roid-injected HIGA mice; n = 6). All mice were sacrificed on day 10. For 24 h before the first injection of LPS or saline, or before sacrificing, urine was obtained from each mouse for the intervention experiment to measure creatinine and protein levels. Kidney tissues were stained for ordinary light microscopy and immunohistological evaluation, and were also evaluated for mRNA expression, as described below.

Measurement of Urinary Protein

To measure urinary albumin excretion, a Mouse Albumin ELISA Quantitation kit (Bethyl Laboratories, Montgomery, Tex., USA) was used according to the manufacturer's protocol. Horseradish peroxidase-labeled goat antibody against mouse albumin was detected with 2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium (Wako Pure Chemical Industries, Osaka, Japan) at an absorbance at 405 nm. Measurement of urinary creatinine was assayed with an enzymatic method. Then, levels of urinary albumin excretion were expressed as values of urinary albumin/urinary creatinine.

Histological Evaluation and Immunoperoxidase Staining for Light Microscopy

Kidney tissues from each animal were processed for evaluation by light microscopy and immunostaining microscopy. For light microscopy, the tissues were fixed in 10% neutral-buffered formalin (pH 7.4) and embedded in paraffin. Then, sections (4 μm) were subjected to periodic acid-Schiff staining, which was evaluated quantitatively by counting the total number of glomerular cells in 20 randomly selected glomerular cross-sections. The sections (4 μm) were also reacted with a mouse monoclonal antibody against proliferative cell nuclear antigen (PCNA; DAKO, Carpinteria, Calif., USA) using an ABC Elite-peroxidase staining kit (Vector Laboratories, Burlingame, Calif., USA). Color was then developed by incubation with a DAB substrate kit (Pierce, Rockford, Ill., USA). The sections were counterstained with hematoxylin. The average number of PCNA-positive cells in a glomerular cross section was evaluated by enumerating the cells in 20 glomeruli in each section.

Immunoperoxidase staining for macrophages was performed using sections (4 μm) fixed in methyl carnoys and embedded in paraffin as previously described [28]. Then, sections were incubated with a rat monoclonal antibody that detects mouse monocytes/macrophages (1:150; F4/80, Serotec, Kidlington, Oxford, UK). The localization of tissue factor, factor V, factor X, PAR2, TLR4, and fibrin were studied using fresh frozen sections (4 μm). Tissue specimens frozen in OCT compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan) were cut into serial sections using a cryostat. Sections for tissue factor staining were fixed in 4% neutral buffered formalin for 8 min followed by 100% ethanol for 2 min, and then treated in 0.025% protease VIII (Sigma, St. Louis, Mo., USA) for 1.5 min at room temperature. Sections for detection of factor V, factor X, PAR2, or fibrin detection were fixed in acetone for 5 min at room temperature. These sections were stained by an indirect method using a goat anti-mouse tissue factor antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), a sheep anti-human factor V antibody (Cedarlane Laboratories, Hornby, Canada), a goat anti-human factor X antibody (Santa Cruz Biotechnology), a goat anti-mouse PAR2 antibody (Santa Cruz Biotechnology), or a goat antibody against mouse fibrinogen (Nordic Immunological Laboratories, Tilburg, The Netherlands) as previously described [22]. Following primary incubation, these sections were incubated with the appropriate secondary antibodies, and color was developed as described above. Sections for TLR4 expression were stained after overnight incubation with a biotinylated rat monoclonal anti-mouse TLR4 antibody (AMS Biotechnology, Oxon, UK) followed by treatment with an ABC Elite-peroxidase staining kit and color development. The glomerular deposition of fibrin was evaluated quantitatively by measuring positively reacting areas in 20 selected glomerular cross sections with NIH Image (NIH, Bethesda, Md., USA), and expressed as the positive area per glomerulus.

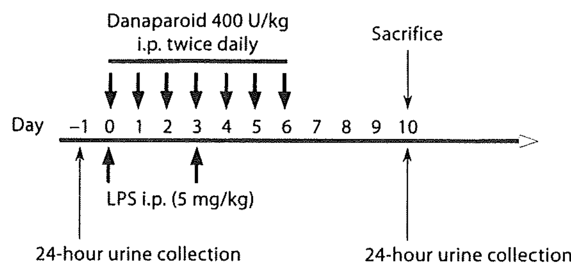

Fig. 1. Experimental design.

The specificity of the anti-PAR2 antibody was previously demonstrated using a blocking test with a specific immunizing blocking peptide [29]. The specificity of the anti-factor X antibody was also confirmed by a blocking test using purified human factor X (Hyphen BioMed, Neuville sur Oise, France). The reactivity of the anti-tissue factor antibody was confirmed using sections of murine uterus [24]. The reactivity of the anti-TLR4 antibody was previously reported [30].

Immunofluorescent Microscopy

Glomerular IgA deposition was evaluated in acetone-fixed fresh frozen sections (4 μm) with FITC-labeled goat anti-mouse IgA (1:100; KPL, Gaithersburg, Md., USA) at room temperature for 2 h. After sections were immersed in PermaFluor aqueous mounting medium (Thermo Fisher Scientific, Kanagawa, Japan), they were observed on an Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany), using LSM 510 software (version 2.3; Carl Zeiss).

Western Immunoblot Analysis

For protein extract preparation, renal cortex tissues were homogenized in lysis buffer with a Polytron (Kinematica AG, Cincinnati, Ohio, USA). The protein extracts were put on ice for 30 min and spun at 12,000 rpm. The supernatant was then collected. Proteins were separated by sodium dodecyl sulfate (8.0%) polyacrylamide gel electrophoresis using 50 μg of protein per sample. Resultant proteins were electroblotted onto polyvinylidene fluoride membranes. Membranes were then incubated for 2 h at 37°C in 10% skim milk solution. The resultant blot was incubated at 4°C overnight with a rabbit specific anti-TLR4 antibody (Imgenex, San Diego, Calif., USA). Blots were washed and incubated

ciated with GBM thickening. These results suggest that obesity could induce GBM thickening independent of IgA nephropathy activity. In the present study, however, all obese patients had mild increase in BMI and there were no severely obese patients with BMI $\geq$35 kg/m^2. In order to make more clearly the relationship between the severity of obesity and glomerular structural changes, a larger study with a broad BMI range will be needed.

The role of ATII is suspected to be great in the pathological state in obese IgA nephropathy patients [17, 19]. In the present study, 7 of 9 obese patients who received only ACE-I or ARB showed ameliorated proteinuria despite the short follow-up period, although the difference did not achieve statistical significance. Of interest, all obese patients whose proteinuria ameliorated by ACE-I or ARB treatment also showed significant body weight reduction during the follow-up period. On the other hand, the BMI of obese patients whose proteinuria did not ameliorate by ACE-I or ARB treatment remained unchanged. Although the number of patients was limited in this study, these results suggest that body weight reduction may be an important factor and essential even under ACE-I or ARB treatment for the amelioration of proteinuria. A large population will be required to determine the potential benefits of drug treatment with or without body weight reduction in obese IgA nephropathy patients.

Our study shows that obesity induces glomerular enlargement and ultrastructural modification of GBM, which would partly contribute to increase proteinuria in IgA nephropathy. In conclusion, obesity may be an independent exacerbating factor for the progression of IgA nephropathy.

Acknowledgment

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with a biotinylated anti-rabbit immunoglobulin followed by staining using an ABC Elite-peroxidase staining kit (Vector Laboratories). For confirmation that equal amounts of the proteins were applied for each lane, the membranes were reprobed with an antibody for β-actin antibody (Abcam, Cambridge, Mass., USA). The antigen-antibody complexes were detected using West Pico Chemiluminescent Substrate (Piece Biotechnology, Rockford, Ill., USA).

RT-PCR for mRNA

Total RNA was extracted from renal cortex that was homogenized with a Polytron (Kinematica), and then isolated by the acid guanidinium thiocyanate-phenol-chloroform method using TRIzol Reagent (Invitrogen, Carlsbad, Calif., USA). For first-strand cDNA synthesis, 1 μg of total RNA was incubated in a 1-μl reaction mixture containing 50 μM Oligo$(dT)_{20}$ as a primer (Invitrogen). The reaction contained 15 units of THERMOSCRIPT RT (Invitrogen), 2 μl of 0.1 M dithiothreitol-10 mM dNTP mixture, and 40 U of RNase inhibitor (Invitrogen). The reaction was performed at 50°C for 1 h. Subsequently, the reverse transcriptase was inactivated by heating the sample at 85°C for 5 min. PCR was performed in 5 μl of 10× PCR buffer with 5 units of pfx50™ DNA polymerase (Invitrogen) containing 1 μl of each primers as follows: tissue factor, 5′-CGGGTGCAGGCATTCCAGAG-3′ sense and 5′-CTCCGTGGGACAGAGAGGAC-3′ antisense primers, expected for 241 bp [31]; mouse monocyte chemoattractant protein 1 (MCP-1), 5′-ACTGAAGCCAGCTCTCTCTTCCTC-3′ sense and 5′-TTCCTTCTTGGGGTCAGCACAGAC-3′ antisense primers, expected for 274 bp [32], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGATGACATCAAGAAGG-TGGTGAAG-3′ sense and 5′-CCTTGGAGGCCATGTAGGC-CAT-3′ antisense primers, expected for 240 bp [33]. Primers for GAPDH were used as the internal standard. The PCR products were separated in 2% agarose gels.

