

Adrenomedullin Is Highly Expressed in Blood Monocytes Associated with Acute Kawasaki Disease: A Microarray Gene Expression Study

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ABSTRACT

Kawasaki disease (KD) is an acute inflammatory disorder of children frequently associated with the development of coronary artery abnormalities. Although a great deal is known about inflammatory and immune responses in acute KD, the mechanisms linking the immune response to vascular changes are not known. To gain further insight into this process, we performed a microarray gene expression analysis on RNA isolated from the peripheral blood mononuclear cells of four patients with KD during both their acute and convalescent phases. Forty-seven genes of 7129 genes examined showed an increased expression in three or all four patients in the acute compared with the convalescent phase of KD. Fourteen of these genes were significantly ($p < 0.05$) up-regulated, including several inflammatory response genes (e.g. S-100 A9 protein) and also anti-inflammatory genes (e.g. TSG-6). Of greatest interest, the adrenomedullin (ADM) gene, known to be associated with coronary artery vasodilation, was up-regulated in the acute phase of KD ($p = 0.024$). Up-regulation of ADM in the acute phase of KD was confirmed in peripheral blood mononuclear cells of 11 additional KD patients by reverse transcriptase-PCR ($p < 0.01$). Isolated blood monocytes but not lymphocytes were demonstrated by real-

time PCR to have increased ADM mRNA ($p = 0.01$). Plasma ADM protein level in 32 additional KD patients was also confirmed to be higher in acute KD compared with convalescent KD ($p < 0.032$). It is interesting that from microarray results, other molecules known to be associated with coronary dilation, including nitric oxide, prostacyclin, acetylcholine, bradykinin, substance P, and serotonin, were not elevated in acute KD. Our current study suggests that ADM-expressing monocytes that infiltrate the coronary vascular wall may be the cause of coronary dilation in the acute phase of KD. (*Pediatr Res* 57: 49–55, 2005)

Abbreviations

ADM, adrenomedullin
EIA, enzyme immunoassay
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
IVIG, intravenous immunoglobulin
KD, Kawasaki disease
PBMC, peripheral blood mononuclear cell
RT-PCR, reverse transcriptase-PCR

Kawasaki disease (KD) is an acute vasculitis that affects mainly infants and young children (1). Ten to 15% of patients with untreated KD develop coronary arterial lesions, and a

greater proportion of patients develop coronary arterial dilation (ectasia) (2,3). Many studies have demonstrated that the acute phase of KD is associated with activation of T cells and monocyte/macrophages (4–6). However, the mechanisms linking the systemic immune response to vascular abnormalities are unknown.

Using new approaches such as the DNA microarray method (7), it is possible to examine thousands of genes, which may be up- or down-regulated during acute compared with convalescent KD, for identification of novel disease processes (mechanisms). Because autopsy cases have demonstrated that the infiltration of macrophages and T cells are prominent in the

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coronary artery and other vascular lesions of patients with KD (8), one can presume that the up-regulated genes in the mononuclear cells found at the site of coronary artery abnormalities can be detected in the gene-expression patterns of peripheral blood mononuclear cells (PBMCs). The current study used the Affymetrix GeneChip technology to identify potential candidate genes that might link the systemic immune response to development of vasculitis and coronary artery disease by examining the gene-expression patterns of PBMCs from patients with acute and convalescent KD.

METHODS

Study patients. For the microarray gene expression study, PBMCs were obtained from four patients who had KD, one girl and three boys, and were treated in Hachioji Metropolitan Children's Hospital (Tokyo, Japan; see Table 1). Because patients with KD are usually infants and young children, insufficiently paired acute and convalescent blood could be obtained from other patients with KD to use in the microarray studies. Blood samples were taken during the acute and convalescent phases in each patient. Immediately after blood sampling was done, i.v. immunoglobulin (IVIG) was administered to the patients. No one showed coronary dilation by echocardiography. This might be because IVIG therapy was performed in the very early stage of the KD and vasculitis was treated before the formation of coronary dilations. All four patients received oral aspirin treatment. Patients 1, 2, and 4 were treated with 5 d of sequential IVIG 400 mg · kg⁻¹ · d⁻¹. Patient 3 was treated with 7 d of sequential IVIG 400 mg · kg⁻¹ · d⁻¹.

