Circulating endothelial cells and vascular cell adhesion molecule-1 correlates with severity of regurgitation lesion and heart failure in rheumatic heart disease

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ABSTRACT

Objective: This study aims to determine the correlation of vascular cell adhesion molecule-1 (VCAM-1) and circulating endothelial cells (CEC) with cardiac valves lesion severity and heart failure (HF) in rheumatic heart disease (RHD).

Methods: A cross-sectional observational study using human peripheral blood samples of 36 children aged 6 to 14 years old, divided into two groups: 18 RHD patients and 18 healthy controls group. The expression of CECs and VCAM-1 on CECs was investigated by using flow-cytometry method while the levels of soluble VCAM-1 (sVCAM-1) were obtained by using ELISA method. The severity of valve lesions was determined in a qualitative manner.

Results: The average of CECs expression, VCAM-1 on CECs expression, and sVCAM-1 levels were significantly higher in the patient group than those of the healthy group. The expression of VCAM-1 on CECs was significantly different among mild, moderate, and severe regurgitation. Correlation analysis showed that the CECs expression had a significant correlation with HF. Moreover, correlation was found between expression of VCAM-1 on CECs and the severity of valve lesions. However, sVCAM-1 in plasma had no correlation either with HF or with valve lesion.

Conclusion: The expression of VCAM-1 on CECs is associated with severe regurgitation while CECs level was correlated with HF. VCAM-1 on CECs may be considered a marker for the severity of functional regurgitation in RHD. CECs could be recommended as a measure for RHD with HF.

KEY WORDS: Circulating endothelial cells, heart failure, rheumatic heart disease, vascular cell adhesion molecule-1, valve regurgitation

INTRODUCTION

Rheumatic heart disease (RHD) is still the main cause of valve diseases and an important cause of cardiovascular morbidity and mortality in developing countries [1]. Approximately 15.6 million people worldwide were affected by RHD whereas its prevalence in Asian children was 1.96 to 2.21 million [2, 3]. Rheumatic heart disease develops in approximately 30-45% of acute rheumatic fever (ARF) cases and results in a permanent heart valve damage. Mitral and aortic regurgitations are the most common valve lesion in RHD that can lead to congestive heart failure (CHF) which causes life-threatening complication for thousands of children worldwide [4-6]. The diagnosis of RHD is determined by using echocardiography [7] that can be affected by machine and operator expertise [8].

Rheumatic fever is the immune sequelae of a β-hemolytic group A streptococcus infection. The streptococcus polysaccharides and heart valves glycoproteins both contain N-acetyl-β-d-glucosamine. The structural similarity causes a cross-reaction between streptococcus and heart valves tissue (molecular mimicry) and plays a role in disease pathogenesis of rheumatic valve disease [9, 10]. The human streptococcal antibodies activate valve endothelial cells and up regulate some molecules adhesion, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1), P- and E-selectin [6]. The adhesion process between endothelial cells and leukocytes is a key factor for leukocytes to enter the sites of inflammation. VCAM-1 binds monocytes and T lymphocytes and then is expressed on the activated vascular endothelial cell surface and inflammatory cells [7, 11]. In RHD inflammation process, VCAM-1 interacts with very late antigen-4 (VLA-4) on lymphocytes and activates the lymphocytes cluster of differentiated CD4 and CD8 to enter the heart valves, leading to a granulomatous response, eventually causing inflammation and scars of the heart valves [6, 7].

Vascular cell adhesion molecule-1 is a glycoprotein which is included into the immunoglobulin superfamily of adhesion molecules. Adhesion molecules are detected low in healthy people serum and elevated in inflammatory condition, autoimmune process, malignancy and cardiovascular disease including heart failure (HF) [1, 11-13]. Several previous studies have described the role of VCAM-1 in rheumatic valve disease [7, 14-16] and implicated patogenesis and progression of HF [17-20].
Recurrent exposures to cardiovascular risk factors lead to the damage and dysfunction of endothelial cells. An inflammation process initiates the damage of endothelial cells and eventually detaches into circulation as circulating endothelial cells (CEC). The endothelial cells detachments are characterized by elevated number of CECs within the bloodstream and it may express some adhesive molecules [21-23]. Elevated number of CECs has been identified in a wide array of diseases and has related to endothelial dysfunction (24). However, the correlation study of CECs with severity of valve regurgitation in RHD has not been established yet. Besides, VCAM-1 has many potential sources of origin [11, 12, 25]. In addition, the soluble form of VCAM-1 (sVCAM-1) may not reliably reflect the endothelial injury (25). Therefore, we are interested in conducting a research to determine whether CECs and the expression of VCAM-1 on CECs have relationship with the severity of valve regurgitation and HF in RHD.

