Toll-like receptor 4 is expressed with enteroviral replication in myocardium from patients with dilated cardiomyopathy

Mamoru Satoh, Motoyuki Nakamura, Tomonari Akatsu, Yudai Shimoda, Ikuo Segawa and Katsuhiko Hiramori

Second Department of Internal Medicine, Iwate Medical University School of Medicine, Iwate, Japan

Expressions of innate immune response proteins, most notably proinflammatory cytokines, against enteroviral (EV) infection have been documented in the heart of human dilated cardiomyopathy (DCM). Toll-like receptor 4 (TLR4) activates signaling pathways leading to the expression of proinflammatory cytokines implicated the etiology of DCM. We sought to determine whether EV replication activates TLR4-dependent immune response in myocardium obtained from patients with DCM. Endomyocardial biopsy tissues were obtained from 56 patients with DCM and 10 controls. Levels of plus- and minus-strand EV RNA and TLR4 mRNA were measured by real-time RT-PCR. Immunohistochemical analysis was performed to identify the cellular source of EV capsid protein VP1 and TLR4. Both plus- and minus-strand EV RNA were detected in 19 DCM patients (34%). Neither strand of EV RNA was detected in controls. TLR4 mRNA levels were higher in DCM patients than in controls ($P < 0.001$). A positive correlation was found between TLR4 levels and each strand type of EV RNA in EV RNA-positive patients (plus-strand vs TLR4: $r = 0.69$, $P < 0.001$; minus-strand vs TLR4: $r = 0.65$, $P = 0.002$). VP1/TLR4 double staining showed extensive colocalization of VP1 and TLR4 proteins in cytoplasm of cardiac myocytes in myocardium obtained from DCM patients. EV RNA-positive patients showed lower systolic function and larger ventricular volume compared with EV RNA-negative patients left ventricular ejection fraction (LVEF): $P = 0.002$; left ventricular end-systolic diameter (LVESD): $P = 0.004$). The DCM subgroup with high TLR4 levels showed lower LVEF and larger LVESD than the subgroup with TLR4 levels (both $P < 0.001$). This study suggests that myocardial expression of TLR4 associates with EV replication in human DCM. EV RNA and TLR4 mRNA levels may correlate with LV dysfunction in DCM. The expression of TLR4 against EV replication may be involved in the pathogenesis of DCM.

Laboratory Investigation (2004) 84, 173–181, advance online publication, 15 December 2003; doi:10.1038/labinvest.3700031

Keywords: cytokines; immunology; infection/inflammation; signal transduction; viral diseases

Enteroviral (EV) RNA has been detected in a proportion of patients with dilated cardiomyopathy (DCM).^{1,2} It has been demonstrated that the detection of EV RNA in myocardium of DCM patients is associated with adverse prognosis.³ In particular, the presence of minus-strand EV RNA indicates active viral replication, and plays a role in the development of myocardial injury in DCM.⁴ Our previous study has reported that myocardial expression levels of tumor necrosis factor- α (TNF- α) and EV RNA were increased in patients with DCM regardless of etiology of left ventricular (LV) dysfunction.^{5,6} It is likely that an immune response against viral

pathogens is important in the pathogenesis of DCM. However, the precise mechanism underlying tissue injury in virus-mediated cardiomyopathy is not clear.

A family of toll-like receptors (TLRs) has recently been identified as a key component of pathogen-associated molecular pattern recognition machinery.⁷ At least nine types of human TLRs have recently been identified.⁷ It has been shown that active TLR4 led to expression of nuclear factor- κ B (NF- κ B)-controlled genes for proinflammatory cytokines that are required for activation of the immune response.⁸ TLR4 may therefore be an important factor in the signaling pathway in the host immune system in response to infectious disease. It has recently been reported that activation of antiviral immune response via TLR4 is important in the pathogenesis of viral infection.⁹

The purpose of this study was to determine whether TLR4 was expressed with EV replication,

Correspondence: M. Satoh, Second Department of Internal Medicine, Iwate Medical University School of Medicine, Uchi-marumaru 19-1, Morioka 020-8505, Iwate, Japan.
E-mail: m_satoh@imu.ncvc.go.jp

Received 11 July 2003; revised 14 October 2003; accepted 17 October 2003; published online 15 December 2003

and to analyze the relationship between levels of TLR4 expression and clinical severity of DCM.