For the reverse transcriptase-PCR (RT-PCR) study, 11 patients who had KD (4 girls and 7 boys; mean age 26 mo ± 20 SD) and also were treated in Hachioji Metropolitan Children's Hospital were enrolled. Blood samples were taken during the acute (5.0 d ± 1.4 SD), subacute (10 d ± 1.4 SD), and convalescent (104.7 d ± 34.4 SD) phases in each patient. The mean white blood cell count in the acute phase was 13,300/mm³ ± 2,900. The mean value of C-reactive protein in the acute phase was 6.6 mg/dL ± 2.7.

For plasma adrenomedullin (ADM) concentration, another 32 patients who were treated in Hachioji Metropolitan Children's Hospital (Japan) were enrolled. All patients fulfilled the diagnostic criteria for KD as defined by the American Heart Association Committee on Rheumatic Fever, Endocarditis, and KD (9).

Two-dimensional echocardiography was performed before treatment with i.v. gamma globulin and during the convalescent phase. A coronary artery with a diameter of 3 mm or more (4 mm if the patient was older than 5 y) was defined as abnormal. Parental informed consent was obtained for each child enrolled in this study. This study was approved by the Research Ethics Committee of the Hachioji Metropolitan Children's Hospital (Japan).

Affymetrix GeneChip protocol. Five-microgram samples of total RNA isolated from PBMCs were individually converted to double-stranded cDNA (ds-cDNA) using the Superscript Choice system (Life Technologies, Rockville, MD). An oligo-dT primer that contained a T7 RNA polymerase promoter (Promega Corp., Madison, WI) was used. After second-strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol, and the ds-cDNA was recovered by ethanol precipitation. *In vitro* transcription and biotin-labeling were then performed with an RNA transcript labeling kit (Enzo Biochem, Inc., New York, NY). A ds-cDNA template was transcribed in the presence of a mixture of unlabeled ATP, CTP, GTP, UTP, and biotin-labeled

CTP and UTP (Enzo Biochem, Inc.). Synthesized cRNA was fragmented in a fragmentation buffer at 94°C for 35 min. Samples were then used to prepare a hybridization solution that contained 100 mM of 2-[N-Morpholino] ethanesulfonic acid, 1 M of NaCl, and 20 mM of EDTA in the presence of 0.01% Tween 20. Each of the four acute samples and four convalescent samples was loaded individually into respective (total eight) human FL GeneChip probe arrays that contained 7129 gene probes (Affymetrix Inc., Santa Clara, CA) and hybridized at 45°C for 16 h. After washing and staining with streptavidin-phycoerythrin, the probe arrays were scanned using a Gene-array scanner (Hewlett Packard, Palo Alto, CA).

The data were analyzed using MicroArray Suite Version 4.0 software (Affymetrix Inc.). Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described (10,11) (Affymetrix Microarray Suite, version 4.0, User's guide). Genes were grouped as "increase" or "decrease" in the acute phase in comparison with the convalescent phase of each patient. For the normalization of fluorescence intensity, raw data from array scans were averaged across all gene probes on each array, and a scaling factor was applied to bring the average intensity for all probes on the array to a user preset level (2500). This allows any sample to be normalized for comparison with any other comparable sample, e.g. acute phase *versus* convalescent phase. We used a paired, two-tailed *t* test to assess whether gene expression differences were significant (*p* < 0.05).

Monocyte/lymphocyte isolation procedures. Peripheral blood monocytes and T cells were separated using a RosetteSep Monocyte or T cell Enrichment Cocktail (StemCell Technologies Inc., Vancouver, British Columbia, Canada) according to the manufacturer's recommendations (12). Briefly, 2 mL of heparinized blood was mixed with 20 μL of 100 mM of EDTA and 100 μL of RosetteSep cocktail that contained antibodies to human CD2, CD3, CD8, CD19, CD56, and CD66b for monocyte, or human CD16, CD19, CD36, and CD56 for T cell. After incubation for 20 min at room temperature, the sample was diluted with an equal volume of PBS that contained 2% fetal bovine serum and 1 mM of EDTA and layered on top of 4 mL of Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). The tubes were then centrifuged at 2000 rpm at room temperature for 20 min. The interface between plasma and Ficoll-Paque was collected, washed, and stored in liquid nitrogen until RNA extraction. The CD14(+) monocytes and CD3(+) T cells typically represented ~75 and 93.5%, respectively, of the total cells by flow cytometry analysis after this enrichment procedure.

