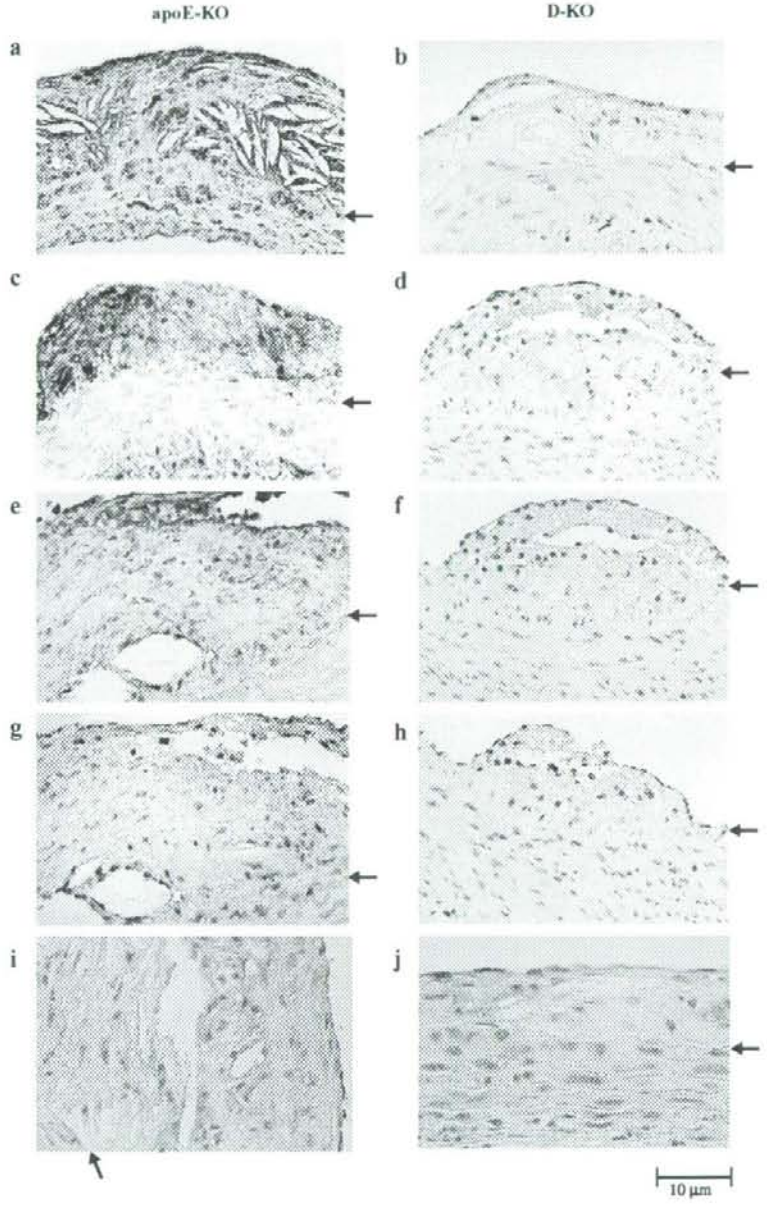

Fig. 5. *Immunohistochemical staining for p22phox (a, b), SAP (c, d), MMP-2 (e, f), MMP-9 (g, h), and AT2 (i, j) of apoE-KO (a, c, e, g, i) and D-KO mice (b, d, f, h, j) at 60-week-old. The immunoreactivities of p22phox, SAP, MMP-2, and MMP-9 of D-KO mice (b, d, f, h) in atherosclerotic lesions were less than those of apoE-KO mice (a, c, e, g). However, the immunoreactivities of AT2 of D-KO mice (j) in atherosclerotic lesions did not differ from those of apoE-KO mice (i). The arrows indicate internal elastic lamina.*

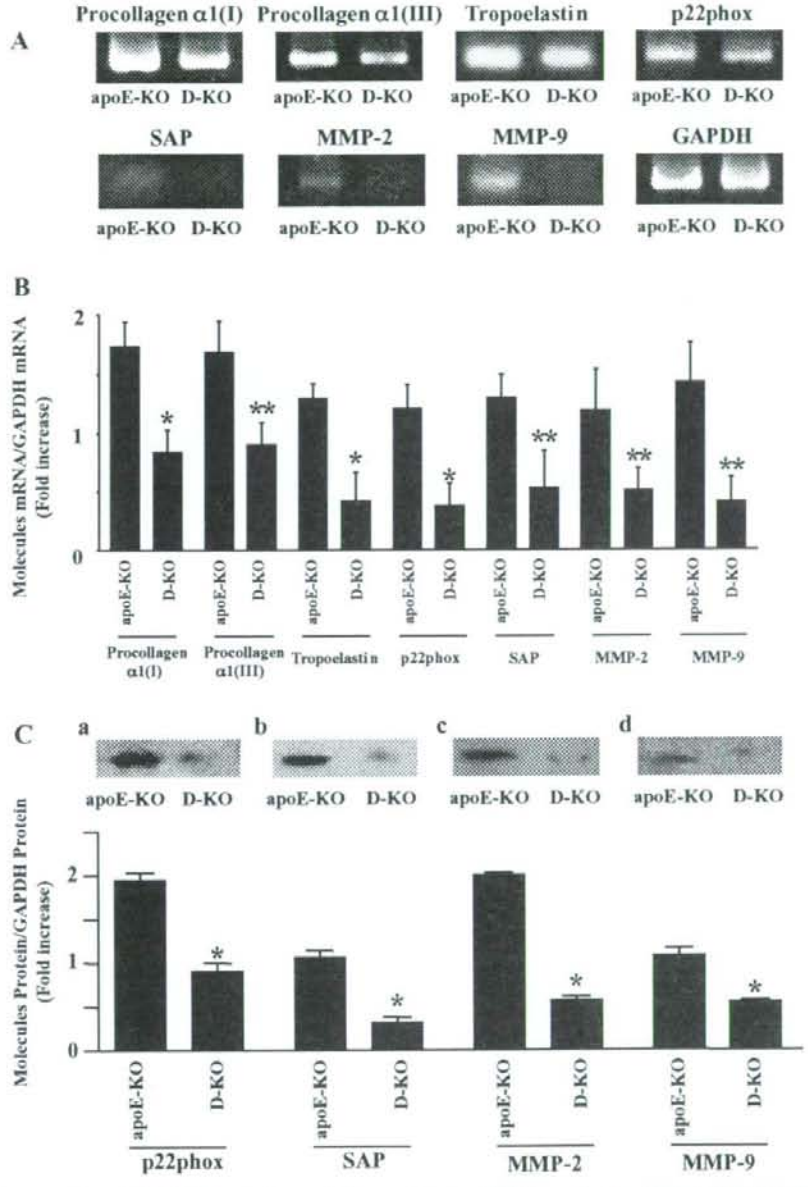

Fig. 6. *A: The expression of mRNA for procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 in cultured VSMCs from apoE-KO and D-KO mice by RT-PCR. B: Expression of mRNA for procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 in cultured VSMCs from apoE-KO and D-KO mice by real-time PCR. Decreases in the mRNA expression of these molecules were seen in VSMCs from D-KO mice in comparison with apoE-KO mice. C: Western blotting for p22phox (a), SAP (b), MMP-2 (c), and MMP-9 (d) in cultured VSMCs from apoE-KO or D-KO mice. C shows that the level of protein expression of these molecules decreased in the D-KO mice in comparison with the apoE-KO mice.* $^{*}p < 0.01$ *vs. apoE-KO mice,* $^{**}p < 0.05$ *vs. apoE-KO mice.*

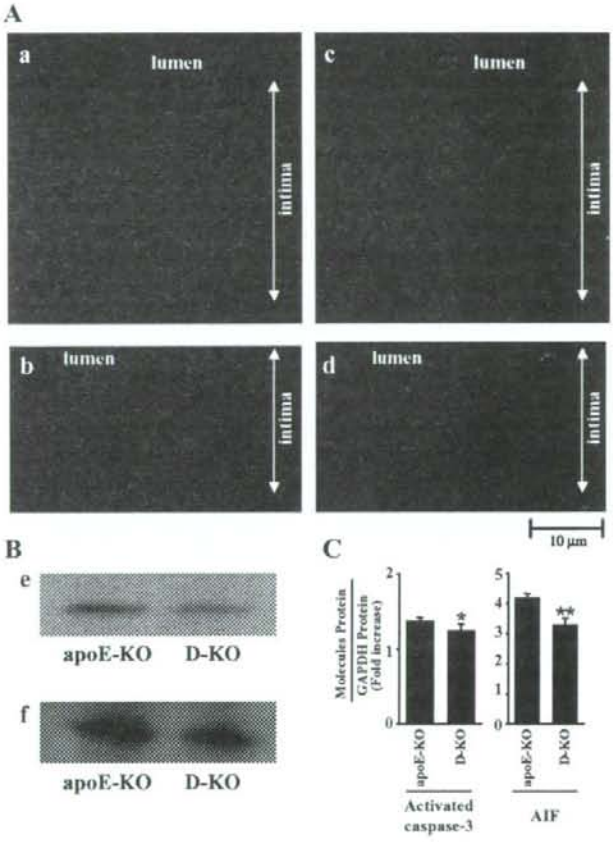

Fig. 7. *A: Immunofluorescence staining for the analysis of apoptosis in atherosclerotic lesions of apoE-KO (a, c) and D-KO mice (b, d) at 60-week-old. The expression of activated caspase-3 (a, b) and AIF (c, d) decreased in D-KO mice compared with apoE-KO mice. B: Western blotting for activated caspase-3 (e) and AIF (f) of cultured VSMCs from apoE-KO or D-KO mice. C: Densitometry of Western blots for activated caspase-3 and AIF of cultured VSMCs from apoE-KO or D-KO mice. The level of protein expression of these molecules decreased in D-KO mice in comparison with the apoE-KO mice. *$p < 0.05$ vs. apoE-KO mice, **$p < 0.01$ vs. apoE-KO mice.*

which is composed of p22phox and gp91phox subunits and three cytosolic components, rac, p47phox, and p67phox (*29*). Ang II was found to activate p47phox *via* AT1 (*30*). In addition, ARB was reported to inhibit p22phox mRNA expression caused by mechanical stretching and in patients with diabetes mellitus (*31, 32*). We also showed that AT1a deficiency in apoE-KO mice inhibited the expression of p22phox at 60 weeks of age.

