(C57BL/6 background) mice were purchased from KBT Oriental, Charles River Grade. Nod1 $^{-/-}$ mice in C57BL/6 background were a gift from Tak Mak, University Health Network. All mice were 5- to 9-week-old female, and were housed in a specific pathogen-free environment. Experiments were performed three times independently under barrier conditions at the animal facility of the biosafety level P1A. These animal experiments were performed according to the protocol approved by the Kyushu University Institutional Animal Care and Use Committee.

Immunohistochemistry. All sections for immunohistochemistry were 4% paraformaldehyde-fixed and paraffin-embedded. Primary antibodies used were Nod1-specific antibody (1:1000, IMGENEX)[6], F4/80 (macrophage)-specific antibody (1:100, Abcam), NIMP-R14 (neutrophil)-specific antibody (1:1500, Abcam), CD3 (T cell) -specific antibody, (1:400, Abcam), alpha smooth muscle actin-specific antibody (1:100, Dako), and CD31-specific antibody (Platelet endothelial cell adhesion molecule 1) (1:50, Abcam) used for the detection of endothelial cells. Deparaffinized sections with or without antigen retrieval (citrate buffer pH6.0 or trypsin) were incubated with 3% nonfat milk to eliminate nonspecific binding and with peroxidase-labeled secondary antibody (Dako and Nichirei) following the primary antibodies. Horseradish peroxidase activity was visualized with Peroxidase Stain DAB Kit (Nacalai Tesque) to give the reaction

product a brown color, and then the sections were counterstained with hematoxylin.

Quantitative real-time RT-PCR. Total RNA was extracted from HCAEC and murine tissues using RNeasy Micro kit and RNeasy Fibrous Tissue (Qiagen), respectively, followed by cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems). Human NOD1 and mouse Nod1 mRNA expression levels were analyzed by TaqMan® Gene Expression Assay Hs00196075_m1 and Mm00805062_m1 (Applied Biosystems), respectively, and TaqMan Gene Expression Master Mix (Applied Biosystems). Human GAPDH (Pre-Developed TaqMan Asssay Reagents GAPDH Control Reagents, Applied Biosystems), mouse GAPDH (primers: CCTGGAGAAACCTGCCAAGTAT, TTGAAGTCGCAGGAGACAACCT; TaqMan probe: VIC-TGCCTGCTTCACCACCTTCTTGATGT-TAMRA) and Cadherin 5 (CDH5, Mm00486938_m1, Applied Biosystems) were used as internal controls. Calibration curve was generated with serial 5-fold dilutions. The mRNA expression levels of the targeted genes were quantified by an ABI PRISM 7700 or Applied Biosystems StepOnePlus sequence detector (Applied Biosystems), as described[4].

Organ Culture. Each tissue of aortic root, pulmonary artery, aortic arch, and abdominal aorta (between roots of right and left renal arteries) sterilely isolated from BALB/c or Nod1$^{-/-}$ mice was cultured for 24 hours in a 96-well plate with EBM-2 medium and

EGM-2 (Lonza) in the presence or absence of an indicated reagent in a CO_2 (5%) incubator at 37°C. We performed these experiments four times independently.

Protein determination. Protein concentrations of each tissue were measured by Bio-Rad protein assay (BioRad) after homogenization with phosphate buffered saline containing Cell Culture Lysis Reagent (Promega) and Protease Inhibitor Cocktail (Nacalai Tesque). Whole protein contents of each tissue were measured to calculate CCL2/IL-6 levels per tissue protein contents.

Microarray analysis. Microarray analysis was performed with *in vivo*-treated and *ex vivo*-cultured organs isolated from 7 mice and *in vitro*-cultured cells (1×10^4 cells). Total RNA was extracted from murine tissues with an RNeasy Fibrous Tissue and from human cell lines with RNeasy Micro Kit (Qiagen). Total RNA was then amplified using Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion). Briefly, double-standard complementary DNA (cDNA) was synthesized from total RNA and *in vitro* transcription was performed to produce multiple copies of amino allyl-labelled complementary RNA (cRNA). Amino allyl-labelled cRNA was purified, reacted with N-hydroxy succinimide esters of Cy3 (GE Healthcare) using Nimblegen's protocol and hybridized for 19h at 42°C to the mouse and human Nimblegen Gene Expression arrays (090901_MM9_EXP_HX12, and 090828_HG18_opt_expr_HX12, Roche NimbleGen) that contain approximately 40,000 genes. The arrays were scanned on Gene Pix 4000B (Molecular Devices Corporation). The averages of triplicate spot intensity data were extracted using NimbleScan v2.5 (Roche NimbleGen) and processed using robust multiarray analysis method[7]. The scaled gene expression values were imported into GeneSpring 11.0 software (Agilent Technologies) for preprocessing and data analysis[4]. The expression value of each gene was normalized to the 75 percentile shift expression of all genes in each chip. Probe sets were deleted from subsequent analysis if they were displayed an absolute value below 30 in all experiments. The normalized data were first $\log_2$ transformed. For each gene, $\log_2$ intensities from stimulated samples were compared by non-stimulated samples. Microarray data were deposited in Gene Expression Omnibus under accession numbers, GSE20929 (*in vitro* gene expression) and GSE20930 (*in vivo* and *ex vivo* gene expression). Microarray experiments of in vivo organs from 7 mice were done once but on 3 different days 2, 4 and 7 after treatment with consistent results.

Statistical Analysis. Data were analyzed by Student's t-test, Dunnett's test or Tukey-Kramer honestly significant difference (HSD) test using a statistical software, JMP version 8.0 (SAS Institute).

Supplementary Figure I: Primary augmenting effects between NOD1 and TLR ligands in HCAEC

A. Effects of innate immune stimulants on HCAEC at day 1.

HCAEC (1 x 10^4 cells for ICAM-expression; 1.6 x 10^4 cells for cytokine production) were incubated with NOD1, NOD2, TLR and other stimulants in various combinations for 24 h. ICAM-1 expression (a) and IL-8 (b) /IL-6 (c) production in the culture supernatants were investigated in triplicate at day 1. The concentrations of stimulants are as follows: iE-DAP, MDP and PGN K12, 1 (▩) or 10 (■) μg/mL; lipid A 10 (▩) or 100 (■) ng/mL. Data are presented as mean ± s.d. *: $P < 0.01$ compared with medium, †: $P < 0.01$ compared with either iE-DAP or Lipid A, ‡: $P < 0.01$ compared with either PGN K12 or Lipid A (Dunnett's test).

B. NOD1 knockdown suppresses the stimulatory effects of NOD1 ligand in the absence or presence of TLR4 ligand in HCAEC.

HCAEC (4 x 10^4 cells) were transfected with either siRNA against NOD1 (#1: HSS115908 or #2: HSS115906) or a non-targeted control siRNA. NOD1 siRNA (#1) and (#2) reduced the expression levels of endogenous NOD1 in HCAEC to 15.9 ± 1.9% and 37.0 ± 7.5% of those treated with control siRNA, respectively, as confirmed by quantitative RT-PCR (data not shown). Secretion levels of IL-8 (a) and IL-6 (b) were

investigated in triplicate at 24 hr after stimulation. The concentrations of stimulants are as follows: iE-DAP and MDP, 10 μg/mL; lipid A 100 ng/mL. Data are presented as mean ± s.d. The effects of NOD1 ligand, iE-DAP, were almost completely inhibited, while those of TLR4 ligand (LipidA) remained uninhibited by NOD1 siRNA (#1). *: $P < 0.01$ compared with those treated with a control siRNA. The additive effect of NOD1 and TLR4 was abolished after NOD1 siRNA (#1) treatment. †: $P < 0.01$ compared with either iE-DAP or Lipid A (Dunnett's test).

Supplementary Figure II. Chemical structures of NOD1 ligands and comparison of the effects on HCAEC.

A. Chemical structures of diaminopimelic acid (DAP)–type peptidoglycan and synthetic NOD1 ligands. iE-DAP: γ-D-Glu-DAP, a synthetic dipeptide with a molecular weight (M.W.) of 319.3 daltons, FK565: heptanolyl-γ-Glu-*meso*-DAP-D-Ala, a synthetic acyltripeptide with a M.W. of 502.6 daltons, FK156: D-lactyl-L-Ala-γ-Glu-*meso*- DAP-Gly, with a M.W. of 519.5 daltons, a synthetic tetrapeptide, originally isolated from culture filtrates of *Streptomyces* strains.

B. HCAEC (4 x 10^4 cells) were incubated with one of Nod1 stimulants, FK565, FK156 or iE-DAP. ICAM-1 expression (a) and CCL2 (b) /IL-8 (c) production in the culture

supernatants were examined at day 3. The concentrations are 1 (▫) or 10 (▪) μg/mL. Data are presented as mean ± s.d. (N=4). *: P <0.01, vs medium; †: P < 0.01, vs. FK 156; ‡: P < 0.01, vs. iE-DAP (Dunnett's test).

Supplementary Figure III. Immunohistochemial studies of coronary arteritis induced by oral administration of FK565.

All the sections were serial ones of aortic root from coronary arteritis model which was orally administrated by FK565 100μg for 6 consecutive days after priming of LPS 20μg i.p. Inflammatory cells infiltrating in the both coronary and aorta including valve consisted of many NIMP-R14-positive neutrophils, some F4/80-positive macrophages, and few CD3-T lymophocytes. Endothelial cells, smooth muscle cells and fibroblasts/myofibroblasts in addition to infiltrating inflammatory cells were apparently positive for NOD1. H&E stain (W: aortic root, x 40 (A: aortic valve (AV), B: coronary artery (CA)); A1: AV, x 400; B1: CA, x 400) and immunohistochemical stainings with Nod1- (A2: AV, B2: CA, x400), F4/80 (macrophage)- (A3: AV, B3: CA, x400), NIMP-R14 (neutrophil)- (A4: AV; B4: CA, x 400), CD3- (A5: AV; B5: CA, x 400), CD31- (B6: CA, x 400), and alpha smooth muscle actin- (B7: CA, x 400) specific antibodies.

Supplementary Figure IV. Microarray analysis of the gene expression of vascular tissues from BALB/c mice and human endothelial cells cultured with or without an innate immune stimulant.

A. The expression patterns of 44,170 genes in aortic root (AR), pulmonary artery (PA), and arch portion of aorta (aorta) *ex vivo* cultured for 24 hours with FK565 (10 μg/mL) by microarray analysis. B. The expression patterns of 44,931 genes in HCAEC and HPAEC *in vitro* cultured for 24 hours with FK565 (10 μg/mL) or lipid A (100 ng/mL) by microarray analysis. Blue-to-red scale indicates expression levels from low (under half) to high (A: over 4-fold, B: over 8-fold), compared with those with no stimulation (no change after stimulation: yellow).

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Histo-blood group gene polymorphisms as potential genetic modifiers of the development of coronary artery lesions in patients with Kawasaki disease

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Summary

Abnormal immunological responses to certain microbial agents may play a crucial role in the pathogenesis of Kawasaki disease (KD). The association studies between histo-blood group genes (Lewis and ABO blood types) and various types of infectious diseases or vasculopathy have been carried out based on the fact that glycosylated antigens could directly mediate microbial infections. We attempted to clarify the role of blood type antigens in the development of KD and coronary artery lesions in KD patients. The subjects included 164 KD patients enrolled from 1998 to 2003 (1st group), 232 patients from 2004 to 2009 (2nd group), and 223 healthy children and 118 patients with growth hormone deficiency as controls. The genotyping of the *FUT2* and *FUT3* genes, and ABO genotypes, was determined with the TaqMan SNP assay and allele-specific polymerase chain reaction. No significant differences were observed in the genotypes and allele frequencies of the *FUT2* and *FUT3* genes between the groups. The frequency of the BB blood genotype was significantly higher in KD patients with coronary artery lesions in the 1st and 2nd groups than in the controls (17% and 14% vs. 5%, $P = 0.0020$). This is the first report to investigate the roles of ABO and Lewis blood types in the development of KD, and in the formation of coronary artery lesions in KD patients. These data suggest that the ABO blood type may play a role in the development of coronary artery lesions in KD patients.

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Introduction

Kawasaki disease (KD) is an acute, self-limited systemic vasculitis which occurs predominantly in infants and young children. Coronary artery lesions (CAL) develop in 5–25% of untreated children with KD (Kawasaki, 1967; Newburger *et al.*, 2004). The aetiology of KD remains unknown. However, it has been elucidated that the development of KD might result from abnormal immunological responses against a wide range of microbial agents. The occurrence of KD has a pronounced seasonality and clustering in every country (Huang *et al.*, 2009), thus suggesting that specific infectious agents may be the contributing factor for the development of KD. On the contrary, clinical and epidemiological features suggest that the disease-inducing pathogens might not be specific micro-organisms but widely distributed infectious agents. We have recently reported that that the unique activation of the innate immune system was detected during the acute phase of KD (Ikeda *et al.*, 2010). From an epidemiological point of view, the differences in the incidence of KD among different ethnic groups and countries and the relatively higher incidence in family members of KD patients suggested that genetic factors might play an important role in the pathogenesis of KD (Newburger *et al.*, 2004). Indeed, we have previously reported that several genetic factors were associated with the development of KD and CAL formation in KD patients (Furuno *et al.*, 2007; Ikeda *et al.*, 2008; Kariyazono *et al.*, 2004).

The association studies between histo-blood group genes [Lewis(Le) and ABO blood types] and various types of infectious diseases have been carried out on the basis of the fact that glycosylated antigens could directly mediate microbial infections (Anstee, 2010). It is now recognized that there are many infectious diseases whose severities are directly linked to Lewis and ABO blood types, such as *Norwalk* virus, *Escherichia coli*, *Helicobacter pylori*, *Campylobacter jejuni*, *Dengue* virus infections and *Malaria* (Lindesmith *et al.*, 2003; Cserti & Dzik, 2007; Holmner *et al.*, 2007; Ilver *et al.*, 1998; Kalayanarooj *et al.*, 2007; Ruiz-Palacios

et al., 2003). In addition, the involvement of ABO(H) blood groups in cardiovascular diseases has also been studied for many years (Hein *et al.*, 1992; Ellison *et al.*, 1999; Hein *et al.*, 1993; Salomaa *et al.*, 2000). A meta-analysis showed that non-O blood groups had a higher odds ratio of 1.25 for myocardial infarction, and 1.79 for venous thromboembolism (Wu *et al.*, 2008).