Semiquantitative RT-PCR for ADM. Two micrograms of total RNA was used for the synthesis of first-strand cDNA, and the expression of ADM and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was examined using specific primer pairs. The sequences of the primers were 5'-TTCCGAGATTTTGCCATTGCCAG-3' (sense) and 5'-TCCTTGTCTTATCTGTGAATCGGT-3' (antisense) for ADM and 5'-CCAGCCGAGCCA-CACTGCTCT-3' (sense) and 5'-GTTGGCAGTGGGACACGGAA-3' (antisense) for GAPDH. PCR was carried out in a 50-μL reaction mixture that contained 1.25 units of *HotStaTaq* DNA polymerase (Qiagen), 1.5 mM of MgCl₂, 0.2 μM of each dNTP, and 0.3 μM of primer pairs. One microliter of each cDNA was subjected to amplification with 32 cycles of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C for ADM and 26 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C for GAPDH. For quantification of amplified products, ³³P end-labeled 3' primers were added to the reactions. The amplified products were separated on 2% agarose gels, dried, and analyzed with a BAS2000 Bio Image Analyzer (Fujix Film Co., Tokyo, Japan). The percentage of ADM to GAPDH mRNA was calculated after measurement of incorporated radioactivities.

Table 1. Clinical data of four KD patients examined with GeneChip microarray

Patient	Gender	Age (mo)	WBC (cells/μm ³)	CRP (mg/dL)	Coronary dilation	Disease day*	
						Acute	Convalescent
1	F	4	12,500	10.0	—	4	13
2	M	6	23,600	5.4	—	5	40
3	M	26	23,300	12.3	—	3	20
4	M	30	14,900	8.0	—	5	111

WBC, white blood cell count (normal <9000 cells/μm³); CRP, C-reactive protein (normal <0.3 mg/dL).

* The disease day blood was obtained.

Table 2. Results of GeneChip microarray: genes with increased call in at least three patients with acute KD