Statistical Analysis

Values were represented as mean ± SEM. Statistical differences were assessed using the Kruskal-Wallis and the Mann-Whitney tests. The Wilcoxon test for paired samples was used to see whether urinary albumin significantly decreased after danaparoid treatment. A p value <0.05 was considered to be significant.

Fig. 2. Changes in macrophage infiltration and expression of tissue factor, factor V, factor X, PAR2, and TLR4 in glomeruli before and after LPS administration. Although macrophage infiltration was negative or scarce in basal BALB/c (**a**), LPS-injected BALB/c (**b**), and basal HIGA mice (**c**), it was frequently detected in the glomerulus in LPS-injected HIGA mice (**d**). Tissue factor, factor V, factor X, and PAR2 were negative in basal BALB/c mice (**e**, **i**, **m**, **q**, respectively), and they were mildly detected after LPS injection (**f**, **j**, **n**, **r**, respecively). In basal HIGA mice, they were weakly detected (**g**, **k**, **o**, **s**, respectively), but staining became intense after LPS injection (**h**, **l**, **p**, **t**, respectively). In contrast to the above findings, TLR4 expression was intense in mesangial cells in basal HIGA mice (**w**) and reduced after LPS injection (**x**). On the other hand, its expression was mild in both basal (**u**) and LPS-injected BALB/c mice (**v**). Original magnification for all panels, ×400.

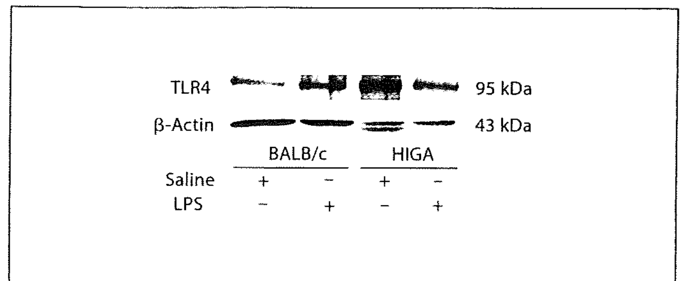

Fig. 3. Representative picture of immunoblotting for TLR4 and β-actin expression. Upper part: expression of TLR4 protein in renal cortex, as detected by Western immunoblotting. Lower part: the levels of β-actin protein (4 mice per group).

Results

Deposition and Expression of Coagulation-Related Factors before and after LPS Administration

Although macrophage infiltration in glomeruli was negative in basal BALB/c, basal HIGA, and LPS-injected BALB/c mice, it was frequently observed in LPS-injected HIGA mice (fig. 2). Staining of factor V, factor X, and PAR2 was weak in basal HIGA mice, but intensely observed in the mesangium after LPS injection. Staining of these coagulation factors and PAR2 was negative in basal BALB/c mice, and mild after LPS injection. Interestingly, TLR4 expression was intense in the mesangial cells of basal HIGA mice, but diminished after LPS injection. On the other hand, TLR4 expression was mild in both basal and LPS-injected BALB/c groups.

Immunofluorescence Microscopic Evaluation of IgA Deposition

Deposition of glomerular IgA was observed mainly in the mesangium and partially along capillary loops, and increased after LPS injection when compared to saline-injected HIGA mice (data not shown).

Western Blot Analysis of TLR4 in the Renal Cortex

Changes in TLR4 expression in the renal cortex were observed in both BALB/c and HIGA mice before and after LPS injection. The specific bands could be clearly detected by immunoblotting both from the BALB/c and HIGA mice. However, TLR4 expression in basal HIGA was greater than basal BALB/c, and reduced in LPS-injected HIGA. These findings were basically the same with the glomerular staining by immunohistochemistry (fig. 3).

proinflammatory and procoagulant activity. The pathophysiology in LPS-injected HIGA mice could therefore be an acute aggravation model for IgA nephropathy that is triggered by enhanced inflammatory response in the standby state.

Acknowledgements

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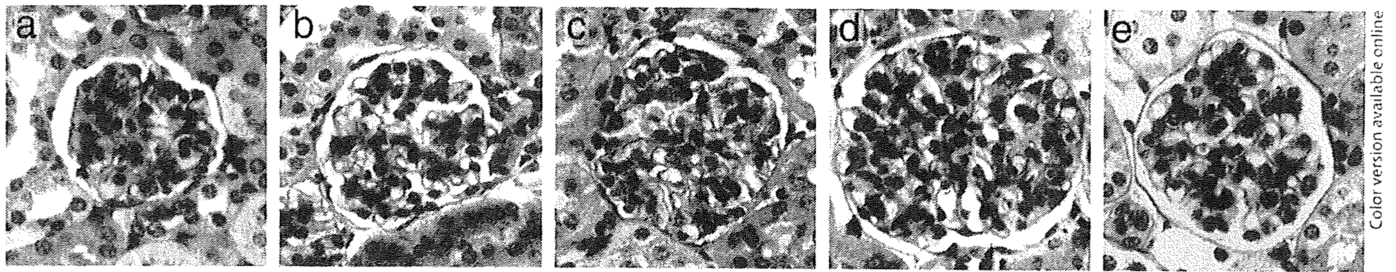


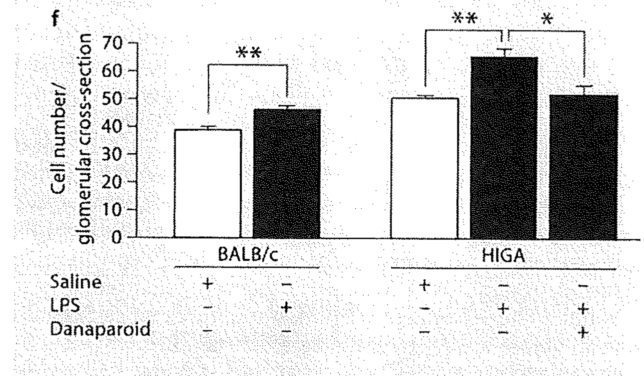
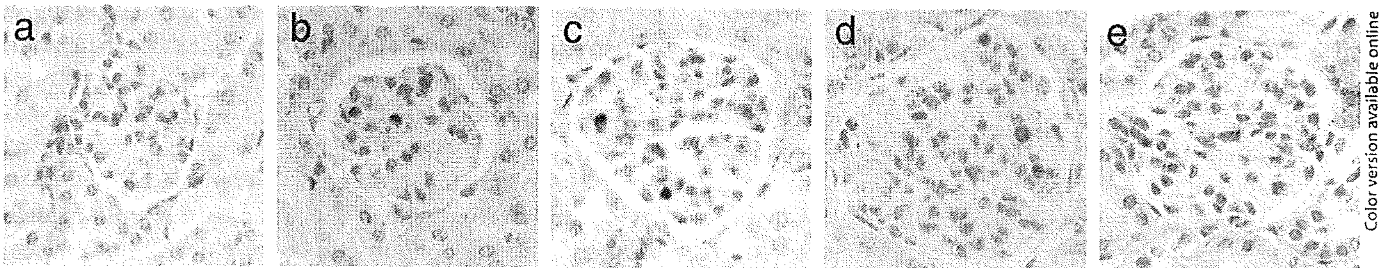


Fig. 5. Change in total glomerular cell number after LPS administration and danaparoid treatment. Representative photomicrographs of glomeruli from basal BALB/c (**a**), LPS-injected BALB/c (**b**), basal HIGA (**c**), LPS-injected HIGA (**d**), and LPS + danaparoid-injected HIGA mice (**e**) are shown. The total glomerular cell number was slightly increased in BALB/c mice after LPS administration (**b**) and amplified in HIGA (**d**). **e** A marked reduction in the total glomerular cell count in danaparoid-treated HIGA mice was observed. Final magnification, ×400. **f** Total glomerular cell number for each group is shown. Values are represented as mean ± SEM. * $p < 0.05$; ** $p < 0.01$ (6 mice per group).