Materials and methods

Subjects

Endomyocardial tissues were obtained from 56 patients with primary DCM by right ventricular endomyocardial biopsy (42 male and 14 female patients; mean age 50.3 ± 2.2 years). The clinical diagnosis of DCM was made according to the World Health Organization/International Society and Federation of Cardiology Task Force criteria.¹⁰ Echocardiography was used to determine left ventricular ejection fraction (LVEF) and diameter immediately before the biopsy.

Control myocardial tissue samples were obtained by endomyocardial biopsy from 10 subjects (seven male and three female subjects; mean age 44 ± 12 years) with suspected cardiac disorder on the basis of ECG abnormality and echocardiographic changes, such as a slight increase in wall thickness without LV dysfunction. The resulting pathology findings showed no evidence of myocardial disease and these subjects were designated as controls. This study protocol was approved by our hospital ethics committee, and written informed consent was obtained from all subjects.

As a positive control of RT-PCR for EV RNA detection, we used HeLa cells incubated with 10^6 plaque-forming units coxsackievirus B3 (CVB3) (Nancy strain).

Extraction of RNA

Total RNA was extracted from endomyocardial tissues and positive control cells by the acid guanidinium thiocyanate–phenol–chloroform method, and treated with DNase I (GIBCO BRL).¹¹

Primers and TaqMan Probes

Plus- and minus-strand EV RNAs were amplified by using two sets of primers and TaqMan probes that recognized the 5' uncoding genomic region (forward primer: 5' GGG CGC TAG CAC TCT GGT AT 3', reverse primer: 5' GCC GGA TAA CGA ACG CTT T 3' and TaqMan probe: 5' ATC AAG CAC TTC TGT TAC CCC GGA CTG A 3').¹² The published sequence for human TLR4 was used for construction of primers and TaqMan probe (forward primer: 5' TGA TTG TTG TGG TGT CCC A 3', reverse primer: 5' TGT CCT CCC ACT CCA GGT AA 3' and TaqMan probe: 5' TCC TGC AGA AGG TGG AGA AGA CCC T 3').⁸ For all myocardial specimens, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified using TaqMan GAPDH control reagents as an internal control (PE Biosystem, Foster City, CA, USA).

Real-time RT-PCR

Total RNA of each biopsy was transcribed into cDNA of TLR4 and GAPDH by MultiScribe RT with random hexamers (TaqMan Gold RT-PCR kit, PE Biosystem). In separate experiments to determine the polarity of EV RNA, the cDNA of plus-strand EV RNA was synthesized in 10 μ l of solution containing 100 ng total RNA, $10 \times$ TaqMan RT-buffer, 10 mM deoxyNTPs mixture, 20 mM EV reverse primer and 1.25 IU MultiScribe RT as described by the manufacturer. The cDNA of minus-strand EV RNA was synthesized in the same way using EV forward primer instead of EV reverse primer. The RT reaction was performed at 37°C for 30 min, and heated at 95°C for 5 min to inactivate RT activity.

PCR mixture was added to each RT mixture containing 0.025 U AmpliTaq Gold DNA polymerase, AmpErase UNG, 25 mM MgCl₂, primer and TaqMan probe, and $10 \times$ TaqMan buffer A. Each of 40 reaction cycles consisted of denaturation for 15 s at 95°C, and annealing and extension for 1 min at 60°C. To improve the accuracy of real-time RT-PCR for quantification, amplifications were performed in triplicate for each RNA sample. To account for variations in input RNA and RT efficiency, EV RNA and TLR4 levels were normalized to GAPDH expression in each sample. To account for PCR amplification of contaminating genomic DNA, a control without RT was included.

