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Full Length Article

Dynamic evaluation of hemostasis in the acute phase of Kawasaki disease using comprehensive coagulation functional assays

Hiroyuki Yoshizawa[a], Keiji Nogami[a,*], Tomoko Matsumoto[b], Nobuyuki Tsujii[a], Toshiyuki Sakai[c], Toshio Takase[d], Ichiro Tanaka[d], Midori Shima[a]

[a] Department of Pediatrics, Nara Medical University, Kashihara, Nara, Japan
[b] Course of Hemophilia Treatment and Pathology, Nara Medical University, Kashihara, Nara, Japan
[c] Pediatrics, Kokuho Central Hospital, Tawaramoto, Nara, Japan
[d] Pediatrics, Yao Municipal Hospital, Yao, Osaka, Japan

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ABSTRACT

Introduction: Kawasaki disease (KD) is a systemic vasculitis involving coronary arteries, sometimes resulting in aneurysms and myocardial infarction. Hyper-coagulability in the acute-phase of KD is indicated in some circumstances based on changes of individual clotting factors. Comprehensive coagulation assays, clot waveform analysis (CWA) and thrombin/plasmin generation assay (T/P-GA), have been developed to assess physiological hemostasis, but these techniques have not been applied in KD.

Methods: We utilized both assays to analyze coagulation function in KD children (n = 42) prior to intravenous-immunoglobulin (IVIG) treatment (Pre), 1-week (1W) and 1-month (1M) post-IVIG.

Results: In CWA, the clot time (CT) pre-treatment was prolonged, and was significantly shortened at 1W and 1M. However, the maximum coagulation velocity (|min1|) and acceleration (|min2|) were ~2-fold greater relative to controls, indicating an overall hypercoagulable tendency. These parameters were related to fibrinogen concentration, and were decreased at 1W and declined to normal at 1M. In T/P-GA, the endogenous potentials of thrombin and plasmin were greater relative to control at each of three time-points, and measurements at 1W were greater than those Pre-treatment. The ratios of TG and PG relative to control were similar, however, suggesting well-balanced dynamic coagulation and fibrinolysis. In non-responders to IVIG, the |min1| and |min2| measurements were greater than those in responders at 1W and 1M, suggesting that non-responders remained hypercoagulable after primary treatment.

Conclusion: The coagulation data observed in KD were consistent with hypercoagulability, although fibrinolytic function appeared to be well-balanced. Comprehensive assays of this nature could provide valuable information on coagulation potential in KD.

1. Introduction

Kawasaki disease (KD), is an acute systemic vasculitis of unknown etiology that occurs mainly in infants and young children, affects the skin, mucous membranes, lymph nodes, and blood vessels, and is typically manifested by fever [1]. This acute vasculitis involves particularly the coronary arteries, and may be complicated with the development of aneurysms that lead to life-threatening coronary thrombosis and myocardial infarction [2,3]. The vasculitis is associated with an increase in inflammatory cells and cytokines induced by unidentified pathogens [4,5]. The disorder is classified, therefore, as immune-mediated, and is generally responsive to intravenous-immunoglobulin (IVIG) and immunosuppressive therapy. IVIG is the first line choice for treatment, with a high rate of responsiveness, although the risk of cardiovascular damage appears to be increased in IVIG-resistant patients [6].

The levels of inflammatory mediators, including tumor necrosis factor alpha, interleukin-6, and interleukin 1β, are elevated in the acute phase of KD (acute-KD) [5,7]. Inflammatory cytokines are the major mediators involved in coagulation activation, and vascular endothelial cell (VEC) damage associated with vasculitis may lead to hyper-coagulability in the main coronary artery [8–10]. Von Willebrand factor (VWF) levels are elevated, likely reflecting the acute-phase reaction [11]. Coagulation-mediated thrombocytopenia or disseminated

* Corresponding author at: Department of Pediatrics, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan.
E-mail address: roc-noga@naramed-u.ac.jp (K. Nogami).

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intravascular coagulation, and the presence of anti-cardiolipin antibodies also have been reported [12,13]. Early studies based on changes of individual factors, including factor (F)VIII, antithrombin, and β-thromboglobulin provided evidence of hyper-coagulability and platelet activation in acute-KD [8–10]. Using conventional measurements of individual plasma clotting factors, however, Lin et al., concluded that hypercoagulability was not a dominant feature of acute-KD [14]. These assays are based on the classical concepts of intrinsic and extrinsic cascade mechanisms, and are widely accepted to reflect coagulation in a non-physiological environment. Hence, laboratory analyses of this type may not fully represent hemostasis *in vivo* [15]. In particular, the commonly utilized routine, activated partial thromboplastin time (aPTT), simply reflects the formation of a small amount of fibrin, and may not sufficiently reflect dynamic changes in blood coagulation. Recent interests have focused, therefore, on so-called 'comprehensive' coagulation assays, developed from a better understanding of clotting mechanisms centered on cell-based models generating thrombin activity on activated platelet membranes and other phospholipid (PL) surfaces [16].