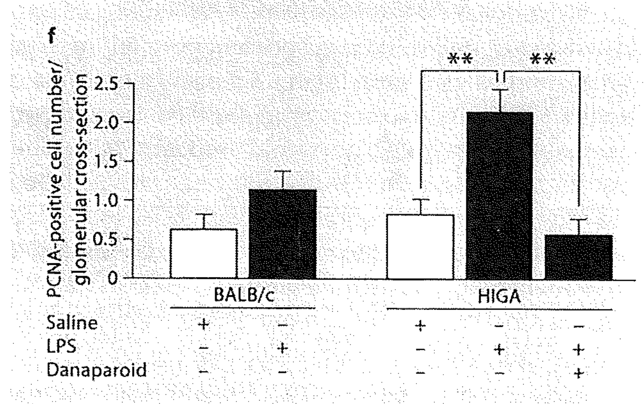
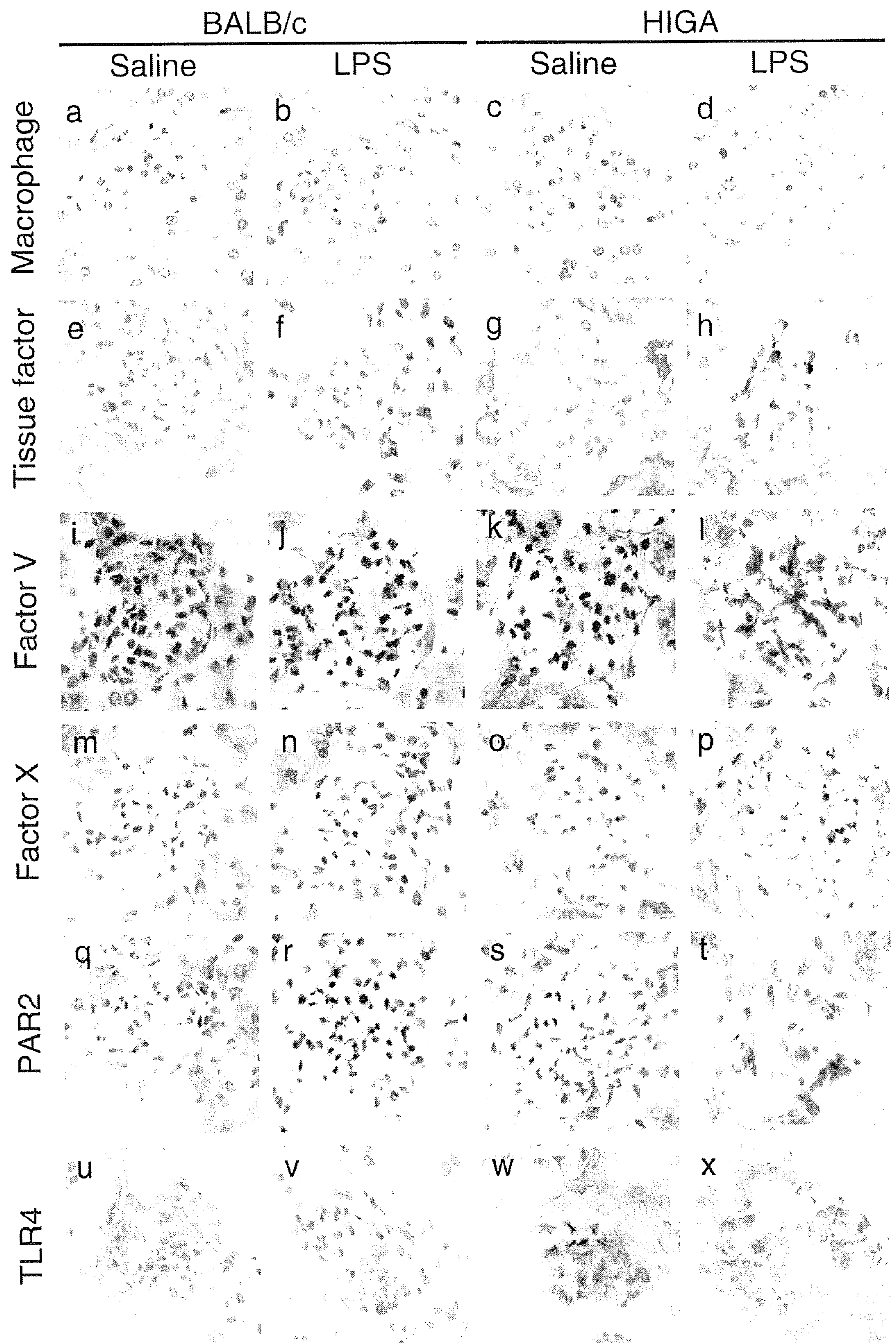


Fig. 6. Change in PCNA-positive cells after LPS administration and danaparoid treatment. Representative photomicrographs of glomeruli from basal BALB/c (**a**), LPS-injected BALB/c (**b**), basal HIGA (**c**), LPS-injected HIGA (**d**), and LPS + danaparoid-injected HIGA mice (**e**) are shown. The PCNA-positive cell number was slightly increased in BALB/c after LPS administration (**b**) and amplified in HIGA (**d**). **e** A marked reduction in the PCNA-positive cell count in danaparoid-treated HIGA mice was observed. Final magnification, ×400. **f** The number of PCNA-positive cells for each group is shown. Values are represented as mean ± SEM. ** $p < 0.01$ (6 mice per group).


BALB/c
HIGA
Saline
LPS
Saline
LPS
Macrophage
Tissue factor
Factor V
Factor X
PAR2
TLR4
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terleukin-6 and -8 in endothelial cells [40]. Thrombin, which is activated from prothrombin by the factor Xa/Va complex (prothrombinase), is also known to be a mitogenic factor in mesangial cells [41]. Thrombin and factor Xa activate a novel family of cell-surface receptors, PARs, which are G protein-coupled receptors [42, 43]. After selective cleavage of the receptors by these serine-proteases, PARs exert various cellular effects that induce inflammatory responses to tissue injury [44–46]. Several in vitro studies have demonstrated that PAR2 can mediate factor Xa signaling, but not thrombin signaling [47]. In our recent study, we reported that PAR2 was expressed in cultured human mesangial cells, and that factor Xa induced cellular proliferation through the activation of extracellular regulated kinase via PAR2 [25]. In our present study, PAR2 was negative in basal BALB/c mice, weakly expressed in basal HIGA mice, and markedly upregulated in LPS-injected HIGA mice.

In a previous study, it was reported that glomerular IgA deposition was suppressed in ddY mice by periodic LPS injections during an observation period of 13 months [48]. In our previous study using IL-12 administration in HIGA mice for 3 weeks, mesangial IgA staining became faint, whereas crescent formation was marked [49]. In the present study, IgA staining increased during the experimental period of 10 days in HIGA mice after the LPS injection, and was still negative in LPS-injected BALB/c mice (data not shown). This could be due to the acute nature of the LPS exposure. Although Wei et al. [50] reported that LPS treatment (200 μg/head) induced nephritic syndrome in mice 24 h after injection, we did not find massive proteinuria in our experiment. Regarding this point, two reasons could be influenced: the use of lower doses in the present study of 5 mg/kg body weight (approximately 150 μg/head in the HIGA group) and the time point of urinary protein measurement (after 7 days of second LPS injection).

It is known that danaparoid has clinically little effect on routine coagulation tests (activated partial thromboplastin time, prothrombin time, and thrombin time) in the patients under treatment with this antithrombotic agent [51]. Recently, it has been reported that danaparoid suppressed the serum levels of inflammatory cytokines (tumor necrosis factor-α and interleukin-6) and high mobility group box 1 in the LPS-induced rats [52]. Consistent with this report, treatment with danaparoid abolished the increases in PCNA-positive cells and macrophage infiltration in LPS-injected HIGA mice and returned them to the basal levels of the saline-injected BALB/c group in our present study. This likely occurred because HIGA mice already have the basic features of MsPGN and show mesangial cell proliferation in contrast to BALB/c mice [14]. The quantitation of fibrin staining in this study suggested that LPS was the major stimulant to glomerular fibrin deposition, because fibrin was markedly higher in LPS-injected BALB/c mice than in basal HIGA mice without LPS in this study. On the other hand, response of glomerular cell proliferation after LPS injection was prominent in HIGA mice compared with BALB/c mice. Therefore, it was suspected that the injury in HIGA mice was not solely due to the effects of LPS. The hyperreaction to LPS in HIGA mice may be due to increased responses to secondary stimulation via constitutive expression of TLR4 and upregulation of PAR2. In this context, it is known that endotoxin tolerance in macrophages correlates with downregulation of surface TLR4 expression [53]. Taken together, it was suspected that related mechanisms might occur in HIGA mice after double administration of LPS at an interval of 3 days.

In this study, danaparoid was administered intraperitoneally as shown in figure 1 for 7 days at the dose of 400 U/kg BW twice daily, which is nearly ten times larger than the dose of clinical use for heparin-induced thrombocytopenia in the European countries (maintenance, 200 U/h). Takahashi et al. [54] compared clinical doses of unfractionated heparin, dalteparin, and danaparoid in the factor Xa activity and bleeding time in mice with tumor zenografts. Although all these anticoagulants inhibited factor Xa activity, prolonged bleeding time was noted only in the treatment with unfractionated heparin. In this context, danaparoid hardly increased bleeding in intravenous doses up to 1,600 anti-Xa U/kg, in contrast to elevated bleeding risk from intravenous doses of 90 anti-Xa U/kg heparin in rats [55]. In consideration of the results in the present experimental design to potential human therapy, factor Xa regulation may be promising for the treatment of the aggravation of IgA nephropathy after microbial infection. However, danaparoid undergoes renal clearance, and the half-life of elimination of plasma anti-Xa activity may be prolonged in patients with severe renal impairment, i.e. chronic kidney disease stage 4–5. Therefore, close monitoring of such patients is recommended when using danaparoid [56]. In the near future, novel factor Xa inhibitors with the characteristics of oral administration, biliary elimination, and usability for patients with renal impairment may appear.