The ABO blood type gene encodes a glycosyltransferase, and nucleotide substitutions in the gene determine the enzyme activity, *N*-acetyl D-galactosaminyl transferase (group A), D-galactosamyl transferase (group B) or a nonfunctional one (group O)(Yamamoto *et al.*, 1990). The Lewis antigen system is determined by the *FUT2* and *FUT3* genes. There are two main types of Lewis antigens, Lewis a and Lewis b, which are secreted from epithelial cells and located on the surface of erythrocytes. The distribution of the ABO blood types and *FUT2*, *FUT3* genotypes are quite different among ethnic groups and countries (Cserti & Dzik, 2007; Koda *et al.*, 2003, 1996).

The aim of this study was to evaluate the genetic contributions of histo-blood group gene polymorphisms to the development of KD, and CAL formation in KD patients. The seasonality of KD development for every blood type was also investigated on the hypothesis that independent infectious pathogens might trigger the development of KD in different seasons.

Materials and methods

Patients and controls

The KD patients who were admitted to Kyushu University Hospital or its affiliated hospitals between 1998 and 2009 were enrolled in this study. All patients met the criteria for the diagnostic guidelines of KD (http://www.kawasaki-disease.org/diagnostic/index.html). CAL was defined when the lumen diameter of the coronary artery was at least 3 mm (4 mm in cases when the patient were older than 5 years old) or the internal diameter of one or more segments was at least 1.5 times larger than that of the adjacent normal-size segments (Akagi *et al.*, 1992). Informed consent was obtained from the patients or their parents. The study population comprised 164 Japanese patients with KD enrolled from 1998 through 2003 including 71 patients with CAL and 93 patients without CAL (the 1st group), 232 patients enrolled from 2004 through 2009 including 28 patients with CAL and 204 patients without CAL (the 2nd group), and 223 healthy children (mean age 7.7 years, male : female = 114:109) or 118 patients with growth hormone deficiency (mean age 9.8 years, male : female = 87:31) as controls.

The 1st group consisted of KD patients with severe clinical phenotype, whose acute symptoms were intractable to intravenous immunoglobulin treatment, or who developed persistent CAL and referred to Kyushu University Hospital from the Kyushu District, whereas the KD patients in the 2nd group were with various grades of severity and most of them lived in Fukuoka City or surrounding areas and admitted to Fukuoka Children's hospital or Kyushu University Hospital. All patients were treated with intravenous immunoglobulin and oral aspirin. Clinical and laboratory data of KD patients in each group are shown in Table 1. This study was approved by the Ethical Committee of Kyushu University. Written informed consent was obtained from all the subjects.

DNA extraction

Whole blood was collected with sodium citrate or EDTA from all patients and controls. Genomic DNA was extracted from whole-blood leucocytes with the QIAamp Blood Kit (Qiagen, Tokyo, Japan).

ABO genotyping

According to a previous report, more than 90% of the major alleles for the ABO blood genotype can be determined by four nucleotide positions of the ABO gene for Japanese individuals: position 261 (del G) for the group O phenotype, and positions 796, 802 and 803 for the group A or B phenotype (Itoh, 2006). Hence, the ABO blood type was determined by a combination of two sets of TaqMan single nucleotide polymorphism (SNP) assays (Ogata *et al.*, 2007), using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan). The primers for polymerase chain reaction (PCR)-1 detect the SNP 261G>del (O allele) and those for PCR-2 detect the SNPs 796C>A and 803G>C (B allele). The sequences of primers and probes used for this assay were as follows (all oligonucleotide sequences are given 5′ to 3′): primers of PCR-1: O-forward, CCTGTGTGGATGTG CAGTAGGA; O-reverse, CGTTGAGGATGTCGATG TTGAA; probes of PCR-1: AB-Fam, Fam-TCCTCGT GGTGACCCCTTGGC-TAMRA; O-Vic, Vic-ATGTC CTCGTGGTACCCCTTGGCT-TAMRA; primers of PCR-2: B-forward, CTGCACCTCTTGCACAGGA; B-reverse, AGGCCTTCACCTACGAGCG; probes of PCR-2: AO-Fam, Fam-CCCGAAGAACCCCCCCAG GTAGTAGAAA-TAMRA; B-Vic, Vic-CCCGAAGAA CGCCCCCATGTAGTAGAAA-TAMRA. The accuracy of the ABO blood genotyping was verified with the comparison of serologic testing of the data for 34 patients or controls.

Table 1. Clinical and laboratory data of Kawasaki disease (KD) patients in each group

	1st group (n = 164)	2nd group (n = 232)	
Variables	Mean	Mean	P value*
Age (months)	28.1 ± 26.0	28.4 ± 21.6	0.901
Gender (male : female)	105:59	125:107	0.0378
Start of IVIG (day of illness)	5.2 ± 1.8	5.1 ± 1.3	0.370
White blood cell count (10^3/μL)	14.27 ± 4.85	15.18 ± 4.85	0.105
C-reactive protein (mg/dL)	9.6 ± 5.9	8.7 ± 5.1	0.0863
Serum Na (mmol/L)	135.3 ± 3.1	134.5 ± 2.8	0.0134
AST (IU/L)	99.2 ± 178.0	101.0 ± 201.8	0.926
ALT (IU/L)	99.0 ± 156.0	93.4 ± 154.6	0.735
Platelet count (10^4/μL)	35.4 ± 12.2	33.9 ± 10.5	0.196

IVIG, intravenous immunoglobulin therapy.
*Student's t test.

FUT2 genotyping

Polymorphisms with loss of function in the *FUT2* (nonsecretor) gene were found in about 25% of Japanese, and the 385A>T SNP in the *FUT2* gene (rs1047781: se2) and the deletion polymorphism (se5) accounted for about 95% of the nonsecretor phenotypes in the Japanese population (Koda *et al.*, 1996). The 385C>T polymorphism (se2) was genotyped by the TaqMan SNP Genotyping Assay. The fusion/deletion polymorphism (se5) was analysed by an allele-specific polymerase chain reaction.

FUT3 genotyping

The nonfunctional alleles of the *FUT3* gene (Lewis negative) were found in 10% of Japanese patients, and the G allele at the 59T>G and the A allele at the 508G>A SNPs (59T>G, rs28362459; 508G>A, rs 874232: le1) account for 90% of the Lewis negative phenotype in the Japanese population (Nishihara *et al.*, 1994). These polymorphisms were also genotyped using the TaqMan SNP Genotyping Assay.

Lewis blood typing

The Lewis blood type was determined based on the combination of the *FUT2* and *FUT3* genotypes. A lack of expression of FUT3 (le/le genotype) will result in a Le(a-b-) phenotype, irrespective of the *FUT2* genotypes. The Le(a+b-) phenotype is determined by the combination of positive expression of FUT3 (Le/Le or Le/le) and negative expression of FUT2 (se/se), whereas the Le (a-b+) phenotype is determined by the combination of positive expression of FUT3 (Le/Le or Le/le) and positive expression of FUT2 (Se/Se or Se/se).

Seasonality of the KD onset

As detailed information from the clinical record was available for the KD patients in the 2nd group, we evaluated the seasonal trend of KD onset for the 2nd group patients divided by ABO genotype and Lewis blood types.

Statistical analysis

The genotype or allele distribution and frequencies of blood types were analysed by a Chi-square test analysis with contingency tables. The seasonality of the KD onset for every blood type was analysed with a goodness-of-fit Chi-square test. To account for multiple comparisons, we used a strictly conservative approach by applying the Bonferroni correction to the genotype numbers, and the corrected P-values (Pc) were calculated as follows: $Pc = P\text{-value} \times 6$ (for ABO genotype) or $\times 3$ (for Lewis type). All statistical operations were performed by using the JMP 8 statistical software package (SAS Institute Inc., Cary, NC).

Results

ABO genotype

The frequencies of each blood group are presented in Table 2. The frequency of the BB blood type was significantly higher in KD patients with CAL in the 1st group than that in controls [17% vs. 5%; P = 0.00091; OR, 3.88 (95% CI, 1.76–8.53); Pc = 0.0055]. Regarding the 2nd group of KD patients, the frequency of the BB blood type was similarly higher in the KD patients with CAL than that in controls, although the difference in these patients did not reach a statistical significance [14% vs. 5%; P = 0.11, OR = 3.18 (95% CI, 0.99–10.19)], probably because the number of patients with CAL in the 2nd group was considerably smaller than in the 1st group.

The frequency of the AB blood type was significantly lower in the KD patients in the 2nd group than in controls [4% vs. 14%; P = 0.00076, OR = 3.20 (95% CI, 1.58–6.51); Pc = 0.0046], whereas the frequency of the AB blood type in KD patients in the 1st group did not show the same tendency.

Lewis blood type

As shown in Table 3, no significant differences were observed in the genotypes and allele frequencies of the *FUT2* and *FUT3* genes between the groups. The

Table 2. Frequencies of ABO genotypes in Kawasaki disease (KD) patients with and without coronary artery lesions (CAL) in the 1st and 2nd groups and in control subjects

		KD 1st group			KD 2nd group		
	Control	Total	CAL(+)	CAL(-)	Total	CAL(+)	CAL(-)
Genotype	*n* = 341	*n* = 164	*n* = 71	*n* = 93	*n* = 232	*n* = 28	*n* = 204
AA	0.12	0.11	0.11	0.12	0.07	0.11	0.07
AO	0.29	0.22	0.21	0.23	0.33	0.25	0.34
BB	0.05[a,b,c]	0.11	0.17[a,c]	0.08	0.06	0.14[b,c]	0.05
BO	0.15	0.20	0.18	0.20	0.16	0.04	0.19
AB	0.13[d,e]	0.13[d]	0.10	0.16	0.04[e]	0.07	0.04
OO	0.27	0.22	0.23	0.22	0.32	0.39	0.31

[a]BB in the KD 1st group, CAL(+) (*n* = 71) vs. in Control (*n* = 341): *P* = 0.00091; OR 3.88 95% CI 1.76–8.53; *Pc* = 0.0055.
[b]BB in the KD 2nd group, CAL(+) (*n* = 28) vs. in Control (*n* = 341): *P* = 0.11; OR 3.18; 95% CI 0.99–10.19; *Pc* = 0.66.
[c]BB in the KD 1st and 2nd groups, CAL(+) (*n* = 99) vs. in Control (*n* = 341): *P* = 0.0020; OR 3.67; 95% CI 1.78–7.58; *Pc* = 0.012.
[d]AB in the KD 1st group, total (*n* = 164) vs. in Control (*n* = 223): *P* = 0.86; OR 0.95 95% CI 0.55–165; *Pc* > 1.0.
[e]AB in the KD 2nd group, total (*n* = 232) vs. in Control (*n* = 223): *P* = 0.00076; OR 3.20; 95% CI 1.58–6.51; *Pc* = 0.0046.

Table 3. Frequencies of FUT2 and FUT3 genotypes in Kawasaki disease (KD) patients with and without coronary artery lesions (CAL) in the 1st and 2nd groups and in control subjects

				KD 1st group			KD 2nd group		
			Control	Total	CAL(+)	CAL(-)	Total	CAL(+)	CAL(-)
Gene	Polymorphisms	allele	*n* = 341	*n* = 164	*n* = 71	*n* = 93	*n* = 232	*n* = 28	*n* = 204
FUT2	Se/se2	Se allele	0.56	0.54	0.53	0.56	0.62	0.50	0.64
		se2 allele	0.44	0.46	0.47	0.44	0.38	0.50	0.36
	W/se5	W allele	0.97	0.97	0.96	0.97	0.96	0.96	0.96
		se5 allele	0.03	0.03	0.04	0.03	0.04	0.04	0.04
FUT3	G/t	G allele	0.63	0.64	0.62	0.65	0.65	0.63	0.65
		t allele	0.37	0.36	0.38	0.35	0.35	0.37	0.35

W, wild type.

Table 4. Frequencies of Lewis blood types in Kawasaki disease (KD) patient with and without coronary artery lesions (CAL) in 1st and 2nd groups and in control subjects

		KD 1st group			KD 2nd group		
	Control	Total	CAL(+)	CAL(-)	Total	CAL(+)	CAL(-)
Lewis blood type	*n* = 341	*n* = 164	*n* = 71	*n* = 93	*n* = 232	*n* = 28	*n* = 204
a-b+	0.69[a,b,c]	0.59	0.55[a,c]	0.64	0.73	0.64[b,c]	0.74
a+b-	0.18	0.22	0.24	0.20	0.15	0.18	0.15
a-b-	0.13	0.19	0.21	0.16	0.12	0.18	0.11

[a]Lewis(a-b+) in the KD 1st group, CAL(+) (*n* = 71) vs. in Control (*n* = 341): *P* = 0.0231; OR 0.550 95% CI 0.327–0.925; *Pc* = 0.069.
[b]Lewis(a-b+) in the KD 2nd group, CAL(+) (*n* = 28) vs. in Control (*n* = 341): *P* = 0.610; OR 0.812 95% CI 0.363–1.82; *Pc* > 1.0.
[c]Lewis(a-b+) in the KD 1st and 2nd groups, CAL(+) (*n* = 99) vs. in Control (*n* = 341): *P* = 0.0355; OR = 0.612 95% CI 0.339–0.970; *Pc* = 0.11.

frequency of the Le(a-b+) phenotype tended to be lower in the KD patients with CAL in the 1st group than in controls, although the difference was not statistically significant after the multiple comparison correction by Bonferroni method. [55% vs. 69%; *P* = 0.0231, OR = 0.550 (95% CI, 0.327–0.925); *Pc* = 0.069] (Table 4). Among the KD patients with CAL in the 2nd group, the frequency of Le(a-b+) was slightly lower than that in controls [64% vs. 69%; *P* = 0.610, OR = 0.812 (95% CI, 0.363–1.82)]. The trend of low frequency of Le(a-b+) seemed consistent in both KD groups, but we concluded that this result was not statistically significant after the Bonferroni correction (*P* = 0.0335; *Pc* = 0.11).