**PATIENTS AND METHODS**

**Study design and study population**

This cross-sectional study was conducted from May to August 2014. The study enrolled 36 children, divided into two groups as patient healthy control groups. The patient group consisted of 18 RHD patients who came for their regular follow up at Dr. Saiful Anwar Pediatric Cardiology Clinic in Malang, Indonesia, during the period of the study (consecutive sampling). The control group consisted of 18 healthy volunteer children.

**Inclusion and exclusion criteria**

The Inclusion criteria for patient group were children with age range of 6 to 14 years, who were diagnosed with RHD and had taken therapy for less than 2 years depending on the severity of the disease: mild carditis treated by oral acetyl salicylic 100 mg/kg for 2-4 weeks; moderate carditis treated by oral acetyl salicylic 100 mg/kg for 6-8 weeks plus prednisone 1-2 mg/kg for 2-4 weeks; severe carditis treated by oral acetyl salicylic mg/kg for 2-4 months plus prednisone 1-2 mg/kg for 2-6 weeks. Subjects who had acute inflammation, infection, autoimmune disease, hematologic disorder and other cardiovascular diseases were excluded from this study.

**Definition used in this study**

The diagnosis of RHD was determined according to Jones criteria which were modified by the world health organization (WHO) in 2002-2003 for the diagnosis of rheumatic fever (RF) and RHD [26]. The valve abnormalities are identified by echocardiography, using color jet flow area to determine the severity of valve regurgitation. The severe valve abnormalities were classified into mild, moderate, and severe valve regurgitation. Heart failure is a clinical syndrome characterized by the inability of the myocardium to pump the blood throughout the body to fulfill metabolic requirements of the body [27]. In the present work, the heart failure severity was classified using Ross score classifications when the patient was admitted. Heart failure is characterized by shortness of current clinical activities, failure to thrive, tachycardia, cardiomegaly on physical examination, chest photo and gallop rhythm: total score less than 2 = no presentation of HF; total score of 3 to 6 = mild HF; total score of 7 to 9 = moderate HF; and total score of 10 to 12 = severe HF [28].

CECs expression was analyzed by flow-cytometry as CD146 expression in peripheral blood mononuclear cells (PBMC) using ‘PE anti-human CD146 (P1H12)’ antibody (BioLegend; San Diego, CA, USA). The expression of VCAM-1 was assayed as CD106 on CECs surface using ‘PE/Cy5 anti-human CD106’ antibody (BioLegend). Soluble VCAM-1 was assayed by enzyme-linked immunosorbent assay (ELISA) methods using ‘human soluble VCAM-1/CD-106’ kit.

**Ethical consideration**

The study was approved by the Research Ethics Committee of the Faculty of Medicine, Brawijaya University Dr. Saiful Anwar Hospital. Informed consents were obtained from mothers or legal guardians of all children.

**Blood collection**

Peripheral blood samples were aseptically taken from the patient and control group subjects in Pathology Clinic Laboratories Dr. Saiful Anwar Hospital; 3 ml for flow-cytometry and 1 ml for ELISA tests. Blood samples were collected in vacutainer tube containing 1.8 mg/ml ethylenediaminetetraacetic acid (EDTA), shipped in containers equipped with maintained temperature at approximately 4°C, immediately sent to Biomedical Laboratories, Faculty of Medicine, Brawijaya University.

