Increased Expression of Human 63-kD Heat Shock Protein Gene in Kawasaki Disease Determined by Quantitative Reverse Transcription-Polymerase Chain Reaction

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ABSTRACT. To investigate the possible role of heat shock protein (HSP) in the pathogenesis of Kawasaki disease (KD), we determined the expression of 63-kD HSP (P1 protein) gene in the leukocytes from the peripheral blood of 20 patients with KD, 20 patients with various other febrile diseases, and 10 healthy children using the quantitative reverse transcription-polymerase chain reaction technique. Although a low but constitutive expression of HSP was observed in the control patients and healthy children, the expression of HSP63 mRNA was significantly higher during the acute phase of KD. Because mycobacterial HSP65, a cognate of human HSP63, is a common immunogen in bacterial species, and because the major immunopathologic feature of KD is reportedly an abnormally activated immune system, it suggests that an increased level of HSP63 gene transcription may be associated with the pathogenesis of KD. (*Pediatr Res* 35: 179-183, 1994)

Abbreviations

HSP, heat shock protein
KD, Kawasaki disease
CAL, coronary artery lesions
RT-PCR, reverse transcription-polymerase chain reaction

KD is an acute febrile illness of infants and young children that is characterized by multisystem vasculitis (1). CAL develop in approximately 15 to 20% of KD patients and may lead to myocardial infarction (2). Although the origin of KD is not known, epidemiologic features suggest it is caused by an infectious agent (3). Immunologic abnormalities during the acute phase of KD are characterized by marked activation of the immune system, leading to an increase in cytokine production by activated macrophages/monocytes, T cells, and B cells (4, 5). Localized erythema at the site of a previous (bacille Calmette-Guérin) inoculation is a relatively specific and early manifestation of KD. Recently, the antibody response to bacille Calmette-Guérin antigens was demonstrated in the convalescent sera of patients with KD by the IgG immunoblotting method (6). A marked increase in the serum antibody level was detected against both the mycobacterial 65-kD HSP (HSP65) and its human

homologue (HSP63, P1 antigen) using the synthetic peptide enzyme-linked immunoassay (7). This finding suggests that molecules that cross-react with some infectious agents and mycobacterial bacille Calmette-Guérin antigens and with exogenous HSP65 and endogenous HSP63 may contribute to the inflammatory process in KD.

HSP are evolutionarily highly conserved proteins that are expressed in response to stressful stimuli, including heat, oxidative free radicals, and toxic metal ions (8). HSP have several families, but their functions are still obscure. The amino acid sequence of HSP65 and HSP70 shows a strong similarity to a 40–70% amino acid identity between HSP of human beings and some bacteria (e.g. *Mycobacterium tuberculosis* or *Escherichia coli*) (9, 10). Human HSP63, a mitochondrial protein designated P1, is the human homologue of bacterial HSP65. Mycobacterial HSP65 is being studied intensively because of its strong immunogenicity as a common antigen of bacteria and because of its possible involvement in the autoimmune response directed against self-epitopes shared by the mycobacterial and human HSP (11–13).

We investigated the expression of the HSP63 gene using the quantitative RT-PCR with β -actin internal standard in the acute and convalescent phase of KD as compared with both control patients and various diseases and with healthy children.

MATERIALS AND METHODS

Patients. We evaluated 20 patients with KD. They were hospitalized at the National Defense Medical College Hospital between August 1991 and December 1992. The group comprised 12 males and eight females, aged 5 mo to 6 y. All patients met the diagnostic criteria for KD established by the Japanese Kawasaki Disease Research Committee (14). All were scheduled to receive i.v. Ig. Three patients had CAL. Blood was obtained from 20 patients in the acute phase (median days after onset of fever, 5.1 ± 1.4 d; mean $\pm$ SD) before initiating the administration of Ig and aspirin and from 16 of these patients during the convalescent phase of KD as paired samples (45.0 ± 8.1 d) (Table 1).