Table 5. Seasonality of the ABO genotypes and Lewis blood types in the 2nd group (*n* = 232)

	ABO genotypes						
	AA	AO	BB	BO	AB	OO	Total
Winter	4 (0.017)	16 (0.069)	2 (0.009)	10 (0.043)	3 (0.013)	21 (0.091)	56 (0.241)
Spring	3 (0.013)	16 (0.069)	8 (0.034)	10 (0.043)	3 (0.013)	21 (0.091)	58 (0.250)
Summer	4 (0.017)	24 (0.103)	2 (0.009)	11 (0.047)	0 (0.000)	13 (0.056)	53 (0.228)
Autumn	6 (0.026)	21 (0.091)	2 (0.009)	8 (0.034)	4 (0.017)	20 (0.086)	65 (0.280)
Total	17 (0.073)	77 (0.332)	14 (0.060)	39 (0.168)	10 (0.043)	75 (0.323)	232 (1.000)

	Lewis blood types			
	Le (a-b+)	Le (a+b-)	Le (a-b-)	Total
Winter	40 (0.172)	8 (0.034)	8 (0.034)	56 (0.241)
Spring	40 (0.172)	7 (0.030)	11 (0.047)	58 (0.250)
Summer	40 (0.172)	8 (0.034)	5 (0.022)	53 (0.228)
Autumn	49 (0.211)	12 (0.052)	4 (0.017)	65 (0.280)
Total	169 (0.728)	35 (0.151)	28 (0.121)	232 (1.000)

Winter: January to March, Spring: April to June, Summer: July to September, Autumn: October to December.

Seasonal trend

We also examined the distributions of ABO genotypes and Lewis blood types in the 2nd KD group divided by seasons of KD onset. There was no significant seasonal trend in KD development regarding the ABO genotypes or Lewis blood phenotypes (Table 5).

Discussion

To the best of our knowledge, this is the first report to investigate the roles of ABO and Lewis blood types in the development of KD, and in CAL formation in KD patients. This study showed that the BB blood type was a facilitating factor for CAL formation in patients with KD. Interestingly, epidemiological data have shown that B blood type was more frequent in the Asian population than in Western countries (22% in Japan; 23% in Taiwan; 11% in U.S.; 7% in France) (http://www.bloodbook.com/world-abo.html. Accessed 18 July 2011) and that the prevalence of KD was higher in Japanese or Chinese populations than in other ethnic ancestries (Dean *et al.*, 1982). In addition, the previous data from the United States demonstrated that the incidence of CAL or coronary artery abnormalities in children of Asian origin was the highest than those of other ethnic backgrounds (Porcalla *et al.*, 2005). The results of this study may provide a clue to elucidate why the incidences of KD and CAL are higher in Asian children, especially in Japanese children, than that in Western children (Newburger *et al.*, 2004).

Our study showed a significantly lower frequency of the AB blood type in the 2nd group, but the same tendency was not detected in the 1st group. This discrepancy may imply the possibility that pathogenic substances from bacteria might be different during every outbreak of KD. To investigate this possibility, we examined the seasonal trend of KD onset divided by ABO genotype and Lewis blood types with the hypothesis that different pathogens might trigger the development of KD in every season. However, our data revealed no significant differences in the ABO genotype or Lewis blood type in KD patients among seasons. This result supports the recent hypothesis that specific pathogens with seasonality or population clustering would be less likely than otherwise nonspecific normal microorganisms to be the potentially relevant pathogens related to the development of KD or to CAL formation in KD patients.

In this study, the frequency of Le(a-b+) was lower in KD patients with CAL, and the frequency of Le(a-b-) tended to be higher, although it was not statistically significant. A previously reported paper demonstrated that Le(a-b-) was a high-risk marker and Le(a-b+) was a resistance (low-risk) marker for the development of ischaemic heart disease (Zhiburt *et al.*, 1997). In the Copenhagen Male Study, the males with Le(a-b-) had a 4.4 times high risk of death from ischaemic heart disease, and a higher serum triglyceride level, higher body mass index and a lower high-density lipoprotein (HDL) cholesterol concentration than those with other Lewis phenotypes (Hein *et al.*, 1992). The genes encoding the *FUT2* and *FUT3* are located on chromosomes 19q13.3 and 19p13.3, respectively (Clausen *et al.*, 1995). Interestingly, the glycogen synthase1 (GYS1) and LDL receptor (LDLR) genes are also located on the chromosome 19q13.3 and 19p13.3, and in particular, the genetic distance between the *FUT2* and *GYS1* genes is only 200kb (Couture *et al.*, 1999; Majer *et al.*, 1996). It might be possible that the development of CAL in patients with KD and ischaemic heart disease in adulthood have common genetic factors (Couture *et al.*, 1999) and that the adjacent location of susceptibility genes might have synergistic effects on the development of the diseases.

Alternatively, the results from genetic study of *FUT2* and *FUT3* genes might be essentially affected by the SNPs or haplotypes in any other genes under linkage disequilibrium, therefore the secondary effects by neighbour genes should be taken into account. Further study is mandatory to investigate the validity of this speculation.

There is limitation that needs to be acknowledged and addressed regarding the present study. The sample size in this study did not have a sufficient power for statistical analysis of CAL formation in patients with KD. Because there were only 28 patients with CAL in the 2nd group, this evaluation may be under powered to detect a difference, although the frequency of the BB blood type and Le (a-b+) in the 2nd group had a similar tendency as in the 1st group.

In conclusion, our study has suggested the role of ABO and Lewis blood group gene polymorphisms as a genetic modifier of the development of CAL in patients with KD. Further evaluation with a larger study population is necessary to confirm the results of this study.

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Lecture

解　説

川崎病血管炎の病態*

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Key Words：Kawasaki disease, vasculitis, pathophysiology, coronary arterial lesion

はじめに

川崎病は1967年に川崎富作博士により，小児急性熱性皮膚粘膜淋巴腺症候群(acute febrile mucocutaneous lymph node syndrome：MCLS)として報告された[1]．川崎病の本態は主に中小動脈を中心とする小児期の全身性血管炎症候群であり，特に冠動脈を傷害するが，その病態はいまだに明らかではない．

川崎病の病因と病態に関するさまざまな報告がなされているが，川崎病の発症に関する因子および血管炎，特に冠動脈病変への進展機序に関する因子の研究に大別される(表1)．

本稿では，川崎病の冠動脈病変の経時的変化から血管炎への進展に関与するとされる代表的な因子の研究について概説したい．

川崎病冠動脈病変の経時的変化

急性期の冠動脈病変については，心エコー上，平均5.4病日に冠動脈壁のエコー輝度の増強が全症例で観察される[2]．病理組織学的には，まず急性期早期に流血中の好中球が著増し，単球・マクロファージの血管内膜への細胞浸潤を認め，この結果，第6～8病日には動脈中膜の水腫性疎開性変化をきたす．続いて，第8～10病日には冠動脈内外膜両側から単球，マクロファージなどの炎症細胞浸潤が起こり，第12病日頃には血管全周に細胞浸潤をきたす汎血管炎へと進展する．病変内に浸潤する細胞は単球・マクロファージを主とするが，好中球も相当数出現しており，細胞浸潤部に一致して中膜は浮腫状の間隙を形成し，平滑筋細胞のアクチンフィラメントや基底膜コラーゲンは消失している．このことから，発病初期の動脈傷害過程には単球・マクロファージとともに好中球が産生，分泌する蛋白分解酵素や活性酸素などが関与していると推測される．その後，汎動脈炎は速やかに動脈全周に波及する．内弾性板や中膜など動脈構築を保つ上で重要な成分は著しく傷害され，発症後12病日頃に風船が膨らむように動脈の拡張が生じる．動脈瘤は肉眼的に球状，紡錘状の瘤として認識され，多くの場合，血栓が内腔に充満する[3]．炎症極期を迎えた冠動脈には単球・マクロファージの著明な浸潤とともに，少数のリンパ球，好中球を混ずる増殖性肉芽腫性炎症がみられる．激しい炎症は25病日頃まで継続したあとに，徐々に消退し始め，40病日頃にはほぼ消失する(図1)．

これまでに，川崎病の急性期ではさまざまな炎症性サイトカインやケモカイン，接着分子，血管作動物質などが活性化あるいは亢進していることが示されているが，病変局所においても炎症に関する諸因子が発現していることが示されている[4](表1)．

川崎病の病因と病態の仮説

川崎病の病因および病態に関しては，発見から半世紀を過ぎた現在においてもなお未知の部分が多い．しかし，発症機序は不明であるが，川崎病の炎症の進展に関しては次のような仮説が考えられるようになってきている．

まず，なんらかの環境因子や微生物の感染が引き金となり，自然免疫担当細胞においてNF-κBのシグナリングが活性化され，それに伴いpro-inflammatory cytokineであるTNF-α，IL-1，IL-6，IL-8，MCP-1，G-CSF，M-CSFなどが分泌される高サイトカイン血症となる[5]．それらによって細胞の相互作用が促進され，endothelial cell specific cytokinesであるVEGF，S100A12，myeloid-related protein(MRP)8/MRP14などが分泌されると，全身の血管炎が惹起され川崎病が発症する．さらに，人種差やITPKC遺伝子のSNPなどの遺伝的素因が川崎病の発症および進展に大きく関与していると考えられている[6,7](図2)．

NF-κB

川崎病急性期には血清中に多くの炎症性サイトカイン，ケモカイン，細胞間接着因子が上昇しているが，これらの発現・産生には遺伝子からmRNAの転写にNF-κBの活性化を必要とする．NF-κBは細胞質内に非活性の状態で存在しており，

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表1　冠動脈病変形成との関連が検討された候補遺伝子

遺伝子	遺伝子座	文献
ACE	17q23	Takeuchi(1997)，Fukazawa(2004)，Wu(2004)*，Shim(2006)*
AGTR1	3q21-q25	Fukazawa(2004)
BAT2	6p21.3	Hsieh(2010)
BAT3	6p21.3	Hsieh(2010)
BAT5	6p21.3	Hsieh(2010)
BTNL2	6p21.3	Hsueh(2010)
CASP3	4q34	Kuo(2010)
CD14	5q31.1	Nishimura(2003)
CD40L	Xq26	Onouchi(2004)，Huang(2008)*
COL11A2	6p21.3	Sheu(2010)
CRP	1q21-q23	Cheng(2008)*
CTLA4	2q33	Kuo(2010)
eNOS	7q36	Khajoee(2003)*
FCGR2Aa	1q22	Taniuchi(2005)，Biezeveld(2007)*
IL-10	1q31-q32	Jin(2007)，Weng(2010)
iNOS	17cen-q11.2	Khajoee(2003)*，Hirono & Ichida(2004)
ITPKC	19q13.1	Onouchi(2008)
ITPR3	6p21	Huang(2010)
MBL	10q11.2-q21	Biezeveld(2003)，Biezeveld(2006)
MICA	6p21.3	Hunag(2000)
MIF-173	22q11.23	Simonini(2009)
MMP3	11q23	Park(2005)，Ikeda(2008)*，Simuzu(2010)
MMP9	20q11.2-q13.1	Lau(2009)
MMP12	11q22.3	Simuzu(2010)
MMP13	11q22.3	Ikeda(2008)
MTHFR	1p36.3	Tsukahara(2000)
RAGE	14q32	Hirono & Ichida(2007)
S100A12	1q21	Hirono & Ichida(2003)(2004)(2007)
S100A8	16q12.1	Hirono & Ichida(2005)(2006)(2009)，Abe(2005)
S100A9	1q21	Hirono & Ichida(2005)(2006)(2009)，Abe(2005)
TGF-β	19q13.1	Shimizu(2010)
TIMP2	17q25	Furuno(2007)
TNF-α	6p21.3	Quansney(2001)，Cheung(2008)*，Hirono & Ichida(2009)*
VEGF	6p12	Hirono & Ichida(2001)(2009)，Kariyazono(2004)，Huang(2008)*
VEGFR2	4q12	Kariyazono(2004)

* 関連を否定する内容の論文

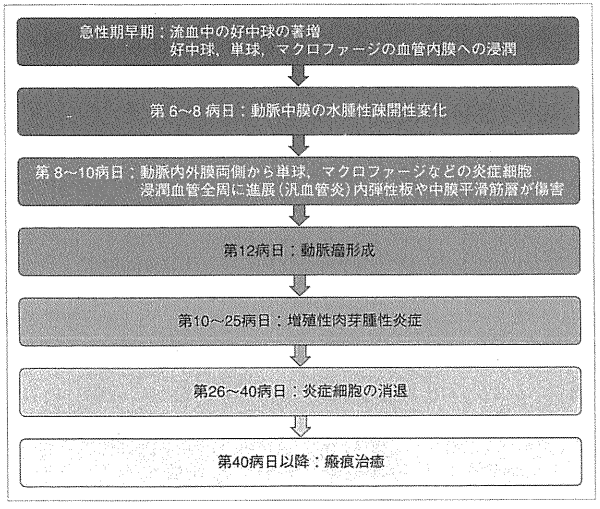

図1　冠動脈病変の経時的変化

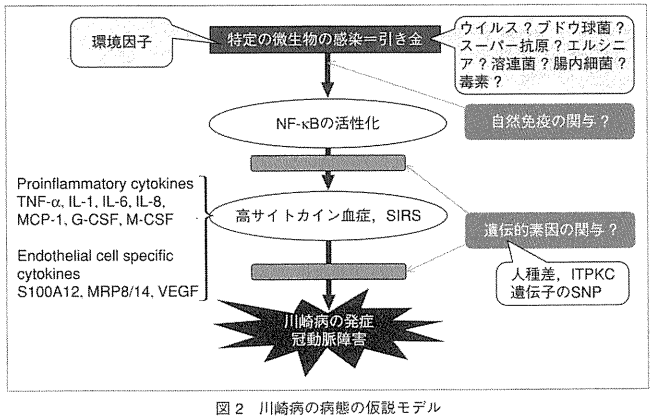

図2　川崎病の病態の仮説モデル

なんらかの刺激により活性化を抑制する蛋白IkBαのリン酸化による分解消滅が起こると核内に移行し，特異的なDNA配列に結合しNF-κBは活性化され，サイトカイン遺伝子の転写が始まりサイトカインの産生が起こる．

