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Noninvasive Determination of Endothelial Cell Function in the Microcirculation in Kawasaki Syndrome

Gregory H. Kurio,

Department of Pediatrics, University of California San Diego School of Medicine, La Jolla, CA 92093, USA, Rady Children's Hospital San Diego, San Diego, CA 92123, USA

Katrine A. Zhiroff,

Department of Pediatrics, University of California San Diego School of Medicine, La Jolla, CA 92093, USA

Lily J. Jih,

Department of Pediatrics, University of California San Diego School of Medicine, La Jolla, CA 92093, USA

Arnost S. Fronek, and

Department of Surgery, University of California San Diego School of Medicine, La Jolla, CA 92093, USA

Jane C. Burns

Department of Pediatrics, University of California San Diego School of Medicine, La Jolla, CA 92093, USA, Rady Children's Hospital San Diego, San Diego, CA 92123, USA

Jane C. Burns: jcburns@ucsd.edu

Abstract

The aim of the study was to noninvasively assess endothelial cell (EC) function in the microcirculation using laser Doppler fluximetry (LDF) in acute and convalescent Kawasaki syndrome (KS) patients and healthy controls. KS is an acute, self-limited vasculitis of childhood that affects the EC of medium-sized arteries. No studies have addressed EC function in the peripheral microcirculation. LDF preacetylcholine and postacetylcholine (ACh) iontophoresis estimates microcirculation EC nitric oxide production leading to smooth muscle relaxation and vasodilatation, which are blunted in EC dysfunction. We studied a total of 97 subjects: 36 acute and 27 convalescent KS patients and 34 normal children. Change in blood flow was measured by LDF for 10 min post-ACh iontophoresis. Acute KS patients had significantly lower average flux when compared to convalescent KS patients and controls in the first 5 min postiontophoresis. However, there was no difference in flux or area under the curve (AUC) between convalescent KS patients and healthy controls. Despite a reduced response of the microvascular EC to ACh in acute KS patients, convalescent patients with and without coronary aneurysms had microvascular EC function similar to normal controls. This suggests that the EC injury in KS is confined to the endothelium of medium-sized arteries and that microvascular EC function is normal after acute KS.

Keywords

Coronary artery aneurysm; Vasculitis; Pediatric heart disease

Correspondence to: Jane C. Burns, jcburns@ucsd.edu.

Present Address: G. H. Kurio, Division of Cardiology Children's Hospital and Research Center at Oakland, 747 52nd Street, Oakland, CA 94609, USA

Despite 40 years of research on Kawasaki syndrome (KS), the extent and nature of endothelial damage are still uncertain for those who have recovered from what is now the most common cause of acquired coronary artery disease in the pediatric population in the United States and Japan. Whereas 15–20% of untreated children will develop coronary artery aneurysms or ectasia as a complication of the vasculitis, current therapy with intravenous immune globulin (IVIG) and high-dose aspirin (ASA) has reduced this figure to approximately 3–5% [1]. However, autopsy studies from Japan have suggested that accelerated atherosclerotic changes might be present even in those children without coronary artery abnormalities detected by echocardiography during the acute disease [2]. Increased arterial stiffness of both central and peripheral medium-sized arteries has recently been documented by direct measurement during cardiac catheterization in patients with both regressed and persistent coronary artery abnormalities [3].

Endothelial cell (EC) function is impaired by systemic inflammation [4] and has been studied in KS patients by several methods. Intracoronary injection of acetylcholine (ACh) in children with both persistent and regressed coronary artery aneurysms has demonstrated EC dysfunction with paradoxical vasoconstriction [5–7]. However, EC function after recovery from KS in children without coronary artery abnormalities in the acute stage is less clear. Studies of brachial artery flow-mediated dilation in young adults who have recovered from KS have yielded conflicting results [8–10]. More recently, EC function as measured by carotid artery flow-mediated dilatation was abnormal in KS patients regardless of the presence or absence of coronary artery abnormalities during the acute phase [11]. Whereas these studies have focused on EC function in medium-sized muscular arteries, no studies have addressed the function of the EC in the microvasculature.