In conclusion, the results of the present study suggest that tissue factor and factor V induction by LPS may at least in part aggravate MsPGN through activation and accumulation of factor X, which could produce further

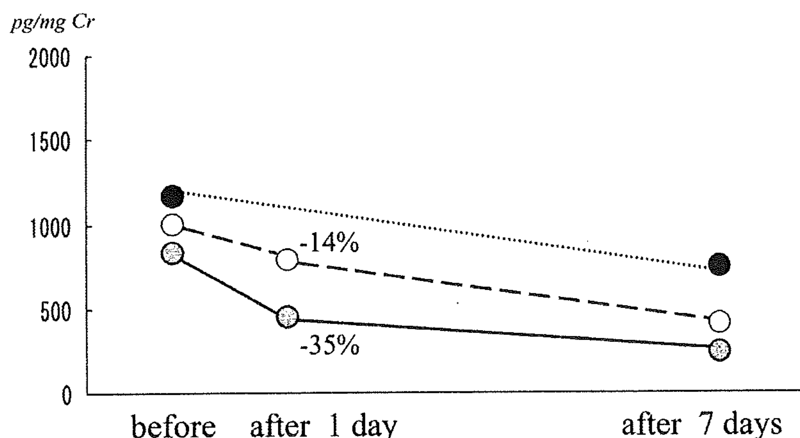

Figure 3. The 8-iso-prostaglandin F2α levels in patients given 3 regimens of intravenous immunoglobulin. (●) 400 mg·kg^{-1}·day^{-1} for 5 days, (○) 1 g/kg for 1 day (◎) 2 g/kg for 1 day.

with the single 2 g/kg administration compared with those who received 1 g/kg of IVIG infusion for 1 day (1 g/kg vs 2 g/kg, –214 pg/mg Cr –14% vs –329 pg/mg Cr –35%; P= 0.2 ANOVA), although this was not statistically significant (**Figure 3**).

We had only 2 cases of CAL and could not evaluate the 8-iso-PG level in those patients.

Discussion

Cardiovascular disease is related to increased systemic OS; established risk factors of systemic vascular disease, such as diabetes, have been associated with elevated levels of markers of OS. Measurement of the urinary excretion of 8-iso-PG has been characterized as a method of investigating lipid peroxidation in vivo and has been shown to reflect enhanced OS, regardless of the underlying pathophysiological trigger. Systemic vascular disease in which endothelial dysfunction is postulated on the basis of recent data has been reported to be associated with increased 8-iso-PG levels.[3–5]

Palombo et al demonstrated that hypercholesterolemic animals had increased circulating levels of 8-iso-PG and increased deposition on the intimal surface of vessels.[12] The association between increased circulating levels and increased intimal deposition of 8-iso-PG supports the pathogenetic role of 8-iso-PG in vascular damage. This finding leads to a hypothesis of intimal disease as a dynamic process involving the arterial wall in the early stages of atherosclerosis, where the morphologic abnormality may be related to possibly reversible biochemical changes more than to permanent structural abnormalities.[12]

Kato et al reported that baseline percent flow-mediated vasodilation (FMD) was lower and the baseline 8-iso-PG level higher in smokers than in nonsmokers, that %FMD decreased and the 8-iso-PG level increased in nonsmokers to the levels in smokers after exposure to 30 min of passive smoking, and the %FMD negatively correlated with the 8-iso-PG level.[13] It has already been established that vascular endothelial function is impaired and plasma lipid peroxidation products are elevated in nonsmokers exposed to passive smoke.[13] This data showed of 8-iso-PG products immediately after exposure to OS. Our results demonstrate that isoprostane products are present in the acute phase of KD. In our group of KD patients, the urinary 8-iso-PG level was higher than that of healthy subjects, which suggests that in the acute phase of KD there is endothelial peroxidation, which may be associated with increased OS in this setting. Moreover, the normal range of 8-iso-PG in childhood is less than 650 pg/mg Cr and our results showed that the 8-iso-PG level in children was higher than that in healthy adults.[14–16] However, we did not differentiate between infants and older children. The 8-iso-PG level in the KD patients was also higher than in those with a febrile disease caused by a mild viral infection. The 8-iso-PG level in patients with a severe bacterial infection may be higher than in those patients.

We observed that IVIG reduced the 8-iso-PG levels in patients with acute phase of KD who had significantly increased 8-iso-PG levels before treatment, which suggests that IVIG may be an anti-oxidant, as are vitamins E and C.

Measurement of the 8-iso-PG concentration has proven to be valuable in assessing OS in vivo, because it is a specific product of lipid peroxidation.[15–18] Although an increase in the 8-iso-PG level is indirect evidence of endothelial dysfunction, 8-iso-PG is a specific marker of oxidative injury in vasculitis.[19–22] Our study demonstrated that OS activation plays an important role in the pathological process of the acute phase of KD. In addition, 8-iso-PG is also a reliable marker of OS in the acute phase of KD and may be a sensitive marker of the effects of IVIG therapy.

Interestingly, our results showed that there is no correlation between 8-iso-PG and inflammatory markers such as CRP, WBC count and ESR. This observation may indicate that 8-iso-PG is an independent parameter of endothelial injury in the acute phase of KD. Furthermore, we hypothesize that IVIG may have antioxidative effects that prevent the generation of 8-iso-PG.

We had 2 patients with coronary artery abnormalities, but only 2 cases are too few to assess OS in patients with CAL and further study of such patients should be needed.

In a recent study, 8-iso-PG promoted platelet aggregation and induced platelet adhesion. Moreover, 8-iso-PG is a powerful constrictor of the vasculature and so increased generation of bioactive 8-iso-PG may be responsible for damage.

Another important point is that measurement of urinary 8-iso-PG is a totally noninvasive method and is superior to measurement of 8-iso-PG in serum, because urinary 8-iso-PG is a stable molecule.[22–24]

Our data showed that measurement of an OS marker could represent oxidative capacity in vascular disease. Further studies are needed to evaluate 8-iso-PG against antioxidants such as vitamin C in acute KD.

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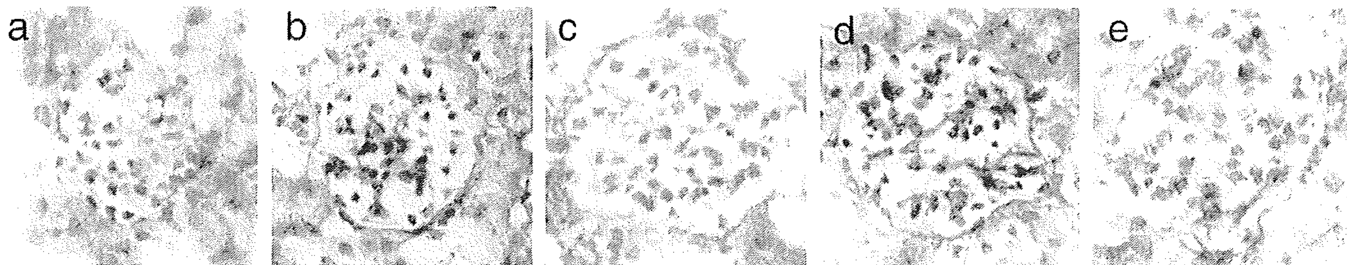

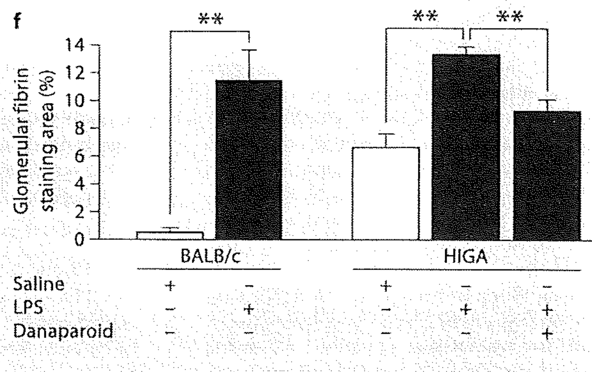

Fig. 7. Change in fibrin deposition after LPS administration and danaparoid treatment. Representative photomicrographs of glomeruli from basal BALB/c (**a**), LPS-injected BALB/c (**b**), basal HIGA (**c**), LPS-injected HIGA (**d**), and LPS + danaparoid-injected HIGA mice (**e**) are shown. The PCNA-positive cell number was markedly increased in BALB/c (**b**) and HIGA (**d**) mice after LPS administration. **e** A marked reduction in fibrin deposition in danaparoid-treated HIGA mice was observed. Final magnification, ×400. **f** The percentage of fibrin-stained area for each group is shown. Values are represented as mean ± SEM. ** $p < 0.01$ (6 mice per group).

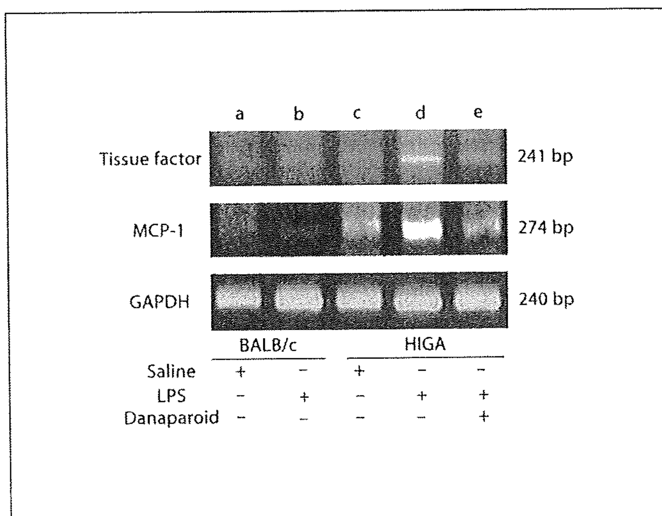

Fig. 8. Inhibitory effects of danaparoid on tissue factor and MCP-1 mRNA expression. Representative picture is shown. Although tissue factor and MCP-1 mRNA expression was negative in basal BALB/c mice (**a**), it was mildly detected after LPS administration (**b**). Compared with basal HIGA mice (**c**), LPS injection resulted in upregulation of tissue factor and MCP-1 mRNA (**d**) that was both suppressed to basal levels in danaparoid-treated HIGA mice (**e**). GAPDH mRNA served as an internal control (3 mice per group).

with fibrin deposition was suggested to be involved in the development of glomerular injury [34]. Tissue factor and factor V are produced in mesangial cells upon stimulation by proinflammatory cytokines [7, 35]. In our previous study, factor V mRNA and protein expression was upregulated in mesangial cells of active IgA nephropathy [22, 36], and cross-linked fibrin deposition with accompanying macrophage infiltration was also observed [35]. It is known that monocytes/macrophages contain small amounts of tissue factor, which is strongly induced upon cellular activation [37]. It has been reported that mesangial cells express both tissue factor and MCP-1 after LPS exposure [7, 38]. MCP-1 mRNA expression, macrophage infiltration, and tissue factor accumulation were all observed in LPS-injected HIGA mice in the present study. Factor X is produced mainly by hepatocytes in vivo, and circulates in blood vessels [39]. Factor Xa forms a complex with its cofactor, factor Va. Therefore, it is likely that in the present study factor X was supplied from the blood circulation, activated by tissue factor expression in mesangial cells and macrophages, and then maintained in the mesangial area by factor V.

