Original Article

Persistence of endothelial cell damage late after Kawasaki disease in patients without coronary artery complications

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Abstract

Background: Recent studies proposed an increased risk of atherosclerosis in patients with a history of Kawasaki disease. This study aimed to investigate the persistence of vascular injury after an acute phase of the Kawasaki disease.

Materials and Methods: We determined the number of circulating endothelial cells (CEC) in the peripheral blood of 13 patients with a history of Kawasaki disease within four to ten years, in comparison with 13 healthy relative controls. The CECs were counted as CD146+/CD34 + cells by the standard flow cytometry technique, and the independent t-test was employed to compare the mean number of CECs in the two groups.

Results: The mean number of CECs was significantly higher in patients than in controls (12 ± 3.03 vs. 2.38 ± 0.87, respectively, $P < 0.001$).

Conclusion: This study elucidates the persistence of vascular injury late after Kawasaki disease. This finding suggests that prolonged administration of vascular anti-inflammatory agents might be beneficial for preventing atherosclerosis in the subsequent years, in these patients.

Key Words: Atherosclerosis, circulating endothelial cells, endothelial dysfunction, Kawasaki disease, vasculitis

INTRODUCTION

Kawasaki disease (KD) is an acute, self-limited vasculitis in the pediatric age group.[1] There is now accumulating evidence on the adverse endothelial

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function characterized by the decrease in elasticity of the brachial and carotid arteries and increased intima media thickness (IMT) of the carotid artery, late after Kawasaki disease, particularly in patients with coronary artery complications.[2-10] Whether it is the sequela of an acute vascular injury or the result of persistent vascular damage is unclear. Some studies indicate persistently elevated serum markers of inflammation, such as high-sensitivity C-reactive protein (hs-CRP), in coronary complicated KD patients, late after the involvement, which suggests chronic low-grade vascular inflammation in these patients.[8,11,12] Studies on uncomplicated KD patients sometimes show no significant increase in

these markers.[4] Endothelial cells (ECs) consist of a huge population of cells that play a crucial role in preserving vascular homeostasis. Certain conditions with vascular injury can induce EC detachment from the vascular walls into the circulation.[13] In these conditions, the number of circulating ECs (CECs) in the peripheral blood can directly reflect the severity of the endothelial damage.[14]

Immune system activation during the acute phase of KD can cause severe EC damage, resulting in an increased number of CECs in the bloodstream.[13]

To the best of our knowledge, no previous study has reported a number of CECs in uncomplicated KD patients late after the acute phase of the illness.

With regard to the preliminary evidence of progressive arterial dysfunction in KD patients,[3] the link between the level of CECs and the existence and severity of the endothelial damage,[14] and the lack of evidence about chronic vascular damage following coronary uncomplicated KD, we hypothesized that the persistent increase in the CEC level may be the cause of progressive systemic arterial dysfunction in patients, late after the acute phase of KD. In this case, controlling EC damage in KD may play a crucial role in preventing the generation and progression of vascular lesions and consequently late atherosclerosis.

To test this hypothesis, we compared the level of CECs in patients with a history of uncomplicated KD in the previous four to ten years with that of the healthy controls.

MATERIALS AND METHODS

Patients and Design

Patients with a history of KD in the previous four to ten years were recruited from the Alzahra Hospital, Isfahan, Iran, which is the only tertiary care referral hospital for the pediatric age group, affiliated to the Isfahan University of Medical Sciences. Patients with complete KD established by the Japanese Kawasaki Disease Research Committee[1] were included in the study. The control group was selected from children of the first and second relatives of KD patients, such as, her/his siblings or cousins. Those patients and control subjects with any underlying disease, such as, connective tissue, cardiovascular, metabolic, hepatic, hematological, renal or endocrine disorders were excluded. Subjects on drugs with any influence on the endothelial function, such as, calcineurin esterase inhibitors, were also excluded from the study. Moreover, patients with any viral

or bacterial infection within one week to the time of the study were excluded from the study. Such disorders were assessed with the help of history, physical examination, measurement of blood pressure, as well as laboratory tests, including fasting blood sugar, alanine transaminase, complete blood count, creatinine, and thyroid stimulating hormone level with standard commercial kits. Furthermore, the following information was extracted from their hospital records: Age, sex, symptoms at presentation, administration of intravenous immunoglobulin during the acute phase, coronary artery involvement during hospitalization, and duration from the onset of disease to the time of the study. The parents of all participants had endorsed a written informed consent form and the Institutional Ethics Committee of the university had approved the study.