Immunohistochemistry

EV capsid protein VP1/TLR4 double staining was performed on serial paraffin sections to determine the cellular source of EV capsid protein VP1 and TLR4. Monoclonal mouse anti-enterovirus against EV capsid protein VP1 (Dako, Glostrup, Denmark) and mouse monoclonal IgG_{2a} against human TLR4 (Santa Cruz Biotechnology) were used as primary antibodies. The tissue sections were deparaffined and thoroughly dehydrated. After inhibition of endogenous peroxidase and blocking of non specific reactions, mouse monoclonal IgG_{2a} against human TLR4 was applied. Biotinylated mouse immunoglobulin was used as a secondary antibody. Peroxidase-labeled streptavidin (Histofine, MAX-PO kit, Nichiren Corp) was applied and visualized using diaminobenzidine as a chromogen.

For double-staining, mouse monoclonal IgG_{2a} against human TLR4 was first stained as mentioned above. Thereafter, the sections were incubated with monoclonal mouse anti-enterovirus against EV capsid protein VP1 at 4°C overnight, followed by incubation for 30 min with rabbit anti-mouse immunoglobulin and alkaline phosphatase anti-alkaline phosphatase, and visualized with FastBlue (Histofine, SAP-AP kit, Nichiren Corp). The specificity of the immunohistochemistry was confirmed by substituting the primary antibodies with mouse

IgG1 negative control (Dako) on control sections from patients with DCM.

Statistical Analysis

All values are presented as mean $\pm$ s.e. Statistically, the differences in EV RNA and TLR4 expression levels between the DCM and control groups were analyzed by unpaired *t* test. Pearson's correlation coefficients were used to examine the relationship between levels of mRNA expression and clinical parameters. A value of $P < 0.05$ was considered statistically significant.

Results

EV RNA and TLR4 mRNA Levels in Myocardial Tissues

Plus- and minus-strand EV RNAs were detected in 19 (34%) of the 56 patients with DCM (mean levels of plus-strand EV RNA/GAPDH ratio and minus-strand EV RNA/GAPDH ratio in EV RNA-positive patients with DCM: 1.63 ± 0.37 and 1.27 ± 0.30 , respectively). All patients who were positive for minus-strand EV RNA were also positive for plus-strand EV RNA. A positive correlation was found between plus- and minus-strand EV RNA levels in EV RNA-positive patients with DCM ($r = 0.90$, $P < 0.001$) (Figure 1). Neither strand of EV RNA was detected in controls. In infected HeLa cells, the level of plus-strand EV RNA was higher than that of minus-strand EV RNA (plus-strand EV RNA/GAPDH ratio: 24076.68 ± 1830.32 , minus-strand EV RNA/GAPDH ratio: 285.61 ± 14.15).

Figure 1 Correlation plot between plus- and minus-strand EV RNA levels in myocardium obtained from patients with DCM. Significant correlation: $r = 0.90$, $P < 0.001$.

TLR4 mRNA levels were higher in patients with DCM than in controls (TLR4/GAPDH ratio: 0.24 ± 0.03 vs 0.01 ± 0.004 , $P < 0.001$). TLR4 mRNA levels were higher in EV RNA-positive DCM patients than in EV RNA-negative DCM patients (TLR4/GAPDH ratio: 0.38 ± 0.06 vs 0.16 ± 0.03 , $P = 0.001$) (Figure 2). A weak positive correlation was found between each strand of EV RNA and TLR4 mRNA levels in 19 EV RNA-positive patients with DCM (plus-strand EV RNA vs TLR4: $r = 0.69$, $P < 0.001$; minus-strand EV RNA vs TLR4: $r = 0.65$, $P = 0.002$).

Immunohistochemistry for TLR4 and EV Capsid Protein VP1

EV capsid protein VP1 was detected in 17 of the 56 patients with DCM (30.3%). As shown in Figure 3a, immunostaining of VP1 was observed in cytoplasm of cardiac myocytes in myocardium from patients with DCM. VP1 signals were found in scattered cardiac myocytes and myofibers in DCM. Both plus- and minus-strand EV RNA were detected in all VP1-positive patients with DCM. TLR4 immunostaining was positive in 35 patients with DCM (62.5%). VP1/TLR4 double staining showed extensive colocalization of VP1 and TLR4 proteins in cytoplasm of cardiac myocytes in myocardium obtained from DCM patients (Figure 3a). TLR4 mRNA levels were

Figure 2 Comparison of TLR4 mRNA levels between EV RNA-positive DCM patients and negative DCM patients.