Techniques of this nature, including clot waveform analysis (CWA) and simultaneous thrombin and plasmin generation assays (T/P-GA), have been especially established for the assessment of hemorrhagic and thrombotic diseases [17–22]. The CWA parameters evaluate coagulation velocity and acceleration of fibrin formation in plasma, and appear to be especially informative [18]. In addition, thrombin and plasmin are known to be the terminal representative enzymes of the coagulation and fibrinolysis cascades, and in general, increased thrombin generation is believed to indicate a hypercoagulable tendency, and increased plasmin generation is understood to reflect enhanced fibrinolysis. Consequently, well-balanced thrombin and plasmin generation is necessary to maintain homeostasis in the normal circulation [21]. The T/P-GA techniques provide an appropriate means to determine this critical balance of coagulation and fibrinolysis.

Hyper-coagulability is a significant risk factor of coronary thrombosis and appears likely to contribute to coagulation-related pathology in KD. Limited information is available, however, on the application of these comprehensive methods for investigating possible dynamic changes of coagulation and fibrinolysis in patients with acute-KD. The present report describes for the first time the use of these assays to characterize coagulation potential in pediatric patients with acute-KD that received IVIG treatment.

2. Materials and methods

2.1. Reagents

Thrombocheck APTT-SLA® (Sysmex Corporation, Kobe, Japan), recombinant human tissue factor (TF; Innovin®, Dade, Marburg, Germany), recombinant tissue-type plasminogen activator (tPA; American Diagnostica Inc., Stamford, CT), plasma-derived fibrinogen (Hematologic Technologies Inc., Burlington, VT), thrombin-specific fluorogenic substrate (*Z*-Gly-Gly-Arg-AMC, Bachem, Bubendorf, Switzerland), plasmin-specific fluorogenic substrate (BOC-Glu-Lys-Lys-MAC, Peptide Institute Inc., Osaka, Japan), Immunoglobulin (Venilon-I®, Kaketsuken, Kumamoto, Japan), Fibriquik® reagent (Trinity Biotech, Dublin, Ireland), were purchased from the indicated vendors. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, 30% phosphatidylethanolamine (Sigma-Aldrich, St Louis, MO) were prepared as previously described [23].

2.2. KD patients

Pediatric patients admitted to Nara Medical University Hospital, Kokuho Central Hospital, and Yao Municipal Hospital, in Japan between January 2011 and January 2014, who had been diagnosed with KD according to Japanese diagnostic guidelines for KD were enrolled in this study [24]. Therapeutic management during acute-KD were standardized. All patients received IVIG together with anti-platelet agents as first-line therapy. IVIG was administered as a single infusion of 2 g/kg or two infusions of 1 g/kg daily. Aspirin was given as an anti-platelet agent using 30–50 mg/kg/day during the acute phase, and subsequently at 3–5 mg/kg/day during convalescence. In patients with elevated levels of liver enzymes, flurbiprofen was given at 3–5 mg/kg/day. Individuals that required additional treatment for fever lasting > 24 h after the end of IVIG infusion or recrudescent fever associated with KD symptoms after an afebrile period were defined as non-responders [25]. Coronary artery abnormalities were assessed by two-dimensional echocardiography by attending physicians with special skills for this examination. Coronary arteries were defined as abnormal if the internal luminal diameter was > 3.0 mm in a child aged younger than 5 years or > 4.0 mm in those aged 5 years and older. Cardiovascular lesions were graded if the lumen diameter of an arterial segment was at least 1.5-fold as large as that of the adjacent segment, or if the lumen was irregular [26].

2.3. Blood samples

This study was approved by the Medical Research Ethics Committee of Nara Medical University, and blood samples were obtained after written informed consent from the child's family. Blood was obtained by venipuncture at three time-points; immediately before treatment with IVIG (termed by 'Pre'), one week post-IVIG (termed by '1W'), and one month post-IVIG (termed by '1M'). The blood samples were collected into plastic tubes containing 3.2% sodium citrate at a 9:1 ratio. Fifteen control patients without medication included age-matched consecutive patients with trivial congenital heart diseases ($n = 13$) and those with past history in KD with no coronary artery abnormality over 1 year after onset ($n = 2$) as a control plasma. Furthermore, normal pooled plasma was prepared from twenty normal healthy individuals who agreed to participate in this study and was used in the experiments on *ex vivo* addition of fibrinogen or immunoglobulin. Platelet poor plasma was recovered after centrifugation of citrated whole blood for 15 min at 1500 g. All plasmas were stored at −80 °C, and thawed at 37 °C immediately prior to the assays.

2.4. Fibrinogen measurement

Plasma fibrinogen was measured by the Clauss method using the MDA-II Haemostasis™ system (Trinity Biotech) with Fibriquik reagent. A standard curve was prepared using Coagtrol N (Sysmex Corp.) [27].

2.5. Clot waveform analysis (CWA)

CWA was performed on the MDA-II Haemostasis™ system and the CS-2000i™ instrument (Sysmex Corporation; Kobe, Japan) using the same aPTT reagent [18,20]. Both automated coagulometers are based on identical principles, and determine clotting end points photo-optically. The CWA in KD patients were examined using the MDA-II and in control samples were assessed using the CS-2000i. The CWA measurements in control samples were converted to equivalent MDA-II data (Nogami and Matsumoto, unpublished data). Fig. 1A-a illustrates a representative aPTT-based clot waveforms obtained by MDA-II, the first and *sec*ond derivative curves reflecting the dynamic process of fibrin formation in age-matched control plasma. The clot waveforms obtained were computer-processed using the commercial kinetic algorithm. The horizontal axis shows the running time (*sec*), and the vertical axis shows the transmittance (%) defined as transmitted light intensity from the pre-coagulation to post-coagulation phase. Clot formation was initiated by the addition of $CaCl_2$ (20 mM). The clot time (CT) was defined as the time until the start of coagulation. The minimum value of the first derivative (min1) was calculated as an index of the maximum velocity of coagulation achieved. The minimum value of second derivative