Superoxide dismutase (SOD) prevents increases in vascular ROS. In addition, activation of AT1 has been reported to contribute to a decrease in the expression of extracellular SOD, and ARB increased the serum SOD activity in patients with hypertension (*33, 34*). Although we did not measure the SOD activity in the present study, AT1a deficiency might increase the SOD activity and prevent age-related atherosclerosis in the apoE-KO mice.

AT1 deficiency was reported to have a striking effect in reducing hypercholesterolemia-induced atherosclerosis in apoE-deficient mice at 19-week-old (*10*) and LDL receptor deficient mice at 20-week-old (*11*). However, the effect of AT1a deficiency on apoptosis, matrix production, or MMP in atherosclerosis of apoE-KO mice has not been previously determined. The present study is the first report demonstrating that AT1 deficiency reduces atherosclerosis in apoE-KO

mice *via* a reduction of oxidative stress, apoptosis, matrix production, and MMP expression at 60-week-old.

Ang II stimulation of AT1 has been reported to inhibit apoptosis in cultured VSMCs, and we expected that AT1 deficiency would increase apoptosis in atherosclerotic lesions. However, the expression of activated caspase-3 and AIF decreased in atherosclerotic lesions of D-KO mice in comparison with apoE-KO mice at 60-week-old. In addition, there is no report that demonstrates stimulation of apoptosis in atherosclerotic lesions by the clinical use of ARB. We speculate that the reduction of oxidative stress may lead to the inhibition of apoptosis in the atherosclerotic lesions of D-KO mice.

Acute coronary syndromes result from fissure, erosion, or rupture of vulnerable atherosclerotic plaques. The characteristics of a vulnerable plaque include a large lipid pool, an abundance of inflammatory cells and mediators, a reduced smooth muscle cell and collagen content, and a thin overlying fibrous cap (*35*). Not only do lipids in the atheroma create mechanical instability, but biologically active lipids participate in promoting oxidative stress and inflammatory responses. Keidar *et al.* (*36*) reported that an AT1 antagonist inhibited LDL lipid peroxidation and atherosclerosis in apoE-KO mice. Blocking AT1 may play an important role in the attenuation of atherogenesis.

Although the reduction of ECM production in the aorta leads to aortic distensibility, decreased ECM production in atheromas may cause plaque vulnerability. However, a reduction of oxidative stress, apoptosis, and MMP in atheromas by AT1a deficiency may contribute to plaque stabilization.

Daugherty *et al.* (*11*) reported that AT1 deficiency reduces hypercholesterolemia-induced atherosclerosis in LDL receptor deficient mice. Absence of AT1a has the potential to lead to a compensatory change in AT2 abundance. However, AT2 mRNA expression was not altered, and Ang II type 1b receptor (AT1b) mRNA expression was increased in the aorta of AT1a-deficient mice. They showed that AT1a deficiency in LDL receptor deficient mice had no significant effect on systolic blood pressure. Our study also demonstrated that AT1a deficiency in apoE-KO mice had no significant effect on blood pressure. We hypothesize that AT1a deficiency may reduce hypercholesterolemia-induced atherosclerosis independent of blood pressure changes.

Similar to the results reported by Daugherty *et al.* (*11*), we also observed no compensatory up-regulation of the AT2 mRNA expression in the atherosclerotic lesions of D-KO mice. Moreover, lack of any effect of AT2 deficiency on the atherosclerotic lesion area in the LDL receptor deficient mice has also been reported (*11*). Therefore, we speculate that AT2 does not contribute significantly to atherosclerosis in mice.

In conclusion, AT1a deficiency reduced atherosclerotic lesion size of apoE-KO mice and protected against the age-related progression of atherosclerosis. Reduction of oxidative stress, apoptosis, and MMP in atheromas by AT1a deficiency may contribute to plaque size.

References

1. Libby P, Ridker PM, Maseri A: Inflammation and atherosclerosis. *Circulation* 2002; **105**: 1135–1143.
2. Nicenig G, Harrison DG: The AT_1-type angiotensin receptor in oxidative stress and atherogenesis. Part I: Oxidative stress and atherogenesis. *Circulation* 2002; **105**: 393–396.
3. Miura S, Saku K, Karnik SS: Molecular analysis of the structure and function of the angiotensin II type 1 receptor. *Hypertens Res* 2003; **26**: 937–943.
4. Griendling KK, Ushio-Fukai M, Lassegue B, Alexander RW: Angiotensin II signaling in vascular smooth muscle. New concepts. *Hypertension* 1997; **29**: 366–373.
5. Kranzhofer R, Schmidt J, Pfeiffer CA, Hagl S, Libby P, Kubler W: Angiotensin induces inflammatory activation of human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1623–1629.
6. Hernandez-Presa M, Bustos C, Ortego M, *et al*: Angiotensin-converting enzyme inhibition prevents arterial nuclear factor–kappa B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation* 1997; **95**: 1532–1541.
7. Tummala PE, Chen XL, Sundell CL, *et al*: Angiotensin II induces vascular cell adhesion molecule-1 expression in rat vasculature: a potential link between the renin-angiotensin system and atherosclerosis. *Circulation* 1999; **100**: 1223–1229.
8. Ferrario CM: Use of angiotensin II receptor blockers in animal models of atherosclerosis. *Am J Hypertens* 2002; **15**: 9S–13S.
9. Takai S, Jin D, Sakaguchi M, *et al*: Comparative effects of candesartan and amlodipine in a monkey atherosclerotic model. *Hypertens Res* 2004; **27**: 517–522.
10. Wassmann S, Czech T, van Eickels M, Fleming I, Bohm M, Nickenig G: Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circulation* 2004; **110**: 3062–3067.
11. Daugherty A, Rateri DL, Lu H, Inagami T, Cassis LA: Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the AT1A receptor. *Circulation* 2004; **110**: 3849–3857.
12. Nyby MD, Abedi K, Smutko V, Eslami P, Tuck ML: Vascular angiotensin type 1 receptor expression is associated with vascular dysfunction, oxidative stress and inflammation in fructose-fed rats. *Hypertens Res* 2007; **30**: 451–457.
13. Smith JD, Trogan E, Ginsberg M, Grigaux C, Tian J, Miyata M: Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc Natl Acad Sci U S A* 1995; **92**: 8264–8268.
14. Tangirala RK, Rubin EM, Palinski W: Quantification of atherosclerosis in murine model: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor–deficient and apolipoprotein E–deficient mice. *J Lipid Res* 1995; **36**: 2320–2328.
15. Miyata M, Biro S, Kaieda H, *et al*: Apolipoprotein J/clus-

terin is induced in vascular smooth muscle cells after vascular injury. *Circulation* 2001; **104**: 1407–1412.
16. Obata H, Biro S, Arima N, *et al*: NF-kappa B is induced in the nuclei of cultured rat aortic smooth muscle cells by stimulation of various growth factors. *Biochem Biophys Res Commun* 1996; **224**: 27–32.
17. Eto H, Biro S, Miyata M, *et al*: Angiotensin II type 1 receptor participates in extracellular matrix production in the late stage of remodeling after vascular injury. *Cardiovasc Res* 2003; **59**: 200–211.
18. Bohm M, Raghunath M, Sunderkotter C, *et al*: Collagen metabolism is a novel target of the neuropeptide alpha-melanocyte–stimulating hormone. *J Biol Chem* 2004; **279**: 6959–6966.
19. Bunda S, Kaviani N, Hinek A: Fluctuations of intracellular iron modulate elastin production. *J Biol Chem* 2005; **280**: 2341–2351.
20. Rupin A, Paysant J, Sansilvestri-Morel P, *et al*: Role of NADPH oxidase–mediated superoxide production in the regulation of E-selectin expression by endothelial cells subjected to anoxia/reoxygenation. *Cardiovasc Res* 2004; **63**: 323–330.
21. Wei W, Parvin N, Tsumura K, *et al*: Induction of C-reactive protein, serum amyloid P component, and kininogens in the submandibular and lacrimal glands of rats with experimentally induced inflammation. *Life Sci* 2001; **69**: 359–368.
22. Kodali R, Hajjou M, Berman AB, *et al*: Chemokines induce matrix metalloproteinase-2 through activation of epidermal growth factor receptor in arterial smooth muscle cells. *Cardiovasc Res* 2006; **69**: 706–715.
23. Abe N, Osanai T, Fujiwara T, Kameda K, Matsunaga T, Okumura K: C-reactive protein–induced upregulation of extracellular matrix metalloproteinase inducer in macrophages: inhibitory effect of fluvastatin. *Life Sci* 2006; **78**: 1021–1028.
24. Eto H, Miyata M, Kume N, *et al*: Expression of lectin-like oxidized LDL receptor-1 in smooth muscle cells after vascular injury. *Biochem Biophys Res Commun* 2006; **341**: 591–598.
25. Suzuki J, Otsuka F, Inagaki K, *et al*: Primary aldosteronism caused by a unilateral adrenal adenoma accompanied by autonomous cortisol secretion. *Hypertens Res* 2007; **30**: 367–373.
26. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK: Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 2002; **91**: 406–413.
27. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H: Role of oxidative stress in atherosclerosis. *Am J Cardiol* 2003; **91**: 7A–11A.
28. Nakano D, Hayashi T, Tazawa N, *et al*: Chronic hypoxia accelerates the progression of atherosclerosis in apolipoprotein E–knockout mice. *Hypertens Res* 2005; **28**: 837–845.
29. Zalba G, San José G, Moreno MU, *et al*: Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. *Hypertension* 2001; **38**: 1395–1399.
30. Clempus RE, Griendling KK: Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc Res* 2006; **71**: 216–225.
31. Hitomi H, Fukui T, Moriwaki K, *et al*: Synergistic effect of mechanical stretch and angiotensin II on superoxide production *via* NADPH oxidase in vascular smooth muscle cells. *J Hypertens* 2006; **24**: 1089–1095.
32. Kusaka I, Kusaka G, Zhou C, *et al*: Role of AT1 receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury. *Am J Physiol Heart Circ Physiol* 2004; **286**: H2442–H2451.
33. Naya M, Tsukamoto T, Morita K, *et al*: Olmesartan, but not amlodipine, improves endothelium-dependent coronary dilation in hypertensive patients. *J Am Coll Cardiol* 2007; **50**: 1144–1149.
34. Chabrashvili T, Kitiyakara C, Blau J, *et al*: Effects of ANG II type 1 and 2 receptors on oxidative stress, renal NADPH oxidase, and SOD expression. *Am J Physiol Regul Integr Comp Physiol* 2003; **285**: R117–R124.
35. Rabbani R, Topol EJ: Strategies to achieve coronary arterial plaque stabilization. *Cardiovasc Res* 1999; **41**: 402–417.
36. Keidar S, Attias J, Smith J, Breslow JL, Hayek T: The angiotensin-II receptor antagonist, losartan, inhibits LDL lipid peroxidation and atherosclerosis in apolipoprotein E–deficient mice. *Biochem Biophys Res Commun* 1997; **236**: 622–625.