Figure 3 EV capsid protein VP1/TLR4 double staining of serial sections of myocardial tissue obtained from patients with DCM and controls. (a) Immunostainings of EV capsid protein VP1 (arrow, blue coloring) and TLR4 (arrow head, brown coloring) were positive in cardiac myocytes in biopsy samples obtained from DCM patients. VP1/TLR4 double staining showed colocalization of VP1 and TLR4 proteins in cardiac myocytes. (b) Immunostainings of VP1 and TLR4 in myocardial tissue obtained from control subjects. Neither VP1 nor TLR4 immunostaining was present in any specimens. Magnification: $\times 250$.

higher in DCM patients with TLR4 immunostaining than in those without TLR4 immunostaining (TLR4/GAPDH ratio: 0.33 ± 0.04 vs 0.06 ± 0.02 , $P < 0.001$). TLR4 immunostaining was also positive in all EV capsid protein VP1-positive patients with DCM. There was no evidence of nonspecific immunostaining in myocardial tissues obtained from patients with DCM. Neither VP1 nor TLR4 immunostaining was present in any specimens from control subjects (Figure 3b).

Clinical Outcome

Table 1 summarizes results of RT-PCR and immunohistochemistry, and clinical outcomes in DCM patients. EV RNA-positive patients with DCM ($n = 19$) showed significantly lower LVEF compared with EV RNA-negative patients with DCM ($n = 37$) ($27.3 \pm 1.2\%$ vs $34.6 \pm 1.5\%$, $P = 0.002$) (Figure 4a).

LV end-systolic diameter (LVESD) was larger in EV RNA-positive patients than in EV RNA-negative patients (54.4 ± 1.4 mm vs 50.3 ± 0.6 mm, $P = 0.004$) (Figure 4b). There was weak negative correlation between plus-strand EV RNA levels and LVEF in EV RNA-positive patients with DCM ($r = -0.52$, $P = 0.02$). Both plus- and minus-strand EV RNA levels were also correlated positively with LVESD in EV RNA-positive patients with DCM (plus-strand EV RNA vs LVESD: $r = 0.68$, $P = 0.001$; minus-strand EV RNA vs LVESD: $r = 0.59$, $P = 0.007$).

We examined the differences in LVEF and LVESD between two groups with DCM divided relative to the mean TLR4 mRNA/GAPDH ratio ($= 0.24$). The group with high TLR4 (≥ 0.24) showed lower LVEF and larger LVESD than the group with low TLR4 (< 0.24) (LVEF: 24.6 ± 1.3 vs $36.1 \pm 1.2\%$, $P < 0.001$; LVESD: 56.7 ± 1.1 vs 49.1 ± 0.5 mm, $P < 0.001$) (Figure 5). TLR4 mRNA levels were negatively and weakly positively correlated with LVEF and LVESD respectively (TLR4 mRNA vs LVEF: $r = -0.72$, $P < 0.001$; TLR4 mRNA vs LVESD: $r = 0.69$, $P < 0.001$).

Discussion

The most important finding of the present study is high levels of TLR4 mRNA and EV RNA in myocardium obtained from DCM patient with advanced LV dysfunction. Both strand types of EV RNA were positive in DCM patients, suggesting the possibility of EV replication in myocardium. Furthermore, there was a positive correlation between TLR4 mRNA and EV RNA levels in DCM patients. Both VP1 and TLR4 immunostaining were seen in scattered cardiac myocytes in DCM. These data therefore support a link between the toll signaling pathway and EV replication in human DCM.

Transcription of minus-strand RNA from the plus-strand EV genomic template is the essential first step of EV replication. This minus-strand RNA is then used as a template to generate multiple copies of plus-strand genomes that are translated into EV structural proteins and ultimately packed into new virions.¹³ Therefore, detection of minus-strand EV RNA is an indicator of active EV replication. In this study, minus-strand EV RNA was detected in all plus-strand-positive DCM patients. These findings indicate that active EV replication occurs in the myocardium in a significant proportion of cases of DCM.