Laser Doppler fluximetry (LDF) is a simple, noninvasive method that measures red blood cell (RBC) flux ($[\text{RBC volume fraction}] \times [\text{RBC velocity}]$) in the microvasculature. The incident laser angles to the RBC motion as well as RBC velocity are averaged, thus obviating the need to consider the structure and angulations of the microvascular tree [12]. The result of these assumptions is an average flux in the area of interest that can be measured before and after iontophoresis of ACh. This technique has been used in the past to study adults with conditions known to affect the microcirculation such as diabetes. To study the ability of the endothelium to release nitric oxide, LDF is coupled with ACh iontophoresis to produce vascular smooth muscle relaxation in response to nitric oxide. The resultant vasodilation can be measured as a change in flux. Limited experience in older pediatric patients suggested that the technique might be adaptable for the study of infants and young children. We used LDF before and after ACh iontophoresis to study EC function in the microvasculature of healthy children and in children during the acute and convalescent stages of KS.

Materials and Methods

Patients

Three populations of subjects were studied: acute and convalescent KS patients and healthy pediatric controls. Acute KS patients were studied during their admission to the hospital for treatment with IVIG and high-dose ASA. Acute patients were studied when their rectal or oral temperature was less than 100.4°F so that elevated body temperature would not complicate the interpretation of the study. Patients older than 3 years of age who were able to cooperate with the study were evaluated without sedation. The remaining acute KS patients were studied by LDF after receiving chloral hydrate sedation for echocardiograms that were performed either during or shortly after IVIG infusion. When possible, studies were performed before beginning IVIG, but no therapy was delayed for the purposes of this study. Convalescent KS patients were recruited from the KS Clinic at Rady Children's Hospital San Diego. Siblings of KS

patients who had never had symptoms of KS were enrolled as healthy controls. Additional healthy control subjects were infants and children undergoing outpatient echocardiograms either with or without chloral hydrate sedation. Patients whose diagnoses included abnormalities of the coronary arteries or complex congenital heart disease were excluded. Subjects were also excluded if they had had a fever in the 24 h prior to study or were on vasoactive medication other than ASA. Participants were asked to refrain from caffeine-containing foods (e.g., chocolate, coffee, tea, or cola) on the day of the test. This study was reviewed and approved by the investigational review boards of the University of California, San Diego, and Rady Children's Hospital San Diego. Informed consent was obtained in writing from the parents of all subjects.

The KS patients enrolled in the study had fever for at least 3 days and met at least four of five standard criteria for KS or had three criteria plus echocardiographic evidence of dilated right or left anterior descending coronary arteries (z-score >2.5) [13]. The internal diameter of the coronary arteries assessed by echocardiogram was classified as normal (z-score <2.5), dilated (z-score ≥ 2.5 but <4), or ectatic or aneurysmal (z-score ≥ 4) [13]. z-Scores were normalized based on body surface area [14].

LDF Procedure

The flexor surface of one forearm was cleaned with iso-propyl alcohol. The forearm length was measured and a point 20% of the total length from the antecubital fossa was marked for the positive electrode. The negative electrode was placed 10 cm distally or just proximally to the wrist flexion point in patients with forearms less than 12 cm. An LDF probe (#408; Perimed, Stockholm, Sweden) was affixed to the positive electrode point and flux readings were obtained using a PF-5010 unit (Perimed, Stockholm, Sweden) connected to a continuous-strip recorder. Although studies in adults utilize an occlusion reading taken by rapidly inflating a pneumatic cuff to suprasystolic pressures proximal to the probe to establish a baseline, there was concern that children under chloral hydrate sedation would be awakened by such a procedure, thus jeopardizing the concurrent acquisition of quality echocardiographic data. Therefore, a flux reading was determined prior to iontophoresis of ACh and used as a baseline.

For the iontophoresis of ACh, single-layer gauze pads were secured to both the positive and negative electrode points using paper tape. The positive electrode was saturated with 1 ml of 1% ACh solution and the negative electrode was saturated with 1 ml of normal saline. An Iontophor II 6111 PM/DX iontophoresis unit (Life-Tech, Inc., Stafford, TX) was used to deliver 0.2 mA for 10 min or a maximum total dose of 2 mC.

The gauze pads were then removed, the areas were dried with clean gauze, and the LDF probe was once again applied to the positive-electrode point. Flux was measured over 10 min and the resulting curve was recorded on graph paper.

Statistical Analysis

The primary outcome measures were peak flux and area under the curve (AUC) at 5 and 10 min in the three patient groups. Peak flux and AUC at 5 min and 10 min were measured as a distance in millimeters from the baseline that was obtained prior to ACh iontophoresis. Attenuation of flux at 5 and 10 min was defined as the difference between peak flux and the flux measured at the specified time point. Data were analyzed using analysis of covariance (ANCOVA) with age as a covariate. Repeated measures analysis (RMA) was conducted to compare the three groups at 10 time points (minute 1–10) to determine if the profile of the acute KS group differed from that of the convalescent KS and control groups. Time was treated as the within-subject factor and the group as the between-subject factor. In the assessment of

test reproducibility, the coefficient of variability was calculated for 18 repeated measures on 8 subjects.

