Expression of Myeloid-Related Protein-8 and -14 in Patients With Acute Kawasaki Disease

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BACKGROUND
Both MRP-8 and -14, which are S100-proteins secreted by activated neutrophils and monocytes, bind specifically to endothelial cells and induce thrombogenic and inflammatory responses in a variety of disease conditions.

METHODS
We investigated 61 patients with acute KD and examined sequential changes in serum levels of MRP-8/MRP-14, messenger ribonucleic acid (mRNA) expression of MRP-8 and -14 in circulating granulocytes and monocytes, and amounts of MRP-8/MRP-14 bound to circulating endothelial cells.

RESULTS
The serum MRP-8/MRP-14 levels as well as mRNA expressions of MRP-8 and -14 in granulocytes were strongly upregulated during the early stage of acute KD, and decreased dramatically within 24 h of intravenous immune globulin therapy (p < 0.05) in 45 responders. In contrast, in 16 nonresponders both of these increased after the initial treatment. The number of MRP-8/MRP-14–positive circulating endothelial cells was higher in patients with acute KD than in control patients and increased significantly by 2 weeks after the onset of KD, especially in patients in whom coronary artery lesions developed.

CONCLUSIONS
We show for the first time that MRP-8/MRP-14 are exclusively secreted by granulocytes in patients with acute KD, and intravenous immune globulin treatment suppresses their gene expression. Serum levels of MRP-8/MRP-14 may be useful markers of disease activity, and the levels of MRP-8/MRP-14–positive circulating endothelial cell may predict the severity of vasculitis, confirming an important role for distinct inflammatory reactions in endothelium. (J Am Coll Cardiol 2006;48:1257–64) © 2006 by the American College of Cardiology Foundation

Kawasaki disease (KD) is the most common systemic vasculitis syndrome primarily affecting small and medium-sized arteries, particularly the coronary artery (1). Although timely treatment with high-dose intravenous immune globulin (IVIG) is now accepted as reducing the incidence of coronary artery lesions (CALs), approximately 15% of the patients do not have a response to IVIG treatment and have a higher incidence of CALs (2,3). Histopathological studies have shown that the infiltration of mononuclear cells, suggesting that granulocytes act as a trigger in the pathogenesis of CALs (5). A multicenter randomized controlled study in Japan resulted in guidelines for the use of IVIG in acute KD, including the criterion of platelet counts of <35 × 10^4/μL, the so-called Harada score (6). Patients with acute KD with low platelet counts were found to have a high incidence of CALs and acute myocardial infarction, suggesting that a low platelet count is the result of intravascular coagulation (7).

Myeloid-related protein (MRP)-8 and -14, 2 S100-proteins, form stable heterodimers and are the major calcium-binding proteins secreted by activated granulocytes and monocytes (8). The protein complex is found in inflammatory fluid in a variety of inflammatory lesions, including rheumatoid arthritis and inflammatory bowel and lung diseases (9,10). However, little is known of the role of MRP-8/MRP-14 in inflammatory processes. The MRP-8/MRP-14 binds specifically to human microvascular endothelial cells via heparan sulfate proteoglycans (11) and via novel carboxylated N-glycans (12), suggesting an important interaction between MRP-8/MRP-14 and endothelial cells, although the functional consequences and underlying molecular mechanisms are unresolved. One recent study showed that MRP-8/MRP-14 induces a thrombogenic and
inflammatory response in human microvascular endothelial cells in vitro (13).

We hypothesized that MRP-8/MPR-14 could directly induce a distinct inflammatory and thrombogenic response in the microvascular wall in acute vasculitis syndromes. In the present study we investigated patients with acute KD who were differentiated by responsiveness to IVIG therapy to validate MRP-8/MPR-14 as a marker of disease activity and severity of CAL development. For this purpose, we examined sequential changes in serum levels of MRP-8/MPR-14, messenger ribonucleic acid (mRNA) expression of MRP-8 and -14 in circulating granulocytes and monocytes, and amounts of MRP-8/MPR-14 bound to circulating endothelial cells.

METHODS

Study population and blood samples. Sixty-one patients with acute KD (40 male, 21 female), ages 2 months to 7 years (median 2.6 years), were enrolled between April 2001 and April 2005. Thirty-three age-matched healthy control patients were enrolled during the same period. We confirmed that there is no evidence showing that the levels do vary with age. The patients were seen at the Toyama University Hospital or at one of five affiliated hospitals. All patients fulfilled the diagnostic criteria and were treated with IVIG (2 g/kg body weight for 1 day) as well as oral aspirin (30 mg/kg/day) (14). The responders were designated as the patients whose fever subsided within 48 h of IVIG treatment. Nonresponders received a second treatment of IVIG (2 g/kg body weight for 1 day). Two-dimensional echocardiography was performed before and after treatment with IVIG as well as at 2, 3, and 4 weeks 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 years) by echocardiogram was considered abnormal (14).