Levels of the two strand types of EV RNA were comparable (plus-strand: 1.63 ± 0.37 , minus-strand: 1.27 ± 0.30), and were positively correlated in DCM patients. No report has yet shown myocardial genome levels of both-strand EV RNA in human DCM. In a mouse model with persistent coxsackievirus infection, actual levels of plus- and minus-strand EV RNA were nearly equal in persistence in

Table 1 Levels of each-strand EV RNA and TLR4 mRNA, immunohistochemistry of EV capsid protein VP1 and TLR4, and clinical outcomes in DCM patients

Patient No.	Age (years)	Sex	Levels of EV RNA and TLR4 mRNA			Immunohistochemistry		LVEF (%)	LVESD (mm)
			Plus-strand	Minus-strand	TLR4	VP1	TLR4		
1	40	M	3.22	0.40	0.16	+	+	22	48
2	22	M	3.54	2.36	0.47	+	+	22	64
3	56	M	5.21	2.89	0.66	+	+	24	55
4	49	M	0.56	0.56	0.13	—	+	26	49
5	68	M	—	—	0.06	—	—	35	49
6	68	M	1.40	1.56	0.25	+	+	29	62
7	52	M	3.15	2.98	0.32	+	+	30	56
8	22	F	0.51	0.39	0.47	—	+	25	51
9	48	M	2.42	2.62	0.10	+	+	22	56
10	70	M	3.48	1.86	0.81	+	+	22	54
11	65	F	3.75	2.68	0.43	+	+	28	57
12	66	F	7.34	6.34	0.58	+	+	22	56
13	67	M	5.31	4.55	0.66	+	+	33	66
14	65	M	1.77	1.42	0.53	+	+	27	50
15	50	F	0.36	0.29	0.13	+	+	38	52
16	42	M	1.01	0.94	0.19	+	+	33	46
17	39	M	4.82	5.09	0.88	+	+	20	64
18	73	M	1.61	1.77	0.13	+	+	28	49
19	38	F	0.27	0.46	0.19	+	+	33	49
20	66	M	—	—	0.08	—	+	39	50
21	54	M	—	—	0.11	—	+	42	51
22	15	M	—	—	0.10	—	+	45	46
23	41	M	—	—	0.19	—	—	36	49
24	65	M	—	—	0.11	—	—	43	48
25	70	M	—	—	0.02	—	—	39	47
26	42	F	—	—	0.01	—	—	38	48
27	65	M	—	—	0.02	—	—	44	48
28	70	M	—	—	0.01	—	—	41	47
29	16	M	—	—	0.02	—	—	39	48
30	25	M	—	—	0.10	—	+	42	46
31	28	M	—	—	0.11	—	+	38	47
32	55	F	—	—	0.07	—	—	45	49
33	43	F	—	—	0.01	—	—	46	47
34	20	M	—	—	0.11	—	+	21	49
35	28	M	—	—	0.27	—	+	27	54
36	64	M	—	—	0.28	—	+	30	55
37	66	M	—	—	0.33	—	+	32	59
38	53	M	—	—	0.22	—	+	19	60
39	48	F	—	—	0.04	—	—	39	48
40	49	M	—	—	0.05	—	—	37	49
41	68	F	—	—	0.02	—	—	38	51
42	29	M	—	—	0.10	—	—	41	45
43	73	M	—	—	0.19	—	—	44	45
44	66	M	—	—	0.22	—	—	29	49
45	62	F	—	—	0.03	—	—	37	49
46	31	F	—	—	0.09	—	—	39	52
47	44	M	—	—	0.44	—	+	18	55
48	60	M	—	—	0.30	—	+	22	49
49	50	M	—	—	0.23	—	+	24	50
50	59	M	—	—	0.58	—	+	19	59
51	53	M	—	—	0.15	—	+	29	49
52	66	M	—	—	0.39	—	+	26	53
53	35	M	0.85	0.42	0.99	+	+	11	59
54	39	F	—	—	0.01	—	—	36	52
55	55	F	—	—	0.02	—	—	44	50
56	41	M	—	—	0.03	—	—	43	50

EV=enteroviral; TLR4=Toll-like receptor 4; DCM=dilated cardiomyopathy; LVEF=left ventricular ejection fraction; LVESD=left ventricular end-systolic diameter; M=male; F, female.

the infectious phase (1 month after infection).¹⁴ In human skeletal muscle, Cunningham *et al*¹⁵ have demonstrated a persistent EV infection of skeletal muscle in patients suffering from chronic fatigue

syndrome. Wessely *et al*¹⁶ have demonstrated that EV viral genome alone, in the absence of infectious virus, can exert direct pathological effects on cultured cardiac myocytes. These observations

Figure 4 Comparison of LVEF (a) and LVESD (b) between EV RNA-positive DCM patients and EV RNA-negative DCM patients. LVEF = left ventricular ejection fraction; LVESD = left ventricular end-systolic diameter.