Factor Xa is not only a key factor in coagulation cascades, but also a potent mitogen for endothelial and smooth muscle cells; it stimulates the production of inflammatory cytokines such as MCP-1 together with in-

Table. Clinical Characteristics of Patients and Controls

	KD group	FI group	HC group	P value
n	62	20	20	NS
Age (median)	2 months–11 years (4 years)	1–10 years (5 years)	1–11 years (4 years)	NS
M/F	37/25	12/8	10/10	NS

KD, Kawasaki disease; FI, febrile illness; HC, healthy children.

Results

Clinical Characteristics of Patients and Controls

The baseline characteristics of the patients and control subjects are detailed in **Table**. No significant differences between patients with KD, FI and HC were found. Two patients in the KD group had coronary artery abnormalities (1 had a coronary artery lesion (CAL), 1 had coronary artery dilatation).

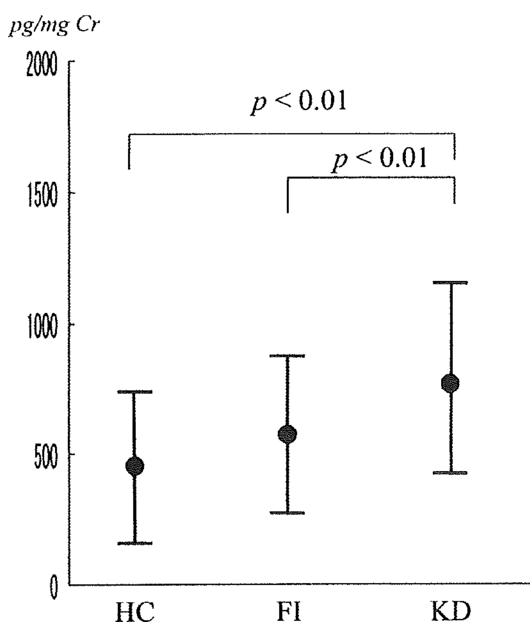

Figure 1. The 8-iso-prostaglandin F2*α* in patients with Kawasaki disease (KD) or febrile illness (FI) and in healthy controls. Gray zone shows the normal range in children. HC, healthy children.

8-iso-PG Levels in the KD and Control Groups

The 8-iso-PG was significantly elevated in the 62 KD patients (719±335 pg/mg Cr) before IVIG administration compared with the 20 patients with FI (583±213 pg/mg Cr) and the 20 HC (443±288 pg/mg Cr) (P<0.01 1-way ANOVA) (**Figure 1**). The normal value of 8-iso-PG in children is <650 pg/mg Cr.

8-iso-PG and Inflammatory Parameters

We evaluated inflammatory parameters such as CRP, albumin, WBC count, neutophil count and ESR, but there were no significant correlations between 8-iso-PG and any of these.

8-iso-PG Levels in the Acute Phase of KD After IVIG Therapy

We evaluated 8-iso-PG levels in 40 patients with acute KD before and after IVIG treatment. The 8-iso-PG levels at 7 days after a single administration of IVIG were significantly decreased compared with before treatment (707± 356 pg/mg Cr vs 426±214 pg/mg Cr; P<0.01 paired t-test) (**Figure 2**). The 8-iso-PG levels before IVIG administration tended to be decreased compared with after 14 days (707± 356 pg/mg Cr vs 556±324 pg/mg Cr; P=0.05 paired t-test).

We also evaluated the 8-iso-PG levels in 40 patients given 3 different regimens of IVIG: 16 patients received 2 g/kg for 1 day, 17 patients received 1 g/kg for 1 day and 7 patients received 400 mg·kg^{-1}·day^{-1} for 5 days. All treatment regimens of IVIG reduced the 8-iso-PG level at 7 days after administration. The 8-iso-PG levels before IVIG administration were not significant different between the 3 regimens. The 8-iso-PG tended to decrease in the patients

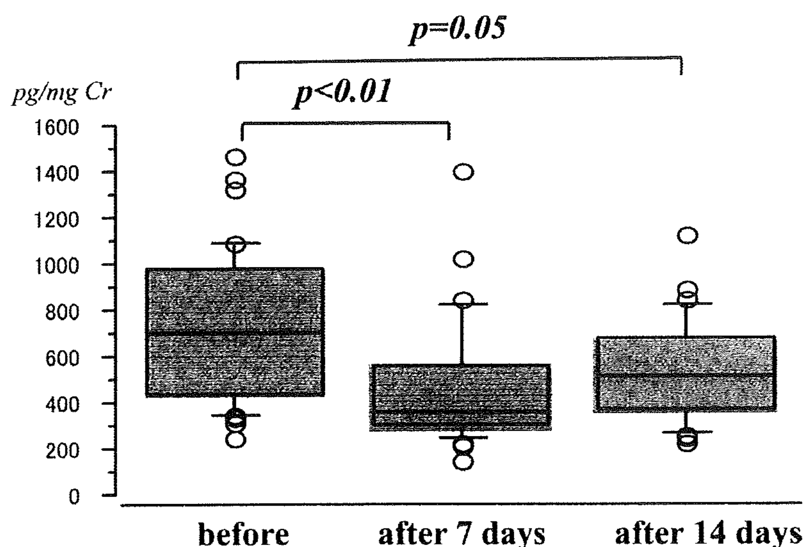

Figure 2. The 8-iso-prostaglandin F2*α* levels before and after intravenous immunoglobulin therapy (7 and 14 days).

Table 2. *Clinical baseline characteristics and outcome of refractory KD patients treated with infliximab*

Patient no.	Age	Sex	Illness day of 1st IVIG	IVIG dose	Other treatment	Infliximab dose	Illness day of infliximab	Fever duration	Efficacy	CAL	Adverse effect
1	1.2	M	4	4 g/kg	UTI	5 mg/kg	9	9	Yes	No	No
2	7.1	M	3	4	UTI	5	8	8	Yes	No	No
3	2	F		0*	IVMP, UTI	5	12	12	Yes	Yes*	No
4	2	F	5	4	IVMP, CyA, UTI	10†	8	32	No	No	No
5	4	M	3	3	UTI	5	8	8	Yes	No	No
6	4	F	5	5	IVMP	5	12	12	Yes	No	No
7	1	M	3	3	IVMP	5	9	9	Yes	Yes*	No
8	2	M	6	6	IVMP	5	12	14	Yes	No	No
9	1	M	3	4	IVMP	5	12	16	No	Yes*	No
10	1	F	7	4	IVMP	5	12	15	No	No	No
11	3	F	4	4	IVMP	5	11	12	Yes	Yes*	No

KD, Kawasaki disease; IVIG, intravenous immune globulin; UTI, ulinastatin; IVMP, intravenous methylpredonisolone pulse; CyA, cyclophosphamide A; CAL, coronary artery lesion.

* Patient No. 3 did not use IVIG because she had a history of allergic reaction to immunoglobulin.

† Patient No. 4 received 5 mg/kg infliximab twice.

Table 3. *Serum concentrations of pro-inflammatory cytokines and endothelial cell specific cytokines in refractory KD patients treated with infliximab, comparing with responders and non-responders to IVIG*

	Healthy controls	Refractory patients treated with infliximab	Responders to IVIG	Nonresponders to IVIG	*p*
No. patients	33	11	18	14	
Proinflammatory cytokines cytokines					
CRP (mg/dL)					
Before treatment		13.9 ± 9.5	7.1 ± 3.8	12.3 ± 5.6	0.00590
After treatment		10.6 ± 10.3	4.5 ± 4.2	14.6 ± 8.8	0.01595
STNFR (ng/mL)					
Before treatment	0.217 ± 0.080	0.714 ± 0.161	0.580 ± 0.180	0.600 ± 0.241	0.2155
After treatment		0.391 ± 0.161	0.326 ± 0.147	0.474 ± 0.220	0.0402
IL-6 (pg/mL)					
Before treatment	20 ± 10	1013 ± 1386	245 ± 321	276 ± 167	0.01786
After treatment		233 ± 561	69 ± 217	182 ± 387	0.14883
Endothelial cell specific cytokines specific cytokines					
MRP8/MRP14 (ng/mL)					
Before treatment	220 ± 40	4859 ± 2997	3261 ± 1724	4818 ± 3983	0.03907
After treatment		5860 ± 5468	2063 ± 1499	4588 ± 4397	0.00459
S100A12 (ng/mL)					
Before treatment	52 ± 32	1027 ± 615	412 ± 315	1148 ± 1837	0.00165
After treatment		1180 ± 1201	244 ± 286	651 ± 574	0.00201
sRAGE (pg/mL)					
Before treatment	1794 ± 368	868 ± 613	1495 ± 834	637 ± 404	0.00210
After treatment		1224 ± 497	3212 ± 1597	864 ± 553	0.00023
VEGF (pg/mL)					
Before treatment	92 ± 12	970 ± 1030	525 ± 607	790 ± 674	0.00809
After treatment		814 ± 946	425 ± 426	975 ± 636	0.07437

KD, Kawasaki disease; IVIG, intravenous immune globulin; CRP, C-reactive protein; STNFR, soluble tumor necrosis alpha receptor; IL-6, interleukin 6; MRP, myeloid-related protein; sRAGE, soluble receptor for advanced glycation end products; VEGF, vascular endothelial growth factor.