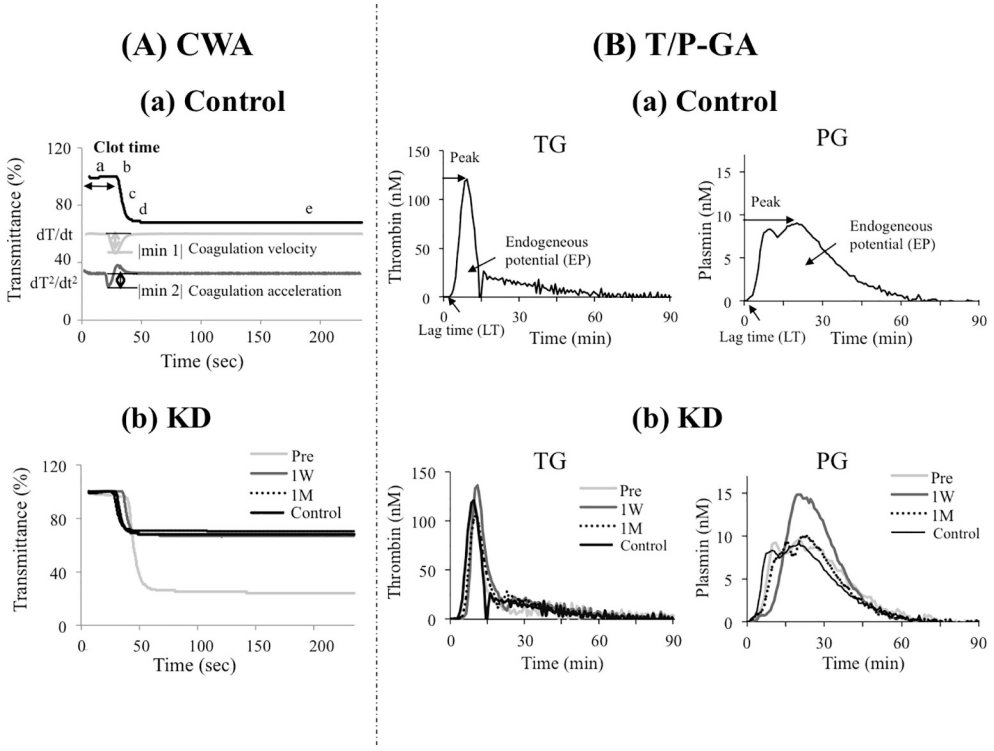

Fig. 1. Representative CWA and T/P-GA in control individuals and acute-KD patients.
(*Panel* A) aPTT-based CWA; Control plasma was incubated with aPTT reagent prior to the addition of $CaCl_2$ at the start of the assay as described in Methods. (*Panel* a) The lines represent the clot waveform (*black*), |min1| (dT/dt; *light gray*), and |min2| (dT^2/dt^2; *gray*) waveforms. The segments a–b, b–d, and d–e show the pre-coagulation phase, coagulation phase, and post-coagulation phase, respectively. (*Panel* b) A representative clot waveform of patient's plasma from acute-KD is illustrated. Pre; immediately before IVIG (*light gray*), 1W; one week post-IVIG (*gray*), 1M; one month post-IVIG (*dotted*), and control plasma (*black*).
(*Panel* B) T/P-GA; Mixtures of TF, tPA, and PL (f.c. 1 pM, 3.2 nM, and 4 μM, respectively) was added to control plasma as described in Methods. (*Panel* a) The figures show the curves of thrombin generation (TG) and plasmin generation (PG) and the calculated parameters. (*Panel* b) Representative TG and PG curves of patient's plasma from acute-KD are illustrated. Pre; immediately before IVIG (*light gray*), 1W; one week post-IVIG (*gray*), 1M; one month post-IVIG (*dotted*), and control plasma (*black*).

(min2) was calculated as an indicator of the maximum acceleration of the reaction. Since the min1 and min2 measurements were derived from negative changes, the data were expressed as |min1| and |min2|, respectively.

2.6. Simultaneous thrombin and plasmin generation assay (T/P-GA)

T/P-GA was assayed using our established protocol [21]. Briefly, trigger reagent (20 μL) and plasma sample (80 μL) were manually pipetted into wells of flat-bottomed, black polystyrene, 96-well plates (Nunc; Thermo Scientific, Waltham, MA). Mixtures of TF (final concentration; f.c., 1 pM), PL vesicles (4 μM), and tPA (3.3 nM) were used as a trigger reagent. Thrombin generation (TG) and plasmin generation (PG) were determined using two specific fluorometric substrates, *Z*-Gly-Gly-Arg-AMC and Boc-Glu-Lys-Lys-MCA, respectively. Assays were performed using a Fluoroskan Ascent™ microplate reader (Thermo Electron Co., Waltham, MA) with an excitation filter at 390 nm and an emission filter at 460 nm. Standard curves were prepared using serially diluted purified α-thrombin and plasmin as previously described by Hemker, *et al* [28]. Data analyses were performed using excel software. Fig. 1B-a illustrates a representaive generation curves obtained in control plasma. From the first derivative (representing velocity) of thrombin and plasmin generation obtained above, lag time (LT; time until the onset of TG and PG), peak height (Peak; the maximum amounts of TG and PG), and endogenous potential (EP; the total amounts of TG and PG) were recorded. The simultaneous T/P-GA confirmed that TG was a prerequisite for the initiation of PG and that overall PG was governed by fibrin concentration [21].