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Toll-like receptor 2 expression level on monocytes in patients with viral infections: Monitoring infection severity

Takashi Kajiya [a], Koji Orihara [a,*], Shuichi Hamasaki [a], Ryutaro Oba [b], Hiroyuki Hirai [b], Kinya Nagata [b], Tomoaki Kumagai [b], Sanemasa Ishida [a], Naoya Oketani [a], Hitoshi Ichiki [a], So Kuwahata [a], Shoji Fujita [a], Nobuhiro Uemura [c], Chuwa Tei [a]

[a] *Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima, Japan*
[b] *Department of Advanced Medicine and Development, BML, INC. Saitama, Japan*
[c] *Kouseikai Uemura Hospital, Kagoshima, Japan*

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Summary For viral infectious diseases, reliable biomarkers capable of monitoring recovery and therapeutic effects and that simultaneously discriminate between viral and bacterial infection are necessary. In this study, by using flow-cytometric quantification system, Toll-like receptor 2 (TLR2) expression levels on monocytes of influenza patients ($n = 47$) were compared with those of healthy volunteers ($n = 50$). Subsequently, throughout their acute, convalescent and healed phases, TLR2, C-reactive protein (CRP), serum amyroid A (SAA), and neopterin levels were followed. Additionally, TLR2 levels in other viral infectious diseases were assayed. The results showed that TLR2 level in influenza patients was remarkably up-regulated in acute phase compared to healthy volunteers ($p < 0.001$). Thereafter, TLR2 levels normalized in good accordance with their recovery processes. CRP and neopterin levels were relatively widely distributed from normal to abnormally high levels in acute phase in spite of similar disease severity among the patients. SAA levels did not necessarily reflect the patients' clinical course during their recovery. Clinical observations of other viral infections also indicated that TLR2 levels were compatible with infection severity. TLR2 expression level on monocytes might serve as a unique biomarker useful in viral infectious diseases.

* Corresponding author. Tel: +81 99 275 5318; fax: +81 99 265 8447.
E-mail address: orip@po.synapse.ne.jp (K. Orihara).

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Introduction

Emerging viral infections have posed an imminent threat to public health in all ages. Reliable serological/hematological markers capable of monitoring recovery from viral infectious diseases and the progression, such as pneumonia,[1-3] myositis,[4] and myocarditis,[5,6] are desired especially in pandemic crisis. Currently, many potential markers for early assessment of viral infection have been developed, of which neopterin and serum amyloid A (SAA) are noteworthy. Neopterin is generated and released in increased amounts by macrophages upon activation by interferon-gamma (INF-γ).[7] Chan et al. showed that mean neopterin concentrations in patients with dengue fever, measles and influenza were significantly higher than that in healthy controls.[8] Alternatively, Falsey et al. indicated that elevated SAA and C-reactive protein (CRP) levels in influenza patients were associated with the presence of dyspnea, wheezing, and fever especially in elderly patients who required hospitalization.[9] However, neopterin level is raised in various disorders besides viral infections, such as chronic renal failure and acute coronary syndrome.[10,11] Thus, it is lacking in specificity for viral infections. Also, SAA concentration seems too sensitive to use in clinical practice as it is often increased even in trivial and asymptomatic viral infections.[12]

Toll-like receptor (TLR) family members, which are key regulators of both innate and acquired immunity, have been studied not only in its functional aspects,[13-15] but also in its expression characteristics in various disorders.[16-18] Recently, it has been recognized that unlike other TLRs, which are active as homodimers, TLR2 has evolutionarily developed its unique ability to form heterodimers with TLR1 or TLR6 to attain specificity for the diverse bacterial lipopeptides repertoire, and that the expression appears to be up-regulated on monocytes during bacterial infectious diseases.[17-19] We corroborated this by using a new quantitative flow-cytometric analysis system for Toll-like receptor 2 (TLR2).[20]

A study on TLR2 modulation in viral infection was previously reported, in which Lee et al. showed that influenza A virus causes rapid up-regulation of TLR2 expression on neutrophils *in vitro*.[21] However, it has been unknown how TLR2 expression on monocytes is modulated in patients with viral infection and how the level is transited during the clinical courses. Some studies showed that T-helper type 1 (Th1) cytokines, such as INF-γ, strongly enhance TLR2 expression *in vitro*.[22-24] In contrast, Mueller and coworkers pointed out that interleukin-4 (IL-4), a typical T-helper type 2 (Th2) cytokine, has a tendency to attenuate Th1-cytokine-dependent TLR2 mRNA enhancement in intestinal epithelial cells.[25] In general, acute viral infections, such as influenza, are shown to bring about a robust Th1-type immune response while prolonged chronic viral infections, such as chronic hepatitis C virus (HCV) and chronic hepatitis B virus (HBV) infections, are associated with Th2-skewed responses.[26,27] Based on these findings, we hypothesized that in viral infections TLR2 expression level on monocytes may be modulated complicatedly, depending on the type of causative viruses, the phase of infections, the type of Th1/Th2 polarization they elicited, the viral load, and so on.

In this study, we examined how TLR2 levels on circulating monocytes are modulated at the onset of influenza viral infections. Next, in addition to TLR2 levels, we followed CRP, SAA and neopterin concentrations from onset to the healed stage with influenza patients. Finally, we assayed TLR2 levels of patients with other acute viral infections, virus carriers with HBV/human T-lymphotropic virus I (HTLV-I) and chronic hepatitis patients due to HBV/HCV, and furthermore compared TLR2 levels between healthy volunteers and patients with infectious/non-infectious inflammatory diseases for differential diagnosis.

Patients and methods

Patient population and blood collection

The "Control" group consisted of 50 healthy volunteers (25 male and 25 female) ranging from 15 to 81 years old (mean = 41). Healthy volunteers were confirmed at the time of blood sampling that they did not have any infectious diseases for at least a month and they did not have a fever within a week. Simultaneously, white blood cell (WBC) and CRP levels and other inflammatory/biochemical parameters were checked, and volunteers with abnormal WBC and/or CRP levels were excluded.

Patients with influenza A/B viral infections were diagnosed with immunochromatography (Rapid Influenza Diagnostic Kit; SYSMEX CORPORATION, Japan) and were categorized in "Flu (onset)" group. "Onset" in this study was defined as acute phase till providing medical treatment and within a time interval of 48 h after subjective symptoms started. "Flu (onset)" group consisted of 47 patients (24 male and 23 female), ranging from 13 to 82 years old (mean = 42). Thirty-eight patients suffered from influenza A (18 male and 20 female, mean age = 43) and the remainder ($n = 9$) from influenza B (6 male and 3 female, mean age = 36). Among these 47 influenza patients, 2 exacerbated patients and 18 normally recovered patients sincerely offered blood samples for the follow-up study. The "Flu (onset)" group was age- and sex-matched to the "Control" group. Other viral infections were diagnosed with specific IgG antibody values for the paired sera from the acute and convalescent phases and with the clinical features. They were categorized into "Other virus infection" group that consisted of 12 patients (3 male and 9 female), ranging from 17 to 89 years old (mean = 63) (Table 1). The classes of etiologic viruses varied. Three patients with cytomegalovirus infection and a patient with respiratory syncytial (RS) virus infection had suffered from malignant lymphoma, adult T-cell leukemia, myelodysplastic syndrome (MDS) and Down's syndrome as underlying diseases, respectively. Moreover, chronic hepatitis patients/carriers with HBV ($n = 3$), chronic hepatitis patients with HCV ($n = 5$) and carriers with human T-lymphotropic virus I (HTLV-I) ($n = 9$) were enrolled in this study (Table 2). These patients and carriers were confirmed to be without other apparent infectious diseases.

Bacterial infectious diseases were diagnosed with the respective clinical features, leukocytosis (neutorophilia), bacteriological tests and the change for the better in physical and serological findings after antibiotics

Table 1 Other viral infections

"Other virus" Group (n = 12)							
Diagnosis	Age	Sex (M/F)	Infecting virus		Underlying disease	WBC (/μl)	CRP(mg/dl)
			Virus	Class			
CP	56	M	Cytomegalo	I	ATL	4200	0.90
CP	88	M	Cytomegalo	I	ML	2400	1.20
CP	88	F	Cytomegalo	I	MDS	2200	0.31
Bronchitis	50	F	RS	V	DS	2000	0.80
EP	18	M	Mumps	V	(−)	3000	1.19
EP	30	F	Mumps	V	(−)	8800	0.72
H-Z	17	F	V-Z	I	(−)	6260	0.08
H-Z	89	F	V-Z	I	(−)	4500	0.90
H-Z	76	F	V-Z	I	DCM	4920	0.29
H-Z	86	F	V-Z	I	(−)	3700	0.72
Enteritis	77	F	Norovirus	IV	(−)	4000	1.00
Myocarditis	80	F	Epstein–Barr	I	(−)	4200	0.25
Mean	63					4182	0.70
Range	17–89					2000–8800	0.25–1.20
(Median)	(77)					(4100)	(0.76)

Note. CP, cytomegalovirus pneumonia; EP, epidemic parotiditis; H-Z, Herpes zoster; Cytomegalo, cytomegalovirus; RS, respiratory syncytial virus; V-Z, varicella-zoster virus; Epstein–Barr, Epstein–Barr virus; ML, malignant lymphoma; ATL, adult T-cell leukemia; MDS, myelodysplastic syndrome; DS, Down's syndrome; DCM, dilated cardiomyopathy; class I, viruses with double stranded DNA; class IV, viruses with positive single-stranded RNA; class V, viruses with negative single-stranded RNA.

administration. As shown in Table 3, the patients enrolled in this study were categorized into "Bacteria (onset)" and "Bacteria (serious)" groups. "Bacteria (onset)" group consisted of 20 patients (12 male and 8 female), ranging from 28 to 89 years old (mean = 63), whose blood samples were taken at onset before administering antibiotics (Table 3, middle column). The "Bacteria (serious)" group consisted of 14 patients (10 male and 4 female), ranging from 55 to 89 years old (mean = 72), whose blood samples were collected during antibiotic administrations. At the time of blood collection, the administrating antibiotics were ineffective and they were suffering from refractory and severe bacterial infections for more than a week after the symptoms started (Table 3, right column). No patient was duplicated between "Bacteria (onset)" group and "Bacteria (serious)" groups.