Data are mean ± SD. *p* value is derived from comparison of refractory patients and IVIG responders.

Serum cytokine levels. The changes in serum levels of cytokines in refractory KD, and responders and nonresponders are shown in Table 3 and Figures 1 and 2.

Serum CRP levels were higher in the refractory KD and nonresponders groups than the responders before treatment (Fig. 1). Levels decreased in both the refractory and responders after treatment but increased further in the nonresponders. Before treatment, SNTFR was increased in all three groups by comparison with controls before treatment and declined to the normal level after treatment in all three groups. Serum IL6 was increased in all three patient groups by comparison with controls before treatment and was significantly higher in the refractory group than the other two groups. After treatment, all three groups decreased but remained higher than controls and were indistinguishable from each other.

Serum levels of MRP8/MRP14 and S100A12 were higher than controls in all three patient groups before treatment with the levels in the refractory patients and nonresponders higher than the responders (Fig. 2). After treatment, levels decreased in the responders, but increased further in the refractory patients and nonresponders. Serum levels of sRAGE were lower than controls in the refractory and nonresponder groups before treatment, whereas they were similar to controls in the responder group. Levels of sRAGE increased in all three

Conclusions

Our study revealed OS provokes vasculitis in KD, the activation of which was reduced by IVIG administration. We conclude that the urinary 8-iso-PG level is a sensitive and useful marker of the effectiveness of IVIG therapy in the acute phase of KD.

Acknowledgments

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IVIG Reduced Vascular Oxidative Stress in Patients With Kawasaki Disease

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Background: Oxidative stress (OS) contributes to the acute phase of Kawasaki disease (KD) in a manner that is as yet unknown. In the present study OS in the acute phase of KD was investigated by measuring urinary 8-iso-prostaglandin F2α (8-iso-PG) and evaluating its correlation to the efficacy of intravenous immunoglobulin (IVIG) administration.

Methods and Results: The 62 patients with acute phase of KD were enrolled, as well as 20 healthy children (HC) and 20 with acute febrile illness (FI). Urinary samples were obtained before and after administration of IVIG. The HC and FI groups also had inflammatory markers evaluated at the same time. The 8-iso-PG was significantly elevated in the 62 KD patients (719±335 pg/mg Cr) without IVIG administration compared with those with FI (583±213 pg/mg Cr) as well as HC (443±288 pg/mg Cr) ($P<0.01$). 40 patients were given 3 different regimens of IVIG: 16 received 2 g/kg for 1 day; 17 received 1 g/kg for 1 day; 7 received 400 mg·kg^{-1}·day^{-1} for 5 days. All regimens of IVIG reduced the 8-iso-PG level at 7 days after initiation.

Conclusions: OS provokes vasculitis in KD, the activation of which was reduced by IVIG. The urinary level of 8-iso-PG is a useful marker of the effectiveness of IVIG in the acute phase of KD. (*Circ J* 2009; **73:** 1315–1318)

Key Words: Intravenous immunoglobulin; 8-iso-prostaglandin F2α; Kawasaki disease; Oxidative stress

Oxidative stress (OS) plays an important role in vascular diseases. Endothelial dysfunction activates the pathway that leads to elevated OS.[1] In particular, oxidative damage occurs when the delicate balance between pro- and antioxidant molecules, which act against free radical injury, is upset. This balance may be destroyed by certain risk factors; for example, atherosclerosis, hypertension, hyperlipidemia, diabetes and cigarette smoking.[2–6]

The 8-isoprostaglandin F2α (8-iso-PG) is a nonenzymatic oxidation product of arachidonic acid and is widely recognized as a reliable marker of lipid peroxidation both in vitro and in vivo.[3,4,7] Enhanced endothelial dysfunction, as reflected by increased 8-iso-PG excretion, has been reported previously.[3–5]

Kawasaki disease (KD) is a systemic vasculitis and an acute febrile illness (FI) in children. Recent studies have shown activation of the immune system is involved and multiple factors are likely to cause the pathologic changes seen in KD.[8–11] How OS contributes to the acute phase of KD is as yet unknown.

In the present study, we investigated OS in the acute phase of KD by measuring urinary 8-iso-PG and evaluated its correlation to the efficacy of intravenous immunoglobulin (IVIG) treatment.

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Methods

Patients

All subjects of this study were typical KD patients admitted to Toho University Hospital between March 2002 and September 2004. They were given IVIG (1–2 g/kg) therapy in the acute phase: 62 patients (37 boys, 25 girls, median age: 4 years, range: 2 months to 11 years) were enrolled. None of the patients had other disorders, such as infection, collagen disease or congenital heart disease. We also studied 20 healthy children (HC, median age: 4 years, range: 1–11 years) and 20 patients with an acute FI such as viral pharyngitis and tonsillitis (median age: 5 years, range: 1–10 years). Informed consent was given by the parents of the patients participating in the study.

Biochemical Measurements

Urinary 8-iso-PG was measured by enzyme immunoassay and the concentration was corrected by urinary creatinine concentration. The samples were obtained before and after IVIG administration. In the HC and FI groups inflammatory markers such as C-reactive protein (CRP), white blood cell (WBC) count, neutrophil count, albumin level and erythrocyte sedimentation rate (ESR) were measured at the same time. Echocardiography was performed in the patients in the acute phase of KD.

Statistical Analysis

Differences between continuous variables were evaluated by t-test. The data for each group were analyzed using ANOVA. The relationship between 2 different parameters was obtained by simple regression analysis. A P-value <0.05 was considered statistically significant.

products (RAGE) and induces cell activation and cytokine production through the nuclear factor-kappa-B signaling pathway (10,11). Transcript and protein levels of these three S100 proteins are reported to be strongly up-regulated during the acute stage of KD and decrease significantly in response to IVIG treatment and are possibly involved in the pathophysiology of acute vasculitis (8–11,15).

Here, we report the distinct effects of infliximab on systemic inflammation and local vasculitis, evaluating both proinflammatory and DAMP molecules and VEGF expression during infliximab treatment.

METHODS

Study population and blood samples. Patients with acute KD seen at the University Hospitals of Toyama University and Toho University School of Medicine between January 2005 and December 2007 were enrolled. All patients fulfilled the diagnostic criteria for KD and were initially treated with IVIG (2 g/kg body weight for 1 d) and oral aspirin (30 mg/kg/d) (16). Patients whose fever subsided within 48 h of IVIG treatment (2 g/kg body weight for 1 d) were considered responders. Patients whose fever did not subside within 48 h of the first IVIG treatment (2 g/kg body weight for 1 d) but responded to the second IVIG were designated nonresponders. Refractory KD was defined as the persistence or recrudescence of fever at least 48 h after the end of the multiple administrations of IVIG or IVMP infusion. Infliximab was used to the patient who was more than 1-y-old and received BCG vaccine. Thirty-one age-matched healthy control patients (16 males and 15 females), aged 3 mo to 7 y 5 mo (median 3.8 y), were enrolled during the same period.

Two-dimensional echocardiography was performed before and after treatment with IVIG as well as at 2 and 4 wk after the onset of KD, which was defined as the day on which fever developed. A coronary artery with a diameter of 3 mm or more (4 mm if the subject was over the age of 5 y) by echocardiogram was considered abnormal (16). The presence of CAL was assessed 1 mo after the onset of KD.

Patient demographic characteristics, therapies administered for KD before and after infliximab treatment, C-reactive protein (CRP) levels, dose, patient response to infliximab, and coronary artery outcome were recorded for all patients. Blood samples were collected from patients before and within 48 h after infliximab treatment in refractory KD and before and within 48 h after the first IVIG treatment in responders and nonresponders to IVIG. Simultaneously, blood samples were collected from healthy control patients on a single occasion. Parental informed consent was obtained for each child enrolled in this study, which was approved by the Research Ethics Committee of Toyama University Hospital.

Determination of serum cytokine levels. Serum concentrations of soluble tumor necrosis factor-alpha receptor I (sTNFRI), IL-6, VEGF, MRP 8/MRP 14, S100A12, and soluble receptor for advanced glycation end product (sRAGE) were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). Commercial kits to quantitative human sTNFRI (Bender MedSystem, Vienna, Austria), human IL-6 (BioSource International, CA), human VEGF (Immuno-Biologic Laboratories, Fujioka, Japan), and sRAGE (Quantikine; R&D Systems, Minneapolis, MN) were used according to the manufacturer-recommended procedures (17,18). Serum concentrations of S100A12 and MRP8/MRP14 were determined by a double-sandwich ELISA using a specific antibody without cross reactivity to other S100 proteins, as described previously (19).