Figure 5 Comparison of LVEF and LVESD in two groups of patients with DCM divided by mean TLR4 mRNA expression levels.

suggest that plus-strand RNA synthesis could diminish as RNA polymerase activity subsides, leading to double-stranded EV RNA replication. EV RNA replication may therefore persist in myocardium in human DCM. The mechanism of equal synthesis of the two strands of EV RNA in these *in vitro* situations is unclear, but it is possible that downregulation of RNA polymerase activity occurs

in infected myocardium through a host immune response.

This study has shown advanced LV dysfunction in EV RNA-positive patients with DCM compared to EV RNA-negative patients. There was a negative correlation between EV RNA levels and LVEF. Wessely *et al*¹⁷ have demonstrated that the transgenic expression of replication-restricted EV genome in the heart could induce LV dysfunction. In this model, defective-contraction coupling and a decrease in the magnitude of isolated myocyte shortening were observed.¹⁷ It has been reported that the presence of active EV replication can be a marker for poor outcome in patients with DCM.⁴ VP1 protein can be found in all DCM patients with both-strand EV RNA. These results suggest the possibility of viral protein synthesis in host cells during virus persistence. Li *et al*¹⁸ have demonstrated that the pattern of VP1 detection may correlate with disease stage and severity of DCM. Therefore, detection of VP1 protein and both plus- and minus-strand EV RNA may indicate the persistence of infection in the heart, and may facilitate LV dysfunction in DCM.

At least two TLRs, including TLR2 and TLR4, have recently been implicated in lipopolysaccharide (LPS)-induced signal transduction.^{7,8} Although TLR2 expression is essential for responses to Gram-positive bacteria, mycobacteria and spirochetes, it is not required for responses to viral protein.¹⁹⁻²¹ The activation of TLR4 induces a variety of cytokines and costimulatory molecules that are crucial to immune responses.⁸ Recent study has reported that activation of antiviral immune response via TLR4 and CD14 is important in the pathogenesis of viral infection.⁹ A TLR4-deficient mouse model has shown that inflammatory cells did not produce any detectable levels of proinflammatory cytokines in response to LPS.²² Fairweather *et al*²³ have reported that TLR4 influenced coxsackievirus replication in mouse model with CVB3-induced myocarditis. This model has also shown that TLR4-deficient mice had significantly reduced levels of myocarditis, viral replication and inflammatory cytokine levels.²³ Our previous study has shown that increased expression of TLR4 is associated with EV replication in human myocarditis.²⁴ These reports suggest that activation of TLR4 may play an important role in host immune response against viral infection. In the present study, high levels of TLR4 mRNA expression were seen in DCM patients positive for both plus- and minus-strand EV RNA. A positive correlation was also found between TLR4 mRNA and levels of each strand type of EV RNA. TLR4 immunostaining was found in VP1-positive myocytes obtained from DCM patients. In agreement with these findings, Frantz *et al*²⁵ have reported that TLR4 was abundantly expressed in dysfunctional cardiac myocytes in failing human hearts. However, the trigger for toll expression in failing heart was not clearly identified in Frantz's

report. The present study has shown that EV replication may produce both viral protein and RNA in the heart. A mouse model of TLR4 expression has demonstrated that viral protein was an important factor for TLR4 activation during the immune response to a viral pathogen (respiratory syncytial virus) and that TLR4 was an important contributor to the immune response against viral replication.²⁶ It is therefore likely that activation of antiviral immune response via TLR4 is triggered by EV replication, and is important in the pathogenesis of DCM.