**Identification of CECs and VCAM-1 on CECs by flow-cytometry**

CECs were isolated from PBMCs. For this reason, PBMCs ring was separated from the blood using Histopaque 1077, placed in a 15 ml new centrifuge tube, washed with a solution of 10 ml of PBS and then centrifuged at room temperature at 1200 rpm of speed for 10 min. The supernatant was discarded and the pellet was washed with PBS by centrifugation at room temperature at 1200 rpm of speed for 10 min. The process is done twice. After centrifugation, the pellet was added with CD146 or CD106 antibodies and then was incubated in the dark for 20 min. After incubation, pellet cells were added 400 ml of staining buffer, transferred into a flow-cytometry cuvette and then read.
Measurement of soluble VCAM-1

The peripheral blood was centrifuged to separate plasma from blood and stored at -20°C until assay. The sVCAM-1 from plasma concentration was quantified using sandwich ELISA kit (BioLegend) according to the instructions of the manufacturer. In brief, the plasma was diluted 1:40 with assay buffer B, the human sVCAM-1 basic standard was made using an assay buffer B which was dissolved in 1730 ml, left at room temperature for 15 min, and then vortexed using before being mixed completely. Five-hundred milliliters of sVCAM-1 basic standard solution was transferred into a new tube. A new solution was made from basic standard solution at a different tube, then sequentially diluted until 1.56 ng/ml concentration. A 100 ml avidin-HRP and detection antibody B (which has been previously mixed) was added into each well, then 50 ml sample or standard solution was added into each well. The wells seal plate was closed, incubated for 1 h in room temperature and then shaken at 200 rpm on a microplate shaker. After that, each well was washed with 300 ml wash buffer for five times. In the last washing, the rest solution in the wells was tapped using absorbent papers. The 100 ml substrate solution was added to each well and incubated for 20 min in a dark room. The wells containing human sVCAM-1 will change into the color of blue, the color intensity was proportional according to the concentration of sVCAM-1 in wells. The process was stopped by adding 100 ml of a stop solution into each well. The color of solution will change from blue to yellow. Absorbance was read at wavelengths of 450 and 570 nm.

Statistical analysis

Statistical analysis was performed using SPSS (version 22). Kolmogorov-Smirnov test was used to evaluate the normality of quantitative data. All data were found to be normally distributed. T-test was performed to compare the expression of CECs, VCAM-1 on CECs, and sVCAM-1 levels among control and patient groups. Independent T-test was used to compare the expression of CECs between RHD with HF and non-HF. Analysis of variance (ANOVA) was performed to compare the expression of VCAM-1 on CECs among mild, moderate and severe valve regurgitation. Pearson correlation test was used in order to determine the correlation between variables. Significance was set at 95% confidence level.

RESULTS

Baseline characteristic of subjects

There were 18 children suffering from RHD and 18 healthy children. From the 36 study subjects, 15 were boys and 21 girls. In the patient group, there were 7 boys and 11 girls while in the control group there were 8 boys and 10 girls. The age range of subjects was 6 to 14 years. From out of 18 RHD patients, 6 patients had mild valve lesion, 6 patients had moderate valve lesion and the other 6 had severe valve lesion. There were 11 patients who had lesion on mitral valve, 4 patients who had lesion on aortic valve and 3 patients who had tricuspid valve lesion. However, only 15 patients suffered from heart failure. Therapy was administered in various time from 2 months to 2 years to each subject, but there was no significant difference of the expression of CECs, expressions of VCAM-1 on CECs, and level of sVCAM-1 between patient and control group for difference of treatment period.

The CECs expression is shown as R2 percentage gate while the expression of VCAM-1 at CECs was displayed in upper quadrant as an expression of the CD106 on CD146 (Figure 1). The expression of CECs and VCAM-1 on CECs were higher in the patient group than that in control group. The R2 gate percentage was the expression of CD146 in PBMCs. The CECs expression in the patient group was 3.38 to 7.4%, while the healthy group was 1.28 to 4.79%. The upper right gate percentage, which represented VCAM-1 expression on CECs surface, in the patient group had a range of 4.03 to 19.11% while in healthy group a range of 0.76-3.84%. The average CECs expression in patient group was 4.41% while in the control group only 2.47%. The average expression of VCAM-1 on CECs in patient group was 9.15% compared to 2.31% in control group. There was a significant difference between patient and control groups (P < 0.001). The average of sVCAM-1 level in patient group was 561.75 ng/ml compared to 334.23 ng/ml in control group. Statistically significant difference was recorded between patient and control groups (P < 0.001). However, there was no significant difference for the expression of CECs, VCAM-1 on CECs and sVCAM-1 levels between boys and girls as well as among different ages.

