Receptor for advanced glycation end products involved in circulating endothelial cells release from human coronary endothelial cells induced by C-reactive protein

Shulai Zhou 1, Lichao Gao 1, Fangqi Gong 1*, Xiaoyang Chen 1

1 Children’s Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310003 China

Objective(s): This study was designed to investigate the effect of receptor for advanced glycation end products (RAGE), S100A12 and C-reactive protein (CRP) on the release of circulating endothelial cells (CECs) from human coronary artery endothelial cells (HCAECs).

Materials and Methods: HCAECs were cultured in increasing concentration of CRP (0, 12.5, 25, 50μg/ml) or S100A12 protein (0, 4, 10, 25μg/ml) for 24 hr. CECs were measured by flow cytometry. Small interfering RNA (siRNA) was designed to decrease RAGE level. Fluorescence microscopy and real-time quantitative polymerase chain reaction were used to assess the efficiency of siRNA silencing RAGE. The release of CECs from HCAECs was further evaluated by flow cytometry.

Results: CRP caused a significant increase in the release of CECs from HCAECs. The number of CECs increased by about 2-fold in 25 μg/ml CRP-treated group compared to the control group (12.22% compared to 6.82%, P=0.032). But S100A12 failed to increase the release of CECs from HCAECs. Blockade of RAGE by siRNA significantly decreased the release of CECs induced by CRP (13.22% of CRP group compared to 8.77% of CRP+siRNA group, P=0.017).

Conclusion: RAGE is involved in the release of CECs induced by CRP, and the effect can be attenuated by silencing RAGE. RAGE may play an important role in endothelial dysfunction in cardiovascular disease. Inhibition of RAGE may be a therapeutic target for coronary artery lesions in Kawasaki disease.

Introduction

Kawasaki disease is an acute childhood vasculitis of unknown etiology that most frequently affects infants and children younger than 5 years. Coronary artery lesions are the most critical complication (1).

Receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules, capable of interacting with a broad repertoire of ligands including advanced glycation end products (AGEs), amyloid fibrils, S100 / calgranulins and amphoterin (2). RAGE is expressed on the surface of various cells such as endothelium, mononuclear phagocytes, lymphocytes and smooth muscle cells. Numerous studies found that RAGE may play an important role in inflammatory processes and endothelial dysfunction in cardiovascular disease (3-5). S100A12 is an inflammatory ligand of RAGE that can interact with RAGE on the endothelium and trigger endothelium activation through generation of the key pro-inflammatory mediators (such as interleukin-1 and tumor necrosis factor). The pro-inflammatory role of RAGE and S100A12 interaction is well established in chronic cellular activation and tissue injury (6).

Endothelial cells (ECs) play a crucial role in the physiological and pathological processes of hemostasis, inflammation, and angiogenesis. Endothelial dysfunction is not only the early phenomenon of vascular injury, but also plays an extremely important role in its development (7). Circulating endothelial cells (CECs) are sensitive biomarkers of endothelial dysfunction. A high CEC count is found in widespread vascular damages associated with rickettsial vasculitis, sickle cell crisis, or cytomegalovirus infection (8). High CEC levels in patients with acute coronary syndrome showed high risk of subsequent cardiovascular events (9). Our previous study found that CEC levels were increased in patients with Kawasaki disease. Furthermore, RAGE and its ligand, S100A12 on CECs increased in these patients, especially in patients with coronary artery lesions (CALs) (10, 11). However, the relationship between CECs and the S100A12-RAGE axis

*Corresponding author: Fangqi Gong, Children’s Hospital, Zhejiang University School of Medicine, 57 Zhugan Lane, Hangzhou 310003, China. Tel: +86-571-87033296; Fax: +86-571-87073271; Email: gongfangqi@zju.edu.cn