We evaluated 20 patients with other illnesses as febrile controls. Six had acute bronchitis, three had acute pneumonia, three had EBV infection, two had acute urinary tract infection, two had aseptic meningitis, and one patient each had exanthema subitum, scarlet fever, measles, and rubella. Blood was obtained from 20 patients at the acute febrile phase (median days after onset of fever, 4.8 ± 1.8 d; mean $\pm$ SD) and from eight of these patients during the convalescent phase as paired samples (13.1 ± 2.9 d). Three had acute bronchitis, and one patient each had acute pneumonia, urinary tract infection, EBV infection, aseptic meningitis, and scarlet fever. Blood was obtained from 10 healthy

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Table 1. Characteristics of patients and controls

	KD		Control patients		
	Acute	Convalescent	Acute	Convalescent	Healthy children
Number	20	16	20	8	10
Median age (range)	19 mo (5 mo–6 y)	21 mo (6 mo–6 y)	27 mo (8 mo–9 y)	27 mo (8 mo–9 y)	3 y (10 mo–5 y)
Sex (male/female)	12:8	9:7	9:11	4:4	5:5
Median days after onset of fever (range)	5.1 ± 1.4 (3–8 d)	45.0 ± 8.1 (32–58 d)	4.8 ± 1.8 (1–9 d)	13.1 ± 2.9 (8–18 d)	

children between the ages of 10 mo and 5 y (median, 3 y) after informed consent had been obtained from the parent or guardian.

Quantitative RT-PCR. Peripheral blood mononuclear cells and neutrophils were isolated from heparinized blood by density gradient centrifugation using Mono-Poly Resolving Medium (ICN Biochemicals, Costa Mesa, CA). Total RNA was isolated by acid guanidium thiocyanate-phenol-chloroform method (AGPC protocol) (15). RNA samples thus isolated were then treated with RNase-free DNase I (Takara Shuzo Co., Kyoto, Japan) for 1 h at 37°C to remove any possible contaminating genomic DNA. Two pairs of oligonucleotide primers were prepared for human HSP63-specific sequences (sense: 5' ATAATCGCTGAAGATGTTGA 3' map position 818–837; antisense: 5' TGCAAGCCGTTTCATTGAGTT 3' map position 1186–1167) and β -actin-specific sequences (sense: 5' AACTGGGACGACATGGAGAA 3' map position 273–292; antisense: 5' ATACCCTCGTAGATGGGCA 3' map position 548–529). Accuracy of respective 369-bp and 276-bp PCR products has been confirmed by nucleotide sequencing after cloning into plasmid vectors.

Both antisense primers (5 pmol) were annealed to RNA samples in 20 mM Tris-HCl, 0.2 mM EDTA, and 50 mM KCl for 15 min at 65°C and then incubated for cDNA synthesis with 2 units of reverse transcriptase (RAV-2, Takara Shuzo Co.), 0.4 mM deoxyribonucleoside triphosphate, 50 mM Tris-HCl, 5 mM MgCl₂, and 50 mM KCl for 1 h at 37°C in total reaction volume of 15 μ L. PCR was carried out with 2.5 μ L of cDNA, 1 unit of Taq DNA polymerase (Pharmacia, Uppsala, Sweden), two pairs of primers (20 pmol), 0.2 mM deoxyribonucleoside triphosphate, 50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl and 10 μ Ci [α -³²P]deoxycytidine triphosphate (total volume, 50 μ L) in a thermal cycler (Program Temp Control System PC-700, ASTEC, Tokyo, Japan). Each PCR cycle included 1 min of denaturation at 94°C, 2 min of primer annealing at 54°C, and 3 min of extension at 72°C. PCR products were separated on 4% polyacrylamide gels, dried after electrophoresis, exposed overnight to x-ray film (Hyperfilm-MP, Amersham, Arlington Heights, IL), and analyzed with a laser densitometer (LKB 2222-020 Ultroskan XL, Pharmacia).

To evaluate the adequacy of quantitative RT-PCR, we used it to study HeLa cells in which the HSP63 and β -actin mRNA are both abundantly expressed. The PCR products of HSP63 and β -actin increased linearly within the range of 20–30 PCR cycles (Fig. 1A and B). The amount of PCR product was proportional to that of template RNA, in the range of 0.001 and 1 μ g (Fig. 1C and 1D). Therefore, we used 25 cycles of PCR amplification. The level of mRNA for HSP63 was expressed as the ratio of HSP63 RT-PCR products to β -actin (HSP63/ β -actin ratio). The quantitative differences of HSP63 mRNA present in peripheral leukocytes were compared using the ratio.

Statistical analysis. We used the two-tailed *t* test where appropriate; *p* < 0.05 was considered significant.

RESULTS

The total RNA was extracted from the peripheral leukocytes of patients with KD, patients with other febrile diseases (control patients), and healthy children, and RT-PCR was carried out.

Figure 2 shows the autoradiographic profiles of quantitative RT-PCR for representative cases. HSP63 (369 bp) was expressed at much higher levels in patients in the acute phase of KD than in control patients and healthy children. This finding was clarified using the ratio against β -actin (276 bp) estimated by densitometer. HSP63 was expressed constitutively in healthy children at very low levels.