The presence of CALs was assessed 1 month after the onset of KD. Parental informed consent was obtained for each child enrolled in this study, which was approved by the Research Ethics Committee of Toyama Medical and Pharmaceutical University Hospital. Blood samples were collected at the time of diagnosis before the initiation of IVIG treatment (mean ± SD, 5.7 ± 1.5 illness days), then immediately after the first IVIG infusion and at 2 and 4 weeks after disease onset. Blood samples were collected from healthy control patients on a single occasion.

Determination of serum MRP-8/MPR-14 levels. The concentrations of MRP-8/MPR-14 were determined by a sandwich enzyme-linked immunosorbent assay as described previously (15). Briefly, rabbit antisera against recombinant MRP-8 and -14 were produced, and their monospecificity was tested by immunoreactivity against recombinant MRP-8 and -14 by Western blot analysis of lysates of monocytes and granulocytes, as well as by immunoreactivity against MRP-8- and/or MRP-14–transfected fibroblastoid cell lines. For calibration, different amounts (0.25 to 250 ng/ml) of the native complex of human MRP-8 and -14 were used, which were isolated from human granulocytes. The assay has a sensitivity of <0.5 ng/ml and a linear range between 1 and 30 ng/ml. Data are expressed in ng/ml MRP-8/MPR-14 and represent the mean of duplicates of each of 3 dilutions within the linear range.

Cell preparation. Granulocytes and mononuclear cells were isolated from the heparinized peripheral blood of patients and control patients by dextran sedimentation and Ficoll-Paque density gradient centrifugation as described previously (16). Granulocytes were more than 98% pure as assessed by May–Gruwald–Giemsa staining. The cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% (v/v) charcoal/dextran-treated fetal bovine serum (HyClone, Logan, Utah), 10 mmol/l glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, Maryland) and diluted to 1 × 10⁶ cells/ml.

RNA extraction. Total RNA was extracted from the cell pellets of granulocytes and mononuclear cells from the patients’ peripheral blood using Trizol reagent (Invitrogen Life Technologies, Carlsbad, California). The purity and quantity of RNA were determined based on ultraviolet absorbance at 260 and 280 nm on a Beckman spectrophotometer DU530 (Beckman Coulter, Fullerton, California).

Real-time reverse-transcriptase (RT)-polymerase chain reaction (PCR). The primers used for PCR analysis were as follows: MRP-8 forward, 5’-AATTGCTAGAGACCGAGTGTCTCCTA-3’, reverse, 5’-TGCCACCACCCA-TCTTTTATCA-3’, MRP-14 forward, 5’-TCCACAAT-ACCTGTGGAAGCTG-3’, reverse, 5’-CTTCCATGATGTGTTCTATGACC-3’, glyceraldehyde phosphate dehydrogenase forward, 5’-GACCCGTCAAGGCTGAGA-C-3’, reverse, 5’-ATGGTGATGAGACGCGCA-GT-3’. For real-time RT-PCR, RNA from every experimental group was analyzed in duplicate. The complementary deoxyribonucleic acid was synthesized from 0.5 μg of total RNA. Real-time RT-PCR reactions were performed on an ABI PRISM 7000 Sequence Detection System (Applied
Biosystems, Foster City, California) using the SYBR RT-PCR kit (Takara, Tokyo, Japan). The baseline emission was calculated by the average fluorescence intensity from cycles 3 to 15. The threshold for determining the threshold cycle value was set at 10 standard deviations (SDs) above the baseline. The relative gene expression was calculated by using the comparative threshold cycle method as described (17). Gene expression was normalized with respect to the endogenous housekeeping gene, glyceraldehyde phosphate dehydrogenase, which was determined not to have significantly changed in the different experiments.