Please cite this paper as:
Zhou Sh, Gao L, Gong F, Chen X. Receptor for advanced glycation end products involved in circulating endothelial cells release from human coronary endothelial cells induced by C-reactive protein. Iran J Basic Med Sci 2015; 18:610-615.
is not clear. On the other hand, C-reactive protein (CRP), a classical acute-phase reactant in response to inflammatory condition, is not only a biomarker of cardiovascular disease, but also a contributor to development and progression of cardiovascular disease via promoting arterial endothelial activation and dysfunction (12). Children with Kawasaki disease often have elevated levels of CRP, and high levels of CRP suggest higher risk of coronary arterial lesions. It was reported that CRP induced CECs-release from Human aortic endothelial cells (HAECs) as further evidence of the endothelial dysfunction (13). Increasing number of evidence suggests that CRP has considerable connections to RAGE (14). Previous studies demonstrated that CRP upregulated RAGE expression in human endothelial cells (15), altered antioxidant defenses, and decreased nitric oxide (NO) production in rat endothelial progenitor cells (16). Blockade of RAGE expression by small interfering RNA (siRNA) could block this effect (17). However, down-regulation caused by RAGE on CRP-induced increase of CECs has not been studied.

In order to investigate the effect of RAGE, S100A12 and CRP on the release of CECs from human coronary endothelial cells (HCAECs), we tested the hypothesis that RAGE is involved in CEC release induced by S100A12 or CRP.

**Materials and Methods**

**Cells culture**

HCAECs (ATCC, Manassas, USA) were grown in Endothelial Cell Growth Supplement (Sciencell, SanDiego, USA) with 1% Endothelial Cell Growth Supplement (Sciencell) and 5% fetal bovine serum (Sciencell) at 37 °C in a humidified atmosphere containing 5% CO₂. HCAECs were used at passages 4 to 6 for all experiments. CRP was purchased from Sigma (C4063, St. Louis, USA). Human recombinant S100A12 was purchased from Sino Biological Inc. (Beijing, China). The cells were treated with increasing concentration of CRP (0, 12.5, 25, 50 μg/ml) or S100A12 (0, 4, 10, 25 μg/ml) for 24 hr when they grow up to 70% to 80% confluence. In siRNA group, HCAECs were incubated for 24 hr when they grow up to 70% to 80% confluence. In siRNA transfection group, HCAECs were incubated for 24 hr when they grow up to 70% to 80% confluence.

**Small interfering RNA for RAGE**

siRNA for RAGE was purchased from RiboBio Inc. (Guangzhou, China). Lipofectamine2000 reagent and OptimEM Reduced Serum Medium were purchased from Invitrogen (Carlsbad, USA). The sequence of the siRNA molecules was as follows: 3′ dTdT CGGUCAUCAUCCAGAUU 5′ and 3′ dTdT CGGUCAUCAUCCAGAUU 5′. siRNA transfection was carried out according to the manufacturer protocol for the siRNA transfection kit. In brief, HCAECs were transferred into T-25 flask and cultured in endothelial cell medium supplemented with 1% Endothelial Cell Growth Supplement and 5% fetal bovine serum, without antibiotics. After overnight growth, cells at 30-50% confluence were treated with 1 ml of siRNA-lipofectamine 2000 complexes (100 nmol/l siRNA final concentration) at each T-25 flask. Transfection medium was removed after 24 hr, and cells were washed with PBS and then maintained in complete medium for 24 hr. Cells were then collected for real-time quantitative PCR to assess the RAGE mRNA level. Scramble siRNA was used as a control. In addition, Cy3-labeled negative control siRNA was used to determine the transfection efficiency.