Table 2 Virus carrier and chronic hepatitis

Diagnosis	Age	Sex (M/F)	TLR2 (sites/cell)	WBC (/μl)	CRP (mg/dl)	AST (U/l)	ALT (U/l)
HBV carrier	70	F	4101	4160	0.70	16	12
HBV carrier	42	M	4135	4000	0.10	24	26
HBV chronic hepatitis	57	M	4555	6320	0.07	34	48
HCV chronic hepatitis	66	M	3800	4800	0.08	40	55
HCV chronic hepatitis	55	M	5380	5820	0.35	32	43
HCV chronic hepatitis	76	M	5826	4580	0.11	24	16
HCV chronic hepatitis	60	F	6098	3800	0.10	21	14
HCV chronic hepatitis	52	M	5294	6690	0.05	24	27
HTLV-I carrier	66	M	6064	3710	0.07	38	35
HTLV-I carrier	61	F	2930	5100	0.08	34	43
HTLV-I carrier	79	F	5982	3900	0.05	18	10
HTLV-I carrier	88	F	5776	5040	1.87	32	13
HTLV-I carrier	79	M	4900	4280	1.73	18	13
HTLV-I carrier	72	M	5500	6250	0.81	26	25
HTLV-I carrier	68	M	4910	10150	0.68	24	21
HTLV-I carrier	75	M	4820	6130	0.37	24	22
HTLV-I carrier	57	M	5581	9410	0.48	19	13

Note. HBV, hepatitis B virus; HCV, hepatitis C virus; HTLV-I, human T-cell lymphocytic virus type I; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 3 Patient population of "Non-infectious inflammation", "Bacteria (onset)" and "Bacteria (severe)" groups

Non-infectious inflammatory disease				Bacteria (onset)			Bacteria (serious)		
Diagnosis	*n*	WBC	CRP	Diagnosis	*n*	Organism	Diagnosis	*n*	Organism
Collagen diseases				Pneumonia	8	*S. pneumoniae* *P. aeruginosa* *S. aureus* *H. influenzae*	Pneumonia	4	*Bacteroides* spp. *S. pneumoniae* MRSA *Serratia* spp.
RA	7	(7109)	(4.56)						
Arteritis	4	(9680)	(6.18)						
SLE	1	4800	2.40						
SLE + Arteritis	1	12200	2.80						
Psoriasis	1	7350	6.22	Pyelitis	4	*Escherichia coli* *K. pneumoniae*	Pyelitis	1	*Escherichia coli*
Ischemic diseases							Cholecytitis	1	Unknown
AMI	4	(10,030)	0.87	Tonsillitis	2	*Streptococcus* spp.			
Cardiac shock	1	10500	4.00						
				Enteritis	2	*Salmonella* Unknown	Sepsis	2	*Enterobacter* spp. Polymicrobial
Cancers									
Colon cancer	2	(8000)	(3.00)						
Esophageal cancer	1	12260	1.64	Respiratory infection	1	*Streptococcus* spp.	Pneumonia/sepsis	1	*P. aeruginosa*
Prostatic cancer	1	10150	0.68						
Meta. liver cancer	1	8900	14.90	Paranasal sinusitis	1	*M. catarrhalis*/*H. influenzae*	ICD infection/sepsis	2	*S.aureus* *S. epidermidis*
Ovarian cancer	2	(6230)	(1.71)						
Lung cancer	1	4280	0.55	Dermato-cellulitis	1	*Streptococcus* spp.	Infectious endocarditis	1	*Streptococcus* spp.
Allergosis				Periostitis of the jaw	1	Unknown	Pyelitis/sepsis	1	*Escherichia coli*
Anaphylactic	1	22590	1.63						
Asthma	1	7200	3.20				Pneumonia/septic arteritis	1	*S. aureus*
Drug eruption	1	5100	2.37						
Injury									
Surgical injury	4	(7413)	(1.96)						

Note. RA, rheumatoid arteritis; SLE, systemic lupus erythematosus; AMI, acute myocardial infarction; ICD, Implantable Cardioverter Defibrillator; *S. pneumoniae*, *Streptococcus pneumoniae*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. aureus*, *Staphylococcus aureus*; *H. influenzae*, *Haemophilus influenzae*; *K. pneumoniae*, *Klebsiella pneumoniae*; *M. catarrhalis*, *Moraxella catarrhalis*; *S. epidermidis*, *Staphylococcus epidermidis*; MRSA, methicillin-resistant *Staphylococcus aureus*; Polymicrobial, polymicrobial pattern. Numbers in parentheses represent the mean values of WBC and CRP levels of the patients classified into same group.

"Non-infectious inflammation" group consisted of patients with collagen diseases, ischemic diseases, cancers, allergies and surgical injury, whose WBC and/or CRP levels were abnormal (Table 3, left column).

Informed consent was obtained from all patients and volunteers, which was in accordance with a protocol approved by the Kagoshima University Ethics Committee. Ten millilitres of peripheral blood were taken from each subject with a heparinized blood collecting tube.

TLR2 assay

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by density gradient centrifugation using Ficoll–Paque plus liquid (Amersham Bioscience, GE Healthcare, UK) and were subjected to the TLR2 assay. The TLR2 assay was performed as described in our previous report.[20] In brief, PBMCs were stained with phycoerythrin (PE)-conjugated mAbs to TLR2, CD14 and control IgG2a (clones T2.1, M5E2 and eBM2a, respectively, all from eBIOSCIENSE) in separate tubes for 30 min at room temperature. In parallel, a mixture of recombinant TLR2-coupled standard beads was stained for TLR2 in the same conditions. The stained cells and beads were analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences). For each sample, monocytes were first identified using the forward/side scatter properties and CD14 staining, and the same gate setting for monocytes was applied to the analysis of TLR2- and control-stained PBMC samples. Net mean fluorescence intensity (MFI) for TLR2 was obtained by subtracting the MFI value of control staining. A calibration curve was generated in each assay by using net MFI values of the standard beads and their values of antibody-binding sites per bead, which had been determined by Scatchard plot analysis with ^{125}I-labeled T2.1 antibody. Using this calibration curve, the net MFI value of monocytes was converted to the number of antibody-binding sites per cell. In this assay system, inter-assay variation was within acceptable levels (CV < 6.8%).

Measurement of SAA and neopterin levels

SSA and neopterin levels in sera were determined with commercially available ELISA kits for SSA (BioSource International, CA, USA) and neopterin (IBL-Hamburg GmbH, Hamburg, Germany), according to the manufacturer's instructions. The minimum detectable concentrations in the ELISA kits for SSA and neopterin were 1.9 μg/ml and 2.7 nmol/l, respectively.

Statistics

Given two data sets, we assessed whether two samples of observations had the same distribution. If the distributions were almost normal and homoscedastic, comparison of two groups was performed using a paired or unpaired Student's *t*-test. These data were expressed as the mean value ± SD. If the distributions were not normal and/or homoscedastic, a Mann–Whitney *U* test (two groups) and Steel–Dwass test (more than two groups) was performed. These data were expressed as median values and 25–75th percentile in the distribution. All differences were considered significant at $p < 0.05$. All statistical analyses were performed with Excel Statistics 2006 for Windows® (Social Survey Research Information Co., Japan).

Results

Influenza infections

Although influenza viruses are well known to trigger robust Th1-type immune reactions, it has not been studied to what extent TLR2 expression levels on circulating monocytes are modulated. At the onset of influenza viral infections, TLR2 expression levels on circulating monocytes, serum CRP, and WBC were examined (Fig. 1a–c). In this study, the normal range of CRP level and WBC in men and women was set as less than 0.5 mg/dl, 3500–9700/μl, and 3500–9300/μl, respectively. The measurable lower limit of CRP was 0.05 mg/dl in this study. In the "Control" group, TLR2 expression levels were distributed from 2422 sites/cell to 6000 sites/cell, with a median value of 4567 sites/cell. Overall, the distribution of TLR2 levels appeared normal, and the mean and the mean + 2 SD values were 4454 sites/cell and 6108 sites/cell, respectively. Based on these results, we set the upper limit of the normal TLR2 level at 6108 sites/cell (mean + 2SD) for this study (Fig. 1a). As shown in Fig. 1a, b, expression levels of TLR2 and CRP in the "Flu (onset)" group were significantly higher than those in the "Control" group [median (25–75th percentile), 8057 (7372–9152) vs. 4567 (3838–4989) sites/cell, $p < 0.001$ and 0.92 (0.44–2.35) vs. 0.06 (0.05–0.10) mg/dl, $p < 0.001$, respectively]. In the "Flu (onset)" group, abnormally high values were seen in 93.6% (44/47) of patients for TLR2 and 70.2% (33/47) of patients for CRP. Whereas, there was no significant difference in WBC between these two groups [median (25–75th percentile), 6000 (4705–7435) vs. 5460 (4408–6418)/μl, $p = 0.13$, Fig. 1c].

Influenza A and influenza B infections

There were no significant differences in TLR2 levels between influenza A and B patients [median (25–75th percentile), 8063 (6985–9159) vs. 7946 (7783–8114) sites/cell, $p = 0.77$]. On the other hand, influenza A patients had significantly higher CRP and WBC levels than influenza B patients [median (25–75th percentile), 1.25 (0.55–2.62) vs. 0.46 (0.31–0.90) mg/dl, $p = 0.022$ and mean ± SD, 6366 ± 1788 vs. 4883 ± 1509/μl, $p = 0.026$, respectively].

