)	TNF-α	IL-1	IL-6	IL-10
ACS	76.3±9.9*	137.2±14.1*	40.5±8.9*	596±9.8*	21.2±6.8*
SAP	25.4±8.2	55.2±9.4	26.0±7.3	33.2±11.9	36.9±10.2
CSF	26.9±7.9	57.8±8.7	26.9±6.9	34.8±12.4	35.5±9.3
HC	23.1±6.2	53.9±9.7	24.5±7.9	31.7±10.9	38.1±11.6

*P<.05 vs HC group.

(SD). One-way analysis of variance (ANOVA) was used to analyze differences in continuous variables. Differences in categorical variables were assessed by Chi-square test. A value of P<.05 was considered statistically significant.

3 | RESULTS

3.1 | Clinical characteristics of the patients

The baseline demographic and clinical characteristics of the patients are summarized in Table 1. No significant differences were observed in patient age, gender, smoking status, alcohol consumption, body mass index (BMI), and waist circumference among the four groups. In addition, the frequencies of dyslipidemia and hypertension did not differ significantly among the ACS, SAP, and CSF groups (Table 1).

3.2 | TLR4 and miR-155 expression and inflammatory cytokine secretion before intervention

Toll-like receptor 4 expression was significantly higher in PBMCs from the ACS group compared with those from the SAP, CSF, and HC groups prior to intervention (Table 2). The level of miR-155 was lower in patients with ACS group than in patients with other three groups (Table 3). No significant differences in TLR4 and miR-155 expression were observed among the SAP, CSF, and HC groups (Tables 2 and 3). The level of TNF-α, IL-1, and IL-6 were significantly higher in the ACS group compared with those in the SAP, CSF, and HC groups prior to intervention. In addition, the level of IL-10 was lower in the ACS group than in the other three groups. The level of TNF-α, IL-1, IL-6, and IL-10 did not differ significantly among the SAP, CSF, and HC groups (Table 2).

3.3 | TLR4 and miR-155 expression and inflammatory cytokine secretion after LPS stimulation

After in vitro stimulation of the PBMCs of the SAP, CSF, and HC groups with LPS, TLR4 expression, miR-155 expression, and the concentrations of TNF-α, IL-6, and IL-1 in the culture medium for the SAP and CSF groups were significantly increased and the concentration of IL-10 in the culture medium was significantly decreased compared with the respective levels in the HC group (Tables 3 and 4). But there were no significantly difference between SAP and CSF groups. In addition, TLR4 expression, miR-155 expression, and the concentrations of TNF-α, IL-6, and IL-1 in the culture medium for the SAP and CSF groups were significantly increased and the concentration of IL-10 in the culture medium was significantly decreased after LPS stimulation compared to the respective levels prior to LPS stimulation (Tables 3 and 4).

3.4 | Inflammatory cytokine secretion after Ad-antagomiR-155 pre-treatment

Peripheral blood mononuclear cells in the SAP and CSF groups were pre-treated with antagomiR-155 for 2 hours, then treated with LPS

TABLE 3 Relative expression of miR-155 before and after LPS intervention

	Relative expression of miR-155 (95% CI)	
Group	Before LPS	After LPS
ACS	0.56 (0.49, 0.64)*	1.68 (1.11, 2.25)**
SAP	0.87 (0.72, 1.02)	1.46 (1.08, 1.84)**
CSF	0.84 (0.67, 1.02)	1.41 (1.04, 1.79)**

Results are expressed relative to miR-155 expression in the HC group, which were given the arbitrary value of 1.
*P<.05 vs SAP and CSF group; **P<.05 vs the respective levels prior to LPS stimulation.

TABLE 4 Toll-like receptor 4 expression and inflammatory cytokine concentrations after LPS stimulation

		Inflammatory cytokine levels (pg/mL)			
Group	TLR4 (%)	TNF-α	IL-1	IL-6	IL-10
SAP	53.2±5.6*,***	95.5±9.7*,***	34.1±7.6*,***	53.2±10.2*,***	28.3±6.5*,***
CSF	55.1±6.7**,***	97.8±9.3**,***	35.9±8.7**,***	54.7±9.8**,***	29.1±9.1**,***
HC	25.6±7.1	53.2±9.8	23.9±7.7	29.5±10.1	35.7±9.2

*P<.05 vs CSF group pre-LPS stimulation; **P<.05 vs SAP group pre-LPS stimulation; ***P<.05 vs HC group post-LPS stimulation.

TABLE 5 Inflammatory cytokine concentrations after Ad-antagomiR-155 pre-treatment

	Inflammatory cytokine concentration (pg/mL)			
Group	TNF-α	IL-1	IL-6	IL-10
SAP	63.9±8.9**	27.1±7.9**	37.4±10.1**	33.1±7.5**
CSF	62.1±8.8*	26.3±6.6*	36.3±10.4*	33.8±8.2*

*P<.05 vs CSF group post-LPS stimulation; **P<.05 vs SAP group post-LPS stimulation.