**Double-labeling immunocytochemistry for circulating endothelial cells.** Circulating endothelial cells were quantitated by immunofluorescence staining ofuffy-coat smears, as described (18). In brief, the resulting buffy-coat pellet was resuspended in RPMI 1640 and transferred to six slides such that each slide would contain cells from the equivalent of 1 ml whole blood. Smears were air-dried overnight, fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, and permeabilized with 0.1% Triton X-100 for 5 min. Cellular autofluorescence was quenched by two 5-min treatments with 1 mg/ml sodium borohydride (NaBH4) freshly prepared in PBS. After 20 min of blocking in 10% normal goat serum, slides were incubated with primary antibodies (mouse antiendothelial cell monoclonal antibody, P1H12, Chemicon International, Temecula, California, at a dilution of 1:600; monoclonal murine antibody 27E10 against the MRP-8/MRP-14 at a dilution of 1:400), which were diluted in blocking solution for 1 h at room temperature. After washing in PBS, a biotin conjugated goat antimouse immunoglobulin G (Vector laboratories, Burlingame, California) at a dilution of 1:200 was applied for 30 min as a second step of the revelation. For the third step, Alexa Fluor 594 or 488 streptavidin was applied for 30 min as a second step of the revelation.

**Stimulation of granulocytes and mononuclear cells.** Granulocytes at 1 × 10⁶ cells/ml or mononuclear cells at 1 × 10⁶ cells/ml from 5 healthy control patients were incubated with tumor necrosis factor (TNF)-alpha (recombinant human TNF-alpha; R&D Systems, Inc, Minneapolis, Minnesota) or with phorbol 12-myristate 13-acetate (PMA: Sigma Chemical Co., St. Louis, Missouri), respectively, at a final concentration of 10 ng/ml, for 15 min, 30 min, 1 h, 2 h, and 4 h at 37°C. At each time point the supernatant was collected for measurement of MRP-8/MRP-14 concentrations by sandwich enzyme-linked immunosorbant assay, and RNA was isolated from the cells and MRP-8/MRP-14 expression analyzed by real-time RT-PCR, as described in the previous text.

**Statistical analysis.** Paired t test and analysis of variance with repeated measures were used to test for significance of the same parameter, within the same group, at 4 points, respectively. If data do not follow normal distribution, paired t test and analysis of variance with repeated measures was replaced by their nonparametric versions. When the data followed a normal distribution determined by Shapiro-Wilks test, comparisons between two groups were performed using an unpaired student t test or a Welch 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 61 KD patients enrolled, comparing responders to IVIG therapy (n = 45) with nonresponders (n = 16). The nonresponders had significantly lower minimum platelets counts, a significantly higher maximum concentration of C-reactive protein (CRP), a higher incidence of CAs, and a longer duration of fever as compared with the responders. There were no significant difference between groups with respect to age and the maximum number of white blood cells (WBCs).

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<tr>
<th>Table 1. Clinical Laboratory Data of Patients With Acute KD, Comparing IVIG Treatment Responders and Nonresponders</th>
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<tr>
<td><strong>Healthy Control Patients</strong></td>
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<td>Number of patients</td>
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<td>Coronary artery lesion</td>
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<tr>
<td>Gender (male)</td>
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<td>Age, yrs (median, range)</td>
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<tr>
<td>Minimum platelet × 10⁴/mm³</td>
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<td>Maximum CRP, mg/dl</td>
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<td>Maximum WBC, × 10⁴/mm³</td>
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<td>Granulocytes (%)</td>
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<td>Duration of fever, days</td>
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Data are mean ± SD. p Value is derived from comparison of responders and nonresponders.

CRP = C-reactive protein; IVIG = intravenous immune globulin; KD = Kawasaki disease; WBC = white blood cells.
Maximal WBC counts were observed before IVIG treatment, and approximately 70% of the WBCs were granulocytes in both groups.

**Serum MRP-8/MPR-14 levels.** The initial serum levels of MRP-8/MPR-14 were elevated in all 61 patients with KD, exceeding the levels seen in healthy control patients (mean ± SD 260 ng/ml). As shown in Table 2 and Figure 1, the initial concentration of MRP-8/MPR-14 in the group of responders decreased from 3,251 ± 1,981 ng/ml to 1,265 ± 1,012 ng/ml within 24 h of IVIG treatment (p < 0.01) and reached normal levels within 4 weeks (Table 2). In contrast, the serum MRP-8/MPR-14 levels in nonresponders increased after the initial treatment (4,220 ± 2,669 ng/ml to 4,900 ± 4,519 ng/ml, p < 0.01), but trended to reduce after the second treatment. Although no differences in MRP-8/MPR-14 concentration were observed between responders and nonresponders before treatment, they were significantly higher in nonresponders than responders after IVIG treatment (p < 0.01). When patients with and without CALs were compared with each other, it was found that the former had higher maximal concentrations of MRP-8/MPR-14 and CRP than the latter (mean difference 1,580 ng/ml, p < 0.01 for MRP-8/MPR-14; mean difference 10.7 mg/dl, p < 0.01 for CRP). The CRP levels varied considerably from patient to patient and increased in number after treatment, but had reached normal values in 32 of 60 (53%) patients 2 weeks after therapy. In all patients, a decrease in MRP-8/MPR-14 concentration was associated with subsiding fever, whereas the CRP level continued to increase even after fever subsided in some patients.