Influenza patient follow-up

In the "Flu (onset)" group, 20 patients were followed-up from onset to convalescence (5–14 days after onset), among whom 9 patients were further tracked to the completely healed phase (14–30 days after onset). At onset, clinical conditions were equally serious for all of these 20 patients. Later, 18 out of these 20 patients were in near-perfect condition at the convalescent phase with some patients having only a slight cough and sore throat. In the remaining 2 patients, complications set in; one case

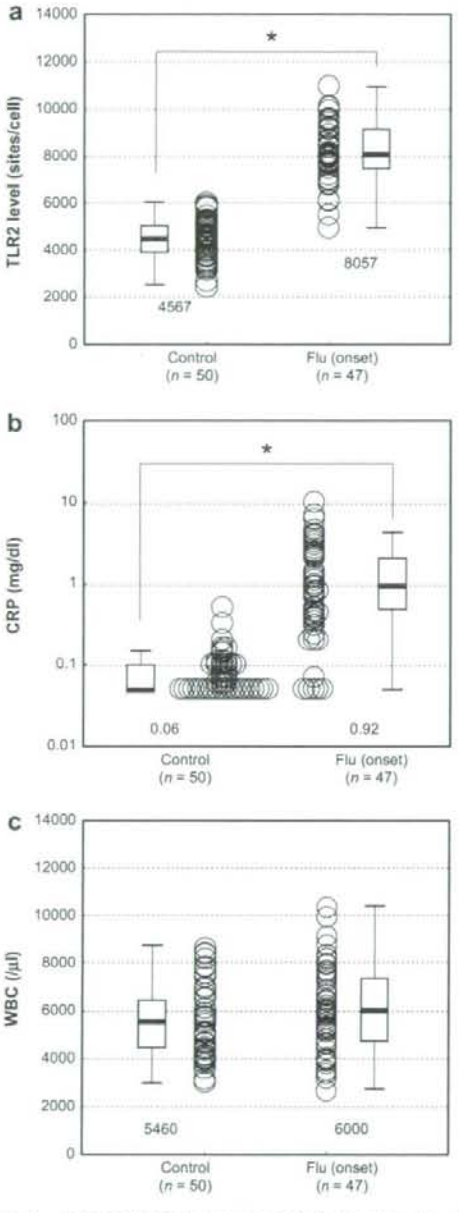

Figure 1 Comparison between the "Control" group ($n = 50$) and "Flu (onset)" group ($n = 47$). (a) TLR2 levels were compared between the two groups. Circles represent individual TLR2 expression values. (b) CRP levels were compared between the two groups. Circles represent individual CRP levels. (c) WBC values were compared between the two groups. Circles represent individual WBC values. The box plot and the horizontal bar show the interquartile range and median value, respectively. The whiskers extend to at most 1.5 times the box width (the interquartile range) from either or both ends of the box. p Value estimates are based on the Mann–Whitney U test; $^*p < 0.001$. Control, healthy volunteers; Flu, influenza patients.

progressed to an episode of proximal muscle weakness and the other developed bacterial pneumonia. All 20 patients were completely cured at the healed phase. During the individual clinical courses, CRP, SAA and neopterin concentrations in sera and TLR2 expression levels on monocytes were assayed. Here, normal ranges of neopterin and SAA levels were less than 12 nmol/l and 10 μg/ml, respectively.[8,9] As shown in Fig. 2a, TLR2 levels were abnormally high in 95% (19/20) of patients at the acute phase. Subsequently, the levels were normalized until the convalescent phase in all patients except for the two complicated cases. In the complicated cases, TLR2 values thereafter declined to normal at the healed phase (Fig. 2a, closed symbols). In Fig. 2b, CRP levels are line-plotted at the respective phases. At onset, values were distributed broadly from normal to abnormal levels. Abnormal CRP values (≥0.50 mg/dl) were seen in 85% (17/20), 20% (4/20, including one complication case) and 0% (0/9) of patients at the acute, convalescent and healed phases, respectively (Fig. 2b). With the SAA values, all 20 patients had high levels well over the normal range at onset. Later, 45% (9/20) and 56% (5/9) of patients had abnormal SAA levels at convalescence and at the healed phase, respectively (Fig. 2c). The two complication cases showed abnormally high SAA values throughout the observation period. In Fig. 2d, transitions of neopterin concentrations are shown. At onset, 80% (16/20) of patients had abnormal levels. Thereafter, 5% (1/20) of patients had abnormal neopterin levels at the convalescent phase, and in all patients they were within the normal range at the healed phase. Complications were not reflected in their neopterin levels.

Statistical analysis showed that at onset TLR2 expression level was not correlated with none of CRP ($r = -0.0065$, $p = 0.98$), SAA ($r = -0.12$, $p = 0.62$) and neopterin ($r = 0.17$, $p = 0.46$) levels.

Other viral infections and virus carriers

Next, TLR2 levels in other viral infections were examined. Their clinical pictures were severe at onset corresponding to the respective viral infectious disease. As shown in Table 1, the TLR2 and CRP levels in "Other viral infection" group were significantly higher than those in "Control" group [median (25–75th percentile), 7738 (7115–8313) vs. 4567 (3838–4989) sites/cell, $p < 0.001$ and 0.76 (0.31–0.93) vs. 0.06 (0.05–0.10) mg/dl, $p < 0.001$, respectively]. Meanwhile, WBC in "Other viral infection" group was significantly lower than those in "Control" group [median (25–75th percentile), 4100 (2850–4605) vs. 6000 (4705–7435)/μl, $p = 0.008$].

Subsequently, we examined the TLR2 levels in the carrier state/chronic hepatitis due to HBV/HCV and in the carrier state due to HTLV-I (Table 2). TLR2 levels were within the normal range [median (25–75th percentile), 5294 (4555–5776) site/cells] in all tested subjects ($n = 17$).

Bacterial infections and non-infectious inflammatory diseases

To further characterize TLR2 levels in clinical settings, we performed multiple comparisons in the TLR2 levels between the two paired groups out of "Control", "Non-infectious inflammation", "Bacteria (onset)", "Bacteria (serious)", "Flu (onset)" and "Other virus infection" groups (Fig. 3a). As a result, there was no significant difference in TLR2 levels between "Control" and "Non-infectious inflammation" groups ($p = 0.31$). TLR2 levels in "Bacteria (onset)" group were significantly higher than those in "Control" group and "Non-infectious inflammation" group [median (25–75th percentile), 5879 (5241–6595) vs. 4567 (3838–4989) sites/cell and 3792 (3188–4910) sites/cell, $p < 0.001$, respectively]. Furthermore, TLR2 levels were remarkably higher in "Flu (onset)" group [median (25–75th percentile), 8057 (7372–9152) sites/cell] and in "Other virus infection" group [median (25–75th percentile), 7813 (7176–8313) sites/cell] than in "Bacteria (onset)" group ($p < 0.001$, respectively). However, there was no significant difference in TLR2 levels between all pairs out of "Bacteria (serious)" [median (25–75th percentile), 7758 (7331–9991) sites/cell], "Flu (onset)", and "Other virus infection" group ($p > 0.9$ for all pairs).

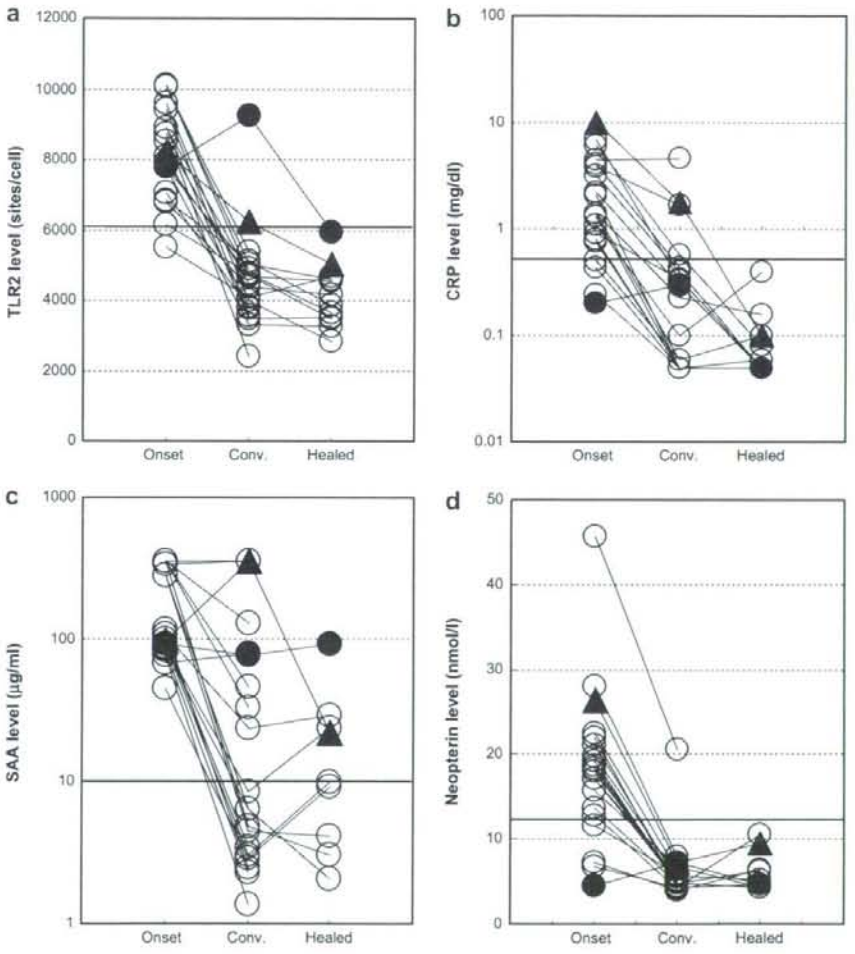

Figure 2 Follow-up of influenza patients ($n = 20$). (a) Plot lines of the transition of TLR2 levels on monocytes. (b) Plot lines of the transition of CRP levels. Vertical axis is logarithmic scale. (c) Plot lines of the transition of SAA levels. Vertical axis is logarithmic scale. (d) Plot lines of the transition of neopterin levels. Onset, onset of influenza; Conv., convalescent phase (5–14 days after the onset); Healed, completely healed phase (15–30 days after the onset). Open circle, smoothly recovered patients; Solid circle, patient with proximal muscle weakness; Solid triangle, patient with subsequent bacterial pneumonia. Horizontal solid lines in charts (a)–(d) are the respective upper limits of normal.