Another important finding of this study was high levels of both TLR4 mRNA and EV RNA with advanced LV dysfunction in DCM. An EV genome-transgenic mouse model has demonstrated that the presence of both strands of EV RNA may activate an intracellular signaling mechanism such as the NF- κ B signaling pathway.¹⁷ TLR4 activates a host immune response that is critical for upregulating the synthesis of a variety of inflammatory mediators and endogenous proteins, such as chemokines, cell-adhesion molecules, proinflammatory cytokines, inducible nitric oxide synthase (iNOS) and heat-shock proteins.^{8,27,28} Our previous studies have demonstrated that TNF- α cascade is activated in human DCM via expression of its converting enzyme and iNOS and that this activation may be related to the clinical severity of DCM.^{5,6,29} Although this study could not confirm a direct causal relationship between activation of the TLR4-signaling pathway and LV dysfunction induced by inflammatory mediators, the LPS-challenged mouse model has demonstrated that TLR4 is tightly linked with NF- κ B activation and expression of proinflammatory cytokines such as interleukin 1 and TNF- α in the heart.³⁰ These inflammatory mediators have been shown in various reports to produce LV dysfunction.^{5,6,29,31,32} Nemoto *et al*³³ have recently reported that LPS challenge resulted in depression of LV systolic function in wild-type mice, whereas no significant changes in LV systolic function were noted in TLR4-deficient mice. The activated TLR4-signaling pathway in the heart may therefore be linked to inflammatory mediators (eg proinflammatory cytokines and iNOS) and to cardiac dysfunction. These observations suggest that activation of the toll-signaling pathway via EV replication may mediate inflammatory mediators and may induce LV dysfunction in human DCM. Therefore, TLR4-mediated-immune response to EV replication may allow new approach for treatment of human DCM.

In conclusion, the present study has demonstrated TLR4 expression with the detection of EV RNA and EV protein suggesting the activation of the toll signaling pathway via EV replication in human DCM. In addition, EV RNA and TLR4 mRNA levels may be related to the clinical severity of DCM. The activation of the toll signaling pathway against EV replication may therefore be involved in the pathogenesis of DCM.

Acknowledgement

This work was supported by a Grant-in-Aid for General Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture (No. 12770348).