Expression of CECs, of VCAM-1 on CECs and level of sVCAM-1 among mild, moderate, and severe valve regurgitation

Analysis by ANOVA test showed there were a significant difference in the expression of VCAM-1 on CECs among mild, moderate and severe valve regurgitation (P < 0.001, Figure 2) and there was no significant difference among the expression of CECs and sVCAM-1 level. The VCAM-1 on CECs had a strong correlation with regurgitation lesion severity (r = 0.793, P = 0.01; Figure 2); but, CECs expression and sVCAM-1 level had no correlation with regurgitation lesion severity.

Table 1. The average of CECs expressions, VCAM-1 on CECs expression and sVCAM-1 level between patient and control groups (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Patient group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CECs (%)</td>
<td>2.47 ± 0.87</td>
<td>4.41 ± 1.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>VCAM-1 on CECs (%)</td>
<td>2.31 ± 0.97</td>
<td>9.15 ± 4.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>sVCAM-1 (ng/ml)</td>
<td>334.23 ± 93.92</td>
<td>561.75 ± 212.99</td>
<td>&lt; 0.001</td>
</tr>
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CECs: circulating endothelial cells; VCAM-1: vascular cell adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1.
Expression of CECs, expression of VCAM-1 on CECs and level of sVCAM-1 in HF and non-HF RHD patients

The CECs expression in HF group was 4.76% compared to non-HF as 2.67% (Figure 3). The CECs expression had a strong correlation with HF ($r = 0.633$, $P = 0.005$). The expression of VCAM-1 on CECs and level of sVCAM-1 in plasma had no correlation with HF and non-HF RHD patients.

DISCUSSION

RHD is still the main cause of valve disease in developing countries, leading to more than 233,000 deaths annually [1, 5]. Rheumatic valve disease is a chronic complication of ARF and mostly occurs in children of 5-15 years old [10, 26]. Previous studies suggested that the pathogenesis of ARF is a combination of three factors: (i) streptococcus virulence, (ii) host susceptibility and (iii) environmental influences [2, 29]. Environmental factors such as low socioeconomic status, overcrowding, low education, insufficient nutrition and unqualified health care management, which are often found in developing countries, promote recurrent pharyngeal streptococcus infection. Moreover, insufficient treatment compliance and incomplete treatment lead to subclinical RHD [29]. In developing countries such as Indonesia, the last incidence of ARF or RHD is unknown. The last record in 1981 showed the ARF incidence varying from 0.3 to 0.8 per 1000 children [30].

Streptococcus infection occurs through the binding of the surface of specific bacterial receptors in the host.
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Streptococcus antigens have a similar structure to the host tissue, such as the M protein, polysaccharide, hyaluronic acid, and protoplasmic membrane. So the antibodies cross-react with cardiac tissue, joints, skin and other tissues, leading to a multisystem inflammatory reaction [9]. Antibodies also up-regulate the expression of VCAM-1 resulting in a chronic inflammation and CD4+ and CD8+ T cells infiltration in the valves, which is a vascular structure [6, 32]. A cross reaction makes T cell-mediated attack on the heart valves, followed by the rise of intracellular protein to invade immune cells. T helper-1 immune response will continue to initiate the granulomatous response in the heart valve, causing additional inflammation and valve scarring, defined as the Aschoff nodule. The Aschoff nodule is considered a pathognomonic sign of ARF, consisting of macrophages and T cells beneath the endocardium [32-34].