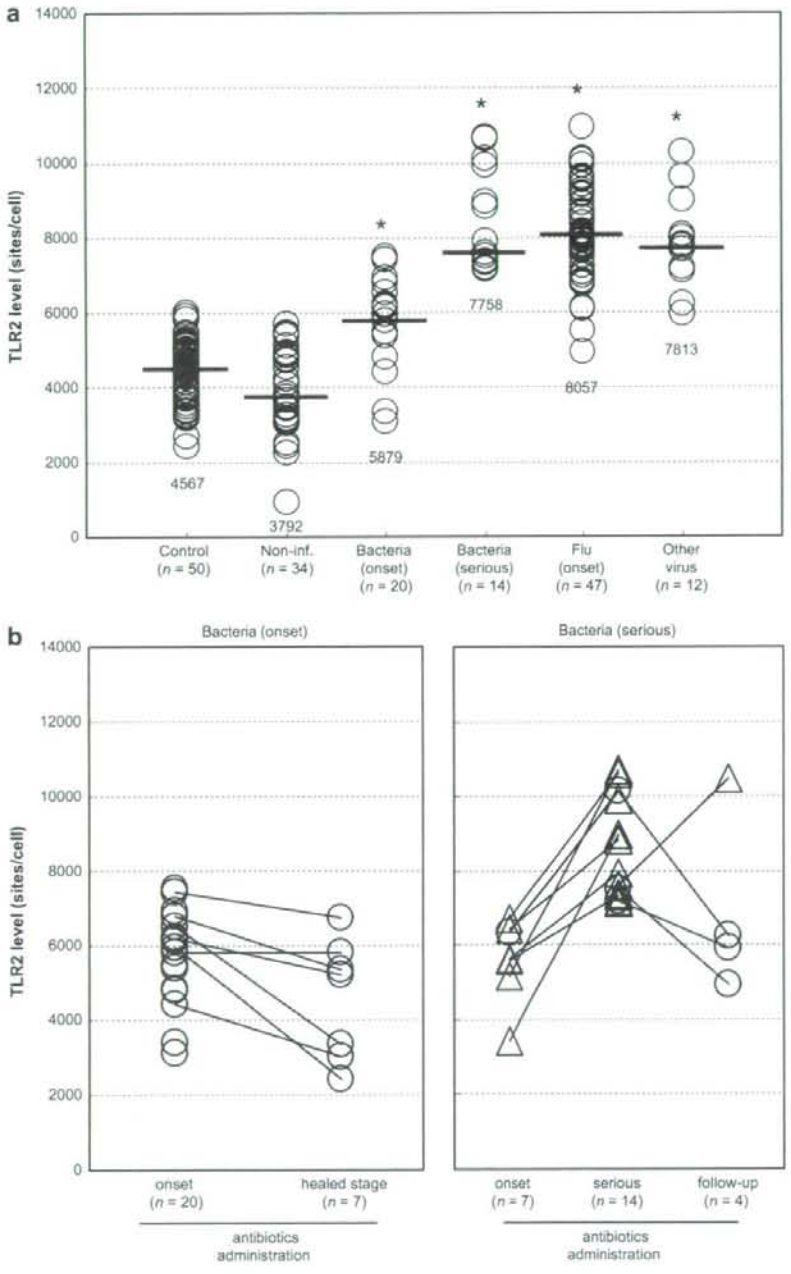

Figure 3 Comparison among "Control", "Non-infectious inflammation", "Bacteria (onset)", "Bacteria (serious)", "Flu (onset)" and "Other virus infections" groups. (a) Up-regulation of TLR2 levels in infectious diseases. Circles represent individual TLR2 expression values. Horizontal bars represent the median per group. Numbers under the circles indicate the median value per group. Multiple comparisons were performed using the Steel–Dwass test. Non-inf., "Non-infectious inflammation" group; $^{*}p < 0.001$, Control vs. Bacteria (onset)/Bacteria (serious)/Flu (onset)/Other virus, Non-inf. vs. Bacteria (onset)/Bacteria (serious)/Flu (onset)/Other virus, Bacteria (onset) vs. Bacteria (serious)/Flu (onset)/Other virus. (b) Transition of TLR2 levels of patients in "Bacteria (onset)" group (left panel). Transition of TLR2 levels of patients in "Bacteria (serious)" group (right panel). Circle, recovered patient; triangle, mortality. *X*-axis is not common in time scale, but corresponding to the individual clinical condition.

All patients of "Bacteria (onset)" group were confirmed to be completely cured, of whom 7 patients could be followed-up till their healed stage (Fig. 3b, left). In these patients, administrating antibiotics at the onset resulted in the rapid normalization of WBC and CRP levels, and their TLR2 levels transited without significant up-regulation throughout the clinical courses. In contrast, in "Bacteria (serious)" group, 3 patients recovered completely after treatment with the second/third-choice antibiotic agents, while remaining 11 patients died by exacerbated bacterial infections. Some of these patients were also examined for TLR2 levels at their onset and the follow-up phase (Fig. 3b, right). The TLR2 levels at onset in these patients ($n = 7$) were comparable to those in patients ($n = 20$) of "Bacteria (onset)" group [median (25–75th percentile), 5636 (5398–6377) vs. 5879 (5341–6595), $p = 0.74$]. The TLR2 levels of the recovered 3 patients were normalized according to the effectiveness of antibiotics (Fig. 3b, open circles), as we previously reported with other patient population.[20]

Discussion

In this study, we found that TLR2 levels on monocytes from patients with viral infectious diseases including influenza were significantly up-regulated as compared to those from healthy volunteers. We and others previously reported that TLR2 levels were markedly up-regulated in patients with advanced bacterial infections.[16–20] However, it remained unclear to what extent TLR2 levels are modulated in bacterial infections at their acute phase before antibiotic administration. Clinically, it is often difficult to discriminate between viral and bacterial infections at their onset. Therefore, in the present study, we attempted to examine TLR2 levels at bacterial infection onset, and compared them with those of viral infections. The result demonstrated that patients at the onset of serious viral infections had much higher TLR2 expression levels than patients at the onset of bacterial infections. However, there was no longer significant difference in TLR2 levels between serious bacterial and viral infections. As shown in Table 3, no critical difference was recognized in causative organisms and infective focuses between our studied "Bacteria (onset)" and "Bacteria (serious)" groups. Also, although 50% (7/14) of "Bacterial (serious)" group developed to sepsis, their TLR2 levels at onset were on a par with those of "Bacteria (onset)" group. Hence, TLR2 levels at onset of bacterial infections, which were significantly higher than those of healthy subjects, seem to be more enhanced after some exacerbating period. These findings imply that TLR2 levels may be more promptly up-regulated in viral infections than in bacterial infections, suggesting a possibility to use TLR2 as one of indications for discriminating between bacterial and viral infections at the time of their onset. On the other hand, non-infectious inflammatory diseases, such as rheumatoid arteritis (RA) and arteritis, sometimes bring about similar clinical symptoms to infectious inflammatory diseases. As we previously reported[20] and confirmed again in this study (Fig. 3a), TLR2 levels in patients with such non-infectious inflammatory diseases were in the normal range, whereas TLR2 levels were markedly enhanced in patients with sustained and/or severe infections, whether viral or bacterial. Thus, TLR2 levels might provide useful information to discriminate between infectious and non-infectious inflammatory diseases, when the disease activity sustained for a prolonged period.

To characterize TLR2 expression levels in viral infections, we followed the individual patients with influenza viral diseases from onset to completely healed phase, assaying TLR2 levels together with pre-existing serum parameters CRP, SAA, and neopterin. The results showed that at onset TLR2 levels were more clustered in the abnormal range as compared with CRP and neopterin levels, and that SAA was most sensitive among the four parameters. Simultaneously, our results showed that, in the case of patients with a smooth recovery, TLR2 levels responded most promptly to patient's clinical conditions and converged within the normal range until the convalescence period. In contrast, SAA levels remained abnormal at convalescence and even at healed phase in several patients who lacked any symptoms, such as a cough or sore throat, as reported previously.[12] Focused on the 2 complicated patients, these parameters are further characterized. The patient with bacterial pneumonia had abnormal CRP levels until the convalescent phase, but the patient with muscle weakness could not be discriminated from smoothly recovered patients by CRP levels. In addition, their neopterin levels did not reflect the individual complications at convalescence. SAA levels indicated a development to bacterial pneumonia by the surge at the convalescent phase, whereas it could not represent an episode of muscle weakness at convalescent phase and spontaneous recovery. On the other hand, TLR2 expression levels were abnormal in both patients at the convalescent phase, and provided evidence to detect these complications early and with specificity. Taken together, these results demonstrate that TLR2 expression level on monocytes appears more compatible with clinical conditions of influenza patients than other three parameters.

In this study, we confirmed that patients with other viral infections, such as cytomegalovirus pneumonia and bronchitis due to RS virus, also had high TLR2 levels equal to influenza A/B patients. It is noteworthy that the remarkable effectiveness of ganciclovir against cytomegalovirus pneumonia was promptly reflected by the normalization of TLR2 levels a week after administration, whereas TLR2 levels remained to be high in two patients with fatal RS virus and cytomegalovirus infections (data not shown). In addition, we confirmed that TLR2 levels were in the normal range in asymptomatic HBV/HTLV-1 carriers and in patients with inactive HCV chronic hepatitis. Notably, Wei et al. showed that there was a gradual increase of TLR2 expression levels from healthy controls to chronic and severe hepatitis B patients.[28] Furthermore, several investigators observed up-regulation of TLR2 protein and mRNA in monocytes from patients with chronic and active hepatitis C.[29,30] All these findings indicated that TLR2 expression levels on monocytes seem to be modulated according to viral infection severity. In order to clarify the association between TLR2 modulation and viral activity further studies will be required from the view of virology.

What mechanisms modulate the TLR2 expression levels on monocytes? It has been reported in an *ex vivo* study that

some cytokines could alter TLR2 expression levels on various kind of cells.[22–25] We also observed that INF-γ and IL-10 have a potency to up-regulate dramatically TLR2 levels, while IL-4 down-regulates TLR2 levels in human monocytes *in vitro* (data not shown). However, as mentioned above, TLR2 levels were in the normal range in organ-specific autoimmune diseases, such as RA and psoriasis, even though they are well known to be under Th1-cytokine dominant polarization like viral infections.[31,32] Thus, Th1-skewed condition alone is not always satisfactory to promote TLR2 expression on monocytes. Stimuli elicited by different kinds of factors might cooperatively modulate TLR2 expression levels on monocytes *in vivo*.