Results

To define the reliability and reproducibility of the LDF procedure, we studied eight normal subjects on each arm on two occasions separated by at least 36 h. The coefficient of variation (CV) for the measurement of peak flux was 18.2% and the CV for AUC over 10 min was 15.7% (Table 1). Attenuation over both a 5- and 10-min interval was highly variable (Table 1) and thus poorly reproducible. Therefore, this variable was not utilized in subsequent analyses. Studies showed that using either the subject's right or left arm or varying the time of day did not influence flux readings (data not shown). The irritant effect of iontophoresis and its contribution to the hyperemia was investigated by performing serial LDF studies following iontophoresis of normal saline and then ACh on the same subject. The results were compared using RMA. Iontophoresis with normal saline consistently produced hyperemia that was approximately 20% of that produced by iontophoresis with ACh, which agrees with previously published data [15,16].

A total of 97 subjects were enrolled in the study: 36 acute KS (Illness days 4–14, 4 patients were studied before receiving IVIG), 27 convalescent KS (36 days to 11 years postacute disease; median: 30 months), and 34 normal controls (Table 2). Age ranges and percent of subjects sedated with chloral hydrate were similar across all three groups. All acute KS patients were afebrile at the time of the study. One unusual acute KS patient was an 18-year-old Vietnamese female studied following IVIG administration on illness day 14. This patient had five clinical criteria for KS but was diagnosed late due to her age. Echocardiography demonstrated coronary artery aneurysms.

The responses to ACh iontophoresis were compared among the two patient groups and the normal controls. No differences were noted between LDF profiles of patients who received sedation versus those who did not. Acute KS patients had significantly decreased AUC in the first 5 min after ACh administration compared to convalescent KS patients and healthy controls (Table 3 and Fig. 1). Results were similar using either RMA ($p = 0.04$) or ANCOVA using age as a cofactor ($p = 0.04$). There was a trend suggesting decreased peak flux (Fig. 1) as well as increased flux attenuation in the 10-min analysis (data not shown) in the acute KS patients compared to the other groups. However, these differences were not statistically significant ($p = 0.33$ and 0.07 , respectively). Of the four subjects studied before IVIG administration, there was no significant difference in average flux readings when compared to acute KS patients studied during or immediately following IVIG administration (data not shown).

No difference was seen in measures of flux between convalescent KS patients and normal controls. Of the 27 convalescent KS patients, 6 (22.2%) had persistent coronary artery aneurysms. LDF studies of these children did not differ from the studies of either convalescent KS children with normal coronary arteries ($n = 13$) or children with transient dilatation that had resolved by the time of their LDF study ($n = 8$).

Discussion

We explored the use of LDF to study EC function in the microvasculature in children during and following acute KS. Children during the acute febrile phase of the illness demonstrated significantly less vasodilatation in response to ACh iontophoresis than either healthy pediatric controls or otherwise healthy convalescent KS children. In contrast, the hyperemic response to ACh iontophoresis was similar in healthy children and children in the convalescent phase of KS, regardless of coronary artery status. There were no adverse effects from the ACh or any

part of the LDF protocol. Thus, LDF in this population of pediatric subjects was a well-tolerated, reliable, and reproducible method of noninvasively studying microvasculature EC function. LDF can be a valuable tool in the assessment of microcirculation EC function in infants and children with other disease states that affect this vascular bed, including diabetes and hyperlipidemia.

This study addresses for the first time the EC function in the microvasculature of KS patients. EC dysfunction in medium-size muscular arteries in children after recovery from KS has been documented invasively by angiography with ACh infusion and arterial input impedance [3] as well as noninvasively by measurement of brachial artery flow-mediated dilation [5–7,10]. Studies have demonstrated elevated levels of von Willebrand factor, selectins (both E and P), and cellular adhesion molecules (ICAM-1, VCAM-1) as potential biomarkers of EC activation or damage during acute KS [17]. However, direct evaluation of EC function in the microvascular circulation of KS has not been previously reported. We sought to address the question of how global the EC dysfunction after KS might be. Serum biomarkers of EC dysfunction were not incorporated into our study because these biomarkers cannot discriminate between EC dysfunction in the microcirculation versus larger vessels. Our data suggest that EC function in the microcirculation is transiently impaired in the acute phase of KS but normalizes in the convalescent phase. This transient EC dysfunction might be the result of anti-EC antibodies [18–20] or the plethora of pro-inflammatory cytokines that circulate during acute KS. Whatever the cause, the effect appears to be self-limited and to resolve following the acute illness. These findings suggest that permanent endothelial cell injury or dysfunction following KS is confined to the EC of medium-sized or larger arteries and does not occur in the microcirculation.