Gene name	Gene accession	Patient								Paired <i>t</i> test
		1		2		3		4		
		FC	Call	FC	Call	FC	Call	FC	Call	
Cytokine, chemokine										
CXCL-2	M57731	32	I	4.1	I	-1.3	NC	5.8	I	
IL-8	M28130	7.4	I	10.4	I	-2.4	D	19.1	I	
Enzyme										
SOD-2	X65965	4.7	I	8.4	I	-1.2	NC	2.1	I	
Spermine N1-acetyltransferase	U40369	6.2	I	3.8	I	1.9	I	1.3	NC	
Lysozyme	M21119	11.4	I	1.7	I	1.6	I	-1	NC	
Glycogen phosphorylase	M14636	6.5	I	1.7	NC	1.9	I	3	I	
Long-chain acyl-CoA synthetase	L09229	1.9	I	2.1	I	2.2	I	1.4	NC	0.012
α -1-antitrypsin	K01396	16.7	I	1.4	NC	2.2	I	2.5	I	
IP-30	J03909	6.3	I	-1.3	NC	2.4	I	1.6	I	
Cathepsin B	HG417-HT417	3.4	I	1	NC	1.7	I	1.8	I	
Long-chain acyl-CoA synthetase	D10040	9.1	I	2.4	I	2.5	I	1.5	NC	
Housekeeping										
Cytochrome c oxidase										
Subunit VIIIb	Z14244	2	I	2	I	1.1	NC	1.6	I	
Immunoglobulin										
Ig lambda locus	X57809	2	I	-3.2	D	2.8	I	2	I	
IgA heavy-chain allotype 2	S71043	1.7	I	-3.6	D	4.8	I	1.7	I	
Ig heavy-chain constant γ	M87789	2.4	I	-4.8	D	5.1	I	2.9	I	
IgJ chain for IgM, IgA	M12759	3.5	I	-2.8	D	2.8	I	2	I	
Membrane-bound receptor										
IL-13R α	Y10659	2	I	2.1	NC	3.2	I	2	I	0.034
CD32, low-affinity IgG2a receptor	X68090	3.8	I	3.9	NC	6.4	I	3.2	I	0.023
Leukocyte immunoglobulin-like receptor 7	U82275	10.6	I	2.2	I	1.6	I	1.8	NC	0.037
CD64, high-affinity IgG Fc receptor I	M63835	13.5	I	-3.3	D	6.7	I	9.7	I	
C5a anaphylatoxin receptor	M62505	27.4	I	1.9	I	1.2	NC	3.7	I	
fMLP-receptor	M60626	5	I	2.8	I	2.2	I	2.9	I	
G-CSFR	M59820	16.7	I	10.3	I	1.8	I	1.8	NC	
LDL receptor	L00352	2	I	1.8	I	2.3	I	1.1	NC	0.048
CD16, low-affinity IgG Fc receptor	J04162	4.3	I	1.2	I	2	I	1.5	I	
HM74, chemokine receptor	D10923	4.4	I	15	I	-1.6	NC	4.4	I	
Nuclear, transcription										
TCF-4	Y11306	5.2	I	-2.3	NC	4.1	I	2.3	I	
TTG-2	X61118	14.7	I	-1.5	NC	3.1	I	1.9	I	
c-fos	V01512	2.2	I	2.1	I	2.1	I	1.1	NC	0.043
CPBP	U44975	1.8	I	2.6	I	1.9	I	1.4	NC	0.032
bcl-6	U00115	3.2	I	2	I	1.8	I	1.2	NC	0.043
NFKBIA	M69043	2	I	3.2	I	1	NC	1.6	I	
DNA-binding protein A	M24069	4.8	I	1.4	NC	1.9	I	2.2	I	
Histone H2A.2	L19779	2.3	I	2	I	4.3	I	2.1	I	0.019
ETS2	J04102	4.4	I	2.1	I	3.5	I	1.5	NC	
Nf-II6, CEBPB	HG3494-HT3688	5.3	I	2.2	I	2.3	I	1.4	NC	
Signaling protein										
Cyclooxygenase-2	U04636	15.2	I	7.1	I	-3	D	4	I	
Tyrosine kinase	M16591	10.9	I	-2.1	D	2.1	I	1.9	I	
Miscellaneous										
TSG-6	M31165	2.9	I	3.4	I	3.2	I	2	NC	0.024
S-100 calcium binding protein A9	M26311	17.3	I	2.5	I	2.3	I	4.8	I	0.046
S-100 calcium binding protein A12	D83657	75.7	I	-1.1	NC	5.4	I	3.9	I	
Palmitoylated erythrocyte memb. protein	M64925	1.9	I	-1.1	NC	1.5	I	1.6	I	
Neutrophil cytosolic factor 1	M55067	1.8	I	1.9	I	1.6	I	1.3	NC	0.001
Ficolin-1	D83920	37.9	I	-1.1	NC	2.2	I	1.9	I	
CFLAR/antiapoptosis	AF005775	1.3	I	2	I	1.7	I	-1.2	NC	
Allograft-inflammatory factor-1	U19713	3.5	I	1.3	NC	1.8	I	2.1	I	0.028
ADM	D14874	11.8	I	8.5	I	6.7	I	2.1	NC	0.024

FC, fold change in acute-phase KD compared with convalescent phase; I, increase (increased gene in acute phase compared with convalescent phase); NC, not changed; D, decreased gene.

Real-time PCR for ADM. Total RNA was isolated from the monocyte-enriched *versus* lymphocyte-enriched PBMCs using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions and reverse-transcribed to cDNA. The expression of ADM and GAPDH was determined by a real-time PCR using the ABI 7700 sequence detector

system (PE Applied Biosystems, Foster City, CA). The primers and probes for GAPDH and ADM were purchased from PE Applied Biosystems (Assays-on-Demand Gene Expression Products). The assay ID (catalog number) of GAPDH was Hs99999905_m1, and the assay ID of ADM was Hs00181605_m1. PCR was carried out in a 25- μ L reaction mixture that contained 12.5 μ L

of TaqMan Universal PCR Master Mix (PE Applied Biosystems), 1.25 μ L of 20 \times Assays-on-Demand Gene Expression Assay Mixture (PE Applied Biosystems), and 11.25 μ L of cDNA diluted in RNase-free H₂O. Samples were preincubated for 10 min at 95°C and then subjected to 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min for denaturing and annealing-extension, respectively. The expression of ADM relative to GAPDH was calculated using a comparative threshold cycle method described in the "User Bulletin #2" provided by the manufacturer (PE Applied Biosystems) and was determined for each sample.