As shown in Figure 3, the HSP63/ β -actin ratio in the acute phase of KD (0.33 ± 0.14 ; mean $\pm$ SD) was significantly higher than that of the control patients and healthy children (0.13 ± 0.10 and 0.11 ± 0.05 , respectively; *p* < 0.001). Thus, mRNA of HSP63 was expressed to a greater extent by peripheral leukocytes of acute KD than those of other febrile disorders studied. The mean ratio of patients with acute-phase KD and CAL (*n* = 3) did not differ significantly from that of patients with KD but without CAL. One of the control patients, a 4-y-old girl with infectious mononucleosis caused by EBV, had a high ratio titer (0.47); the ratios of two other patients with this disease were less than 0.15 (0.12 and 0.10). We also observed that the expressions of HSP63 mRNA were low in two patients with recurrent systemic juvenile rheumatoid arthritis (the HSP63/ β -actin ratio, 0.11 and 0.13), one patient with virus-associated hemophagocytic syndrome (0.10), and three patients with anaphylactoid purpura (range, 0.07–0.13).

We compared the expression of HSP63 in the acute *versus* the convalescent phase of KD (Fig. 4). Samples were obtained from 16 of the patients with KD and from 8 of the control patients during their convalescent phase. The ratios (mean $\pm$ SD) of the convalescent phase were significantly lower than those of the acute phase (acute *versus* convalescent: KD, 0.35 ± 0.14 *versus* 0.23 ± 0.13 , *p* < 0.01; control patients, 0.19 ± 0.13 *versus* 0.13 ± 0.06 , *p* < 0.05), but the ratios in the convalescent phase of KD were still elevated. We also observed that the mean ratio 20 d after the onset of fever in five patients with KD was high (0.30 ± 0.13). The ratio was less than 0.20 in two of three samples obtained 3 mo after the onset of KD (data not shown). This finding indicates that a high level of HSP63 gene expression in KD was maintained for more than 1 mo and then decreased gradually.

DISCUSSION

We demonstrated that HSP63 mRNA was expressed at a significantly higher level in patients in the acute phase of KD than in the control patients and the healthy children. Although the origin of KD is obscure, Yokota (6) and Yokota *et al.* (7) recently reported that the anti-HSP65 antibodies in sera from patients with KD during convalescence reacted with both synthetic peptides of mycobacterial HSP65 and human HSP63 and that peripheral T lymphocytes from patients with KD were sensitized to recombinant HSP65. This finding suggested that both bacterial HSP65 and human HSP63 play an important role in KD pathogenesis at the protein level in an autoimmune fashion. Because no firm evidence exists that the expression of HSP63 protein is increased in KD, the direct proof for the central role of HSP63 is still missing. If the increased synthesis of HSP63 protein is induced, it is assumed that intracellular HSP63 (self-HSP) would be processed and presented in the context of MHC class I molecule on the cell surface (11, 16).

Fig. 1. *A*, Autoradiogram of the HSP63 and β -actin PCR products by RT-PCR in HeLa cells. HSP63 mRNA was reverse-transcribed simultaneously with the β -actin internal standard and amplified in the presence of [α -³²P]deoxycytidine triphosphate for 20, 25, and 30 cycles. After PAGE, the gel was dried and exposed to x-ray film overnight at room temperature. Arrows indicate the specific product bands for HSP63 (369 bp) and β -actin (276 bp) sequences. *B*, Absorbance units (AU) of HSP63 and β -actin increased linearly within 20–30 PCR cycles. *C*, Autoradiogram of the HSP63 and β -actin RT-PCR products amplified for 25 cycles in HeLa cells RNA (10⁻³, 10⁻², 10⁻¹, 1 μ g). *D*, Absorbance units of HSP63 and β -actin increased in proportion to the RNA concentration on a logarithmic scale.

Fig. 2. Autoradiogram of quantitative RT-PCR. Total RNA was extracted from peripheral leukocytes obtained in paired samples (acute and convalescent phase) from three patients with KD, five control patients, and four healthy children, and RT-PCR was carried out. After PAGE and autoradiogram, the expression of HSP63 mRNA was determined as HSP63/ β -actin ratio with a laser densitometer. The presence of KD was associated with increased expression of the HSP63 gene.