**mRNA expression of MRP-8 and MRP-14 in granulocytes.** In 10 healthy control patients, the relative quantities of MRP-8 and -14 mRNA were 18.6 ± 15.1 and 8.9 ± 2.3, respectively, in granulocytes. Both were significantly higher in the granulocytes of acute KD responders before treatment with IVIG, decreasing significantly within 24 h of IVIG.
treatment (MRP-8: from 74.4 ± 41.3 to 29.1 ± 26.4, p < 0.05; MRP-14: from 50.5 ± 43.2 to 11.2 ± 5.4, p < 0.05), reaching normal levels within 1 month (Fig. 2). In contrast, the expression of both MRP-8 and -14 was similar to control patients initially in nonresponders, and then increased after the initial treatment and then decreased after the second IVIG treatment (Fig. 2). There were significant differences between responders and nonresponders in the expression of both MRP-8 and -14 2 weeks after initial IVIG treatment (p < 0.05). The MRP-8 and -14 mRNA in mononuclear cells were below detection limit during the course of acute KD (data not shown).

Quantitation of circulating endothelial cells. Peripheral blood samples from healthy control patients contained a very small number of circulating endothelial cells, whereas peripheral blood samples from patients with acute KD had significantly elevated numbers of circulating endothelial cells at the time of diagnosis (2.5 ± 5.9 cells/ml vs. 1.0 ± 1.7 cells/ml, p < 0.05). The number of circulating endothelial cells continued to increase (Fig. 3B), even after IVIG administration, and peaked at 2 weeks, which is the time when CALs usually develop (Fig. 3). Further, there were significantly increased numbers of circulating endothelial cells in patients with CALs compared with those without CALs at 2 weeks after onset (94.6 ± 68.5 cells/ml vs. 20.7 ± 20.4 cells/ml, p < 0.05). Although endothelial cells were negative for MRP-8/MRP-14 (Fig. 3A) in healthy control patients (data not shown), most of the endothelial cells were positive for MRP-8/MRP-14 in patients with acute KD, and the proportion of endothelial cells that were MRP-8/MRP-14–positive cells remained relatively constant (Fig. 3B).

Stimulation of granulocytes by TNF-alpha. To show that MRP-8/MRP-14 is actively secreted by granulocytes, granulocytes were incubated with TNF-alpha. As shown in Figure 4A, minimal basal secretion of MRP-8/MRP-14 was detected in unstimulated granulocytes, with a time-dependent increase in MRP-8/MRP-14 secretion after stimulation with TNF-alpha. In contrast, little MRP-8/MRP-14 was secreted by mononuclear cells incubated with PMA (Fig. 4A). The MRP-8– and -14–specific mRNA transcription increased in a time-dependent manner, peaking at 1 h (Fig. 4B). In mononuclear cells, no MRP-8– or -14–specific mRNA was detected during the course of PMA stimulation of mononuclear cells (data not shown).

DISCUSSION

This study shows that the number of circulating endothelial cells increases in patients with acute KD, particularly if CALs developed, and that most of the P1H12-positive endothelial cells were positive for MRP-8/MRP-14. These data are in agreement with previous studies that have reported increased numbers of activated circulating endothelial cell in patients with acute KD (18,19). It seems that the MRP-8/MRP-14 protein complex can bind to endothelium via the interaction of MRPI4 with heparan sulfate proteoglycans or by the MRP-8/MRP-14 complex interacting with carboxylated N-glycans, which are exclusively expressed by endothelial cells after inflammatory activation (11,12). Here we show that the vast majority of P1H12-positive cells show binding of the 2 S100 proteins MRP-8 and -14, which may point to a novel molecular mechanism during the process of tissue damage in the coronary artery wall. It has been shown in vitro that MRP-8/MRP-14 induce thrombogenic and inflammatory responses in human microvascular endothelial cells, leading to platelet adhesion...
and aggregation under shear stress (13). In our study, the nonresponders had significantly lower minimum platelet counts, as well as a significantly higher maximum serum concentration of MRP-8/MRP-14, suggesting that the low platelet counts are a result of severe vasculitis and intravascular coagulation (7).