Enzyme immunoassay of plasma ADM. Enzyme immunoassay (EIA) of ADM was performed using Adrenomedullin Enzyme immunoassay kit (Phoenix Pharmaceuticals, Belmont, CA). Two-hundred microliters of each plasma sample was acidified and extracted with C18 separation column. Samples were then reacted with primary antibody and biotinylated secondary antibody and reacted subsequently with streptavidin horseradish peroxidase solution. After incubation with substrate solution, the reaction was stopped with 2 N of HCl and OD450 was measured in a microplate reader. Because interference of EIA reaction by plasma factor was not eliminated, standard peptides were mixed with plasma from healthy controls and extracted as in the same method as patients' plasma samples, and then standard curve was determined.

Statistical methods. For GeneChip microarray, batch analysis was performed using GeneChip Suite Version 4.0 to assess differences in call (increase, marginal increase, no change, marginal decrease, and decrease) and fold change between acute and convalescent samples. Two-tailed paired *t* test was also done to determine statistically significant differences between acute and convalescent samples after normalized values averaged by intensity of all probes on the array was logarithmically transformed.

RT-PCR of ADM in the PBMCs and plasma ADM concentration were examined with one-factor ANOVA and Scheffe F test as a *post hoc* test by using the STATVIEW IV program. Real-time PCR of ADM was examined with two-tailed unpaired *t* test after raw value was logarithmically transformed. Statistically significant difference was set at $p < 0.05$.

RESULTS

In each GeneChip oligonucleotide microarray study, we included an analysis of GAPDH as internal control. We verified the integrity of our RNA by checking the hybridization intensity of the 5', mid-portion, and 3' regions of GAPDH and β -actin gene. In all samples used in this study, the hybridization intensity of these three portions was similar, indicating that the integrity of the RNA used was intact (data not shown). Next, we focused on genes that demonstrated the greatest differences in hybridization signal intensity between acute and convalescent KD samples.

Table 2 shows the Affymetrix Microarray Suite data analysis results of our KD PBMC samples. "Increase call" represents increased expression of the designated gene in the acute phase

of KD compared with the convalescent phase. Of 7129 genes, >85% of the genes showed NC (not changed) call in the acute compared with the convalescent phase of KD. Only 0.9–9.5% of genes showed an increased call in the acute compared with the convalescent phase in each patient pair. Four genes showed increase call in all four patient samples. Forty-three genes showed increase call in three patients. Table 2 shows the results of these 47 genes. Table 3 shows the results of genes that showed a decreased call in the acute compared with the convalescent phase of KD.

Fourteen of the 47 genes in Table 2 showed a statistically significant ($p < 0.05$) increase. The genes with known functions included tumor necrosis factor–stimulated gene-6 (TSG-6), which has anti-inflammatory effects in various models of inflammation (13). This suggests that it is a component of a negative feedback loop that is capable of down-regulating the inflammatory response. Calgranulin B (S-100 protein A9) was significantly increased in acute KD and is known to be a small calcium-binding protein that is highly expressed in the cytosol of neutrophils and monocytes. It is found at high levels in the extracellular milieu during inflammatory conditions, inducing neutrophil chemotaxis and adhesion (14). S-100A12, another S-100 protein family member, has already been reported to be increased in acute-phase KD and is a marker of inflammation (15).

Of greatest interest were genes expressed in immune effector cells that may have vasodilatory effects. These genes are shown in Table 4. A number of genes that are known to be involved in vasodilation did not demonstrate increased expression in the acute compared with the convalescent phase of KD. These gene products include induced nitric oxide synthase involved in the production of nitric oxide (16); prostacyclin synthase, an enzyme involved in production of prostacyclin (17); kininogen, which is known to be converted into bradykinin (18); and tryptophan hydroxylase, which catalyzes the reaction to produce serotonin (19). Importantly, ADM was the only gene that was involved in vasodilation and was significantly ($p = 0.024$) up-regulated in the acute compared with the convalescent phase of KD (20).