(1 mg/L) for 24 hours, The concentrations of TNF-α, IL-6, and IL-1 in the culture medium were significantly decreased and the concentration of IL-10 in the culture medium was significantly increased in the SAP and CSF groups compared with the respective levels with LPS stimulation only (Table 5).

4 | DISCUSSION

Multiple studies have demonstrated that inflammation is a key factor in the initiation and progression of atherosclerosis and the onset of ACS,[4,5] with inflammatory cells, especially monocytes/macrophages and T lymphocytes, playing important roles.[6,7] Mononuclear cells express toll-like receptors (TLRs) on their surface,[8] and TLR4, among other TLRs, is involved in the initiation of an inflammatory response and, most likely, in the pathogenesis of atherosclerosis.[9-11] MiR-155 had recently emerged as a novel component of inflammatory signal transduction in the pathogenesis of atherosclerosis, overexpression of miR155 in macrophages enhanced their inflammatory response to lipopolysaccharide, miR155 deficiency attenuated atherogenesis in apoE(-/-) mice by reducing inflammatory responses of macrophages.[12] Several studies have demonstrated increased expression of pro-inflammatory cytokines, such as TNF-a, IL-1β, and IL-6, as well as deficiency in the anti-inflammatory cytokine IL-10 during the development of atherosclerosis.[13,14] The results of the present study were consistent with these previous studies with greater TLR4 expression and lower miR-155 expression on PBMCs from the ACS groups compared with the SAP, CSF, and HC groups. However, the level of TLR4 and miR-155 on PBMCs did not differ significantly among the SAP, CSF, and HC groups. Moreover, the level of TNF-α, IL-1, and IL-6 were significantly higher and that of IL-10 was significantly lower in the ACS group compared with the SAP, CSF, and HC groups.

Although some research have focused on microRNA-155 as an atherosclerosis regulator, the pro-atherosclerosis mechanisms are largely unknown. Circulating levels of miR-155 are significantly down-regulated in patients with coronary artery disease compared with healthy controls.[15] The expression of miR-155 in the PBMCs of ACS patients was decreased by approximately 60%, showed no significant difference between unstable angina group and chest pain syndrome group.[16] In vitro experiments confirmed that miR-155 expression in macrophages was increased in response to LPS stimulation.[17] The results of the present study also confirmed that the level of miR-155 was significantly lower in the ACS group.

Plaque rupture has been shown to be a major contributing factor in the initiation of the occurrence of ACS.[18] Notably, increased levels of inflammatory cytokines are associated with plaque rupture.[19] TLR4 expressions of PBMCs and plaque debris from ACS patients was significantly higher than those from SAP patients.[20,21] MiRNAs have been shown to be important regulators of TLR signaling. MiR-155 was up-regulated by several Toll-like receptor ligands through myeloid differentiation factor 88- or TRIF- dependent pathways.[22] MiR-155 also induced the expression of the pro-inflammatory cytokines (IL-12, IL-6, IL-1b) in LPS-treated RAW264.7 cells.[23] Coronary atherosclerotic plaque burden was significantly correlated with transcoronary concentration gradients of miR-155.[24] But the results of the present study confirmed that the level of TLR4 was significantly higher and that of miR-155 was significantly lower in the ACS group. The reduction of miR-155 in the PBMCs of ACS patients might be explained by the uptake of miR-155 by atherosclerotic lesions.[15] In the present study, PBMCs from SAP and CSF groups were stimulated with LPS, the TLR4, miR-155 expression, and TNF-α, IL-6, and IL-1 secretion were significantly increased, whereas the IL-10 level was significantly decreased. But when over-expressing antagomiR-155 pre-transfected into PBMCs from SAP and CSF groups, the TLR4, miR-155 expression, and TNF-α, IL-6, and IL-1 secretion increase induced by LPS were restrained. This observation may imply that acute coronary events are possible upon activation of TLR4 and miR-155 in SAP and CSF patients, and miR-155 may be an important key point during the onset of ACS.

The mechanism of the CSF phenomenon may be closely associated with inflammation,[25,26] with one study even proposing it may be the early phenomenon in coronary atherosclerosis.[27] Previous studies have reported that the levels of inflammatory markers may be associated with pathogenesis of CSF.[26,28] Here, we also found that the TNF-α, IL-1, and IL-6 levels were slightly higher for the CSF group compared

with the HC group and were comparable to those of the SAP group. When the PBMCs of CSF group were treated with LPS or with over-expressing antagomiR-155 and LPS, the level of TLR4, miR-155 and inflammatory reaction were the same as those of SAP group. Therefore, no matter the findings of previous studies or our study all strengthen the hypothesis that the CSF phenomenon was closely linked to the inflammatory response, CSF represented a potential form of "ACS", with the same clinical importance as SAP.

Our results further illustrate that inflammation is a key factor in the onset of ACS, miR-155 can induce the expression of the pro-inflammatory cytokines in LPS-treated PBMCs. Effective inhibition of the inflammatory response by depressing miR-155 may represent a target for therapies to stabilize plaques and thus improve patients' clinical prognosis.

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