**Detection of the efficiency of siRNA silencing RAGE**

For the determination of the efficiency of siRNA silencing RAGE, fluorescence microscope and real-time quantitative PCR were used. HCAECs transplanted with Cy3-labeled siRNA were detected by fluorescence microscopy after 6 hr of transfection. The effect of siRNA on the RNA expression of RAGE was assessed by real-time quantitative PCR after 48 hr of transfection. For the determination of the effect of siRNA on the RNA expression of RAGE, we assayed the efficiency of siRNA silencing RAGE by real-time quantitative PCR after 48 hr of transfection. The efficiency of siRNA silencing RAGE was measured by real-time quantitative PCR after 48 hr of transfection. The efficiency of siRNA silencing RAGE was measured by real-time quantitative PCR after 48 hr of transfection. The efficiency of siRNA silencing RAGE was measured by real-time quantitative PCR after 48 hr of transfection. The efficiency of siRNA silencing RAGE was measured by real-time quantitative PCR after 48 hr of transfection. The efficiency of siRNA silencing RAGE was measured by real-time quantitative PCR after 48 hr of transfection. The efficiency of siRNA silencing RAGE was measured by real-time quantitative PCR after 48 hr of transfection. The efficiency of siRNA silencing RAGE was measured by real-time quantitative PCR after 48 hr of transfection.

**Flow cytometry for CECs**

HCAECs were detached using 0.25% trypsin, and harvested by centrifugation. Then, cells were diluted with 100μl PBS and stained with antibodies of FITC-Carlsbad, USA). The sequence of the siRNA molecules was as follows: 3′ dTdT CGGUCAUCAUCCAGAUU 5′ and 3′ dTdT CGGUCAUCAUCCAGAUU 5′. siRNA transfection was carried out according to the manufacturer protocol for the siRNA transfection kit. In brief, HCAECs were transferred into T-25 flask and cultured in endothelial cell medium supplemented with 1% Endothelial Cell Growth Supplement and 5% fetal bovine serum, without antibiotics. After overnight growth, cells at 30-50% confluence were treated with 1 ml of siRNA-lipofectamine 2000 complexes (100 nmol/l siRNA final concentration) at each T-25 flask. Transfection medium was removed after 24 hr, and cells were washed with PBS and then maintained in complete medium for 24 hr. Cells were then collected for real-time quantitative PCR to assess the RAGE mRNA level. Scramble siRNA was used as a control. In addition, Cy3-labeled negative control siRNA was used to determine the transfection efficiency.

**Statistical analysis**

Statistical differences between groups were analyzed using ANOVA followed by least significant difference multiple comparison for normal data and Mann–Whitney test for non-parametric data. The difference was considered significant with a P-value < 0.05. Analysis was done using SPSS 18.0 Software.

**Results**

**CRP induced release of CECs from HCAECs**

CRP increased the number of CECs released from HCAEC, with significant increase at CRP concentrations of 25 and 50μg/ml as compared to control (P=0.039
Zhou et al. RAGE involved in CECs release induced by CRP

Iran J Basic Med Sci, Vol. 18, No. 6, Jun 2015

612

Figure 1. Flow cytomeric study of circulating endothelial cells
HCAECs were stained with the antibodies of FITC-CD146, APC-CD105, Perp-cy5.5-CD45 and PE-CD34 for 30 min at room temperature. Cells were then washed with PBS for 3 times and immediately assessed by flow cytometry. After excluding cell debris with a morphological gate, cells which expressed as CD146 (+), CD105 (+), CD45 (−) and CD34 (+) (Q4-1 of C) were identified as CECs and 0.032, respectively, Figure 2A). S100A12 failed to increase the number of CECs released from HCAEC (Figure 2B).

Efficiency of siRNA silencing RAGE
The transfection efficiency of siRNA was detected by fluorescence microscopy. HCAECs transplanted with Cy3-labeled siRNA were used to detect the transfection efficiency. Most of the HCAECs (>80%) showed uptake of Lipofectamine 2000, and these cells displayed red fluorescence in the cytoplasm and blue fluorescence in the cell nucleus (Figure 3A). The expression of RAGE mRNA was measured by quantitative RT-PCR to assess the inhibitory effect of siRNA. siRNA decreased the expression of RAGE by about 70% in mRNA levels compared to the control (33.62%±8.56% compared to control, Figure 3B).

Blockade of RAGE by siRNA and the relationship between CECs release and CRP
HCAECs were pretreated with siRNA and then incubated with 25 μg/ml purified human CRP for 24 hr. The number of CECs was assessed by flow cytometry. CRP (25 μg/ml) significantly increased CEC production, and this effect was significantly inhibited by siRNA (P=0.03 for CRP compared to control, P=0.017 for CRP+siRNA compared to CRP, Figure 4).