Limitations of our study include the small number of subjects studied and the inability to study all acute subjects before IVIG infusion. With our small sample of subjects studied prior to IVIG infusion, we cannot assess the role, if any, of this therapy in the reversal of the transient EC dysfunction in the microcirculation during the acute illness. In addition, LDF studies of febrile children without KS were not possible within the constraints of the Institutional Review Board, as this would have necessitated sedation for research purposes only or delayed patient flow through the acute care setting. Without such a control group, we cannot conclude whether the EC dysfunction in acute KS patients resulted from circulating mediators of inflammation that might be seen in any child with fever or whether this EC dysfunction is more specific to patients with acute KS.

In summary, ACh-induced vasodilatation of the microcirculation monitored by LDF was a reliable and reproducible technique that was well tolerated in infants and children. Transient EC dysfunction of the microvasculature was documented during the acute phase of KS, but microvascular EC function was similar to controls after recovery from KS. These findings support the concept that EC damage in KS is confined to medium-size or larger arteries and does not involve the microcirculation.

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Fig. 1.

Plot of mean flux over 10 min as measured by LDF following ACh iontophoresis in three study populations. Flux recordings were made at 1-min intervals and an average value for each population was plotted for each time point. The AUC between 1 and 5 min was significantly reduced in acute KS patients compared to both healthy controls and convalescent KS children ($p = 0.04$)

Table 1

Reproducibility of laser Doppler fluximetry with Ach iontophoresis in eight normal control subjects each studied on two separate occasions

Hyperemia post-ACh iontophoresis	Coefficient of variability (%) (mean $\pm$ SD)
Peak response	18.2 $\pm$ 8.2
Area under curve over 10 min	15.8 $\pm$ 11.1
Attenuation over 5 min	34.3 $\pm$ 24.9
Attenuation over 10 min	38.0 $\pm$ 39.3

Table 2

Demographic characteristics of study populations

	Acute KS (<i>n</i> = 36)	Convalescent KS (<i>n</i> = 27)	Controls (<i>n</i> = 34)
Median age, years (range)	2.25 (0.25–18.7)	4.26 (0.33–16)	6.74 (0.33–19.2)
% Male	53	85	38
Race or ethnicity: <i>n</i> (%)			
Asian	9 (25)	5 (18)	10 (29)
African American	3 (8)	5 (18)	1 (3)
Caucasian non-Hispanic	11 (30.5)	8 (30)	16 (47)
Caucasian Hispanic	11 (30.5)	7 (26)	7 (21)
Mixed ancestry	1 (3)	1 (4)	0
Hawaiian/Pacific Islander	1 (3)	1 (4)	0
CA status: <i>n</i> , (%)			
Normal	20 (55.5)	13 (48)	NA
Aneurysm/ectasia	5 (14)	6 (22)	
Dilated	11 (30.5)	8 (30)	

Note: Status based on echocardiographic measurement of internal diameter z scores normalized for body surface area for the LAD and RCA: normal ($z < 2.5$), dilated ($z \geq 2.5$ and < 4), aneurysm/ectasia ($z \geq 4$)

CA = coronary artery; NA = not applicable

Table 3

Response to ACh iontophoresis in KS subjects and controls as assessed by LDF

	Acute KS (mean $\pm$ SD)	Convalescent KS (mean $\pm$ SD)	Controls (mean $\pm$ SD)
Peak flux	3.3 $\pm$ 1.9	4.0 $\pm$ 1.9	3.9 $\pm$ 2.3
AUC 1–5 min	8.8 $\pm$ 6.0*	12.7 $\pm$ 7.0	12.7 $\pm$ 8.6
AUC 1–10 min	17.7 $\pm$ 13.5	25.9 $\pm$ 15.0	25.3 $\pm$ 17.3

*
 $p = 0.04$ compared to convalescent KS and controls

AUC = area under curve; SD = standard deviation