Statistical analysis. All results were expressed as mean ± SD. Paired t test was used to test for significance of the same parameter, within the same group, respectively. Analysis of variance or χ^2 test was used to test for significance of variables among three different groups. If data did not follow a normal distribution, the paired *t* test was replaced by a Wilcoxon signed rank test. When the data followed a normal distribution determined by Shapiro-Wilks test, comparisons between the two groups were performed using an unpaired t test or a Welch's test depending on equal or unequal variance. If the data did not follow a normal distribution, then a Mann Whitney *U* test was used.

RESULTS

Patient clinical characteristics and laboratory data. Table 1 shows the clinical characteristics of the 43 KD patients enrolled, comprising 18 responders, 14 nonresponders to the first IVIG, and 11 refractory KD patients treated with infliximab. The refractory KD patients and nonresponders had a significantly higher maximum concentration of CRP, a higher incidence of CALs, and a longer duration of fever compared with the responders. There were no significant difference between the groups with respect to age and the maximum number of white blood cells (WBCs).

In the refractory KD group, 10 patients received three or more IVIG infusions and eight patients received one to three doses of pulse methylprednisolone (30 mg/kg/dose i.v.) (Table 2). One patient did not use IVIG because she had a history of allergic reaction to immunglobulin. In addition, CyA was used in one patient (Table 2). All failed to become persistently afebrile after these treatments.

Patient outcome. Of the 11 refractory patients, 10 received a single infusion of 5 mg/kg of infliximab: one patient received a second infusion of 5 mg/kg. In eight of the 11 patients, fever subsided dramatically in response to the infliximab treatment and other symptoms disappeared (Table 2). In three patients, fever persisted and needed additional therapy after infliximab treatment. Four patients had coronary artery abnormalities documented by echocardiography before infliximab therapy and had transient dilatation that resolved postinfliximab infusion (Table 2); these patients continued to have CAL after treatment. Seven patients had normal coronary arteries preinfliximab and postinfliximab treatment. All 11 patients (excluding Patient 1, who died) were followed for 6–26 mo (median follow-up 17 mo) with no apparent complications of their infliximab therapy. Tuberculin reactions before and 6 mo after infliximab treatment disclosed no evidence of tuberculosis in all patients. Chest CT after 6 mo of infliximab treatment revealed normal findings in all patients.

Table 1. *Clinical demographic data of refractory KD patients treated with infliximab, comparing with patients responding to IVIG treatment and those who did not*

	Healthy controls	Refractory patients treated with infliximab	Responders to IVIG	Nonresponders to IVIG	*p*
No. patients	31	11	18	14	
Coronary artery lesion	0	4 (37%)	4 (9%)	6 (40%)	0.0984
Sex (male)	16 (52%)	6 (55%)	9 (50%)	10 (69%)	0.6833
Age in y (median, range)	3.8 (0.3–7.5)	4.0 (1.0–7.1)	2.6 (0.4–7.2)	2.8 (0.2–6)	0.6890
Max. CRP, mg/dl		14.3 ± 9.1	9.9 ± 5.2	15.5 ± 7.4	0.0068
Max. WBC, $\times 10^3$/mm^3		24.6 ± 4.7	15.4 ± 4.1	14.6 ± 8.7	0.0001
Duration of fever (d)		13.4 ± 6.8	6.7 ± 1.5	10.3 ± 3.1	0.0002

KD, Kawasaki disease; IVIG, intravenous immune globulin; CRP, C-reactive protein; WBC, white blood cells.
Data are mean ± SD. *p* value is derived from comparison of refractory patients and responders.

Risk Stratification in the Decision to Include Prednisolone With Intravenous Immunoglobulin in Primary Therapy of Kawasaki Disease

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Background: We reported previously that intravenous immunoglobulin (IVIG) plus prednisolone for initial therapy for Kawasaki disease (KD) prevented coronary artery abnormalities (CAA) more effectively than IVIG alone. However, questions remain as to whether PSL has potential benefit in all KD patients. The present study was designed to explore the possibility of stratified initial therapy including PSL in patients with and without a high predicted risk of being an IVIG nonresponder.

Methods: We retrospectively analyzed data from KD patients who received IVIG (n = 896) or IVIG + PSL (n = 110) by scoring the likely risk of being an IVIG nonresponder. We compared clinical and coronary outcomes between treatment-defined groups separately for high- and low-risk patients.

Results: Among low-risk patients (score 0–4), clinical and coronary outcomes were similar. Among high-risk patients (score 5 or more), incidences of treatment failure and coronary artery abnormalities until 1-month follow-up were more frequent in the IVIG than in the IVIG + PSL group. Sex- and score point-adjusted odds ratios for IVIG + PSL were 0.17 (95% confidence interval, 0.08–0.39) for treatment failure and 0.27 (95% confidence interval, 0.07–0.85) for coronary artery abnormalities A among high-risk patients.

Conclusions: IVIG + PSL treatment was associated with improving clinical and coronary outcomes in patients at high risk of being IVIG nonresponders.

Key Words: risk stratification, treatment failure, prednisolone, intravenous immunoglobulin, coronary artery abnormalities

(*Pediatr Infect Dis J* 2009;28: 498–502)

Kawasaki disease (KD) is an acute febrile illness of childhood characterized by clinical, biochemical, and histopathologic manifestations of systemic vasculitis.[1] Echocardiographic and cardiac angiographic data indicate that 20% to 25% of untreated KD patients develop coronary artery abnormalities (CAA).[2] Use of high-dose intravenous immunoglobulin (IVIG) together with aspirin is clearly effective in resolving inflammation in KD and reducing the occurrence of CAA.[3] However, about 10% to 20% of patients still have persistent or recurrent fever after completion of IVIG treatment, while CAA occur in about 10% of KD patients despite this therapy.[1] KD is presently considered to be the most common pediatric cause of acquired heart disease in developed countries.

Although corticosteroids are considered as a treatment option for various types of vasculitides, many physicians were uncomfortable with the use of corticosteroids in KD because of an early report,[4] showing a high incidence of CAA in a group that received a prolonged course of oral prednisolone (PSL) alone. Subsequent retrospective studies on effects of corticosteroids in KD, however, have shown either no ill effects or possible benefits.[5,6] Wooditch and Aronoff[7] concluded from a metaanalysis that inclusion of corticosteroids in aspirin-containing regimens for primary therapy for KD reduced the incidence of CAA. Recently, we reported a multicenter, prospective, randomized controlled trial demonstrating that primary therapy with IVIG plus PSL had a significant advantage over IVIG alone with respect to prevention of CAA and rapid resolution of inflammation.[8] Nonetheless, corticosteroid therapy may be associated with potential adverse reactions.[9–11] Additionally, a large fraction of IVIG responders, who account for 80% of KD patients, ultimately remain free of CAA.[12] Whether or not corticosteroids should be administered to all KD patients, therefore, is uncertain.

Recently, we developed a risk score that identified IVIG nonresponders in advance, with high sensitivity and specificity, based on 7 laboratory and demographic variables available before initiation of primary therapy.[13] This risk score could define 2 risk strata in patients with KD, indicating high- or low-risk for IVIG unresponsiveness. The risk score thus might enable us to identify KD patients who require more intensive primary therapy. We hypothesized that the addition of PSL to IVIG as primary therapy for KD patients would offer important therapeutic benefits to patients in this high-risk stratum. To explore the possibility of stratified primary therapy in KD patients, we retrospectively introduced risk stratification to identify the benefits of primary therapy, including PSL, in patients with and without a predicted high risk of IVIG unresponsiveness.

MATERIALS AND METHODS

Study Patients and Outline of Therapies

Data used for the present study were obtained from consecutive KD patients from August 2000 to August 2007 at 13 medical institutions in Gunma and Saitama prefectures in Japan. KD was diagnosed using the Japanese Diagnostic Guidelines for KD (fifth revised edition).[14] The first day of illness was defined as the first day of fever. Patients in the IVIG group received IVIG (1 g/kg/d

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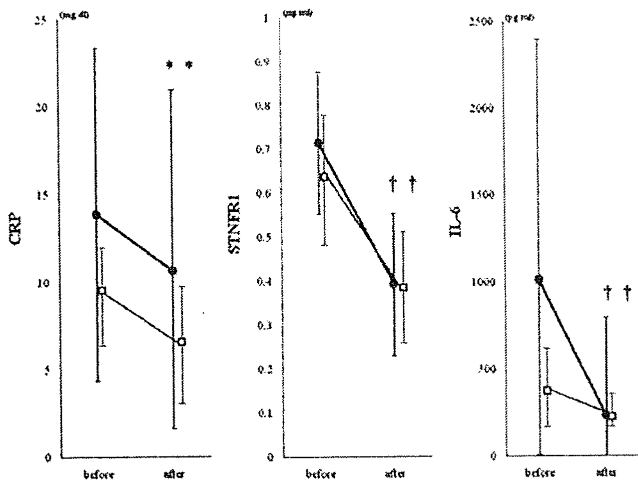

Figure 1. Sequential changes of proinflammatory cytokines, CRP (*left*), sTNFR I (*middle*), and IL-6 (*right*), in refractory KD patients who were treated with infliximab (●), compared with responders (□). Blood samples were collected from patients before and within 48 h after infliximab treatment in refractory KD and before and within 48 h after the first IVIG treatment in responders to IVIG. The p value shows changes between before and after treatment in each group. $*p < 0.05$, $\dagger p < 0.01$.