2.7. Statistical analyses

The results were expressed as mean and standard deviations. Analyses were performed using JMP 10.0.2 (SAS Institute Inc., Cary, NC). Significant differences of the continuous variables at the three time-points were evaluated using repeated measurement analysis of variance, and post-*hoc* comparisons were adjusted with the Bonferroni correction. The Bonferroni correction set the significance cut-off at the calculated *p*-value × n. Comparisons between the two study groups were performed using the Student's *t*-test. The correlations between parameters were analyzed with the Pearson correlation coefficient. Two-sided tests were used for all analyses. *P* values < 0.05 were considered as statistically significant.

3. Results

3.1. Patients' characteristics

A total of 43 patients with KD and 15 control age-matched patients were initially screened. One female KD case received anticoagulant drugs for coronary artery aneurysm and was excluded from the final study. The remaining 42 patients with acute-KD (22 male, 20 female) were enrolled in the study. The clinical characteristics of the KD patients are shown in Table 1. The age at onset ranged from 3 to 60 months (mean ± SD; 24 ± 16, median; 21 months old). Among all children who received IVIG, 29 cases received aspirin orally, and 13 cases received flurbiprofen orally because of elevated levels of liver enzymes. Eight cases poorly responded to the IVIG. A risk score (Kobayashi score) for non-responder to IVIG was 3.5 ± 2.5 points (≥5 points; 76% sensitivity, 80% specificity) [25]. Two and 6 of these patients subsequently received additional IVIG without and with prednisolone, respectively. Transient dilation of the coronary artery was detected in 3 cases but returned to normal size within one month of onset. No coronary aneurysms were observed during the period of study. The age of control patients ranged from 4 to 80 months (mean ± SD; 27 ± 23, median; 17 months old), and included 10 males and 5 females.

3.2. Comprehensive coagulation evaluation using CWA in acute-KD

Comprehensive clotting function in acute-KD was first assessed using CWA. Representative clot waveforms observed in the patient's plasma, 'Pre', '1W', and '1M', are illustrated in Fig. 1A-b. In Pre-

Table 1
Clinical characteristics of acute-KD patients.

Patients (n)	42
Male/Female (n)	22/20
Age at onset (month)	24 ± 16 (21; 3–60)
Days of illness at IVIG (day)	5 ± 1 (3–8)
IVIG (n)	42
1 g/kg × 2 days	24
2 g/kg × 1 day	18
Kobayashi score (points)	3.5 ± 2.5 (3; 0–8)
Non-responder for IVIG (n)	8
Additional therapy	
IVIG only (n)	2
IVIG + PSL (n)	6
Coronary artery lesion (n)	3
Dilation	3
Aneurysm	0
Laboratory data	
White-cell count ($\times 10^3/\mu L$)	14.5 ± 5.3
Platelet count ($\times 10^4/\mu L$)	33.7 ± 9.7
C-reactive protein (mg/dL)	7.5 ± 4.5
Aspartate aminotransferase (U/L)	75 ± 87

Values are mean ± SD, (); median; min-max, IVIG; intravenous immunoglobulin.
PSL; prednisolone.

samples, the initiation of coagulation was delayed and light transmittance was markedly diminished compared to control. Fig. 2 shows CWA parameters at the three time-points in acute-KD. The Pre CTs were clearly prolonged compared to age-matched control plasmas ($p < 0.001$), but were close to normal at 1W and 1M. In contrast, the Pre |min1| and |min2|, reflecting the maximum coagulation velocity (min1) and acceleration (min2), respectively, were significantly increased by ~2-fold compared to control ($p < 0.001$). Both parameters were lower at 1W than those at Pre ($p < 0.05$) but remained high relative to control ($p < 0.05$). Normal levels were recorded at 1M. These results indicated that the initiation of coagulation in acute-KD patients before treatment was moderately delayed, but the dynamics of fibrin formation, represented by |min1| and |min2|, paradoxically appeared to suggest a hypercoagulable state.

3.3. Dynamics of coagulation and fibrinolysis evaluated by T/P-GA

We utilized the T/P-GA to evaluate the pivotal balance between coagulation and fibrinolysis in acute-KD patients. Representative TG and PG curves in acute-KD plasma samples at the three time-points are illustrated in Fig. 1B-b. The changes in individual dynamic parameters are shown in Fig. 3. TG analyses demonstrated that the LT at 1W was prolonged compared to control, and was not significantly different at any of the three time-points. Little difference in Peak levels relative to control was seen in these samples. The EP levels at Pre and 1W were increased to the greatest extent, and were slightly decreased at 1M but remained much higher than control ($p < 0.001$) (Fig. 3A). Simultaneous measurements of PG demonstrated that the LT was not significantly different from control, but the EP and Peak levels were increased. The EP and Peak levels at 1W were significantly increased relative to Pre ($p < 0.001$), but both were modestly decreased at 1M ($p < 0.001$) (Fig. 3B). These results suggested that both TG and PG remained enhanced in acute-KD for at least one month post-IVIG.