川崎病の末梢血では急性期に単球/マクロファージでのNF-κB活性化が顕著であり，免疫グロブリン静注（IVIG）投与後に活性化が速やかに低下する[8]．血管病変においても単球/マクロファージが多数浸潤し，NF-κBの活性化により炎症をひき起こしていることが考えられる．

TNF-α

川崎病急性期では血中TNF-αが上昇し，血中濃度と冠動脈瘤の形成に有意な相関があることが知られている．1988年に古川らは川崎病の血中TNF-α値の上昇を報告し，さらに1994年に血中TNF receptor（TNF-R）type 1の上昇と血中TNF-R1の高い症例では冠動脈病変の併発が高いことを明らかにした[9][10]．

これらの事実に基づき，難治性川崎病においても抗TNF-α抗体による抗サイトカイン療法が行われるようになってきている．2004年にWeissらが，2005年にBurnsらが，難治川崎病症例への抗TNF-α抗体であるインフリキシマブの投与例を発表して以来，国内においても，IVIG療法不応例に対してインフリキシマブ投与例が散見され，IVIG療法不応例に対する追加治療の一つとして選択されるようになってきている[11][12]．

われわれは，実際の川崎病の血管炎に対するTNF-αの関与を検討するために，インフリキシマブ投与前後のサイトカインの動態を検討した．11人のIVIG療法またはIVMP療法不応の難治性川崎病患者の検討では，インフリキシマブ投与例ではSTNFR，IL-6はインフリキシマブ投与前に有意に高値を示し，投与後に低下したが，血管炎を示唆するMRP8/MRP14およびS100A12はむしろインフリキシマブ投与後に増加し，同様にVEGFは変化なく高値を示し続けた．その結果，抗TNF-α抗体の投与では血管炎をひき起こすサイトカインであるMRP8/MRP14やS100A12，VEGFが抑制されないことが明らかとなり，血管炎においてはTNF-α以外の因子の関与が示唆された[13]．

VEGF

VEGFは細胞分裂や遊走，分化を刺激したり，微小血管の血管透過性を亢進させる作用を持ち，単球/マクロファージの活性化にも関与する糖蛋白である．主に血管内皮細胞表面にあるVEGF受容体（VEGFR）にリガンドとして結合し，機能を発揮する．その分子としてはVEGF-A，VEGF-B，VEGF-C，VEGF-D，VEGF-E，PlGF（胎盤増殖因子：placental growth factor）-1，PlGF-2の7種があり，それぞれ臓器特異性のある受容体に結合する（表2）．

表2　VEGFの種類

種類	機能
VEGF-A	血管新生 血管内皮細胞の遊走↑ 内皮細胞の有糸分裂↑ メタンオキシゲナーゼ活性↑ αvβ3活性 血管管腔形成 マクロファージおよび顆粒球の走化性亢進 血管拡張（間接的なNOの放出による）
VEGF-B	胎児期の血管新生
VEGF-C	リンパ脈管新生
VEGF-D	肺気管支を取り巻くリンパ血管の発達
PlGF	血管発生，虚血，炎症，創傷治癒時の血管新生

VEGFRにはVEGFR-1～3の3種類が同定されている．VEGFR-2（Flk-1/KDR）はほとんどすべての内皮細胞表面に発現しており，VEGF-A, E, Cが結合する．VEGFR-1（Flt-1）およびVEGFR-3（Flt-4）は特定の一部の内皮細胞に発現しているのみであり，Flt-1にはVEGF-A, Bが，Flt-4にはVEGF-C, Dがそれぞれ結合する．

川崎病急性期においてVEGFの血中濃度はIVIG治療前後において高値を示し，とりわけ冠動脈病変例においてIVIG治療後も高値を示すことが報告されている[14]．また，急性期に血漿蛋白が血管外へと漏出するや血中VEGF濃度と血中アルブミン濃度が負の相関を示すことが報告され，川崎病の血管炎においてVEGFが蛋白の漏出と炎症細胞の浸潤に関与し，冠動脈病変への進展を促進させていることが考えられている[15]．

川崎病における末梢血の好中球および単核球細胞中のVEGFの発現の検討では，IVIG治療前は好中球でのVEGFの発現が優位であり，IVIG治療後では単核球細胞においてVEGFの発現が優位であり，発症後2週までピークを示した．また，冠動脈病変合併例では単核球細胞でのVEGFの発現が高い傾向を認めた[16]．さらに，冠動脈瘤を形成する時期と考えられている発症後10～12病日前後では，血管内膜へVEGFの発現が亢進した単核球細胞の浸潤を多数認めた（図3, 4）．

また，川崎病回復期以後の冠動脈の病理組織

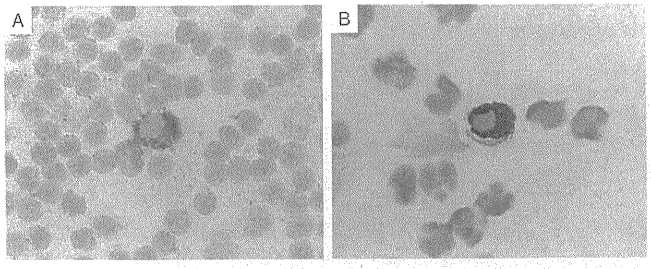

図3　流血中血管内皮細胞におけるiNOSの発現
A：P1H12陽性細胞（血管内皮細胞），B：iNOS陽性細胞（文献[16]より引用改変）

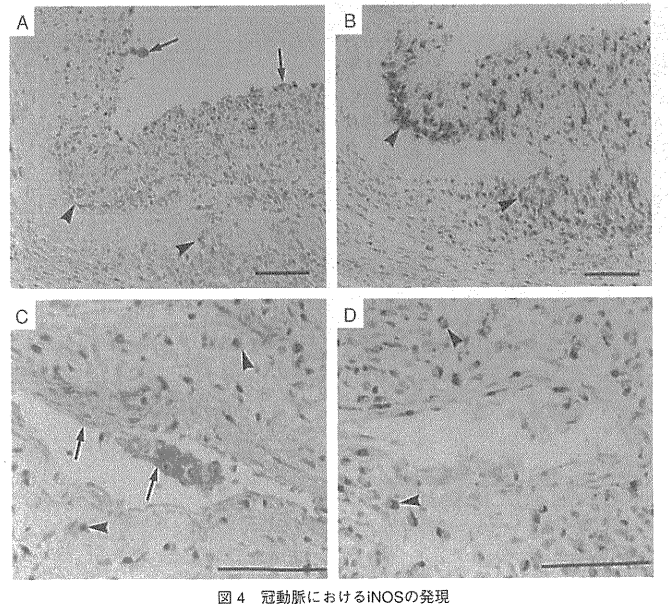

図4　冠動脈におけるiNOSの発現
A：症例1，B：症例2，C：症例1の強拡大，D：症例2の強拡大．矢印：血管内皮細胞，矢頭：iNOS陽性細胞
（文献[16]より引用改変）

の検討では，冠動脈病変を有しない例においても，VEGF，TGF-β，iNOS，PDGF-Aを発現した平滑筋細胞が内膜および中膜に認められることが報告されている[17]．このことから，回復期以後も，冠動脈においてもリモデリングが活性化されいてることが示唆されている．

iNOS

一酸化窒素合成酵素（NOS）は3つに大別される（表3）．このうちendothelial NOSは血管内皮

表3　NOSの種類

名称	遺伝子	発現	機能
内皮型NOS（eNOS）	NOS3	血管内皮細胞，骨髄細胞，血小板	血管拡張作用
誘導型NOS（iNOS）	NOS2A, NOS2B, NOS2C	免疫系，心血管系，肺	病原体に対する生体防御
神経型NOS（nNOS）	NOS1	神経組織，肺，腎臓	細胞間情報伝達

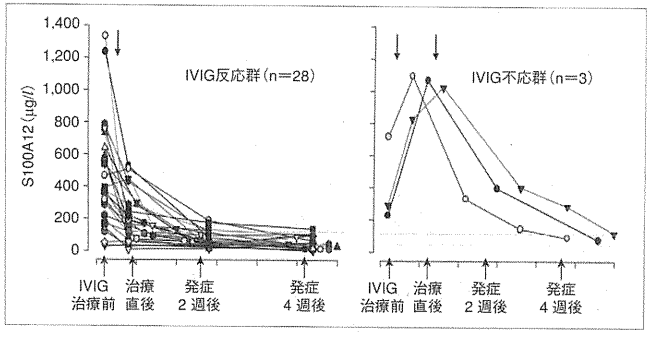

図5　血清中のS100A12濃度（文献[20]より引用改変）

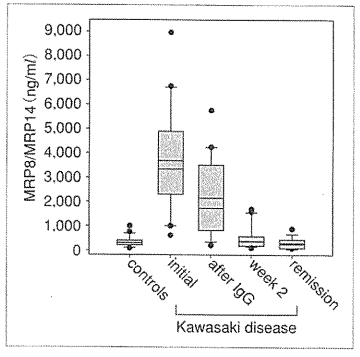

図6　血清中のMRP8/MRP14濃度
（文献[22]より引用改変）

細胞からカルシウムイオン依存性に恒常的に発現し，血管におけるさまざまな刺激に対する負の調節因子として働き，白血球の接着や血小板の凝集の阻害，平滑筋細胞の増殖や遊走の抑制などを誘導する．しかし，inducible NOSは炎症時に活性化されたIFN regulatory factor-1やNF-κBがiNOSのプロモーター領域に結合することにより炎症細胞から産生され，血管壁の変性やショックをひき起こすと考えられている．

末梢血におけるiNOSの発現に関する検討では，病初期は主に好中球に発現がみられ，その後，単核球細胞に発現がみられ，VEGFと同様の発現パターンを示し，冠動脈病変例においてiNOSの発現は強い傾向がみられた[18]．さらに，末梢血ではiNOS陽性の血管内皮細胞の増加がみられ，冠動脈の免疫染色では浸潤している炎症細胞および血管内皮細胞において，iNOSが発現していた（図3，4）．これらの所見は血管内皮細胞が，VEGFによりNOの分泌が誘導され，血管に傷害が与えられていると考えられる．

S100蛋白

S100蛋白ファミリーは炎症性組織に高濃度で認められ，自然免疫における重要なproinflammatory factorsである．その中でも特にMRP8（S100A8），MRP14（S100A9）やS100A12はdamage-associated molecular pattern（DAMP）moleculesとして知られ，これらは自然免疫反応における内在性のligandとして活性化することによって細胞内のシグナル伝達にかかわっている．

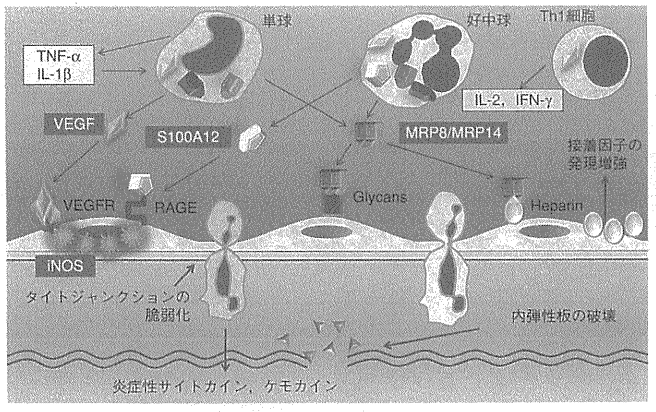

図7　川崎病における血管炎の仮説モデル（文献[13]より引用改変）

S100蛋白の一つであるS100A12は，川崎病急性期において冠動脈瘤形成期である発症2週をピークに好中球から大量に遊離している．分泌されたS100A12は血管内皮細胞に存在する受容体であるreceptor for advanced glycation end products（RAGE）に結合し，血管内皮細胞に炎症のシグナルを活性化することで，冠動脈病変の進行に関与していると考えられている[19][20]（図5）．

MRP8/MRP14（S100A8/S100A9）はプロテオグリカンやカルボキシNグリカンを介して血管内皮細胞に結合し，特異的な炎症性の反応を示し，MRP8/MRP14の発現が血管炎の炎症の活動性と相関する[21]．川崎病急性期では，顆粒球が主体となって産生されたMRP8/MRP14が血管内皮細胞に結合し，血栓形成および炎症反応が惹起され，血管炎が生じると考えられる（図6）．また，冠動脈病変群において流血中のMRP8/MRP14が結合した血管内皮細胞の増加がみられ，冠動脈病変の進展に重要な役割を担っていると考えられる[20][22]．また，MRP8/MRP14はToll-like receptor（TLR）4の内在性ligandの一つであり，この結合は正のフィードバックとなり，炎症性サイトカインの発現を著明に活性化し，血管炎を助長させる[23]．これらのS100蛋白は川崎病の血管炎において重要な増悪因子と考えられる（図7）．

おわりに

これまでに行われた川崎病の血管炎に関する研究を概説した．現在まで血管炎および冠動脈病変に関してさまざまな因子が報告されているが，川崎病の発症から，血管炎さらには冠動脈病変への進展機序についてはいまだに解明されていない部分が多い．今後は免疫学的な素因に加えて，遺伝的な素因とあわせて多方面からの検討が必要であると思われる．

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EDITORIAL

Possible New Role of Vascular Endothelial Growth Factor-D During the Acute Phase of Kawasaki Disease

Keiichi Hirono, MD, PhD; Fukiko Ichida, MD, PhD

Kawasaki Disease (KD) was first reported in 1967, yet the cause has not been established. A relationship between KD and vascular endothelial growth factor (VEGF) was initially established 13 years ago.[1–3] In this issue of the Journal, Ebata et al describe a role for VEGF-D during the acute stage of KD and report that its expression increased during lymphangiogenesis.[4] Here we review the role of VEGF in KD and its possible role during the acute phase of KD, especially in the development of coronary artery lesions (CAL).