Avian influenza virus (H5N1) is regarded as an emerging pathogen with the potency to cause great harm to humans.[33] This virus is a subtype of the influenza A virus species of the Orthomyxoviridae family, which consists of negative-stranded, segmented RNA viruses. Like all other influenza A subtypes, the viral replication strategies, which are closely intertwined with normal cellular processes, will induce similar host immune responses, including some defensive Th1-type cytokine secretion. TLR2 levels on monocytes might provide information about the severity of such a new pathogenic influenza virus infection.

In this study, we elucidated some characteristics of TLR2 expression levels on monocytes by examining patients suffering from various viral infections, focusing on influenza patients. Our results demonstrated that TLR2 expression level on monocytes might serve as a unique biomarker useful in the field of viral and bacterial infectious diseases.

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References

1. Oseasohn R, Adelson L, Kaji M. Clinicopathologic study of thirty-three fatal cases of Asian influenza. *N Engl J Med* 1959;**260**:509–18.
2. Louria DB, Blumenfeld HL, Ellis JT, Kilbourne ED, Rogers DE. Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza. *J Clin Invest* 1959; **38**:213–65.
3. Murata Y, Walsh EE, Falsey AR. Pulmonary complications of interpandemic influenza A in hospitalized adults. *J Infect Dis* 2007;**195**:1029–37.
4. Middleton PJ, Alexander RM, Szymanski MT. Severe myositis during recovery from influenza. *Lancet* 1970;**2**:533–5.
5. Guarner J, Paddock CD, Shieh WJ, Packard MM, Patel M, Montague JL, et al. Histopathologic and immunohistochemical features of fatal influenza virus infection in children during the 2003–2004 season. *Clin Infect Dis* 2006;**43**:132–40.
6. Bowles NE, Ni J, Kearney DL, Pauschinger M, Schultheiss HP, McCarthy R, et al. Detection of viruses in myocardial tissues by polymerase chain reaction. evidence of adenovirus as a common cause of myocarditis in children and adults. *J Am Coll Cardiol* 2003;**42**:466–72.
7. Weiss G, Murr C, Zoller H, Haun M, Widner B, Ludescher C, et al. Modulation of neopterin formation and tryptophan degradation by Th1- and Th2-derived cytokines in human monocytic cells. *Clin Exp Immunol* 1999;**116**:435–40.
8. Chan CP, Choi JW, Cao KY, Wang M, Gao Y, Zhou DH, et al. Detection of serum neopterin for early assessment of dengue virus infection. *J Infect* 2006;**53**:152–8.
9. Falsey AR, Walsh EE, Francis CW, Looney RJ, Kolassa JE, Hall WJ, et al. Response of C-reactive protein and serum amyloid A to influenza A infection in older adults. *J Infect Dis* 2001;**183**:995–9.
10. Godai K, Uemasu J, Kawasaki H. Clinical significance of serum and urinary neopterins in patients with chronic renal disease. *Clin Nephrol* 1991;**36**:141–6.
11. Ray KK, Morrow DA, Sabatine MS, Shui A, Rifai N, Cannon CP, et al. Long-term prognostic value of neopterin: a novel marker of monocyte activation in patients with acute coronary syndrome. *Circulation* 2007;**115**:3071–8.
12. Whicher JT, Chambers RE, Higginson J, Nashef L, Higgins PG. Acute phase response of serum amyloid A protein and C reactive protein to the common cold and influenza. *J Clin Pathol* 1985;**38**:312–6.
13. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997;**388**:394–7.
14. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999;**11**:443–51.
15. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunol* 2004;**4**:499–511.
16. Pons J, Sauleda J, Regueiro V, Santos C, López M, Ferrer J, et al. Expression of Toll-like receptor 2 is up-regulated in monocytes from patients with chronic obstructive pulmonary disease. *Respir Res* 2006;**7**:64.
17. Harter L, Mica L, Stocker R, Trentz O, Keel M. Increased expression of toll-like receptor-2 and -4 on leukocytes from patients with sepsis. *Shock* 2004;**22**:403–9.
18. Armstrong L, Medford AR, Hunter KJ, Uppington KM, Millar AB. Differential expression of Toll-like receptor (TLR)-2 and TLR-4 on monocytes in human sepsis. *Clin Exp Immunol* 2004;**136**:312–9.
19. Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, et al. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *J Leukoc Biol* 2007;**3** [Epub ahead of print].
20. Orihara K, Nagata K, Hamasaki S, Oba R, Hirai H, Ishida S, et al. Time-course of Toll-like receptor 2 expression, as a predictor of recurrence in patients with bacterial infectious diseases. *Clin Exp Immunol* 2007;**148**:260–70.
21. Lee RM, White MR, Hartshorn KL. Influenza a viruses upregulate neutrophil toll-like receptor 2 expression and function. *Scand J Immunol* 2006;**63**:81–9.
22. Mita Y, Dobashi K, Shimizu Y, Nakazawa T, Mori M. Toll-like receptor 2 and 4 surface expressions on human monocytes are modulated by interferon-gamma and macrophage colony-stimulating factor. *Immunol Lett* 2001;**78**:97–101.
23. Faure E, Thomas L, Xu H, Medvedev A, Equils O, Arditi M. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. *J Immunol* 2001;**166**:2018–24.
24. Pivarcsi A, Bodai L, Réthi B, Kenderessy-Szabó A, Koreck A, Széll M, et al. Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *Int Immunol* 2003;**15**:721–30.
25. Mueller T, Terada T, Rosenberg IM, Shibolet O, Podolsky DK. Th2 cytokines down-regulate TLR expression and function in human intestinal epithelial cells. *J Immunol* 2006;**176**:5805–14.
26. Waggoner SN, Hall CH, Hahn YS. HCV core protein interaction with gC1q receptor inhibits Th1 differentiation of CD4 + T cells via suppression of dendritic cell IL-12 production. *J Leukoc Biol* 2007;**82**:1407–19.
27. Milich DR. Influence of T-helper cell subsets and crossregulation in hepatitis B virus infection. *J Viral Hepat* 1997;**4**:48–59.

28. Wei XQ, Wen ZF, Zheng FP, Yao JL. Changes of Toll-like receptor (TLR) 2 and TLR4 on the peripheral blood mononuclear cells in patients with chronic hepatitis B and chronic severe hepatitis B. *Zhonghua Gan Zang Bing Za Zhi* 2007;**15**:354–7.
29. Riordan SM, Skinner NA, Kurtovic J, Locarnini S, McIver CJ, Williams R, et al. Toll-like receptor expression in chronic hepatitis C: correlation with pro-inflammatory cytokine levels and liver injury. *Inflamm Res* 2006;**55**:279–85.
30. Shehata MA, Abou El-Enein A, El-Sharnouby GA. Significance of toll-like receptors 2 and 4 mRNA expression in chronic hepatitis C virus infection. *Egypt J Immunol* 2006;**13**:141–52.
31. Yudoh K, Matsuno H, Nakazawa F, Yonezawa T, Kimura T. Reduced expression of the regulatory CD4 + T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. *Arthritis Rheum* 2000;**43**:617–27.
32. Jacob SE, Nassiri M, Kerdel FA, Vincek V. Simultaneous measurement of multiple Th1 and Th2 serum cytokines in psoriasis and correlation with disease severity. *Mediators Inflamm* 2003;**12**:309–13.
33. Sedyaningsih ER, Isfandari S, Setiawaty V, Rifati L, Harun S, Purba W, et al. Epidemiology of cases of H5N1 virus infection in Indonesia, July 2005–June 2006. *J Infect Dis* 2007;**196**:522–7.

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ORIGINAL ARTICLE

No reflow-like pattern in intramyocardial coronary artery suggests myocardial ischemia in patients with hypertrophic cardiomyopathy

Woo-Shik Kim (MD)[a], Shinichi Minagoe (MD, FJCC)[b,*], Naoko Mizukami (RCS)[a], Xiaoyan Zhou (MD)[a], Keiichiro Yoshinaga (MD)[a], Kunitsugu Takasaki (MD)[a], Toshinori Yuasa (MD)[a], Koichi Kihara (MD)[a], Shuichi Hamasaki (MD, FJCC)[a], Yutaka Otsuji (MD, FJCC)[a], Akira Kisanuki (MD, FJCC)[a], Chuwa Tei (MD, FJCC)[a]

[a] *Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, Kagoshima, Japan*
[b] *Department of Cardiology, National Hospital Organization Kagoshima Medical Center, Shiroyama-cho 8-1, 892-0853 Kagoshima, Japan*

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Hypertrophic

Abstract
Background and purpose: To evaluate intramyocardial coronary flow velocity pattern by transthoracic Doppler echocardiography and its clinical significance in patients with hypertrophic cardiomyopathy (HCM).
Methods and results: In 48 patients with HCM who had angiographically normal coronary artery, coronary flow velocity in the left anterior descending coronary artery (LAD) and intramyocardial coronary artery (IMCA) derived from LAD were evaluated using transthoracic Doppler echocardiography. Two clearly different flow patterns in the IMCA were observed in patients with HCM. Twenty-seven HCM patients (group A) had slow deceleration slope in the IMCA flow (average diastolic deceleration time, 989 ± 338; range, 585–1680) and the remaining 21 patients (group B) had steep deceleration slope with diastolic deceleration time <300 ms, resulting in a no reflow-like pattern in the IMCA flow (average diastolic deceleration time, 166 ± 67; range, 55–280). There were no significant differences in the clinical characteristics and LAD flow velocity profiles between the two groups. The incidence of cardiovascular symptoms (chest pain or syncope) was significantly higher in group B than in group A (67% vs. 26%, $p < 0.01$). Additionally, exercise-induced ischemia as detected by thallium-201 scintigraphy was significantly more frequent in group B than in group A (6 of 9 (67%) vs. 0 of 9 (0%), $p < 0.01$).

* Corresponding author. Tel.: +81 99 275 5318; fax: +81 99 265 8447.
E-mail address: minagoe@kagomc2.hosp.go.jp (S. Minagoe).

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Conclusions: Two different intramyocardial coronary flow velocity patterns are observed in patients with HCM using transthoracic Doppler echocardiography. No reflow-like pattern in the IMCA is strongly related to myocardial ischemia in the absence of epicardial coronary artery stenosis, suggesting that coronary microvascular dysfunction may be a causative mechanism.

Introduction

Clinical signs and symptoms suggesting myocardial ischemia frequently develop in patients with hypertrophic cardiomyopathy (HCM) despite anatomically normal epicardial coronary artery [1,2]. Although there is growing evidence that abnormalities of the coronary microvasculature can result in myocardial ischemia in patients with HCM with normal epicardial coronary artery [2–4], clinical evaluation of the coronary microcirculation has not been fully investigated due to technical difficulties.