The delicate relationship between TG and PG was further assessed by calculating the ratios of the parameters for acute-KD relative to control (Fig. 3C). The ratios for EP were similar for both TG and PG at each time-points. The LT ratio for PG was less than that for TG on each occasion, however, and the Peak ratio for PG was greater than that for TG at all points. These results suggested that and the initiation and potential for fibrinolysis appeared to be more dominant than those of coagulation, but that the overall balance between coagulation and fibrinolysis was maintained throughout the period of study. Relationships between T/P-GA parameters and other clinical indicators, including CRP, liver function, IVIG regimen, or steroid therapy were also investigated. None of these clinical data correlated with the dynamic changes of TG and PG, however (data not shown).

3.4. Impact of fibrinogen and immunoglobulin on CWA and T/P-G parameters

The results described above were consistent with enhanced hemostatic potential in children with acute-KD. The principles of two comprehensive coagulation assays are different, however, especially that CWA measurements are dependent on blood clotting whilst T/P-GA predominantly monitors reactions upstream of fibrin formation. Circulating levels of fibrinogen and immunoglobulin in acute-KD are generally increased by inflammatory cytokines [29] and by IVIG administration, and could have influenced our CWA and T/P-GA parameters. Actually, our patients' plasmas also showed that before IVIG treatment, fibrinogen levels (684 ± 174 mg/dL) were increased > 2-fold compared to control (230 ± 33 mg/dL, $p < 0.001$), but markedly decreased in 1W (345 ± 118 mg/dL, $p < 0.05$) and returned to normal at 1M (275 ± 115 mg/dL). Our studies were extended, therefore, to assess the direct influence of fibrinogen or immunoglobulin on these assays.

Incremental concentrations of commercially available plasma-derived fibrinogen were added to normal pooled plasma before performing CWA. This *ex vivo* addition of fibrinogen prolonged the CT and increased |min1| and |min2| dose-dependently (Fig. 4Aa–c). These results were similar to the Pre-CWA parameters seen in our patients, and were in keeping with the influence of fibrinogen on coagulation dynamics. Similarly, immunoglobulin concentrations up to 50 mg/mL (corresponding to a therapeutic dose of 2 g/kg) were added to normal pooled plasma prior to analysis. The immunoglobulin at these concentrations appeared to prolong the CT but did not significantly affect the |min1| and |min2| (Fig. 4Ba–c). These results were not fully

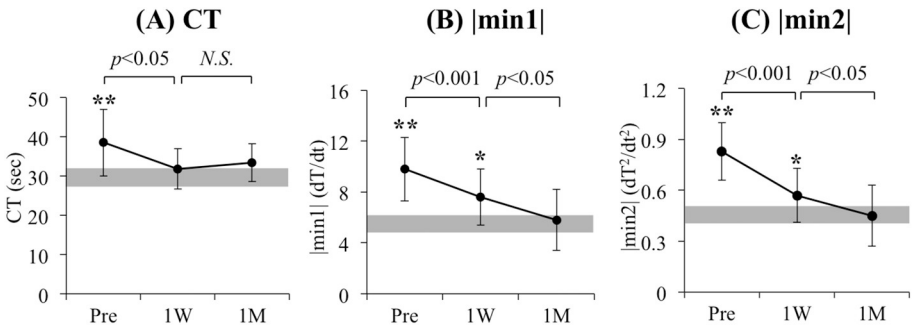

Fig. 2. Dynamic changes of CWA parameters at the three time-points in acute-KD.
The parameters (Clot time (CT), |min1|, and |min2|) at Pre, 1W, and 1M in all patients obtained by an aPTT-based CWA are shown. The gray horizontal bars illustrate the reference range obtained from controls (CT; 29.5 ± 2.6 s, |min1|; 5.5 ± 0.6 and |min2|; 0.42 ± 0.03). All values and bars indicate the average and standard variation. Significant differences of continuous variables among the three time-points are indicated by $p < 0.05$, and are shown by p value. In addition, the significant differences at each of three-time point relative to age-matched control are indicated by *; $p < 0.05$, **; $p < 0.001$, and are shown by the asterisk. *N.S.*; not significant.