Article p1455

KD is the most common systemic vasculitis syndrome primarily affecting small and medium sized arteries, particularly the coronary artery. Although timely treatment with high-dose intravenous immune globulin (IVIG) is now accepted as reducing the incidence of CAL, approximately 15% of patients do not respond to IVIG treatment and are at the highest risk for developing CAL.

The etiologic nature of KD and the pathogenesis of CAL largely remain to be determined. Previous histopathologic studies revealed that early coronary vascular lesions are infiltrated by a large number of mononuclear cells, such as macrophages and lymphocytes, leading to the assumption that these cells play a key role in the progression of CAL. In acute KD, the migration and proliferation of endothelial cells have been demonstrated to be markedly enhanced by cytokines. As a matter of fact, during the acute phase of KD, serum levels of proinflammatory cytokines are elevated.[5] In addition to these proinflammatory cytokines, expression of VEGF, and of markers of local inflammation, including the family of damage-associated molecular pattern molecules, such as myeloid-related protein (MRP) 8/MRP14 and S100A12, which respond to initiation of the innate immune response, increases during acute KD and plays a crucial role in inflammation.[3,6,7]

VEGF and their endothelial tyrosine kinase receptors are central regulators of vasculogenesis, angiogenesis and lymphangiogenesis. VEGF is expressed by a variety of cell types, including aortic smooth muscle cells, macrophages, and myocytes. In addition, lymphocytes, neutrophils, and platelets have recently been shown to express VEGF. Currently, 7 members in the VEGF family have been identified: VEGF-A, -B, -C, -D, -E (viral VEGF analogs), -F (snake venom VEGFs), and placental growth factor. VEGFs belong to the platelet-derived growth factor/VEGF superfamily of secreted dimeric glycoprotein growth factors that contain a cysteine knot motif consisting of 8 regularly spaced cysteine residues. The VEGF ligands bind with differing specificities to 3, mostly endothelial transmembrane tyrosine kinase, receptors: VEGFR-1/fms-like tyrosine kinase (Flt) 1, VEGFR-2/fuman kinase insert domain receptor/mouse fetal liver kinase 1 and VEGFR-3/Flt 4 (**Figure**). All VEGFRs have a conserved intracellular split tyrosine kinase domain and a series of extracellular Ig-like domains. Neuropilins-1 and -2, originally identified as semaphoring receptors, function as coreceptors for specific VEGFs. They share a common structure, yet they bind VEGFs with distinct affinities and specificities.

VEGF-D (also known as *c-fos*-induced growth factor) is secreted into the extracellular space as a full-length VEGF-D homodimer. After secretion, proprotein convertases cleave the C- and N-terminal propeptides from the VEGF-homology domain to form the mature VEGF-D, which binds to VEGFR-2 and -3 with higher affinities than the full-length unprocessed VEGF-D (**Figure**). However, as VEGF-D seems to be largely dispensable for the development of the lymphatic system, its physiological role remains unclear. Vascular endothelial cells express VEGFR-2, and its expression is upregulated inter alia by angiogenesis. VEGF-D exerts angiogenic effects on binding to VEGFR-2, whereas when bound to VEGFR-3 on lymphatic endothelium it stimulates lymphangiogenesis (**Figure**). VEGFR-3 is initially widely expressed in endothelial cells during murine embryogenesis, but later in development its expression becomes largely restricted to the lymphatic endothelium.[8] In adult human tissues, VEGFR-3 is specific to the lymphatic endothelium, with the exception of some fenestrated and discontinuous blood capillary bed.[9] VEGFR-3 phosphorylation has been shown to lead to PI3K-dependent Akt activation and PKC-dependent activation of the p42/p44 MAPK pathway.[10]

It has been suggested in several studies[1–3] that VEGF has a key role in the deterioration of vasculitis in the acute phase of KD. The studies have shown that serum and plasma levels of VEGF are significantly elevated in both the acute and subacute phases of KD and that circulating levels could be associated with the risk of developing CAL. It has been hypothesized that the high levels of VEGF could contribute to the pathologic findings observed in vascular tissue in KD, such as subendothelial edema, gap formation, and fenestrations of endothelial cells.[11] Several single-nucleotide polymorphisms in the *VEGF* gene have been found to be associated with differences in VEGF protein production.[12]

There have been many studies reporting a role for VEGF in KD, but the study by Ebata et al represents the first report of a relationship between VEGF-D and KD. They conclude that serum VEGF-D levels increased after IVIG therapy in patients with acute KD and were significantly lower in patients with CAL compared to those without CAL. They also showed that the cross-sectional areas of lymphatic vessels in cardiac tissues were enlarged in patients with acute KD while VEGF-D protein was detected on the endothelium of the enlarged lymphatic vessels. Lymphatic vessels are thought to be maintained in a collapsed state and are important for the drainage of interstitial fluid under pathological conditions.[13] Therefore, these new findings might reflect disruption or insufficiency of the regulation of lymphangiogenesis mediated by VEGF-D during the acute stage of KD.

Although further studies are required to elucidate the mechanism of how VEGF-D affects the outcome of KD, the evidence so far suggests that VEGF-D plays an important role and may serve as a biomarker for KD, as well as providing a novel therapeutic target.

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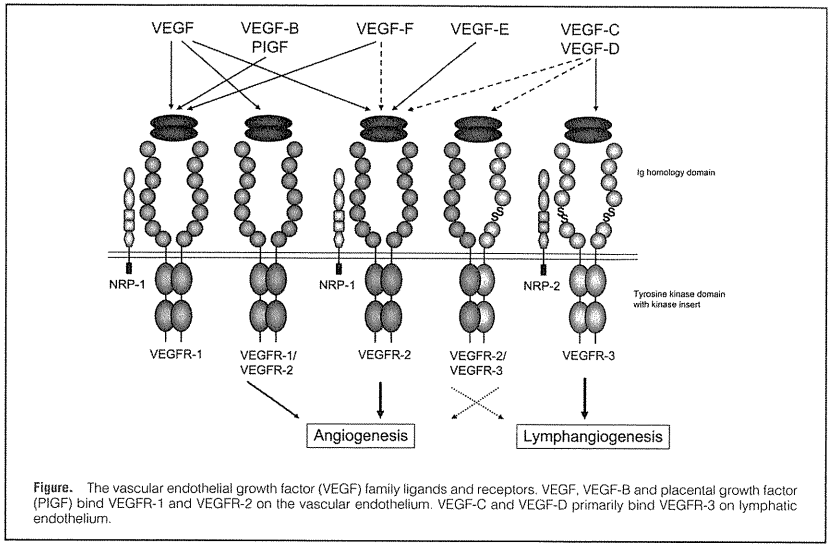

Figure. The vascular endothelial growth factor (VEGF) family ligands and receptors. VEGF, VEGF-B and placental growth factor (PIGF) bind VEGFR-1 and VEGFR-2 on the vascular endothelium. VEGF-C and VEGF-D primarily bind VEGFR-3 on lymphatic endothelium.

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特集　川崎病の本態にせまる－古くて新しい研究から－

Ⅰ．病態にせまる（研究成果から本態にせまる）

病原体から

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要旨　川崎病の感染症仮説について概説した．スーパー抗原が川崎病の原因かどうかはいまだ不明である．次世代シークエンサーなどの高度なゲノム解析技術とゲノムデータベースの拡張により未知の病原微生物が見つかる可能性は高まっているが，感染因子の探索だけではおそらく不十分であろう．血管炎発症の機序や免疫細胞の役割，遺伝子多型の関与といった要素を総合的に理解することが，川崎病の本態の解明には必要である．

Key words　スーパー抗原，毒素性ショック症候群，PCR法，次世代シークエンサー，ゲノムデータベース

はじめに

1987年12月，筆者はクリスマス・イルミネーションの灯るボストンにいた．前年にハーバード大学のBurnsらが提唱した川崎病レトロウイルス説の追試をするために，小林登国立小児病院長（当時）の紹介でボストン小児病院に患者検体を持参したのだった．もっと正確にいうと，筆者の指導者だった平間稔先生のかばん持ちとして特命使節に仲間入りさせてもらっただけだったが．結局，この時はレトロウイルス説を証明することはかなわなかった．しかし，末梢血細胞の培養上清を使って逆転写酵素活性を測定するという斬新な実験が，初めて訪れたボストンの街の静かな佇まいとともに研究生活に対する筆者の憧れを掻き立てたことは今も記憶に新しい．これが，筆者にとっての川崎病の病因研究の始まりだった．

川崎病の感染症仮説

いきなりモノローグから始めてしまったが，1967年に川崎富作先生が「急性熱性皮膚粘膜リンパ節症候群」を報告されて以来，川崎病の病因は今なおミステリーである．その臨床像や疫学調査からは，病原微生物による感染が強く疑われてきた．その根拠としては，①急激に発症し発熱がほぼ全例にみられる，②皮膚の発疹，粘膜の発赤，結膜充血，リンパ節腫脹など，感染症に多い症状が主体である，③これらの症状は前後して現れるが，一過性の経過をたどることが多く再燃はまれである，④一般的なウイルス感染で生じる上気道炎症状や胃腸炎症状が発症の数日前にみられることが多い，⑤年間を通じて発症するが，冬から春にかけて季節的な発症のピークがある，⑥全国的な流行あるいは地域的な小流行がみられる，などがあげられる[1]．

さらに感染症らしさを裏づける事象として，①発症が乳幼児期に集中している，②母親からの受動免疫が残る新生児期や乳児期早期には発症が少ない，などの疫学調査の結果がある．乳児の代表的なウイルス感染症である突発性発疹症などと比べ川崎病の患者数が少ないのは，大半の小児は病原体に曝露する機会は多いが川崎病を発症せずに免疫を獲得し，その免疫は成人以降も残存するためではないかと考えられる．あるいは，川崎病を発症しないままで病原体を終生保持し続ける可能性も考えられる．しかし一方で，川崎病を感染症と考えると矛盾する事象もある．しばしば指摘されることだが，ヒトからヒトへの感染が証明されないことである．家族内での同時感染が少ない理由は，年長の同胞や両親はすでに病原体に対する免疫を獲得しているからとも考えられるが，保育所での集団発症や院内感染がみられないことは，この年齢層での感染症としては特異なことである．

さて，このように複雑な臨床像，疫学像を示す川崎病の条件に合致するような感染症ははたして存在するのだろうか．**表1**に，これまでに川崎病の病因として提唱されてきた感染仮説を示す．これらの仮説を証明するために用いられている実験法は，細菌学的な菌株の分離やウイルスの分離をはじめとして，血清診断（seroconversion）やその他の宿主側の反応（白血球の表面抗原やリンパ球の抗原レセプター，細胞増殖能など）を調べたもの，さらに逆転写酵素活性の測定やPCR（polymerase chain reaction）法を使って微生物の核酸を増幅して検出する方法など，実に多様である．いずれも，急性期の川崎病患者と健常対照あるいは発熱対照患者とを比べて有意差があると報告されたが，他のグループによる追試の結果は必ずしも一致していない．多くの研究者が創意工夫して病原体探しに挑戦してきたにもかかわらず，川崎病の感染症仮説を証明することはいまだできていないのが現実である．

本稿では，最近も研究報告が続いている川崎病のスーパー抗原仮説について概説するとともに，近年，発展の著しいゲノム解析技術を用いた病原体探索の可能性について私見を述べたい．

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表1　川崎病の病因仮説（感染症関連）

細菌・病原生物による感染	*Rickettsia* *Candida* ダニ抗原 *Streptococcus pyogenes* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Propionibacterium* *Streptococcus sanguis* *Yersinia*偽結核菌 *Mycoplasma* *Chlamydia*，など
ウイルス感染	ロタウイルス Epstein-Barrウイルス サイトメガロウイルス RSウイルス アデノウイルス レトロウイルス パルボウイルスB19 ヒトコロナウイルス，など
スーパー抗原	*Staphylococcus aureus*由来（TSST-1） *Streptococcus pyogenes*由来（SPECなど） 腸内細菌由来（未同定）
スーパー抗原以外の微生物由来産物	LPS（グラム陰性菌） 熱ショック蛋白（おもにグラム陰性菌）

TSST：toxic shock syndrome toxin，SPEC：streptococcal pyogenic exotoxin type C，LPS：lippolysaccharide

川崎病のスーパー抗原仮説

スーパー抗原は，*Staphylococcus aureus*や*Streptococcus pyogenes*などが分泌する外毒素の一種で，強力な炎症惹起作用をもつ蛋白である．スーパー抗原という命名は，抗原提示細胞のHLA（human leukocyte antigen）分子とT細胞の抗原レセプターとに直接結合することにより，二つの細胞を仲介して活性化することに由来する．スーパー抗原によって活性化されたT細胞と抗原提示細胞は，TNF（tumor necrosis factor）-α やIFN（interferon）-γなどのサイトカインを産生して生体の炎症反応を増強する．このように炎症反応を増強させることが，スーパー抗原を産生する*S. aureus*や*S. pyogenes*にとってどのような生活環上の利点があるのかは明らかでない．血流が増加することで好気性菌にとっては利用できる酸素量が

表2　スーパー抗原を産生する細菌と関連疾患

スーパー抗原	産生菌	関連疾患
黄色ブドウ球菌腸管毒素（SE toxin）	*Staphyrococcus aureus*	食中毒，ショック症状
毒素性ショック症候群毒素（TSST-1）	*Staphyrococcus aureus*	毒素性ショック症候群 新生児TSS様発疹症
表皮剥奪毒素（exfoliative toxin）	*Staphyrococcus aureus*	皮膚熱傷症候群
発熱性外毒素（SPE toxin）	*Streptococcus pyogenes*	猩紅熱，ショック症状
溶連菌由来マイトジェン（SSA，SMEZ）	*Streptococcus pyogenes*	猩紅熱
エルシニア由来マイトジェン（YPM）	*Yersinia pseudotuberculosis*	急性腸炎，急性腎不全

SE：staphylococcal enterotoxin，TSST：toxic shock syndrome toxin，SPE：streptococcal pyrogenic exotoxin，SSA：streptococcal suprantigen，SMEZ：streptococcal mitogenic exotoxin Z，YPM：Yersinia pseudotuberculosis derived mitogen