We obtained evidence that the expression of HSP63 increased at mRNA level in peripheral leukocytes of acute KD (Figs. 2 and 3), indicating that an increase in HSP63 gene expression may not be induced by a rise in temperature (heat shock) but may be relatively specific to the acute phase of KD. We also observed that the expressions of HSP63 mRNA were low in patients with recurrent systemic juvenile rheumatoid arthritis, virus-associated hemophagocytic syndrome, and anaphylactoid purpura. These results suggest that the expression of HSP63 mRNA is not necessarily high in the diseases with a high level of C-reactive protein or with vasculitis.

Previous attempts have been made to quantitate PCR amplification of mRNA with an internal standard such as β -actin or β_2 -microglobulin (17, 18). We analyzed HSP63 gene expression quantitatively with the relatively invariant β -actin mRNA as internal standard. Although it is difficult to measure the absolute amount of HSP63 mRNA, one can compare quantitatively the relative amounts of HSP63 mRNA present in peripheral leukocytes using the HSP63/ β -actin ratio. However, this approach is

semiquantitative and provides only comparative data. The PCR technique must be carefully performed to find the proper concentration for the primers Mg^{2+} and deoxyribonucleoside triphosphate and to find the optimal PCR cycles because small differences in efficiency could lead to large differences in the amount of PCR product as a result of its sensitivity. The [α -³²P] deoxycytidine triphosphate-labeled PCR was highly sensitive because the low-level expression of HSP63 as seen in healthy controls barely stained with ethidium bromide after 20–30 PCR cycles.

HSP65, which belongs to the HSP60 family, has important physiologic functions, including protein folding, unfolding, and translocation (16). It has been identified as a major antigen in a variety of bacterial pathogens. HSP63 (mitochondrial protein, P1 antigen) is the human homologue of HSP65; it shares about 65% of the protein sequence homology with the HSP65 of *M. tuberculosis* (3). A possible role of HSP65 in autoimmunity is suggested by several findings: 1) the presence of T cells and antibodies against the epitopes shared by bacterial and human

Fig. 3. Expression of HSP63 gene in peripheral leukocytes from patients in the acute phase of KD, control patients, and healthy children. Data are the ratio of the amount of labeled PCR products (HSP 63/ β -actin ratio). The mean HSP63/ β -actin ratio of patients in the acute phase of KD was significantly higher than that of control patients and healthy children. Filled circles indicate the three patients with KD and CAL.

Fig. 4. Expression of HSP63 during the acute and convalescent phase of KD and control patients. The mean HSP63/ β -actin ratio in the acute phase of KD decreased during the convalescent phase but remained persistently high. Filled circles indicate three patients with KD and CAL.

HSP, 2) the activation of these T cells or antibodies during microbial infection, 3) the expression of self-HSP, and 4) the recognition of these stressed host cells by T cells or antibodies (16). Our finding that the increased expression of self (autologous) HSP is detected in KD is compatible with the autoimmune role of HSP65. HSP65 has also been reported to be a potent immunogen. It induces the production of IL-1 and tumor necrosis factor (19). HSP65 is a target molecule to cytotoxic T cells (11, 20). Therefore, it is suggested that the increased expression of endogenous HSP (HSP63) contributes to the abnormally activated immune system of patients with acute KD via a molecular mimicry between HSP65 and HSP63. The synthesis of HSP has also been reported to be induced by various cytokines, including IL-1, IL-2, interferon, and tumor necrosis factor (21). The mRNA of HSP63 may be consequently transcribed after the increase in cytokine production during the acute phase of KD and lead to the further production of cytokines. Both findings

suggest that HSP63 would presumably be involved in the pathogenesis of KD as the endogenous stimulus that triggers the immune activation or would have some roles in the cytokine network as an endogenous mediator that affects the host response. In the group of control patients, one of the three patients with EBV infection showed an elevated expression of HSP63 mRNA. This finding may reflect the activation of cytokines because some of the EBV-transformed B cell lines secrete IL-1, IL-6, and tumor necrosis factor (22). This elevation also may be associated with an alteration in mononuclear cell subpopulation.

The observation that the levels of HSP63 mRNA were still elevated in the convalescent phase of KD indicated that the peripheral leukocytes are under stressed conditions during the acute and convalescent phases of this disease. Other factors, such as a suspected infectious agent, rise in body temperature, activation of the cytokine network, and presence of superoxides, can induce the synthesis of HSP63. The transcription factors that regulate HSP63 gene should be studied to explain the influences of these inducers.

In summary, the expression of human HSP63 (self-HSP) was significantly increased in the peripheral leukocytes from patients in the acute phase of KD. An augmented transcription of HSP63 may play an important role in enhancing the immune activation of KD as endogenous mediator and may thus be involved in the pathogenesis of KD.

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