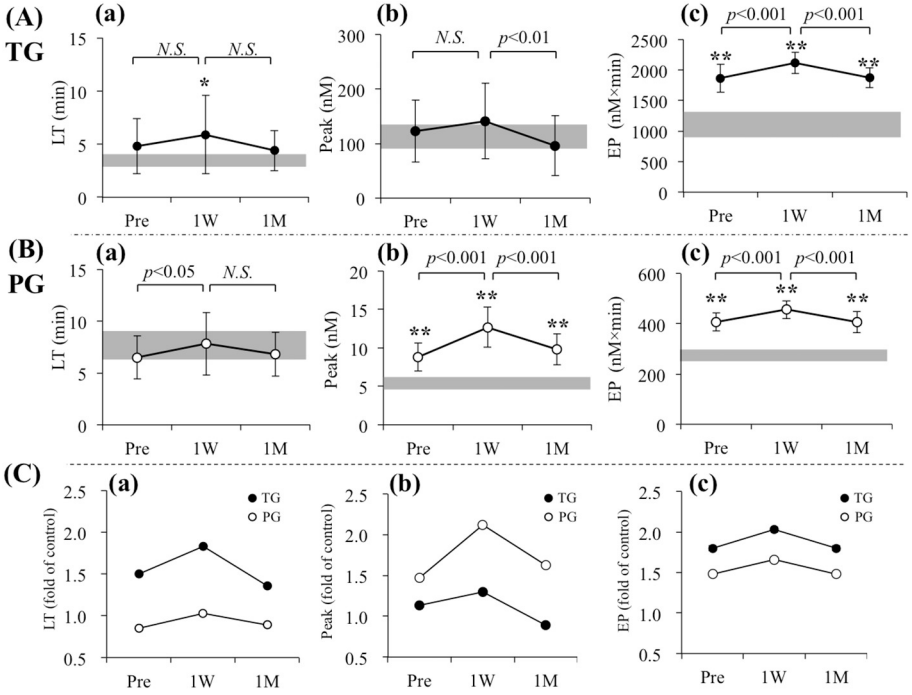

Fig. 3. Dynamic changes of T/P-GA parameters at the three time-points in acute-KD.
The T/P-GA parameters, lag time (LT), peak height (Peak), and endogenous potential (EP) of TG (*panel* A) and PG (*panel* B) at Pre, 1W, and 1M are illustrated. The gray horizontal bars show the reference range obtained from control in TG (LT; 3.2 ± 0.6 min, Peak; 108.0 ± 22.9 nM, EP; 1038 ± 247 nM × min) and in PG (LT; 7.6 ± 1.4 min, Peak; 6.0 ± 0.8 nM, EP; 275 ± 23 nM × min). All values and bars indicated the average and standard deviation. Significant differences of continuous variables at the three time-points are calculated at $p < 0.05$, and are shown by p value. In addition, the significant differences at each of three-time point relative to age-matched control are indicated by *; $p < 0.05$, **; $p < 0.001$, and are shown by the asterisk. *N.S.*; not significant. (*Panel* C) The balance between coagulation and fibrinolysis represented by T/P-G parameters at the three time-points - The ratios of LT, Peak and EP in TG (*closed circles*) and PG (*open circles*) of acute-KD patients' plasmas to those of control are shown.

consistent with our clinical laboratory findings. The laboratory data in our KD patients at 1W indicated that the immunoglobulin concentration remained high after IVIG treatment, and it seemed unlikely that the presence of immunoglobulin directly affected our CWA parameters.

Similar experiments were repeated with T/P-GA. The LT and EP for both TG and PG were not affected by the different concentrations of added fibrinogen or immunoglobulin *ex vivo*. Both Peak TG and Peak PG increased dose-dependently in proportion to the amount exogenous fibrinogen. In contrast, Peak TG values were inversely related to immunoglobulin concentration but Peak PG results were not influenced by immunoglobulin (data not shown). These data again were not in keeping with the *in vivo* laboratory findings, and overall, the results indicated that high fibrinogen and immunoglobulin concentrations were unlikely to have directly affected the T/P-GA. In addition, the parameters obtained by CWA and T/P-GA assays were not significant difference between patients treated with aspirin and flurbiprofen (data not shown).

3.5. Comparisons between IVIG responders and non-responders

Thirty-four patients demonstrated a good response (responders) and 8 patients appeared to respond poorly to IVIG (non-responders). No significant differences were observed between the two groups in any of the T/P-GA parameters (data not shown). The CWA parameters varied, however, and are summarized in Table 2. The Pre CT in responders was prolonged compared to control. All CTs were close to normal at 1W and