増加する，全身に播種しやすくなる，などの利点があるのかもしれない．ともあれ，宿主であるヒトにとっては，スーパー抗原は侵入した菌の病原性を増大して炎症反応に油を注ぐ迷惑な存在である．

スーパー抗原が感染した宿主の病態に関与する疾患を**表2**に示す．もっとも代表的な疾患は，毒素性ショック症候群（toxic shock syndrome：TSS）である．毒素性ショック症候群毒素（TSS toxin-1：TSST-1）を産生する*S. aureus*感染により発症する．同じくTSST-1を産生する*S. aureus*の感染で発症するのが，新生児毒素性ショック様発疹症（neonatal TSS-like exanthematous disease：NTED）である．二つの疾患ともに発熱と皮膚の発疹・潮紅を症状とするが，NTEDが自然治癒もありうる良好な経過をたどるのに対してTSSは抗菌薬による治療が必須で，死に至ることもある重篤な疾患である．同じスーパー抗原が新生児（NTED）と成人（TSS）とで異なった病態をひきおこすメカニズムはよくわかっていないが，新生児では菌の侵襲が体表に近い部位（鼻腔や臍）に限られること，スーパー抗原で活性化された新生児のリンパ球はアポトーシスによる細胞死をおこしやすいことなどの理由によるのかもしれない．これらの疾患以外にも，*S. aureus*による皮膚熱傷症候群や*S. pyogenes*による猩紅熱などは，おのおのの菌が産生するスーパー抗原活性が病態に関連すると考えられている．さらに，*Yersinia*感染症に合併する急性腎不全も，スーパー抗原性毒素（*Yersinia pseudotuberculosis* derived mitogen：YPM）によって刺激されたT細胞やマクロファージの活性化が発症に関与する．

川崎病のスーパー抗原仮説は，前述した川崎病の臨床像がスーパー抗原性疾患の臨床症状によく似ていることに由来する．TSSや*Yersinia*感染症では，発熱や皮膚粘膜の発赤，頸部リンパ節腫脹など川崎病の主要症状が一過性に出現するし，心嚢液の貯留や冠動脈障害を合併することもある．しかし，スーパー抗原仮説のなかで，川崎病の発症にスーパー抗原が実際にかかわることを明確に証明したものはまだない．

スーパー抗原仮説に対する疑問の第一は，川崎病患者からスーパー抗原を産生する菌が分離される頻度が高くないことである．たとえば，*S. pyogenes*は，スーパー抗原仮説〔発熱性外毒素C（Streptococcal pyogenic exotoxin type C：SPEC）が発症に関与する〕以前から川崎病の原因菌として注目されていたが，実際に川崎病患者の咽頭などから分離される頻度はきわめて低い[2]．第二は，スーパー抗原を産生する菌を分離しただけでは，そのスーパー抗原が患者の体内に侵入して川崎病をひきおこした証拠にはならないことである．たとえば，*S. aureus*は川崎病患者の咽頭からしばしば分離される．*S. aureus*の多くはTSST-1をはじめとするなんらかのスーパー抗原遺伝子をもっているので，培養上清にリンパ球増殖刺激活性を検出できる菌株も多い．しかし，これらのスーパー抗原が実際に患者の体内に侵入し炎症を惹起したことを証明するデータは今のところない[3]．スーパー抗原に対する抗体価の測定も以前から報告されているが，川崎病ではサイトカインによるB細胞の全般的な活性化が生じること，治療としてIgGが大量に投与されること，スーパー抗原の種類が非常に多いので限られた項目の測定では十分ではないことなどの事情があるため，エビデンスレベルはウイルス感染症における抗体価の上昇ほど期待できないのではないだろうか[4]．スーパー抗原が川崎病の原因かどうかはいまだ不明である．

ゲノム解析技術の進歩と川崎病

前項で述べたように，病原体探しのゴールデン・スタンダードは，やはり病原微生物の分離と同定であり，それを用いた感染実験である．しかし，ヒトの消化管や皮膚にはスタンダードな培養法では成育しない．したがって同定もされていない未知の細菌やウイルスが既知の種よりもはるかに多数存在することはよく知られている．本項では，川崎病の病原体をこのような未同定の細菌あるいはウイルスの中から探そうとする試みについて紹介する．

培養によらない病原微生物の検出法として，微生物由来の核酸，DNAやRNAを増幅して検出するPCR法は，現在ではさまざまな分野で広く用いられている．川崎病の病因研究では早くも1992年に，KikutaらがEBウイルスのDNAを血液から検出するためにPCR法を用いている[5]．Rowleyらは，1994年にヘルペスウイルス属とパルボウイルス属におのおの共通な塩基配列をもつDNAが川崎病患者の血液にないかをPCR法で調べている．さらに，細菌のリボソームRNA（16S rRNA）の遺伝子に共通な塩基配列を利用して，未知の細菌を検出する目的で16S rRNAの遺伝子をPCR法で増幅しようとしたが，結果はどちらも陰性であった[6]．1999年にShibataらは，Rowleyらと同じく細菌の16S rRNAの遺伝子に着目して，通常のPCR法よりも検出感度の高いnested PCRとよばれる方法で川崎病患者の血液を調べた．今回は見事にヒットして，nested PCRで増幅された16S rRNAの遺伝子断片が複数得られた．DNA断片の塩基配列を決定し実験過程で混入したと考えられるものを除外していくと，*Corynebacterium*属の新種と推測される遺伝子断片だけが最終的に残ったことを報告している[7]．

2000年代になって，核酸の塩基配列を決定するゲノム解析技術の急速な進歩により，多種の生物のゲノムの塩基配列が解読され，その情報がデータベース化された．これまで知られなかった微生物の生態系や感染症の病態を，これらの高度な技術を使って研究しようという機運も高まってきた．国立感染症研究所の水谷，黒田らは，PCRを応用したRDV（rapid determination system of viral nucleic acid sequences）法や次世代シークエンサーを使って，川崎病患者の検体から未知のウイルスや細菌を網羅的に検出しようと試みている[8]．次世代シークエンサーは従来の電気泳動を必要とする塩基配列の解読法とは異なり，不特定の核酸の塩基配列（リード）を何万個も網羅的に読み取ることができるシステムである．解読された塩基配列を外部のゲノム・データベースと比較することにより，どの生物（あるいは細菌やウイルス）のどのような遺伝子の塩基配列にもっとも近いかを決定することができる．

昨年の日本川崎病学会では，急性期の患者血清から特定のウイルス・シークエンスが高頻度に検出されたこと，急性期の患者の頸部リンパ節組織から腸内細菌叢を構成する多種類のグラム陰性菌の塩基配列が検出されたことが報告された．印象的だったのは，われわれが本来無菌的と考えていた血清中からも細菌由来の核酸が多数見出されたことである．これらが本当に川崎病の急性期に特徴的な所見なのか，また核酸が検出された微生物

が川崎病の病態形成に直接関係するものなのか，あるいはたまたま貪食されて「その場にいた」だけなのか，今後，検討されなければならない疑問は多く残されている．しかし，これらの新技術が切り拓く研究はその方法論からして，先入見に左右されずにまったく新規な微生物と川崎病との関係を明らかにする可能性を秘めている．今後，網羅的なデータの山のなかから特定の微生物を抽出し，川崎病の病態との関連を明らかにしていけるかどうか，研究の進展に期待したい．

おわりに

前述のスーパー抗原仮説の項ではかなり辛口の評価になったが，筆者自身，この説に組みしながら十分に証明しえなかった無念があるので，自然にそうなってしまうのだと思う．スーパー抗原仮説が提唱された頃はT細胞の抗原受容体遺伝子がクローニングされ，獲得免疫システムへの理解が飛躍的に深まった時代だった．その後，Toll様受容体など非自己を認識するシステムが次々に明らかにされて，現在は自然免疫全盛の時代といえるかもしれない．微生物由来の「非自己」が川崎病のトリガーになるのであれば，スーパー抗原仮説もまったく的外れではなかったのかもしれない．いずれにしても，川崎病の病因を明らかにするためには感染因子の探索だけでは不十分であり，血管炎発症のメカニズム，免疫細胞の役割，遺伝子多型の関与といった要素を総合的に理解する必要があると思う．

川崎病の本態は，これらの諸因子のベクトルが「発症」に向けて打ち揃うことで初めて可能になる病態ではないだろうか．今後の研究で，これまで「好発年齢」とよばれてきたことの内実を明らかにすることが，原因解明にとって大きな一歩となると思う．

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Original Article

Kawasaki disease patients with six principal symptoms have a high risk of being a non-responder

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Abstract

Background: A diagnosis of Kawasaki disease (KD) is established using six principal symptoms. Because the principal symptoms are deeply connected with KD, it is thus important to investigate the usefulness of the principal symptoms for evaluating the disease severity of KD.

Methods: Patients with definite KD or suspicion of KD were retrospectively examined. Blood test data and the incidence of patients who failed to respond to the initial i.v. immunoglobulin treatment (non-responders) were compared between patients with six principal symptoms, including fever of ≤4 days, before treatment of KD (six-symptom patients), and those with five or fewer symptoms (five-symptom patients).

Results: The study group of 207 patients who were treated with immunoglobulin consisted of 121 six-symptom patients and 86 five-symptom patients. The six-symptom patients were older and had higher neutrophil proportion and total bilirubin, and lower serum sodium at diagnosis than the five-symptom patients. Although the treatments did not differ between the groups, the six-symptom patients had a higher incidence of non-responders than the five-symptom patients (17% vs 5%; $P = 0.008$). Logistic regression analysis showed that six-symptom status was related to the risk of being a non-responder (odds ratio [OR], 5.3; 95% confidence interval [95%CI]: 1.6–17.4). This association was still significant after adjustment for the effect of age, neutrophil proportion, and total bilirubin and sodium (OR, 4.4; 95%CI: 1.4–17.3).

Conclusions: The number of principal symptoms before treatment is a useful guide to KD disease severity. Six-symptom patients have a higher risk of being a non-responder than five-symptom patients.

Key words　coronary artery abnormality, immunoglobulin treatment, non-responder, principal symptoms.

Kawasaki disease (KD) is an acute febrile vasculitis of childhood.[1–3] A diagnosis of KD is established using six principal symptoms: (i) fever persisting ≥5 days (inclusive of patients in whom the fever has subsided before the fifth day in response to therapy); (ii) bilateral conjunctival congestion; (iii) changes of lips and oral cavity; (iv) polymorphous exanthema; (v) changes of peripheral extremities; and (vi) acute non-purulent cervical lymphadenopathy.[3,4]

The most serious problem in patients with KD is coronary artery abnormality (CAA).[1,2] Treatment with aspirin and high-dose i.v. immunoglobulin (IVIG) has reduced the prevalence of CAA from 20–25% in patients treated with aspirin alone to 2–4%.[1–3] Despite this improvement, some patients develop CAA, and many risk factors for these complications have been reported to help screen for problematic patients.[3,5–7] These risk factors consist of laboratory findings, but do not include any of the principal symptoms. Because the principal symptoms are deeply connected with KD, it is reasonable to consider that a relationship between the development of the principal symptoms and the disease severity may exist. Nakamura *et al.* examined a nationwide epidemiologic survey on KD between 2001 and 2002. They reported that patients with the six principal symptoms were more problematic for CAA than those with five symptoms among the definite cases.[8] The principal symptoms in their report were those observed during the whole disease course, and not at the time of diagnosis of KD. Their report suggested that the number of principal symptoms seen at diagnosis may be helpful for evaluating the severity of KD.

The aim of the present study was to investigate the usefulness of the principal symptoms for evaluating the severity of KD.

Methods

Patients

The clinical records of consecutive patients with definite KD or suspicion of KD who were referred and then admitted to Kagoshima Medical Association Hospital between January 2002 and June 2009 were retrospectively reviewed. The

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diagnosis of KD was made using the Japanese criteria.[3,4] The principal symptoms observed between the onset and the day when KD treatment was initiated were counted. Regarding the principal symptom of fever, this was defined as lasting for ≥5 days, or ≤4 days when shortened by early IVIG treatment.[4] The patients were treated with aspirin and IVIG (2 g/kg for 1–2 days). Patients who had low Harada scores[9] (scores of 1 or 2) or who had tendencies for peak body temperature decrease upon diagnosis were treated with aspirin alone. To examine patients in the same treatment conditions, patients treated without IVIG were excluded from the study. Patients admitted after 7 days of illness were also excluded. When the probability of KD was considered to be high by the medical team, the treatment for KD was initiated even if the patient had not fully met the criteria for KD. Patients who had resolution of fever (<37.5°C) within 24 h of finishing the initial IVIG treatment were considered as IVIG responders. Patients who did not meet the responder criteria were considered as non-responders and were given additional IVIG treatment.

The relationship between the number of principal symptoms and disease severity was examined. Disease severity was estimated using routine blood examination, risk score, and incidence of non-responders and patients with CAA. With regard to risk scores, the Harada score (score of 0–7, with 7 being the highest score)[9] and the Gunma score (score 0–11, with 11 being the highest score)[7] were used. CAA was evaluated on 2-D echocardiography. It was performed at the time of diagnosis and repeated after 1 or 2 days, and then subsequently performed several times a week during hospitalization or at an outpatient clinic. CAA were classified at 1 month of illness according to the criteria of the Japanese Ministry of Health and Welfare.[3] These criteria classify the coronary artery as abnormal when the internal lumen diameter is >3 mm in children aged <5 years or >4 mm in children aged ≥5 years; when the internal diameter of any segment measures at least 1.5-fold the diameter of an adjacent segment; or when the coronary artery lumen is clearly irregular. Coronary artery z-scores (standard deviations from a predicted normal mean) were also assessed using the methods described by Kurotobi *et al.*[10] The largest z-score among the scores for the right, left main and left anterior descending arteries was used to evaluate CAA at admission and at 1 month of illness.

This study was approved by the Institutional Review Board of Kagoshima University Hospital. Informed consent was obtained from each child's parents before treatment.