To confirm the GeneChip result of ADM, we performed semiquantitative RT-PCR. Figure 1 shows the results of ADM

Table 3. Results of GeneChip microarray: genes with decreased call in at least three patients with acute KD

Gene name	Gene accession	Patient								Paired <i>t</i> test
		1		2		3		4		
		FC	Call	FC	Call	FC	Call	FC	Call	
PAS domain containing serine/threonine kinase	D50925	-2.5	D	1.3	NC	-2.7	D	-1.4	D	
CDC25Hu2	S78187	-2.1	D	-1.4	NC	-1.6	D	-3	D	
Nonerythroid α -spectrin	J05243	-3.2	D	-2.2	D	-1.7	D	-1.4	NC	0.04
Major nuclear matrix protein, matrin 3	M63483	-1.8	D	-1.9	D	-2.1	D	-1	NC	
T-cell receptor T3 γ polypeptide	X04145	-2.5	D	-3.6	D	-4.2	D	1.5	NC	
Diacylglycerol kinase	X62535	-1.8	D	-3.4	D	-1.8	D	-1.4	NC	
T-cell receptor active α -chain	M12959	-1.9	D	-1.6	D	-2.3	D	1.2	NC	
Perforin gene	M31951	-7.6	D	-6.7	D	1.8	I	-3.3	D	
KIAA0192	D83783	-2	D	-2.8	D	-1.1	NC	-2.6	D	0.0498
Src-like kinase	M14676	-1.6	D	-1.5	D	-1.4	NC	-1.6	D	0.0483
T-cell receptor Ti rearranged γ -chain mRNA V-J-C region	M30894	-2.9	D	-1.6	D	-1.2	NC	-2.2	D	

Table 4. Results of GeneChip microarray of vasoactive genes

Gene name	Gene accession	Patient								Paired <i>t</i> test
		1		2		3		4		
		FC	Call	FC	Call	FC	Call	FC	Call	
Vasodilator										
ADM	D14874	11.8	I	8.5	I	6.7	I	2.1	NC	0.024
Calcitonin gene-related peptide	X02404			Under detection limit						
iNOS	D29675	-1.5	NC	1	NC	-1.6	NC	1.4	NC	
Prostacyclin synthase	D38145			Under detection limit						
Kininogen	M11437			Under detection limit						
Tryptophan hydroxylase	L29306			Under detection limit						
Vasoconstrictor										
Endothelin 2	M65199			Under detection limit						
Endothelin 3	X52001			Under detection limit						
Thromboxane synthase	M80647	14.3	I	-1.2	NC	2.2	NC	2.5	NC	

