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 recrudescence fever after

IVIG is to institute additional therapies, which may include one or more repeat doses of IVIG, high-dose pulse methylprednisolone, 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; sTNFR I, soluble tumor necrosis factor- α receptor I; DAMP, damage associated molecular pattern molecules

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- α receptor I (sTNFRI), IL-6, VEGF, MRP 8/MPR 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/MPR14 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 $\pm$ 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 immunoglobulin. 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	<i>p</i>
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 $\pm$ 9.1	9.9 $\pm$ 5.2	15.5 $\pm$ 7.4	0.0068
Max. WBC, $\times 10^3/\text{mm}^3$		24.6 $\pm$ 4.7	15.4 $\pm$ 4.1	14.6 $\pm$ 8.7	0.0001
Duration of fever (d)		13.4 $\pm$ 6.8	6.7 $\pm$ 1.5	10.3 $\pm$ 3.1	0.0002

KD, Kawasaki disease; IVIG, intravenous immune globulin; CRP, C-reactive protein; WBC, white blood cells.

Data are mean $\pm$ SD. *p* value is derived from comparison of refractory patients and responders.

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 methylprednisolone 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	<i>p</i>
No. patients	33	11	18	14	
Proinflammatory 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					
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, STNFR 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

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, †*p* < 0.01.

Figure 2. Sequential changes of endothelial cell specific cytokines and markers of local tissue damage, myeloid-related protein (MRP8/MPR14) (*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, †*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 sTNFR I 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/MPR14 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 up-

regulated and changed dynamically during IVIG and infliximab treatment in refractory KD patients (Table 3, Fig. 1 and 2). In refractory KD, the serum sTNFR I and IL-6 levels reacted to infliximab treatment, but VEGF and DAMP-molecules like MRP8/MPR14 and S100A12 as well as sRAGE were not significantly affected.

Previous studies have shown that sTNFR I 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 sTNFR I 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/MPR14, S100A12, and VEGF remained high after infliximab treatment. MRP8/MPR14, 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/MPR14 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/MPR14 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 MRP8/MPR14 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- κ 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.

cally, these proteins regulate adhesion of neutrophils and monocytes to endothelial cells and are implicated in their transmigration into the vessel wall (25). MRP8/MRP14 is believed to have an important functional role in vasculitis syndromes and serves as a marker identifying patients at risk for developing CAL during acute phase of KD (8). Interestingly, MRP8/MRP14 has recently been identified as novel endogenous ligands of TLR4, which directly promote inflammation. MRP14 $-/-$ mice are protected from LPS-induced shock due to a lack of TNF- α production compared with wild-type mice indicating that MRP8/MRP14 act up-stream of TNF- α in the inflammatory cascade (12). This hypothesis is supported by our data showing that blockade of TNF- α efficiently inhibits down-stream pathways of systemic inflammation, such as the expression of IL-6, but does not completely block expression of local proinflammatory mechanisms. Whether this lack of action has negative consequences with respect to the long-term prognosis of KD-patients after infliximab treatment cannot be answered by this relatively small cohort but requires a prospective follow-up study including a larger number of patients.

Another member of the S100 family, S100A12, is also released by neutrophils, binds to the RAGE on endothelial cells and leukocytes, and induces cell activation and cytokine production through the nuclear factor-kappa-B signaling pathway (26) (Fig. 3). Transcript and protein levels of all three of these S100 proteins are increased in circulating leukocytes and in serum during the acute phase of KD (8–11,15,23). Taken together, these observations support the hypothesis that MRP8/MRP14 heterodimers and S100A12 participate directly in the pathogenesis of the coronary artery vasculitis in KD and contribute to endothelial cell damage and transmigration of leukocytes into the arterial wall independent of TNF- α activity. Moreover, during acute KD, sRAGE may block the binding of S100A12 to RAGE on the cell surface by neutralizing this proinflammatory protein. The fact that concentrations of MRP8/MRP14 and S100A12 did not change during infliximab treatment indicates that MRP8/MRP14 and S100A12 maintain signaling of local vascular injury despite the blockade of TNF- α . Lower levels of sRAGE detected in KD patients might thus confer susceptibility to hyperinflammation (27). Lower levels of sRAGE may reflect insufficient compensation of S100A12 because of the occurrence of dramatic and strong inflammation in refractory KD patients during infliximab treatment.

VEGF enhances proliferation and migration of endothelial cells in collaboration with nitric oxide and may contribute to later vascular remodeling after the acute phase of KD (7). Because systemic overproduction of VEGF has been demonstrated in acute KD, VEGF is considered to be involved in the pathophysiology of KD, especially in the development of CAL (28,29).

This study shows that serum IL-6 and sTNFRI levels dramatically decreased after treatment and correlated with serum CRP levels and fevers; however, the serum levels of VEGF, MRP8/MRP14, and S100A12 remained high after infliximab treatment, especially in patients with CAL. In contrast, in IVIG responders all cytokines decreased markedly

after IVIG treatment. These data indicate that proinflammatory cytokines decrease in response to infliximab treatment, but VEGF and local inflammatory proteins of the DAMP-family such as MRP8/MRP14 and S100A12, which were reported to be important factors in development of CAL during acute stage of KD, do not. Thus, it seems that infliximab is effective for the suppression of systemic inflammation, but could not completely block the local vasculitis in KD.

We have now successfully treated eight of the 11 refractory KD patients. Three patients who did not respond to infliximab treatment needed additional therapy. Four patients developed CAL despite infliximab therapy: of note, these patients were treated later than the patients without CAL and had signs of CAL before treatment. Histopathological studies have shown that transient infiltration by granulocytes occurs in the very early stage of acute KD, before infiltration of mononuclear cells, suggesting granulocytes act as a trigger in the pathogenesis of CAL (30). In light of our data, to prevent the progression of CAL and to prevent the secretions of MRP8/MRP14 and S100A12 from inflammatory cells such as neutrophils and monocytes, early administration of infliximab, or combination therapy with IVIG may lead to the successful cessation of the inflammatory response and vasculitis.

Study limitations. Limitations of this study include its retrospective nature, small number of patients, and the administration of multiple different therapies after the first IVIG infusion failure. Different IVIG preparations were used at different centers and concomitant or sequential antiinflammatory therapies administered to several of these patients precluded a final assessment of the effect of infliximab infusion. In conclusion, in this study, we show for the first time that inflammatory cytokines are up-regulated and changed dynamically during Infliximab treatment in refractory KD patients. Infliximab was effective for suppression of systemic inflammation, but could not completely block the local vasculitis in refractory KD patients. The early administration of infliximab or combination therapy with IVIG might be recommended for refractory KD patients.

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