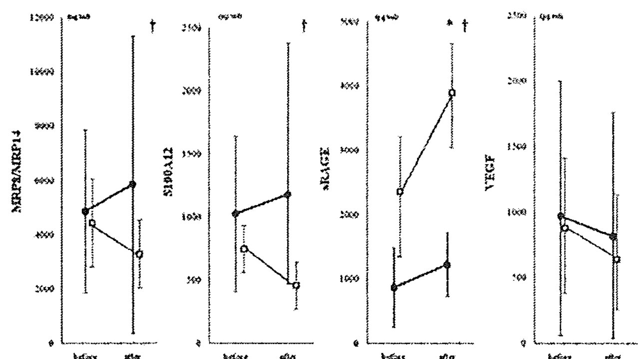

Figure 2. Sequential changes of endothelial cell specific cytokines and markers of local tissue damage, myeloid-related protein (MRP8/MRP14) (*left*), S100A12 (*left middle*), sRAGE (*right middle*), and VEGF (*right*), in refractory KD patients who were treated with infliximab (●), compared with responders (□). p value shows changes between before and after treatment in each group. $*p < 0.05$, $\dagger p < 0.01$.

groups after treatment but remained below normal in the refractory and nonresponder groups. Serum levels of VEGF were higher than controls in all three patient groups before and after treatment with only moderate changes seen after treatment.

As a result, each of the cytokines was elevated compared with healthy controls before treatment in all patients. Although serum IL-6 and sTNFRI levels dramatically decreased after infliximab treatment and correlated with serum CRP levels and fevers, the serum levels of VEGF and DAMP-molecules such as MRP8/MRP14 and S100A12 remained high after infliximab treatment in refractory KD. This pattern of cytokines in refractory KD is different from responders or nonresponders after IVIG treatment; all cytokines decreased markedly in responders and did not decrease in nonresponders.

DISCUSSION

In this study, we show for the first time that different cytokines and proinflammatory DAMP-molecules are upregulated and changed dynamically during IVIG and infliximab treatment in refractory KD patients (Table 3, Fig. 1 and 2). In refractory KD, the serum sTNFRI and IL-6 levels reacted to infliximab treatment, but VEGF and DAMP-molecules like MRP8/MRP14 and S100A12 as well as sRAGE were not significantly affected.

Previous studies have shown that sTNFRI is the natural homeostatic regulator of TNF-α activity and may reflect the true biologic activity of TNF-α more closely than serum TNF-α level (20). IL-6 causes many of the clinical and laboratory features of KD and is a reflection of a vigorous acute phase response (21). A recent case study reported that serum IL-6 was elevated and then decreased after infliximab treatment in a refractory KD patient (22). In this study, the fact that the serum levels of sTNFRI and IL-6 are elevated and decrease after infliximab treatment suggest infliximab blocks systemic inflammation and inhibits the process of signaling of cytokines *via* TNF-α in KD.

In contrast, the serum levels of MRP8/MRP14, S100A12, and VEGF remained high after infliximab treatment. MRP8/MRP14, a complex of two calcium-binding proteins of the S-100 family, form heterodimers and are secreted by neutrophils and monocytes in response to inflammatory signaling cascades (Fig. 3) (8,9,23). The MRP8/MRP14 heterodimer binds to microvascular endothelial cells and phagocytes and may participate in the genesis of a proinflammatory and prothrombotic state during systemic vasculitis (24). Specifi-

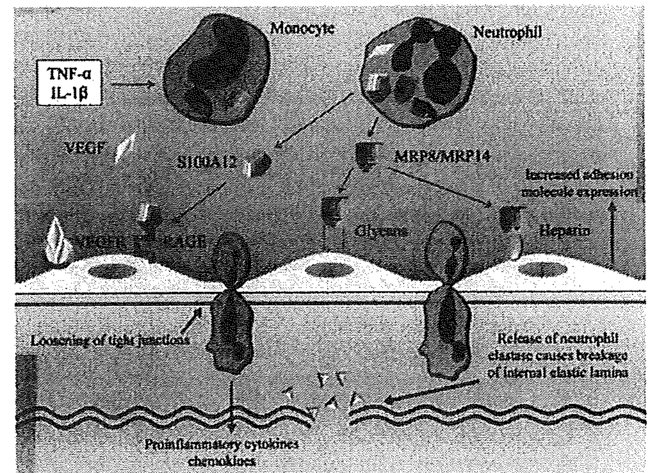

Figure 3. TNF-α and other proinflammatory cytokines activate endothelium and lead to the expression of carboxylated *N*-glycans. MRP8/MRP14 are released in high amounts at local sites of inflammation and have been recently described as novel members of the DAMP-family acting as endogenous ligands of TLR4. Activated neutrophils and monocytes secrete MRP-8/MRP-14 heterodimers, which bind to the carboxylated *N*-glycans and heparin sulfate on the endothelial cell surface. Leukocytes also secrete S100A12, which binds to the RAGE expressed on endothelial cells, lymphocytes, and macrophages. This receptor signals through the nuclear factor-kappa-B pathway and induces expression of many proinflammatory molecules. The net result of S100 protein binding is platelet aggregation and adherence to endothelium, increased expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, adhesion of neutrophils and monocytes, loosening of endothelial cell junctions, and trafficking of inflammatory cells across the endothelial cell barrier. Adapted from Burns J, J Am Coll Cardiol 48:1265–1267, copyright © 2006 American College of Cardiology Foundation Published by Elsevier Inc., with permission.

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Infliximab Reduces the Cytokine-Mediated Inflammation but Does Not Suppress Cellular Infiltration of the Vessel Wall in Refractory Kawasaki Disease

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ABSTRACT: The aim of our study was to evaluate the efficacy of infliximab for the treatment of patients with refractory Kawasaki disease (KD) and investigate the dynamic changes of cytokines during infliximab treatment. We have performed a study of cytokine and proinflammatory molecule levels in 43 KD patients including 18 responders to IVIG, 14 nonresponders, and 11 patients treated with infliximab. We determined serum levels of soluble TNF receptor I (sTNFR I) and IL-6, as well as VEGF, damage associated molecular pattern (DAMP) molecules; myeloid-related protein (MRP)8/MRP14 and S100A12 sequentially. In eight patients, fever subsided immediately upon infliximab treatment. Four patients, who started infliximab after 12 d of illness, developed coronary artery lesions. Each of the cytokines was elevated before infliximab treatment in all patients. Although serum levels of proinflammatory cytokines decreased dramatically after infliximab treatment, DAMP molecules and VEGF and markers of local tissue damage were not suppressed. In contrast, in IVIG responders all cytokines decreased markedly after IVIG treatment. We show that infliximab is one of the adoptive therapies in refractory KD patients. Different behaviors of proinflammatory cytokines and DAMP molecules and VEGF after infliximab treatment suggest that infliximab is effective for suppression of cytokine-mediated inflammation, but could not completely block local vasculitis. ***(Pediatr Res* 65: 696–701, 2009)**

Kawasaki disease (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 i.v. immune globulin (IVIG) is now accepted as reducing the incidence of coronary artery lesions (CAL), approximately 15% of the patients do not respond to IVIG treatment and have persistent fever as a manifestation of ongoing inflammation. These patients are at highest risk for development of CAL (1). The current practice for patients with KD and persistent or recrudescent fever after IVIG is to institute additional therapies, which may include one or more repeat doses of IVIG, high-dose pulse methylprednisoline, cyclophosphamide, methotrexate, ulinastatin, cyclosporine A (CyA), or plasmapheresis (2,3). Recently, potential new therapeutic approaches with infliximab (Remicade), a chimeric mouse-human MAb against tumor necrosis factor (TNF)-α, have been reported in refractory KD patients (4).

During the acute phase of KD, serum levels of proinflammatory cytokines such as TNF-α are elevated (5). In experimental studies of this syndrome, characterized by vasculitis resulting in coronary, as well as extracoronary, aneurysms, and stenosis, the attenuation of cytokine responses, especially IL-6, after infusions of IVIG may play an integral role in the rapid resolution of most of the symptoms in children with KD (6). In addition to these proinflammatory cytokines, VEGF, and markers of local inflammation of the family of damage-associated molecular pattern molecules (DAMPs) such as myeloid-related protein (MRP) 8/MRP14 and S100A12 have been reported to increase in acute KD and to play a crucial role in inflammation and are probably involved in the pathophysiology of acute vasculitis (7–13).

MRP8/MRP14, two calcium-binding proteins in the S-100 family, binds to microvascular endothelial cells and may participate in the genesis of a proinflammatory and prothrombotic state during systemic vasculitis (9,13). MRP8/MRP14 are released in high amounts at local sites of inflammation and have been recently described as novel members of the DAMP-family acting as endogenous ligands of toll-like receptor 4 (TLR4) (12,14). We reported that MRP8/MRP14 levels closely correlate with disease activity in acute KD and potential biomarker to predict both responses to IVIG therapy and coronary artery sequelae in the acute stage of KD (8,9). Another member of the S100 family, S100A12, also binds to endothelial cells *via* the receptor for advanced glycation end

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Abbreviations: CAL, coronary artery lesions; **CRP,** C-reactive protein; **IVIG,** intravenous immune globulin; **KD,** Kawasaki disease; **MRP,** myeloid-related protein; **sRAGE,** soluble receptor for advanced glycation end product; **sTNFRI,** soluble tumor necrosis factor-alpha receptor I; **DAMP,** damage associated molecular pattern molecules

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