We have already shown that VCAM-1 expression on CECs corresponds to the severity of valve regurgitation in RHD patients treated less than 2 years. All patients had regurgitation valve lesion varying from mild to severe. No one had valve stenosis. We rarely found pure cases of ARF. We suggest this phenomenon due to the initial incidence of ARF manifested as mild, subclinical or undiagnosed. Therefore, the patient did not get prophylaxis to prevent recurrence, especially in conditions of low socio-economic states. Recurrence causes further damage to the heart, resulting in symptomatic RHD with the involvement of heart valves and CHF, identified as severe occurrence of the first ARF [2, 29].

During inflammatory process in RHD, integrin, another adhesion molecule such as VCAM-1, ICAM-1, E-selectin and chemokine, is involved in the recruitment of leukocytes into the tissues [6]. The role of adhesion molecules on RHD has been widely revealed in several previous studies [1, 6, 7, 15, 16]. The VCAM-1 is up-regulated by anti-streptococcal antibodies and leads to the extravasations of activated CD4 and CD8 lymphocytes into the valve tissue [6]. A number of adhesion molecules are expressed mainly on endothelial cells and implicated in the pathogenesis of HF [18]. The expression of adhesion molecules is associated with a variety of cardiovascular diseases and plays a role in both

Figure 2. Bar chart shows the average expression of CECs and VCAM-1 on CECs among mild, moderate, and severe valve lesions; the CECs expression was not significantly different among mild, moderate and severe lesions (P = 0.228)

Figure 3. Bar chart shows that CECs expression was higher RHD patients with heart failure (HF) compared to non-HF.
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devlopment and progression of HF [20]. Previous study reported that sVCAM-1 may has the potential role for risk assessment in patients end stage HF [35]. Heart failure patients with a worse functional stage have higher levels of VCAM-1 [19].

The adhesive molecules allow leucocytes to adhere and start a cascade of inflammation in endothelial cells and cause endothelial cells damage so that the endothelial cells detach into the circulation as CECs [22, 24], resulting in an increase in the number of CECs in the blood [23]. CECs are a marker of vascular damage and has been reported to increase in a variety of cardiovascular disease [35-37]. Some studies indicated a strong correlation between CECs and several plasma markers of endothelial injury, suggesting some degree of disturbance of the endothelium, dysfunction or activation and also indicating adverse cardiovascular outcome [13, 21, 23, 38]. Kassem et al [13] found a strong positive correlation between CEC count and disease activity; VCAM-1 expression has significantly increased with disease activity and correlate positively with CEC count. Increase in CECs has been described in a wide spectrum of cardiovascular diseases, such as acute myocardial infarction, unstable angina and CHF, in which severe endothelial alterations were implicated [15, 35-37]. HF is a disorder associated with activation of the immune system and inflammation through endothelial injury [39]. Chong et al [36] measured the increase of CECs in acute HF. The study found that CECs were significantly increased in both acute and chronic HF compared to healthy controls. The level of CECs increased approximately three times than normal in HF patients which can be concluded that CECs may be used as a novel measurement of endothelial damage in heart failure. Martinez-Sales et al [37] evaluated the evolution of CECs at different stages of HF patients and found that CECs were associated with acute phase of HF and could be used as a marker of the worsening in HF.

In the present study, we found there was a significant difference between CECs expression in RHD patients with and without HF. Interestingly, although the clinical symptoms of HF were not acute anymore, the CECs still presented correlation with HF. Unfortunately, this study did not compare the CECs expression before and after treatment; therefore, it is ascertained whether CECs was used as a predictor for worsening or progression of HF. However, the results support inflammation and endothelial damage theory in heart failure and it gives an allegation that CECs could be used as a new biomarker for vascular endothelial damage in RHD. This finding gives new information that CECs also plays a role in RHD. CECs may be recommended to be measured in RHD with HF.

We found sVCAM-1 and VCAM-1 on CECs were higher in patients than those in control group. Our findings support the previous report regarding the role of VCAM-1 in RHD [1, 6, 7, 15, 16]. Hafez et al [7] found serum level of ICAM-1, VCAM-1 and E-selectin were higher in RHD patients than in healthy controls and could predict residual valve lesions in RHD patients. Chen et al [15] found that levels of nuclear factor-kappa (NF-κB), ICAM-1 and VCAM-1 were higher in RHD patients in comparison to control subjects. Zhang et al [16] measured the levels of ICAM-1, VCAM-1 and von Willebrand factor (vWF) in patients suspected RHD and found that VCAM-1 played a role in pathogenic mechanisms of heart valve damage in RHD. Moreover, Altener et al [1] found that level of VCAM-1 was higher in rheumatic valve disease compared to healthy controls.