Statistical analysis

The mean ranks between the two groups were compared using the Mann–Whitney U-test. Fisher's exact probability test was used to assess the frequencies in the two groups. To examine the risk of being a non-responder, multivariate logistic regression analysis was applied using potential confounding factors as covariates. All P presented are two-sided, and $P < 0.05$ was considered to be statistically significant. The data were processed using SPSS Statistics 17.0 (SPSS Japan, Tokyo, Japan).

Results

During the study period, 229 patients were referred and then admitted to Kagoshima Medical Association Hospital and were suspected of having KD. Of these, 20 patients who were treated with aspirin alone and two patients who were admitted after 7 days of illness were excluded from the study.

Two hundred and seven patients (M/F: 120/87) were treated with IVIG, and 182 patients were responders and 25 patients (12%) were non-responders. At 1 month of illness, 13 patients (6%) had CAA, consisting of nine with dilatation, three with small aneurysms (diameter of the maximum lesion: <5 mm) and one with a middle-size aneurysm (<8 mm). None of the patients had a giant aneurysm (>8 mm). Of these 13 patients, eight patients were non-responders and remaining five patients were responders. All of the patients with small or middle size aneurysms were non-responders. At 1 month of illness, 21 patients (10%) had a maximum z-score >3.0 and 10 patients (5%) had a maximum z-score >5.0.

Between disease onset and initiation of KD treatment, 121 patients had six principal symptoms including fever, 78 patients had five symptoms, and eight patients had either three or four symptoms. During the entire disease course, 203 patients were confirmed to have five or six principal symptoms. The remaining four patients were eventually confirmed with four symptoms and two of these patients had coronary artery findings (slight dilatations). Therefore, a diagnosis of KD was established in 205 patients, and the remaining two patients were diagnosed as having incomplete KD.

Comparison between six-symptom and five-symptom patients

Comparisons between patients with six principal symptoms (six-symptom patients) and those with five or fewer symptoms (five-symptom patients) before the initiation of treatment for KD are given in Table 1. The six-symptom patients were significantly older than the five-symptom patients. White blood cell count (WBC) was slightly higher in the six-symptom patients than in the five-symptom patients ($P = 0.103$), while the neutrophil proportion was significantly higher in the six-symptom patients. Total bilirubin was significantly higher and sodium was significantly lower in the six-symptom patients than in the five-symptom patients. Disease severity based on Harada score did not differ between the two groups (six-symptom vs five-symptom: median, 25th–75th percentile: 4, 3–5 vs 4, 3–5). With regard to Gunma score, the six-symptom patients had a significantly higher score than the five-symptom patients (3, 2–5 vs 2, 1–4; $P = 0.006$).

The KD treatment was initiated on the same day of illness in both groups (Table 1) and they received the same dosage of IVIG (2.0, 1.9–2.1 g/kg vs 2.0, 1.9–2.1 g/kg). There was a significantly higher prevalence of non-responders among the six-symptom patients than the five-symptom patients (Table 2). The total duration of fever was longer in the six-symptom patients than in the five-symptom patients, but the difference was not statistically significant (6, 5–8 days vs 6, 5–7 days; $P = 0.096$). Regarding the

Table 1 Patient characteristics vs no. symptoms before KD treatment

Group	Six-symptom group Median (25th–75th percentile)	Five-symptom group Median (25th–75th percentile)	P
No. patients (M/F)	121 (70/51)	86 (50/36)	
Age (years)	2.2 (1.1–3.8)	1.3 (0.7–2.3)	0.001
Days of illness at admission	4 (3–5)	4 (3–5)	
Days of illness at IVIG initiation	5 (4–5)	5 (4–5)	
Laboratory findings			
WBC ($\times 10^3/\mu L$)	140 (112–175)	130 (104–157)	
Neutrophil proportion (%)	72.8 (62.3–71.5)	58.7 (50.0–70.0)	<0.001
Hematocrit (%)	34.9 (33.3–36.8)	34.8 (32.6–36.8)	
Platelet counts ($\times 10^4$ /μL)	33.6 (22.8–39.9)	34.6 (28.4–41.6)	
AST (IU/L)	41 (27–128)	36 (27–61)	
ALT (IU/L)	32 (12–151)	29.5 (17–92)	
LD (IU/L)	444 (302–584)	456 (307–628)	
Total bilirubin (mg/dL)	0.7 (0.5–1.1)	0.5 (0.4–0.8)	0.003
Sodium (mEq/L)	134 (132–136)	135 (134–137)	0.020
Albumin (g/dL)	3.9 (3.6–4.1)	3.9 (3.6–4.1)	
C-reactive protein (mg/dL)	6.3 (3.8–9.5)	5.9 (3.2–8.7)	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; IVIG, i.v. immunoglobulin; KD, Kawasaki disease; LD, lactate dehydrogenase; six-symptom group, patients with six principal symptoms before KD treatment; five-symptom, patients with five or fewer principal symptoms before KD treatment; WBC, white blood cells.

risk of being a non-responder, it was found that six-symptom status was significantly related to the risk (odds ratio [OR], 5.3; 95% confidence interval [CI]: 1.6–17.4). This association was still significant in a multivariate analysis using the factors that were significantly different between the six-symptom and five-symptom patients (age, neutrophil proportion, and total bilirubin and sodium) as covariates (OR, 4.4; 95%CI: 1.4–17.3).

The maximum z-scores of the coronary arteries did not differ between the two groups at admission (1.0, 0.4–2.7 vs 0.9, 0.3–1.6). Among the six-symptom patients, 10 patients (8%) had CAA at 1 month of illness. This incidence was higher than that in the five-symptom patients, but this difference was not statistically significant (Table 2). The largest z-scores at 1 month of illness did not differ between the two groups (1.0, 0.2–1.9 vs 0.9, 0.3–2.1). The incidence of patients with a z-score >3.0 at 1 month of illness was higher in the six-symptom patients than in the five-symptom patients, but this was not statistically significant (Table 2).

All of the eight patients who had three or four principal symptoms before KD treatment responded to the initial IVIG treatment. A CAA was observed in one patient, specifically a 10-month-old girl with a mild dilatation (right coronary artery dimension of 3.2 mm). This abnormality became normal within 3 months of the illness.

Table 2 Principal symptoms before Kawasaki disease treatment vs outcome

Principal symptoms	n	Non-response n (%)	CAA n (%)	z-score >3.0 n (%)
Six symptoms	121	21 (17)#	10 (8)	14 (12)
≤Five symptoms	86	4 (5)#	3 (3)	7 (8)
Acute non-purulent cervical lymphadenopathy				
Yes	149	23 (15)*	10 (7)	16 (11)
No	58	2 (3)*	3 (5)	5 (9)
Change of peripheral extremities				
Yes	194	23 (12)	12 (6)	19 (10)
No	13	2 (15)	1 (8)	2 (15)
Changes of lips and oral cavity				
Yes	196	25 (13)	13 (7)	21 (11)
No	11	0	0	0
Polymorphous exanthema				
Yes	197	25 (13)	13 (7)	20 (10)
No	10	0	0	1 (10)
Bilateral conjunctival congestions				
Yes	204	25 (12)	13 (6)	21 (10)
No	3	0	0	0

#$P = 0.008$; *$P = 0.017$. CAA, coronary artery abnormality at 1 month of illness; non-response, lack of response to initial treatment with i.v. immunoglobulin; z-score, largest z-score among the right and left coronary arteries at 1 month of illness.

Table 3 Prediction of non-response to initial IVIG treatment and presence of CAA

Patient category	*n*	Non-responder	CAA	*z*-score > 3.0
High risk	76			
Six symptoms	54	14 (26)	8 (15)	10 (19)
Five symptoms	22	2 (9)	1 (9)	3 (14)
Low risk	131			
Six symptoms	67	7 (10)	2 (3)	4 (6)
Five symptoms	64	2 (3)	2 (3)	4 (6)

CAA, coronary artery abnormality at 1 month of illness; high risk, high risk of being a non-responder (Gunma score ≥4); IVIG, i.v. immunoglobulin; low risk, low risk of being a non-responder (Gunma score ≤3); non-response, lack of response to initial IVIG treatment; z-score, largest z-score among the right and left coronary arteries at 1 month of illness.

Principal symptom and outcome

Cervical lymphadenopathy was the most infrequently observed symptom, and the 58 patients without cervical lymphadenopathy had a significantly lower incidence of non-response than those with cervical lymphadenopathy (Table 2). The incidence of CAA was higher in the cervical lymphadenopathy cohort, but this difference was not statistically significant. Non-response was not observed among the patients who lacked symptoms of the lips and oral cavity, exanthema or conjunctival congestion. There were 149 patients who had cervical lymphadenopathy. Among these patients, 23 non-responders were noted among the 128 six-symptom patients (18%), whereas there were no non-responders in the five-symptom group consisting of 21 patients. This difference was statistically significant ($P = 0.045$).

Presence of CAA and score prediction of non-response to IVIG

Using the Gunma score, which predicts non-response to IVIG, 37% of patients had high Gunma scores (≥4; Table 3). There was a significantly higher incidence of six-symptom patients in that group than in the group with low Gunma scores (71% vs 51%; $P = 0.006$). The cohort with high Gunma scores included 16 non-responders, of whom 14 patients (88%) were six-symptom patients. Similar proportions were observed for patients with CAA or *z*-scores >3.0 at 1 month of illness. Among the patients with low Gunma scores, the six-symptom patients had a higher proportion of non-responders than the five-symptom patients, while the incidences of CAA and *z*-score >3.0 did not differ.

Discussion

Compared with the five-symptom patients, the six-symptom patients had a significantly different neutrophil proportion, and bilirubin and serum sodium levels. It has been reported that these differences were observed in non-responders and in patients with CAA.[6,7] The six-symptom patients also had higher Gunma scores and a higher prevalence of non-response. Logistic regression analysis showed that six-symptom status was related to the risk of being a non-responder. These findings suggest that six-symptom patients have more severe illness conditions than five-symptom patients, and that the development of the principal symptoms is related to the severity of the vasculitis in KD. We believe that the number of principal symptoms before treatment is a useful guide for evaluating the severity of KD. Six-symptom patients have a higher risk of being a non-responder than five-symptom patients; this may be emphasized in patients who are assessed as being at high risk using a predictive score. This further suggests the possibility of a beneficial effect of combined use of the six-symptom status and a predictive score, such as the Gunma score, to improve the accuracy of predicting non-response. Improvement of this prediction will help in discussions of alternative treatments for high risk-patients, such as combination treatment involving IVIG and steroid.[11,12]

We previously reported that KD presenting with only fever and cervical lymphadenopathy at admission indicated a severe form associated with increased risk of non-response and of CAA.[13] In that report, the presence of cervical lymphadenopathy was also related to the risk of being a non-responder. Further to our previous study, the present study found a lower risk of being a non-responder among patients who lacked symptoms, not only the symptom of cervical lymphadenopathy but also symptoms of the lips, oral cavity, exanthema and conjunctival congestion.

Nakamura *et al*. examined nationwide survey data for 14 068 patients with definite KD and reported that the proportion of patients with CAA was higher in six-symptom patients than in five-symptom patients.[8] In the present study, the incidence of patients with CAA was also higher in the six-symptom patients than in the five-symptom patients. Although this difference was not statistically significant, the difference between the incidences of CAA in the five-symptom and six-symptom patients was greater in the present study than in the Nakamura *et al*. study, and we considered that the statistical power difference may have been caused by the smaller number of patients (207 patients) examined in the present study. We have shown that having six symptoms at presentation confers a significant risk of being a non-responder. Because non-responders to initial IVIG are at an increased risk of developing CAA,[14,15] screening of high-risk patients who fail to respond to the initial IVIG treatment and discussion of alternative treatments for the screened patients are important for further reducing the incidence of CAA. With regard to adequate alternative treatments, further studies are necessary.

The incidences of non-response and CAA were lower in the five-symptom patients than in the six-symptom patients. These findings may suggest that disease severity decreases as the number of principal symptoms decreases. Sonobe *et al*., however, analyzed nationwide survey data for 15 857 patients and reported that patients with incomplete KD had a higher prevalence of CAA than definite KD patients.[16] In that report, only 61.6% of patients with incomplete KD were treated with IVIG. In contrast, all the patients in the present study were treated with 2 g/kg IVIG. The treatment difference between the Sonobe *et al*. study and the present study may have caused the difference in the disease severity of fewer-symptom patients. From the viewpoint of the diagnostic process for incomplete KD, the identification of CAA is a key feature. Therefore, it is reasonable that few patients are diagnosed as having incomplete KD without a CAA. There could be many patients who are not diagnosed as having incomplete KD, and the number of patients with incomplete KD may be greater than the number being currently observed. The present study included only two patients with incomplete KD. It is, therefore, not clear whether the relationship between disease severity and number of principal symptoms is preserved in the patients with incomplete KD.

Although we focused on the principal symptoms in the present study, the grades of the principal symptoms were not examined, and there was no distinction between minor and major findings based on severity. The grades of the principal symptoms may be related to the disease severity in the same fashion as the number of the symptoms. Further studies on evaluation of disease severity using grades of symptoms are necessary. Another limitation was that the present study was a single-center retrospective study. A multicenter prospective study is necessary to determine the risk of being a non-responder or developing a CAA in six-symptom patients.

Conclusions

The number of principal symptoms before treatment is a useful guide for evaluating the severity of KD. Six-symptom patients are at greater risk of being a non-responder than five-symptom patients.

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Potential Role of Autoantibody in Severe Neutropenia of a Patient with Kawasaki Syndrome

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Abstract

Neutropenia associated with Kawasaki Syndrome (KS) has been rarely reported, and the detailed mechanisms responsible for this state are not yet elucidated. The aim of this study was to clarify the mechanisms of neutropenia in KS. We examined antibodies to known neutrophil antigens (HNA1a, HNA1b, HNA null, HNA2, HNA3, HNA4 and non-HLA antigen 9a) in a KS patient with neutropenia. We also performed the granulocyte immunofluorescence test (GIFT) using patient or control neutrophils incubated with the patient's serum at serial time points over the patient's clinical course. No specific antibody to known neutrophil antigens was detected. Flow cytometric analysis showed that autoantibodies bound to immature CD13-positive myeloid cells, which resulted in myeloid lineage maturation arrest in the bone marrow. GIFT showed that neutrophil-specific autoantibodies were produced by the patient, and the amount of autoantibody inversely correlated with the patient's neutrophil counts. The presence of an autoantibody to a novel antigen on immature myeloid cells or neutrophils is the likely the cause of severe neutropenia in this patient with KS.