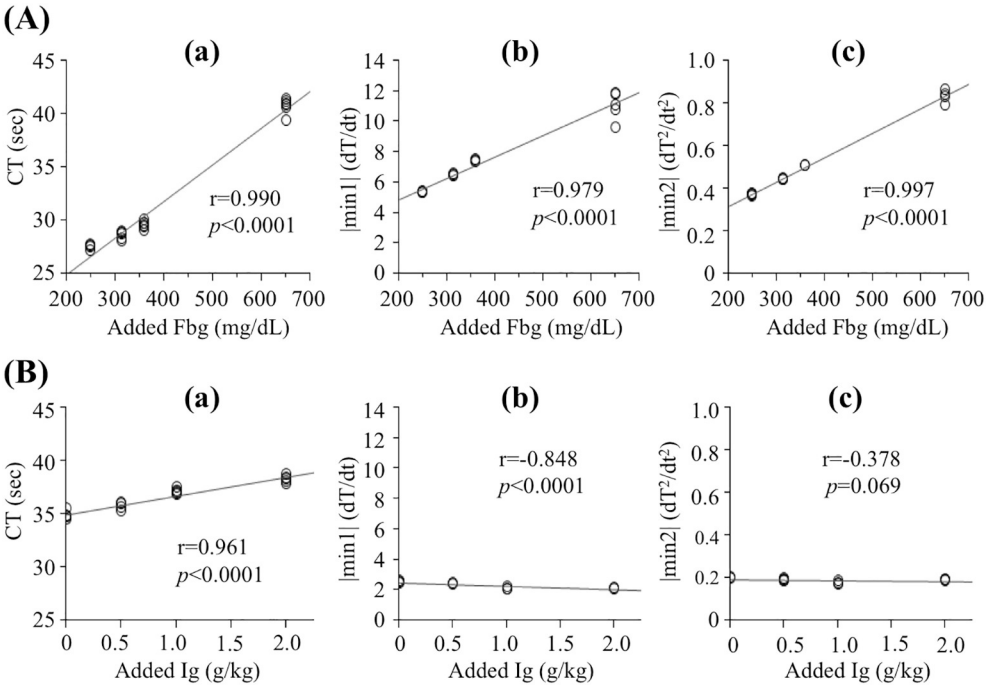

Fig. 4. Correlation of CWA parameters in the presence of various concentrations of fibrinogen and immunoglobulin.
Various concentrations of plasma-derived fibrinogen (*panel* A) or immunoglobulin (*panel* B) were added to normal pooled plasma *in vitro* prior to CWA as described in Methods. Fibrinogen concentration in normal pooled plasma was approximately 250 mg/dL. The addition of 50 mg/mL of immunoglobulin corresponded to the initial therapeutic dose (2 g/kg) of IVIG treatment in acute-KD. The CT (*panel* a), |min1| (*panel* b), and |min2| (*panel* c) data are presented on the vertical axis. The final concentrations of fibrinogen and immunoglobulin in normal pooled plasma are given on the horizontal axis. The coefficient of correlations (r) of these parameters with fibrinogen and immunoglobulin are shown (*line*). Significant differences were calculated at $p < 0.05$. All experiments were performed at least four separate times. Fbg; fibrinogen, Ig; immunoglobulin.

Table 2
Comparisons in CWA parameters and plasma fibrinogen levels in responders and non-responders of KD patients.

Parameters		Responders (n = 34)	Non-responders (n = 8)	P value
CT (sec)	Pre	39.2 ± 9.1**	35.2 ± 4.8	*N.S.*
	1W	32.2 ± 4.8	30.0 ± 6.5	*N.S.*
	1M	34.3 ± 4.8*	29.2 ± 1.9	< 0.05
|min1| (dT/dt)	Pre	9.6 ± 2.5**	10.8 ± 2.2**	*N.S.*
	1W	6.9 ± 1.6*	10.1 ± 2.3**	< 0.001
	1M	5.5 ± 1.3	8.1 ± 3.7*	< 0.05
|min2| (dT^2/dt^2)	Pre	0.82 ± 0.17**	0.89 ± 0.14**	*N.S.*
	1W	0.51 ± 0.11*	0.77 ± 0.16**	< 0.001
	1M	0.44 ± 0.11	0.62 ± 0.26*	< 0.05
Fibrinogen (mg/dL)	Pre	675 ± 177**	721 ± 164**	*N.S.*
	1W	305 ± 71*	510 ± 132**	< 0.001
	1M	250 ± 73	362 ± 189*	< 0.05

Values are mean ± SD. Significant differences were expressed as $p < 0.05$. *N.S.*; no significant difference, CT; clot time, Pre; just before IVIG, 1W; one week post IVIG, 1M; one month post IVIG. The differences with values in Pre, 1W, and 1M relative to those in age-matched control plasmas are shown as follows; * $p < 0.05$, ** $p < 0.001$ (shown by the asterisk).

1M. In contrast, the |min1| and |min2| in non-responders at 1W and 1M were greater than in responders and control, although no significant differences had been evident between the two groups Pre-treatment. In addition, the concentrations of plasma fibrinogen showed a similar pattern to the |min1| and |min2|. The results suggested that non-responders remained hypercoagulable one month post-IVIG.

4. Discussion

The pathology of KD centers on systemic vasculitis, mainly in the coronary artery, and prognosis is determined by any accompanying thrombus formation. Thrombogenesis is generally mediated by VEC damage and platelet activation in a hypercoagulable environment, resulting in fibrin formation, modified fibrinolytic potential and a dynamic change in blood flow. Some studies have suggested that hemostasis in KD patients may be influenced by a range of coagulant and fibrinolytic factors including fibrinogen, FVIII, and VWF [8–10]. Investigations have commonly utilized conventional individual coagulation assays, however, and do not consider the overall assessment of global hemostasis. We have examined, therefore, comprehensive coagulation potential in acute-KD using CWA and T/P-GA. The results particularly indicated differences in CWA and T/P-GA parameters prior to IVIG (Pre) and 1 week after treatment (1W), respectively. Additionally, the findings confirmed that serial changes in coagulation and fibrinolysis occurred in response to the initial hypercoagulability, and consequently the complementary mechanisms appeared to be mostly well-balanced.

CWA reflects clotting dynamics from initiation to fibrin clot formation. Prior to treatment (Pre) in acute-KD, the CT paradoxically appeared to be prolonged, but the coagulation process proceeded rapidly after initial fibrin formation. These findings would not be possible to identify using conventional assay. In this context, Sakurai et al. demonstrated a prolonged PT and APTT in KD that were significantly shortened after IVIG [10]. In an earlier study, Yu et al. demonstrated excessively increased levels of fibrinogen-related proteins in acute-KD, and suggested that an abnormal fibrinogen cascade might be good biomarker of KD [29]. We examined the effects of added fibrinogen *ex-vivo* on clotting dynamics in CWA. The results demonstrated that the CT was prolonged at high concentrations of fibrinogen. Braun et al. examined CWA parameters related to the concentration of a range coagulation proteins or anticoagulants, and reported that the |min2| was decreased but the CT was unaffected at fibrinogen levels below normal [27]. Little information was provided, however, on the impact of higher levels of fibrinogen. Our results showed that |min1| and |min2| depended on fibrinogen concentrations. In addition, our data suggests that increased fibrinogen tended to obstruct the initiation of clotting dynamics and enhance the coagulation potential.