Circulating endothelial cell count

Seven milliliters of fresh blood was drawn from the cubital vein of KD patients and healthy controls. The first 4 ml were discarded. The CECs were counted immediately by the standard flow cytometry technique, as has been previously described.[15]

Briefly, 2 ml of Ethylenediaminetetraacetic acid (EDTA)-treated blood was put in each 15 ml Falcon tube and red blood cells (RBCs) were lysed using 10 ml of FACS lysing solution (Becton Dickinson, 10x, diluted 1:10) according to the manufacturer's instructions. The cells were washed with cell buffer solution [PBS + Bovine serum albumin (BSA) 1%+ sodium azaide 0.05%] and centrifuged at 500 × g to replete the cells. The white cells were then incubated with10 μL Fc receptor block (*Miltenyi* Biotec, California, USA) for 20 minutes at room temperature. Next, the samples were incubated with fluorochrome-labeled monoclonal anti-human mouse antibodies, namely FITC-CD45, PE-CD146, and PE-Cy5-CD34 (Becton Dickinson, Oxford, UK) for 30 minutes in a dark place, at room temperature. The cells were washed, repleted, and made up to a final volume of 1 ml, with cell buffer solution and analyzed immediately. Each sample was analyzed on a FACSCalibur flow cytometer (BD), using the CellQuest™software (BD). The cells were plotted according to the forward scatter and side scatter profiles (a measure of size and granularity of an event, respectively) and gated to include only mononuclear cell events, excluding cell doublets, platelets, dead cells/debris, microparticles, and high-side scatter events. A second gate was used to include only those cells that were negative for CD45 (FITC) and were with low-to-medium side scatter singlets. A third gate was used to analyze the cells doubly positive for CD146 (PE) and CD34 (PE-Cy5) expression and

only high intensity doubly fluorescent cells were defined as CECs [Figure 1]. The sample was analyzed for a minimum of 105 mononuclear cellular events. Flourochrome-matched isotype controls (FITC-IgG1, PE-IgG1, PE-Cy5-IgG1, Becton Dickinson) as well as non-stained samples were used to set the appropriate gate parameter and served as negative controls.[15]

Statistical analysis

Results of the CECs, ALT, white blood cells, hemoglobin, platelets, creatinine, and thyroid stimulating hormone (TSH) were presented as mean ± SD. The independent *t*-test was employed to compare the mean number of CECs in two groups.

With regard to the average of CECs in the patient group, it was significantly higher than that in the controls. We used the Receiver Operating Characteristic (ROC) curve and area under the curve for processing and analyzing our data.

Statistical analysis was performed with the SPSS version 16 (SPSS Inc, Chicago, IL, USA). A *P* value of less than 0.05 was considered as significant.

RESULTS

The study comprised of 26 participants (13 patients and an equal number of controls). The case group consisted of 13 patients with a history of KD, 4.8-9.6 (median 6.6) years at the time of the study; all of them had received 2 g/kg of intravenous immunoglobulin and 80-100mg/kg/d of aspirin for three to five days during the acute phase of the illness, and 3-5 mg/kg/d of aspirin for six to eight weeks thereafter. None of the cases had coronary artery involvement at any time. The control group comprised of 13 age- and sex-matched healthy children, who were siblings or cousins of the KD patients. The demographic characteristics of the subjects are shown in Table 1. There wa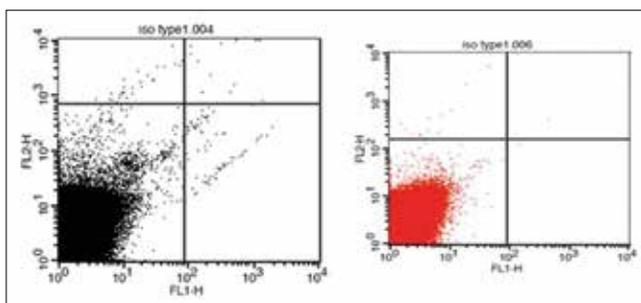s no significant difference in the studied variables between the two groups. Other than the CEC number, the mean value of other laboratory tests was in the normal range in all participants, without any significant difference between the two groups [Table 1].

The mean number of CECs in patients and control group was significantly different (12 ± 3.03 vs. 2.38 ± 0.87, respectively, *P* < 0.001).

In the ROC curve [Figure 2], the area under the curve (AUC) and the cut-off point obtained were 100% and 6.5, respectively. These results showed 100% CEC sensitivity and specificity for endothelial cell dysfunction, late after KD.

DISCUSSION

The present study demonstrated a higher number of CECs, late after an acute phase of uncomplicated KD, in the case group than in the control group. It has been shown previously that vascular elasticity

Table 1: Demographic characteristics and laboratory data of Kawasaki disease patients and controls

Demographic characteristics	Patients (*n*=13)	Controls (*n*=13)	*P* value
Age at study (years) mean±SD	11.4±3.3	12.7±4.8	0.42
Male gender number (percent)	5 (38.5%)	6 (46.2%)	0.7
Laboratory data mean±SD			
ALT	19.7±8.5	20.7±7	0.7
WBC	5800±1500	5900±1300	0.78
Hb	12.7±1.2	12.8±0.9	0.88
Plt	261000±66000	240000±47000	0.38
Cr	0.7±0.1	0.73±0.1	0.57
TSH	3.2±1.5	3.2±2	0.85