References

- Bowles NE, Richardson PJ, Olsen, *et al.* Detection of coxsackie-B-virus-specific RNA sequence in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. *Lancet* 1986;1: 1120–1122.
- Satoh M, Tamura G, Segawa I. Enteroviral RNA in endomyocardial biopsy tissues of myocarditis and dilated cardiomyopathy. *Pathol Int* 1994;44:345–351.
- Why HJ, Meany BT, Richardson PJ, *et al.* Clinical and prognostic significance of detection of enteroviral RNA in the myocardium of patients with myocarditis or dilated cardiomyopathy. *Circulation* 1994;89: 2582–2589.
- Fujioka S, Kitauro Y, Ukimura A, *et al.* Evaluation of viral infection in the myocardium of patients with idiopathic dilated cardiomyopathy. *J Am Coll Cardiol* 2000;36:1920–1926.
- Satoh M, Tamura G, Segawa I, *et al.* Expression of cytokine genes and presence of enteroviral genomic RNA in endomyocardial biopsy tissues of myocarditis and dilated cardiomyopathy. *Virchow Arch* 1996;427: 503–509.
- Satoh M, Nakamura M, Saitoh H, *et al.* Tumor necrosis factor- α -converting enzyme and tumor necrosis factor- α in human dilated cardiomyopathy. *Circulation* 1999; 99:3260–3265.
- Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol* 2001;1:135–145.
- Medzhitov R, Preston-Hurlburt P, Janeway Jr CA. A human homologue of the *Drosophila* toll protein signals activation of adaptive immunity. *Nature* 1997; 388:394–397.
- Kurt-Jones E, Popova L, Kwinn L, *et al.* Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 2000;5:398–401.
- Bradenberg RO, Chazov E, Cherian G. Report of the WHO/ISFC task force on the definition and classification of cardiomyopathies. *Circulation* 1980;64: 1397–1399.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987;162: 156–159.
- Zhang HY, Yousef GE, Cunningham L, *et al.* Attenuation of a reactivated cardiocytotropic coxsackievirus B3: The 5'-nontranslated region does not contain major attenuation determinants. *J Med Virol* 1993;41: 129–137.
- Klingel K, Rieger P, Mall G, *et al.* Visualization of enteroviral replication in myocardial tissue by ultrastructural *in situ* hybridization: identification of target cells and cytopathic effects. *Lab Invest* 1998;78: 1227–1237.
- Tam PE, Messner RP. Molecular mechanisms of coxsackievirus persistence in chronic inflammatory myopathy: viral RNA persists through formation of a double-stranded complex without associated genomic mutations or evolution. *J Virol* 1999;73: 10113–10121.
- Cunningham L, Bowles NE, Lane RJM, *et al.* Persistence of enteroviral RNA in chronic fatigue syndrome is associated with the abnormal production of equal amounts of positive and negative strands of enteroviral RNA. *J Gen Virol* 1990;71:1399–1402.
- Wessely R, Henke A, Zell R, *et al.* Low-level expression of a mutant coxsackieviral cDNA induces a myocytopathic effect in culture: an approach to the study of enteroviral persistence in cardiac myocytes. *Circulation* 1998;98:450–457.
- Wessely R, Klingel K, Santana LF, *et al.* Transgenic expression of replication-restricted enteroviral genomes in heart muscle induces defective excitation-contraction coupling and dilated cardiomyopathy. *J Clin Invest* 1998b;102:1444–1453.
- Li Y, Bourlet T, Andreoletti L, *et al.* Enteroviral capsid protein VP1 is present in myocardial tissues from some patients with myocarditis or dilated cardiomyopathy. *Circulation* 2000;101:231–234.
- Heine H, Kirschning CJ, Lien E, *et al.* Cutting edge: cells that carry A null allele for toll-like receptor 2 are capable of responding to endotoxin. *J Immunol* 1999;162:6971–6975.
- Kirschning CJ, Wesche H, Merrill Ayres T, *et al.* Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J Exp Med* 1998;188: 2091–2097.
- Yang RB, Mark MR, Gray A, *et al.* Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling. *Nature* 1998;395:284–288.
- Hoshino K, Takeuchi O, Kawai T, *et al.* Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999; 162:3749–3752.
- Fairweather D, Yuseung S, Frisancho S, *et al.* IL-12 receptor beta1 and toll-like receptor 4 increase IL-1beta- and IL-18-associated myocarditis and coxsackievirus replication. *J Immunol* 2003;170: 4731–4737.
- Satoh M, Nakamura M, Akatsu T, *et al.* Expression of toll-like receptor 4 is associated with enteroviral replication in human myocarditis. *Clin Sci (Lond)* 2003;104:577–584.
- Frantz S, Kobzik L, Kim YD, *et al.* Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. *J Clin Invest* 1999;104:271–280.
- Haynes LM, Moore DD, Kurt-Jones EA, *et al.* Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *J Virol* 2001;75: 10730–10737.
- Brightbill HD, Libraty DH, Krutzik SR, *et al.* Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* 1999;188: 2091–2097.
- Dybdahl B, Wahba A, Lien E, *et al.* Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. *Circulation* 2002;105:685–690.
- Satoh M, Nakamura M, Tamura G, *et al.* Inducible nitric oxide synthase and tumor necrosis factor- α in myocardium in human dilated cardiomyopathy. *J Am Coll Cardiol* 1997;29:716–724.

- 30 Baumgarten G, Knuefermann P, Nozaki N, *et al*. *In vivo* expression of proinflammatory mediators in the adult heart after endotoxin administration: the role of toll-like receptor-4. *J Infect Dis* 2001; 183:1617–1624.
- 31 Finkel MS, Oddis CV, Jacob TD, *et al*. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 1992;257:387–389.
- 32 Yokoyama T, Vaca L, Rossen RD, *et al*. Cellular basis for the negative inotropic effects of tumor necrosis factor- α in the adult mammalian heart. *J Clin Invest* 1993;92:2303–2312.
- 33 Nemoto S, Vallejo JG, Knuefermann P, *et al*. *Escherichia coli* LPS-induced LV dysfunction: role of toll-like receptor-4 in the adult heart. *Am J Physiol Heart Circ Physiol* 2002;282:H2316–H2323.