Our T/P-GA showed that EPs in TG and PG before treatment (Pre) were greater than those of control, and were further increased at 1W. Vasculitis itself could have been expected to enhance both TG and PG. IVIG therapy is expected to moderate inflammatory mechanisms, however, and the persistently elevated EPs at 1W and 1M appeared likely to be caused by some factor(s) other than inflammation. VEC damage could contribute to these mechanisms, in which the coronary artery lesions in acute-KD lead to an influx of neutrophils in an early stage (7–9 days after onset of KD). This type of pathogenetic process has been well described, and the primary response is believed to follow by a rapid influx of large mononuclear cells, lymphocytes, *etc*. At this stage, destruction of the internal elastic lamina and fibroblastic proliferation occurs, leading to the formation of coronary aneurysms [4,30,31]. Moreover, the release of cytokines promotes VEC damage, which stimulates the release of ultra-large VWF multimers from VEC [7,32]. High levels of VWF and FVIII are indices of VEC damage, and persist 10–15 days after IVIG [8,10]. Thrombin provides a link between coagulation with inflammation, and increased inflammatory cytokines mediate enhancement of thrombin generation in the presence of VEC damage. A trace amount of thrombin activates FVIII and platelets at the cellular interface, resulting in consolidation of thrombin generation and an increased risk of thrombosis. These mechanisms may be pivotal to the persistence of elevated EPs at 1W and 1M after IVIG.

The present findings suggest that in patients with acute-KD without coronary lesions thrombotic risk might be moderated by well-balanced control of coagulation and fibrinolysis, even though hemostasis potential may be greater than normal. Mechanisms of coagulation and fibrinolysis are complex and intertwined, and well-balanced coagulation and fibrinolytic mechanisms are central to the maintenance of homeostasis and blood flow at sites of vascular damage. TG is responsible for the initiation of PG, and increases in TG promote an adequate PG response [21]. We have demonstrated the importance of the balance between coagulation and fibrinolysis associated with the life-threatening coagulopathy in pediatric acute leukemia after hematopoietic stem cell transplantation [33,34] and in patients with acquired hemophilia A with major hemorrhage [35]. Our current results confirmed that the global coagulation assays could provide meaningful data for the clinical management of difficult patients with acquired disorders of hemostasis.

Failure to responder to IVIG in 10–20% of patients with KD appears to be significantly associated with a high risk of developing coronary artery lesions [3,25,36]. Our data indicated that non-responders had significantly higher |min1| and |min2| levels in CWA compared to responders. This accelerated coagulation potential seemed to persist for at least one month after the initiation of treatment. It may be that the coronary arteries in these children were more sensitive to damage creating a higher risk of thrombosis. In contrast, T/P-GA parameters were not significantly different between the two groups, possibly due to limited VEC damage in this relatively small number of individuals. The precise reason remains unclear, however, and further studies with a larger number of cases are needed to clarify coagulation and fibrinolysis-related mechanisms in unresponsive KD.

Our present study is limited, however, by the moderately small number of children available for investigation. Most of the enrolled cases had no complications, and the data did not identify any reliable coagulation differences in acute-KD patients with coronary artery abnormalities. In addition, control studies with febrile children were not included, and relationships between infection and hypercoagulability in acute-KD were not thoroughly investigated. Nevertheless, we considered that continued enhanced coagulation potential at 1M after defervescence appeared likely to be a significant feature in the pathophysiology of KD. Moreover, blood samples were initially obtained on the first day prior to treatment with IVIG. Non-responders received

additional treatment, and of necessity, therefore, time intervals were shorter relative to the responders. Also, supplementary treatment in most of the non-responders included steroids. The potential influence of steroid therapy was compared in the two groups, and no significant differences were evident (data not shown), but further studies might be required to confirm that this medication does not affect coagulation and/or fibrinolysis in these circumstances.

CWA, which is performed by some automatic coagulometers, is a versatile and readily available technique, but the analysis method in all instruments remains to be established. Similarly, since T/P-GA requires the technical skills for use, the limited institutions are available. Both assays can provide to evaluate the comprehensive coagulation and fibrinolysis potentials and their balancing. Therefore, to transit these tests from research to clinical practice, we have to spread that the use of these assays offers potential for application of assessment of thrombotic as well as hemorrhagic conditions in routine hemostasis laboratories.

5. Conclusion

The novel findings using comprehensive coagulation assays in our patients without coronary artery lesions provided strong evidence for an increase of the coagulation potential in acute-KD. Further studies are in progress to validate the application of these techniques in overt KD-related coronary lesions, and in the chronic phase of KD.

Authorship

Contribution

HY; designed the research, performed experiments, interpreted the data and wrote the paper, KN; designed the research, interpreted the data, wrote the paper, edit the manuscript, and approved the final version to be published, TM; performed experiments, interpreted the data, NT; TS, TT, IT; clinical supports in all patients, MS; supervised the manuscript.

Conflict-of-interest disclosure

T. Matsumoto teaches a course endowed by Shire Japan Co. Ltd. The other authors declare that they have no conflicts of interest.

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