TSH: Thyroid stimulating hormone, SD: Standard diviation

Figure 1: Flow cytometric analysis for circulating endothelial cells. Blood cells were plotted according to forward scatter and side scatter profiles and gated to include only mononuclear cells. A second gate was used to exclude CD45-positive cells. Cells doubly positive for a high intensity of CD146 (PE) and CD34 (PE-Cy5) expression (highlighted for clarity) were counted as CECs
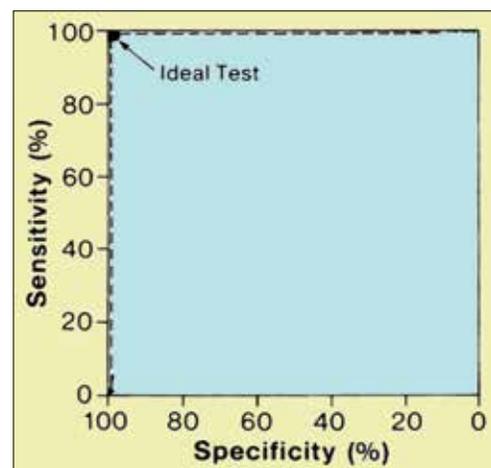

Figure 2: ROC curve of the circulating endothelial cells in Kawasaki disease and normal control subjects showing sensitivity and specificity of 100 percent for increased circulating cells in Kawasaki disease patients

documented as a decreased pulse wave velocity and flow mediated dilatation, which are good biomarkers for the risk of atherosclerosis, are impaired in patients with a history of KD.[3-9] Furthermore, increased intima media thickness of the carotid artery, which reliably indicates the presence of atherosclerosis, has been shown in patients with a history of coronary complicated KD in many studies.[3,5,10] One of the most convincing questions in the prognosis of KD patients is that if the progression to atherosclerosis can be prevented by vascular anti-inflammatory medications. To respond to this question, we need to understand whether the process of vascular inflammation is restricted to the acute and sub-acute phases of KD or will persist lifelong. Some researches indicate that vascular inflammation manifests as elevated levels of high sensitive C- reactive protein (hs-CRP) and persists for two many years after an acute phase of KD in coronary complicated cases.[8,11,12] Although others revealed no evidence of vascular inflammation years after uncomplicated KD.[4] To the best of our knowledge, the present study is the first one that has investigated the persistence of vascular cell damage by the number of CECs, several years after subsiding, in the acute phase of the disease. According to the fact that the number of the CEC count is a good marker for the existence and severity of endothelial cell damage,[14] the present study reveals that a low-grade endothelial cell injury can last for several years after the subsiding of KD, even in patients without a coronary adverse outcome. This finding proposes that the process of atherosclerosis may be prevented by prolonged anti-inflammatory medications in these patients.

All patients who participated in the study had received an adequate amount of IVIG and Aspirin. The elevated number of CECs several years after KD showed that the standard anti-inflammatory medications used for KD, with confirmed influence upon reducing coronary aneurysms,[1] were not effective in controlling prolonged vascular inflammation and subsequent atherosclerosis in these patients.

None of our patients had aneurismal coronary disorder during the early phases of KD. Therefore, the present study cannot compare the magnitude of endothelial cell damage late after KD in those patients who had aneurismal coronary complications and those who had not suffered from that. In a previous study, there was a significant increase in the number of CECs in the early phases of KD in those with aneurismal coronary sequels compared to individuals without the complication.[13]

In the current study, the mean number of CECs in the control group was 2.38 ± 0.87, which was comparable to the reported counts in other studies.[13] Therefore, the baseline number of these cells in the studied population is similar to other populations. It seems the demographic parameters such as race, age, and sex had no effect on the amount of baseline CECs in the circulation.

The average number of CECs in KD patients was 12 ± 3.03, which was close to the mean number of these cells in the convalescent phase of KD, in patients without coronary artery complication in another study (7.3+/- 0.9). In that study, the mean number of CECs in the convalescent phase of KD in patients with coronary artery aneurysm was 15.7+/- 4.9.[13] Hence, there is a possibility that the number of CECs late after KD is higher in patients with coronary complications than in those without it.

Study limitations and strengths: The sample size of our study was small, and we could recruit few KD patients; however, the considerable difference documented between the minimum number of CECs in the subjects and maximum number in the controls justifies that the sample size has been appropriate. Repeating the study in other populations and with more participants would confirm the important finding of the research. The main strengths of this study are its novelty and recall of KD patients after a long period of time. The other strength is considering relatives of patients as the control group, to minimize the genetic and sociodemographic differences between the two groups studied.

CONCLUSION

The present study shows that the number of CECs, which is a sensitive marker of vascular injury, is apparently higher in KD patients who received standard treatment and has no coronary complications. This finding suggests that prolonged administration of vascular anti-inflammatory agents may be effective for preventing atherosclerosis in the subsequent years, in these patients.

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