The IVIG treatment is clearly effective in the rapid resolution of KD inflammation and in reducing the incidence of CALs. However, the mechanisms of IVIG action remain unknown. In the acute stage of KD, the serum levels of inflammatory cytokines are elevated, and decrease after successful IVIG treatment (20). A recent study (21) reported that various gene expressions change in patients with acute KD in peripheral mononuclear cells after IVIG treatment, and suggested that IVIG therapy downregulated various functional genes. It may reflect that the effect of IVIG was mediated by suppression of an array of immune activation genes in monocytes. Here we show for the first time that the expression of MRP-8 and -14 in granulocytes is increased in patients with acute KD, and rapidly decreased after successful IVIG treatment in responders. In contrast, in nonresponders the expression of MRP-8 and -14 in granulocytes increased after the initial IVIG treatment and only decreased after the second IVIG treatment. It had previously been reported that MRP-8 and -14 form stable heterodimers and are secreted by activated granulocytes and monocytes (8,22). However, in patients with acute KD, mRNA expression of MRP-8 and -14 were below the detection limit in peripheral blood mononuclear cells in our present study. These data suggest that granulocytes have an important role in triggering the vasculitis in acute KD. Interestingly, a neutrophil-elastase inhibitor, ulinastatin, has been reported to be an alternative therapeutic approach for KD, especially in nonresponders to IVIG treatment (23).

The present study confirms that MRP-8 and -14 are potential markers for monitoring KD and are more reliable than CRP in determining the response to IVIG treatment because serum levels correlated with disease activity in individual patients. Further, nonresponders to IVIG treatment had higher initial and maximum MRP-8/MRP-14 and CRP levels than responders, suggesting that MRP-8 and -14 may not only serve as diagnostic markers, but also that they are involved in the pathophysiology of systemic vasculitis in acute KD. Although the function of MRP-8/MRP-14 has not been fully elucidated, there is evidence that it plays a pivotal role during inflammatory conditions. Supporting this idea, we showed that TNF-alpha promotes MRP-8 and -14 gene transcription in granulocytes directly

Figure 3. Immunocytochemical analysis of circulating cells. (A) Buffy-coat smear-stained with P1H12 showing a circulating endothelial cell (red staining) (magnification ×1,000) (left). A circulating endothelial cell stained for myeloid-related protein (MRP)-8/MRP-14 (green staining) (magnification ×1,000) (center) in acute Kawasaki disease (KD) patients with coronary artery lesions (CAL). Nuclei stained with DAPI (magnification ×1,000) (right). (B) Quantitation of circulating endothelial cells (left) and cells positive for MRP-8/MRP-14 (right) in patients with acute KD with or without CAL. *p < 0.05 patients without CAL versus patients with CAL. Black columns = patients with CAL (n = 9); white columns = patients without CAL (n = 15).
and increases the secretion of MRP-8/MRP-14 from granulocytes in vitro. Thus, we hypothesize that serum MRP-8/ MRP-14 levels and MRP-8 and -14 expression in granulocytes may serve as markers confirming responsiveness to IVIG treatment. Elevated MRP-8/MRP-14 serum levels in active disease, sustained MRP-8/MRP-14 mRNA expression in nonresponders, and increased numbers of circulating MRP-8/MRP-14–positive endothelial cells in patients with CALs point to a role of these proteins during the pathogenesis of KD.

Study limitations. We were unable to perform immunohistochemical analyses of myocardium and coronary arteries because autopsy cases of acute KD were not available. A previous study showed that the endothelium of the vessels are almost completely coated with MRP-8 and -14, and showed a massive adhesion of MRP-8/MRP-14–positive granulocytes to the endothelium (13). In the study of MRP-8/MRP-14 transcription, samples from just three nonresponders were studied; they had similar expression profiles, however.

Conclusions. Our results suggest that serum MRP-8/ MRP-14 levels closely correlate with disease activity in acute KD, and that IVIG treatment suppresses the expression of the MRP-8 and -14 genes in granulocytes. Moreover, the fact that MRP-8/MRP-14–positive endothelial cells are significantly different already at 2 weeks after onset in KD could well serve as a meaningful marker identifying patients at risk for developing CALs. Our data imply an important functional role for MRP-8/MRP-14 and suggest that novel, directed inhibitory agents may be useful in treating human vasculitis syndromes.

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