Kawasaki syndrome (KS) is an acute febrile illness that presents with systemic vasculitis and is associated with a high incidence of coronary artery abnormalities (CAA) [1, 2]. High-dose intravenous immunoglobulin (IVIG) therapy is effective and reduces the incidence of CAA [3]. Although haematological abnormalities, including leukocytosis, thrombocytosis and anaemia associated with KS, have been reported [4], there are only a few publications reporting severe neutropenia [5–7].

Neutropenia is defined as an absolute neutrophil count (ANC) of $<1500/mm^3$, while severe neutropenia, observed in 1.0% of patients with KS [6], has an ANC of $<500/mm^3$. Neutropenia was observed approximately 3–4 weeks after onset of KS [7]. Neutropenia during the subacute phase of KS has been ascribed to the transient inhibition of GM-CSF production [7], downregulation of inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (neutrophil apoptosis inhibitors) [8, 9], the administration of aspirin or IVIG therapy [10, 11] and the possible relation of the production of antibodies that bind to neutrophils [12]. However, the detailed mechanisms behind neutropenia in KS have not been fully elucidated.

Here, we describe a patient with KS whose disease was complicated with severe transient neutropenia. Bone marrow examination revealed developmental arrest at the early myelocyte stage, and flow cytometric analysis showed the presence of autoantibodies that bound to immature CD13-positive myeloid cells. We speculated that this specific antibody bound to premature myeloid cells or peripheral neutrophils and contributed to the transient severe neutropenia of the patient.

The aim of this study was to clarify the mechanisms of neutropenia in KS, using a combination of the granulocyte immunofluorescence test (GIFT) and flow cytometry.

Materials and methods

Patient report. A previously healthy 2-year-old boy was admitted to a neighbourhood hospital suffering with fever, lymphadenopathy and fatigue (Fig. 1). Laboratory findings revealed a white blood cell count (WBC) of $24{,}700/mm^3$ and C-reactive protein (CRP) of 19.8 mg/dl. He was diagnosed with bacterial lymphadenitis and treated with Panipenem/Betamipron (PAPM/BP). On the fifth day of illness, he developed a skin rash, reddening of lips and conjunctival injection and was then diagnosed with KS. He was referred to Kagoshima Medical Association Hospital and treated with IVIG (2 g/kg) for 1 day and oral aspirin (30 mg/kg), and the antibiotics were changed to flomoxef sodium (FMOX). As his clinical symptoms gradually subsided, the antibiotic treatment was terminated by day 11 and aspirin was reduced to 5 mg/kg per day on day 12, when the serum CRP level decreased to the normal range. However, on the 13th day of illness, he developed a fever of 39 °C and a systemic rash. As drug-induced erythema exsudativum multiforme was suspected, aspirin was discontinued. He was then treated with hydrocortisone (5 mg/kg) for 7 days. His erythema gradually subsided and left pigmentation on the trunk. He was discharged on day 21 with no signs of CAA with a WBC of $7700/mm^3$, ANC of $2310/mm^3$ and CRP of 0.1 mg/dl. He was scheduled for follow-up appointments at the outpatient clinic on day 25. Laboratory findings showed agranulocytosis, although he had no clinical symptoms. On the 30th day of illness, he developed high fever and fatigue. He was then referred to Kagoshima University Hospital. At referral, bone marrow aspiration showed a nucleated cell count of $15.5 \times 10^4/mm^3$, normocellularity, no phagocytosis of granulocytes and no leukaemic cells. Normal development up to the early myelocyte stage was observed. Flow cytometric analysis showed high levels of early myeloid precursor marker profiles ($CD13^+/CD33^+/CD71^+/HLADR^-$), but low expression of late stage/mature myeloid markers (CD16 and CD11b) (Fig. 2A). Furthermore, we observed that immunoglobulin G (IgG) was bound to premature CD13-positive myeloid cells (Fig. 2B). The patient was diagnosed with febrile neutropenia and was treated with Cefozopran. His fever slowly subsided when the peripheral blood WBC gradually increased on day 33. On the 38th day of illness, he was discharged with complete recovery after an increase in leukocytes. The presence of known anti-neutrophil antibodies (HNA1a, HNA1b, HNA null, HNA2, HNA3, HNA4 and non-HLA antigen 9a) was not detected by flow cytometry. The drug lymphocyte stimulation tests (DLST) for immunoglobulin, aspirin,

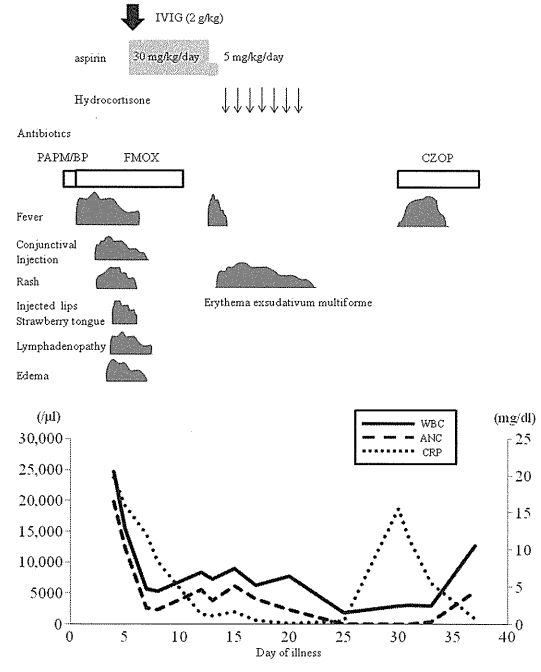

Figure 1 Clinical course and laboratory findings of neutropenic KS patient. IVIG, intravenous immunoglobulin; PAPM/BP, panipenem/betamipron; FMOX, flomoxef sodium; CZOP, cefozopran; WBC, white blood cell count; ANC, absolute neutrophil count; CRP, C-reactive protein.

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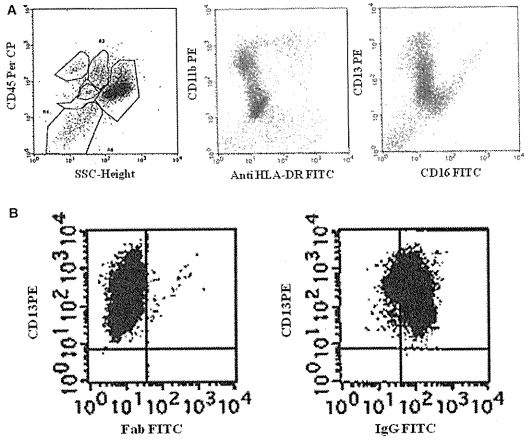

Figure 2 Flow cytometric analysis of bone marrow. (A) Lower expression of CD11b and CD16 on the surface of neutrophils. (B) Strong antibody binding to immature CD13-positive myeloid cells.

PAPM/BP and FMOX were evaluated using the conventional method [13] by a commercial laboratory testing service company (SRL, Inc. Tokyo, Japan). Briefly, the patient's mononuclear cells and antigen solution were incubated for 48 h. They were then pulsed for an additional 24 h with ^{3}H-thymidine. After washing and lysing the cells, incorporation of ^{3}H-thymidine was measured. The stimulation index (SI) was calculated using the following formula, and an SI value beyond 180% was defined as positive: SI = ^{3}H-thymidine incorporation with antigen/^{3}H-thymidine incorporation without antigen. The SI of PAPM/BP was 397%, while the others were negative (Veniron; lot SSV700 99%, aspirin 97%, FMOX 149%).

Subjects. We studied the KS patient with neutropenia (case A), another KS patient without neutropenia (case B) as a disease control (obtained at 18 days after onset of KS) and three healthy age-matched controls (case C through E) with no evidence of infection, inflammation, allergy, medication or previous blood transfusion (Table 1). Approval of this study was obtained by the Institutional Review Board at Kagoshima University Hospital. All patients or their guardians gave informed consent and assent before the blood collection.

Leukocyte isolation and serum samples. Whole blood was collected into tubes containing ethylene diamine tetraacetic acid and kept for 1 h at room temperature. The tubes from five subjects were standardized to the number of WBC in 3000/μl. The cellular and cell-free serum fractions were separated, and cells were washed twice in 2 ml of phosphate buffer saline (PBS), followed by centrifugation at 300 *g* for 5 min. The leukocyte pellets were resuspended in 100 μl of PBS and were incubated with 10 μl of 2% rabbit serum (Dako) for 30 min at 4 °C to block Fc receptors. The supernatant was removed, and the remaining pellets were resuspended in 2 ml of PBS. The leukocyte preparation was hemolysed in erythrocyte lysing solution at room temperature for 10 min, followed by centrifugation at 300 *g* for 5 min. The leukocyte pellets were washed twice and finally resuspended in 2 ml

Table 1 Clinical data and laboratory findings.

Case	Age	Sex	Sample	WBC counts (/μl)	ANC (/μl)	IgG (mg/dl)	C3 (mg/dl)
A	2	M	Present case[a]	5250	2078	571	103
B	1	M	Control patient[b]	12,300	7134	1409	94
C	1	M	Normal	8960	3763	477	110
D	3	M	Normal	7810	2968	655	129
E	2	M	Normal	9640	3856	552	105

ANC, absolute neutrophil counts; C3, complement component 3; IgG, immunoglobulin G; KS, Kawasaki Syndrome; WBC, white blood cell;.
[a]KS associated with neutropenia (89 days after onset of KS).
[b]Control KS patient (18 days after onset of KS).

Table 2 Laboratory findings of the Kawasaki syndrome patient with neutropenia (case A)

Serum Sample	WBC counts (/μl)	ANC (/μl)	Hb (g/dl)	Plt counts (/μl)	CRP (mg/dl)	IgG (mg/dl)
Day 5	15,400	12,320	11.2	18.0	16.0	403
Day 13	7200	3852	11.2	45.6	1.1	
Day 30	2900	29	9.4	56.2	15.6	966
Day 64	5350	2889	10.9	33.5	0.1	571

ANC, absolute neutrophil counts; CRP, C-reactive protein; Day, Day of illness after onset of Kawasaki syndrome; Hb, hemoglobin; IgG, immunoglobulin; Plt, platelet; WBC, white blood cell.

of PBS. To avoid variability in the flow cytometric analysis, the serum and the leukocytes prepared from the same controls were used throughout this study. Laboratory findings of the neutropenic patient with KS are shown in Table 2. Serum samples were separated by centrifugation at 700 *g* for 15 min at room temperature and were stored at −40 °C until time of assay.

Flow cytometry. Flow cytometric analysis of cell specimens was performed on a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA). Neutrophils were initially gated by their characteristic forward scatter (FSC) and side scatter (SSC) profiles, which represent size and granularity, respectively. Cells in these gates were then analysed for fluorescence intensity. Within the neutrophil cluster, a minimum of 10,000 cells were analysed.

Flow cytometric analysis of GIFT. Anti-neutrophil antibodies on the surface of neutrophils were tested by the direct granulocyte immunofluorescence test (D-GIFT). Anti-neutrophil antibodies in serum were tested by the indirect granulocyte immunofluorescence test (I-GIFT). D-GIFT was performed on the leukocytes described in Table 1 (case A through E) in PBS, incubated with FITC-conjugated goat F(ab')2 anti-human IgG (Biosource) and PE-conjugated mouse anti-human CD13 (BD Biosciences) for 30 min at 4 °C. After washing, neutrophils were analysed on a FACSCalibur (Becton Dickinson Biosciences). I-GIFT was performed by the addition of 10 μl of serum from the patient, disease control or normal controls to treated leukocytes, incubation for 30 min at 4 °C, followed by centrifugation at 300 *g* for 5 min. After washing once with 2 ml of PBS containing 0.2% bovine serum albumin, the following monoclonal antibodies were used for staining: 2.5 μl of FITC-conjugated goat F(ab')2 anti-human IgG (Biosource) and 2.5 μl of PE-conjugated mouse anti-human CD13 (BD Biosciences) for 30 min at 4 °C. After washing PBS, 10,000 neutrophils were analysed on a FACSCalibur (Becton Dickinson Biosciences).

Results

Laboratory findings

The laboratory data of the disease control were different from the other controls as he had undergone treatment with IVIG and aspirin. All blood samples were confirmed as blood group A, RhD positive. The laboratory findings during the disease course of case A are shown in Table 2. At day 30, ANC values were significantly decreased and platelet counts had contrastingly increased.

Granulocyte immunofluorescence test analysis

The presence of autoantibodies to neutrophils was tested by D-GIFT and I-GIFT. D-GIFT was negative in all subjects. Fig. 3B shows a representative I-GIFT result using the leukocytes of case C and the serum of case A. The M2 gate shows the levels of the neutrophil-associated antibody attaining an arbitrary level of fluorescence. No antibodies were present on day 5, before IVIG treatment. There was a direct correlation between increase in neutrophil-associated antibody levels and neutrophil counts of case A: as the amount of antibody increased, neutrophil counts of case A were further decreased, followed by an agranulocytic stage (serum on day 13 and day 30); then, as the amount of antibody gradually decreased, neutrophil counts of case A increased, resulting in recovery from neutropenia (serum on day 64). Similar results were observed using different neutrophils (present case, control patient and other normal volunteers) with serum from the present case (case A). The percentage of cells within the M2 gate is shown in Fig. 3C, which represents the changes in the relative antibody level and the ANC of the case A. The neutrophil counts of case A inversely correlated with the level of autoantibody during the patient's clinical course. No positive results using I-GIFT were observed among the serum from the disease or normal healthy controls. Examination of the same lots of immunoglobulin used for IVIG treatment also revealed an absence of antibodies to neutrophils.

Discussion

Neutropenia associated with KS patients is reported to be complicated with various autoimmune disorders [6]. In this study, an autoantibody to a novel antigen on immature myeloid cells or neutrophils was produced in a patient with KS and revealed as the possible cause of severe neutropenia.