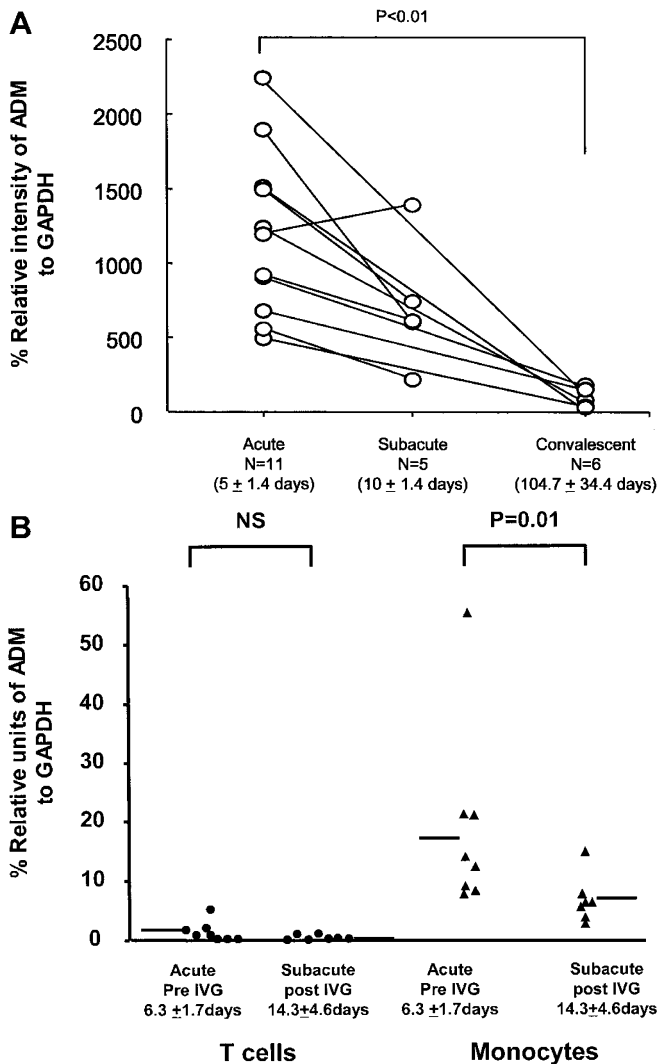


Figure 1. Results of ADM RT-PCR from the RNA of PBMCs from patients with acute and convalescent KD. All results are calculated regarding the densitometric intensity of GAPDH as 100%. From this conventional RT-PCR method, confirming GeneChip microarray result, expression of ADM is high in the acute phase of KD and diminished in the convalescent phase.

RT-PCR from the RNA of PBMCs in patients with acute and convalescent KD. All results are calculated using the densitometry intensity of GAPDH as 100%. These RT-PCR results confirmed the results from the microarrays.

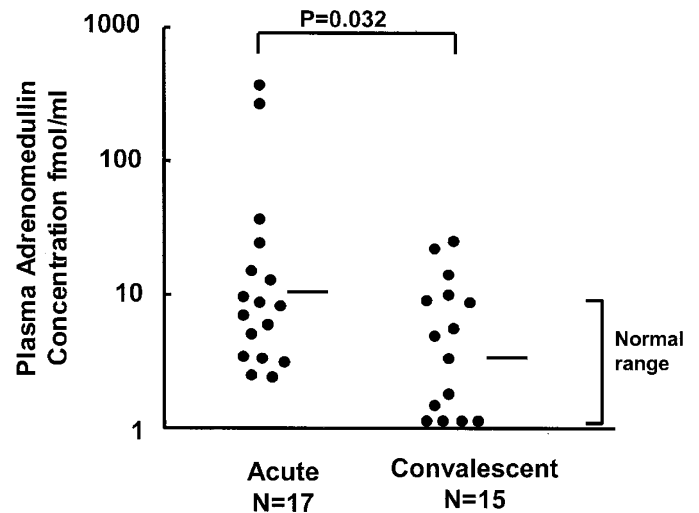


Figure 2. Monocytes and lymphocytes were separated individually from PBMCs, and real-time PCR of ADM was performed. Monocytes showed higher expression of ADM genes in the acute phase compared with the convalescent phase.

We next determined which cell type in PBMCs was the main producer of ADM. Monocytes and lymphocytes were separated individually from PBMCs, and real-time PCR was performed for the ADM gene. Monocytes but not lymphocytes showed significantly higher expression ($p = 0.01$) of the ADM gene in the acute phase compared with the convalescent phase (Fig. 2). Thus, monocytes seem to be the major producer of ADM mRNA.

EIA results of plasma ADM from KD patients were performed to examine expression of ADM at the protein level. Plasma ADM concentrations of acute-phase patients were significantly ($p = 0.032$) higher than in the convalescent phase. The levels in convalescent KD are similar to levels previously reported in normal controls (21).

DISCUSSION

Acute KD is a systemic vasculitis associated with activation of T cells and macrophages/monocytes in the circulation as well as cardiovascular tissues. A significant proportion of untreated patients with acute KD have coronary artery dilation, and suppression of cardiac contractility is a common finding in

this illness (3). Immunohistology of autopsy tissues has revealed the infiltration of activated mononuclear cells including macrophages and the up-regulation of adhesion molecules on the vascular endothelium (22). Increased production of macrophage/monocyte-derived cytokines, *e.g.* tumor necrosis factor- α , IL-1, and IL-6, have been associated with development of coronary artery disease (23–25).

The current study was carried out to identify novel molecules that could link the activation of immune effector cells with the development of cardiovascular changes in acute KD. A GeneChip oligonucleotide microarray study that screened 7129 genes in PBMCs from four KD patients in both the acute and the convalescent phases of their illness was carried out. Although we have shown all of the data found in this microarray study to stimulate further research in this area, we were primarily interested in molecules with known vasodilatory effects or rate-limiting enzymes to catalyze production of vasodilation molecules. These included ADM (20), induced nitric oxide synthase (16), prostacyclin synthase (17), kininogen (18), and tryptophan hydroxylase (19).