Discussion
Patients with Kawasaki disease usually have elevated CRP. Koyanagi et al. reported that CRP levels in children with Kawasaki disease reached 110 μg/ml (18). High levels of CRP often amplify the risk of coronary artery lesions (19). In the present study, we showed that CRP increased the release of CECs from HCAEC at a dose as low as 25 μg/ml. Therefore, CRP may have a direct effect on endothelial dysfunction and may promote coronary artery lesions in patients with Kawasaki disease. CECs are sensitive biomarkers of endothelial dysfunction and are defined by the expression of the membrane glycoprotein, CD146 in peripheral blood. Multiple definitions and methods have been developed to

Figure 2. Effect of C-reactive protein or S100A12 on the release of circulating endothelial cells
HCAECs were treated with increasing concentrations of (A) purified human CRP (0, 12.5, 25, 50 μg/ml) or (B) human recombinant S100A12 (0, 4, 10, 25 μg/ml) for 24 hr. The number of CECs was assessed by flow cytometry. *P<0.05 compared to control. MFI: Mean fluorescence intensity
evaluate and measure CEC levels by various research groups. Devaraj et al reported that CRP increased the release of CECs from HAEs, and demonstrated that the effect of CRP on the increase in the number of CECs is due to CRP-induced NO deficiency (13). Our study highlights this effect of CRP on human coronary artery endothelial cells. CRP caused a significant increase in the release of CECs at a dose as low as 25 μg/ml, an almost 2-fold increase compared to control, but we did not find significant differences between CRP concentrations of 25 μg/ml and 50 μg/ml. In the present study, S100A12 failed to increase the number of CECs which may be because of two reasons. First, recombinant human S100A12 may have had an insufficient affinity to bind RAGE (20, 21). Second, S100A12-RAGE interaction may only amplify the pro-inflammatory response rather than initiating endothelial cell injury (2).

This study was the first of its kind to show that RAGE is involved in the release of CRP-induced CECs. Our previous studies reported that CECs are increased in the acute stages of Kawasaki disease. RAGE and its ligand, S100A12 on CECs are increased in these patients as well, especially in the patients with coronary artery lesions (10, 11). There is an increasing number of evidence which suggest an important link between CRP and RAGE. Zhong et al demonstrated that CRP upregulated RAGE expression in human endothelial cells (15). Chen et al reported that CRP altered antioxidant defenses and decreased NO production in rat endothelial progenitor cells. Inhibition of RAGE expression by siRNA could block this effect (16, 17). Here, we assessed the efficiency of siRNA silencing RAGE by real-time quantitative PCR, which did not represent the protein level of RAGE (22, 23).

Previous studies suggested that CRP decreased eNOS expression and bioactivity in endothelial cells, and CRP could induce endothelial dysfunction by decreasing NO production (24, 25). RAGE involvement in endothelial dysfunction induced by CRP may be related to the increase in intracellular reactive oxygen species (ROS) and the decrease in NO production. Various studies show that the most significant pathological consequence of RAGE appears to be cellular activation, which leads to the induction of oxidative stress (26, 27). One of the best documented consequences of RAGE activation is the generation of ROS. Increased intracellular ROS has a direct effect on damaging endothelial cells and scavenging NO (28).

**Conclusion**

RAGE is involved in the release of CECs induced by CRP, and the effect can be attenuated by silencing RAGE. RAGE may play an important role in endothelial dysfunction in cardiovascular disease. Inhibition of
RAGE may be a therapeutic target for coronary artery lesions in patients with Kawasaki disease.

Acknowledgment
This work is supported, in part, by grants from The National Natural Science Foundation of China (81270177), Ministry of Health Research Foundation of China (WQJ-ZJ-020), and Special Major Science and Technology Project of Zhejiang Province (2013C03043-1). The results described in this paper were part of student thesis.

References
the interaction of advanced glycation end products with their receptors/binding proteins. J Biol Chem 1994; 269:9889-9897.