Since sVCAM-1 has many potential sources of origin and is produced by a variety of cell types, including leucocytes, endothelium, epithelium and smooth muscle cells, the increase in plasma levels of sVCAM-1 may not reliably reflect the endothelial injury [11, 25]. The theory supports our finding in which although the sVCAM-1 is higher in patient group than that in control group, sVCAM-1 was not associated with valve regurgitation. Otherwise, VCAM-1 on CECs had correlation with valve regurgitation severity. Moreover, we considered any influence from the valve lesion measurement methods. The methods could not determine the anatomic valve lesion, because we did not measure regurgitant volume (Rvol), regurgitant fraction (RF) and the effective regurgitant orifice area (EROA) [8];only the functional valve lesion was measured.

Previous study reported that sVCAM-1 may have the potential role in HF. Chiang et al [17] found VCAM-1 for risk assessment in patients with end stage HF. HF patients with a worse functional stage had higher levels of VCAM-1 [19]. We found a different result that VCAM-1 had no correlation with HF. We suggest the different result is due to the HF symptoms that were no longer marked when this study was conducted, since all the patients already treated with medicine.

The present work is a preliminary study that used observational cross-sectional design. Some samples were taken from outpatients to reflect the results obtained had less acute conditions RHD. Although the inflammatory process in RHD is a chronic process, further study is needed in the form of a cohort study to compare the conditions at the start of illness and after receiving treatment for a specific time period. Additional studies are needed to compare the increase in the expression of CECs, the expression of VCAM-1 on CECs and sVCAM-1 which induced inflammatory process by other disease. A second limitation was in obtaining the data accurate to classification of the severity of the valve. Data obtained from the documentation of echocardiography retrograde past 6 months, in which the measurement and classification of the severity of the valve lesion was performed using a qualitative method using color Doppler flow mapping. Doppler flow mapping is widely used to assess the mitral regurgitation, but it can be profoundly affected by instrument setting and hemodynamic variable or,
in other words, depends on the operator and/or the machine. However, color Doppler flow mapping does offer some potential ways to assess the severity of regurgitation [8, 40].

Recent studies are no longer using qualitative measurements but quantitative examination by measuring the volume of regurgitation, regurgitate fraction and effective regurgitate orifice area [40]. Using the qualitative method makes it difficult to determine whether changes in the expression of VCAM-1 on the valve due to the anatomical heart valve damage or inflammation caused by RF, and also difficult to rule out the influence of hemodynamic conditions that exist in patients, especially in patients with HF, hypertension, etc.

From the results of the present study it could be assumed that the valve regurgitation is associated with anatomical abnormalities of the valve while the CECs better reflect systemic conditions, because VCAM-1 was only significantly associated with mitral valve while CECs only associated with HF. However, this study did not analyze the changes in the expression of CECs in conditions other than HF patients with RHD or without mitral valve regurgitation, and this has become our third limitation because all subjects with HF also have a valve disorder; so it cannot be certain that the changes occur purely because of HF and are not influenced by valve regurgitation. The limitation is that most patients coming to Dr. Saiful Anwar General Hospital had valve regurgitation. Also, the early symptoms of ARF are not so typical that patients might not feel them, resulting in their already chronic medical condition when coming to hospital. In further studies, if possible, quantitative method should be used in assessing the severity of valve regurgitation.

In conclusion, the present work demonstrated that VCAM-1 on CECs is associated with the severity of regurgitation of valve lesion in RHD and supports the theory of inflammation and endothelial damage in RHD. The CECs were higher in RHD and correlated with HF, providing a new biomarker candidate of inflammation that can predict the presence of the severity of valvular regurgitation and worsening HF in RHD.

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