Although PBMCs may not always be the relevant cell type for analysis of disease-relevant genes and the microarray approach may not detect low expression genes, the current studies are still useful in the investigation of prominently up-regulated genes. Surprisingly, ADM was the only vasodilator gene in the microarray study that was found to be increased. This was not a spurious finding because analysis of ADM gene expression by RT-PCR of independent samples of PBMCs from other patients in the acute and convalescent phases of KD also confirmed significantly increased gene expression of ADM in acute compared with convalescent KD. This was also confirmed at the protein level because plasma concentrations of ADM protein were significantly increased in acute compared with convalescent KD. Our current observation is consistent with another report that plasma ADM protein levels are elevated in acute KD (26) and confirmed our former presentation (in part) at the Seventh International Kawasaki Disease Symposium that ADM mRNA is highly expressed in PBMCs of acute KD (27,28).

This is interesting because ADM is known to be a potent vasodilator and therefore may contribute to the dilation of coronary arteries and other middle-sized arteries in acute KD. ADM is also known to have several other properties that may contribute to the clinical features of acute KD. Recent studies indicate that ADM can cause a significant decrease in contractility of the heart (29). ADM has also been found to induce expression of adhesion molecules, including E-selectin and intracellular adhesion molecule-1, on human vascular endothelial cells (30) and endothelial cell proliferation (31). These actions likely contribute to the vascular inflammation associated with acute KD.

The elevation of plasma ADM, however, cannot completely explain coronary arterial dilation in KD. This is because there are other disease states, such as septic shock (21) and Behçet's disease (32), in which ADM plasma concentrations are elevated but there is no evidence for coronary arterial dilation. Further prospective echocardiography studies are needed to be certain that septic shock and Behçet's disease are not acutely associated with coro-

nary artery dilation, however, before we can conclude that ADM does not fully explain coronary artery dilation in acute KD. In our current study, we therefore also examined the potential cell source of ADM in PBMCs. We found that ADM mRNA was highly up-regulated in monocytes/macrophages but not lymphocytes from patients with acute KD. We postulate that these monocytes/macrophages may infiltrate into the walls of coronary arteries and other blood vessels to deliver ADM to vascular endothelial cells and smooth muscle cells. ADM produced by monocytes/macrophages therefore may be a potential coronary artery vasodilator in acute KD and reflects a general activation of monocytes/macrophages in this disease. Future research is needed to examine coronary artery-infiltrating macrophages as a potential source of ADM.

In addition to ADM, a number of other genes were found to be up-regulated in the acute compared with convalescent phase of KD. Several of these genes have known proinflammatory effects, including the nuclear transcription factor c-fos, a member of AP-1 complex that activates the expression of many proinflammatory genes. S-100 A9, a small calcium-binding protein found in the neutrophil and monocyte cytosol, is known to be highly expressed under conditions of inflammation associated with monocyte neutrophil activation. The current study is the first report of the up-regulation of S-100 A9 in acute KD. This molecule functions as a neutrophil chemoattractant and therefore may contribute to the infiltration of neutrophils in sites of vasculitis (33). The other calcium-binding protein S100A12 that we found increased causes inflammation through its interaction with the multiligand receptor for advanced glycation end products and has already been reported to be up-regulated in acute KD (34). S-100 A12 enhances monocyte migration (35). It is interesting that TSG-6 was also increased in acute-phase KD. TSG-6, however, has anti-inflammatory effects in various models of inflammation (13). This suggests that in addition to the up-regulation of proinflammatory genes, anti-inflammatory proteins are made. Although it was beyond the scope of this study to examine this response in more detail, future studies may be of interest to examine whether patients with persistent fever or tissue inflammation as a result of KD have an inadequate anti-inflammatory response.

CONCLUSION

In conclusion, the current study has demonstrated an increase of ADM in acute KD. This up-regulation was observed using gene microarray, RT-PCR, and ELISA. Up-regulation of ADM was found by real-time PCR in monocytes but not lymphocytes from patients in the acute phase of KD. These data suggest that ADM produced by monocytes/macrophages that infiltrate the coronary vascular wall may contribute to coronary artery dilation in acute KD.

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