Characteristic phasic coronary flow velocity patterns have been reported in patients with HCM. However, there was no relationship between phasic flow abnormalities and those with symptoms [5–7]. Since the phasic blood velocity pattern in penetrating coronary arteries is different than that in large epicardial arteries, epicardial coronary flow velocity profiles may not accurately reflect intramyocardial coronary perfusion [8]. Recent advances in transthoracic Doppler echocardiography allow evaluation of flow velocity not only in the left anterior descending coronary artery (LAD) but also in the intramyocardial coronary artery (IMCA, 500–1000 μm) [9–12]. Therefore, we hypothesized that transthoracic Doppler echocardiography would potentially allow evaluation of an impaired flow velocity profile associated with myocardial ischemia in the absence of epicardial coronary stenosis. On this basis, the primary aim of this study was to evaluate the characteristics of the coronary flow velocity profiles in the IMCA using transthoracic Doppler echocardiography and to define the relationship between the coronary flow velocity pattern and clinical manifestations in patients with HCM. Furthermore, we compared transthoracic Doppler echocardiographic measurements of coronary flow velocity profiles with exercise thallium-201 scintigraphy results in patients with HCM.

Subjects and methods

Study patients

Sixty-eight consecutive patients with HCM who underwent evaluation of cardiac function and echocardiography at Kagoshima University Hospital or Nanpuh Hospital were enrolled in the study. The diagnosis of HCM was based on echocardiographic evidence of myocardial hypertrophy, defined as a maximal septal thickness of at least 13 mm, in the absence of any other cardiac or systemic cause of left ventricular hypertrophy [13].

Twenty patients were excluded from the analysis because of the presence of atrial fibrillation ($n = 13$) or an artificial pacemaker ($n = 7$). Consequently, the study population consisted of the remaining 48 patients (33 men, 15 women; mean age 53 years). Exercise thallium-201 scintigraphy was performed in 18 patients with HCM within 6 months before and after the echocardiographic examination as previously described [14]. Patient medications were not changed during the study. Nine subjects with normal echocardiograms and no known cardiovascular disease served as the control subjects (8 men and 1 woman, mean age 39 years). The study protocol was approved by the institutional committee of Kagoshima University, and each patient gave written informed consent prior to enrollment.

Transthoracic Doppler echocardiography

Transthoracic Doppler echocardiography with a 2.5–3.5-MHz transducer was performed using a commercially available echocardiographic system (ATL HDI 5000CV, GE Logiq 500 MR, or Acuson Sequoia C256). Left ventricular end-diastolic and end-systolic dimension and left atrial dimension were measured in the parasternal long axis view. The peak instantaneous left ventricular outflow tract gradient was estimated under basal conditions with the use of continuous-wave Doppler echocardiography.

Recording of LAD and IMCA flow by transthoracic Doppler echocardiography

Using a 4–7-MHz transducer, the short axis of the anterior interventricular groove was visualized from the echo window between the left parasternal fourth or fifth intercostal space and the apex. Next, the transducer was rotated to visualize the long axis of the groove, and color flow mapping with a Nyquistic limit ≤25 cm/s was applied to visualize the mid-to-distal LAD flow with relatively slow flow velocities.

To image the IMCA flow, the left parasternal short axis and the classical or modified two-, three- and four-chamber views were used. First, through the left parasternal short axis view at the level of the mitral valve, chordae, and papillary muscle, we searched for IMCA flows in the anteroseptal left ventricular wall using color Doppler mapping with a low Nyquistic limit. Next, using classical or modified two-, three- and four-chamber views, IMCA flow from the epicardium to the endocardium was also evaluated in the apical half of the left ventricular wall using color flow mapping with a low Nyquistic limit. The flow velocity profiles in the LAD and IMCA were recorded by pulsed-wave Doppler echocardiography with angle correction.

The peak and mean diastolic velocity diastolic velocity time integral, and deceleration time of diastolic flow were measured and averaged over three consecutive beats in each subject.

Thallium-201 scintigraphy

Each patient performed graded bicycle exercise starting at 25 W, with an increment of 25 W every 3 min. At peak exercise, 110 MBq (3 mCi) of thallium-201 chloride was injected and the exercise was continued for another 1 min. Stress thallium-201 perfusion scanning was begun within 10 min after the tracer injection. Delayed thallium-201 perfusion scanning was performed 4 h later.

Only anteroseptal and apical segments were evaluated by thallium-201 scintigraphy, because transthoracic Doppler echocardiographic evaluation of coronary flow abnormalities was limited to the territory of the LAD. Two experienced observers who had no knowledge of the clinical or echocardiographic data individually analyzed the images. The patients were considered to have myocardial ischemia when a perfusion defect was seen on the stress study but was absent in the redistribution images, or when a defect on the stress study was larger than that in the redistribution study. The images were considered negative when no perfusion defect was seen on either image, or when the defect on the stress study was the same as that in the redistribution.

Reproducibility of measurements

Interobserver variability of the measurements was assessed from 10 randomly selected recordings, with an observer blinded to previous echocardiographic data. For the assessment of intraobserver variability, 10 subjects were examined twice on different days by the same sonographer and cardiologist, who were blinded to prior Doppler echocardiographic data. All measurements were calculated as the standard deviation of the differences between the two measurements divided by the mean measurement and were expressed as the percentage of the average value.

Statistical analysis

Continuous variables are expressed as mean ± standard deviation, and categorical variables are given as absolute values, percentages, or both. Differences between continuous variables were assessed with the unpaired *t*-test. Proportions were compared by the chi-square or Fisher exact test. A *p*-value of <0.05 was considered statistically significant.

Results

Patients' characteristics

Clinical characteristics and general echocardiographic findings are shown in Table 1. Left ventricular wall thickness, left atrial dimension, and fractional shortening were significantly greater in patients with HCM than in normal subjects, whereas left ventricular end-systolic dimension was significantly smaller. Twenty-one (44%) patients with HCM had clinical symptoms of chest pain or syncope with angiographically normal coronary arteries.

Comparison of LAD flow velocity profiles between HCM and normal subjects

Adequate spectral Doppler recordings of diastolic coronary flow in the LAD were obtained in all study populations. The peak and mean diastolic velocity and velocity time integral of LAD flow were significantly increased and diastolic deceleration time was significantly prolonged in patients with HCM compared to those in normal subjects (Table 1).

Table 1 Baseline characteristics of the study population

Characteristics	Controls ($n=9$)	Patients with HCM ($n=48$)	p value
Age (years)	39 ± 13	53 ± 16	0.015
Men/women (n)	8/1	33/15	NS
Heart rate (beats/min)	68 ± 8	61 ± 11	NS
Echocardiographic measurements			
Maximal wall thickness (mm)	10 ± 2	24 ± 5	<0.001
IVS thickness (mm)	10 ± 2	21 ± 7	<0.001
PW thickness (mm)	9 ± 2	12 ± 3	0.004
LV end-diastolic dimension (mm)	48 ± 4	44 ± 7	NS
LV end-systolic dimension (mm)	32 ± 5	26 ± 5	0.006
Fractional shortening (%)	35 ± 5	42 ± 8	0.024
LA dimension (mm)	31 ± 4	39 ± 6	<0.001
LVOT pressure gradient ≥ 30 mmHg	0 (0%)	3 (17%)	NS
Incidence of moderate to severe MR	0 (0%)	0 (0%)	NS
LAD flow			
Peak diastolic velocity (cm/s)	26 ± 6	49 ± 23	<0.001
Mean diastolic velocity (cm/s)	18 ± 4	32 ± 15	<0.001
Diastolic velocity time integral (cm)	10 ± 3	23 ± 11	<0.001
Diastolic deceleration time (ms)	840 ± 144	1008 ± 353	0.028
IMCA flow			
Peak diastolic velocity (cm/s)	31 ± 10	95 ± 43	<0.001
Mean diastolic velocity (cm/s)	24 ± 8	61 ± 31	<0.001
Diastolic velocity time integral (cm)	12 ± 4	34 ± 17	<0.001
Diastolic deceleration time (ms)	657 ± 100	613 ± 485	NS

Data are presented as number (%) or mean ± 1 S.D. HCM = hypertrophic cardiomyopathy; IVS = interventricular septum; PW = posterior wall; LV = left ventricular; LA = left atrial; LVOT = LV outflow tract; MR = mitral regurgitation; LAD = left anterior descending coronary artery; IMCA = intramyocardial small coronary artery.

Comparison of IMCA flow velocity profiles between HCM and normal subjects

Adequate spectral Doppler recording of the diastolic flow in the IMCA were obtained in all study populations. The peak and mean diastolic velocity and velocity time integral of IMCA flow were significantly increased in patients with HCM compared to those in normal subjects. However, there was no significant difference in diastolic deceleration time for IMCA flow between patients with HCM and normal subjects (Table 1).

Two different IMCA flow velocity patterns

Two characteristic flow patterns in the IMCA were observed in patients with HCM (Figs. 1B, 2B and 3B). One flow pattern in the IMCA showed peak velocity is observed in early diastole and then gradually decreased during the remainder of diastole (Fig. 1B). The other flow pattern was characterized by steep decelerated diastolic flow with deceleration time <300 ms. Overall, the latter flow velocity pattern has a no reflow-like appearance (Figs. 2B and 3B). Therefore, the 48 study patients were divided into two groups according to their IMCA flow pattern. Group A consisted of 27 patients with HCM who showed slow deceleration slope in the IMCA (average deceleration time, 989 ± 338; range, 585–1680). Group B consisted of the remaining 21 patients with HCM who showed a no reflow-like pattern in the IMCA (average deceleration time, 166 ± 67; range, 55–280).

Comparison of LAD and IMCA flow velocity profiles between two groups in HCM patients

LAD and IMCA flow velocity profiles are summarized in Table 2. Measurements of LAD flow velocity profiles were not significantly different between the two groups. However, the mean diastolic velocity and velocity time integral in the IMCA were decreased in group B compared to group A ($p<0.05$), and the diastolic deceleration time of IMCA flow was significantly shortened in group B by definition ($p<0.001$).

Comparison of clinical symptoms between two groups in HCM patients

There were no significant differences in age, sex, type of HCM, and echocardiographic data between